A Serine Oligopeptidase from African Trypanosomes

by

Rory Edward Morty
B.Sc. (Hons) (Natal)

Submitted in fulfilment of the
academic requirements for the degree of
Doctor of Philosophy
in the
Department of Biochemistry
University of Natal
Pietermaritzburg

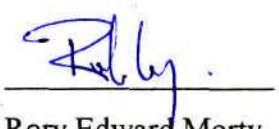
31 January 1998

This thesis is dedicated to my parents

Ken and Lyn Morty

Preface

The experimental work described in this dissertation was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg from June 1995 to December 1997 under the supervision of Dr Theresa Coetzer and co-supervision of Professor John Lonsdale-Eccles. These studies represent original work by the author and have not been submitted in any other form to another university. Where use has been made of the work of others, it has been duly acknowledged in the text.



Rory Edward Morty

31 January 1998

Abstract

Protozoan parasites of the genus *Trypanosoma* are responsible for chronic and widespread disease in livestock and humans in Africa. This study describes the purification and characterisation of a serine oligopeptidase from *Trypanosoma brucei brucei* and from *T. congolense*. Serine peptidase activity has previously been described for *T. b. brucei* although the responsible enzyme was not purified to electrophoretic homogeneity. In the present study this enzyme was purified from bloodstream-form *T. b. brucei* by a combination of three-phase partitioning, ion-exchange, affinity and molecular exclusion chromatography. Characterisation of the enzyme revealed that it closely resembled a bacterial serine oligopeptidase, *Escherichia coli* oligopeptidase B, in terms of cleavage-site specificity, inhibition characteristics and molecular mass. Its overall properties indicate that it is probably a serine oligopeptidase and we have called it OP-Tb (oligopeptidase from *Trypanosoma brucei*). Antibodies to OP-Tb were prepared in chickens. These antibodies were used in the purification of a similar enzyme, designated OP-Tc, from *T. congolense*. OP-Tc closely resembled OP-Tb in its enzymatic properties.

OP-Tb appears to be monomeric, with an apparent molecular mass of 80 kDa. Activity is optimal between pH 8.0 and 10.0, and is enhanced in the presence of reducing agents. Inhibition by 4-(2-aminoethyl)benzenesulfonylfluoride, 3,4-dichloroisocoumarin and diisopropylfluorophosphate indicates that the enzyme may be classified as a serine protease. While various natural and synthetic fluorogenic peptide substrates were hydrolysed by OP-Tb, larger potential substrates (proteins) were not. Studies of the digestion of naturally occurring bioactive peptides suggested that substrates were restricted to peptides smaller than

approximately 4 or 5 kDa. These peptides were cleaved at the carboxy side of basic amino acid residues such as arginine and lysine. This is characteristic of a trypsin-like specificity. Because the enzyme is known to be readily released from the parasites, and because it was possible to detect OP-Tb-like activity in the blood of *T. b. brucei*-infected mammalian hosts, it appears that the enzyme is released into the host bloodstream where it remains uninhibited by endogenous protease inhibitors. Indeed, OP-Tb was not inhibited by mammalian plasma serpins or α_2-macroglobulin *in vitro*. This, and the degradation of host peptide regulatory hormones *in vitro*, suggests that OP-Tb may have secondary, but important, extracellular roles in the pathogenesis of African trypanosomiasis.

A variety of serine protease inhibitors, including inhibitors of OP-Tb were tested for their potential as trypanocidal agents. The results from both *in vitro* and *in vivo* studies, suggest that inhibitors of trypanosome oligopeptidases are promising new lead targets for drug development. Furthermore, data presented here also shows that OP-Tb is efficiently inhibited by several of the currently employed trypanocidal drugs. Thus, OP-Tb may already be a cellular target for trypanocidal drugs. If correct, this may represent an important step towards understanding the biochemical mechanisms of the trypanocidal activity of these drugs, as well as providing valuable clues as to how to improve their efficacy.

Acknowledgements

I would like to extend my thanks to the following people and organisations for their substantial contributions to this study:

To Dr Theresa Coetzer, for being an outstanding supervisor; for her expert advice, good humour and unfailing support, and thorough and efficient proof-reading of this manuscript.

To Professor John Lonsdale-Eccles, for his faith in me, for initially agreeing to take me on as a student, for giving me *carte blanche* as his student; for his infectious enthusiasm for his field, and for introducing me to the world of biochemical parasitology. Furthermore, for his very efficient and thorough trans-Atlantic proof-reading of the manuscript.

To Dr Rob Pike, friend and post-doctoral student, for initiating this study, for his friendship, excellent humour and invaluable guidance.

To Ron Berry, for his good humour, and for always being a willing source of assistance in the face of technical crises.

To the academic staff of the Department of Biochemistry: Dr Edith Elliott, Dr Romilla Maharaj, Dr Trevor Anderson and Dr André van der Hoven, for providing an interesting and entertaining working environment, and in particular to Professor Clive Dennison, for his good humour and many interesting discussions on aspects of this and other projects.

To Lesley Brown for highly efficient ordering of reagents and equipment, Jenny Schwartz and Melody Webber for performing numerous administrative tasks and John Geyser for repairing and modifying technical apparatus.

To my laboratory colleagues, in particular to Linda Troeberg, for her friendship, patience and constant willingness to teach me new techniques; Niccy Scholefield and Nicolette Shearer for their moral support and Omalokoho Tosomba for his thorough instruction in raising and isolating African trypanosomes.

To fellow students in the Department: Scott Davies, Thembile Dalasile, Adam Dawe, Kevin Dennehy, Jonathan Edwards, Andrew Howes, Liesl Howes, Peter Lomo, Richard Meinesz, Lizette Moolman, Thunicia Moodley, Mark Ramsden, Ché Pillay, Brendon Price, Kerry Slatter and Kerry Taylor. I would in particular like to thank Frieda Dehrmann for a thorough introduction to many protein chemistry techniques.

To Prof. Dr Hans Fritz and Dr Ennes Auerswald, Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgische Klinik und Poliklinik der Ludwig-Maximilians-Universität, Munich, Germany, for facilitating attendance at the *Vth International Symposium on Proteinase Inhibitors and Biological Control* at Brdo, Slovenia; and for the invitation to visit their department.

To colleagues Ulrich Rester and Reinhardt Mentele, Abteilung Strukturforschung, Max-Planck-Institut für Biochemie, Martinsreid-Planegg, Germany, for facilitating the visit to their facility.

To Philip Fortgens for his sincere friendship and unparalleled integrity, and his insight and expert technical advice on many aspects of this study. Furthermore, to Philip Fortgens and Suzanne Harvey for providing a home away from home in Pietermaritzburg.

To Michael Vorster and Nabeelah Ismail, for their friendship and for being constant companions throughout my university career.

To Jan-Philipp Gelzer for his much valued friendship and advice, and to Jan-Philipp Gelzer and the *Akademische Verbundung IGEL* for providing a home away from home in Tübingen, Germany.

To my parents, Lyn and Ken Morty, my sisters, Lisa and Kirsty, my brother-in-law, Vaughan, and my uncle Arthur for their constant moral and financial support, interest and encouragement, without which I would never have been able to reach this point in my career, and to Calsi and Suzanne Adams, for being the shining stars in our family that they are.

To Louis Beaumont and Gary Preston, and the rest of the staff at Knight Alarms, for their flexibility, and their understanding of student financial constraints.

To the Foundation for Research Development, University of Natal Research Fund, and the University of Natal Graduate Assistant Programme for financial assistance.

Last, but not least, to the Almighty, in whom I trust, for guidance and inspiration.

Contents

Preface iii
Abstract iv
Acknowledgements vi
List of Figures xxvii
List of Tables xx
Abbreviations xxi

CHAPTER 1. Introduction 1
1.1 African trypanosomiasis 1
1.1.1 Trypanosome morphology 2
1.1.2 Classification of African trypanosomes 2
1.1.3 Life cycle of African trypanosomes 3
1.1.4 Control of trypanosomiasis 4
1.1.5 Treatment of African trypanosomiasis 7
1.1.6 Pathogenesis of African trypanosomiasis 7
1.2 Structure and classification of proteolytic enzymes 11
1.2.1 Serine proteases 12
1.2.2 Oligopeptidases 14
1.3 Proteolytic enzymes and disease 16
1.3.1 Proteases of parasitic protozoa 16
1.3.2 Microbial proteases and disease pathogenesis 19
1.3.2.1 Tissue destruction 19
1.3.2.2 Kinin generation 20
1.3.2.3 Cell and tissue invasion 21
1.3.2.4 Inactivation of host protease inhibitors 21
1.3.2.5 Receptor destruction 22
1.3.2.6 Inactivation of immune system components 22
1.3.3 Protease inhibitors as drugs 23
1.4 Objectives of the current study 25

CHAPTER 2. Materials and Methods 27
2.1 Materials 27
2.2 Protein assays 29
2.2.1 Bradford dye-binding assay 29
2.2.1.1 Materials 29
2.2.1.2 Method 30
2.2.2 Bicinchoninic assay 30
2.2.2.1 Materials 30
2.2.2.2 Method 31
2.2.3 Extinction coefficient 31
2.3 Synthetic peptide substrate enzyme assays 31
2.3.1 Materials 32
2.3.2 Standard assay 33
2.3.3 Microplate assay 33
2.4 Precipitation techniques 34
2.4.1 Three-phase partitioning 34
2.5 Column chromatographic techniques 35
2.5.1 Ion-exchange chromatography 35
2.5.1.1 Diethylaminoethyl-cellulose 36
2.5.1.2 Q-Sepharose 36
2.5.2 Molecular exclusion chromatography 36
2.5.3 Affinity chromatography 37
2.5.3.1 *Para*-aminobenzamidine-Sepharose 38
2.5.3.2 Poly-(L-lysine)-Sepharose 38
2.5.3.3 Cibacron Blue F3GA-Sepharose 38
2.6 Sample concentration 39
2.6.1 Concentration by ultrafiltration 39
2.6.2 Concentration by dialysis 39
2.7 Electrophoretic techniques 40
2.7.1 Tris-Tricine SDS-PAGE 41
2.7.1.1 Materials 41
2.7.1.2 Method 43
2.7.2 Substrate gel electrophoresis 43
2.7.2.1 Materials 44
2.7.2.2 Method 44

2.7.3 Coomassie Blue R-250 staining of proteins 44
2.7.3.1 Materials 45
2.7.3.2 Method 45
2.7.4 Silver staining of proteins 45
2.7.4.1 Materials 46
2.7.4.2 Method 46
2.7.5 Electroblotting of proteins 47
2.7.5.1 Materials 47
2.7.5.2 Method 47
2.8 Cell culture 47
2.8.1 Materials 48
2.8.2 Method 49

CHAPTER 3. Isolation of an oligopeptidase from *T. b. brucei* 50
3.1 Introduction 50
3.2 Growth and harvesting of trypanosomes 52
3.2.1 Materials 52
3.2.2 Methods 53
3.3 Three-phase partitioning 54
3.3.1 Materials 54
3.3.2 Optimisation of three-phase partitioning 54
3.3.3 Purification using three-phase partitioning 55
3.4 Q-Sepharose anion-exchange chromatography at pH 5.5 55
3.4.1 Materials 55
3.4.2 Method 55
3.5 *p*-Aminobenzamidine-Sepharose chromatography 56
3.5.1 Method 56
3.6 Q-Sepharose anion-exchange chromatography at pH 8 56
3.6.1 Method 56
3.7 Poly-(L-lysine)-Sepharose chromatography 57
3.7.1 Method 57
3.8 Sephacryl S-100 HR chromatography 57
3..8.1 Method 57
3.9 Active-site titration of OP-Tb 57
3.9.1 Materials 58

3.9.2 Method 58
3.10 N-terminal sequence and amino acid analysis of OP-Tb 59
3.10.1 Materials 60
3.10.2 Methods 60
3.11 Results 60
3.11.1 Three-phase partitioning 60
3.11.2 Q-Sepharose chromatography at pH 5.5 61
3.11.3 *p*-Aminobenzamidine-Sepharose chromatography 62
3.11.4 Q-Sepharose chromatography at pH 8 62
3.11.5 Poly-(L-lysine)-Sepharose chromatography 63
3.11.6 Sephacryl S-100 HR chromatography 64
3.12 Evaluation of purification 64
3.12.1 Active-site titration 65
3.12.2 N-terminal and amino analysis 66
3.13 Discussion 67

CHAPTER 4. Immunochemical studies 71
4.1 Introduction 71
4.2 Production of anti-OP-Tb antibodies 72
4.2.1 Materials 73
4.2.2 Method 73
4.3 Enzyme-linked immunosorbent assay 74
4.3.1 Materials 74
4.3.2 Method 75
4.4 Western blotting 75
4.4.1 Materials 76
4.4.2 Method 76
4.5 Inhibition of OP-Tb activity by anti-OP-Tb antibodies 77
4.5.1 Method 77
4.6 Effects of antibodies on trypanosomes in culture 77
4.7 Immunoaffinity purification of OP-Tc 77
4.7.1 Materials 78
4.7.2 Preparation of immunoaffinity matrix 78
4.7.3 Purification procedure 79
4.8 Do trypanosome-infected hosts produce anti-OP-Tb antibodies? 79

4.8.1 Materials 79
4.8.2 Method 80
4.9 Results and discussion 80
4.9.1 Enzyme-linked immunosorbent assay 80
4.9.2 Western blotting 81
4.9.3 Evaluation of inhibition of OP-Tb activity by anti-OP-Tb antibodies 83
4.9.4 Effects of antibodies on trypanosomes in culture 86
4.9.5 Immunoaffinity purification of OP-Tc 88
4.9.6 Do trypanosome-infected hosts produce anti-OP-Tb antibodies? 92
4.10 Concluding remarks 94

CHAPTER 5. Enzymatic characterisation of trypanosome oligopeptidases 96
5.1 Fluorogenic peptide specificity 96
5.1.1 Materials 97
5.1.2 Method 98
5.2 Effect of pH on oligopeptidase activity and stability 98
5.2.1 Materials 98
5.2.2 Method 99
5.3 Effect of ionic strength on OP-Tb activity 99
5.3.1 Materials 99
5.3.2 Method 99
5.4 Reductive activation of oligopeptidases 99
5.4.1 Materials 100
5.4.2 Method 100
5.5 Rate of activation with dithiothreitol 100
5.5.1 Method 101
5.6 Inhibitor profile of trypanosome oligopeptidases 101
5.6.1 Materials 101
5.6.2 Method 102
5.7 Potential activators of oligopeptidase activity 104
5.7.1 Materials 104
5.7.2 Method 104
5.8 Influence of metal ions on oligopeptidase activity 104
5.8.1 Materials 104
5.8.2 Method 105

5.9 Hydrolysis of protein substrates 105
5.9.1 Materials 105
5.9.2 Methods 105
5.10 Digestion of gelatin in substrate SDS-PAGE 106
5.10.1 Methods 106
5.11 Results and discussion 106
5.11.1 Fluorogenic peptide substrate specificity 106
5.11.2 Effect of pH on OP-Tb activity 108
5.11.3 Effect of ionic strength on OP-Tb activity 110
5.11.4 Effect of reducing agents on oligopeptidase activity 111
5.11.5 Rate of activation with dithiothreitol 112
5.11.6 Inhibitor profile of trypanosome oligopeptidases 113
5.11.7 Potential activators of oligopeptidase activity 118
5.11.8 Influence of divalent metal ions 119
5.11.9 Hydrolysis of protein substrates 120
5.11.10 Digestion of gelatin in substrate SDS-PAGE 122
5.12 Concluding remarks 123

CHAPTER 6. Interaction of OP-Tb with host molecules 125
6.1 Introduction 125
6.2 Serpin purification 128
6.2.1 Antichymotrypsin activity assay 128
6.2.1.1 Materials 129
6.2.1.2 Method 129
6.2.2 Antitrypsin activity assay 129
6.2.2.1 Materials 130
6.2.2.2 Method 130
6.2.3 α_1-Antichymotrypsin purification 130
6.2.3.1 Materials 130
6.2.3.2 Methods 131
6.2.4 α_1-Protease inhibitor purification 131
6.2.4.1 Materials 132
6.2.4.2 Methods 132
6.3 Inhibition of OP-Tb by serpins 132
6.3.1 Classical serpins 132

6.3.1.1 Method 132
6.3.2 α_2-Macroglobulin 133
6.3.2.1 Materials 133
6.3.2.2 Method 133
6.4 Detection of OP-Tb activity in host plasma 134
6.4.1 Materials 134
6.4.2 Method 134
6.5 Hydrolysis of Cbz-Arg-Arg-AMC by live trypanosomes 135
6.5.1 Materials 135
6.5.2 Method 135
6.6 Inactivation of serpins by OP-Tb 135
6.6.1 Materials 136
6.6.2 Methods 136
6.7 Interaction with regulatory peptides 136
6.5.1 Materials 136
6.5.2 Methods 137
6.8 Results and discussion 137
6.8.1 Purification of α_1-antichymotrypsin 137
6.8.2 Purification of α_1-protease inhibitor 140
6.8.3 Inhibition of OP-Tb by host protease inhibitors 141
6.8.4 Detection of OP-Tb activity in host plasma 144
6.8.5 Hydrolysis of Cbz-Arg-Arg-AMC by live trypanosomes 145
6.8.6 Inactivation of serpins by OP-Tb 146
6.8.7 Interaction of OP-TB with regulatory peptides 148
6.9 Concluding remarks 151

CHAPTER 7. OP-Tb as a therapeutic target 152
7.1 Introduction 152
7.2 Interaction of trypanocidal drugs with OP-Tb 156
7.2.1 Materials 157
7.2.2 Method 158
7.3 Trypanocidal activity of general protease inhibitors 159
7.3.1 Materials 159
7.3.2 Method 159
7.4 Trypanocidal activity of peptidyl methylketones 159

7.4.1 Materials 161
7.4.2 Method 161
7.5 Trypanocidal activity of peptidyl phosphonate diphenyl esters 162
7.5.1 Materials 163
7.5.2 Method 163
7.6 Results and discussion 165
7.6.1 Interactions with trypanocidal drugs 165
7.6.2 Trypanocidal activity of general protease inhibitors 175
7.6.3 Trypanocidal activity of peptidyl methylketones 178
7.6.4 Trypanocidal activity of peptidyl phosphonate diphenyl esters 181
7.7 Concluding statements 185

CHAPTER 8. General discussion 187
8.1 The structure and enzymatic properties of trypanosome oligopeptidases 188
8.2 The physiology and pathophysiology of trypanosome oligopeptidases 191
8.3 Are trypanosome oligopeptidases potential compounds for vaccine development? 195
8.4 Are trypanosome oligopeptidases potential chemotherapeutic targets? 196
8.5 Cloning and hyperexpression of OP-Tb 199
8.6 Conclusion 200

References 201

Appendix 1. Triple and single-letter amino acid codes 227
Appendix 2. Publications 228

List of Figures

Figure 1. Distribution of disease-carrying tsetse flies in Africa 3
Figure 2. General morphology of an African trypanosome 4
Figure 3. Current classification of the genus *Trypanosoma* 5
Figure 4. The life-cycle of an African trypanosome 6
Figure 5. Subsite classification of the active-site of a protease 12
Figure 6. The catalytic mechanism of a serine protease 13
Figure 7. Generation of kinins by the proteases of invading micro-organisms 20
Figure 8. Flow-diagram depicting the OP-Tb purification procedure 51
Figure 9. Optimisation of three-phase partitioning 61
Figure 10. Anion-exchange chromatography of OP-Tb on Q-Sepharose at pH 5.5 61
Figure 11. Affinity chromatography of OP-Tb on *p*-aminobenzamidine-Sepharose 62
Figure 12. Anion-exchange chromatography of OP-Tb on Q-Sepharose at pH 8 63
Figure 13. Affinity chromatography of OP-Tb on poly-(L-lysine)-Sepharose 63
Figure 14. Molecular exclusion chromatography of OP-Tb on Sephacryl S-100 HR 64
Figure 15. Evaluation of OP-Tb purification by Tris-Tricine SDS-PAGE 65
Figure 16. Sequence of OP-Tb-derived peptides 67
Figure 17. Monitoring of chicken anti-OP-Tb antibody production by ELISA 81
Figure 18. Characterisation of anti-OP-Tb antibodies by western blotting 82
Figure 19. Inhibitory activity of anti-OP-Tb antibodies raised in chickens 85
Figure 20. Titration of inhibitory week 13 anti-OP-Tb IgY antibodies 85
Figure 21. Effect of anti-OP-Tb IgY antibodies on trypanosomes in culture 86
Figure 22. Optimisation of three-phase partitioning for OP-Tc purification 88
Figure 23. Anion-exchange chromatography of OP-Tc on Q-Sepharose at pH 5.5 89
Figure 24. Affinity chromatography of OP-Tb on *p*-aminobenzamidine-Sepharose 89
Figure 25. Immunoaffinity chromatography of OP-Tc on anti-OP-Tb-Sepharose 90
Figure 26. Evaluation of OP-Tc purification 91
Figure 27. Characterisation of IgG response to trypanosome infection in cattle 93
Figure 28. Characterisation of IgM response to trypanosome-infection in cattle 94
Figure 29. Effect of pH on the activity and stability of trypanosome oligopeptidases 109
Figure 30. Effect of ionic strength on OP-Tb activity against Cbz-Arg-Arg-AMC 110
Figure 31. Effect of reducing agents on OP-Tb activity against Cbz-Arg-Arg-AMC 111

Figure 32. Optimum time of OP-Tb activation with dithiothreitol 112
Figure 33. Tertiary structure of protein inhibitors of serine proteases 116
Figure 34. Alkylation of free thiol groups in proteins by iodoacetate and iodoacetamide 118
Figure 35. Digestion of protein substrates by OP-Tb 121
Figure 36. Digestion of gelatin by OP-Tb in a substrate gel 122
Figure 37. Three-dimensional structure of plasma serpins 127
Figure 38. Chromatography of α_1ACT on Cibacron Blue-Sepharose at pH 6.8 138
Figure 39. Rechromatography of α_1ACT on Cibacron Blue-Sepharose at pH 8 138
Figure 40. Chromatography of α_1ACT on Sephacryl S-100 HR 139
Figure 41. Evaluation of α_1ACT purification by Tris-Tricine SDS-PAGE 139
Figure 42. Chromatography of α_1PI on DEAE-cellulose 140
Figure 43. Chromatography of α_1PI on Sephadex G-75 140
Figure 44. Evaluation of α_1PI purification by Tris-Tricine SDS-PAGE 141
Figure 45. Interaction of OP-Tb with α_2M 143
Figure 46. Detection of serine oligopeptidase activity in infected rat plasma 144
Figure 47. Hydrolysis of Cbz-Arg-Arg-AMC by live trypanosomes 145
Figure 48. Proteolytic digestion of serpins 146
Figure 49. Reactive-site loop cleavage sites of serpins 147
Figure 50. Degradation of neurotensin by OP-Tb 148
Figure 51. Peptide hormones cleaved by OP-Tb 149
Figure 52. Peptide hormones not cleaved by OP-Tb 150
Figure 53. Chemical structure of trypanocidal drugs 153
Figure 54. Chemical formula of suramin and designated nomenclature for suramin analogues 157
Figure 55. Mechanism of inhibition of a serine proteases by a peptidyl chloromethyl-ketone 160
Figure 56. Mechanism of inhibition of serine proteases by a peptidyl α-aminoalkyl phosphonate diphenyl ester derivative 162
Figure 57. Diagnosis of inhibition mechanisms by trypanocidal drugs 167
Figure 58. Effect of suramin on substrate hydrolysis by serine proteases 169
Figure 59. Symmetrical analogues of suramin 172
Figure 60. Asymmetrical analogues of suramin 174
Figure 61. Trypanocidal activity of protease inhibitors 176
Figure 62. Active-site blot of *T. b. brucei* lysates and purified OP-Tb 180

Figure 63. Active-site labelling with fluorescent peptidyl phosphonate diphenyl ester derivatives 183
Figure 64. Alignment of sequences surrounding the catalytic triad of members of the subtilisin family of serine proteases 190
Figure 65. Alignment of sequences surrounding the catalytic triad of the prolyl oligopeptidase subclass of serine proteases 190

List of Tables

Table 1. The four currently-recognised classes of proteases 12
Table 2. Serine oligopeptidases identified to date 15
Table 3. Proteases of pathogenic protozoans 16
Table 4. Protease inhibitors as drugs 23
Table 5. Preparation of Tris-Tricine SDS-PAGE gels 43
Table 6. Purification table for the isolation of OP-Tb from *T. b. brucei* lysates 65
Table 7. Amino acid composition of OP-Tb 66
Table 8. Purification table for the isolation of OP-Tc from *T. congolense* lysates 90
Table 9. Amidolytic activity of trypanosome oligopeptidases 107
Table 10. Inhibition of trypanosome oligopeptidase activity by competitive reversible inhibitors 113
Table 11. Inhibition of trypanosome oligopeptidase activity by irreversible inhibitors of cysteine and serine proteases 116
Table 12. Effect of potential activators on oligopeptidase activity 119
Table 13. Effect of divalent metal ions on oligopeptidase activity 120
Table 14. Serpins present in human plasma 127
Table 15. Inhibition of OP-Tb activity by host protease inhibitors 142
Table 16. Inhibition of OP-Tb by trypanocidal drugs 165
Table 17. Inhibition constants and EC_{50} values for suramin analogues 170
Table 18. Peptidyl methylketone inhibitors of OP-Tb 178
Table 19. Peptidyl phosphonate diphenyl ester inhibitors of OP-Tb 182
Table 20. Effect of administration of Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ on the progression of trypanosomiasis in BALB/c mice 184
Table 21. Potential peptide hormone substrates for OP-Tb in the mammalian host bloodstream 194

Abbreviations

A_{280}	absorbance at 280 nm
ABTS	2,2′-azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulfonate]
Abz	*o*-aminobenzoyl
Ac	acetyl
ACE	angiotensin-converting enzyme
α_1ACT	alpha$_1$-antichymotrypsin
ACTH	adrenocorticotropic hormone
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
AFU	arbitrary fluorescence units
AIDS	acquired immunodeficiency syndrome
AMC	7-amino-4-methylcoumarin
AMK	acyloxymethylketone
4AmPhGly	4-amidinophenylglycine
AMT	acetate-Mes-Tris
ANF	atrial natriuretic factor
α_2AP	alpha$_2$-antiplasmin
ATIII	antithrombin III
BCA	bicinchoninic acid
BCDSA	bathocuproinedisulfonic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCT	*Bureau Central de la Trypanosomiase*
Bis	N,N′-methylenebisacrylamide
BSA	bovine serum albumin
BSA-TBS	bovine serum albumin in Tris-buffered saline
Bz	benzoyl
c	concentration
C-terminal	carboxy terminal
Caps	3-[cyclohexylamino]-1-propanesulfonic acid
Cbz	carbobenzoxy
CD	cluster determinant

cDNA	complementary deoxyribonucleic acid
CH_2Cl	chloromethylketone
CH_2F	fluoromethylketone
CHN_2	diazomethylketone
CNS	central nervous system
CSF	cerebrospinal fluid
DCI	3,4-dichloroisocoumarin
ddH_2O	distilled, deionised water
DDT	dichlorodiphenyltrichloroethane
DEAE	diethylaminoethyl
DFMO	α-DL-difluoromethylornithine
DFP	diisopropylfluorophosphate
dH_2O	distilled water
DIC	disseminated intravascular coagulation syndrome
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ε	extinction coefficient
$[E]_0$	active enzyme concentration
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
EC_{50}	effective concentration of inhibitor reducing parasite population by 50%
EDDnp	*N*-(2,4-dinitrophenyl)ethylenediamine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycolbis(ß-aminoethylether)*N*,*N*,*N*′,*N*′-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
g	relative centrifugal force
ΔF	change in fluorescence
F_{ab}	fragment, antigen-binding
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
F_c	fragment, crystallisable

fMLP	formyl-methionyl-leucyl-phenylalanine peptide
H-kininogen	high molecular weight kininogen
Hepes	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
HIV	Huam Immunodeficiency Virus
HNE	human neutrophil elastase
HPLC	high-performance liquid chromatography
HRPO	horse-radish peroxidase
α_2HSGP	α_2-HS-glycoprotein
FSH	follicle-stimulating hormone
I	ionic strength
IAA	iodoacetic acid
IAM	iodoacetamide
ICP	intracranial pressure
IEC	ion-exchange chromatography
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	egg-yolk immunoglobulin
ILRAD	International Laboratory for Research on Animal Diseases
ILRI	International Livestock Research Institute
k_{ass}	rate of complex association
k_{diss}	rate of complex dissociation
K_{av}	availability constant
k_{cat}	turnover number
kDa	kilo-Dalton
kDNA	kinetoplast DNA
K_i	inhibition constant
K_m	Michaelis-Menten constant
K'_m	apparent Michaelis-Menten constant
l	light-path
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength

LD_{50}	50% lethal dose
LH	luteinising hormone
LS	long slender
α_2M	alpha$_2$-macroglobulin
MEC	molecular exclusion chromatography
Mes	2-(*N*-morpholino)ethanesulfonic acid
4MeßNA	4-methoxy-β-naphthylamine
M_r	molecular mass
β-MSH	beta-melanocyte stimulating hormone
MUGB	4-methylumbelliferyl-*p*-guanidobenzoate
MΩ	megaohm(s)
N-terminus	amino terminus
NBT	nitroblue tetrazolium
NEM	*N*-ethylmaleimide
NPGB	4-nitrophenyl-*p*-guanidobenzoate
OP-Tb	oligopeptidase from *Trypansoma brucei*
OP-Tc	oligopeptidase from *Trypanosoma congolense*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
*p*CMB	*para*-chloromercuribenzoate
PCR	polymerase chain reaction
PDP	phenyldimethyl pyrazole
PEG	polyethylene glycol
PGK	phosphoglycerate kinase
α_1PI	alpha$_1$-protease inhibitor
pI	isoelectric point
PITC	phenylisothiocyanate
PMSF	phenylmethylsulfonylfluoride
*p*NA	*para*-nitroanilide
PSG	phosphate-buffered saline containing glucose
PVDF	polyvinylidene difluoride
Q	quaternary ammonium

RP-HPLC	reversed-phase high performance liquid chromatography
RT	room temperature
[S]	substrate concentration
SBTI	soybean trypsin inhibitor
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide-gel electrophoresis
SS	short stumpy
SV8	*Staphylococcus aureus* V8 proteinase
SWR	standard working reagent
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
t-butanol	tertiary butanol
TFA	trifluoroacetic acid
TPP	three-phase partitioning
T_3	3,5,3′,-triiodo-L-thyronine
T_4	3,5,3′,5′-tetraiodo-L-thyronine
Tricine	*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
V_e	elution volume
v_0	initial velocity
V_0	void volume
VIP	vasoactive intestinal polypeptide
V_{max}	maximum velocity
V'_{max}	apparent maximum velocity
VSG	variant surface glycoprotein
V_t	total volume

Chapter 1

Introduction

1.1 African trypanosomiasis

Trypanosomiasis is a term applied to a group of acute or chronic infectious diseases of man and other animals, due to the invasion of the body by unicellular blood parasites of the genus *Trypanosoma* (Protozoa: Kinetoplastida). While the first record of a parasitic organism belonging to this genus was made as early as 1841 (Laveran and Mesnil, 1904), the significance of these parasites as pathogenic micro-organisms in Africa was not realised until Bruce (1895) discovered that the notorious "tsetse fly disease" of cattle in Zululand, South Africa was due to a trypanosome. This organism was subsequently named *Trypanosoma brucei* by Plimmer and Bradford (1899), and descriptions of two related organisms from bovine sources, *T. congolense* from the Congo (Broden, 1904), and *T. vivax*, also from West Africa (Zieman, 1905), followed. During this time, Dutton (1902) described a related parasite, first discovered in the blood of a human by Forde (1902), and named it *T. gambiense*, and eight years later, a second species of human-infective trypansome, *T. rhodesiense*, was described by Stephen and Fatham (1910). Subsequently, while trypanosomes infecting all classes of vertebrates have been described, only those causing disease in man and livestock appear to have a significant medical and economic impact on the tropical regions of the world (Ukoli, 1984).

Human African trypanosomiasis has two forms, chronic and acute African trypanosomiasis. The aetiological agent of chronic African trypanosomiasis is *T. brucei gambiense*. This disease occurs widely in the riverine regions of Zaïre (recently re-named the Democratic Republic of the Congo), the Congo and Nigeria, extending westwards along the African coast as far north as The Gambia (Kuzoe, 1993). The parasite is transmitted by the riverine tsetse fly, *Glossina palpalis* (Swellengrebel and Stermann, 1961) and causes chronic disease in human beings, which initially causes fever and headache (Fripp, 1983). The parasites cross the blood-brain barrier, and enter the cerebrospinal fluid (CSF), causing meningoencephalomyelitis, fatigue, emaciation and loss of nervous control, resulting in a comatose condition, followed by death.

Acute African trypanosomiasis is caused by *T. b. rhodesiense*, which is far more virulent than its Gambian counterpart, rapidly causing severe febrile illness characterised by high fever, hepato- and splenomegaly, followed by death within a few weeks of infection (Hall, 1977; Fripp, 1983). *T. b. rhodesiense* is transmitted by the savannah tsetse fly, *G. morsitans*, which occurs primarily in the open savannah of central east Africa, from as far north as Sudan, and extending as far south as the Okavango delta of Botswana (Kuzoe, 1993). A variety of ungulates serve as the natural reservoir of this parasite (Schmidt and Roberts, 1989).

Cattle trypanosomiasis, which is known as *nagana*, is transmitted by a variety of *Glossina* species. The aetiological agents include *T. vivax*, *T. congolense* and *T. b. brucei*. Infection in cattle is characterised by severe anaemia, elevated pulse-rate, fever, emaciation, impaired neurological function and finally death. Nagana costs Africa about US$ 5 billion per annum (International Laboratory for Research on Animal Diseases, 1994).

Broadly speaking, the distribution of African trypanosomiasis parallels that of the tsetse fly. The tsetse fly and its disease-causing trypanosome parasites currently makes 10 million square kilometres of Africa - an area larger as the continental United States - inhospitable for live-stock (International Laboratory for Research on Animal Diseases, 1991). The distribution of the tsetse fly and the current cattle-producing areas of Africa are illustrated in Fig. 1.

1.1.1 Trypanosome morphology

The generalised morphology of an African trypanosome is illustrated in Fig. 2. Trypanosomes are typically elongate (30 × 2 μm), torpedo-shaped cells. A single flagellum arises from the flagellar pocket at the posterior end, runs laterally to the anterior, where it extends freely beyond the anterior end of the body. The kinetoplast is associated with the single tubular mitochondrion and contains the mitochondrial DNA. The remaining cellular structures are similar to those found in other eukaryotes (Schmidt and Roberts, 1989).

1.1.2 Classification of African trypanosomes

The classification of the genus *Trypanosoma* is given in Fig. 3, and this genus, along with members of the genus *Leishmania*, are the only members of the family Trypanostomatidae of medical and veterinary importance. The genus *Trypanosoma* is divided into two groups on the basis of the development of the parasite within its insect vector. Kleine (1909) first described

the development of the flagellate in the alimentary canal of tsetse flies. The "salivarian" trypanosomes undergo most of their morphological development in the anterior part of the alimentary system of the vector (i.e. in the salivary glands). In contrast, the development of the "stercorarial" trypanosomes takes place in the hindgut of the insect vector.

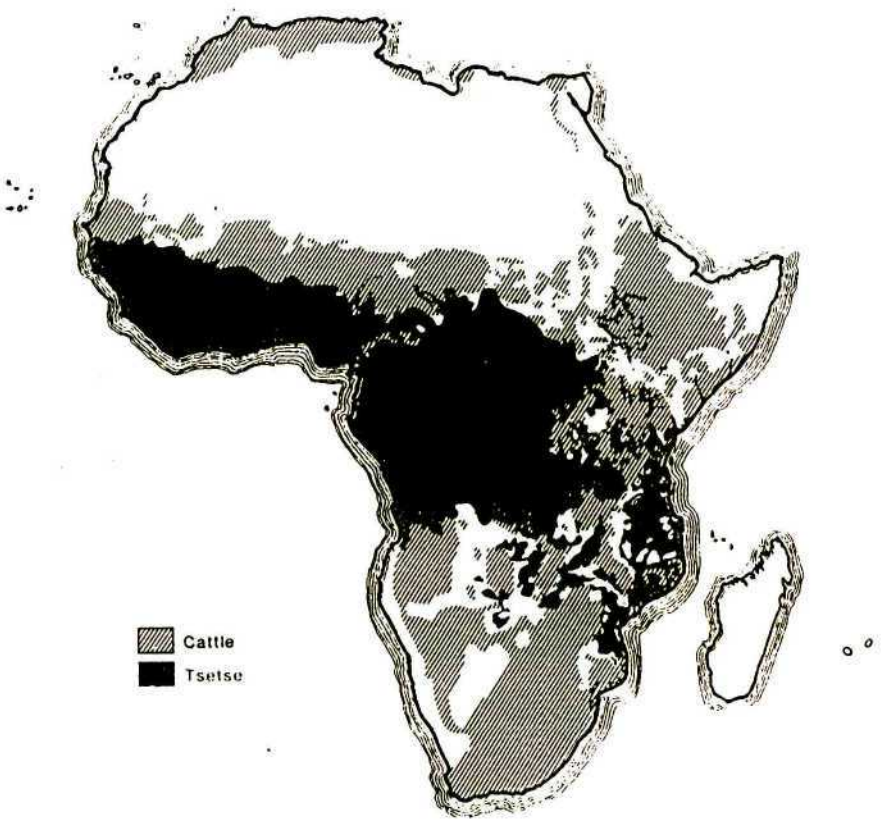


Figure 1. Distribution of disease-carrying tsetse flies in Africa. Tsetse distribution is superimposed on the cattle-producing regions of the continent (after International Laboratory for Research on Animals Diseases, 1991).

1.1.3 Life cycle of African trypanosomes

The life-cycle of *T. b. brucei* is illustrated in Fig. 4. Non-dividing metacyclic forms occur in the salivary glands of the tsetse fly and are introduced into the mammalian host when the fly bites the host to take a blood meal. The metacyclic trypanosomes differentiate into the bloodstream-forms, which rapidly multiply by binary fission once every six to eight hours (Vickerman, 1976). After a period of rapid division, some of these long, slender (LS) bloodstream forms of the parasite stop dividing and differentiate into short, stumpy (SS) non-dividing bloodstream forms (Nelson *et al.*, 1979). It has been hypothesised that these SS

forms have undergone a pre-adaptation for tsetse fly reinfection. When the fly takes a blood meal from an infected host, bloodstream-form parasites are ingested and enter the midgut of the tsetse fly where they differentiate into procyclic forms. The organisms are further transformed into epimastigote forms, and then subsequently into the dormant metacyclic forms, in the salivary glands of the fly.

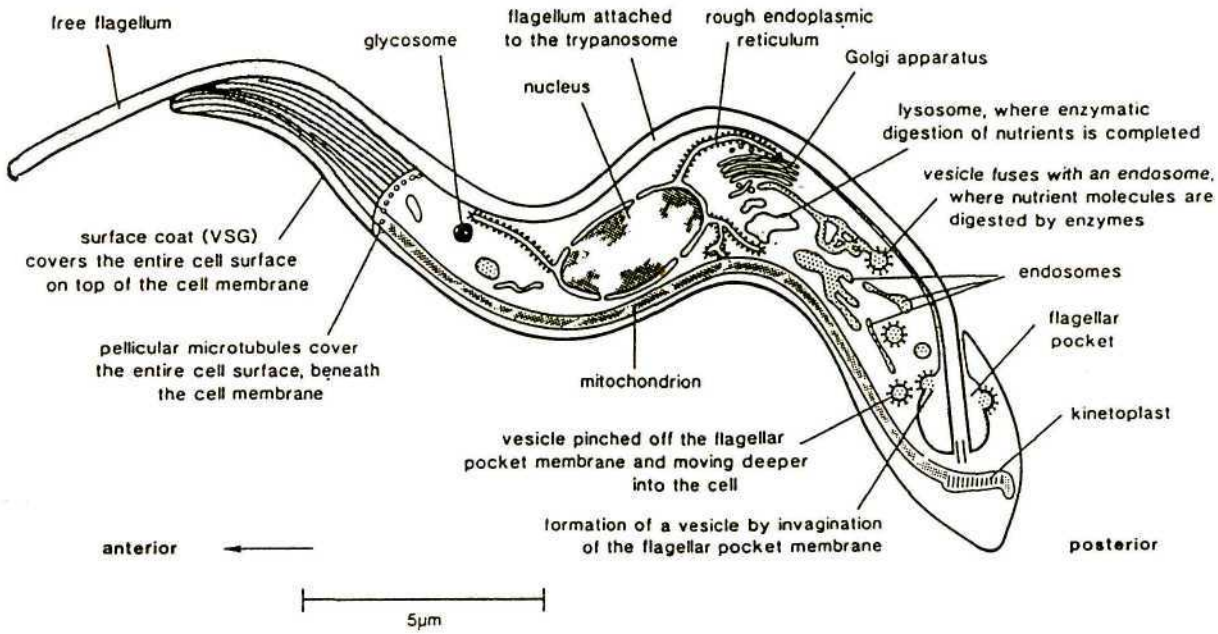


Figure 2. General morphology of an African trypanosome. This figure illustrates the subcellular structures of a bloodstream-form parasite of the *T. brucei* group (after Vickerman and Cox, 1978).

1.1.4 Control of trypanosomiasis

Efforts to control trypanosomiasis generally involve the control and elimination of the insect vector. The most satisfactory means of vector elimination is through spraying pesticides such as dichlorodiphenyltrichloroethane (DDT), by aircraft (first described by Du Toit, 1949). However, there are considerable harmful side effects to the environment with this method of insect control. Elimination of game reservoirs has also enjoyed limited success but is obviously counter to conservation philosophies (Schmidt and Roberts, 1989). As a result of the increasing political and economic instability which currently plagues Africa, tsetse-control programmes have largely become defunct, resulting in a concomitant increase in the incidence

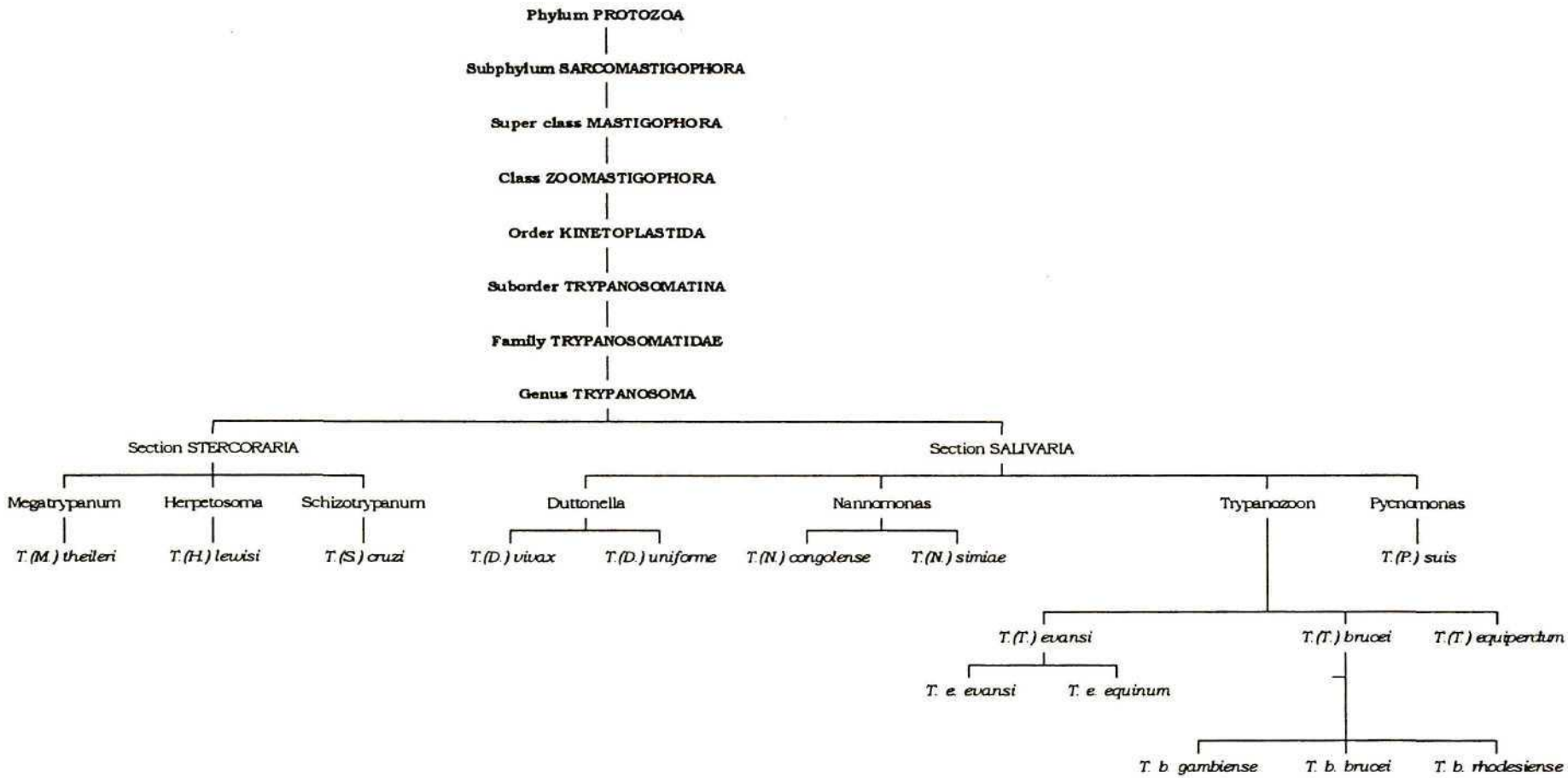


Figure 3. Current classification of the genus *Trypanosoma*. The phylogenetic tree of both stercorarian and salivarian trypanosomes is illustrated (after Vickermann, 1976).

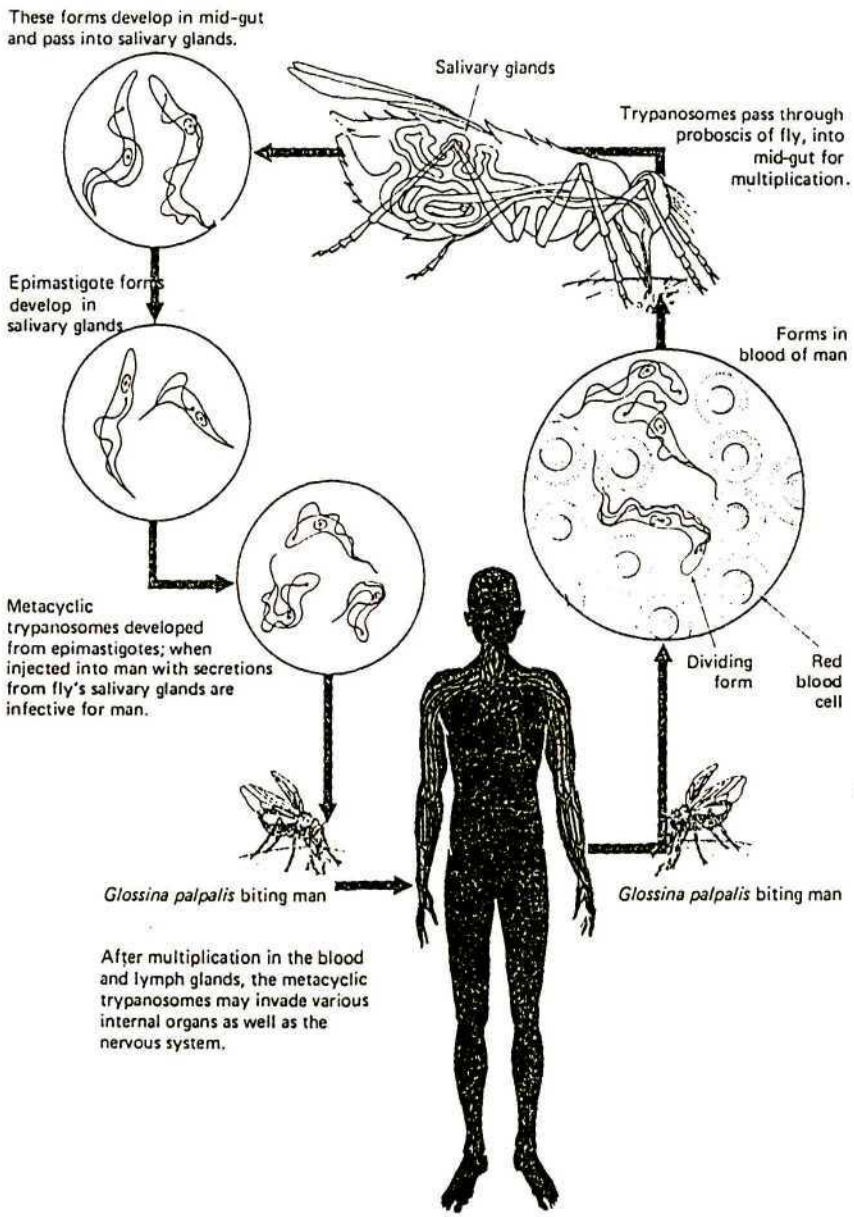


Figure 4. The life cycle of an African trypanosome. Most members of the family Trypanosomatidae are heteroxenous: during one stage of their lives they live in the blood and solid tissues of all classes of vertebrates, and during the other stages they live in blood-sucking vertebrates (after Read, 1972).

of human and bovine African trypanosomiases, particularly in Zaïre and the Congo. For example, in colonial Zaïre, economic interests required healthy, fit labour forces which ensured that disease control was tight. In 1988 the *Bureau Central de la Trypanosomiase* (BCT) was detecting and treating an average of 7 500 patients each year. The political crises in 1990 led the Belgian government to abandon its support for the BCT, and four years later the case-load increased to 150 000 per year, with prevalence levels in some villages of over 70% (Walgate, 1994). Similar trends for both the human and bovine forms of the disease have been reported in 36 African nations (Kuzoe, 1993). Given the considerable agricultural and medical impact of this disease on the African continent, there should be much interest in developing more effective means of controlling and treating African trypanosomiasis.

1.1.5 Treatment of African trypanosomiasis

The inability to control the insect vector (Section 1.1.4), together with recent reports of trypanosome resistance to these drugs (Section 7.1), have prompted considerable interest in identifying targets for the development of new drugs for the treatment of trypanosomiasis. There are a number of drugs currently available for the chemotherapy of African trypanosomiasis. A variety of arsenic-based compounds (e.g. melarsoprol) and the sulfated naphthylamine, suramin, have been used since the middle of this century for the chemotherapy of African trypanosomiasis. Recently, an ornithine analogue, α-difluoromethylorninthine (DFMO) has also been introduced. However, once the disease has progressed to nervous-system involvement, the prognosis is still very poor. These drugs are discussed in detail in Section 7.1 and will therefore not be considered further here.

1.1.6 Pathogenesis of African Trypanosomiasis

The pathogenesis of a disease is the pathophysiological changes that occur during that disease, including the disturbances to the normal physiology, the mechanisms producing these functional abnormalities and the ways in which they are expressed as symptoms and clinical signs (Boreham, 1979). An understanding of the mechanisms of disease processes could lead to better methods of treatment by possibly inhibiting or reversing the important pathological changes, or by alleviating some of the symptoms of the disease.

The pathogenic mechanisms operating during infection with African trypanosomes are very poorly understood, and remain a considerable challenge to scientists (Poltera, 1985).

Pathogenic African trypanosomes are loosely divided into two groups: (1) the *T. brucei* (humoral) subgroup, which includes those species pathogenic to man and are distributed throughout the tissues and fluids of the body; and (2) the *T. vivax* and *T. congolense* (haematic) subgroup, which are confined to the blood and are pathogenic to cattle (Losos and Ikede, 1972). While this separation is not strictly correct it is useful to retain in terms of pathogenesis. The humoral subgroup causes an inflammatory degenerative disease with extensive necrosis and central nervous system (CNS) involvement, whereas the haematic subgroup causes anaemia that is far more severe and pronounced than that of *T. brucei* subgroup infections. The salient features of the pathogenesis of the *T. brucei* and *T. congolense* subgroup infections may be summarised as follows:

Altered blood cell function. Both B-cell and T-cell function are altered during infection. Depression of T-cell responses has been documented in rodents infected with *T. brucei* (Pearson *et al.*, 1978), and in cattle infected with *T. congolense* (Flynn and Sileghem, 1993), although the mechanisms causing this phenomenon have not been elucidated. This effect is reversed with the administration of the trypanocidal drug Berenil® (Roelants *et al.*, 1979). A dominant pathological event is polyclonal activation of B-cells (Greenwood and Whittle, 1980), for which two possible causes have been proposed: deficient T-cell control over B-cells, or a mitogenic factor for B-cells. Elevated eosinophil levels (500%) have also been documented (Basson *et al.*, 1977). Platelet counts are reduced in late-stage infection, which is characterised by disseminated intravascular coagulation (DIC) syndrome (Barrett-Connor *et al.*, 1973) and thrombocytopaenia (Davis, 1980).

Haemolytic anaemia is a consistent feature of human and animal trypanosomiasis and is more severe in *T. congolense* and *T. vivax* infections. This condition is believed to contribute to fatigue and general loss of condition in infected mammals and is characterised by a decreased red-cell count (Facer *et al.*, 1982). It has been suggested that the anaemia may have an immunological basis. Various trypanosome antigens adsorb to the surface of erythrocytes, where antigen-antibody complexes are subsequently formed, resulting in the lysis of the erythrocytes in the presence of complement (Murray and Dexter, 1988). Additionally, Tizard *et al.* (1978) have described a 10 kDa haemolytic factor from *T. b. brucei*, but it is not clear if this operates in infections as such.

Emaciation and nutritional considerations. Within 15 days to 4 months trypanosome infections result in emaciation, and death may rapidly ensue from malnutrition. The reason for this malnourished state is not clear (Schmidt and Roberts, 1989). The presence of trypanosomes of the *T. brucei* subgroup have been detected in the stomach, small intestine, abdominal lymphatics and pancreas (Morrison *et al.* 1981a), and it is possible that toxic products released from trypanosomes may interfere with normal digestive processes (Tizard *et al.*, 1978).

Endocrine system dysfunction. The adrenal medulla exhibits sclerosis and diffuse mononuclear infiltration (Gallais *et al.*, 1953). In the case of *T. brucei* infections, the interstitial presence of parasites in the medulla has been observed (Poltera, 1985). Severe lesions of the pituitary have also been observed, including necrotic foci and trypanosome perivascular cellular infiltrations (Morrison *et al.*, 1981b). Marked sclerosis and cellular infiltration of the thyroid and pancreas have also been observed (Gallais *et al.*, 1953).

Blood pressure fluctuations. Hypotension is a common feature of *T. brucei* subgroup infections. A drop in blood pressure from 70/65 mm Hg in uninfected control rabbits down to 31/25 mm Hg in infected rabbits has been reported, while the heart-rate remained unchanged (Boreham and Wright, 1976). These hypotensive responses can be prevented using the kallikrein inhibitor aprotinin, which led Boreham and Wright (1976) to conclude that hypotension was a result of the conversion of prekallikrein to active kallikrein, which itself has hypotensive properties, and will cause the release of hypotensive bradykinin from high molecular mass kininogen (H-kininogen).

Inflammation. Inflammation incorporates a series of semi-independent reactions. The inflammatory process is usually divided into a number of stages, including increased vascular permeability which causes localised oedema, neutrophil exudation, cell proliferation and tissue repair (Vickerman *et al.*, 1991). *T. brucei* subgroup infections are characterised by gross oedema of all tissues (Nagle *et al.*, 1980), as opposed to *T. congolense* and *T. vivax* infections, where the major inflammatory lesions are in the brain as a result of pressure necrosis caused by an elevated intracranial pressure (ICP). The elevated ICP is caused by oedema resulting from

accumulations of trypanosomes in the capillaries and venules (Losos and Ikede, 1972). One of the major pathological changes that occurs in *T. brucei* subgroup infections is the increase in vascular permeability (Boreham, 1979). This has been attributed to a number of pharmacologically active substances that have been implicated in trypanosomiasis. These include plasma kinins, fibrinogen degradation products, 5-hydroxytryptamine and histamine (Boreham, 1979).

Nervous system dysfunction. During the late stages of infection, trypanosomes invade the central nervous system, in particular the cerebrospinal fluid (Rudin *et al.*, 1983). Neurological involvement results in lethargy, mental dullness, tremors of the hands and trunk, and disturbances of coordination. This is usually followed by paralysis, convulsions and death (Schmidt and Roberts, 1989). The mechanisms producing these symptoms have not been elucidated, although the release of toxic trypanosome metabolites which produce similar effects in experimental animals, are thought to be responsible (Schmidt and Roberts, 1989).

Reproductive system dysfunction. A variety of reproductive disorders have been documented in trypanosome-infected animals, including atrophy of the testes and ovaries, irregular oestrus cycle, infertility and abortion. This is often attributed to impairment of the pituitary-gonadal and pituitary-adrenal axes (Mutayoba *et al.*, 1995a). Hublart *et al.* (1990) reported decreased levels of testosterone (less than 50% compared to control animals) in rats infected with *T. b. brucei*. Furthermore, the administration of trypanosome extracts to uninfected rats reduced the levels of serum follicle-stimulating hormone (FSH) and serum luteinising hormone (LH) by more than 50% five-hours post-administration (Hublert *et al.*, 1990). Depression of plasma progesterone and estradiol-17β during infection has also been observed (Mutayoba *et al.*, 1988a).

Alterations to serum/plasma protein levels. The concentrations of a number of plasma constituents are altered during *T. brucei* subgroup infections. Trypanosomes evade the host immune system by regularly changing their surface antigens, known as variant surface glycoproteins (VSG). The continual presence of high levels of trypanosmal antigens promotes high levels of circulating antibodies. Cross-reactivity of these antibodies is thought to cause a number of autoimmune reactions (Müller *et al.*, 1993), and autoantibodies directed against

smooth-muscle (Wolga *et al.*, 1981) and DNA (Daniel-Ribeiro *et al.*, 1983) have been reported. The depletion of complement levels, in particular C1 (Tizard *et al.*, 1978), C3 and C4 (Basson *et al.*, 1977) down to less than 50% of normal values, has also been reported. Fibrinogen concentrations are raised and total serum lipid concentration is elevated up to 400%. Concentrations of serum albumin are decreased (Boreham, 1979), while IgM levels are elevated (Clarkson and Penhale, 1973).

The nature of most of the above changes cannot be ascribed to any single obvious cause. Indeed, different systems may be operative in different situations. However, many of the above changes clearly involve alterations in peptides and/or proteins and therefore any parasite enzymes that may effect changes in host proteins are obvious targets for examination. Foremost amongst these are proteases, although there are obviously other candidates such as kinases and protein phosphatases. Since proteases are the focus of this research, the remainder of this introduction will focus on proteases and their characteristics.

1.2 Structure and classification of proteolytic enzymes

Proteolytic enzymes (proteases or peptidases), are a broad group of enzymes which hydrolyse peptide bonds in peptides and proteins. These enzymes are further subdivided into two groups: the exopeptidases and endopeptidases. The former group catalyses the sequential removal of amino acid residues from the amino- or carboxy-termini of peptides and proteins. They are accordingly termed aminopeptidases and carboxypeptidases. The endopeptidases, or proteinases, catalyse the cleavage of peptide bonds within a protein. These enzymes usually exhibit a certain degree of specificity, which is often dictated by the nature of the amino acid residues surrounding the peptide bond that is cleaved (the "scissile bond"). Proteases are currently classified into one of five groups, depending on the identity of the amino acid residues that make up the catalytic unit of each group. This classification is given in Table 1.

The active site of an enzyme is divided up into a number of substrate-binding pockets, which determine the specificity of the enzyme (Fig. 5). Those pockets which bind residues of the substrate on the N-terminal side of the scissile bond are designated S_1, S_2, S_3,...S_n, whereas those pockets binding substrate residues on the C-terminal side of the scissile bond are

designated S_1', S_2', S_3',...S_n'. Similarly, the corresponding regions on the peptide substrate are referred to as P_1, P_2, P_3...P_n and P_1', P_2', P_3'...P_n' (Schechter and Berger, 1968).

Table 1. The four currently-recognised classes of proteases.

Class[a]	Representative protease	Active-site residues of the representative protease	Diagnostic inhibitors[b]
Serine	Chymotrypsin	Asp^{102}, Ser^{195}, His^{57}	DFP
Cysteine	Papain	Cys^{25}, His^{159}, Asp^{158}	E-64
Aspartic	Penicillopepsin	Asp^{33}, Asp^{213}	Pepstatin
Metallo	Thermolysin	Glu^{270}, Trp^{248}	EDTA, 1,10-phenanthroline

[a]A fifth class of protease ("proteases of unknown mechanism") are also recognised for proteolytic enzymes for which the catalytic mechanisms have not been elucidated (after Barrett, 1994).
[b]Abbreviations: DFP, diisopropylfluorophosphate; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino) butane, EDTA, ethylenediaminetetraacetic acid.

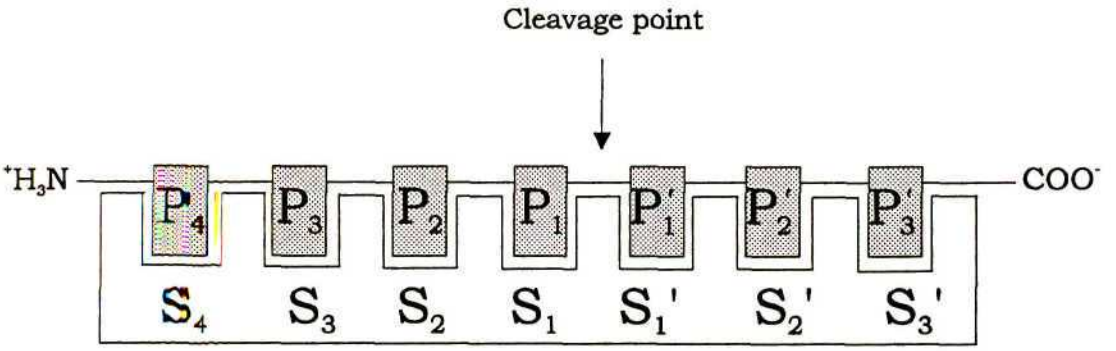


Figure 5. Subsite classification of the active site of a protease. This system describes the substrate binding regions on the enzyme (S) and the corresponding regions on the peptide substrate (P) (after Schechter and Berger, 1968).

As only the serine proteases are of particular interest in this study, the other classes of proteases will not be considered further.

1.2.1 Serine proteases

Serine proteases are the most numerous and diverse group of proteolytic enzymes, being involved in a wide range of physiological processes (Barrett and Rawlings, 1992). Apart from the role played by digestive enzymes such as trypsin and chymotrypsin, the serine proteases are important regulators of enzyme activity, as exemplified by their role in the blood clotting

cascade (Davie *et al.*, 1991). Serine proteases are also believed to play an important role in cell differentiation (Chasan and Anderson, 1989).

Serine proteases are currently divided into about 30 families, which are grouped together into six "clans" (Barrett and Rawlings, 1995). All these proteases are believed to share a common catalytic mechanism which involves the hydrolysis of ester and amide bonds, and proceeds via an acyl transfer mechanism. Michaelis-complex formation is facilitated through the binding of the P and P′ residues of the substrate to the S and S′ sites of the enzyme. General base catalysis by the imidazole of the active-site histidine (His^{57}, Fig 6A) activates the hydroxyl group of the active site serine (Ser^{195}; Fig 6A).

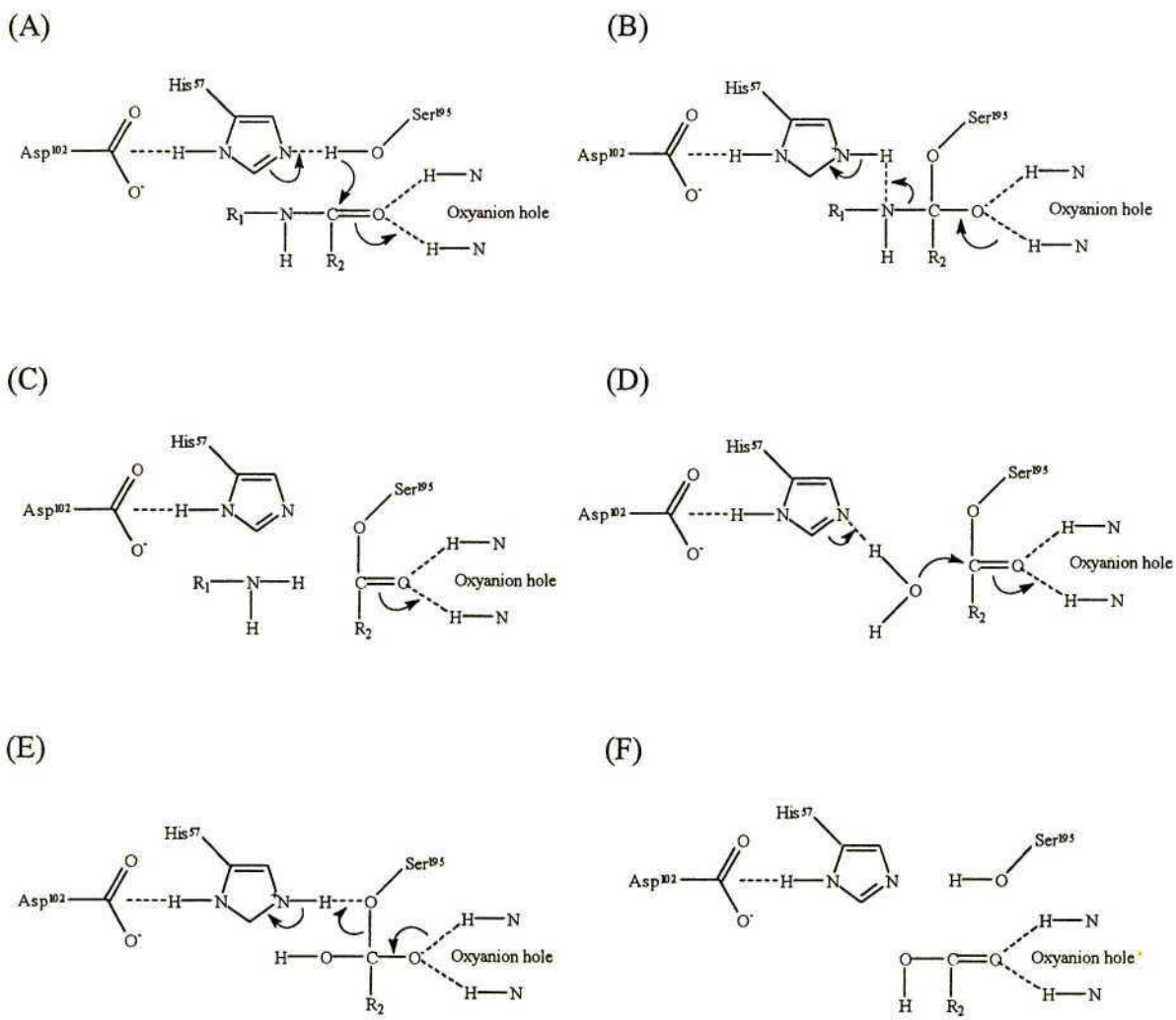


Figure 6. The catalytic mechanism of a serine protease. The catalytic groups of chymotrypsin are shown interacting with oligopeptides over the P_1 to P_3 regions. The numbering of chymotrypsin is shown (after Fink, 1987). R_1 and R_2 represent the C-terminal and N-terminal peptides respectively.

The active-site aspartic acid (Asp^{102}) may assist by permitting the abstraction of a proton from His^{57}. The overall effect is to enhance the nucleophilicity of Ser^{195}. A covalent tetrahedral intermediate is formed after nucleophilic attack by the active-site serine hydroxyl group on the carbonyl carbon of the scissile bond (Fig. 6B), with concomitant loss of the C-terminal peptide (Fig. 6C). In the enzyme-catalysed reaction, this transition state is stabilised by the "oxyanion hole" created by the backbone amino groups of Gly^{193} and Ser^{195}.

Imidazolium-catalysed proton donation (by His^{57}) to the newly formed alcohol results in the dissociation of the amine product and formation of a covalent acyl-enzyme intermediate. Deacylation occurs via the reverse series of events. General base catalysis by the imidazole group of His^{57} activates a H_2O molecule (Fig. 6D), forming a second tetrahedral intermediate, which breaks down *via* imidazolium-catalysed protonation of the serine O^{γ} (Fig. 6E) yielding an acid product (Fig. 6F) (Huber and Bode, 1978; Fink, 1987; Perona and Craik, 1995).

1.2.2 Oligopeptidases

Oligopeptidases form a distinct sub-group of the proteases that are specialised for the degradation of peptides up to 35 amino acid residues in length. Oligopeptidases have no activity against proteins (Barrett, 1994) and their natural substrates are believed to include biologically active peptides. As no three-dimensional structure of any oligopeptidase is presently available, the structural basis of oligopeptidase specificity is not understood. It is speculated that oligopeptidases are somehow sensitive to the conformation of their substrates, and that the active site of oligopeptidases consists of a three-dimensional substrate binding site, like a pit, as opposed to the essentially two-dimensional active-site cleft found in other proteases (Barrett and Rawlings, 1992).

Oligopeptidases are widely distributed amongst eukaryotes and prokaryotes, and are usually serine or metallo-enzymes. Serine oligopeptidases identified to date are presented in Table 2. A variety of metallo-oligopeptidases have also been described, such as thimet oligopeptidase (E.C. 3.4.24.15) from avian (Barrett and Brown, 1990) and mammalian (McKie *et al.*, 1995) sources.

The term oligopeptidase as used here excludes obligate amino- and carboxy-peptidases such as angiotensin converting enzyme (ACE) and lysine aminopeptidase that act on small peptide

substrates. The true oligopeptidases have a defined substrate-size exclusion limit, although this has only been determined for a small number of oligopeptidases, and is generally between 15-35 amino acid residues (reviewed by Barrett and Rawlings, 1992).

Table 2. Serine oligopeptidases identified to date.

Species	Size (kDa)	P_1 residue preference	Reference
Bacteria			
Aeromonas hydrophila	80	basic	Kanatani *et al.* (1993)
Escherichia coli	80	basic	Kanatani *et al.* (1991)
Flavobacterium sp.	80	basic	Yoshimoto *et al.* (1991)
Moraxella lacunata	80	basic	Yoshimoto *et al.* (1995)
Treponema denticola	75	proline	Mäkinen *et al.* (1994)
Treponema denticola	78	basic	Mäkinen *et al.* (1995)
Plants			
Ambrosia artemisiifolia	80	hydrophobic	Bagarozzi *et al.* (1996)
Prosopis velutina	84	basic	Matheson *et al.* (1995)
Protozoa			
African trypanosomes	80	basic	This study
T. cruzi	80	basic	Burleigh *et al.* (1997)
Mammals			
Human	80	proline	Yoshimoto *et al.* (1997)
Pig	80	proline	Rennex *et al.* (1991)

An important characteristic of oligopeptidases that must be of biological significance is their apparent lack of naturally occurring inhibitors (Barrett and Rawlings, 1992). None of the known oligopeptidases identified to date have any known natural inhibitors, including a variety of classical serine proteinase inhibitors (serpins) and the general protease inhibitor, α_2-macroglobulin. This raises the possibility that oligopeptidases released from trypanosomes into the infected hosts may significantly contribute to the pathogenesis of African trypanosomiasis.

1.3 Proteolytic enzymes and disease

Proteolytic enzymes from all four classes of proteases have been implicated in the pathogenesis of a variety of pathological conditions, including bacterial and protozoan infections, hypertension, coagulation disorders, viral infection and cancer (Sharpe *et al.*, 1991). Discussion will be limited here to the proteolytic enzymes of parasitic micro-organisms, and the potential roles played by these enzymes in the pathogenesis of microbe-induced diseases.

1.3.1 Proteases of parasitic protozoa

Proteolytic enzymes have been identified in a wide variety of pathogenic protozoans (Table 3) where they have been ascribed a variety of roles. The roles of proteases in the physiology of these organisms are generally unknown. The lysosomal cysteine and aspartic proteases are speculated to be involved in protein catabolism for nutritional purposes. This is certainly true of the aspartic proteases of the malaria plasmodium, *Plasmodium falciparum*, which are involved in the catabolism of erythrocyte haemoglobin (van der Jagt *et al.*, 1986).

Table 3. Proteases of pathogenic protozoans[a].

Organism	Disease	Protease class			
		Cysteine	Serine	Aspartic	Metallo
Entamoeba histolytica	amoebiasis	✓			✓
T. cruzi	Chagas' disease	✓	✓		
Leishmania sp.	kala-azar	✓			✓
Plasmodium sp.	malaria	✓	✓	✓	
T. brucei	sleeping sickness	✓	✓		✓[b]
Trichomonas vaginalis	vaginitis	✓	✓		
Giardia lamblia	diarrhoea	✓			

[a]Members of all four classes of proteases have been identified in pathogenic protozoans (adapted from McKerrow *et al.*, 1993).
[b]Unpublished observations from the present study.

A variety of physiological roles have been ascribed to the proteases of these organisms. These include:

- metabolism of host proteins for nutritional purposes;
- immune system evasion or modulation by degradation of host immune molecules;
- the degradation of host connective tissue to facilitate invasion;

- parasite remodelling from one morphological stage to another;
- activation or turnover of parasite peptides, enzymes and hormones;
- degradation of host cytoskeletal proteins during invasion or rupture of host cells.

Of particular interest in this study were the proteolytic enzymes of protozoans of the genus *Trypanosoma*. The major proteolytic activity of *T. b. brucei* and *T. congolense* results from a cysteine protease, trypanopain, which resembles mammalian cathepsin L (EC 3.4.22.15) (Lonsdale-Eccles and Mpimbaza, 1986; Robertson *et al.*, 1990; Lonsdale-Eccles, 1991). The trypanopains have molecular masses in the region of 28-30 kDa and optimum activity against proteins at pH 5-6. Trypanopain activity is enhanced by reducing agents and is inhibited by the low-molecular mass cysteine protease inhibitor L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64) as well as by the high-molecular mass proteinaceous inhibitors of cysteine proteases, the cystatins (Troeberg *et al.*, 1996). Trypanopain displays a cathepsin L-like substrate specificity with a preference for basic residues (arginine > lysine) in P_1, and bulky hydrophobic residues (phenylalanine) in P_2 (Lonsdale-Eccles and Grab, 1987). The physiological role of trypanopain in *T. b. brucei* is unknown but the immunolocalisation of trypanopain in the lysosomal system of *T. congolense* by Mbawa *et al.* (1991) suggests a role in protein catabolism analogous to that of the mammalian lysosomal cathepsins. Inhibition studies by Russo *et al.* (1993) also suggest that the enzyme may play a role in assisting the parasites to evade the immune system.

Serine proteases of African trypanosomes have received little attention in comparison to their cysteine counterparts. Kornblatt *et al.* (1992) undertook a preliminary study of a protease that appeared to be a cytosolic enzyme with optimal activity at pH 8. While this enzyme appeared to be inactivated by some typical serine protease inhibitors, for instance diisopropylfluorophosphate (DFP) and 3,4-dichloroisocoumarin (DCI), it was not inhibited by phenylmethanesulfonylfluoride (PMSF). To confound matters further, the enzyme displayed thiol-activatable activity and was inhibited by typical low-molecular mass inhibitors of cysteine proteases, including Hg^{2+}, iodoacetamide and *para*-chloromercuribenzoic acid (*p*CMB). Thus, some ambiguity remained regarding the enzyme's precise classification. It is this enzyme, designated as an "oligopeptidase from *T. b. brucei*", or OP-Tb, that will be the subject of the present study.

Other proteases are also present in these organisms. Recently, active subunits of the proteasome from *T. b. brucei* have recently been purified and characterised (Hua *et al.*, 1996; Lomo *et al.*, 1997). Also, El-Sayed *et al.* (1995) showed that the deduced amino acid sequence from a cDNA expressed sequence tag (EST) from *T. b. rhodesiense* clone T613 displayed substantial homology to Thimet oligopeptidase (EC 3.4.24.15) (see Section 1.3.2). While there are no published reports to date of aspartic or metalloproteinase activity from African trypanosomes, a phenanthroline-sensitive gelatinase activity has been identified in a crude membrane fraction of *T. b. brucei* lysates (unpublished data). The gene encoding the *T. b. rhodesiense* homologue of the major membrane-bound metalloprotease of *Leishmania* has recently been identified (El-Sayed & Donelson, 1997).

The physiological functions of any of these enzymes have not been determined, although Mbawa *et al.* (1991) reported changes in the proteolytic activity of various life-cycle stages of *T. brucei*, *T. congolense* and *T. vivax*. Procyclic forms of *T. brucei* and *T. congolense* typically found in the insect vector demonstrated enhanced activity possibly attributable to OP-Tb. Conversely, activity attributable to trypanopain was enhanced in LS bloodstream forms of the trypanosomes. This activity was elevated further after differentiation into SS bloodstream forms.

As with the African trypanosomes, cysteine and serine protease activities have also been identified in the South American trypanosome, *T. cruzi*, the aetiological agent of Chagas' disease. The major cysteine protease of *T. cruzi*, cruzipain (also called cruzain), closely resembles the trypanopains and mammalian cathepsin L (Bontempi *et al.*, 1984; Cazzulo *et al.*, 1990). Again, the serine proteases of the South American trypanosomes are relatively poorly investigated. Using degenerate oligonucleotide primers and the polymerase chain reaction (PCR), Sakanari *et al.* (1989) isolated eight gene fragments from *T. cruzi* epimastigote genomic DNA which displayed homology to mammalian serine proteases. Furthermore, Burleigh *et al.* (1995) demonstrated the existence of a 120 kDa alkaline peptidase that is believed to be involved in the generation of a Ca^{2+}-signalling factor in mammalian cells and with the penetration of *T. cruzi* into cells. More recently, the gene encoding this enzyme has been cloned, and homology data suggests that the enzyme belongs to the oligopeptidase B family of serine peptidases (Burleigh *et al.*, 1997).

Additional peptidase activities have been identified in South American trypanosomes. A protease has recently been identified that is secreted by *T. cruzi* and is able to degrade collagen types I and IV. However, its precise classification remains to be determined (Santana *et al.*, 1997). In addition, putative membrane-bound metalloproteinase activities in *T. dionisii* and *T. mega* extracts have been detected on gelatin zymograms (Branquinha *et al.*, 1996).

1.3.2 Microbial proteases and disease pathogenesis

Proteases secreted by invading micro-organisms into the host organism exhibit a wide range of pathogenic effects, either directly, through the digestion of host proteins, or indirectly, through the inappropriate activation or inactivation of important signalling factors. As one objective of this investigation was to propose how trypanosome proteases may contribute to disease pathogenesis, some discussion of the role of microbial proteases in disease pathogenesis is warranted.

1.3.2.1 Tissue destruction

Direct digestion and liquefaction of tissues at infection foci is the most direct mechanism whereby the proteases of invading micro-organisms may cause damage to a host. This is evident in the pathology of periodontal disease, where cysteine and metalloproteases (e.g. from *Porphyromonas gingivalis*) cause direct damage to the gum tissues of the mouth (Scott *et al.*, 1993). Similarly, the keratitis that results from the colonisation of the cornea by *Pseudomonas* spp. and *Serratia* spp. is due to microbial-derived proteases (Holder and Haidaris, 1979).

The secretion of digestive enzymes also appears to be involved in the invasion of host tissues by invasive pathogenic micro-organisms. The production of cysteine and metalloproteases by *Entamoeba histolytica*, the causative agent of amoebiasis, correlates with the virulence and invasiveness of these parasites. Patients with invasive disease produce antibodies to amoebapain, the major cysteine protease of the organism, while those with non-invasive infections do not (Reed *et al.*, 1989). The fascinating parallels between the release of a cathepsin B-like proteinase from invasive amoebae and similar enzymes found in the extracellular milieu of invasive cancer cells have been noted by Lushbaugh (1988).

1.3.2.2 Kinin generation

Bradykinin is an endogenous peptide that exerts a wide range of pharmacological activities, including hypotension, vasodilation, pain and shock. Bradykinin is usually released from high-molecular mass kininogen (H-kininogen) by plasma kallikrein, or from kallidin by lysine aminopeptidase (Bhoola *et al.*, 1993). A number of microbial proteases, particularly those from *Vibrio cholerae* and *P. gingivalis*, are known to activate one or more steps of the bradykinin-generating cascade (Fig. 7). Additionally, a serine protease from the house-dust mite *Dermatofagoides farinae* generates bradykinin from H-kininogen (Maruo *et al.*, 1991). Significantly, a number of these proteases generate bradykinin in the presence of plasma protease inhibitors (Maeda and Molla, 1989). The effect on the host of bradykinin generation by proteases of invading micro-organisms includes hypotension, pain and shock.

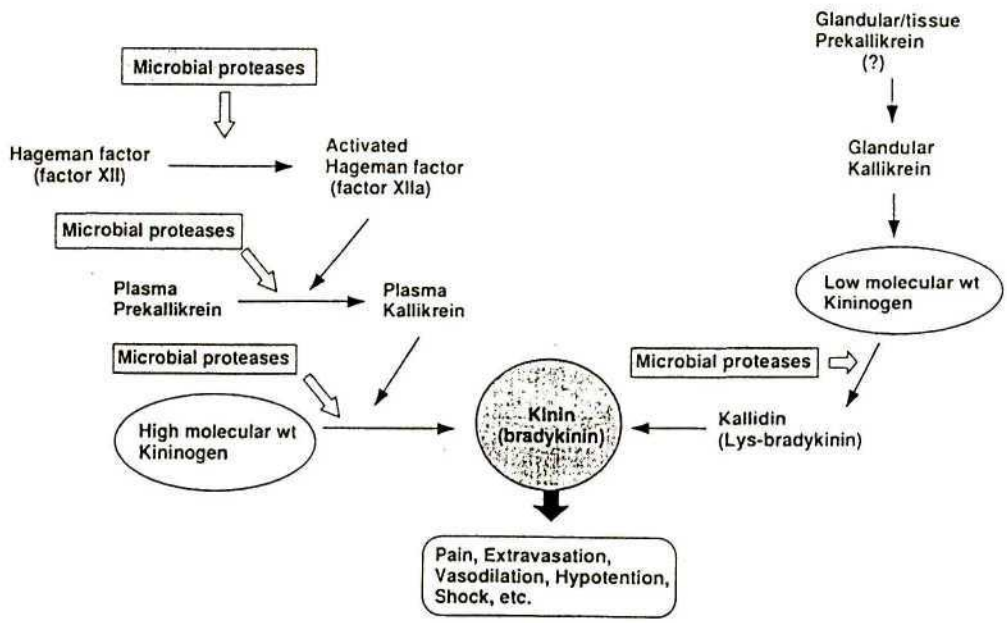


Figure 7. Generation of kinins by the proteases of invading micro-organisms. Microbial proteases may activate the bradykinin generating cascade at four different points (after Maeda and Yamamoto, 1996).

1.3.2.3 Cell and tissue invasion

Plasmodium is an intracellular parasite which invades red blood cells. Serine proteases of *P. falciparum* have been implicated in the invasion of host erythrocytes by the parasite (Rosenthal *et al.*, 1987), and both cysteine and serine proteases have been implicated in the subsequent merozoite-mediated rupture of erythrocytes (Grellier *et al.*, 1989). This erythrocyte rupture is thought to contribute to anaemia and shock in these conditions. A serine protease has also been associated with *T. cruzi* invasion of host cell. Proteases are also believed to play a role in cytoadherance of protozoan parasites to mammalian mucus membranes. Thus, colonisation of the urinogenital tract of women by *Trichomonas vaginalis* results in an acute inflammatory response and the production of a purulent vaginal discharge (Nielsen and Nielsen, 1975). Pre-treatment of parasites with cysteine protease inhibitors results in a loss of cytoadherance of the parasite, which is restored upon addition of the proteases. This, together with the cell-surface localisation of a 43 kDa protease, implicates protease activity in cytoadherance (Arroyo and Alderete, 1989).

1.3.2.4 Inactivation of host protease inhibitors

A variety of pathogen-derived proteases are known to catalytically inactivate host protease inhibitors. Disturbances in the protease-inhibitor balance of host systems are evident in the pathogenesis of a number of diseases, including emphysema and possibly also in malignant cancer (Travis and Salvesen, 1983). Proteases from *Candida* spp. and *Serratia* spp. are known to inactivate antithrombin III (ATIII) leading to uncontrolled thrombus formation within the host. This causes hypercoaggulation and DIC syndrome (Kaminishi *et al.*, 1994). Furthermore, *Candida* spp. and *Serratia* spp. are known to produce proteases that inactivate C1 inhibitor, raising the possibility of uncontrolled activation of the complement system.

α_1-Protease inhibitor (α_1PI) is a serine protease inhibitor that protects the lower respiratory tract against damage by proteases released during inflammation (Travis *et al.*, 1990). Bagarozzi *et al.* (1996) demonstrated that a serine oligopeptidase with a chymotrypsin-like specificity isolated from the pollen of *Ambrosia artemisiifolia* (ragweed) was able to catalytically inactivate α_1PI. As the pollen of *A. artemisiifolia* is the major cause of late summer hayfever in the United States, this led to the speculation that the oligopeptidase may be involved in the degradation of pulmonary neuropeptides and the inactivation on protective

protease inhibitors during pollen-initiated allergic reactions. Similarly, the faecal pellets of the house dust mite, *D. farinae*, contain a cysteine protease designated *Der p*1. It is inhaled into the respiratory tract and *Der p*1 has been shown to catalytically inactivate α_1PI (Kalsheker *et al.*, 1996). Inactivation of the α_1PI in the lungs will exacerbate tissue damage and inflammation and accentuate conditions such as asthma and emphysema.

1.3.2.5 Receptor destruction

Tetanus and botulism toxins are potent neurotoxins that were recently discovered to be proteases. Both proteases rapidly and specifically degrade the acetylcholine receptors on nerve cells, blocking the transmission of signals between nerves (Schiavo *et al.*, 1992).

1.3.2.6 Inactivation of immune system components

A number of pathogen-derived proteases are capable of inactivating various components of the immune system thereby permitting the proliferation of the pathogen. The complement system is a potent defense mechanism against invading micro-organisms, which is coupled to the recruitment of phagocytic cells through the generation of a variety of chemotactic components, including the anaphylatoxins C5a and C3a. *Streptococcus* spp. are known to produce a specific protease that inactivates these anaphylatoxins (Cleary *et al.*, 1992). The major cysteine protease of *E. histolytica* is also known to rapidly, but non-specifically, degrade C3a and C5a (Reed *et al.*, 1995). Furthermore, a *Pseudomonas*-derived elastase destroys the formyl-methionyl-leucyl-phenylalanine (fMLP) peptide, an important bacterial metabolite that acts as a chemotactic factor for host neutrophils and macrophages. This elastase also destroys fMLP receptors on neutrophils (Ijiri *et al.*, 1994).

A number of bacteria that specifically colonise mucosal surfaces, *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*, produce metalloproteases that specifically cleave the hinge-region of immunoglobulin A (IgA) (reviewed by Killian *et al.*, 1996). These pathogens are the leading causes of bacterial meningitis and urinogenital-tract infections. IgA is a class of antibody produced to protect membranous surfaces. The ability of pathogens to cleave this molecule permits the colonisation of such surfaces. Cleavage in the hinge-region of IgA results in the loss of the F_c region of the antibody, and thereby prevents IgA-mediated immune-complex elimination and complement-mediated lysis of opsonised cells.

Additionally, the surface epitopes on the pathogens are masked by F_{ab} regions of the antibodies. This prevents their recognition by antibodies of other classes, for example, serum IgG (Killian *et al.*, 1996).

Pathogen derived proteases may also be cytotoxic to cells of the immune system. Maeda *et al.* (1987) reported that a serine protease from *Serratia* spp. forms a complex with α_2M. This complex is then internalised by macrophages which possess receptors for the conformationally altered α_2M-protease complex. Once endocytosed, this serratial protease gradually inactivates α_2M and is liberated from the complex within the cell. This free protease is thought to then disintegrate intracellular structures leading to cell lysis.

1.3.3 Protease inhibitors as drugs

Proteases are important in many aspects of normal physiology and pathology. If the proteolytic activity is of real clinical importance, the search for a very specific inhibitor is the next logical step. Proteases have been identified as therapeutic targets in a number of pathological conditions, including coagulation disorders, hypertension and acquired immunodeficiency syndrome (AIDS) (reviewed by Sharpe *et al.*, 1991). The use of protease inhibitors as potential drugs is summarised in Table 4 and is under development for a number of protozoan diseases, including kala-azar, Chagas' disease and malaria.

Table 4. Protease inhibitors as drugs[a].

Enzyme	Thrombin	ACE[b]	Renin	HIV Protease
Class	serine	metallo, Zn^{2+}	aspartic	aspartic
Cleavage Site	↓ -Arg-Lys-	many	↓ -Leu-Val-	↓ -Phe-Pro-
Origin	plasma	many	renal cortex	HIV-1
Substrates	fibrinogen	angiotensin I substance P	angiotensinogen	*gag* peptide
Inhibitors	hirudin	captopril	synthetic transition state analogue	synthetic transition state analogue
Application	thrombosis	hypertension	hypertension	AIDS

[a]Modified from Scharpe *et al.* (1991).
[b]Abbreviations: ACE, angiotensin-converting enzyme; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency disease syndrome.

Rosenthal *et al.* (1991) identified a cysteine protease from *P. falciparum* trophozoites called falcipain that is analogous to human lysosomal cathepsin L. The enzyme is responsible for the degradation of host haemoglobin. A panel of peptidyl fluoromethylketone (-CH_2F) protease inhibitors were investigated as inhibitors of falcipain. Effectiveness at inhibiting falcipain was correlated with effectiveness at both blocking haemoglobin degradation and killing of cultured parasites. The most potent inhibitor, carbobenzoxy-phenylalanyl-arginyl-fluoromethylketone (Cbz-Phe-ArgCH_2F), blocked haemoglobin degradation and killed parasites at nanomolar concentrations, far below concentrations which are toxic to cultured mammalian cells, suggesting that falcipain was a promising target for therapeutic intervention.

The inhibition of intraerythrocytic development of *P. falciparum* by peptidyl fluoromethylketones has also been reported (Rockett *et al.*, 1990). Again, inhibitors of falcipain were employed. These included Cbz-Leu-TyrCH_2F and Cbz-Phe-ImNvaCH_2F, where ImNva represents the unnatural amino acid imidazolylnorvaline. These two compounds inhibited intraerythrocytic development of *P. falciparum* at nanomolar IC_{50} concentrations. Rosenthal *et al.* (1991) developed these ideas further, and demonstrated that Mu-Phe-hPheCH_2F was a potent inhibitor of the falcipain homologue of *P. vinckei*, the aetiological agent of murine malaria. Furthermore, daily administration of Mu-Phe-hPheCH_2F elicited long-term cures in 80% of experimentally-infected animals.

Peptidyl halomethylketones have shown considerable promise in the treatment of other parasitic diseases. Harth *et al.* (1993) reported the arrest of intracellular replication and intercellular transmission of *T. cruzi* by Cbz-Phe-AlaCHN_2 and Cbz-Phe-AlaCH_2F, both of which are known to be potent inhibitors of cruzipain (IC_{50} of 2.2 μM for both compounds). In spite of the better IC_{50} of Cbz-Phe-ArgCH_2F for cruzipain (0.18 μM), this inhibitor was considerably less effective at inhibiting the intracellular replication of *T. cruzi* epimastigotes and amastigotes. This is probably attributable to the diminished cell penetration of Cbz-Phe-ArgCH_2F, which contains a highly charged arginine residue, making this inhibitor less likely to diffuse across the cell membrane than the less charged alanine-containing analogues.

Similar observations have been reported by Franke de Cazullo *et al.* (1994) who reported the inhibition of *T. cruzi* growth and differentiation by a peptidyl acyloxymethylketone (AMK), Cbz-Phe-LysAMK, and additional diazomethylketones, including Cbz-Phe-PheCHN$_2$. Again, the activity of these protease inhibitors was attributed to the inhibition of cruzipain. This inhibitor penetrated living parasites, decreased the infection of Vero cells in culture by the parasites, and abolished differentiation into metacyclics, at a concentration of 5 μM.

McKerrow *et al.* (1991) built on these ideas to design a peptide-based drug which appears to have potential in the chemotherapy of Chagas' disease. A high-resolution crystal structure of cruzipain in a complex with a dipeptide fluoromethylketone, Cbz-Phe-AlaCH$_2$F was obtained which provided a structural understanding of how the inhibitor interacted with cruzipain. This facilitated the development of a dipeptide inhibitor, Mu-Phe-hPheCH$_2$F. This compound incorporates an unnatural amino acid side-chain, homophenylalanine (hPhe), to minimise potential cleavage between the two amino acids by mammalian host proteases. A daily dose of 1-2 mg of this novel inhibitor protected mice from experimental infection by *T. cruzi*.

Two other classes of chemical compounds, the chalcones (1,3-diphenyl-2-propen-1-one, and its derivatives) (Li *et al.*,1995) and vinyl sulfones (Rosenthal *et al.*, 1996) have recently shown promise as potential anti-malarial agents. These compounds exhibit potent anti-malarial activity at nanomolar concentrations and this activity is attributed to the inhibition of the malarial trophozoite cysteine protease (Li *et al.*, 1995; Rosenthal *et al.*,1996).

Attempts have also been made to target the highly glycosylated membrane-bound zinc metalloprotease, gp63, of *Leishmania* spp. by synthetic vaccines. Intraperitoneal vaccination of mice with purified gp63 of *L. mexicana* emulsified in Freund's complete adjuvant (FCA) conferred significant protection to the mice upon challenge infection (Russell and Alexander, 1988). Furthermore, synthetic peptides of predicted T-cell epitopes of gp63 protected mice from challenge infections by *L. mexicana* and *L. major* (Jardin *et al.*, 1990).

1.4 Objectives of the current study

Proteases are known to be important virulence factors in parasitic and other diseases (Maeda and Yamamoto, 1996). While the cysteine proteases of African trypanosomes have been well

investigated (reviewed by Lonsdale-Eccles, 1991 and Coombs and Mottram, 1997), the serine proteases have received little attention.

The objectives of this study were to characterise a recently identified oligopeptidase from African trypanosomes, designated OP-Tb from *T. b. brucei* and OP-Tc from *T. congolense*. This first necessitated the purification of OP-Tb to electrophoretic homogeneity, described in Chapter 3.

Antibodies were raised against the enzyme, to examine whether such antibodies have any effect on enzyme activity, whether they kill live parasites *in vivo*, to provide tools for further investigation by immunochemical means, and provide a tool for the immunoaffinity purification of the same enzyme from *T. congolense* (Chapter 4).

Once purified, comprehensive characterisation of OP-Tb and OP-Tc was undertaken (Chapter 5). This included detailed substrate specificity, inhibition and activator profiles in order to determine the precise classification of the protease and determine whether the enzyme would be active in the extracellular milieu of the host.

In order to investigate whether the enzyme may contribute to disease pathogenesis, attempts were made to detect the enzyme in the bloodstream of infected mammals and to determine whether the enzyme is regulated by host plasma protease inhibitors. The interactions of the enzyme with host regulatory molecules and host peptide hormones were examined in detail, in the context of disease pathogenesis (Chapter 6).

Trypanosome oligopeptidases were also evaluated as potential targets for currently available trypanocidal agents, as the mechanism of action of these agents is presently not understood. Finally, the trypanosome oligopeptidases were evaluated as potential targets for anti-trypanosome chemotherapy employing serine protease inhibitors (Chapter 7).

Chapter 2

General materials and methods

A variety of general biochemical techniques which were used throughout this study are described in this chapter. More specific experiments pertaining to specific chapters of this dissertation are described in their appropriate sections.

2.1 Materials

HiLoad™ Q-Sepharose columns and protein M_r standards were from Pharmacia LKB Biotechnology (Lund, Sweden). 2,2′-Azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulphonate] (ABTS), bovine serum albumin (BSA), phenylmethanesulfonylfluoride (PMSF), 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), α_2-macroglobulin (α_2M) and papain were from Boehringer Mannheim (Mannheim, Germany). Human α_2-antiplasmin (α_2AP), human low-molecular mass kininogen (L-kininogen), high-molecular mass kininogen (H-kininogen), human α_2-HS-glycoprotein (α_2HSGP) and human neutrophil elastase (HNE) were from Calbiochem (San Deigo, USA). Sephacryl S-100 HR, Sephacryl S-200, benzamidine and poly-(L-lysine) insolubilised on Sepharose 4B, Percoll, reduced and oxidised glutathione, pentamidine isethionate, 3,3′,5,5′-tetramethylbenzidine (TMB), 5-bromo-4-chloro-3-indolylphosphate (BCIP), nitroblue tetrazolium (NBT), fibrinogen, ovalbumin, trypsin, poly-(L-lysine), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were from Sigma (St Louis, USA). Nunc Immuno Maxisorp F96 multiwell plates and FluorNunc® 96-well fluorimetry plates were from Nunc Intermed (Roskilde, Denmark). Sterile disposable plasticware for cell culture was from Corning/Costar (Cambridge, USA). Sterivex™-GM 0.22 μm filter units were from Millipore (Bedford, USA). Diethylaminoethyl-Cellulose (DE-52, DEAE-Cellulose) was from Whatman International (Kent, UK). Diisopropylfluorophosphate (DFP) and chymotrypsin were from Fluka (Buchs, Switzerland). Peptide aldehydes, fluorogenic peptide substrates, diazomethylketones and chloromethylketones were from Bachem (Bubendorf, Switzerland) or Cambridge Research Biochemicals (Cambridge, UK). Foetal calf serum was from Delta Bioproducts (Kempton Park, South Africa). Penicillin-streptomycin mixtures were from Gibco (Paisley, UK). Germanin® was from Bayer (Leverkusen, Germany) and Berenil® was from Hoechst Veterinär (Munich, Germany). 3 ml

Polystyrene concentrators, containing 10 kDa M_r cut-off membranes, were from Micron Separations Incorporated (Westboro, USA). Common laboratory chemicals were from BDH (Poole, England), Merck (Darmstadt, Germany) or Boehringer Mannheim (Mannheim, Germany) and were of analytical or higher quality.

T. b. brucei strain ILTat 1.1 and *T. congolense* strain IL3000 were obtained from the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya. Human cystatin C was a gift from Dr Magnus Abrahamson (Institutionen för Klinik Kemie, Universitet i Lund, Lund, Sweden). Sheep cystatin B was purified in this department by Dr Theresa Coetzer. Antithrombin III (ATIII) was a gift from Dr Robert Pike (Department of Haematology, University of Cambridge, Cambridge, UK). *E. coli* oligopeptidase B was a gift from Prof. Tadashi Yoshimoto (Faculty of Pharmacy, University of Nagasaki, Nagasaki, Japan). Adult male Wistar rats were obtained from the Biomedical Resource Centre (University of Durban-Westville, Westville, South Africa). Bovine serum, naturally infected with trypanosomes, was a gift from Dr Olivier Matthee (Department of Protozoology, Onderstepoort Veterinary Research Institute, Pretoria, South Africa). Sera from N'Dama and Boran cattle experimentally infected with *T. congolense* was a gift from Dr Edith Authié (International Laboratory for Livestock Diseases, Nairobi, Kenya).

DL-α-difluoromethylornithine (DFMO) was a gift from Dr Andrew Peregrine (International Livestock Research Institute, Nairobi, Kenya). Melarsoprol and melarsen oxide were gifts from Dr Alan Fairlamb (Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, London, UK). Suramin analogues were synthesised by Prof. Dr Peter Nickel (Pharmazeutisches Institut, Universität Bonn, Bonn, Germany). Peptidyl phosphonate diphenyl esters were synthesised by Dr James Powers (School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, USA).

Distilled water (dH_2O) was produced by a Milli-RO® 15 Water Purification System (Millipore, Marlboro, USA). Distilled, deionised water (ddH_2O) was produced by a Milli-Q Plus Ultra-Pure Water System (Millipore, Marlboro, USA). The minimum resistivity of the ddH_2O was 18 MΩ.cm.

2.2 Protein assays

Protein concentration was determined by one of three methods. The modified method of Bradford (Read and Northcote, 1981) was routinely used as it is a rapid and sensitive means of protein determination, that is unaffected by the presence of free thiols. However, in cases where samples contained detergents or high concentrations of salts, or low concentrations of proteins, the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985) was employed. In some cases, protein concentration could be determined directly from the absorbance provided that the molar extinction coefficients were available (Schultze and Heremans, 1966).

2.2.1 Bradford dye-binding assay

The Bradford dye-binding assay for protein concentration employs the dye Coomassie Brilliant Blue G-250. The dye is believed to bind chiefly to arginine residues of the protein via the electrostatic attraction of the dye's sulfonic acid groups (Compton and Jones, 1985). The dye reagent contains the dye in acid solution, where it exists in its cationic (yellow) form with an absorbance maximum of 470 nm. Reduction on exposure to protein in solution converts the dye to an anionic (blue) species with a concomitant shift in absorbance maximum to 595 nm.

The advantage of the Bradford dye-binding assay is that it is compatible with most agents that interfere with other protein assays, including metal ions and reducing agents. However, detergents including Triton X-100 and SDS do interfere with this assay (Friedenauer and Berlet, 1989). Another disadvantage of this assay is the protein-to-protein variation in colour response. Read and Northcote (1981) modified the original method of Bradford (1976) by replacing the original dye, Coomassie brilliant Blue G-250, with Serva Blue G, and altering the dye and phosphoric acid concentrations, at the expense of a loss in sensitivity of the assay.

2.2.1.1 Materials

Dye-reagent. Serva Blue G dye (50 mg) was dissolved in 88% (v/v) phosphoric acid (50 ml) and added to 99.5% (v/v) ethanol (23.5 ml). The solution was made up to 500 ml with dH_2O, stirred (30 min), filtered through Whatman No. 1 filter paper, and stored in an amber bottle at RT. Each batch of reagent was calibrated individually.

Standard protein solution (1 $mg.ml^{-1}$ ovalbumin). Ovalbumin (10 mg) was dissolved in dH_2O (10 ml). This stock solution was diluted to 100 $\mu g.ml^{-1}$ for the assay and used immediately.

2.2.1.2 Method

A standard curve was constructed with the protein standard for the range 1-5 μg of protein. Ovalbumin working solution (10-50 μl of the 1 $\mu g.ml^{-1}$ solution) was diluted to a final volume of 50 μl with dH_2O in a 1.5 ml polyethylene microfuge tube. Dye reagent (950 μl) was added, the solution mixed by inversion, and incubated at RT for 2 min, after which the absorbance at 595 nm was determined in a 1 ml plastic microcuvette with a Pharmacia LKB Ultraspec III spectrophotometer, with dH_20 serving as a blank. Samples of unknown protein concentration were diluted to a total volume of 50 μl in buffer before the addition of dye reagent (950 μl). Buffer (50 μl) treated in the same way served as a blank. The protein concentration of the unknown sample was then calculated from equations generated by linear regression of the standard curve data.

2.2.2 Bicinchoninic acid assay

The bicinchoninic acid (BCA) protein assay relies on the reduction of alkaline Cu^{2+} to Cu^{+} by proteins, biogenic amines and reducing sugars. The cuprous (Cu^{+}) ion is then complexed with the BCA ion in a 1:2 (Cu^{+}:BCA) ratio. BCA specifically chelates the Cu^{+} ion, and the BCA-Cu^{+} complex has an intense purple colour with a strong absorbance at 562 nm, permitting the spectrophotometric quantitation of the complex (Smith *et al.*, 1985).

This assay has the advantage over the Bradford dye-binding assay (Section 2.2.1) of compatibility with ionic and non-ionic detergents and exhibiting less protein-to-protein variation while retaining high sensitivity. While, reducing agents do interfere with the assay they may be removed beforehand by precipitation of the protein with trichloroacetic acid (TCA), and resolubilisation of the protein in alkaline SDS (Brown *et al.*, 1989).

2.2.2.1 Materials

BCA reagent [1% (m/v) sodium bicinchoninate, 2% (m/v) Na_2CO_3, 0.16% (m/v) sodium tartrate, 0.95% (m/v) $NaHCO_3$]. Sodium bicinchoninate (1 g), Na_2CO_3 (2 g), sodium tartrate (0.16 g), NaOH (0.4 g) and $NaHCO_3$ (0.95 g) were dissolved in dH_2O (90 ml), adjusted to pH 11.25 with NaOH and made up to 100 ml with dH_2O.

4% (m/v) Copper sulfate. $CuSO_4.5H_2O$ (0.4g) was dissolved in dH_2O (10 ml).

Standard Working Solution (SWR). BCA reagent (50 ml) and 4% (m/v) $CuSO_4$ (1 ml) were combined and stored for not longer that 1 week at 4°C.

Standard protein solution (1 $mg.ml^{-1}$ ovalbumin). Ovalbumin (10 mg) was dissolved in dH_2O (10 ml). This stock solution was diluted to 250 $\mu g.ml^{-1}$ for the assay and used immediately.

2.2.2.2 Method

A standard curve was constructed with the protein standard for the range 1-5 µg of protein. Ovalbumin working solution (4-20 µl of the 250 $\mu g.ml^{-1}$ solution) was diluted to a final volume of 20 µl with dH_2O in a 1.5 ml polyethylene microfuge tube. SWR (1 ml) was added, the solution mixed by inversion, and incubated at 60°C for 30 min. The absorbance at 562 nm was determined in a 1 ml plastic microcuvette using a Pharmacia LKB Ultraspec III spectrophotometer, with dH_20 serving as a blank. Samples of unknown protein concentration were diluted to a total volume of 20 µl in buffer before the addition of SWR. Buffer (20 µl) treated in the same way, served as a blank. The protein concentration of the unknown sample was then calculated from equations generated by linear regression of the standard curve data.

2.2.3 Extinction coefficient

The concentration of any pure protein solution is determined provided the molar extinction coefficient (ε) and light-path length (*l*) are known (Schultze and Heremans, 1966). The molar extinction coefficient of a compound may be defined as the absorbance of a 1 M solution using a 1 cm light-path (Dawson *et al.*, 1968), although this is often replaced with an extinction coefficient describing the absorbance of a 0.1% (i.e. 1 $mg.ml^{-1}$) solution using a 1 cm light-path. Concentration (c) may thus be defined in terms of absorbance at 280 nm (A_{280}) as:

$$A = \varepsilon l c \qquad \text{(Schultze \& Heremans, 1966).}$$

2.3 Synthetic peptide substrate enzyme assays

Synthetic peptide substrates consist of single amino acids or short peptides which are covalently modified at their amino- (N) and carboxy- (C) termini. The N-terminus may be blocked with moieties such as benzoyl (Bz), carbobenzoxy (Cbz) or acetyl (Ac) groups. The C-terminus is usually coupled by an amide bond to a fluorogenic or chromogenic leaving

group, such as 4-methoxy-β-naphthylamine (4MeβNA), *para*-nitroaniline (*p*NA) or 7-amino-4-methylcoumarin (AMC) (John, 1992; Sarath *et al.*, 1989). Due to their higher sensitivity, fluorogenic leaving groups are generally preferred to their chromogenic counterparts. These synthetic substrates have a low intrinsic fluorescence but after hydrolysis of the amide bond, a highly fluorescent leaving group is released. The intensity of the fluorescence provides a direct indication of enzyme activity (reviewed by Knight, 1995).

Throughout this investigation the substrate Cbz-Arg-Arg-AMC was used (Barrett and Kirschke, 1981), as a preliminary survey of the substrate specificity of OP-Tb (Section 5.11.1 Table 9) revealed a preference for cleavage after paired basic residues. OP-Tb activity is expressed throughout this investigation either as picomoles of AMC released per second ($pmol.sec^{-1}$) or change in fluorescence per min ($\Delta F.min^{-1}$)

The standard synthetic substrate assay (Section 2.3.2) was employed where a high degree of precision was required. For example, in the determination of kinetic constants and the effects of drugs on enzyme activity (Section 5.11.1 *et seq.*). For the simultaneous assay of a large number of samples, for example, to obtain an enzyme activity profile from chromatography column fractions, the standard method (Section 2.3.2) was adapted for use in a 96-well microfluorimeter plate (Section 2.3.3).

2.3.1 Materials

4 × Assay buffer [200 mM Tris-Cl, 40 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. Tris (2.422 g), dithiothreitol (0.616 g) and NaN_3 (0.02 g) were dissolved in dH_2O (75 ml), adjusted to pH 8, and made up to 100 ml.

Substrate stock solution (1 mM Cbz-Arg-Arg-AMC in DMSO). Cbz-Arg-Arg-AMC (1.1 mg) was dissolved in DMSO (1.5 ml) and stored at 4°C.

Substrate working solution [20 μM Cbz-Arg-Arg-AMC in 0.02% (v/v) DMSO in dH_2O]. Stock substrate solution (100 μl) was diluted to 5 ml. This solution was stored at 4°C for no longer that 2 days.

0.1% (m/v) Brij 35. Brij 35 (0.1 g) was dissolved in dH_2O (80 ml) and made up to a final volume of 100 ml.

Stop solution [100 mM monochloroacetate, 20 mM sodium acetate, 70 mM sodium acetate, pH 4.3]. Monochloroacetate (9.45 g), $CH_3COONa.3H_2O$ (4.08 g) and glacial acetic acid (4 ml) were dissolved in dH_2O (950 ml), adjusted to pH 4.3 with NaOH, and made up to 1 litre.

1 mM AMC standard. AMC (1.8 mg) was dissolved in DMSO (10 ml), and stored at 4°C.

2.3.2 Standard assay

All reagents were pre-equilibrated to 37°C. For standard continuous assays, samples (1-5 ng OP-Tb) were diluted in 0.1% (m/v) Brij 35 to 500 µl before assay buffer (250 µl) was added. The enzyme was allowed to activate in a temperature-controlled cell (10 min, 37°C) after which time substrate working solution (250 µl) was added, and fluorescence monitored continuously on a Hitachi F-2000 spectrofluorimeter (λ_{ex} 370 nm, λ_{em} 460 nm), for 10 min. Stopped-time assays were performed in the same manner, except that stop solution (1 ml) was added to the reaction mixture 10 min after the addition of substrate, and a single fluorescence reading taken.

To facilitate quantitation of product, separate calibration graphs were constructed for the standard continuous assays and the stopped-time assays. AMC standard was diluted in assay buffer for calibration of the continuous assay, while for the stopped-time assay, AMC standard was diluted in a 1:1 mixture of assay buffer and stopping reagent. Linear regression analysis of the standard data yielded equations from which the AMC concentration in the standard continuous and stopped-time assays could be determined.

2.3.3 Microplate assay

All reagents were pre-equilibrated to 37°C. Samples (1-5 ng OP-Tb) were diluted in 0.1% (m/v) Brij 35 to 50 µl, before assay buffer (25 µl) was added. The enzyme was allowed to activate on a temperature-controlled heating pad (10 min, 37°C) after which time substrate working solution (25 µl) was added, and fluorescence monitored continuously in a Cambridge Technology 7620 Microplate Fluorimeter (λ_{ex} 370 nm, λ_{em} 460 nm), for 3 min. Stopped-time

assays were performed in the same manner, except that stop solution (100 µl) was added to the reaction mixture 3 min after the addition of substrate, and a single fluorescence reading taken. Separate calibration graphs were constructed for the continuous and the stopped-time microplate assays, as described in Section 2.3.2 to facilitate quantitation of product.

2.4 Precipitation techniques

A variety of precipitation techniques are used in the purification of proteins, particularly as crude fractionation steps in the early stages of purifications. These include precipitation with ammonium sulfate (Melander and Horvath, 1977) or organic solvents (Askonas, 1951), or combinations thereof such as three-phase partitioning (TPP; Odegaard *et al.*, 1984). In this investigation three-phase partitioning was employed in the crude fractionation of trypanosome lysates (Section 3.3).

2.4.1 Three-phase partitioning

Three-phase partitioning is a protein fractionation technique which employs ammonium sulfate, $(NH_4)_2SO_4$, and tertiary-butanol (*t*-butanol) to promote the precipitation of proteins in solution. The technique, first introduced by Odegaard *et al.* (1984), has been developed by Pike and Dennison (1989) and by Jacobs *et al.* (1989) for the purification of the lysosomal proteases cathepsin L and cathepsin D respectively.

The procedure entails the mixing of an aqueous protein sample with a volume of *t*-butanol, which is miscible with water. The aqueous and organic phases are induced to form separate phases by the introduction of a salt, usually $(NH_4)_2SO_4$. Protein present in solution is precipitated out at the organic-aqueous interface, thereby concentrating and dehydrating the sample. The technique is also selective, as different degrees of saturation with $(NH_4)_2SO_4$ will precipitate out different species of proteins. The technique has the advantage of extracting various contaminants such as lipids and phenolics into the organic phase (Pike and Dennison, 1989). In addition, the technique may be performed at RT, in contrast to the low temperatures required for alcohol precipitation.

2.5 Column chromatographic techniques

In order to facilitate the flow of arguments presented in this study, only background and general chromatographic methods will be introduced in this section, and the specific procedures will be described in the relevant chapter.

2.5.1 Ion-exchange chromatography

Ion-exchange chromatography (IEC) relies on the reversible electrostatic binding of ions in solution to an inert support medium to which ionisable functional groups have been covalently bound (Cooper, 1977). The system is equilibrated with a buffer of a pH at which the functional groups are ionised, and will attract counter-ions of an opposite charge to satisfy the requirements of electrical neutrality (Rossomando, 1990). For cation-exchange chromatography, negatively-charged functional groups, such as sulfonate [SO_3^-], are attached to the matrix. Conversely, for anion-exchange chromatography, positively-charged groups such as quaternary ammonium [$CH_2N^+(CH_3)_3$] are be employed. The pH of the system is manipulated to alter the charge of the proteins in solution. As protein molecules contain, *inter alia*, ionisable amine and carboxylate moieties, the degree of protonation, and hence charge, of these moieties is dependent on the pH of the solvent in which the protein is dissolved.

Anion-exchange resins, such as the DEAE-cellulose and Q-Sepharose resins employed in this investigation are positively charged and attract negatively charged counter ions. A mixture of solutes of various charges is applied to the matrix where anionic solutes displace the electrostatically-bound counter ions, and themselves act as counter ions to the charged functional groups. Neutral and cationic solutes do not bind to the cationic matrix and a group fractionation is thus accomplished. Bound ions of different characteristics may be sequentially eluted by washing the matrix with a gradient of increasing concentration of a suitable counter-ion, provided by a salt such as NaCl, which will cause the displacement of bound solutes by mass action. Alternatively, a pH gradient may replace the salt gradient. This will alter the charge of the solutes and the matrix, thereby reducing the electrostatic attraction between the two components of the system. For preparative protein fractionation, a salt gradient is preferred as adverse pH may irreversibly denature protein molecules.

2.5.1.i Diethylaminoethyl-cellulose

Cellulose is a linear polymer of D-glucose residues linked by β-D-(1→4)-glycosidic bonds (Ward and Seib, 1970), and is a popular matrix for IEC involving macromolecules or whole cells, as cellulose matrices have a very open microstructure providing easy accessibility of cells or macromolecules to charged functional groups. Additionally, the highly hydrophilic nature of cellulose minimises the hydrophobic interactions which complicate protein adsorption to IEC resins (Peterson, 1970).

DEAE-cellulose is a weak anion-exchange resin, containing a tertiary amine in the form of a diethylaminoethyl (DEAE) functional group covalently linked to the cellulose matrix (Peterson, 1970). DEAE-Cellulose was employed in this investigation for the isolation of live trypanosomes from infected rat blood (Section 3.2) and in the purification of α_1-protease inhibitor from bovine plasma (Section 6.2.4).

2.5.1.2 Q-Sepharose

Q-Sepharose is a strong anion-exchange chromatography medium, consisting of quaternary amine groups (Q) covalently attached to Sepharose CL-6B. In contrast to cellulose, Sepharose has a microreticular structure, imparting to it a high degree of chemical and physical stability. This prevents the fluctuations in bed volume under conditions of changing ionic strength and permits the use of high flow rates, in excess of 1 $ml.min^{-1}$, which results in enhanced performance in terms of resolution. These reasons, together with the stability of the matrix to pH, ionic strength and organic solvents, make Sepharose-based ion exchange media preferable to cellulose-based media for protein fractionation (Cooper, 1977). Q-Sepharose was employed in this investigation in the isolation of proteolytic enzymes from trypanosome lysates (Sections 3.4 and 3.6).

2.5.2 Molecular exclusion chromatography

Molecular exclusion chromatography (MEC), also termed gel filtration, gel permeation or molecular sieving, is a chromatographic technique that exploits differences in the size of solutes to effect their separation (James and Morris, 1964).

The chromatographic matrix is composed of polymeric particles containing a network of uniform pores into which solvent and solute may diffuse. The average time spent by a solute in

the porous network of the matrix is a function of its size. Molecules smaller than the average pore size penetrate deepest into the pores, and so their passage through the column is hindered, and they elute from the column last. In contrast, molecules significantly larger than the average pore size are excluded from the matrix, hence the term *molecular exclusion*, and elute from the column with the buffer front.

Thus, MEC serves as a method of preparative protein fractionation, as well as an analytical tool used to determine the M_r of proteins (Andrews, 1965). For this purpose, an MEC column is first calibrated by the application of a set of M_r standards. A calibration graph is constructed relating $\log_{10}M_r$ to the elution volume (V_e) of each standard. Alternatively, an availability constant (K_{av}), may be calculated from the equation,

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$ (Ackers, 1970).

The elution volume (V_e) is given by the volume of buffer required to elute that particular protein species. The void volume (V_o) is given by the V_e of a standard with a M_r considerably larger than the exclusion limit of the column and V_t is the volume of the cylindrical gel bed.

A variety of MEC matrices were utilised in this investigation. Sephacryl S-100 HR (fractionation range 1-100 kDa) was utilised in the purification of OP-Tb from *T. b. brucei* lysates (Section 3.8) and for the isolation of α_1-antichymotrypsin from bovine plasma (Section 6.2.3). Additionally, Sephacryl S-200 HR (fractionation range 5-250 kDa) was used to probe the interaction between OP-Tb and α_2-macroglobulin (Section 6.3.2), while Sephadex G-75 (fractionation range 3-80 kDa) was employed in the isolation of α_1-protease inhibitor (Section 6.2.4).

2.5.3 Affinity chromatography

Affinity chromatography relies on the specific, reversible interactions between a protein and a ligand immobilised on a chromatographic matrix. Such ligands may take the form of enzyme inhibitors, antibodies, carbohydrate-binding lectins or cofactors (Hermanson *et al.*, 1992). After application of a sample to such a matrix, only the protein of interest will bind to the immobilised ligand, allowing the remaining contaminants to be washed off the matrix. The

bound protein may then be eluted by applying conditions to the matrix that reduce the affinity between the protein and the ligand. Such conditions include changes in pH, the use of chaotropic agents (e.g. in the case of immunoaffinity chromatography), increasing ionic strength, or supplying a compound which will compete with the protein for the ligand and displace the bound protein by mass action (Cooper, 1977).

2.5.3.1 *Para*-aminobenzamidine-Sepharose

The ligand *p*-aminobenzamidine is an arginine analogue that has been used extensively for the affinity-purification of proteases that possess an arginine-binding site, including trypsin and plasminogen activator (Hermanson *et al.*, 1992). In this investigation, *p*-aminobenzamidine coupled to cyanogen bromide-activated Sepharose 4B via a 6-atom spacer, with a binding capacity of 10 mg trypsin per ml (Sigma, St. Louis, USA), was utilised for the isolation of a OP-Tb (Section 3.5).

2.5.3.2 Poly-(L-lysine)-Sepharose

Poly-(L-lysine) may be employed as an affinity ligand for proteins which are known to bind polyamines, and has been successfully used for the isolation of phosphoprotein phosphatases from rabbit muscle (Gratecos *et al.*, 1977) and *Paramecium* (Friderich *et al.*, 1992). Throughout this investigation, commercially available poly-(L-lysine) (70-150 kDa) immobilised on cyanogen bromide-activated Sepharose 4B (Sigma, St. Louis, USA) was employed. The poly-(L-lysine) was immobilised, via its ε-amino group with a one-atom spacer, at a concentration of 1-2 mg ligand per ml of gel. Poly-(L-lysine)-Sepharose was utilised in this investigation for the isolation of a OP-Tb (Section 3.7).

2.5.3.3 Cibacron Blue F3GA-Sepharose

The use of dye molecules as affinity ligands constitutes a pseudo-affinity chromatography method in which the structure of the dye molecule resembles a biological ligand. Cibacron Blue F3GA is a triazine dye that contains an anthroquinone region which successfully mimics the structure of certain enzyme substrates. The dye is thus able to bind enzymes that require adenylic co-factors and has been used for the purification of a variety of proteins, including serum albumins and α_2-macroglobulin (reviewed in Clonis, 1987). Hydrophobic interactions are also believed to be involved in the interaction between this dye and its ligands (Hermanson *et al.*, 1992). In this investigation, chromatography on Cibacron Blue F3GA-Sepharose was

employed in the isolation of α_1-antichymotrypsin (α_1ACT) and α_1PI from bovine plasma (Sections 6.2.3 and 6.2.4).

2.6 Sample concentration

When necessary, for example, prior to electrophoresis or molecular exclusion chromatography, samples were concentrated by one of two methods. If the volume of sample was small (<3 ml), concentration was achieved by ultrafiltration. For larger sample volumes, dialysis against polyethylene glycol (M_r 20 000) was employed.

2.6.1 Concentration by ultrafiltration

Concentration by ultrafiltration was performed using 3 ml polysulfone concentrators (Millipore, Bedford, USA) containing a 10 kDa M_r cut-off membrane. Samples were then centrifuged (7 000 × *g*, 10°C) in a Beckman J2-HS centrifuge until concentration was achieved, usually in 1-2 h. Buffer ions and water are forced through the membrane while macromolecules such as proteins are retained, thus effecting concentration of the protein sample.

2.6.2 Concentration by dialysis

Concentration by dialysis entails creating a concentration gradient between a dilute sample and a concentrated solution (usually sucrose or polyethylene glycol), through a semi-permeable (dialysis) membrane. The concentration gradient causes water and other small molecules to move by osmosis from the dilute sample into the more concentrated sample, effecting concentration. Dialysis is usually conducted against sucrose or organic polymers, for example, polyethylene glycol (PEG), which is a are water-soluble, high molecular mass polymer. PEG 20 000 has an average M_r of 20 000 (Budavari *et al.*, 1987). PEG 20 000 is the compound of choice as, in contrast to sucrose, its large molecular mass prevents it from entering the dialysis tube and contaminating the sample.

Cellulose membrane dialysis tubing (M_r 12 000 cut-off) was soaked in dH_2O for 10 min at RT before use. Dilute protein samples were placed in the dialysis tube, and the tube was placed on a bed of solid PEG 20 000, with which it was also covered, and the sample allowed to concentrate at 4°C. Once concentration had been achieved, the dialysis tube was briefly rinsed with dH_2O, and the sample squeezed out of the tube.

2.7 Electrophoretic techniques

In this investigation reducing and non-reducing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was used to determine the homogeneity of purified protein preparations, and for the determination of the molecular mass of purified trypanosome oligopeptidases. In conjunction with western blotting (Section 4.4), SDS-PAGE was used to evaluate the specificity of antibodies raised against OP-Tb, and their cross-reactivity with potentially related proteins from other sources. A combination of SDS-PAGE and protein blotting was also used to detect OP-Tb in trypanosome lysates and purified preparations by active-site blots (Section 7.4), and for the preparation of material for N-terminal sequencing (Section 3.10).

The supporting matrix usually take the form of polyacrylamide gels. These are constructed by the vinyl polymerisation of a synthetic acrylamide monomer, with a cross-linking agent, such as N,N′-methylenebisacrylamide (Bis). The polymerisation occurs in the presence of a free-radical catalyst, such as the sulfate radical, $SO_4^{\bullet}$, provided by ammonium persulfate. The free-radicals react with the acrylamide to generate activated acrylamide where the free-radical state is preserved within the acrylamide molecule. These polymeric chains, which are still in a fluid state, are then cross-linked in the presence of Bis to form a gel, which is analogous to a "net" of acrylamide chains. Because of its ability to exist in the free-radical state, the compound N,N,N′,N′-tetramethyl-1,2-diaminoethane (TEMED) is used as an accelerator of polymerisation. The porosity of these gels may be varied by manipulating the relative amount of cross-linking agent to acrylamide monomer (described as %C), and the mass of acrylamide in the total volume of the gel (m/v) (described as %T).

The extent of migration of proteins during native PAGE is a function of the charge-to-mass ratio of that particular protein species and the size and shape of the protein. However, this provides little information about the molecular mass of the protein. For this reason, PAGE conducted in the presence of the anionic detergent, sodium dodecyl sulfate (SDS), using the discontinuous gel and buffer system of Ornstein (1964) and Davis (1964) was introduced by Laemmli (1970). Sodium dodecyl sulfate binds to most proteins at a constant ratio of 1.4 gram SDS per gram of protein (Reynolds and Tanford, 1970). The extensive binding of detergent to protein is sufficient to negate the intrinsic charge carried by the protein, and imparts to that

protein a nett negative charge that is directly proportional to the molecular mass of that protein. To effect complete unfolding of proteins, which may contain tertiary structures that SDS alone cannot disrupt, samples may be denatured (100°C, 10 min) in the presence of reducing agents, such as β-mercaptoethanol or dithiothreitol. These agents permit extended unfolding of the proteins by reducing disulfide bridges which ordinarily restrain the structure, thereby inhibiting unfolding. Proteins may thus be separated on the basis of size alone. In this way, the M_r of a protein may be determined by comparing the migration of the protein of interest with that of a set of standard proteins of known M_r run in parallel (Neville, 1971; Weber and Osborne, 1969).

As most protein species are colourless, they have to be visualised on the gel. A number of means exists for this, including protein staining (Syrovy and Hodny, 1991; Sections 2.7.3 and 2.7.4) and densitometry (Hoffmeister, 1974).

2.7.1 Tris-Tricine SDS-PAGE

Tris-Tricine SDS-PAGE was first described by Schägger and von Jagow (1987) as an alternative to the Tris-glycine system of Laemmli (1970). In this system, Tricine is used instead of glycine as the trailing ion in the stacking phase, at a higher pH. This system is reportedly useful for the resolution of proteins in the range of 5-100 kDa, provides better resolution of protein samples due to enhanced stacking of protein bands, and can tolerate high salt concentrations in protein samples.

2.7.1.1 Materials

SDS Solution [10% (m/v) SDS]. SDS (10 g) was dissolved in a total volume of 100 ml dH_20. This process required gentle heating.

Gel buffer [3 M Tris-Cl, 0.3% (m/v) SDS, pH 8.45]. Tris (72.7 g) and SDS solution (6 ml) were dissolved in dH_2O (150 ml), adjusted to pH 8.45 with HCl and made up to 200 ml.

Monomer solution [49.5% (m/v) acrylamide, 3% (m/v) bisacrylamide]. Acrylamide (48 g) and bisacrylamide (3 g) were dissolved and made up a total volume of 100 ml with dH_2O, filtered through Whatman No. 1 filter paper and stored in an amber bottle at RT.

Initiator [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was dissolved in dH_2O (1 ml), just before use.

Anode buffer [200 mM Tris-Cl, pH 8.9]. Tris (24.22 g) was dissolved in dH_2O (900 ml), adjusted to pH 8.9 with HCl and made up to 1 litre.

Cathode buffer [100 mM Tris-Cl, 100 mM Tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (12.2 g), Tricine (17.9 g) and SDS solution (10 ml) were dissolved in dH_2O (900 ml), adjusted to pH 8.25 with HCl, and made up to 1 litre.

Non-reducing sample treatment buffer [100 mM Tris-Cl, 1% (m/v) SDS, 20% (v/v) glycerol, pH 8.45]. Tris (0.121 g), SDS solution (1 ml) and glycerol (2 ml) were dissolved in dH_2O (4 ml), adjusted to pH 8.45, made up to 10 ml, divided into aliquots and stored at -20°C.

Reducing sample treatment buffer [100 mM Tris-Cl, 1% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, pH 8.45]. Tris (0.121 g), SDS solution (1 ml), glycerol (2 ml) and β-mercaptoethanol (1 ml) were dissolved in dH_2O (4 ml), adjusted to pH 8.45, made up to 10 ml with, divided into aliquots and stored at -20°C.

Tris-Tricine M_r standards [phosphorylase b (97 kDa) , BSA (68 kDa) , ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), α-lactalbumin (14 kDa; each protein at 1 $\mu g.\mu l^{-1}$), in 125 mM Tris-HCl, 4% (m/v) SDS, 25% (m/v) sucrose, pH 8.45 in non-reducing sample treatment buffer]. Lyophilised protein standards, containing sucrose, were obtained as a kit from Pharmacia (Lund, Sweden). The standards were reconstituted in non-reducing sample treatment buffer from which the glycerol had been omitted.

HNO_3 [20% (v/v) HNO_3]. 55% HNO_3 (36 ml) was diluted to 100 ml with dH_20, and stored in an amber bottle at RT.

2.7.1.2 Method

Tris-Tricine SDS-PAGE was carried out as described by Schägger and von Jagow (1987) using a Bio-Rad Mini-Protean® II vertical slab electrophoresis unit assembled as described in the manufacturer's manual. If gels were intended for silver staining, all glassware was soaked overnight in 20% (v/v) HNO_3. Stacking and separating gels were prepared as described in Table 7. Separating gels were cast and allowed to polymerise for 1 h. Stacking gels usually polymerised within 20 min. Gels were cast using 1.5 mm spacers and combs.

Table 5. Preparation of Tris-Tricine SDS-PAGE gels[a].

Component	Running Gel	Stacking Gel
Monomer	3 ml	500 µl
Gel Buffer	5 ml	1.5 ml
ddH_2O	7 ml	4 ml
Initiator	50 µl	30 µl
TEMED	5 µl	12 µl
Final Volume	15 ml	6 ml

[a]After Schägger and von Jagow (1987).

Protein samples (containing at least 2 µg protein per band for Coomassie Blue staining, or 100 ng per band for silver staining) were combined in a 1:1 ratio with the relevant sample treatment buffer, and boiled for 10 min. After cooling, bromophenol blue (1 µl), as a marker dye, was added, and the samples loaded into gel wells with a micropipette (disposable 25 µl tip). Gels were run at unlimiting current and 70 V, until samples reached the separating gel, when the voltage was increased to 100 V.

2.7.2 Substrate gel electrophoresis

Proteases may be visualised after electrophoretic separation by the co-polymerisation of protein substrates (such as fibrinogen or gelatin) with the polyacrylamide gel, as described for urokinase-type plasminogen activator by Heussen and Dowdle (1980). Upon completion of electrophoresis, the denaturing, protein-bound SDS is removed from the gels with several washes of a non-ionic detergent, usually Triton X-100. This is believed to assist in the renaturing of the protease. The gel is then incubated in an appropriate assay buffer containing

any necessary activators of the protease. Areas of proteolytic activity are evident as clear bands against a stained background. This technique has the added advantage of providing the approximate molecular mass at which the proteolytic activity occurs, and is reportedly at least as sensitive as silver staining.

2.7.2.1 Materials

Amido black stain solution [0.1% (m/v) amido black in methanol:acetic acid:dH_2O (30:10:60)]. Amido black (0.1 g) was dissolved in methanol:acetic acid:dH_2O (30:10:60; 100 ml) and filtered through Whatman No. 1 filter paper.

Assay buffer [50 mM Tris-HCl, 10 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. Tris (3 g), dithiothreitol (0.77 g) and NaN_3 (0.03 g) were dissolved in dH_2O (450 ml), adjusted to pH 8, and made up to 500 ml.

Protein solution [1% (m/v) gelatin in separating gel buffer]. Gelatin (0.1 g) was dissolved in gel buffer (10 ml, Section 2.5.1.1) with gentle heating. The solution was prepared freshly.

2.5% Triton X-100. Triton X-100 (5 ml) was diluted to 200 ml with dH_2O.

2.7.2.2 Method

Separating gels were cast as described in Section 2.7.1.2 but a proportion (3.75 ml) of the separating gel buffer (Table 5) was replaced with a mixture of 1% (m/v) gelatin (1.5 ml) and separating gel buffer (2.25 ml). Stacking gels were cast as described in Section 2.5.1.2. Once the electrophoretic run was complete, gels were incubated in 2.5% (v/v) Triton X-100 (2 × 30 min) to remove SDS. Gels were then incubated in assay buffer (3 h, 37 °C) to permit the digestion of protein substrates, and stained in amido black for 1 h. Gels were destained with several changes of methanol:acetic acid:dH_2O (30:10:60) until clear bands were visible against a darkly stained background.

2.7.3 Coomassie Blue R-250 staining of proteins

Coomassie staining is a simple and rapid, yet insensitive, method of protein visualisation. This technique, which employs the dye Coomassie Brilliant Blue R-250, is able to detect down to 1 μg of protein per band (Syrovy and Hodny, 1991). Once the electrophoresis run was

completed, the gel was briefly incubated in stain solution. Background dye was then removed by incubating the gel in a destain solution.

2.7.3.1 Materials

Stain stock solution [1% (m/v) Coomassie Blue R-250]. Coomassie Blue R-250 (1 g) was dissolved in dH_2O (100 ml) by magnetic stirring for 1 h at RT, and filtered through Whatman No. 1 filter paper.

Stain working solution [0.125% (m/v) Coomassie Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock solution (62.5 ml) was diluted with methanol (250 ml) and acetic acid (50 ml), and made up to 500 ml.

Destaining I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) was mixed with acetic acid (100 ml), and made up to 1 litre with dH_2O.

Destaining II [5% (v/v) methanol, 7% (v/v) acetic acid]. Methanol (50 ml) was mixed with acetic acid (70 ml), and made up to 1 litre with dH_2O

2.7.3.2 Method

The gels were removed from the electrophoresis apparatus, and incubated in stain working solution (4 h) with agitation on a Belly Dancer® agitator (Stovall Life Science, Greenboro, USA). Following staining, the gels were rinsed with dH_2O and placed in destain I overnight. Finally, the gels were placed in destain II until the background staining was completely removed. Gels were stored hydrated in polythene zip-seal bags, at 4°C, until photographed.

2.7.4 Silver staining of proteins

Silver staining of proteins is considerably (up to 100-fold) more sensitive than the Coomassie Blue R-250 stain (Section 2.5.4). This technique relies on the reduction of ionic silver in solution to its (insoluble) metallic form. This may be accomplished in strongly acidic or basic solution (Allen and Budowle, 1994). The basic procedure, employing sodium carbonate or sodium hydroxide are more sensitive but generally have high background staining. The inclusion of a pre-treatment step with sodium thiosulfate (Blum *et al.*, 1989) considerably

reduces the intensity of background staining primarily by preventing the precipitation of insoluble silver complexes on the surface of the gel.

2.7.4.1 Materials

Fixative [50% (v/v) methanol, 12% (v/v) acetic acid, 0.1% (v/v) HCOH]. Methanol (100 ml), acetic acid (24 ml) and 37% (v/v) formaldehyde (0.1 ml) were mixed and made up to 200 ml.

Wash solution [50% (v/v) EtOH]. Ethanol (100 ml) was made up to 200 ml with dH_2O.

Pre-treatment solution [0.2 $mg.ml^{-1}$ $Na_2S_2O_3$]. $Na_2S_2O_3.5H_2O$ (40 mg) was dissolved in 200 ml dH_2O.

Impregnation solution [0.2% (m/v) $AgNO_3$, 0.03% (v/v) HCOH]. $AgNO_3$ (400 mg) and 37% formaldehyde (0.15 ml) were dissolved in 200 ml dH_2O.

Developer [60% (m/v) Na_2CO_3, 0.02% (v/v) HCOH, 0.004% (m/v) $Na_2S_2O_3$]. Anhydrous Na_2CO_3 (12 g), pre-treatment solution (4 ml) and 37% formaldehyde (0.1 ml) were combined and made up to a total volume of 200 ml.

Stop solution [50% (m/v) methanol, 12% (m/v) acetic acid]. Methanol (50 ml) and acetic acid (12 ml) were dissolved and made up to a total volume of 200 ml.

2.7.4.2 Method

Gloves were worn during the developing procedure to prevent fingerprints on the gel. Development of gels was carried out on a Belly Dancer® agitator (Stovall Life Sciences, Greensboro, USA), at RT, in glass containers which had been soaked overnight in 20% (v/v) HNO_3 (Section 2.7.1.1). Gels were removed from the electrophoresis apparatus and soaked in fixative (16 h), followed by wash solution (3 × 25 min), to remove residual fixative. The gels were treated with pre-treatment solution (2 min), rinsed with dH_2O (3 × 25 sec) and soaked in impregnation solution (25 min, in the dark). Gels were rinsed with dH_2O (2 × 25 sec), to remove excess $AgNO_3$, and immersed in developer until protein bands became evident against a faintly stained background. The gel was washed with dH_2O (2 × 2 min), and the reaction

terminated in stop solution (10 min). The gel was stored hydrated in polythene zip-seal bags, at 4°C, until photographed.

2.7.5 Electroblotting of proteins

Electroblotting of proteins provides a convenient means of transferring proteins that have been resolved by gel electrophoresis onto membranes. Electroblotting was undertaken in this investigation for the N-terminal analysis of OP-Tb (Section 3.10), the detection of proteases in complex samples by active-site labelling (Section 7.4) and for the characterisation of anti-OP-Tb antibodies by western blotting (Section 4.4).

2.7.5.1 Materials

Electrotransfer buffer [10 mM Caps, 10% (v/v) methanol, pH 11]. Caps (2.21 g) and methanol (100 ml) were dissolved in dH_2O (800 ml), titrated to pH 11 with NaOH, and made up to 1 litre.

2.7.5.2 Method

A variety of membranes were employed, depending on the application of the blot. For N-terminal analysis (Section 3.10) proteins were blotted onto polyvinylidene difluoride (PVDF) membranes, while for western blots (Section 4.4) and active-site blots (Section 7.4) proteins were blotted onto nitrocellulose.

The membranes were cut to a suitable size and floated on top of the blotting buffer before immersion to prevent entrapment of air-bubbles. The membrane was placed on top of the gel, air-bubbles expelled, and sandwiched between three pieces of Whatman No. 4 filter paper and two pieces of Scotchbrite® foam. Both the filter paper and the foam were pre-soaked in blotting buffer. The sandwich was placed into a Hoefer TE Series Transphor western blot apparatus which was pre-filled with blotting buffer. The apparatus was maintained at 8°C by a refrigerated circulator. Electrotransfer of the proteins from the gel to the membrane was accomplished at 30V for 16 h with unlimiting current.

2.8 Cell culture

In contrast to the culture of procyclic salivarian trypanosomes and stercorarian trypanosomes (Brun and Schonenberger, 1979), the *in vitro* culture of bloodstream-form salivarian

trypanosomes was initially highly problematic (Baltz *et al.*, 1985) and only very limited success had been reported with the cultivation of bloodstream-form salivarian trypanosomes on feeder-cell layers of NIH 3T3 fibroblasts (Hirumi *et al.* 1977). Duszenko *et al.* (1985) observed that cysteine eliminated the requirements for feeder-cells, and Duszenko *et al.* (1992) later reported that cysteine was an essential requirement for the *in vitro* culture of bloodstream-form salivarian trypanosomes. However, cysteine is readily oxidised to cystine by Cu^{2+} ions present in the culture medium. With this in mind, Hesse *et al.* (1995) reported a novel cultivation technique for the long-term maintenance of bloodstream-form trypanosomes in culture, through the inclusion of bathocuproinedisulfonic acid (BCDSA), in the culture medium. Bathocuproinedisulfonic acid (2,9-dimethyl-2,4-diphenyl-1,10-phenthrolinedisulfonic acid) was designed as a highly soluble, specific chelator of divalent copper cations (Zak, 1958). While the culture medium is not specifically supplemented with copper, it is present in bovine serum at a concentration of approximately 6 μM (Altman and Dittmer, 1961), and is probably introduced with the addition of foetal calf serum, to a final concentration of approximately 0.9 μM in the culture medium.

In vitro culture of African trypanosomes was employed in this investigation to investigate the effect of antibodies directed against trypanosome proteins on parasite viability (Section 4.6). Furthermore, cultures were employed to determine the trypanocidal activity of trypanocidal drugs and their analogues (Section 7.2) and potentially new therapeutic agents (Section 7.3, 7.4, 7.5).

2.8.1 Materials

Penicillin/streptomycin solution [10 000 $U.ml^{-1}$ penicillin, 10 $mg.ml^{-1}$ streptomycin, 0.9% (m/v) NaCl]. Penicillin/streptomycin was reconstituted in autoclaved ddH_2O (20 ml).

Cysteine stock solution [25 mM cysteine, 1 mM bathocuproinedisulfonic acid (BCDSA)]. Cysteine.HCl (0.22 g) and BCDSA (0.028 g) were dissolved in dH_2O (50 ml) and filter-sterilised through a 0.22 μm filter.

Foetal calf serum (FCS). If necessary, FCS was heat-treated to inactivate complement proteins, by incubation at 57°C for 1 h.

Culture medium [minimal essential medium (MEM), with Earle's salts, 0.3 $g.l^{-1}$ L-glutamine, 0.25 mM cysteine, 0.01 mM BCDSA, 15% (v/v) FCS]. Supplemented MEM was prepared exactly as described by Hesse *et al.* (1995). Supplemented MEM (9.6 g) and $NaHCO_3$ (2.2 g) were dissolved in ddH_2O (850 ml) and filter-sterilised through a 0.45 μm pre-filter and a 0.22 μm filter. FCS (150 ml) was added aseptically after filtration. For each 100 ml aliquot of medium, cysteine stock solution (1 ml) and penicillin/streptomycin solution (1 ml) were added aseptically.

2.8.2 Method

Cell culture was undertaken exactly as described by Hesse *et al.* (1995). Culture medium was pre-warmed to 37°C and divided into 5 ml aliquots in disposable polystyrene tissue culture flasks (25 cm^2 surface area) with a vented-cap containing 0.22 μm filters. Culture medium was inoculated with parasites thawed from cryo-preserved stabilates prepared from infected rat-blood. Stabilates had previously been rapidly thawed and diluted in PSG (Section 3.2.1) to an appropriate cell-number. Cultures were maintained at 37°C in 5% (v/v) CO_2.

Cytotoxicity assays were undertaken exactly as described by Kaminsky and Zweygarth (1989). Cytotoxic agents were dissolved in DMSO and added at various concentrations to trypanosome cultures, with a final DMSO concentration maintained at 1% (v/v). The cultures were incubated as described above for 24 h. Control cultures (without inhibitors, but also containing 1% (v/v) DMSO) were incubated under the same conditions. Cell numbers were determined with a haemocytometer and the data analysed graphically by plotting growth inhibition versus drug concentration. The effective concentration which inhibited growth of trypanosome populations by 50% (EC_{50}) was then obtained from these plots.

Chapter 3

Isolation of an oligopeptidase from *T. b. brucei*

3.1 Introduction

The aim of this study was to fully characterise a trypanosome oligopeptidase from *T. b. brucei* (OP-Tb) and investigate its potential role in the pathogenesis of African trypanosomiasis. It was therefore necessary to obtain a pure preparation of this enzyme. While a trypsin-like proteinase activity from *T. b. brucei* has been identified (Lonsdale-Eccles and Grab, 1987; Mbawa *et al.*, 1991) and partially characterised (Kornblatt *et al.* 1992), the enzyme had not been purified to electrophoretic homogeneity at the outset of the present study.

Working with partially purified preparations, Kornblatt *et al.* (1992) demonstrated that activity against Cbz-Arg-Arg-AMC was the most suitable marker for the enzyme. Therefore, an assay based on this substrate (Sections 2.3.2 and 2.3.3) was selected to follow the purification of the enzyme through the isolation procedure. Initial attempts at purification revealed that the presence of reducing agents was essential to maintain the stability of OP-Tb and consequently 1 mM dithiothreitol was included in all solutions.

Kornblatt *et al.* (1992) employed a combination of ultracentrifugation, ion-exchange and molecular exclusion chromatography as well as preparative isoelectric focusing in an attempt to isolate OP-Tb. In the present investigation, three-phase partitioning (Pike and Dennison, 1989) was used as an initial crude purification step since it has been successfully employed for the purification of several proteases in our department (Jacobs *et al.*, 1989; Pike *et al.*, 1992; Troeberg *et al.*, 1996). Subsequent fractionation was achieved by anion-exchange and molecular exclusion chromatography, and affinity chromatography using *para*-aminobenzamidine (Hermanson *et al.*, 1992) and poly-(L-lysine) (Friderich *et al.*, 1992) as ligands. The purification scheme is summarised in Fig. 8.

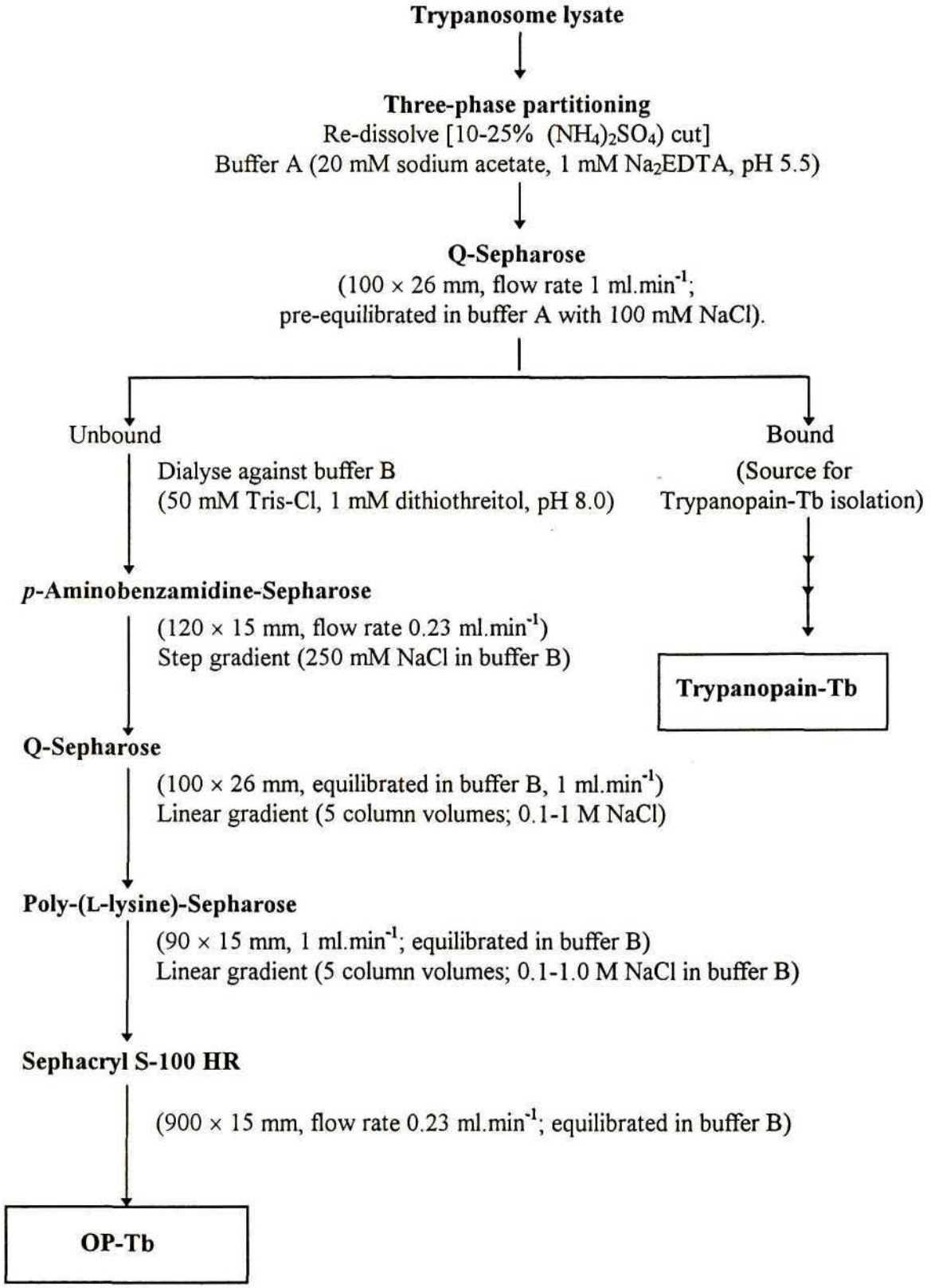


Figure 8. Flow-diagram depicting the OP-Tb purification procedure.

3.2 Growth and harvesting of trypanosomes

Throughout this investigation, *T. b. brucei* clone ILTat 1.1 was used. The first stock was isolated in 1965 from infected bovine blood at Utembo, Kenya (Young, 1985). Trypanosomes were purified from infected rat blood by a combination of isopycnic density gradient centrifugation on a Percoll® gradient (Grab and Bwayo, 1982) and anion-exchange chromatography on DEAE-cellulose (Lanham and Godfrey, 1970); supplemented with hypoxanthine (Lonsdale-Eccles and Grab, 1987).

3.2.1 Materials

Phosphate-buffered saline, containing glucose (PSG) [57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 44 mM NaCl, 56 mM glucose, 0.1 mM hypoxanthine, pH 8.0]. Na_2HPO_4 (8.1 g), $NaH_2PO_4.2H_2O$ (0.36 g), NaCl (2.5 g), D(+) glucose (10 g) and hypoxanthine (0.02 g) were dissolved in dH_2O (950 ml), the solution adjusted to pH 8 with NaOH, and made up to 1 litre.

Elution buffer [100 mM sodium acetate, 1 M NaCl, pH 5]. Glacial acetic acid (5.7 ml) and NaCl (58.4 g) were dissolved in dH_2O (950 ml), adjusted to pH 5 with NaOH, and made up to 1 litre.

Anti-coagulant [2% (m/v) tri-sodium citrate in PSG]. Tri-sodium citrate (2 g) was dissolved in PSG (100 ml).

Percoll® solution [250 mM sucrose, 2% (m/v) glucose in Percoll®, pH 7.4]. Sucrose (8.55 g) and glucose (2 g) were dissolved in Percoll® (80 ml), adjusted to pH 7.4 by the addition of solid Hepes, and made up to 100 ml with Percoll®.

20% (v/v) glycerol in PSG. Glycerol (10 ml) was dissolved in PSG (80 ml) and made up to 100 ml with PSG.

1% (v/v) Triton X-100. Triton X-100 (1 ml) was dissolved in dH_2O (50 ml) with gentle heating (37°C) and made up to 100 ml.

3.2.2 Methods

Trypanosomes were thawed from cryopreserved stabilates, and viability determined microscopically. Trypanosome suspensions were diluted to a concentration of 1×10^6 cells per ml with PSG. Inoculum (1 ml, containing 1×10^6 parasites, per rat) was injected intraperitoneally into large male (4-5 month old) Wistar rats. Blood parasitaemia was monitored microscopically from smears taken from the tail vein, and a high level of parasitaemia was usually attained in less than one week.

Once an acceptable level of parasitaemia was reached (as judged by smears from the tail-vein), rats were euthanased with ether, and anticoagulant (1 ml) was injected into the heart. The thoracic cavity was opened and the heart and lungs macerated with scissors. Clotting was prevented by the addition of more anticoagulant (up to 2 ml). Blood was aspirated out of the thoracic cavity with a syringe and stored on ice, with regular agitation to ensure mixing of the anticoagulant and blood. Infected blood was diluted with 1.5 volumes of Percoll® solution and centrifuged (20 600 × *g*, 40 min, 4°C). The trypanosome layer was aspirated off and diluted 1:1 with PSG to dilute out the Percoll®, and the trypanosomes pelleted by centrifugation (3 000 × *g*, 15 min, 4°C). The pellet was resuspended in PSG (15-20 ml) and evenly layered onto an equilibrated DEAE-cellulose gel bed contained in a glass funnel with a flat sintered-glass disk of porosity 1. The DEAE-cellulose was pre-equilibrated with PSG and the trypanosome preparation washed through the gel with PSG. Platelets and plasma proteins adsorbed to the gel, while trypanosomes passed through unhindered. The eluant was continually examined microscopically until trypanosomes were no longer evident in the eluant. Trypanosomes were concentrated by centrifugation (3 000 × *g*, 15 min, 4°C). Cell numbers were determined with a Neubauer haemocytometer after resuspending the trypanosome pellet in PSG (5 ml), and the remainder of the suspension was frozen at -75°C until required.

Cryopreserved stabilates were prepared as described by Carrington (1993). Stabilate was prepared directly from freshly collected infected rat blood which was combined with 20% (v/v) glycerol in PSG, to give a final concentration of 10% (v/v) glycerol as cryoprotectant. The stabilate was divided into aliquots (1.5 ml), in 2 ml cryopreservation tubes. Stabilate was slowly frozen by placing the cryotubes in an insulated plastic tube which was then immersed in

liquid N_2. Once the stabilate was completely frozen (30 min), the cryotubes were stored directly in liquid N_2 until used.

DEAE-cellulose was regenerated after trypanosome isolation by washing with PSG containing 1 M NaCl, followed by extensive washing alternately with elution buffer and dH_2O. The gel was finally washed with dH_2O (2.5 litres) to remove acetate, suspended in PBS containing 0.02% NaN_3 and stored at 4°C. Before use, the gel was rinsed extensively with dH_2O (≈ 2.5 litres) to remove traces of azide preservative which may harm the trypanosomes during the isolation. As the pH of the gel was crucially important, it was resuspended in PSG, allowed to equilibrate, and the pH adjusted to pH 8 with NaOH.

When required for enzyme purification, trypanosome lysates were prepared from frozen or freshly prepared trypanosome preparations by means of a freeze (-75°C)-thaw (25°C) cycle. Prior to protein purification, Triton X-100 was added to the lysate to a final concentration of 0.1% (m/v), to effect complete solubilisation of trypanosome cell membranes (Neugebauer, 1990).

3.3 Three-phase partitioning

3.3.1 Materials

Buffer A [20 mM acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (1.15 ml), Na_2EDTA (0.37 g) and NaN_3 (0.2 g) were dissolved in dH_2O (950 ml), adjusted to pH 5.5 with NaOH, made up to 1 litre, and filtered through a 0.22 μm filter.

0.1% (m/v) Brij 35. Brij 35 (0.1 g) was dissolved in a final volume of 100 ml dH_2O.

3.3.2 Optimisation of three-phase partitioning

As this technique has not previously been used for the isolation of this enzyme, it was necessary to optimise the TPP conditions. For optimisation, trypanosome lysates (3×10^9 cells) were diluted to 25 ml with 0.1% (m/v) Brij 35, and *t*-butanol (11 ml) added to a final concentration of 30% (v/v) in a total volume of 36 ml. Solid $(NH_4)_2SO_4$ (1.8 g) was added to bring the solution to a concentration of 5% (m/v) $(NH_4)_2SO_4$ and the suspension was centrifuged (10 000 × *g*, 10 min, 25°C). The precipitate was recovered, and the supernatant

volume determined. Additional $(NH_4)_2SO_4$ was added to bring the solution to 10% (m/v) $(NH_4)_2SO_4$. This procedure was repeated, in 5% increments, up to 45% (m/v) $(NH_4)_2SO_4$. The precipitate obtained at each step was resuspended in buffer A (Section 3.3.1) (5 ml) and assayed for activity against Cbz-Arg-Arg-AMC (Section 2.3.3).

3.3.3 Purification using three-phase partitioning

Trypanosome lysates (1.8×10^{10} cells) were diluted to 25 ml with 0.1% (m/v) Brij 35, and a 0-10% (m/v) $(NH_4)_2SO_4$ cut prepared as described in Section 3.2.2. The solution was centrifuged (10 000 × *g*, 10 min, 25°C), the resultant pellet discarded and solid $(NH_4)_2SO_4$ (5.4 g) added to a concentration of 25% (m/v). The solution was centrifuged (10 000 × *g*, 10 min, 25°C), the resultant pellet resuspended in buffer A (Section 3.3.1) (10 ml), and the solution clarified by centrifugation (15 000 × *g*, 30 min, 4°C). The clarified supernatant was retained for further fractionation by Q-Sepharose anion-exchange chromatography at pH 5.5 (Section 3.4).

3.4 Q-Sepharose anion-exchange chromatography at pH 5.5

3.4.1 Materials

20% (v/v) Ethanol. Ethanol (20 ml) was dissolved in ddH_2O to total volume of 100 ml.

Buffer B [50 mM Tris-Cl, 1 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. Tris (6.1 g), dithiothreitol (0.15 g) and NaN_3 (0.2 g) were dissolved in ddH_2O (950 ml), adjusted to pH 8 with HCl, and made up to 1 litre.

3.4.2 Method

Q-Sepharose stored in 20% (v/v) ethanol as preservative was obtained pre-packed as a HiLoad™ Q column (26 × 100 mm) from Pharmacia (Lund, Sweden). Ethanol was removed by washing the column with ddH_2O (500 ml), and the column was equilibrated with buffer A containing 100 mM NaCl (100 ml, 1 $ml.min^{-1}$). Sample [10-25% (m/v) $(NH_4)_2SO_4$ cut from TPP] was loaded, and after elution of the unbound protein fraction with buffer A containing 100 mM NaCl, bound material was eluted with a linear gradient of 0.1 to 1 M NaCl in buffer A, over five column volumes.

Fractions were assayed for activity against Cbz-Arg-Arg-AMC and Cbz-Phe-Arg-AMC (Section 2.3.3), and the unbound fractions active against Cbz-Arg-Arg-AMC were pooled and retained for further fractionation by *p*AB-Sepharose affinity chromatography (Section 3.5). The bound material which had activity against Cbz-Phe-Arg-AMC was used for the purification of trypanopain-Tb in a parallel study.

Buffer exchange was achieved by dialysis against buffer B (16 h, 4°C), with three buffer changes (3 × 1 litre).

3.5 *p*-Aminobenzamidine-Sepharose chromatography

3.5.1 Method

After dialysis into buffer B (Section 3.4.1), the pooled enzyme from Q-Sepharose chromatography at pH 5.5 (Section 3.4) was loaded onto a *p*-aminobenzamidine-Sepharose affinity chromatography column (120 × 15 mm, 1 $ml.min^{-1}$), which had been pre-equilibrated with buffer B. After the elution of unbound material with buffer B, elution of bound protein was achieved in a single step by application of 250 mM NaCl in buffer B. The enzyme-containing fractions were pooled and retained for further fractionation by Q-Sepharose anion exchange chromatography at pH 8 (Section 3.6).

3.6 Q-Sepharose anion-exchange chromatography at pH 8

3.6.1 Method

The active pool from the *p*-aminobenzamidine-Sepharose column (Section 3.5) was diluted two-fold with buffer B (Section 3.4.1), to reduce the NaCl concentration in the sample. The sample was applied to the column and, after elution of the unbound protein fraction with buffer B, bound material was eluted with a linear gradient of 0.1 to 1 M NaCl in buffer B over five column volumes, followed by one column volume of buffer B containing 1M NaCl.

Fractions were assayed for activity against Cbz-Arg-Arg-AMC, (Section 2.3.3), and the bound, active fractions pooled and retained for further fractionation by poly-(L-lysine) affinity chromatography (Section 3.7).

3.7 Poly-(L-lysine)-Sepharose-chromatography

3.7.1 Method

The active pool from Q-Sepharose chromatography at pH 8 (Section 3.6) was diluted five-fold in buffer B (Section 3.4.1), and loaded onto a poly-(L-lysine) affinity chromatography column (90 × 15 mm, 1 ml.min^{-1}), which had been pre-equilibrated with buffer B. After the elution of unbound material with buffer B, bound material was eluted with a linear gradient of 0.1 to 1 M NaCl in buffer B over ten column volumes, followed by 1 column volume of buffer B containing 1 M NaCl.

Fractions were assayed for activity against Cbz-Arg-Arg-AMC (Section 2.3.3), and the bound, active fractions pooled and retained for further fractionation by Sephacryl S-100 HR molecular exclusion chromatography (Section 3.8).

3.8 Sephacryl S-100 HR Chromatography

3.8.1 Method

The active pool from the poly-(L-lysine)-Sepharose column (Section 3.7) was concentrated by ultrafiltration (Section 2.6.1) to 200 µl, and applied to a Sephacryl S-100 HR molecular exclusion chromatography column (900 × 15 mm, 4°C, 0.32 ml.min^{-1}), which had been pre-equilibrated in buffer B (section 3.4.1). Fractions were assayed for activity against Cbz-Arg-Arg-AMC (Section 2.3.3), and the eluted active fractions pooled. The active pool was concentrated by ultrafiltration (Section 2.6.1) and sample purity evaluated by Tris-Tricine SDS-PAGE (Section 2.7.1).

3.9 Active-site titration of OP-Tb

It is often useful to know the active concentration, $[E]_0$, of a pure enzyme preparation, as a proportion of the purified enzyme may become irreversibly denatured during purification. A variety of methods exists for the active-site titration of trypsin-like enzymes. Chase and Shaw (1970) described the use of the nitrophenyl ester of *p*-guanidobenzoate (NPGB) for the active site titration of trypsin, plasmin and thrombin. This compound has found widespread use in this regard, where the enzyme is rapidly acylated with a stochiometric release of nitrophenol, followed by a very slow deacylation of the enzyme due to the instability of *p*-guanidobenzoyl-

enzymes (Kézdy and Kaiser, 1981). The limitation of the NPGB method is a lack of sensitivity. This method has a detection limit of down to 1 nmole of active enzyme, whereas, for quantitative measurements with an accuracy of above 5%, 0.1 μmoles of active enzyme is required. Jameson *et al.* (1973) described the use of a fluorogenic analogue of NPGB, 4-methylumbelliferyl-*p*-guanidobenzoate (MUGB), which operates on the same principle as NPGB but extends the sensitivity of the procedure to a detection limit of 1 pmole of active enzyme. Although this technique theoretically has a detection limit of 1 fmole, MUGB is subject to base-catalysed hydrolysis and this limits the sensitivity of the procedure, and underscores the importance of a blank.

3.9.1 Materials

MUGB titrant [1 mM 4-methylumbelliferyl-*p*-guanidobenzoate in dry DMF]. MUGB (3.7 mg) was dissolved in dry DMF (10 ml). The bottle was wrapped in aluminium foil to protect the reagent from exposure to light.

Assay buffer [100 mM sodium phosphate, 1 mM dithiothreitol, pH 7]. $NaH_2PO_4.2H_2O$ (1.56 g) and dithiothreitol (15 mg) were dissolved in dH_2O (80 ml), titrated to pH 7 with NaOH and made up to 100 ml with dH_2O.

Dilution buffer [100 mM sodium phosphate, pH 6]. $NaH_2PO_4.2H_2O$ (1.56 g) was dissolved in dH_2O (80 ml), titrated to pH 6 with NaOH and made up to 100 ml with dH_2O.

4-methylumbelliferone standard [10 mM 4-methylumbelliferone in dry DMF]. 4-methylumbelliferone (17.6 mg) was dissolved in dry DMF (10 ml). The bottle was wrapped in aluminium foil to protect the reagent from light.

3.9.2 Method

Enzyme (10 μg in 10 μl) was pre-incubated in assay buffer (20 μl) in a 1.5 ml microfuge tube (10 min, 37°C), after which MUGB titrant (2 μl) was added. The preparation was centrifuged in a Hägar HM2 bench-top microfuge to ensure complete mixing of the small volumes, and incubated (10 min, 37°C). A blank was prepared in the same manner, except the enzyme was replaced with buffer in the blank. After incubation, dilution buffer (800 μl) was added to the

preparation, the solution transferred to a spectrofluorimeter cuvette and fluorescence determined on a Hitachi F-2000 spectrofluorimeter (λ_{ex} 323 nm, λ_{em} 446 nm).

To facilitate quantitation of fluorescence, a calibration graph relating fluorescence units to picomoles of 4-methylumbelliferone was prepared. The 4-methylumbelliferone standard was diluted with dilution buffer to the necessary concentrations, and the fluorescence of 800 µl aliquots determined on a Hitachi F-2000 spectrofluorimeter (λ_{ex} 323 nm, λ_{em} 446 nm).

3.10 N-terminal sequence and amino-acid analysis of OP-Tb

Proteins may be sequenced by Edman degradation from their N-termini. During one cycle of this reaction, the N-terminal residue is removed from a polypeptide after labelling with Edman's reagent, phenylisothiocyanate (PITC), and identified by high-performance liquid chromatography (HPLC). The shortened polypeptide is left with a free N-terminus which can undergo further labelling and degradation. This procedure is now performed by automated gas-phase sequenators (Matsudaira, 1993). N-terminal analyses are usually performed on purified proteins which have been resolved by SDS-PAGE, and electroblotted on to PVDF membranes (Hulmes *et al.*, 1989).

Knowledge of the N-terminal sequence of a protein is useful for molecular cloning studies as it permits the construction of oligonucleotide probes with which full-length clones from gene libraries can be isolated. Furthermore, a number of protein sequence databases now exist where an N-terminal sequence may be compared with those held in the database, permitting the identification and classification of the protein of interest (Lottspeich, 1994).

A number of post-translational modifications of proteins may hinder the sequencing process, in particular, N-terminal blocking. The N-termini of up to 50% of eukaryotic cytosolic proteins are believed to be blocked. This may occur by acetylation of N-terminal residues, particularly serine and threonine residues; formylation of N-terminal methionine residues; or the formation of pyroglutamyl groups at the N-terminus. Such N-terminally blocked proteins cannot be sequenced by conventional Edman degradation (LeGendre *et al.*, 1993), and are either enzymatically or chemically fragmented, and the N-termini of these internal fragments are sequenced.

3.10.1 Materials

Stain solution [0.1% (m/v) Coomassie Brilliant Blue G-250 in 50% (v/v) methanol]. Coomassie Brilliant Blue G-250 (0.1g) was dissolved in 50% (v/v) methanol (100 ml).

Destain solution [50% (v/v) methanol]. Methanol (50 ml) was made up to 100 ml.

3.10.2 Methods

Tris-Tricine gels were prepared as described in Section 2.7.1.2 three days in advance, after which no reactive peroxide radicals should remain in the gel which may catalyse the artifactual N-terminal blocking of the enzyme (Matsudaira, 1987). Stacking gels were polymerised a day in advance for the same reason. Electrotransfer was accomplished as described in Section 2.7.5. Thioglycollic acid (0.1 mM) was added to the electro-transfer buffer (Section 2.7.5.1) to reduced the possibility of artifactual N-terminal blocking (LeGendre *et al.*, 1993). After completion of electro-blotting, the PVDF membrane was washed in dH_2O (5 min), immersed in stain solution (2 min), destain solution (10 min), then rinsed in dH_2O (2 × 5 min) and air-dried. N-terminal sequencing and amino-acid analysis were undertaken by the core sequencing facility of the British Medical Research Council Cambridge Centre, University of Cambridge, Cambridge, United Kingdom.

3.11 Results

3.11.1 Three-phase partitioning

The results of the optimisation of the three-phase partitioning for OP-Tb isolation are illustrated in Fig. 9. Activity against Cbz-Arg-Arg-AMC precipitated primarily in the 15-20% (m/v) $(NH_4)_2SO_4$ cuts, which contained 54% of the activity (Fig. 9). Additional activity precipitated in the 10-15% and 20-25% cuts. A 10-25% $(NH_4)_2SO_4$ cut was subsequently used in the purification of OP-Tb to maximise the yield of enzyme. Furthermore, the major lysosomal cysteine proteinase of *T. b. brucei*, trypanopain-Tb also precipitated over this range of $(NH_4)_2SO_4$. Thus, both enzymes were completely precipitated at this stage, facilitating the simultaneous purification of both enzymes from the same starting material. About 11% of the total activity was associated with the membrane and cytoskeletal fractions in the 0-5% cut, introducing the possibility of a membrane-bound form of the enzyme.

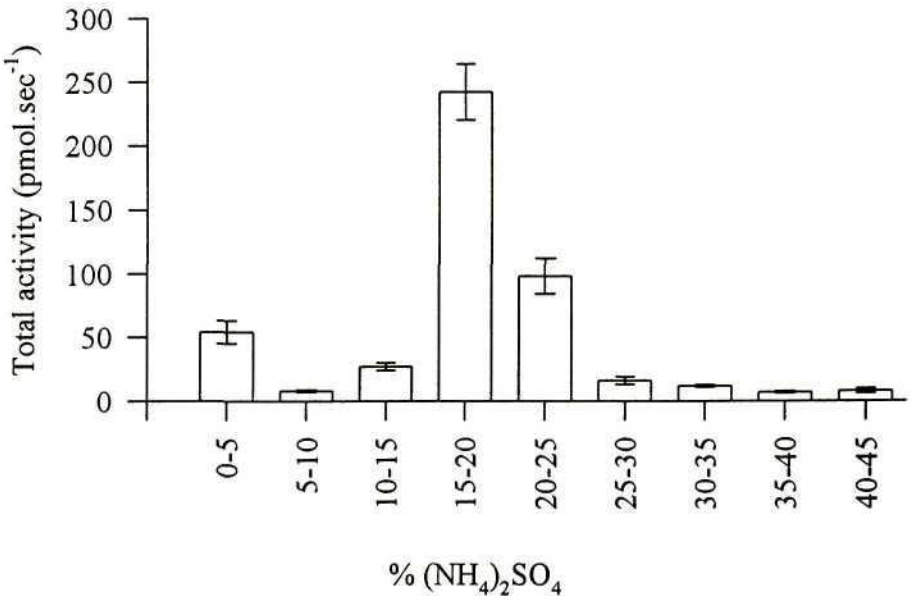


Figure 9. Optimisation of three-phase partitioning. *T. b. brucei* lysates were precipitated at various $(NH_4)_2SO_4$ concentrations, and assayed for activity against Cbz-Arg-Arg-AMC. Error bars represent the mean ± SEM (n = 3).

3.11.2 Q-Sepharose chromatography at pH 5.5

Activity against Cbz-Arg-Arg-AMC eluted in the unbound fraction from a Q-Sepharose column at pH 5.5, while activity against Cbz-Phe-Arg-AMC was retained on the column, eluting at about 250 mM NaCl (Fig. 10).

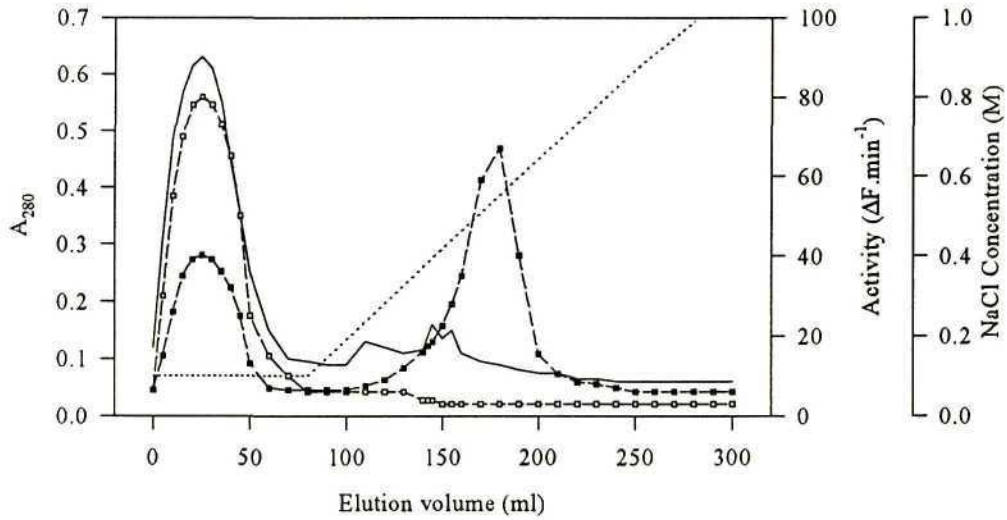


Figure 10. Anion-exchange chromatography of OP-Tb on Q-Sepharose at pH 5.5. Q-Sepharose (26 × 100 mm, 1 ml.min^{-1}) was equilibrated in buffer A [20 mM acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5] containing 100 mM NaCl. After elution of unbound protein with buffer A, bound protein was eluted with a linear gradient (····) of 0.1 to 1 M NaCl in buffer A over 5 column volumes. (——) A_{280}, (–□–) enzyme activity against Cbz-Arg-Arg-AMC, (–■–) enzyme activity against Cbz-Phe-Arg-AMC.

This reflects the separation of a trypsin-like activity attributable to OP-Tb from a cathepsin L-like activity attributable to trypanopain-Tb (Mbawa *et al.*, 1992; Troeberg *et al.*, 1996).

3.11.3 *p*-Aminobenzamidine-Sepharose chromatography

Application of the Q-Sepharose pH 5.5 active fraction (Section 3.4) to a *p*-aminobenzamidine column resulted in the separation of two fractions with activity against Cbz-Arg-Arg-AMC (Fig. 11). The bulk of the activity (84%) remained weakly bound to the column, eluting with a single-step salt gradient. A small amount of activity was also evident in the unbound fraction. This activity did not bind to the matrix, even after repeated passages over a previously unused batch of *p*-aminobenzamidine-Sepharose matrix. This enzyme activity was not characterised further, and probably represents an as yet unknown enzyme.

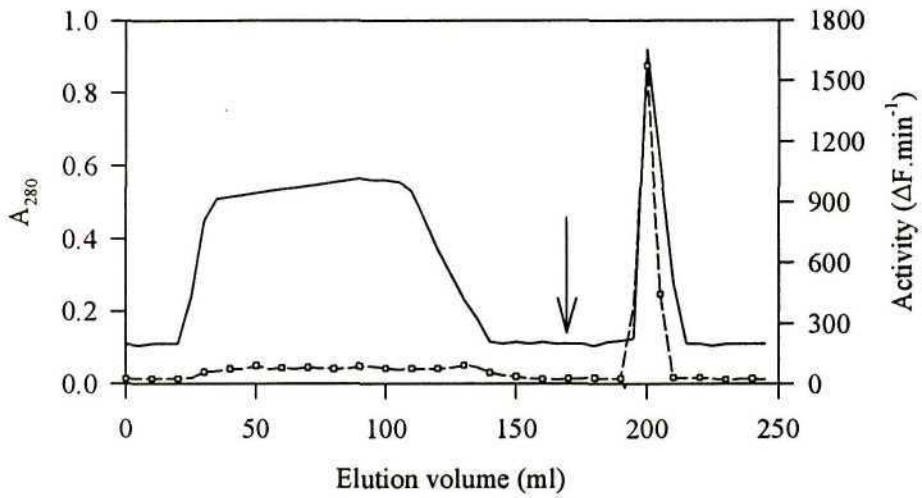


Figure 11. Affinity chromatography of OP-Tb on *p*-aminobenzamidine-Sepharose. *p*-Amino-benzamidine-Sepharose (120 × 15 mm, 0.32 $ml.min^{-1}$) was equilibrated in buffer B [50 mM Tris-Cl, 2 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. After elution of unbound protein with buffer B, bound protein was eluted by application of 250 mM NaCl in buffer B, in a single step, applied at the point indicated by the arrow (↓). (–) A_{280}, (–□–) enzyme activity against Cbz-Arg-Arg-AMC.

3.11.4 Q-Sepharose chromatography at pH 8

In contrast to chromatography on Q-Sepharose at pH 5.5 (Fig. 10), application of the active, bound fraction from the *p*-aminobenzamidine-Sepharose column (Section 3.5) to Q-Sepharose at pH 8 resulted in the Cbz-Arg-Arg-AMC activity binding to the column (Fig. 12). This activity was eluted at approximately 400 mM NaCl.

3.11.5 Poly-(L-lysine)-Sepharose chromatography

Application of the active fraction from Q-Sepharose chromatography at pH 8 (Section 3.6) to a poly-(L-lysine)-Sepharose column resulted in strong binding of the Cbz-Arg-Arg-AMC activity. This activity was eluted in a single, well-resolved peak at approximately 450 mM NaCl (Fig. 13).

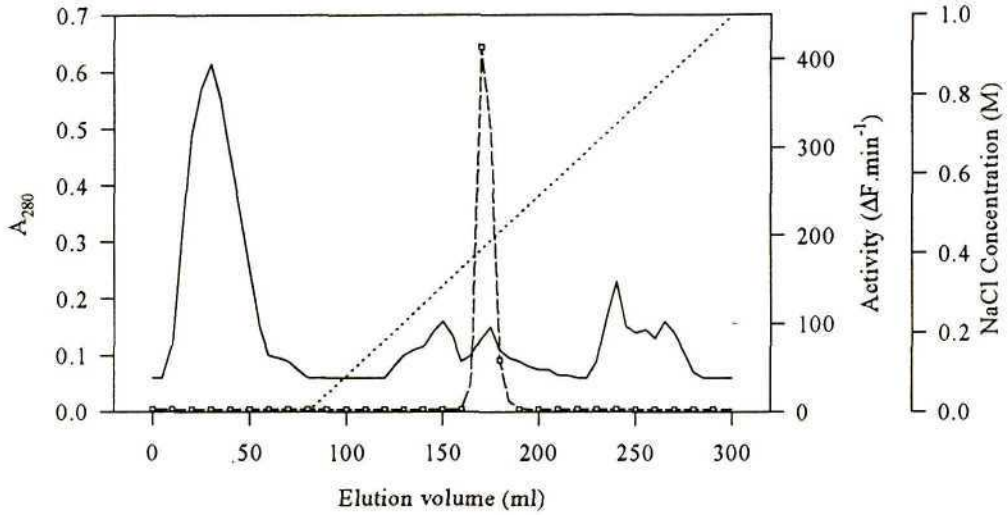


Figure 12. Anion-exchange chromatography of OP-Tb on Q-Sepharose at pH 8. Q-Sepharose (26 × 100 mm, 1 $ml.min^{-1}$) was equilibrated in buffer B [50 mM Tris-Cl, 1 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. After elution of unbound protein, bound protein was eluted with a linear gradient (·····) of 0.1-1 M NaCl in buffer B over 5 column volumes. (——) A_{280}, (–□–) enzyme activity against Cbz-Arg-Arg-AMC.

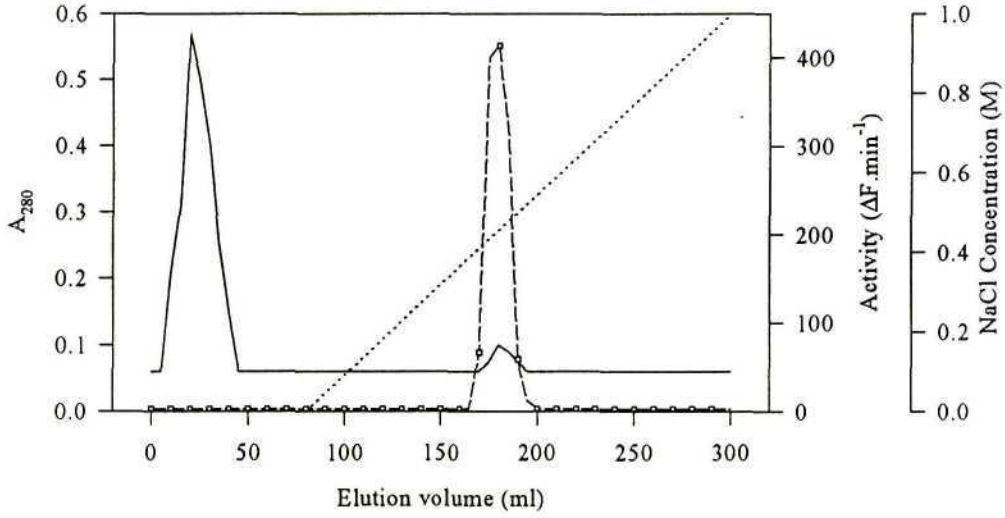


Figure 13. Affinity chromatography of OP-Tb on poly-(L-lysine)-Sepharose. poly-(L-lysine)-Sepharose (90 × 15 mm, 1 $ml.min^{-1}$) was equilibrated in buffer B [50 mM Tris-Cl, 1 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. After elution of unbound protein, bound protein was eluted with a linear gradient (·····) of 0.1-1 M NaCl in buffer B over 5 column volumes. (—) A_{280}, (–□–) enzyme activity against Cbz-Arg-Arg-AMC.

3.11.6 Sephacryl S-100 HR chromatography

Cbz-Arg-Arg-AMC activity was eluted in a single peak from a Sephacryl S-100 HR molecular exclusion column (Fig. 14), well resolved from low molecular mass material which eluted at approximately V_t. Calibration of the column (Section 2.5.2) revealed that the Cbz-Arg-Arg-AMC activity eluted at a molecular mass corresponding to a molecular mass of approximately 100 kDa.

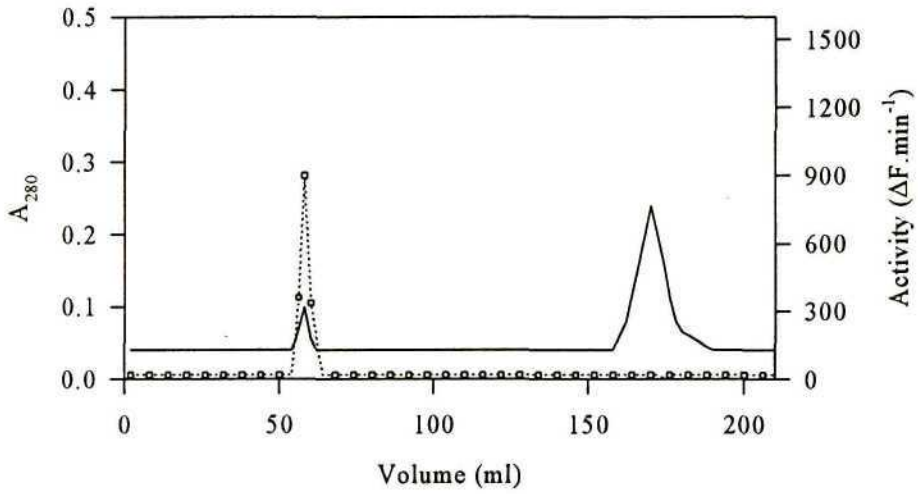


Figure 14. Molecular exclusion chromatography of OP-Tb on Sephacryl S-100 HR. Sephacryl S-100 HR (900 × 15 mm, 4°C, 0.32 $ml.min^{-1}$) was equilibrated in buffer B [50 mM Tris-Cl, 1 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. Sample (200 µl) was loaded, and protein eluted with buffer B. (——) A_{280}, (··□··) enzyme activity against Cbz-Arg-Arg-AMC.

3.12 Evaluation of purification

Evaluation of sample purity by Tris-Tricine SDS-PAGE is illustrated in Fig. 15, on which the active enzyme fraction from Sephacryl S-100 HR chromatography (Section 3.11.6; Fig. 14) gives rise to a single band at approximately 80 kDa. This estimated molecular mass compares well with that of potentially related enzymes from *E. coli* (82 kDa; Kanatani *et al.*, 1991) and *T. cruzi* (80 kDa; Burleigh *et al.*, 1997). A purification table for the isolation of OP-Tb is presented as Table 6. OP-Tb was isolated from *T. b. brucei* lysates in a six-step procedure with a 28% yield.

Table 6. Purification table for the isolation of OP-Tb from *T. b. brucei* lysates.

Fraction	Volume (ml)	Total Protein (mg)	Total Activity ($pmol.sec^{-1}$)	Specific Activity ($pmol.sec^{-1}.mg^{-1}$)	Purification (fold)	Yield (%)
Lysate	17.5	213	3245	15	1	100
TPP	15	9	2044	227	15	63
HiLoad Q (pH 5.5)	52.5	8.6	1947	226	15	60
Benzamidine	31	0.211	1429	6773	451	44
HiLoad Q (pH 8)	20	0.134	1428	10656	710	44
Poly-(L-lysine)	6	0.104	941	9048	603	29
Sephacryl S-100HR	0.2	0.068	909	13367	891	28

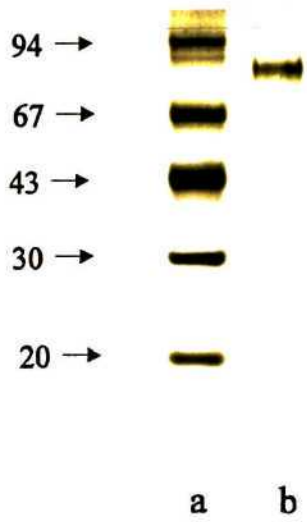


Figure 15. Evaluation of OP-Tb purification by Tris-Tricine SDS-PAGE. (a) molecular mass markers (as in Section 2.7.1.1); (b) Sephacryl S-100 HR active pool (200 ng). Samples were resolved by reducing Tris-Tricine SDS-PAGE (Section 2.7.1) and protein visualised by silver staining (Section 2.7.4).

3.12.1 Active-site titration

When a solution of enzyme containing 10 μg of OP-Tb was incubated with the MUGB, 51 pmoles of 4-methylumbelliferone was liberated. As the number of moles of 4-methylumbelliferone liberated is equivalent to the number of moles of active enzyme, it follows that there are 51 pmole of active enzyme in 10 μg of the OP-Tb sample. Assuming a M_r of 80 kDa, and assuming one active-site per enzyme, 51 pmoles is equivalent to a mass of 4.08 μg of active OP-Tb. The OP-Tb preparation was therefore 40.8% active.

3.12.2 N-terminal and amino acid analysis

N-terminal analysis of OP-Tb revealed that the enzyme was N-terminally blocked and it was therefore not possible to determine the N-terminal sequence. However, an amino acid analysis was obtained for the enzyme, and is illustrated in Table 7, along with the amino acid analyses of two other trypsin-like serine oligopeptidases, for comparison. The amino acid composition of OP-Tb was from the analysis of a protein sample, while the composition of the *T. cruzi* and *E. coli* enzymes was from deduced amino acid sequences obtained from their genes. Hence, no values for methionine, tryptophan and cysteine were obtained for the OP-Tb enzyme.

Table 7. Amino acid composition of OP-Tb.

Amino Acids	Composition[a]		
	OP-Tb	*T. cruzi* oligopeptidase B[b]	*E. coli* oligopeptidase B[c]
Ala	33	43	48
Arg	59	43	49
Asp+Asn	70	74	68
Cys	n.d.	11	6
Glu+Gln	77	59	76
Gly	66	51	47
His	26	20	24
Ile	36	33	28
Leu	83	54	68
Lys	34	32	27
Met	n.d.	21	18
Phe	8	34	27
Pro	29	39	36
Ser	36	43	40
Thr	27	48	38
Trp	n.d.	12	15
Tyr	30	35	50
Val	112	59	42

[a]Composition of OP-Tb was from amino acid analysis of a protein sample, while the composition of the *T. cruzi* and the *E. coli* enzymes is from deduced amino acid sequences from their respective genes. Hence, no values for methionine, tryptophan and cysteine were obtained for the OP-Tb enzyme.
[b]After Burleigh *et al.* (1997).
[c]After Kanatani *et al.* (1991).

Internal sequences were obtained for OP-Tb by digestion of the enzyme with endoproteinase Lys-C (E.C. 3.4.99.30). The resultant OP-Tb-derived peptides were resolved by reverse-phase high-performance liquid chromatography (RP-HPLC), and the individual peptides subjected to N-terminal sequencing. Three OP-Tb-derived peptides were obtained from endoproteinase Lys-C digestion, and the sequences of all three peptides displayed homology to the primary sequences of oligopeptidase B from *T. cruzi*, *E. coli* and *M. lacunata* (Fig. 16).

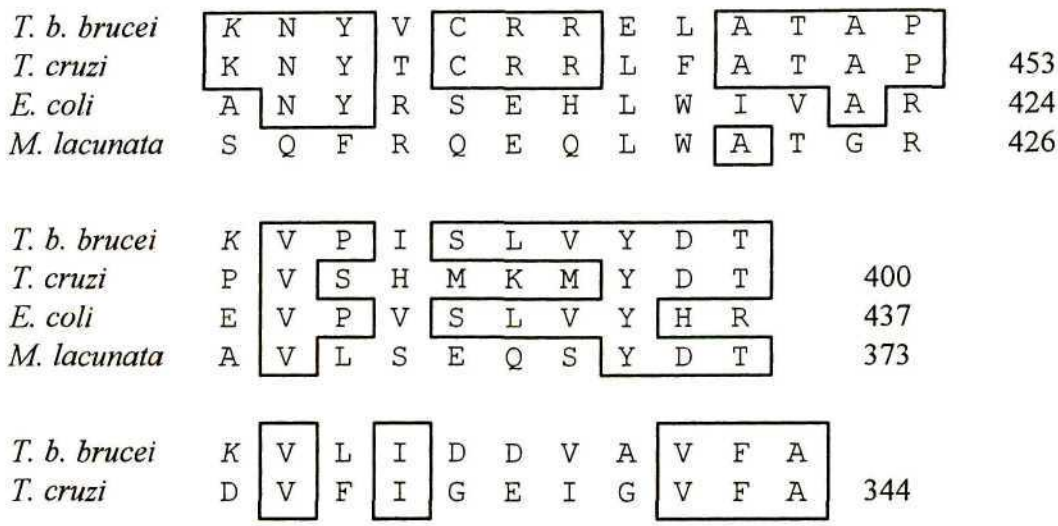


Figure 16. Sequences of OP-Tb-derived peptides. OP-Tb was cleaved with endoproteinase Lys-C, and the resultant peptides were resolved by reverse-phase high-performance liquid chromatography. Peptides sequences were obtained from N-terminal sequencing of the individual peptides. Sequences from the *T. b. brucei* enzyme were compared with those from *T. cruzi* oligopeptidase B (Burleigh *et al.*, 1997), *E. coli* oligopeptidase B (Kanatani *et al.*, 1991) and *M. lacunata* oligopeptidase B (Yoshimoto *et al.*, 1995). The first residue in each *T. b. brucei*-derived peptide is assumed to be lysine and is therefore shown in italics.

3.13 Discussion

OP-Tb was purified to electrophoretic homogeneity for the first time in this study from *T. b. brucei* lysates in a six-step procedure with a 28% yield. A most useful step in the procedure was the crude fractionation of trypanosome lysates by TPP. OP-Tb activity was precipitated in the 10-25% $(NH_4)_2SO_4$ cut. This activity co-precipitated with that of the major cysteine proteinase of *T. b. brucei*, trypanopain-Tb. This was fortuitous as it permitted the simultaneous purification of the two enzymes studied in our laboratory from the same starting material. Due to the difficulty in obtaining sufficient starting material, an effort was made to co-purify the two proteinases from the same limited starting material. For this reason, the first two steps of the OP-Tb purification were performed at pH 5.5, at which trypanopain-Tb is most stable.

Although this pH had no direct adverse affects on OP-Tb, it was found that trypanopain-Tb degraded OP-Tb resulting in a lower yield (results not shown). The next step in the purification was therefore designed to rapidly separate these two proteolytic activities.

The two activities were completely separated by anion-exchange chromatography on Q-Sepharose at pH 5.5. Trypanopain remained bound to the column while OP-Tb eluted in the unbound fraction. Despite the fact that there was no evident increase in the specific activity of OP-Tb (Table 6), this was an essential step as it removed all contaminating trypanopain-Tb early in the purification scheme with only a small loss in yield (3%). This behaviour on Q-Sepharose may be explained in terms of the isoelectric point of OP-Tb. Kornblatt *et al.* (1992) suggested a pI of 5.1 for this enzyme and OP-Tb is thus not expected to bind this matrix at pH 5.5. In contrast, trypanopain-Tb was reported to bind quarternary amine resins at pH 5.5 (Troeberg *et al.*, 1996). Therefore, anion-exchange chromatography at pH 5.5 provided a convenient means of purifying the two enzymes to be purified from the same trypanosome preparation.

Due to the trypsin-like specificity of the enzyme, where cleavage occurs preferentially after arginine and lysine residues, the arginine analogue, *p*-aminobenzamidine, was selected as an affinity ligand for the next step in the purification. OP-Tb bound weakly to *p*-aminobenzamidine-Sepharose, and was eluted at 250 mM NaCl, resulting in a 30-fold increase in the specific activity of the active fraction. Unfortunately, the weak affinity of OP-Tb for the matrix did not permit the loading or washing of the column with a high-salt buffer. Consequently, the *p*-aminobenzamidine-Sepharose also acted as an ion-exchange column, and many protein contaminants were eluted with the bound, active pool. A small amount of activity against Cbz-Arg-Arg-AMC was evident in the unbound *p*-aminobenzamidine-Sepharose fraction. This activity did not bind the affinity matrix after repeated passage over the matrix, or over unused matrix, and resulted in a loss of 16% of the activity. This activity was therefore attributed either to an altered or complexed form of OP-Tb which could not bind to the matrix, or to another proteinase with activity against Cbz-Arg-Arg-AMC. The pro-protein convertases (Lazure *et al.*, 1983; reviewed by Nakayama, 1997) and the proteasome (Hua *et al.*, 1996; Lomo *et al.*, 1997) are potential candidates.

A 68 kDa protein proved to be a persistent contaminant throughout the purification. This contaminant was identified by N-terminal analysis as rat serum albumin. Although chromatography on Cibacron Blue F3GA-Sepharose is a conventional method of removing albumin from protein preparations (Travis *et al.*, 1976), this method did not successfully remove all of the contaminating albumin from OP-Tb preparations (results not shown). This is explained by the observation of Naval *et al.* (1982) that while Cibacron Blue F3GA-Sepharose binds up to 100% of human serum albumin, it will only bind up to 80% of the albumin present in rat sera. Hydrophobic chromatography on phenyl-Sepharose, another frequently used method for removing albumin (Belew *et al.*, 1985) could not be employed as OP-Tb eluted from phenyl-Sepharose at the same salt concentration as albumin (results not shown). The observation that polyamines influence the activity of OP-Tb (Section 5.11.7, Table 12) suggested that a polyamine affinity matrix may be useful in the affinity chromatography of OP-Tb. Therefore, the bound active fraction from Q-Sepharose at pH 8 was applied to a poly-(L-lysine)-Sepharose affinity matrix. OP-Tb bound relatively strongly to this matrix, eluting at 450 mM NaCl, successfully separating the enzyme from albumin and a number of other protein contaminants which were eluted in the unbound fraction. Molecular exclusion chromatography on Sephacryl S-100 HR successfully removed the remaining protein contaminants, and desalted the OP-Tb preparation. The resultant protein preparation was homogenous by SDS-PAGE (Fig. 15), yielding a single band at approximately 80 kDa.

Active-site titration of the purified enzyme with MUGB revealed that the enzyme preparation was 40.8% active. The addition of dithiothreitol to the chromatography buffers considerably (10-fold) enhanced the final yield of enzyme, and it is possible that during the isolation procedure an essential thiol group is oxidised by oxidising agents present in the isolation reagents or liberated from the trypanosomes when they are ruptured. This may account, in part, for the reduced activity of the preparation. This hypothesis is supported by enzymatic studies with OP-Tb, which have revealed that OP-Tb activity is enhanced by reducing agents (Section 5.11.4, Fig. 31). Furthermore, thiol-blocking reagents irreversibly extinguished the activity of the enzyme (Section 5.11.6, Table 11), suggesting an essential free thiol group near the active site which must be maintained in its reduced state if the enzyme is to retain its activity.

N-terminal analysis of OP-Tb revealed that this enzyme had a blocked N-terminus. N-terminal blocking is a common phenomenon amongst cytosolic proteins (Brown and Roberts, 1976). Matheson *et al.* (1995) isolated an oligopepidase from *P. velutina* pollen which was also N-terminally blocked. However, the N-terminal sequences of a number of bacterial oligopeptidases have been obtained, including those from *E. coli* (Kanatani *et al.*, 1991), *M. lacunata* (Yoshimoto *et al.*, 1995) and *F. meningicoseptum* (Yoshimoto *et al.*, 1991). It would therefore appear that the N-termini of prokaryotic oligopeptidases are not blocked. Sequences was obtained, however, for three peptides obtained from OP-Tb, following incubation with endoproteinase Lys-C. All three peptides displayed homology to the primary sequences of oligopeptidase B from *T. cruzi*, and to a lesser degree, to the primary sequences of oligopeptidase B from *E. coli* and *M. lacunata*. These data suggest that OP-Tb is indeed a member of the prolyl oligopeptidase family of serine proteases, to which the oligopeptidase B enzymes belong (Barrett and Rawlings, 1995). This can only be confirmed when the entire OP-Tb sequence is known.

OP-Tb had approximately four-fold less phenylalanine residues, and twice as many valine residues as potentially related enzymes from *T. cruzi* and *E. coli* (Table 7). In contrast, there was approximately the same number of histidine, lysine and isoleucine residues in all three enzymes. While amino acid analysis data were not particularly useful on their own, they did assist in the interpretation of inhibition data for polysulfated naphthylamine inhibitors of OP-Tb, which bind to basic residues in proteolytic enzymes (Section 7.6.1).

The purification of OP-Tb to electrophoretic homogeneity facilitated the generation of polyclonal antibodies against OP-Tb which allowed the immunoaffinity purification of a related enzyme from *T. congolense* (Chapter 4). Following the purification of these enzymes they were comprehensively characterised (Chapter 5), which further facilitated investigations into how the enzyme may contribute to the pathogenesis of African trypanosomiasis through its interaction with host molecules (Chapter 6). The purification of these enzymes also allowed their potential as novel chemotherapeutic targets to be evaluated (Chapter 7).

Chapter 4

Immunochemical studies

4.1 Introduction

As a result of the high degree of specificity of an antibody for its antigen, antibodies are extremely useful reagents for detection of specific molecules. This lends itself to a variety of biological applications. Indeed, antibodies are exploited in many situations. In medicine for sero-diagnostics and immunotherapy, in research for immunoaffinity purification and in immunocytochemistry for localisation of intracellular compounds. It was thus desirable to generate antibodies against OP-Tb.

It is generally accepted that parasitaemia is controlled by anti-VSG antibody responses, stimulated in response to each new VSG variant (reviewed in Roelants and Pinder, 1984). Following antibody-mediated destruction of parasites, a wide variety of invariant antigens are released, and it has been suggested that these antigens may be involved in producing the pathology associated with the disease (discussed in Chapter 6; Mansfield, 1990). It has been proposed for malaria that immune recognition of such parasite "toxins" could protect the host against the pathological effects of infection (Playfair *et al.*, 1990). The cysteine proteinases of parasitic protozoa and helminths are known to be antigenic in human infections. Sera from patients with chronic Chagas' disease contain anti-cruzipain antibodies (Martínez *et al.*, 1991), and IgG_1 and IgG_4 antibodies are generated against schistosome cathepsins B and L in *Schistosoma mansoni*-infected hosts (Grogan *et al.*, 1997). Such antibodies may have an immunoprotective function, as immunisation with cruzipain enhanced the percentage survival of mice subsequently challenged with 10^3 trypomastigotes (Laderach *et al.*, 1996).

Boran cattle (*Bos indicus*), which represents the vast majority of cattle in Africa, are highly susceptible to trypanosomiasis, whilst N'Dama cattle (*Bos taurus*) are known to be resistant or "trypanotolerant" (Roelants, 1986). Trypanotolerance is defined as the genetically determined ability to limit the multiplication of the parasite and to resist the pathogenic effects of infection (reviewed by Trail *et al.*, 1989). Although the mechanisms of trypanotolerance are not understood, an effective immune response to invariant trypanosome antigens may be an

important mechanism of resistance to the disease. In support of this, the antibody response to two trypanosome invariant antigens, with molecular masses of 69 and 33 kDa, appeared to correlate with trypanotolerance (reviewed by Authié, 1994; Authié *et al.*, 1994). Gene sequence analysis revealed that the 69 kDa antigen is homologous to the mammalian immunoglobulin heavy-chain-binding protein (BiP) (Boulangé and Authié, 1994). The 33 kDa antigen has been identified as congopain, the major lysosomal cysteine proteinase of *T. congolense* (Authié *et al.*, 1992; 1993a). N'Dama cattle have a considerably elevated IgG_1 response to both antigens as well as other antigens, when compared to Boran cattle, while there is little difference in the IgM profiles of both species (Authié *et al.*, 1993b). Such antigens may provide a basis for an anti-trypanosome vaccine, and it was therefore of interest to determine whether infected bovine hosts generate antibodies to trypanosome oligopeptidases, and furthermore, whether the generation of anti-oligopeptidase antibodies correlates with natural resistance to trypanosome infections.

In the present study, antibodies were raised in chickens against OP-Tb (Section 4.2), antibody production was monitored by enzyme-linked immunosorbent assay (ELISA, Section 4.3), and antibody specificity was determined by western blotting (Section 4.4). The effects of the anti-OP-Tb antibodies on the activity of OP-Tb (Section 4.5) and on live *T. b. brucei* (Section 4.6) were also investigated, as was their potential use for the purification of a trypanosome oligopeptidase from *T. congolense*. Finally, the generation of anti-oligopeptidase antibodies in *T. congolense*-infected hosts was investigated (Section 4.7).

4.2 Production of anti-OP-Tb antibodies

Chickens are highly suitable experimental animals for the production of polyclonal antibodies (Polson *et al.*, 1980a). Chickens are easily handled, have a very accessible immunisation site on the breast muscle, and conveniently package large quantities of antibodies in their egg-yolks, from which the antibody fraction is easily isolated (Polson *et al.*, 1980b, 1985). Generally, the larger the immunogen, the more immunogenic it is (Catty and Raykundalia, 1988), and no problems were therefore anticipated, or encountered, in preparing antibodies against the 80 kDa OP-Tb.

4.2.1 Materials

100 mM sodium phosphate buffer [100 mM sodium phosphate, 0.02% (m/v) NaN_3, pH 7.6]. $NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) were dissolved in dH_2O (950 ml), titrated to pH 7.6 with NaOH, and made up to 1 litre.

4.2.2 Method

Chickens were immunised by intramuscular injection, at two sites in the breast muscle, with a total of 20 µg OP-Tb emulsified in a 1:1 (v/v) ratio with adjuvant. Chickens were boosted at 3, 7, and 11 weeks after the first immunisation, with 10 µg of OP-Tb per booster. The initial immunisation was done with OP-Tb emulsified in FCA, and subsequent immunisations with OP-Tb emulsified in FIA. Chicken eggs were collected on a daily basis.

Chicken egg-yolk antibodies (IgY) were isolated by a method adapted from those of Polson *et al.* (1980a) and Polson *et al.* (1985). Yolks were separated from the egg-white and washed carefully under running water to remove as much egg-white as possible. The yolk sac was punctured, and the yolk volume determined in a measuring cylinder. Two volumes of 100 mM sodium phosphate buffer were added to the yolk, and mixed thoroughly. Solid PEG 6 000 was added to 3.5 % (m/v) (i.e. 3.5 g per 100 ml yolk-buffer suspension), and dissolved by gentle stirring. The precipitated vitellin fraction was removed by centrifugation (4 420 × *g*, 30 min, RT), and the supernatant filtered through absorbent cotton wool to remove the lipid fraction. The PEG concentration was brought to 12% (m/v) (i.e. 8.5 g PEG 6 000 was added per 100 ml supernatant), and dissolved by gentle stirring. The suspension was centrifuged (12 000 × *g*, 10 min, RT) to pellet the IgY-containing precipitate, and the supernatant was discarded. The precipitate was redissolved in 100 mM sodium phosphate buffer, in a volume equal to the volume obtained after filtration, and the IgY fraction was re-precipitated by the addition of PEG 6 000 to 12% (m/v), which was dissolved by gentle stirring. The IgY fraction was pelleted by centrifugation (12 000 × *g*, 10 min, RT), and the supernatant discarded. The pellet was redissolved in 1/6 of the original egg-yolk volume in 100 mM sodium phosphate buffer, and stored at 4°C. Antibody concentrations were determined directly from the A_{280} of the IgY preparations in 100 mM sodium phosphate buffer, as described in Section 2.2.2.3, using an IgY extinction coefficient of 1.25 $(mg.ml^{-1})^{-1}.cm^{-1}$ (Coetzer, 1985).

4.3 Enzyme-linked immunosorbent assay

Antibody production in experimental animals is conveniently monitored by ELISA, originally introduced by Engvall and Perlmann (1971), as this assay is highly sensitive and is surpassed only by radioimmunoassays (Butler, 1980). Briefly, antigen is coated to the plastic surface of the wells of polystyrene microtitre plates, and the primary antibodies to be quantified are incubated with the insolubilised antigen, and residual primary antibody washed away. Non-ionic detergents such as Tween® 20 are usually included in the washing solution to prevent any non-specific protein interactions. After excess antibody has been washed away, a secondary antibody, raised against the primary antibody, to which a marker enzyme has been conjugated, is added to the wells of the microtitre plate (Clarke and Engvall, 1981). This "sandwich" has the added advantage of amplifying the response, as more than one secondary antibody, and hence more reporter enzyme, may bind to a single primary antibody. The enzyme reacts with a substrate which yields a soluble, coloured, reaction product, which can be quantified spectrophotometrically (Kemeny and Chantler, 1988). In the present study, horseradish peroxidase (HRPO) (EC 1.11.1.7) was employed as the reporter enzyme, and 2,2′-azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulfonate] (ABTS) was used as the substrate. ELISAs were employed in the present study to evaluate the production of polyclonal antibodies generated against OP-Tb in chickens during the immunisation programme.

4.3.1 Materials

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 were dissolved in dH_2O (950 ml), titrated to pH 7.2, and made up to 1 litre.

0.5% (m/v) Bovine serum albumin in PBS (BSA-PBS). BSA (0.5 g) was dissolved in PBS (100 ml).

0.1% Tween® in PBS (PBS-Tween). Tween® 20 (1 ml) was made up to 1 litre in PBS.

0.15 M citrate-phosphate buffer, pH 5.0. A 0.15 M solution of citric acid.H_2O (21.0 $g.l^{-1}$) was titrated with a 0.15 M solution of $Na_2HPO_4.2H_2O$ (35.6 $g.l^{-1}$) to pH 5.0.

Substrate solution [0.05% (m/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]. ABTS (7.5 mg) and H_2O_2 [7.5 µl of a 30% (v/v) solution] were dissolved in citrate-phosphate buffer, pH 5.0 (15 ml).

Stopping buffer [0.1% (m/v) NaN_3 in citrate-phosphate buffer, pH 5.0]. NaN_3 (0.1 g) was made up to 100 ml in citrate-phosphate buffer, pH 5.0.

4.3.2 Method

Nunc Immuno F polystyrene microtitre plates were coated with antigen (1 $\mu g.ml^{-1}$, 100 µl per well) in PBS overnight at 4°C. Uncoated areas of wells were blocked with BSA-PBS (200 µl per well) for 1 h at 37°C and washed 3 times with PBS-Tween. Appropriate dilutions of primary chicken antibodies in BSA-PBS (1 $mg.ml^{-1}$-1 $\mu g.ml^{-1}$) were added (100 µl per well), incubated for 1 h at 37°C and excess antibody removed by rinsing the wells with PBS-Tween (3 × 200 µl). A 1:5 000 dilution of sheep anti-rabbit IgG-horseradish peroxidase (HRPO) conjugate in BSA-PBS, was added (120 µl per well) and incubated for 1 h at 37°C. The ABTS substrate (150 µl per well) was added and incubated in the dark for optimal colour development (usually 10-20 min). The enzyme reaction was stopped by the addition of 0.1% (m/v) NaN_3 in citrate-phosphate buffer (50 µl per well) and the absorbance determined at 405 nm in a Bio-Tek EL307 ELISA plate reader.

4.4 *Western blotting*

Western blotting is a convenient means of determining the specificity of an antibody preparation. Western blotting entails the electroblotting of a purified protein or a complex protein mixture, resolved by SDS-PAGE, onto an insoluble matrix such as nitrocellulose (Towbin *et al.*, 1979). Unoccupied protein binding sites are blocked non-specifically with low-fat milk or BSA, after which the blot is probed with antibodies raised against the proteins of interest (the primary antibody). Antigen-antibody complexes are visualised with a secondary antibody directed against the primary antibody, and conjugated to a reporter enzyme, which catalyses a reaction that leads to the deposit of an insoluble, coloured, product at the site of the reaction (Wilson and Goulding, 1986). In the present study, an alkaline phosphatase (AP) (EC 3.1.3.1) reporter enzyme was employed, using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as substrates (McInnes and Symons, 1989).

The method used in the present study is essentially the same as that of Towbin *et al.* (1979), with the exception that proteins were electroblotted with Caps buffer (pH 11) instead of Tris-glycine blotting buffer (pH 8.3) (Section 2.7.5). In the present study, western blotting was employed to determine the specificity of antibodies raised against purified OP-Tb, and to investigate whether infected hosts generate their own antibodies against trypanosome oligopeptidases.

4.4.1 Materials

Tris-buffered saline (TBS, 20 mM Tris-Cl, 200 mM NaCl, pH 7.4). Tris (2.42 g) and NaCl (11.7 g) were dissolved in dH_2O (950 ml), adjusted to pH 7.4 with HCl and made up to 1 litre.

0.5% (m/v) BSA-TBS. BSA (0.5 g) was dissolved in TBS (100 ml).

Substrate [100 mM Tris-Cl, 0.15 $mg.ml^{-1}$ BCIP, 0.3 $mg.ml^{-1}$ NBT, 5 mM $MgCl_2$]. Substrate was obtained pre-mixed in tablet form from Sigma. One tablet was dissolved in dH_2O (10 ml).

0.1% (m/v) Ponceau S. Ponceau S (0.1 g) was dissolved in 1% (v/v) glacial acetic acid.

4.4.2 Method

Purified proteins or complex protein mixtures were resolved by Tris-Tricine SDS-PAGE (Section 2.5.2) and electroblotted to nitrocellulose membranes (Section 2.5.6). Following electrotranfer of proteins, the nitrocellulose sheet was briefly rinsed with dH_2O (2 × 1 min) air-dried overnight, transiently stained with Ponceau S (30 sec), and rinsed in dH_2O to remove background stain. Positions of M_r standards were marked on the nitrocellulose in pencil, and the blot completely destained by the addition of a few drops of 500 mM NaOH to the dH_2O. Unoccupied protein binding sites were blocked by incubating the nitrocellulose strips in 0.5% (m/v) BSA-TBS (1 h, RT). The blot was washed with TBS (3 × 5 min), incubated with an appropriate dilution of anti-OP-Tb IgY in 0.5% (m/v) BSA-TBS (2 h, RT), rinsed with TBS (3 × 5 min) and incubated with a 1:20 000 dilution of rabbit anti-chicken IgY-AP conjugate in 0.5% (m/v) BSA-TBS. Excess conjugate was removed by rinsing with TBS (3 × 5 min), after which the blots were immersed in BCIP/NBT substrate solution and allowed to react in the dark until dark bands were evident against a lightly-stained background. The reaction was terminated by washing the blots in dH_2O and drying them between sheets of filter-paper.

4.5 Inhibition of OP-Tb activity by anti-OP-Tb antibodies

4.5.1 Method

Inhibition of the OP-Tb-catalysed hydrolysis of Cbz-Arg-Arg-AMC by anti-OP-Tb antibodies was evaluated using assays essentially as described in Section 2.3.2. OP-Tb (≃20 ng in 10 µl, 0.1 pmole, active concentration) was diluted with Brij 35 to 500 µl, and added to antibody solution (1 $mg.ml^{-1}$, 500 µl, yielding a final antibody concentration of 500 $\mu g.ml^{-1}$). This mixture was incubated at 37°C for 10 min, after which an aliquot (500 µl) was removed, and pre-incubated with 4 × assay buffer (250 µl, 37°C, 10 min), prior to the addition of substrate (250 µl). Change in fluorescence was monitored continuously for 5 min at 37°C (Section 2.3.2). Strongly inhibitory antibodies were titrated out in the same way, except that serial two-fold dilutions of antibody preparations replaced the 1 $mg.ml^{-1}$ antibody solution.

4.6 Effects of antibodies on trypanosomes in culture

In order to determine whether anti-OP-Tb antibodies had any affect on live trypanosomes, trypanosomes were cultivated in the presence and absence of the week 13 anti-OP-Tb antibodies, which demonstrated the greatest effect on OP-Tb activity (Section 4.9.3, Fig. 19). Cell culture was undertaken in two different media: one containing heat-inactivated FCS, and another containing native FCS, to explore the effect of active complement components on parasite health. Cell culture was undertaken and parasite numbers determined essentially as described in Section 2.8.2.

4.7 Immunoaffinity purification of OP-Tc

The generation of antibodies to OP-Tb (Section 4.9.1), and their demonstrated cross-reactivity with a related enzyme from *T. congolense* (Section 4.9.2, Fig. 18) suggested that they may be useful tools for the immunoaffinity purification of such an enzyme from *T. congolense* lysates. The diseases caused by the humoral (*T. brucei*-subgroup) and the haematic (*T. congolense*-subgroup) trypanosomes have considerably different pathologies (Section 1.1.6). Since we are investigating potential roles played by trypanosome oligopeptidases in the pathogenesis of African trypanosomiasis, it was interesting to compare the enzymatic characteristics of oligopeptidases from both humoral and haematic trypanosomes. This necessitated the purification of the related oligopeptidase from *T. congolense*, which we have called OP-Tc.

4.7.1 Materials

2 M Na_2CO_3. Na_2CO_3 (21.19 g) was dissolved in dH_2O (100 ml).

1 M ethanolamine-Cl, pH 8.0. Ethanolamine (6.06 ml) was diluted in dH_2O (80 ml), titrated to pH 8.0 with HCl and made up to 100 ml.

Coupling buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3). Na_2CO_3 (5.3 g) and $NaHCO_3$ (4.2 g) were each dissolved separately with NaCl (14.61 g) in dH_2O (500 ml). The $NaHCO_3$ solution (250 ml) was titrated against the Na_2CO_3 solution to pH 8.3.

Cyanogen bromide (1 $g.ml^{-1}$). CNBr (10 g) was dissolved in acetonitrile (10 ml).

Elution buffer [3.5 M NaSCN]. NaSCN (7.1 g) was dissolved in loading buffer (25 ml).

Loading buffer [50 mM Tris-Cl, 1 M NaCl, 10 mM $CaCl_2$, 0.5% (m/v) Brij-35, 0.02% (m/v) NaN_3, pH 7.4]. Tris (6.06 g), NaCl (58.44 g), $CaCl_2$ (1.47 g), Brij-35 (5 g) and NaN_3 (0.2 g) were dissolved in dH_2O (950 ml), titrated to pH 7.4 with HCl and made up to 1 litre.

Wash A (200 mM $NaHCO_3$, pH 9.6). A 200 mM $NaHCO_3$ solution (100 ml) was titrated against the a 200 mM Na_2CO_3 solution to pH 9.6.

Wash B (100 mM $NaHCO_3$, pH 9.2). A 100 mM $NaHCO_3$ solution (100 ml) was titrated against a 100 mM Na_2CO_3 solution to pH 9.2.

4.7.2 Preparation of immunoaffinity matrix

Wet Sepharose-4B (15 ml, packed gel volume) was washed in a Büchner funnel with dH_2O, allowed to settle in a small (50 ml) beaker and the supernatant solution aspirated off. Distilled H_2O (15 ml) and 2 M Na_2CO_3 (30 ml) were added to the gel, and the slurry was put on ice under a fume-hood and slowly mixed with a magnetic stirrer. Once the slurry had cooled, the rate of stirring was increased and the CNBr solution in acetonitrile (2 ml) added, all at once. The slurry was stirred quickly (2 min), transferred to a Büchner funnel and washed successively with 100 ml each of dH_2O, wash A and wash B.

Anti-OP-Tb IgY (15 mg) was dissolved in coupling buffer (10 ml), and dialysed against coupling buffer (100 ml, 16 h, 4°C). The activated Sepharose-4B (10 ml) was resuspended in coupling buffer (10 ml) to which the anti-OP-Tb solution (10 ml) was added, and the suspension was mixed in an end-over-end mixer (24 h, 4°C). To determine coupling efficiency, the A_{280} was determined for the solution before and after coupling. After coupling, free reactive sites on the activated Sepharose were blocked by the addition of 1 M ethanolamine-Cl, pH 8 (10 ml) for 2 h at RT. Coupling efficiency was estimated at 96.7%. Ethanolamine and ionically-bound ligand were removed from the affinity matrix with alternate washes of loading buffer and elution buffer.

4.7.3 Purification procedure

Parasites (*T. congolense* strain IL3000) were grown in rats and harvested exactly as described in Section 3.2. For purification purposes, lysates (6.7×10^9 cells) were subjected to three preliminary clean-up steps before immunoaffinity chromatography. These steps included three-phase partitioning, ion-exchange chromatography on Q-Sepharose, and affinity chromatography on *p*-aminobenzamidine-Sepharose, all performed exactly as described in Sections 3.3, 3.4 and 3.5 respectively. The resultant active fraction from *p*-aminobenzamidine-Sepharose (22 ml) was dialysed against loading buffer (250 ml, 8 h, 4°C), and loaded onto the anti-OP-Tb immunoaffinity column (50×15 mm, 0.32 $ml.min^{-1}$). After elution of unbound proteins, the column was washed with loading buffer, after which bound, active, material was eluted with elution buffer. Thiocyanate was removed by dialysis against buffer B (Section 3.4.1), and protein concentrated by ultrafiltration (Section 2.6.1).

4.8 Do trypanosome-infected hosts produce anti-oligopeptidase antibodies?

In order to investigate the vaccine potential of trypanosome oligopeptidases, and whether or not the immunogenicity of these oligopeptidases is related to trypanotolerance, the sera of infected trypanosusceptible and trypanotolerant bovine hosts were analysed for anti-oligopeptidase antibodies. Due to the paucity of the material, only *T. congolense*-infected material was screened.

4.8.1 Materials

Extravidin®-AP conjugate working solution. Extravidin®-AP conjugate obtained from Sigma (St. Louis, USA) was diluted 1:50 000 with 0.5% (m/v) BSA in TBS prior to use.

Primary antibodies. Chronically infected Boran and N'Dama sera were obtained from Dr. Edith Authié, International Livestock Research Institute, Nairobi, Kenya.

Secondary antibodies. Monoclonal anti-bovine IgM clone BM-23-biotin conjugate and rabbit anti-bovine IgG-alkaline phosphatase conjugate were obtained from Sigma (St. Louis, USA).

4.8.2 Method

Trypanosome lysates and purified OP-Tb were subjected to western blotting (Section 4.4) and probed using infected bovine serum as a source of primary antibody. As whole serum was employed as a source of primary antibody, the precise antibody concentration could not be determined. Whole bovine serum is estimated to contain 12 $mg.ml^{-1}$ IgG, and 1 $mg.ml^{-1}$ IgM (Altman and Dittmer, 1961). Thus, infected sera was diluted to give an estimated 100 $\mu g.ml^{-1}$ when used as a source of primary antibody. As mild increases (10%) in IgG levels (Clarkson and Penhale, 1973) and considerable increases (400%) in IgM levels (Nielsen *et al.*, 1978) of *T. congolense*-infected animals have been reported, this is a very approximate estimate. The IgG antibodies in infected sera were then detected employing rabbit anti-bovine IgG-alkaline phosphatase conjugate at a 1:30 000 dilution as described in Section 4.4.2. The IgM antibodies in infected sera were detected in a two-step procedure. Blots were first incubated with monoclonal mouse IgG_1 anti-bovine IgM clone BM-23-biotin conjugate (1:30 000 dilution, 2 h, RT), after which the blots were washed with TBS (3 × 5 min), incubated with extravidin-AP conjugate working solution (2 h, RT), rinsed with TBS (3 × 5 min) and developed as described in Section 4.4.2.

4.9 Results and discussion

4.9.1 Enzyme-linked immunosorbent assay

Antibody production monitored by ELISA (Section 4.3) showed an increasing antibody response over the immunisation period. Week 13 antibodies gave the highest response when compared to the pre-immune antibodies, and the titre was estimated at approximately 75 $\mu g.ml^{-1}$ (Fig. 17). Titre is defined in the present study as the lowest antibody concentration yielding a significant A_{405} ($\approx$0.3 absorbance units) relative to pre-immune antibodies at the same concentration. The week 13 antibodies were used in western blots (Section 4.9.2) and for immunoaffinity purification of the oligopeptidase from *T. congolense*. Only 50 µg in total

of the OP-Tb immunogen was used to elicit a very good immune response. This suggests that the parasite enzyme is highly immunogenic in the avian immune system, resulting in high titre antibodies suitable for further application (e.g. cross-reactivity studies).

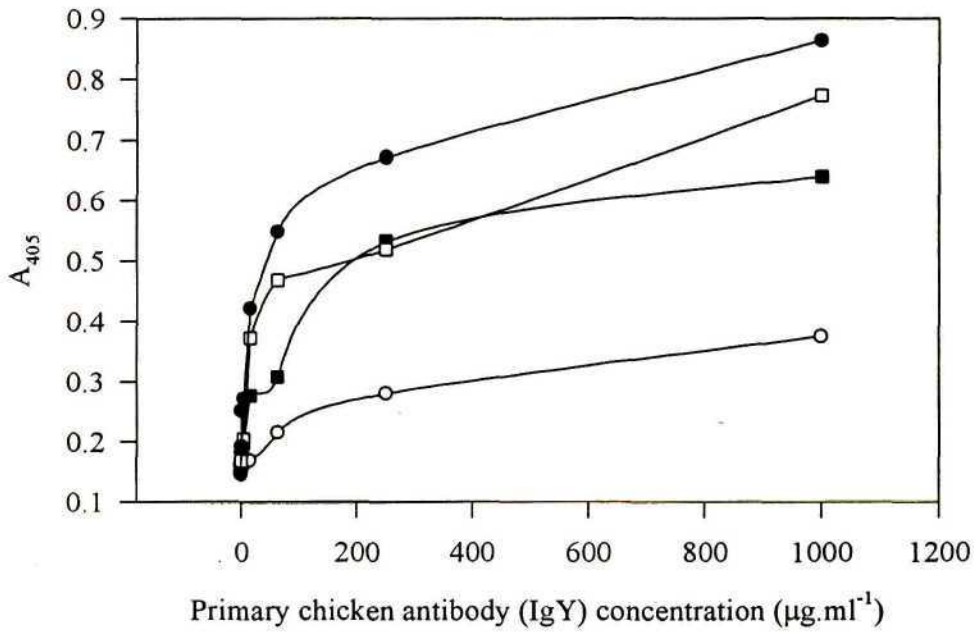


Figure 17. Monitoring of chicken anti-OP-Tb antibody production by ELISA. Microtitre plates were coated with OP-Tb at 1 μg.ml^{-1} and incubated with dilutions of pre-immune IgY (–○–); IgY from week 4 (–■–); 8 (–□–) and 13 (–●–) after the start of the immunisation programme. Binding of antibodies was visualised by incubation with HRPO-linked secondary antibodies, followed by ABTS/H_2O_2 substrate (Section 4.3.2). Each point is the mean absorbance at 405 nm of duplicate samples.

4.9.2 Western blotting

Antibody specificity was determined by western blotting (Section 4.4). Week 13 antibodies targeted single bands in *T. b. brucei* lysates and in a purified OP-Tb preparation. Furthermore, a single band was targeted in *T. congolense* lysate (Fig. 18).

The targeting of a single band in a *T. b. brucei* lysate on the western blot (Fig. 18a) demonstrates the specificity of the antibody preparation for OP-Tb. Cross-reactivity of anti-OP-Tb IgY with a protein in *T. congolense* lysates suggests the presence of a structurally related enzyme in *T. congolense*, and raised the possibility of immunoaffinity purification of this enzyme (Section 4.7).

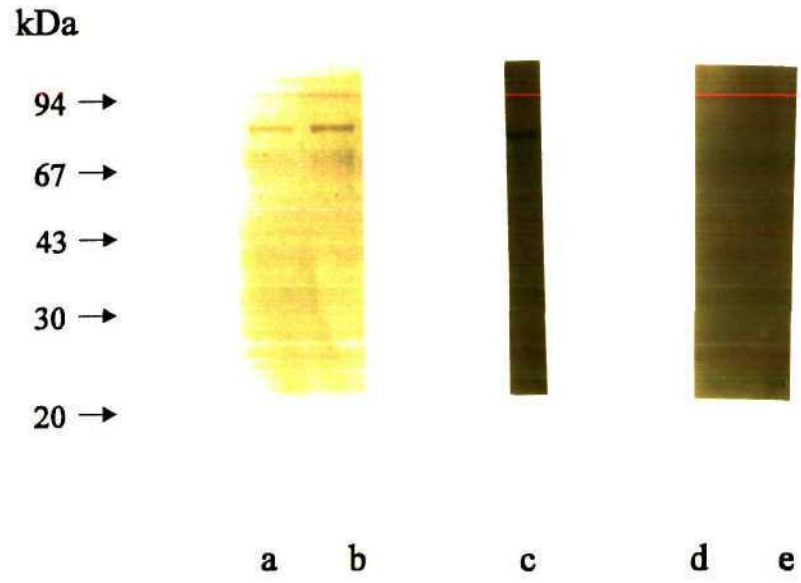


Figure 18. Characterisation of anti-OP-Tb antibodies by western blotting. Protein samples were resolved by Tris-Tricine SDS-PAGE (Section 2.5.2) and electroblotted onto nitrocellulose (Section 2.5.6): (a) *T. b. brucei* lysates (40 μg); (b) purified OP-Tb (200 ng); (c) *T. congolense* lysates (100 μg); each incubated with week 13 anti-OP-Tb IgY (75 $\mu g.ml^{-1}$). (d) *T. b. brucei* lysates and (e) purified OP-Tb; incubated with pre-immune IgY (75 $\mu g.ml^{-1}$). Antibody-antigen complexes were detected with an AP-linked secondary antibody and BCIP/NBT substrate as described in Section 4.4.2.

It is interesting to compare the western blot data with that obtained from the active-site blots (Section 7.6.3, Fig. 62). Active site blots of *T. b. brucei* lysates with biotin-ArgCH_2Cl revealed three prominent high-molecular mass bands, at approximately 68, 80 and 100 kDa (Fig. 62a). While the 80 kDa band is most probably attributable to OP-Tb, the identity of the other two bands is problematic. It has been suggested that the 100 kDa band may represent a complex of OP-Tb with a regulatory protein, although such complexes are usually non-covalent and would not be stable to boiling in the presence of SDS. The absence of a comparable band on the western blot (Fig. 18a) suggests that the protein labelled by biotin-ArgCH_2Cl at 100 kDa is not attributable to OP-Tb, and probably represents a different protease with a similar substrate specificity. Similarly, no band was visible at 68 kDa on the western blot (Fig. 18a). It has been suggested that the 68 kDa protein labelled by biotin-ArgCH_2Cl (Fig. 62a) may be due to a processed form of OP-Tb. The lack of immunological reactivity at 68 kDa suggests that if the 68 kDa band on the active-site blot is indeed a processed form of OP-Tb, this processing has destroyed the epitopes recognised by the anti-OP-Tb antibodies. It is quite probable that the 68 kDa protein labelled on the active-site blot represents a non-specific interaction with rat serum albumin, or a different protease.

4.9.3 Evaluation of inhibition of OP-Tb activity by anti-OP-Tb antibodies

All antibody preparations from chickens immunised with OP-Tb demonstrated varying degrees of inhibition of OP-Tb activity (Fig. 19). Inhibitory antibodies initially peaked at week 5, and then peaked sharply again at week 13, two weeks after the third booster immunisation. Week 13 antibodies were strongly inhibitory, producing 92% inhibition of enzyme activity. By definition, a polyclonal immune response entails the production of a heterogeneous antibody population. These antibodies are produced by a population of B-cells, each B-cell clone producing antibodies to a particular epitope. It is expected that only a proportion of B-cell epitopes will elicit inhibitory antibodies. The initial fluctuation in inhibitory antibody titre over the progression of the immunisation schedule may suggest that different B-cell clones are being stimulated at different stages of the immunisation period. At week 13 there was a dramatic increase in the level of inhibitory antibodies which may indicate maturation of antibody affinity for the active-site epitopes as a result of repeated booster injections (Roitt, 1994). Week 13 antibodies were strongly inhibitory, and inhibitory activity was therefore titrated out (Fig. 20). Half maximal inhibition occurred at approximately 7.5 $\mu g.ml^{-1}$. Maximal inhibition, producing about 90% inhibition relative to controls containing pre-immune antibodies, was attained at an antibody concentration of 250 $\mu g.ml^{-1}$.

To date, the only other oligopeptidase for which inhibition by antibodies has been examined is porcine prolyl oligopeptidase. Moriyama *et al.* (1988) described the production of polyclonal inhibitory antibodies in rabbits. Half-maximal inhibition of activity against Suc-Gly-Pro-AMC was observed at rabbit IgG concentrations of 10 $\mu g.ml^{-1}$. Maximal inhibition (95% of the uninhibited controls) was obtained at IgG concentrations of 100 $\mu g.ml^{-1}$.

The production of polyclonal inhibitory antibodies has been described for a number of proteases, including human and ovine cathepsin L (Coetzer *et al.*, 1991, Coetzer, 1992). However, a much greater excess of antibody over enzyme (based on mass ratios) was required in comparison to that required for the inhibition of OP-Tb (present study) or porcine prolyl oligopeptidase (Moriyama *et al.*, 1988). Coetzer (1992) reported that human cathepsin L (25 ng) was still not fully inhibited in the presence of 1 mg of polyclonal anti-human cathepsin L IgY.

Similar results were obtained with polyclonal IgY raised against ovine cathepsin L where half-maximal inhibition of cathepsin L (25 ng) was obtained in the presence of 250 μg of antibody. This contrasts sharply with the 7.5 μg required to produce half-maximal inhibition of OP-Tb for the same mass of protease. It should be stressed, however, that these figures do not represent the molar ratio of active enzyme to antibody. It was not practical to calculate such a value as the antibody preparations were not affinity purified, and active concentrations of enzyme were not considered. Additionally, OP-Tb has a M_r approximately three times greater than cathepsin L, and the same mass of protein would yield a lower molar concentration of protease.

The active-sites of oligopeptidases are thought to be deeply buried in an active site "pit", as opposed to the active site "cleft" for most other proteases (Barrett and Rawlings, 1992). This introduces steric hindrance access by large peptides and proteins, and consequently, only oligopeptides can gain access to the active-site and are hydrolysed. It follows therefore that a single antibody blocking entry to the active-site pit would completely inhibit access to the active site of the enzyme. For this reason, should the correct B-cell clone be stimulated, polyclonal antibodies directed against oligopeptidases may be more strongly inhibitory than antibodies directed against proteases, which have a more exposed active site.

The results of Moriyama *et al.* (1988) are also interesting as they report the production of strongly inhibitory antibodies by a mammal, against a mammalian oligopeptidase, raising the possibility that chronically infected mammalian hosts may produce antibodies, perhaps even inhibitory antibodies, against OP-Tb. This would be extremely desirable in terms of host defence, as OP-Tb activity has been detected in the bloodstream of infected animals (Section 6.8.4, Fig. 46), presumably being released into the bloodstream during complement-mediated trypanosome-lysis. Circulating anti-OP-Tb antibodies could opsonise OP-Tb released into the bloodstream, thereby hastening its removal from the bloodstream, and possibly inhibiting its activity in the bloodstream, which would neutralise its contribution to disease pathogenesis. For these reasons, the effects of anti-OP-Tb antibodies were examined for their effects on live *T. b. brucei* in culture (Section 4.9.4) and the serum of infected bovine hosts was examined for the presence of circulating antibodies to trypanosome oligopeptidases (Section 4.9.6).

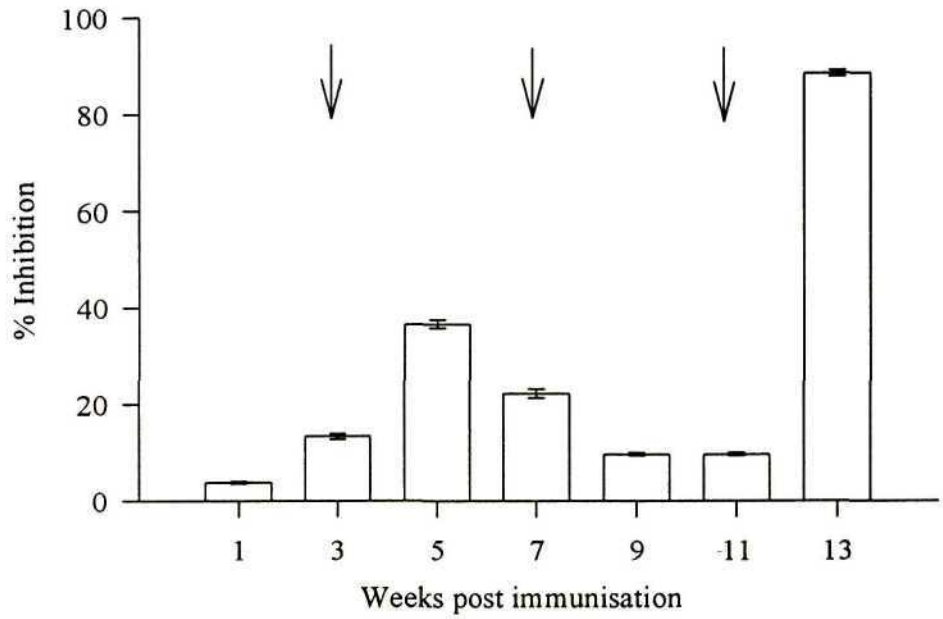


Figure 19. Inhibitory activity of anti OP-Tb antibodies raised in chickens. OP-Tb (25 ng) was pre-incubated in the presence of IgY (500 $\mu g.ml^{-1}$), and then assayed for activity against Cbz-Arg-Arg-AMC. Percentage inhibition was calculated as the activity in the presence of week 13 IgY relative to the activity in the presence of pre-immune IgY at the same concentration. Arrows (↓) indicate administration of booster immunisations. Error bars give the mean ± SEM (n = 3).

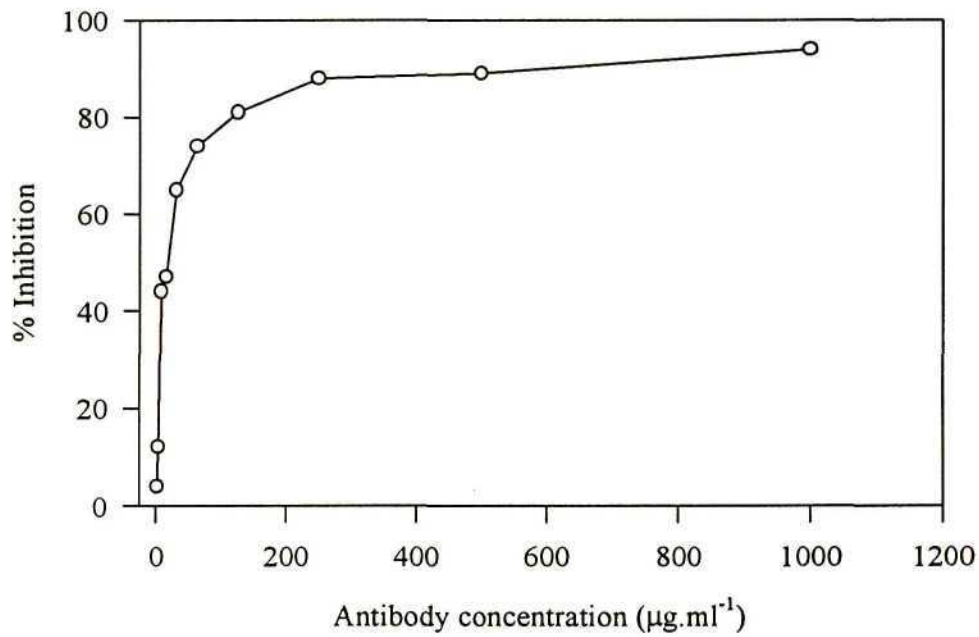


Figure 20. Titration of inhibitory week 13 anti-OP-Tb IgY antibodies. OP-Tb (25 ng) was pre-incubated with an appropriate dilution of IgY (0-1 $mg.ml^{-1}$, 10 min), and then assayed for activity against Cbz-Arg-Arg-AMC (Section 2.3.2). Percentage inhibition was calculated as the activity in the presence of immune IgY relative to the activity in the presence of pre-immune IgY at the same concentration. Standard deviations for the % inhibition were less than ±5% of the mean value.

4.9.4 Effects of antibodies on trypanosomes in culture

Anti-OP-Tb antibodies had little effect on trypanosomes in culture. Trypanosomes cultured in the presence of native (i.e. without prior heat-inactivation) FCS exhibited extremely poor growth, while growth was satisfactory in the presence of heat inactivated FCS containing pre-immune IgY. In the presence of the heat inactivated FCS, week 13 anti-OP-Tb IgY caused a slight (20%) reduction in viable cell number (Fig. 21).

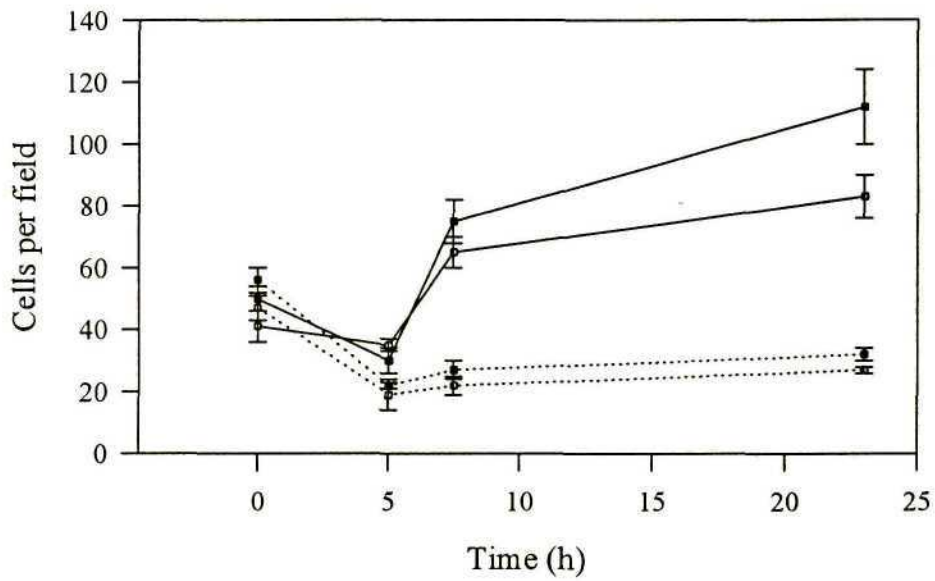


Figure 21. Effect of anti-OP-Tb IgY antibodies on trypanosomes in culture. Trypanosomes were cultured for 24 h in the presence of week 13 inhibitory anti-OP-Tb IgY (□) or in the presence of pre-immune IgY (■) both at a concentration of 1 $mg.ml^{-1}$. Parallel experiments were conducted in the presence of normal FCS (·····) and heat-inactivated FCS (——). Error bars represent the mean ±SEM (n = 3).

Antibody-mediated cell-lysis is usually facilitated by an antibody binding to a surface antigen on a cell, which then activates the classical pathway of complement activation (Joiner *et al.*, 1984). *T. b. brucei* and *T. congolense* are known to activate both the classical (Balber *et al.*, 1979) and alternative (Kierszenbaum and Weinman, 1977) complement pathways *in vivo*, and the activation of the classical pathway, mediated by anti-VSG antibodies, is proposed to be the primary means of parasite clearance from host tissues.

It is possible that anti-OP-Tb antibodies may exert a trypanocidal effect in two ways. We have detected OP-Tb-like activity in a membrane/cytoskeletal fraction of *T. b. brucei* (Section 3.11.1, Fig. 9). Furthermore, live *T. b. brucei* are able to hydrolyse Cbz-Arg-Arg-AMC (Section 6.8.5, Fig. 47), and this hydrolysis is blocked in the presence of anti-OP-Tb antibodies (Section 6.8.5, Fig. 47).

Taken together, these data suggest the possibility of surface-membrane bound OP-Tb. Anti-OP-Tb antibodies may therefore bind to OP-Tb the trypanosome cell-surface and activate the classical pathway of complement activation. Secondly, if the presence of active OP-Tb on the cell-surface is necessary for parasite viability, inhibitory anti-OP-Tb antibodies may bind OP-Tb on the cell-surface and inhibit enzyme function, with deleterious consequences for the parasite.

However, in this experiment, chicken IgY was employed as the antibody, while bovine native and heat-inactivated FCS were employed as a serum source. As it is not known whether IgY can activate the classical pathway of complement activation, and because parasites grew poorly in the presence of native FCS, only the second hypothesis was tested here. Trypanosome growth was satisfactory in the presence of heat-inactivated FCS, and in the presence of anti-OP-Tb antibodies, a small (20%) decrease in viable cell number was observed. As the standard error bars do not overlap for the 24 h time-point in Fig. 21, it is possible that this 20% reduction in cell numbers, caused by anti-OP-Tb antibodies, is significant. The reason for poor parasite growth in the presence of native FCS is probably attributable to the action of complement proteins. The presence of antibodies or antigen-antibody complexes in the native FCS would result in the non-specific activation of the complement cascade, and may cause complement-mediated lysis of trypanosomes. It is for this reason that complement proteins are usually heat-inactivated for use in cell-culture.

Internalised antibodies would probably enter the lysosomal system of the trypanosome, and as OP-Tb is probably a cytosolic enzyme, it is unlikely that these internalised antibodies would encounter OP-Tb. Thus any trypanocidal effect of anti-OP-Tb antibodies must be mediated by antibody interaction with a surface-bound protein.

4.9.5 Immunoaffinity purification of OP-Tc

The results of the optimisation of three-phase partitioning for OP-Tc isolation are illustrated in Fig. 22. The bulk (86%) of the total activity against Cbz-Arg-Arg-AMC precipitated in the 10-25% (m/v) $(NH_4)_2SO_4$ cuts, with 11% remaining associated with the insoluble fraction containing membrane and cytoskeletal components. These findings are consistent with the trends observed with the OP-Tb purification (Section 3.1.1; Fig. 9 *et seq.*). Thus, a 10-25% $(NH_4)_2SO_4$ cut was subsequently used in the purification of OP-Tc to maximise the yield of enzyme. Furthermore, the major lysosomal cysteine proteinase of *T. congolense*, congopain, precipitated over this range of $(NH_4)_2SO_4$ (results not shown). Thus, both enzymes were completely precipitated at this stage, again facilitating the simultaneous purification of both enzymes from the same starting material.

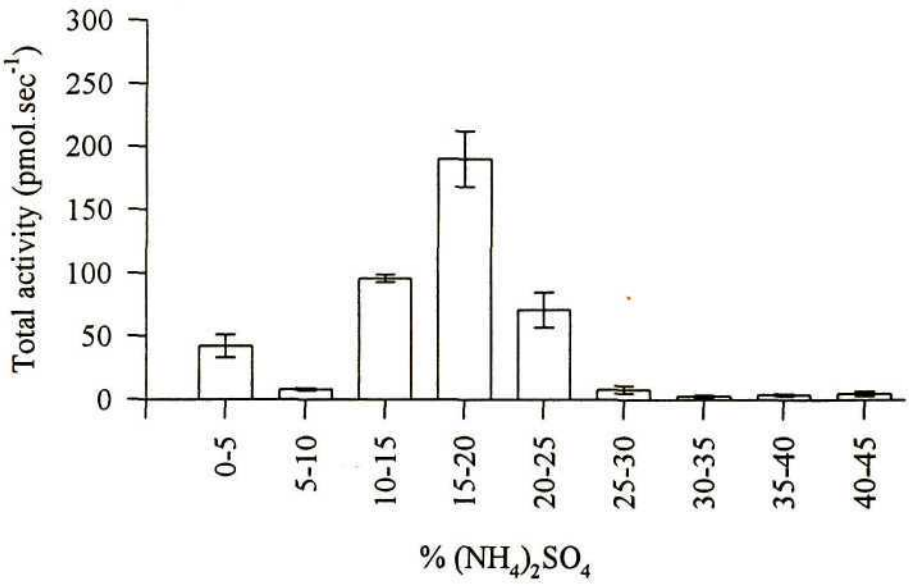


Figure 22. Optimisation of three-phase partitioning for OP-Tc purification. *T. congolense* lysates were precipitated using TPP at various $(NH_4)_2SO_4$ concentrations, and the precipitated fractions were assayed for activity against Cbz-Arg-Arg-AMC. Error bars represent the mean ± SEM (n = 3).

Activity against Cbz-Arg-Arg-AMC eluted in the unbound fraction from a Q-Sepharose column at pH 5.5, while activity against Cbz-Phe-Arg-AMC was retained on the column, eluting at about 200 and 600 mM NaCl (Fig. 23). This reflects the separation of a trypsin-like activity attributable to OP-Tc from a cathepsin L-like activity attributable to congopain (Mbawa *et al.*, 1992).

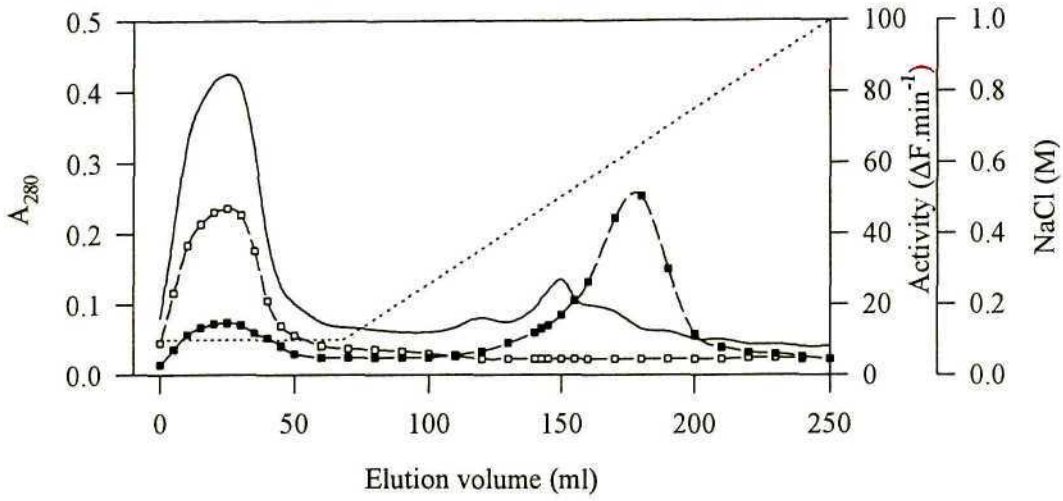


Figure 23. Anion-exchange chromatography of OP-Tc on Q-Sepharose at pH 5.5. Q-Sepharose (26 × 100 mm, 1 $ml.min^{-1}$) was equilibrated in buffer A [20 mM acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5] containing 100 mM NaCl. After elution of unbound protein with buffer A containing 100 mM NaCl, bound protein was eluted with a linear gradient (····) of 0.1 to 1 M NaCl in buffer A over 5 column volumes. (——) A_{280}, (–□–) enzyme activity against Cbz-Arg-Arg-AMC, (–■–) enzyme activity against Cbz-Phe-Arg-AMC.

Application of the Q-Sepharose pH 5.5 active fraction to a *p*-aminobenzamidine-Sepharose column (Section 3.4) resulted in the separation of two fractions with activity against Cbz-Arg-Arg-AMC (Fig. 24). The bulk of the activity (approximately 85%) remained weakly bound to the column, eluting with a single-step salt gradient. A small amount of activity was also evident in the unbound fraction.

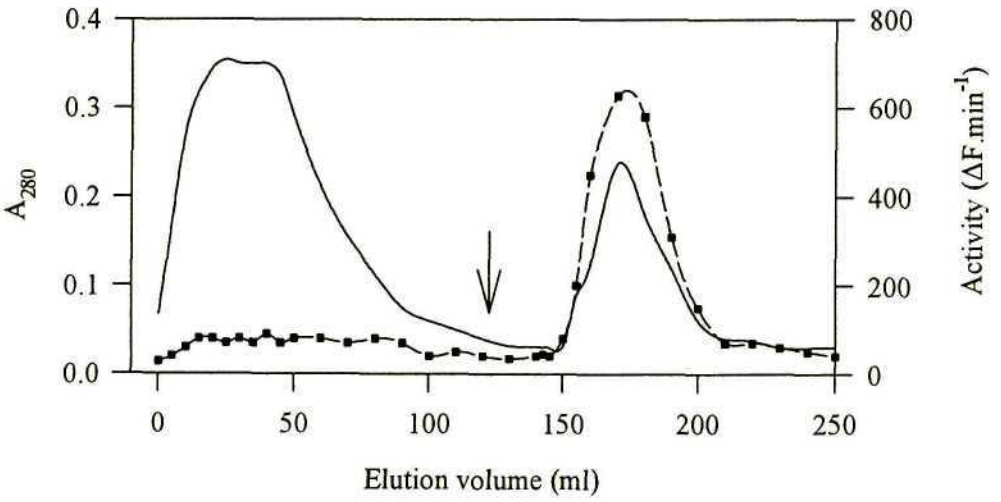


Figure 24. Affinity chromatography of OP-Tc on *p*-aminobenzamidine-Sepharose. *p*-Aminobenzamidine-Sepharose (120 × 15 mm, 1 $ml.min^{-1}$) was equilibrated in buffer B [50 mM Tris-Cl, 2 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. After elution of unbound protein with buffer B, bound protein was eluted by application of 250 mM NaCl in buffer B, in a single step, applied at the point indicated by the arrow (↓). (–) A_{280}, (–■–) enzyme activity against Cbz-Arg-Arg-AMC.

Cbz-Arg-Arg-AMC-hydrolysing activity was eluted in a single well-resolved peak from an anti-OP-Tb-Sepharose immunoaffinity column (Fig. 25), while no activity was observed in the unbound fraction. The purified OP-Tc was homogenous on Tris-Tricine SDS-PAGE, and has a molecular mass of approximately 80 kDa (Fig. 26).

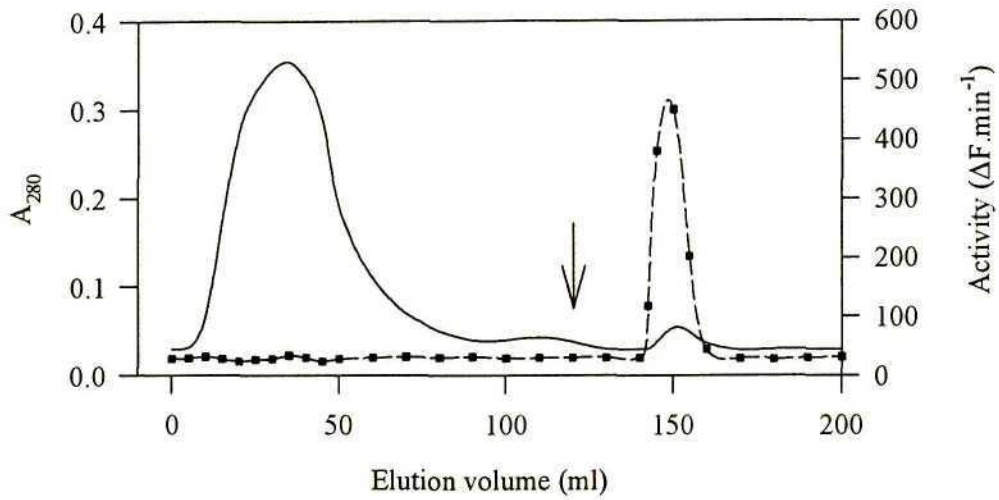


Figure 25. Immunoaffinity chromatography of OP-Tc on anti-OP-Tb-Sepharose. Anti-OP-Tb-Sepharose (50 × 15 mm, 1 $ml.min^{-1}$) was equilibrated in loading buffer [50 mM Tris-Cl, 1 M NaCl, 10 mM $CaCl_2$, 0.5% (m/v) Brij-35, 0.02% (m/v) NaN_3, pH 7.4]. After elution of unbound protein with loading buffer, bound protein was eluted by application of 3.5 M NaSCN in loading buffer, in a single step, applied at the point indicated by the arrow (↓). (–) A_{280}, (–■–) enzyme activity against Cbz-Arg-Arg-AMC.

Table 8. Purification table for the isolation of OP-Tc from *T. congolense* lysates.

Fraction	Volume (ml)	Total Protein (mg)	Total Activity ($pmol.sec^{-1}$)	Specific Activity ($pmol.sec^{-1}.mg^{-1}$)	Purification (fold)	Yield (%)
Lysate	12	196	2228	11	1	100
TPP	15	7.4	1473	198	18	66
HiLoad Q (pH 5.5)	44	6.8	1109	163	15	50
Benzamidine	22	0.413	1012	2450	221	45
Immunoaffinity	4	0.054	754	13962	1269	34

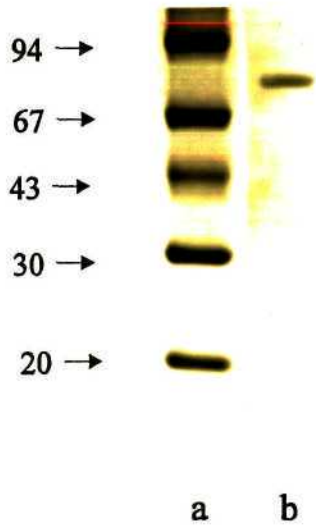


Figure 26. Evaluation of OP-Tc purification (a) M_r markers (as in Section 2.7.1.1); (b) anti-OP-Tb-Sepharose active pool (300 ng). Samples were resolved by reducing Tris-Tricine SDS-PAGE (Section 2.7.1) and protein visualised by silver staining (Section 2.7.4).

OP-Tc was purified to electrophoretic homogeneity for the first time in this study from *T. congolense* lysates in a four-step procedure with a 34% yield. OP-Tc behaved almost identically to OP-Tb in the three-phase partitioning, ion-exchange and *p*-aminobenzamidine-Sepharose affinity chromatography (Sections 3.11.1, Fig. 9; 3.11.2, Fig. 10, and 3.11.3, Fig. 11 respectively). Affinity chromatography on anti-OP-Tb-Sepharose replaced three further column chromatography steps that were used to purify OP-Tb. However, the shortened procedure only resulted in a 6% improvement in enzyme yield, with 11% being lost in the immunoaffinity procedure. It is possible that OP-Tc was not stable in the 3.5 M NaSCN, and this may account for the loss of activity during this procedure. However, elution of the OP-Tc from the immunoaffinity column with 50 mM Glycine-Cl, pH 2.3, instead of 3.5 M NaSCN (results not shown) resulted in an even lower final yield (24%). It is also possible that the high-salt binding conditions required for the immunoaffinity step were unfavourable, however, the stability of trypanosome oligopeptidases under conditions of high ionic strength has not been determined. Despite only a 6% increase in yield, the immunoaffinity step, which replaced three column-chromatography procedures, represented a considerable reduction (approximately 50%) in the duration of the purification procedure.

4.9.6 Do trypanosome-infected hosts produce anti-oligopeptidase antibodies?

Most of the specific antibodies present in N'Dama and Boran cattle sera were directed against trypanosome antigens in the range 20-90 kDa. Both Boran and N'Dama cattle appeared to raise IgG against two primary antigens of approximately 30 and 50 kDa (Fig 27). Furthermore, a 40 kDa trypanosome protein appeared to be a dominant antigen in infections of N'Dama, but not of Boran cattle, and it is likely that the responsible antigen is congopain (L. Troeberg, *pers. comm.*, Authié *et al.*, 1993b). OP-Tc was not a dominant antigen in infections of either Boran or N'Dama cattle, as it was not targeted on western blots by IgG from either source.

In contrast to these results, IgM in both Boran and N'Dama sera primarily targeted antigens at 40 and 80 kDa, although there was a stronger antibody response from the Boran sera, where a greater number of bands which showed much more pronounced staining, was evident (Fig. 28). In spite of the band at 80 kDa targeted in *T. congolense* lysates, purified OP-Tc was not targeted by IgM from either source, again suggesting that OP-Tc was not a dominant antigen in either Boran or N'Dama infection.

These data are generally consistent with the findings of Authié *et al.* (1993b), although no immunodominant 69 kDa antigen was detected in the present study. A 40 kDa antigen was detected, and the IgG response to this antigen appeared to correlate with trypanotolerance, suggesting that the antigen was congopain. Furthermore, Authié *et al.* (1993b) also reported that Boran cattle have higher IgM responses, but weaker IgG responses when compared with N'Dama cattle, which is consistent with the trends observed here. This has been attributed to an impairment in the class-switching from IgM to IgG during infection in trypanosusceptible cattle (Authié *et al.*, 1993b; Musoke *et al.*, 1981), since isotype switching involves T_h-cell participation (Kishimoto and Hirano, 1988), and depression of T_h-cell has been documented in *T. b. brucei* (Pearson *et al.*, 1978) and *T. congolense* (Flynn and Sileghem, 1993).

No inhibitory activity was detected when N'Dama and Boran sera were tested for their ability to inhibit the OP-Tc catalysed hydrolysis of Cbz-Arg-Arg-AMC, even at serum dilutions of 1:10 (results not shown). This suggests that inhibitory antibodies are not generated by the infected hosts.

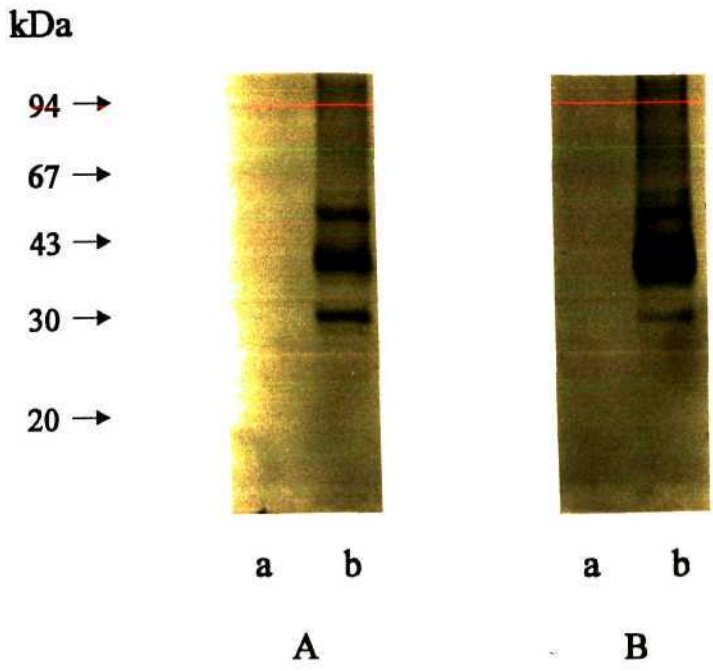


Figure 27. Characterisation of IgG response to trypanosome infection in cattle. Purified OP-Tb (200 ng) (a) and *T. congolense* lysates (50 µg) (b) were resolved by Tris-Tricine SDS-PAGE (Section 2.7.1) and electroblotted onto nitrocellulose (Section 2.7.5). Nitrocellulose strips were then incubated with *T. congolense*-infected N'Dama (A) and Boran (B) serum (1:100 dilution). Antibody-antigen complexes were detected with an anti-bovine IgG-AP-linked secondary antibody as described in Section 4.4.

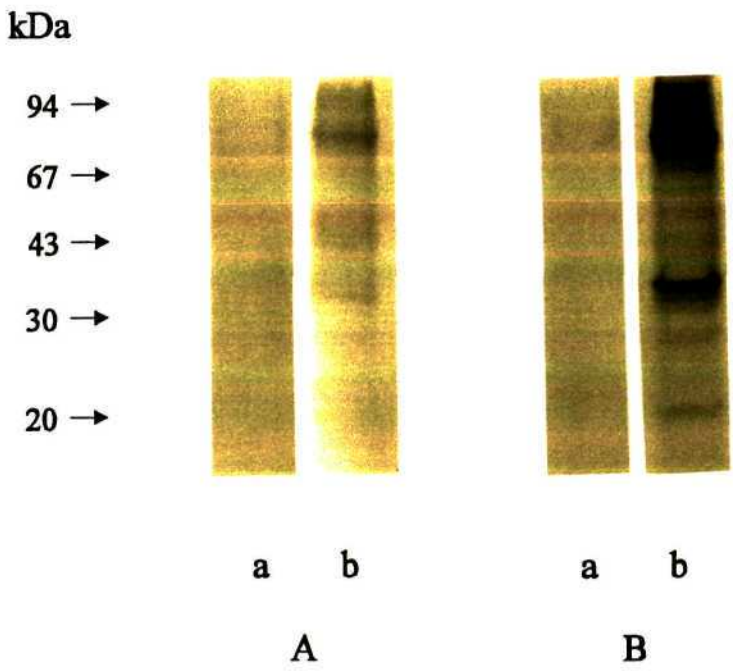


Figure 28. Characterisation of IgM response to trypanosome infection in cattle. Purified OP-Tb (200 ng) (a) and *T. congolense* lysates (50 µg) (b) were resolved by Tris-Tricine SDS-PAGE (Section 2.7.1) and electroblotted onto nitrocellulose (Section 2.7.5). Nitrocellulose strips were then incubated with *T. congolense*-infected N'Dama (A) and Boran (B) serum (1:100 dilution). Antibody-antigen complexes were detected with a monoclonal anti-bovine IgM clone BM-23-biotin conjugate, followed by an extravidin-AP conjugate as described in Section 4.4.

In conclusion, these preliminary data illustrate that OP-Tc is not differentially recognised by the immune system of trypanotolerant versus trypanosusceptible cattle, and inhibitory antibodies are not generated by *T. congolense*-infected hosts. However, whether or not OP-Tc may be a suitable candidate as a target in a vaccine regimen remains to be determined. The fact that trypanosome oligopeptidases are released into infection serum and remain active there suggests that these oligopeptidases may be suitable candidate targets for a vaccine.

4.10 Concluding remarks

Antibodies were raised against OP-Tb in chickens, and antibody production was monitored by ELISA. These antibodies were highly specific for OP-Tb, targeting a single band at 80 kDa in *T. b. brucei* lysates on a western blot. Anti-OP-Tb IgY strongly inhibited enzyme activity, producing half-maximal inhibition of OP-Tb activity at 7.5 $\mu g.ml^{-1}$. This appears to be far more potent than antibodies raised against other proteases by other investigators. This raises the possibility that chronically infected mammalian hosts may produce antibodies against OP-Tb, which would be highly desirable in terms of host defence. Such antibodies would inhibit the activity and hasten the removal of circulating OP-Tb, released by lysed trypanosomes, from the host bloodstream.

Inhibitory antibodies had no major effect on live trypanosomes in culture, but it is not yet known whether they may influence the course of parasitaemia during natural infections. It would be interesting to see if a prior immunisation of mice with OP-Tb had any affect on parasitaemia after a subsequent challenge infection.

The generation of anti-OP-Tb antibodies facilitated the purification of a related enzyme from *T. congolense* by immunoaffinity chromatography. Although this procedure did not result in a greatly improved enzyme yield, it did reduce the duration of the purification procedure by 50%. In addition, the cross-reactivity of the antibody with both *T. brucei* and *T. congolense* derived enzymes raises the possibility that the antibody may prove to be a useful diagnostic probe for trypanosome infections.

The intracellular distribution of eukaryotic oligopeptidases has not been conclusively demonstrated, although subcellular fractionation has suggested that porcine prolyl oligopeptidase is both soluble and membrane bound (O'Leary *et al.*, 1996). Oligopeptidase B

from *T. cruzi* has recently been localised to the parasite cytoplasm by immunoelectron microscopy, with no evidence of surface-membrane association (Burleigh *et al.*, 1997). Similar observations have been made with OP-Tb (Lonsdale-Eccles and Mpimbaza, preliminary unpublished observations). Live *T. b. brucei* do hydrolyse Cbz-Arg-Arg-AMC, and this activity is inhibited in the presence of anti-OP-Tb antibodies. This raised the possibility that OP-Tb is membrane bound, or is released extracellularly by trypanosomes. Furthermore, OP-Tb activity has been detected in the plasma of infected rats and cattle (Section 6.8.4, Fig. 46). It is not known whether the protease is actively or constitutively secreted, present in an active form on the trypanosome cell surface, or simply released into the host bloodstream upon complement-mediated trypanosome lysis. It would thus be useful to determine the precise intracellular localisation of trypanosome oligopeptidases. Should trypanosome oligopeptidases be membrane-bound, anti-oligopeptidase antibodies raised by the host may abrogate any hydrolytic activity of these membrane-bound oligopeptidases. Furthermore, attachment of such anti-oligopeptidase antibodies to the cell surface would facilitate complement-mediated cell-lysis of the parasites. Preliminary investigations suggest that in the case of *T. congolense* infections, anti-OP-Tc antibodies are not elicited in chronically infected bovine hosts.

The diseases caused by *T. congolense* and *T. b. brucei* in infected hosts vary considerably in their pathology (Section 1.1.6), and since proteases are known to be important pathogenic factors in microbial infections (Section 1.3.2), we have proposed that trypanosome oligopeptidases play a role in the pathogenesis of African trypanosomiasis. To explore this hypothesis further, a comprehensive enzymatic characterisation of OP-Tb and OP-Tc was undertaken (Chapter 5).

Chapter 5

Enzymatic characterisation of trypanosome oligopeptidases

Serine proteases from African trypanosomes have only recently been identified, and have not previously been purified to electrophoretic homogeneity. For this reason, no thorough enzymatic characterisation has been undertaken of these enzymes. Thus, the aim of this aspect of the present study was to conduct a thorough enzymatic characterisation of two trypanosome proteases: OP-Tb, from *T. b. brucei* and OP-Tc from *T. congolense*. This characterisation included determination of the substrate specificity and sensitivity to pH and ionic strength. Furthermore, the effect of a variety of potential activators and inhibitors on the activity of these trypanosome oligopeptidases was examined, to further probe the substrate specificity and catalytic mechanism of these enzymes.

5.1 Fluorogenic peptide specificity

The Michaelis constant, K_m, and the maximum velocity of an enzyme-catalysed reaction, V_{max}, are important kinetic constants which reflect the specificity and activity of an enzyme. The V_{max} represents the maximum turnover-rate of an enzyme, a point at which all active sites are occupied by the substrate. It is often useful to express V_{max} in terms of the number of moles of enzyme present, known as the turnover number, or k_{cat}

$$k_{cat} = \frac{V_{max}}{[E]_0}$$ (Salvesen and Nagase, 1986)

where $[E]_0$ represents active enzyme concentration. The K_m represents the amount of substrate required to produce $\frac{1}{2}V_{max}$. In many cases it is an indication of the affinity between enzyme and substrate; the lower the K_m, the more preferred the substrate (Michal, 1983). Determination of these constants is accomplished for single-substrate reactions by the determination of the initial steady-state velocity (v_0) at various substrate concentrations. A direct plot of v_0 versus substrate concentration ([S]) permits an estimation of K_m and V_{max}. These four factors are related by the Briggs-Haldane revision of the Michaelis-Menten equation:

$$v_0 = \frac{V_{max}[S]}{[S] + K_m} \quad \text{(Briggs and Haldane, 1925)}$$

The inherent uncertainty in evaluating the V_{max}, and hence, K_m, from a direct plot prompted a number of investigators to devise alternative plots in which such data is fitted to a straight line, including the Lineweaver-Burk plot (Lineweaver and Burk, 1934), the Eadie-Hofstee plot (Eadie, 1942; Hofstee, 1952), the Hanes plot (Hanes, 1932) and the direct linear plot of Eisenthal and Cornish-Bowden (Eisenthal and Cornish-Bowden, 1974).

Knowledge of the precise substrate specificity of trypanosome oligopeptidases would provide the groundwork for the design of highly specific inhibitors which would be useful to explore the physiological functions of these enzymes both within the trypanosome, and in the pathogenesis of African trypanosomiasis. Such inhibitors could also be used to explore the potential of these enzymes as therapeutic targets. A knowledge of the substrate specificity may also assist in the identification of the *in vivo* substrates of these enzymes. For these reasons, it was desirable to determine the P_4-P_1 subsite specificity of OP-Tb and OP-Tc, and this was undertaken by examining the hydrolysis of a panel of aminomethylcoumaryl peptide substrates by these two proteases.

5.1.1 Materials

1 mM Substrate stock solutions. Cbz-Arg-Arg-AMC (0.9 mg), Boc-Leu-Lys-Arg-AMC (1.1 mg), Cbz-Gly-Gly-Arg-AMC (0.9 mg), Cbz-Phe-Arg-AMC (1 mg), Boc-Leu-Arg-Arg-AMC (1.2 mg), Boc-Leu-Gly-Arg-AMC (0.9 mg), Boc-Gly-Arg-Arg-AMC (1 mg), Cbz-Ala-Arg-Arg-AMC (1.1 mg), Boc-Val-Gly-Arg-AMC (0.9 mg), Cbz-Arg-AMC (0.7 mg), Boc-Gly-Lys-Arg-AMC (1 mg), H-Ala-Phe-Lys-AMC (0.8 mg), Boc-Val-Leu-Lys-AMC (0.9 mg), Boc-Ala-Gly-Pro-Arg-AMC (1 mg), Boc-Val-Pro-Arg-AMC (0.9 mg), Glt-Gly-Arg-AMC (0.8 mg), Ac-Ala-Ala-Pro-Ala-AMC (0.8 mg), H-Gly-AMC (0.3 mg); H-Leu-AMC (0.4 mg); MeoSuc-Gly-Trp-Met-AMC (1 mg); Suc-Leu-Tyr-AMC (1 mg); Cbz-Glt-Gly-Gly-Phe-AMC (0.8 mg) or Gly-Pro-AMC (0.7 mg) were dissolved separately in DMSO (1.5 ml). H-Arg-AMC (0.5 mg) was dissolved in dH_2O (1.5 ml).

5.1.2 Method

The hydrolysis of fluorogenic peptide substrates is discussed in Section 2.3. Oligopeptidase activity against fluorogenic peptide substrates was determined by preincubation of OP-Tb or OP-Tc (1.5 ng, representing $\simeq$18.75 fmol of active enzyme, 5 min) in assay buffer (Section 2.3.2), followed by addition of substrate. The initial steady-state velocity (v_0) was determined by continuous assay over a range of substrate concentrations (usually 45 nM-75 μM but for K_m values above 20 μM, the upper limit was extended to $8K_m$). K_m and V_{max} were determined by hyperbolic regression of the kinetic data using the software package Hyper 1.01 (obtained from Dr. J.S. Easterby, University of Liverpool, UK).

5.2 Effect of pH on oligopeptidase activity and stability

A pH activity profile permits the determination of the pH at which an enzyme is most active, and the pH at which the enzyme no longer functions. It is useful to determine these parameters for the optimisation of an enzyme assay, and as a clue to the *in vivo* localisation of an enzyme. Cytosolic enzymes frequently have alkaline pH optima, as opposed to lysosomal enzymes, which are usually optimally active at acidic pH. This rule has its exceptions, however. For example, the lysosomal enzyme cathepsin B has an alkaline pH optimum (Dehrmann *et al.*, 1996). It was of interest to determine whether oligopeptidases were active and stable at the physiological pH of the mammalian host-organism, as activity and stability at physiological pH (taken as pH 7.4) would suggest that the enzyme may remain active in the host bloodstream, where it may contribute to disease pathogenesis, until eliminated from the host.

As variations in ionic strength (I) may affect enzyme activity independently of pH, it is important to maintain a constant ionic strength over the pH range utilised (Dehrmann *et al.*, 1995). For this reason, pH activity and stability studies were conducted using the constant ionic strength acetate-Mes-Tris (AMT) buffers of Ellis and Morrison (1982).

5.2.1 Materials

AMT buffers (100 mM acetate, 200 mM Tris, 100 mM Mes, 1 mM dithiothreitol, 4 mM Na_2EDTA; I$\simeq$0.1). Glacial acetic acid (1.72 ml), Mes (5.86 g), Tris (7.27 g) and $Na_2EDTA.2H_2O$ (0.45 g) were dissolved in dH_2O (200 ml). This solution was divided into 12 aliquots (20 ml each) which were titrated to pH values in the range 4.0 to 9.0 using HCl or

NaOH, and then made up to 25 ml with dH_2O. Dithiothreitol (4 mg) was added to each 25 ml aliquot on the day of use, and the pH re-adjusted if necessary.

5.2.2 Method

The hydrolysis of Cbz-Arg-Arg-AMC by OP-Tb or OP-Tc (1.5 ng, ≃18.75 fmol active enzyme) was conducted exactly as described for the standard continuous assay (Section 2.3.2), except that AMT buffers replaced the standard assay buffer. OP-Tb or Op-Tc (10 ng, ≃0.125 pmol active enzyme, in 5 µl) was incubated (1 h, 37°C) in the relevant AMT buffer (25 µl). AMT buffer (I=0.1, pH 8.5, 0.72 ml) was then added to bring all samples to pH 8.5, and residual activity against Cbz-Arg-Arg-AMC determined as described in Section 2.3.2.

5.3 Effect of ionic strength on OP-Tb activity

Ionic strength is known to influence the activity of the proteolytic enzymes cathepsin B and cathepsin L (Dehrmann *et al.*, 1995, 1996; Dennison *et al.*, 1992). It was important to determine whether oligopeptidases were active at the ionic strength of the host extracellular milieu, as this would indicate whether the enzyme could possibly be active at the ionic strength of the host bloodstream.

5.3.1 Materials

AMT buffers. AMT buffers were prepared exactly as described in Section 5.2.1, with the exception that they were prepared at 1.33 × nominal ionic strength in the assay, to allow for dilution of the buffer.

5.3.2 Method

The effect of ionic strength on OP-Tb activity was investigated exactly as described by Dehrmann *et al.* (1995), employing constant ionic strength AMT buffers. OP-Tb (1.5 ng, ≃18.75 fmol active enzyme) was pre-incubated in AMT buffer (750 µl) for 2 min, after which substrate solution (250 µl) was added, and fluorescence monitored as described in Section 2.3.2. To facilitate comparison with the work of others, the effect of ionic strength was also investigated by addition of NaCl to 50 mM Hepes, pH 8.0 (used in place of AMT buffers).

5.4 Reductive activation of oligopeptidases

Trypanosome oligopeptidases are sensitive to the presence of reducing agents. The presence of reducing agents was necessary to maintain the stability of the enzyme throughout a

purification protocol (discussed in Section 3.13), and enzyme activity was enhanced in the presence of reducing agents. The effects of various reducing agents on oligopeptidase activity were therefore determined.

5.4.1 Materials

Assay buffer (50 mM Tris-Cl, pH 8). Tris (3 g) was dissolved in dH_2O (450 ml), titrated to pH 8 with HCl, and made up to 500 ml with dH_2O.

Stock 25 mM reduced glutathione. Reduced glutathione (0.77 g) was dissolved in assay buffer (100 ml), and the pH re-adjusted if necessary.

Stock 25 mM dithiothreitol. Dithiothreitol (0.38 g) was dissolved in assay buffer (100 ml), and the pH re-adjusted if necessary.

Stock 25 mM L-cysteine-HCl. L-cysteine-HCl (0.3 g) was dissolved in assay buffer (100 ml) and the pH re-adjusted if necessary.

5.4.2 Method

OP-Tb and OP-Tc activity against Cbz-Arg-Arg-AMC were determined as described in Section 2.3.2, except that various reducing agents, either cysteine-HCl, reduced glutathione, or dithiothreitol were included in the assay buffer at various concentrations. Stock reducing agent solutions were diluted to the necessary concentration with assay buffer. Enzyme (1.5 ng, $\simeq$18.75 fmol active enzyme) was pre-incubated with the respective reducing agents (37°C, 10 min) before addition of substrate.

5.5 Rate of activation with dithiothreitol

As dithiothreitol proved to be the most effective reducing agent for enzyme activation, this was the reagent of choice for the standard enzyme assay. For the standard assay to yield accurate results, particularly for the calculation of kinetic constants, it is necessary to have the enzyme fully activated before the addition of substrate. It was therefore important to know the optimum time of activation of these enzymes by dithiothreitol.

5.5.1 Method

Enzyme (1.5 ng, ≃18.75 fmol active enzyme) was pre-incubated in assay buffer containing 10 mM dithiothreitol (Section 2.3.2) for various lengths of time (0-15 min), and activity against Cbz-Arg-Arg-AMC determined as described in Section 2.3.2.

5.6 Inhibitor profile of trypanosome oligopeptidases

Testing the effect of a broad spectrum of inhibitors on the activity of a protease often permits its classification into one of the four currently recognised classes of proteases (Table 1). Additionally, inhibition data may provide insight into the substrate specificity of the protease by providing information about the binding site specificity.

5.6.1 Materials

2 × Assay buffer (100 mM Tris-Cl, 20 mM dithiothreitol, pH 8). Tris (3 g) and dithiothreitol (0.4 g) were dissolved in dH_2O (200 ml), titrated to pH 8 with HCl and made up to 250 ml with dH_2O. Due to the relative instability of dithiothreitol, the solution was prepared on the day of use.

10 × Inhibitor stock solutions. Inhibitor stock solutions were prepared at 10 × their working concentration in the final assay mixture and stored at -75°C. AEBSF (31.8 mg, 100 mM), benzamidine-HCl (4.2 mg, 10 mM), chymostatin (12.1 mg, 10 mM), EDTA (74.4 mg, 100 mM), EGTA (38 mg, 100 mM), iodoacetate (37.2 mg, 100 mM), iodoacetamide (37 mg, 100 mM), leupeptin (8.6 mg, 10 mM), pepstatin A (13.7 mg, 10 mM), soybean trypsin inhibitor (1 mg, 50 μM), lima bean trypsin inhibitor (1 mg, 0.11 mM) and aprotinin (1 mg, 0.16 mM) were each dissolved separately in dH_2O (1 ml). Antipain (12.1 mg, 10 mM), *N*-ethylmaleimide (25 mg, 100 mM), E-64 (7.2 mg, 100 mM), 1,10-phenanthroline (39.6 mg, 100 mM) and *p*CMB (35.7 mg, 100 mM) were each dissolved separately in 1 ml volumes of DMSO. PMSF (34.8 mg, 10 mM) was dissolved in ethanol (1 ml).

100 mM DFP. Pure DFP (5.43 M) was diluted in assay buffer to a concentration of 100 mM and used immediately. These experiments were performed in a fume hood, and residual DFP was neutralised with 2 M NaOH when necessary.

5.6.2 Method

Enzymes were pre-incubated with the respective compounds (10 min, 37°C) in assay buffer, before assaying residual activity against Cbz-Arg-Arg-AMC over a range of substrate concentrations (45 nM-75 μM). K_m and V_{max} were determined by hyperbolic regression of the kinetic data. The inhibition mechanism was diagnosed from the effect of the inhibitor on the K_m and V_{max} obtained in the presence and the absence of the inhibitor. Where inhibitors were dissolved in inorganic solvents, control experiments were performed in the presence of comparable concentrations of solvent, without the inhibitor.

For competitive reversible inhibitors, the inhibition constant (K_i) was determined by the method of Salvesen and Nagase (1989). The enzyme-catalysed hydrolysis of Cbz-Arg-Arg-AMC was monitored continuously (Section 2.3.2) to establish an uninhibited rate of substrate hydrolysis (v_0), after which a twenty-fold molar excess of inhibitor over enzyme was added (in less than 5% of the total assay volume), and the new steady-state velocity in the presence of the inhibitor (v_i) determined. The apparent inhibition constant in the presence of substrate ($K_{i(app)}$) was calculated from the equation

$$\frac{v_0}{v_i} = 1 + \frac{[I]}{K_{i(app)}} \quad \text{(Salvesen and Nagase, 1989).}$$

The true K_i was calculated for reversible competitive inhibitors, catering for the presence of substrate, from the relationship

$$K_i = \frac{K_{i(app)}}{1 + \frac{[S]}{K_m}} \quad \text{(Salvesen and Nagase, 1989).}$$

The rate constant for complex formation (k_{ass}) between the enzyme and reversible inhibitors was determined by the methods of Lenarčič *et al.* (1996) which is based on methods described by Nicklin and Barrett (1984) and Salvesen and Nagase (1989), where the inhibitor is added to a steady-state enzyme-substrate reaction, and the change in reaction velocity monitored continuously. The pseudo-first-order inhibition rate constant (k_{obs}) was obtained from the

linear section of plots of ln v_t/v_0 versus time, which has a slope of $-k_{obs}$. The apparent second-order inhibition rate constant (k_{ass}) was obtained from the relationship

$$k_{ass} = \frac{k_{obs}}{[I]}\left(1 + \frac{[S]}{K_m}\right) \quad \text{(modified from Salvesen and Nagase, 1989).}$$

The rate constant for complex dissociation, k_{diss}, was determined from the relationship

$$K_i = \frac{k_{diss}}{k_{ass}} \quad \text{(Bieth, 1980).}$$

As non-competitive and irreversible inhibition are difficult to differentiate from simple kinetic data alone, attempts were made to dilute the inhibitory effect of compounds exhibiting either mode of inhibition. This was achieved with a succession of dilution/concentration cycles where enzyme-inhibitor samples were diluted with assay buffer and concentrated to the original volume in 3 ml polysulfone concentrators (7 000 × *g*, 10°C) (Section 2.6.1).

The effects of irreversible protease inhibitors were investigated under pseudo first-order conditions ([I] > 50[E]$_0$), as described by described by Salvesen and Nagase (1989) by adding an aliquot of inhibitor (10 µl) to a buffered enzyme solution (140 µl, containing 50 ng OP-Tb or OP-Tc in 50 mM Tris-Cl, pH 8.0) to initiate the inactivation. Aliquots were removed at timed intervals and residual activity determined against 5 µM Cbz-Arg-Arg-AMC (v_t). Again, k_{obs} was obtained from plots of ln v_t/v_0 versus time, which have a slope of $-k_{obs}$, facilitating the calculation of k_{ass} from the relationship

$$k_{ass} = \frac{k_{obs}}{[I]} \quad \text{(Salvesen and Nagase, 1989)}$$

where [I] represents the inhibitor concentration. The time required for the free enzyme concentration to decrease by 50% (half-life, $t_{½}$) is given by

$$t_{½} = \frac{0.693}{k_{ass}[I]} \quad \text{(Salvesen and Nagase, 1989).}$$

5.7 Potential activators of oligopeptidase activity

A variety of effector molecules are known to be involved in the regulation of enzyme activity *in vivo*. Some serine proteases, such as the Clp protease of *E. coli* are known to be ATP-dependent (Barrett and Rawlings, 1992), while others such as kexin are strongly calcium-dependent (Fuller *et al.*, 1986). Polyamines are also known to regulate the activity of enzymes *in vitro* (Lim Tung *et al.*, 1985). The effect of these molecules on the activity of OP-Tb and OP-Tc was therefore investigated.

5.7.1 Materials

10 mM Stock solutions. Putrescine.2HCl (16 mg), spermine.4HCl (35 mg), spermidine.3HCl (25.4 mg), ornithine.HCl (17 mg), ATP (55 mg) and GTP (52 mg) were each dissolved separately in dH_2O (10 ml). As heparin does not have a well-defined molecular mass, it was made up as a stock solution of 1 $mg.ml^{-1}$.

5.7.2 Method

Assays were performed exactly as described in Section 2.3.2, with the exception that the activator was included in the assay buffer at 1.33 × final concentration, to allow for dilution following addition of substrate.

5.8 Influence of metal ions on oligopeptidase activity

The influence of metal ions on the activity of an enzyme may give an indication of the catalytic mechanism of the enzyme. Enhancement of protease activity by metal ions, in particular by Zn^{2+} often suggests the action of a metalloprotease, such as a carboxypeptidase or matrix metalloprotease (Matrisian, 1992), while a number of serine proteases are activated by Ca^{2+} (Fuller *et al.*, 1986). Similarly, most cysteine proteases are reversibly inactivated in the presence of Hg^{2+} (Barron, 1951).

5.8.1 Materials

10 mM metal ion stock solutions. $MgCl_2.6H_20$ (20 mg), $CaCl_2.2H_2O$ (15 mg), $ZnCl_2$ anhydrous (14 mg), $MnCl_2.4H_2O$ (20 mg), $HgCl_2$ anhydrous (27 mg), $CuCl_2.2H_2O$ (17 mg), $FeCl_2.4H_2O$ (12 mg), $NiCl_2.2H_2O$ (23 mg), $CdCl_2.2½H_2O$ (23 mg) and $BaCl_2.2H_2O$ (24 mg) were each dissolved in assay buffer (10 ml). Because of the potential formation of complexes between dithiothreitol and heavy-metal ions, dithiothreitol was omitted from the assay buffers.

As dithiothreitol is a non-essential activator of OP-Tb and OP-Tc activity (Sections 5.11.4 and 5.11.5), there was sufficient enzyme activity in its absence to conduct these experiments.

5.8.2 Method

Assays were performed as described in Section 5.7.2, with the exception that activator was replaced with the relevant metal ion, in the absence of dithiothreitol.

5.9 Hydrolysis of protein substrates

OP-Tb was incubated with a number of mammalian host plasma proteins in order to determine whether protein substrates were hydrolysed. For this investigation, four of the most prominent mammalian plasma proteins were selected as potential substrates. Bovine pancreatic trypsin was employed as a positive control as it has a similar cleavage specificity to OP-Tb.

5.9.1 Materials

Protein stock solutions (1 $mg.ml^{-1}$). BSA (1 mg), bovine fibrinogen (1 mg), human α_2-HS-glycoprotein (1 mg) were each dissolved separately in dH_2O (1 ml). Bovine IgG stock solution (11.2 $mg.ml^{-1}$) and the 1 $mg.ml^{-1}$ protein stock solutions were diluted further with dH_2O when required.

Trypsin stock solution (1 $mg.ml^{-1}$). Bovine pancreatic trypsin (1 mg) was dissolved in 1 mM HCl (1 ml).

5.9.2 Methods

OP-Tb (60 ng, $\simeq$0.75 pmol) was incubated (37°C) in separate experiments with 100-fold molar excesses of BSA (5.1 µg, $\simeq$75 pmol), fibrinogen (3.75 µg, $\simeq$75 pmol), bovine IgG (12 µg, $\simeq$75 pmol) or α_2-HS-glycoprotein (3.6 µg, $\simeq$75 pmol). Aliquots were removed at 0 min, 60 min, 120 min and 180 min, and proteolytic activity terminated by the addition of reducing treatment buffer and boiling (10 min). Positive controls were conducted in the same manner using bovine pancreatic trypsin at a 1:100 molar ratio. Samples were resolved by reducing Tris-Tricine SDS-PAGE (Section 2.7.1), and proteins visualised by Coomassie-blue staining (Section 2.7.3).

5.10 Digestion of gelatin in substrate SDS-PAGE

Substrate SDS-PAGE provides a sensitive and convenient means of detecting proteolytic activity on polyacrylamide gels. This technique is discussed fully in Section 2.7.2.

5.10.1 Methods

Purified OP-Tb (100-500 ng) and *T. b. brucei* lysates (2-10 μg) were subjected to electrophoresis on gelatin-containing SDS-PAGE gels (Section 2.5.3) and the gels developed as described in Section 2.7.2.2.

5.11 Results and discussion

5.11.1 Fluorogenic substrate specificity

Peptide hydrolysis by OP-Tb indicated that the presence of basic amino acid residues in the P_1 position was obligatory, thereby suggesting that OP-Tb has a trypsin-like specificity (Table 9). A variety of residues were acceptable in the P_2 position, including Phe, Leu, Gly, Pro, Lys and Arg. A comparison of the hydrolyses of Cbz-Arg-Arg-AMC and Cbz-Phe-Arg-AMC indicates that Arg is preferred over Phe in P_2, with a 6.5-fold decrease in k_{cat}/K_m compared to the Phe-containing substrate. A comparison of the hydrolysis of Boc-Val-Gly-Arg-AMC versus Boc-Val-Pro-Arg-AMC illustrates that substitution of Pro with Gly in P_2 is accompanied by a 8.5-fold increase in k_{cat}/K_m (i.e. Gly is preferred over Pro in the P_2 position). Substitution of Gly with Arg in Boc-Leu-**Gly**-Arg-AMC (to give Boc-Leu-**Arg**-Arg-AMC) caused a 20% increase in k_{cat}/K_m, indicating that Arg is preferred over Gly in P_2. However, substitution of this P_2-Arg with Lys in this substrate (to give Boc-Leu-**Lys**-Arg-AMC) has little (0.01% increase) effect on k_{cat}/K_m, indicating that Lys and Arg are equally acceptable in P_2. In contrast, substitution of Lys with Arg in Boc-Gly-**Lys**-Arg-AMC (to give Boc-Gly-**Arg**-Arg-AMC) resulted in a four-fold increase in k_{cat}/K_m, suggesting that Arg is preferred over Lys in P_2 in this case.

The poor k_{cat}/K_m for H-Arg-AMC (0.07 $s^{-1}.\mu M^{-1}$) and lack of activity against H-Gly-AMC and H-Leu-AMC suggests that OP-Tb has poor aminopeptidase activity. In contrast, blocking of the N-terminus with a Cbz group (i.e. Cbz-Arg-AMC) elevated the k_{cat}/K_m approximately 150-fold. This may indicate that substrate binding is more successful when both P_1 and P_2 are occupied. This is further supported by the results obtained with reversible competitive

inhibitors (Section 5.11.6, Table 10) and trypanocidal drugs (Section 7.6.1, Table 16). Thus, the P_2 preference appears to be (Arg/Lys) > Gly > Phe > Pro. There appears to be little specificity in P_3, as substitution of Val for Leu in Boc-**Leu**-Gly-Arg-AMC (to give Boc-**Val**-Gly-Arg-AMC) and Gly for Leu in Boc-**Leu**-Arg-Arg-AMC (to give Boc-**Gly**-Arg-Arg-AMC) has little (<0.001%) effect on k_{cat}/K_m.

Table 9. Amidolytic activity of trypanosome oligopeptidases

Substrate	OP-Tb			OP-Tc		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.\mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.\mu M^{-1}$)
Cbz-Arg-Arg-AMC	0.21	111.0	528.6	0.72	96.0	133.3
Cbz-Gly-Gly-Arg-AMC	0.91	142.0	157.8	1.19	114.0	95.8
Cbz-Phe-Arg-AMC	1.12	92.0	82.1	1.09	67.0	61.5
Cbz-Ala-Arg-Arg-AMC	2.21	120.0	54.3	2.07	92.0	44.4
Boc-Leu-Lys-Arg-AMC	0.84	44.0	52.4	1.04	37.0	33.6
Boc-Leu-Arg-Arg-AMC	1.14	60.0	52.3	1.69	54.0	40.0
Boc-Gly-Arg-Arg-AMC	1.87	97.0	51.9	2.21	49.0	39.6
Boc-Leu-Gly-Arg-AMC	1.27	54.0	42.5	1.11	44.0	33.3
Boc-Val-Gly-Arg-AMC	2.21	86.0	38.9	2.10	40.0	19.1
H-Ala-Phe-Lys-AMC	3.13	57.0	18.2	3.00	27.0	9.0
Boc-Gly-Lys-Arg-AMC	3.12	39.0	12.5	2.29	16.0	7.0
Boc-Val-Leu-Lys-AMC	4.04	50.0	12.4	4.34	29.0	6.7
Cbz-Arg-AMC	2.73	30.0	11.0	3.03	12.0	3.4
Boc-Ala-Gly-Pro-Arg-AMC	7.05	51.0	7.2	5.79	22.0	3.8
Boc-Val-Pro-Arg-AMC	9.89	46.0	4.6	9.89	17.0	1.7
Glt-Gly-Arg-AMC	59.4	25.0	0.42	67.1	8.0	0.12
H-Arg-AMC	61.6	4.6	0.07	54.4	2.7	0.05

[a]No activity was detected against Ac-Ala-Ala-Pro-Ala-AMC, H-Gly-AMC; H-Leu-AMC; MeoSuc-Gly-Trp-Met-AMC; Suc-Leu-Tyr-AMC; Glt-Gly-Gly-Phe-AMC or H-Gly-Pro-AMC after 1 h.

The k_{cat} was consistently lower for OP-Tc activity against fluorogenic peptides, and this suggests that OP-Tc may be catalytically less efficient than OP-Tb. It also cannot be discounted that errors in the determination of the $[E]_0$ for OP-Tb and OP-Tc may be

responsible for this apparent difference. Similar trends in substrate-specificity were observed for OP-Tc, suggesting that the substrate specificities of these two enzymes are very similar. The indistinguishable values of K_m observed for many of the substrates also points to this conclusion. However, the 3.4-fold difference in K_m observed between OP-Tb and OP-Tc with their best substrate (Cbz-Arg-Arg-AMC) indicates that the enzymes are not identical.

The K_m values for the hydrolysis of Cbz-Arg-Arg-AMC by OP-Tb and OP-Tc compare well with those obtained for potentially analogous enzymes from other sources, including an "alkaline peptidase" from *Crithidia fasciculata* (1 μM; Ashall *et al.*, 1990a) and a trypsin-like proteinase from soybeans, with a K_m of 2.2 μM against Cbz-Arg-Arg-*p*Na (Nishikata, 1984). Furthermore, an enzyme resembling the oligopeptidases of African trypanosomes has recently been isolated and cloned from *Trypanosoma cruzi* (Burleigh and Andrews, 1995; Burleigh *et al.*, 1996) for which the recombinant enzyme has a K_m of 5 μM for Cbz-Arg-Arg-AMC. As the K_m is not an absolute constant and depends on pH, temperature, presence of effector molecules and buffer composition (Michal, 1983), these small variations in K_m values may be attributable to different reaction conditions.

5.11.2 Effect of pH on oligopeptidase activity

OP-Tb activity against Cbz-Arg-Arg-AMC peaked at pH 9 (Fig. 29A), and the enzyme was still considerably active (75% of maximal activity) at physiological pH (pH 7.4). At pH 6, OP-Tb activity was less than 20% of that obtained at pH 9, although at pH 10 the enzyme still remained about 40% active. Almost identical results were obtained for OP-Tc.

The effect of pH on K_m and k_{cat} respectively was only investigated for OP-Tb, as these studies are expensive in terms of enzyme and substrate. The K_m appeared to be relatively unaffected by pH with a 1.6-fold variation in K_m over the pH range 4 to 12 (Fig. 29B). The shape of the curve suggested pK_a values of approximately 6 and 10. This is probably due to ionisation of the imidazole group of an active-site histidine, and the hydroxyl group of an active-site serine, respectively. These data are consistent with the pH-dependence trends observed for serine proteinases (Dunn, 1989). In contrast, changes in pH exerted a dramatic effect (100-fold increase) on the k_{cat} of OP-Tb.

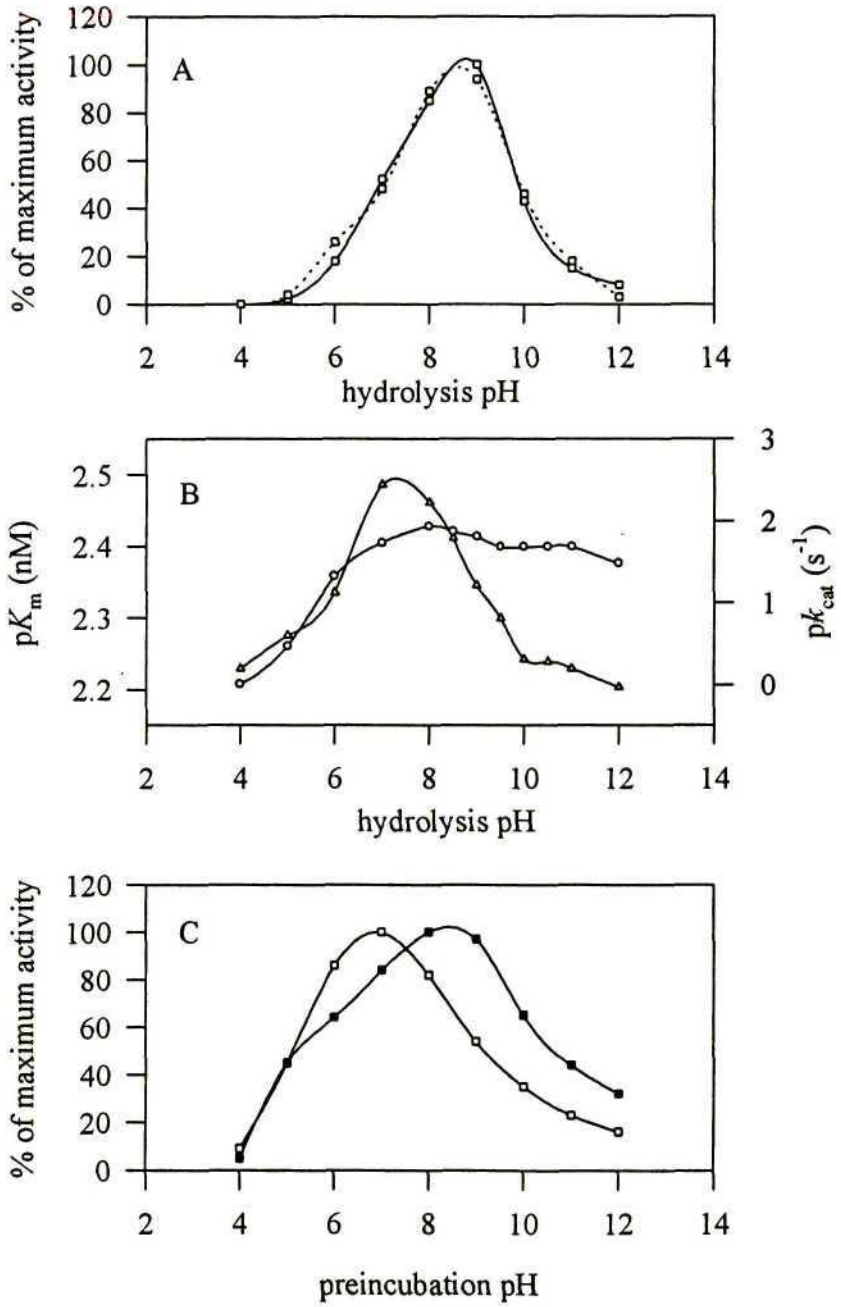


Figure 29. Effect of pH on the activity and stability of trypanosome oligopeptidases. (A) OP-Tb (—) and OP-Tc (·····) (1.5 ng) was assayed in AMT buffers (I = 0.1) over the pH range 4.0 to 12.0, containing 10 mM dithiothreitol. (B) Effect of pH on the K_m (Δ) and k_{cat} (○) of OP-Tb, constructed from the data presented in (A). (C) pH stability of OP-Tb was determined by incubating OP-Tb (10 ng) for 1 h at 37°C in AMT buffers (I = 0.1) over the pH range 4.0 to 12.0 in the absence (□) and presence (■) of 10 mM dithiothreitol. Residual enzyme activity against Cbz-Arg-Arg-AMC was then determined in the pH 8 AMT buffer.

This suggests that the enzyme will bind substrates over the pH-range tested, but can only hydrolyse them at neutral or alkaline pH. Thus, the arrangement of the catalytic residues (Asp-His-Ser triad, or oxyanion hole) is being disrupted, possibly through the titration of one or more of the residues.

Both in the presence and absence of the reducing agent dithiothreitol, OP-Tb was stable over a wide pH range, between pH 6 and 10 (Fig. 29C). In the absence of dithiothreitol, pH stability peaked at approximately pH 7, while in the presence of 10 mM dithiothreitol, OP-Tb was optimally stable at approximately pH 8.5. OP-Tb was nearly 100% irreversibly inactivated below pH 4, both in the absence and presence of dithiothreitol. However, OP-Tb appeared to be more stable in alkaline solution, retaining up to 40% of its activity at pH 12, in the presence of 10 mM dithiothreitol.

5.11.3 Effect of ionic strength on OP-Tb activity

OP-Tb activity was sensitive to ionic strength (Fig. 30), where increasing ionic strength resulted in a decrease in OP-Tb activity irrespective of the buffer system employed. OP-Tb activity was optimal at low ionic strength ($I \simeq 0.025$) and about half-maximally active at physiological ionic strength ($I \simeq 0.1$) at pH 7. At $I \simeq 0.2$, OP-Tb was less than 20% active, and activity was completely abolished at $I \simeq 0.5$.

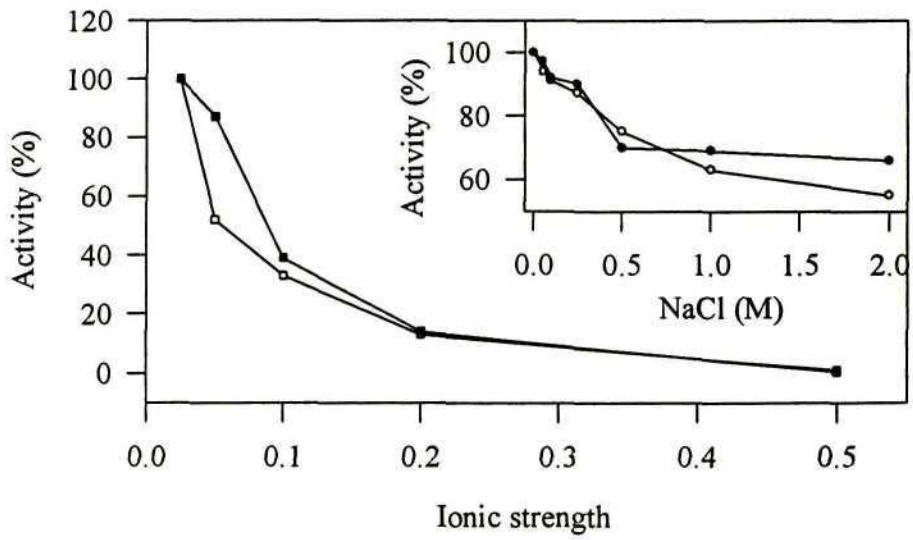


Figure 30. Effect of ionic strength on OP-Tb activity against Cbz-Arg-Arg-AMC. OP-Tb (1.5 ng) activity was assayed in the absence (□) an presence (■) of 10 mM dithiothreitol in constant-ionic-strength AMT buffers. *Inset*, Activity assayed in the absence (○) an presence (●) of 10 mM dithiothreitol in 50 mM Hepes, pH 8, containing sodium chloride as indicated on the abscissa. Standard deviations were less that ±10%.

While similar trends have been observed for the lysosomal cysteine proteinases (Dehrmann *et al.*, 1995; 1996, Dennison *et al.*, 1992), these observations contrast with the finding that increasing the sodium chloride concentrations of up to 2 M NaCl enhances the activity of prolyl oligopeptidase from porcine brain (Polgár, 1982) and *Treponema denticola* (Mäkinen *et al.*, 1994). Turk *et al.* (1994) have proposed a mechanism for the destabilisation of the tertiary structure, and hence active-site, of cathepsin B by elevated ionic strength. However, in the absence of a three-dimensional structure for OP-Tb, it is not possible to comment further at present on the extent to which this model may apply to OP-Tb.

5.11.4 Effect of reducing agents on oligopeptidase activity

Dithiothreitol, cysteine and reduced glutathione (GSH) all enhanced OP-Tb activity (Fig. 31), and a parallel situation was observed for OP-Tc (results not shown). At all concentrations tested, dithiothreitol proved to be the best reducing agent, with maximal activation occurring at approximately 10 mM. Cysteine and GSH were comparatively poor activators of OP-Tb and OP-Tc, with maximal activation occurring between 5 and 10 mM. Above these concentrations, cysteine and GSH became inhibitory. Therefore 10 mM dithiothreitol was routinely employed as an activator.

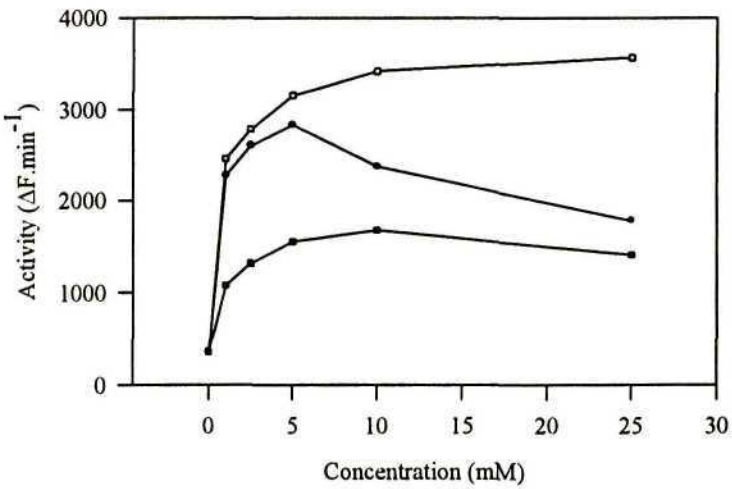


Figure 31. Effect of reducing agents on OP-Tb activity against Cbz-Arg-Arg-AMC. OP-Tb (1.5 ng) was assayed as described in Section 2.3.2 except that dithiothreitol (–□–), GSH (–●–) or cysteine-HCl (–■–) were used as reducing agents at various concentrations. Standard deviations for the activities were less than ±10%.

The activation of serine protease activity by reducing agents suggests that an essential cysteine residue, which must be maintained in its reduced state for catalysis to proceed, may occur in the vicinity of the active site. The enhancement of oligopeptidase activity by reducing agents has also been observed for prolyl oligopeptidase (EC 3.4.21.26), a serine oligopeptidase from mammalian and porcine brain tissue, which exhibits 150% enhancement of activity in the presence of 50 μM dithiothreitol or 50 μM β-mercaptoethanol. However, these reducing agents became inhibitory at concentrations above 0.5 mM (Moriyama *et al.* 1988). Prolyl oligopeptidase possesses two cysteine residues in the immediate vicinity of the catalytic aspartic acid residue (Rennex *et al.*, 1991). However, as the amino acid sequences of OP-Tb and OP-Tc are not yet known, it is not possible to investigate this hypothesis further. Furthermore, essential cysteine residues that are far removed from the catalytic apparatus in the primary structure of the enzyme may be brought into close proximity to the catalytic site by the tertiary structure of the enzyme. As the cytosol is a reducing environment, this introduces the possibility that intracellular reducing agents such as glutathione and trypanothione may act as *in vivo* regulators of OP-Tb activity within trypanosomes.

5.11.5 Rate of activation with dithiothreitol

OP-Tb was rapidly activated (approximately two-fold increase in activity) in the presence of 10 mM dithiothreitol after 1 min (Fig. 32). The rate of activation then slowed down considerably, and plateaued after about 10 min. In all subsequent assays, OP-Tb was activated for 10 min in 10 mM dithiothreitol.

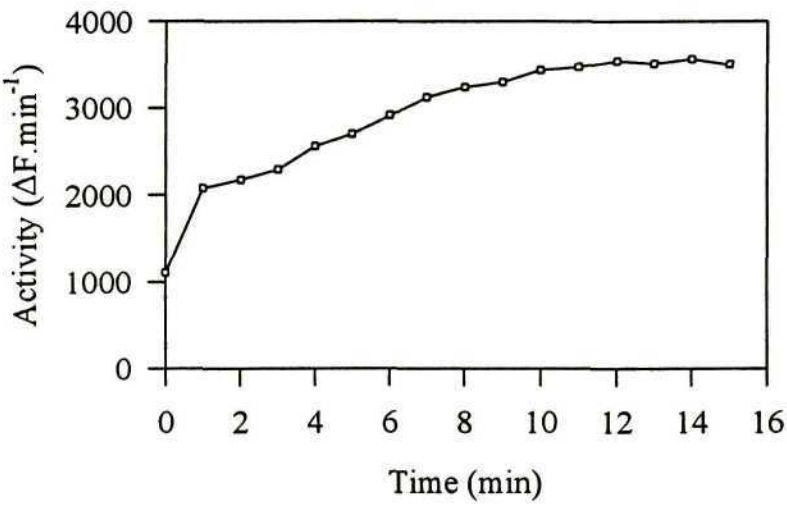


Figure 32. Optimum time of OP-Tb activation with dithiothreitol. OP-Tb (1.5 ng) was assayed as described in Section 2.3.2, except that the enzyme was pre-incubated with dithiothreitol (10 mM) for 0-15 min before the addition of Cbz-Arg-Arg-AMC. Standard deviations for the activities were less than ±10%.

5.11.6 Inhibitor profile of trypanosome oligopeptidases

The inhibition of trypanosome oligopeptidases by reversible, competitive inhibitors is illustrated in Table 10. Both OP-Tb and OP-Tc were inhibited by the peptide aldehydes antipain and leupeptin, which contain the aldehyde on the P_1-Arg residue. The lower K_i and higher k_{ass} of antipain are probably attributable to the comparatively more basic nature of antipain, whose tripeptide contains two arginine residues, compared to leupeptin that has only one arginine residue (Umezawa, 1976). No inhibition was observed for chymostatin, where the aldehyde is present on the P_1-Phe residue. This further supports the findings with fluorogenic peptide substrates that phenylalanine is not acceptable in P_1 (Table 9). Similar trends in inhibitory potency of tripeptide argininals (antipain > leupeptin) have also been reported for oligopeptidase B from *M. lacunata* (Kanatani *et al.*, 1991), a trypsin-like protease from soybeans (Nishikata, 1984), and by a related oligopeptidase from *T. cruzi* (Burleigh *et al.*, 1996). As the argininals (arginine aldehydes) probably exist primarily as inactive cyclic carbinolamines in aqueous solution (Schultz *et al.*, 1989), the actual [I] is probably below the predicted [I], resulting in an observed K_i that is higher and an observed k_{ass} that is lower than the real values.

Table 10. Inhibition of trypanosome oligopeptidase activity by competitive reversible inhibitors[a,b].

Inhibitor	OP-Tb			OP-Tc		
	K_i (μM)	k_{ass} ($M^{-1}.s^{-1}$)	k_{diss} (s^{-1})	K_i (μM)	k_{ass} ($M^{-1}.s^{-1}$)	k_{diss} (s^{-1})
leupeptin	30×10^{-3}	4.76×10^{4}	1.43×10^{3}	21×10^{-3}	5.39×10^{4}	1.13×10^{3}
antipain	1.81×10^{-3}	1.08×10^{6}	1.96×10^{3}	2.11×10^{-3}	0.89×10^{6}	1.88×10^{3}
aprotinin	0.59×10^{-3}	n.d.	n.d	0.89×10^{-3}	n.d.	n.d
benzamidine	254	n.d.	n.d.	247	n.d.	n.d.
E-64	62.5	n.d.	n.d.	73.7	n.d.	n.d.

[a]No inhibition was observed with amastatin, bestatin, chicken ovomucoid, chymostatin, EDTA, EGTA, elastinal, lima bean trypsin inhibitor, pepstatin, 1,10-phenanthroline or soybean trypsin inhibitor.
[b]n.d., not determined. In these cases, the k_{ass} was too fast to be measured experimentally.

Both OP-Tb and OP-Tc were inhibited competitively by E-64, with K_i values of 62.5 μM and 73.7 μM respectively. This contrasts with the widely-held view that E-64 is a class-specific inhibitor of cysteine proteinases (Barrett *et al.*, 1982), although the inhibition of bovine β-trypsin by E-64, by a reversible competitive mechanism with a K_i of 36 μM, has recently been reported (Sreedharan *et al.*, 1996). These K_i values are, however, considerably higher than those reported for the inhibition of cathepsin L-like cysteine proteinases by E-64, which are generally below 10 μM (Barrett *et al.*, 1982).

Benzamidine, a low molecular mass inhibitor of trypsin-like proteases, was a comparatively poor inhibitor of OP-Tb and OP-Tc, with a K_i of 254 μM and 247 μM respectively, compared to a K_i of 36 μM for bovine β-trypsin and 12 μM for mast-cell tryptase (Caughey *et al.*, 1993). In contrast to these proteinases, oligopeptidase B from microbial sources is relatively unaffected by benzamidine (Kanatani *et al.*, 1991; Yoshimoto *et al.*, 1995). The poor K_i for benzamidine may account for the relatively weak binding of OP-Tb and OP-Tc to *p*-aminobenzamidine-Sepharose, from which they eluted at about 250 mM NaCl. In contrast, trypsin requires much harsher conditions for elution from this matrix (e.g. 100 mM CH_3COOH) (Hermanson *et al.*, 1992).

Classical reversible aspartic and metalloprotease inhibitors had no affect on the activity of OP-Tb or OP-Tc (Table 10).

Aprotinin (bovine pancreatic trypsin inhibitor) is a competitive inhibitor of OP-Tb and OP-Tc with sub-nanomolar K_i values, indicating that it was a better inhibitor of OP-Tb than plasma kallikrein (K_i = 30 nM) and plasmin (K_i = 1 nM) (Fritz and Wunderer, 1983). While oligopeptidase B from soybeans is inhibited by aprotinin (Nishikata, 1984), recombinant oligopeptidase B from *T. cruzi* is apparently not (Burleigh *et al.*, 1997). Neither OP-Tb nor OP-Tc were inhibited by any other protein inhibitors of serine and cysteine proteases, including soybean trypsin inhibitor (SBTI), lima bean trypsin inhibitor, chicken ovomucoid (Table 10), or the mammalian plasma serpins, kininogens and cystatins (Section 6.8.3, Table 15), supporting the hypothesis that these enzymes are oligopeptidases with considerable size constraints on their ability to bind substrates and/or inhibitors.

If indeed the catalytic-site of oligopeptidases is buried in an "active-site pit" as has been proposed by Barrett and Rawlings (1992), the differential effects of aprotinin and SBTI may be explained in terms of the size and tertiary structure of these two inhibitors. Aprotinin has a highly cross-linked, very compact pear-shaped tertiary structure, about 29Å in length and 19Å in diameter at its widest and about 10Å at its narrowest point (Huber *et al.*, 1971; Deisenhofer and Steigmann, 1974) (Figs 33A and 33B). The region of interaction of aprotinin with its target protease, containing the Lys^{15}-Ala^{16} reactive-site bond, is located in this narrow region, at the "top" of the pear (see arrows in Fig. 33B).

In contrast, SBTI (Fig. 33C) has a far more globular structure, and the overall structure of this inhibitor is that of a sphere of about 35 Å in diameter (Sweet *et al.*, 1974). Here the reactive-site Arg^{65}-Phe^{66} bond is located on a curved loop slightly protruding from the bulk of the molecule (see arrow in Fig 33C). It is evident from the structures presented in Fig. 33 that the reactive-site region of SBTI would have greater difficulty gaining access to a catalytic site buried in an active-site "pit" than would be the case for the almost rod-shaped aprotinin molecule. Furthermore, the regions of contact between aprotinin and trypsin span a single domain of aprotinin, all located in the "top" of the pear-shaped molecule (shading, in Fig. 33A). In contrast, the interaction of SBTI with trypsin involves interactions within and outside the reactive-site region, which are located in two separate domains of SBTI. In addition to the Pro^{60}-Phe^{66} region containing the reactive-site bond, the N-terminal Asp^{1}-Phe^{2} region is believed to form an important interaction with trypsin, where the carboxyl group of Asp^{1} forms an ion-pair with Lys^{60} of trypsin (Sweet *et al.*, 1974). Again, it is unlikely that the proposed active-site "pit" of oligopeptidases could accommodate two separate domains of a protein inhibitor. It seems likely that the dimensions of this "pit" in OP-Tb and OP-Tc may lie somewhere between 10Å and 35Å.

OP-Tb and OP-Tc were inactivated by irreversible organophosphate, sulfonylfluoride and isocoumarin inhibitors of serine proteinases (Table 11). 3.4-dichloroisocoumarin (DCI) was the most effective of the non-peptide irreversible inhibitors, with k_{ass}-values of 142.1 $M^{-1}.s^{-1}$ for OP-Tb and 167 $M^{-1}.s^{-1}$ for OP-Tc. These k_{ass} values approximate those reported by Harper *et al.* (1985) for bovine trypsin (198 $M^{-1}.s^{-1}$), human factor D (192 $M^{-1}.s^{-1}$) and human plasmin (133 $M^{-1}.s^{-1}$).

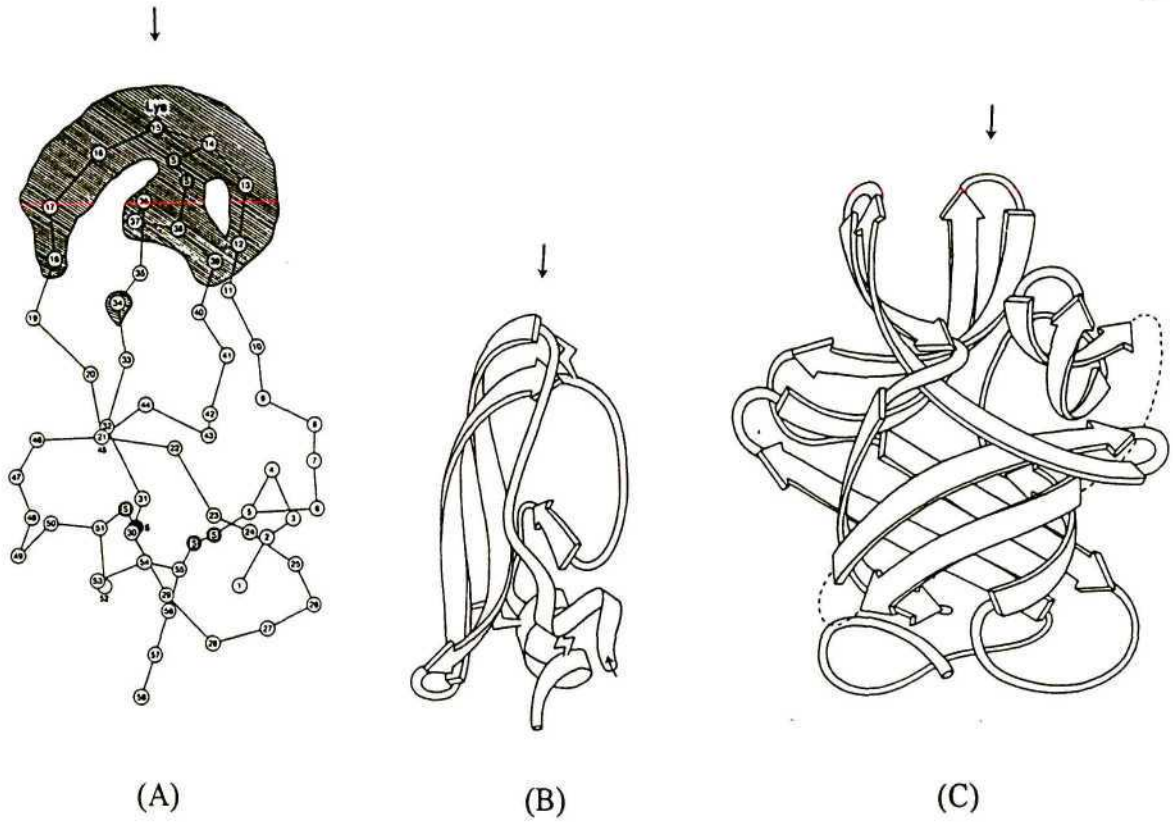


Figure 33. Tertiary structure of protein inhibitors of serine proteases. (A) C_α-skeletal model of aprotinin. Shaded region indicates regions in close contact in the trypsin-aprotinin complex (Deisenhofer and Steigmann, 1974). Ribbon-diagrams illustrating the tertiary structure of aprotinin (B) and SBTI (C). Arrows indicate the reactive-site bond (after Richardson, 1981).

Table 11. Inhibition of trypanosome oligopeptidase activity by irreversible inhibitors of cysteine and serine proteinases.

Inhibitor	OP-Tb		OP-Tc	
	k_{ass}[a] ($M^{-1}.s^{-1}$)	$t_{½}$[b] (s)	k_{ass}[a] ($M^{-1}.s^{-1}$)	$t_{½}$[b] (s)
AEBSF	14.00 ± 2.07	196	22.02 ± 4.05	126
DCI	142.10 ± 11.90	18	167.00 ± 23.60	16
DFP	7.40 ± 0.79	375	13.09 ± 2.17	213
iodoacetamide[c]	1.27 ± 0.71	2182	1.11 ± 0.71	2497
iodoacetic acid[c]	1.91 ± 0.08	1451	2.19 ± 0.08	1265
N-ethylmaleimide[c]	1.57 ± 0.11	1765	1.72 ± 0.11	1611
*p*CMB[c]	21.90 ± 4.47	126	29.10 ± 4.47	95
PMSF	0.60 ± 0.02	4620	0.91 ± 0.02	3046

[a] Data reflect the mean k_{ass} ± SD (n = 3).
[b] $t_{½}$ at 250 μM inhibitor concentration.
[c] Assays conducted in the absence of dithiothreitol. In the presence of 10 mM dithiothreitol, the $t_{½}$ for the inhibiton of OP-Tb by thiol-reactive agents was elevated as follows: iodoacetamide (2520 s), iodoacetic acid (2559 s), *N*-ethylmaleimide (1900 s) and *p*CMB (1854 s).

AEBSF is an irreversible inhibitor of serine proteases, inactivating trypsin and chymotrypsin at a rate comparable to that of DFP. Both OP-Tb and OP-Tc were more rapidly inactivated by AEBSF than is serum kallikrein (k_{ass} = 0.68 $M^{-1}.s^{-1}$). Similarly, both oligopeptidases were inactivated more rapidly by DFP and PMSF than is serum kallikrein, which has k_{ass} values of 0.3 $M^{-1}.s^{-1}$ and 0.07 $M^{-1}.s^{-1}$ for these inhibitors respectively (Markwardt *et al.*, 1974).

As AEBSF has enhanced stability at physiological pH values ($t_{½}$ = 2 h, compared with 20 min for DCI, and 1 h for DFP and PMSF) and much lower toxicity [LD_{50} = 2.8 $g.kg^{-1}$, oral dose in mice, as opposed to 3.7 $mg.kg^{-1}$ for DFP (Markwardt *et al.*, 1974)], AEBSF was routinely employed in this investigation to inhibit trypanosome oligopeptidases, and in conjunction with SBTI, to distinguish oligopeptidase activity from trypsin-like serine protease activity, primarily of plasma kallikrein (see later).

The inhibition of OP-Tb activity by thiol-reactive agents (iodoacetate, iodoacetamide, *N*-ethylmaleimide and *p*CMB) supports the reductive activation data, suggesting that an essential cysteine residue is either implicated in catalytic activity or located in close proximity to the active site. Alkylating reagents attached to this residue could either create an unfavourable charge environment at the catalytic site or block access of the substrate to the catalytic apparatus.

In order to test this hypothesis, a variety of thiol-reactive agents with differing physico-chemical properties were examined for inhibitory potency. *N*-ethylmaleimide and *p*CMB would both introduce relatively large bulky groups at the site of covalent attachment which may sterically interfere with the binding of substrates in the active site. In contrast, iodoacetamide and iodoacetate are both small molecules which carry opposite charges. Their presence in the active-site may either electrostatically interfere with substrate binding, or may interfere with the catalytic mechanism of the enzyme. Iodoacetamide would introduce a neutral amide group at the site of covalent attachment, whereas iodoacetate would introduce a negatively-charged acetate group (Fig. 34).

$$\text{Protein}\sim\text{SH} + \text{ICH}_2\text{CONH}_2 \rightleftharpoons \text{Protein}\sim\text{SCH}_2\text{CONH}_2 + \text{H}^+ + \text{I}^-$$

$$\text{Protein}\sim\text{SH} + \text{ICH}_2\text{COO}^- \rightleftharpoons \text{Protein}\sim\text{SCH}_2\text{COO}^- + \text{H}^+ + \text{I}^-$$

Figure 34. Alkylation of free thiol groups in proteins by iodoacetate and iodoacetamide. Alkylation of OP-Tb by iodoacetamide (ICH_2COONH_2) introduces a neutral amide, whereas iodoacetate (ICH_2COO^-) introduces a negatively charged acetate group (after Benesch and Benesch, 1962).

All four thiol-reactive agents demonstrated inhibitory activity, albeit with different rates of association with the enzyme. The fact that these compounds do inhibit oligopeptidase activity may suggest that both steric hindrance, or an unfavourable charge environment at the catalytic site may interfere with substrate binding and/or catalysis. The presence of competing thiols such as dithiothreitol elevated the $t_{½}$, probably by mopping up the inhibitor in solution. In the absence of a three-dimensional structure of OP-Tb, it is not possible to predict where these inhibitors may bind to the enzyme, and thus impirair its activity.

These findings parallel those of Polgár (1991) who reported a similar trend (*p*CMB > *N*-ethylmaleimide > iodoacetamide) in the inhibition of porcine prolyl oligopeptidase by thiol-reactive agents. Prokaryotic members of the prolyl oligopeptidase family including oligopeptidase B from *E. coli* (Kanatani *et al.*, 1991), *F. meningosepticum* (Yoshimoto *et al.*, 1991) and *M. lacunata* (Yoshimoto *et al.*, 1995) also display sensitivity to these thiol-reactive agents, albeit to a lesser extent than their eukaryotic counterparts.

5.11.7 Potential activators of oligopeptidase activity

Oligopeptidase activity was enhanced in the presence of the polyamines spermine and spermidine, and to a lesser degree, putrescine (Table 12). Polyamines are known to influence the activity of various enzymes, for example, the serine/threonine protein phosphatases where micromolar concentrations of spermine enhance activity, but higher concentrations (i10 mM) abolish the activity (Lim Tung *et al.*, 1985).

In contrast to the results reported here, Ashall (1990) reported the inhibition of alkaline peptidase activity from *T. cruzi* and *C. fasciculata* by spermine and spermidine. These investigators possibly did not use the polyamines at sufficiently low concentrations to recognise the enhancing effects of these compounds. In this investigation, both spermine and spermidine (50 µM) enhanced the activity of OP-Tb against Cbz-Arg-Arg-AMC. This

introduces a possible *in vivo* mechanism of regulation of oligopeptidase activity within trypanosomes as trypanosomes contain micromolar concentrations of a number of polyamines, including spermine and spermidine (Fairlamb *et al.*, 1987).

Neither ATP nor GTP had any effect on the activity of OP-Tb which suggested that it was not related to the ATP-dependent serine proteases. Heparin was also shown to enhance OP-Tb activity, raising the possibility that any heparin circulating in the host bloodstream may also enhance the activity of OP-Tb when released by trypanosomes into the bloodstream.

Table 12. Effect of potential activators on oligopeptidase activity[a].

Compound	Concentration (μM)	Activity relative to control (%)	
		OP-Tb	OP-Tc
Heparin	30 $\mu g.ml^{-1}$	158	166
Spermine	50	177	179
Spermidine	50	162	177
Putrescine	50	119	108

[a]No effect on activity was observed in the presence of ornithine, ATP or GTP.

A number of ATP-dependent serine proteases from *E. coli* are currently recognised, including the Clp endopeptidase and endopeptidase La (reviewed by Gottesman and Maurizi, 1992). However, neither ATP nor GTP had any effect on the activity of OP-Tb or OP-Tc (Table 12), which suggested that trypanosome oligopeptidases are not related to the ATP-dependent serine proteases.

5.11.8 Influence of divalent metal ions

The influence of divalent metal ions on OP-Tb activity is illustrated in Table 13. None of the metal ions tested had an activating effect on the activity of OP-Tb. Heavy metal ions, in particular, iron, zinc, cadmium and mercury were strongly inhibitory, abolishing between 90% and 100% of the activity. Copper and nickel were also inhibitory, albeit to a lesser extent.

The divalent metal ions of heavy metals and some metalloids can combine with free thiols to form mercaptides. Zn^{2+}, Hg^{2+}, Cd^{2+} and Fe^{2+} all completely inhibited oligopeptidase activity at

1 mM, and each of these metals are known to form mercaptides of varying degrees of reversibility (Barron, 1951). Furthermore, Cu^{2+}, which partially extinguished oligopeptidase activity, is known to catalyse the oxidation of free thiols (Barron, 1951).

It would therefore appear that OP-Tb contains a cysteine residue which must be maintained in its free and reduced state to preserve the activity of the enzyme. The lack of activation by divalent metal ions (Table 13), together with the lack of inhibition of oligopeptidase activity by EDTA, EGTA and 1,10-phenanthroline (Table 10), suggests that these oligopeptidases do not posses metal ion requirements.

Table 13. Effect of divalent metal ions on oligopeptidase activity[a].

Ion	Parent compound	Activity (% relative to control)	
		OP-Tb	OP-Tc
Cd^{2+}	$CdCl_2.2½H_2O$	3	0
Cu^{2+}	$CuCl_2.2H_2O$	55	66
Fe^{2+}	$FeCl_2.4H_2O$	7	4
Hg^{2+}	$HgCl_2$ anhydrous	0	0
Ni^{2+}	$NiCl_2.6H_2O$	37	12
Zn^{2+}	$ZnCl_2$ anhydrous	4	0

[a]No effect on activity was observed in the presence of $BaCl_2.2H_2O$, $CaCl_2.2H_2O$, $MgCl_2.2H_2O$ or $MnCl_2.2H_2O$.

5.11.9 Hydrolysis of protein substrates

OP-Tb did not hydrolyse a number of major plasma proteins, including BSA, IgG, fibrinogen or α_2-HS-glycoprotein at 1:100 (enzyme:substrate) molar ratios (Figs 35A-35D), despite their rapid hydrolysis by trypsin, a serine protease with some substrate specificity similarities to OP-Tb. Furthermore, OP-Tb was not inhibited by high molecular mass inhibitors of trypsin-like serine proteases, including SBTI (reviewed in Birk, 1976a) and lima bean trypsin inhibitor (reviewed in Birk, 1976b) (Table 10). All these data together suggested that OP-Tb was in fact an oligopeptidase, rather than a proteinase.

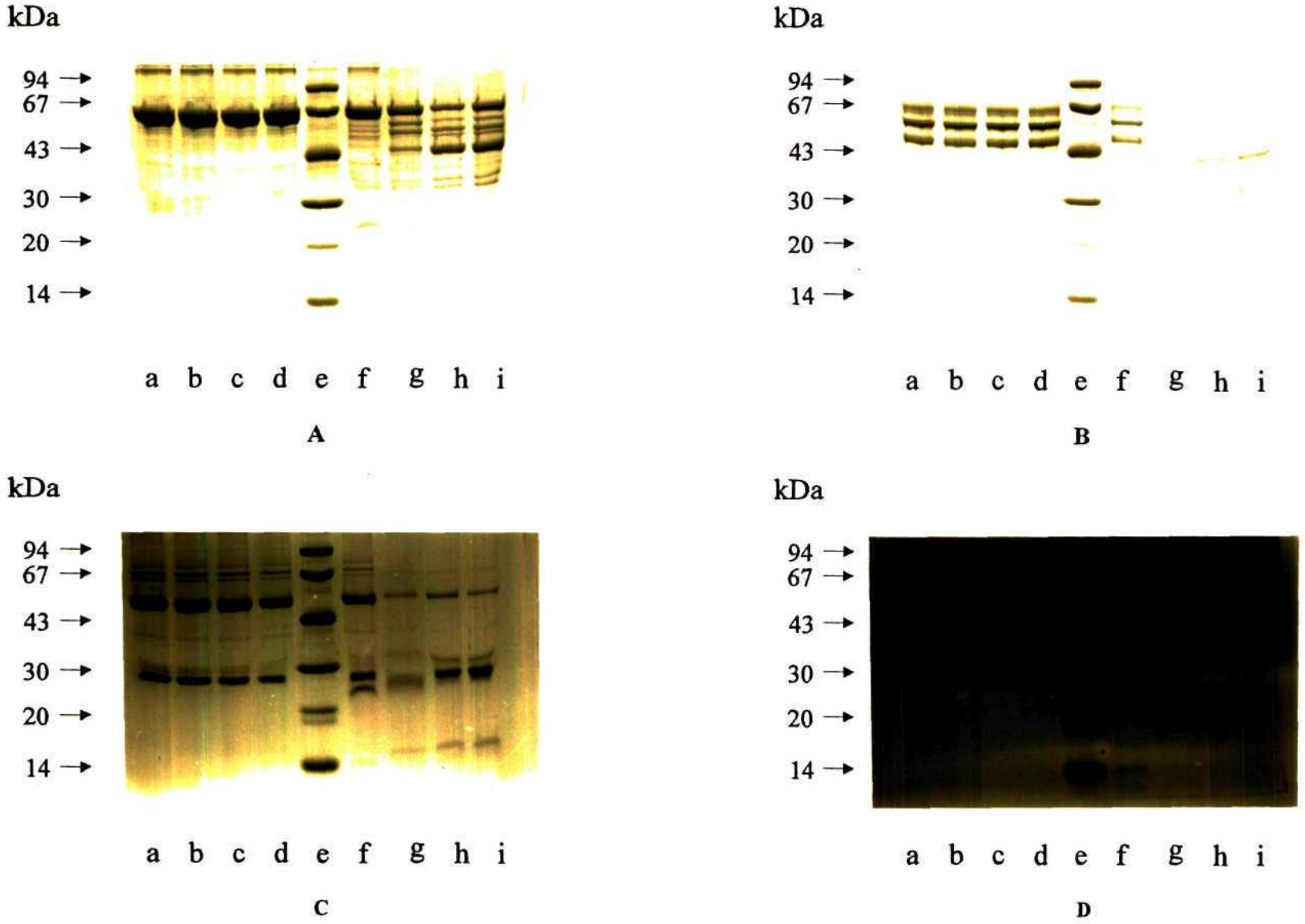


Figure 35. Digestion of protein substrates by OP-Tb. BSA (A), fibrinogen (B), rabbit IgG (C) and α_2-HS-glycoprotein (D) (1:100 molar ratio) were incubated with OP-Tb and aliquots were removed at 0 min (a), 60 min (b), 120 min (c) and 180 min (d). Lane (e) contains M_r markers as in Section 2.7.1.1. Similarly, each protein (1:100 enzyme:substrate molar ratio) was incubated with bovine pancreatic trypsin and aliquots were removed at 0 min (f), 60 min (g), 120 min (h) and 180 min (i). Samples were resolved by reducing Tris-Tricine SDS-PAGE as described in Section 2.7.1.

5.11.10 Digestion of gelatin in substrate SDS-PAGE

OP-Tb did not degrade gelatin in substrate SDS-PAGE (Fig. 36). *T. b. brucei* lysates gave rise to a number of zones of proteolytic activity against gelatin in gelatin-containing SDS-PAGE gels (Fig. 36).

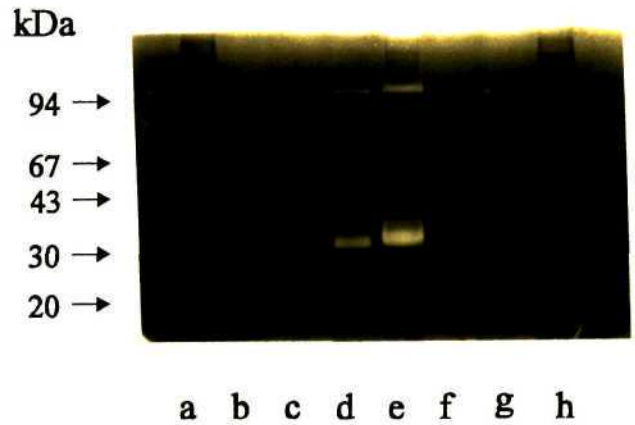


Figure 36. Digestion of gelatin by OP-Tb on a substrate gel. Gelatin-containing SDS-PAGE of OP-Tb-containing preparations was performed at pH 8 as described in Section 5.10. M_r markers as in Section 2.7.1.1 (lanes a and h); OP-Tb (100 ng) (b); OP-Tb (200 ng) (c); *T. b. brucei* lysate (2 µg) (d); *T. b. brucei* lysate (10 µg) (e); OP-Tb (400 ng) (f) and OP-Tb (500 ng) (g).

A number of bands of activity are evident between 30 and 45 kDa, with a major band of activity at 30 kDa. These activities are probably attributable to the major cysteine protease of *T. b. brucei*, trypanopain-Tb. This enzyme yields multiple bands of activity in samples which have not been boiled or reduced prior to electrophoresis (L. Troeberg, *pers. comm.*), as was the case with substrate SDS-PAGE. Furthermore, additional bands of activity are evident at approximately 85 and 100 kDa. Despite the activity at 85 kDa in *T. b. brucei* lysates, no activity was observed in lanes containing purified OP-Tb. This high molecular mass activity may be attributable to enzymatically active trypanopain-Tb-L-kininogen complexes. The formation of such complexes has been reported for *T. b. brucei*, *T. congolense* and *Leishmania* sp (Lonsdale-Eccles *et al.*, 1995) and for *T. cruzi* (Wiser *et al.*, 1997). From the data presented here, it appears that OP-Tb does not hydrolyse gelatin in substrate SDS-PAGE.

5.12 Concluding remarks

Enzymatic characterisation of the trypanosome oligopeptidases OP-Tb and OP-Tc revealed that they exhibited a preference for basic amino acid residues in the P_1 and P_2 positions. While only basic residues were acceptable in P_1, a variety of residues were tolerated in the P_2 position. These included basic residues (arginine and lysine); small, uncharged residues (glycine); hydrophobic residues (phenylalanine and leucine) and bulky residues (proline), in that order of preference. There was little preference exhibited at the P_3 position, and only a very weak arginine aminopeptidase activity was detected. These observations were supported by data obtained for the inhibition of OP-Tb activity by reversible, competitive inhibitors such as peptide aldehydes.

OP-Tb was optimally active at neutral to basic pH, and displayed a similar pH stability profile. The effects of varying pH on the K_m and k_{cat} were consistent with those observed for other serine proteases. As OP-Tb and OP-Tc were active and stable at the pH and ionic strength of the host bloodstream, the possibility was raised that the enzyme may contribute to disease pathogenesis through the digestion of host peptide hormones if it is released into the host bloodstream.

OP-Tb and OP-Tc were sensitive to the presence of reducing agents (dithiothreitol > β-mercaptoethanol > cysteine-HCl), and were inhibited by a number of thiol-reactive agents. Taken together, these data suggest that an essential cysteine residue within the oligopeptidase molecule must be maintained in its reduced state for substrate binding and/or catalysis to proceed. The activation of OP-Tb and OP-Tc by reducing agents and polyamines may constitute two mechanisms of *in vivo* regulation of the activity of these enzymes.

In spite of the weak inhibition of OP-Tb and OP-Tc by E-64 and their activation by reducing agents (which would normally suggest the activity of a cysteine protease) OP-Tb was also inhibited by mechanism-based inhibitors of serine proteases, including DCI, AEBSF and PMSF. This led us to conclude that OP-Tb and OP-Tc were thiol-sensitive serine oligopeptidases.

No activity was evident against a variety of protein substrates tested, and these data, together with the apparent lack of inhibition by high-molecular-mass serine protease inhibitors suggested that these enzymes were oligopeptidases, and without activity against large protein molecules. Furthermore, the sensitivity of OP-Tb and OP-Tb to reducing agents, and the inhibition of these

two enzymes by thiol-reactive agents, together with their apparent lack of activity against proteins, strongly suggests that OP-Tb and OP-Tc are members of the prolyl oligopeptidase family of serine proteases (the "S9" family of serine proteases in the nomenclature of Barrett and Rawlings, 1995).

The results of a detailed study of the interactions of OP-Tb with host system molecules are presented in Chapter 6. Furthermore, determination of the substrate specificity of these oligopeptidases facilitated the identification of various inhibitors which were used to evaluate the potential of trypanosome oligopeptidases as therapeutic targets (Chapter 7).

Chapter 6

Interaction of OP-Tb with host molecules

6.1 Introduction

There are no known endogenous inhibitors of oligopeptidases (Barrett and Rawlings, 1992). Furthermore, trypanosome oligopeptidases are active at the physiological pH and ionic strength of the mammalian host bloodstream (Sections 5.11.2, Fig. 29 and 5.11.3, Fig. 30). This raises the possibility that if trypanosome-derived oligopeptidases enter the host bloodstream or cerebrospinal fluid (CSF), the enzyme may remain active and thereby contribute to disease pathogenesis through the digestion of host peptides. It was therefore decided to investigate whether trypanosome oligopeptidases were inhibited by endogenous host serine protease inhibitors, and whether oligopeptidase activity could be detected in the bloodstream of infected hosts.

Host plasma proteases are usually regulated *in vivo* by host plasma protease inhibitors, which are generally high molecular mass proteins (50-750 kDa) (Travis and Salvesen, 1983). However, due to the restricted substrate size accommodated by oligopeptidases, it was not certain whether these inhibitors would exhibit any inhibitory activity against oligopeptidases.

Protease inhibitors represent more than 10% of the total protein in human plasma. The majority of these inhibitors are involved in the regulation of serine protease activity, and are collectively referred to as serpins (serine protease inhibitors) (Travis and Salvesen, 1983) (Table 14). *In vivo*, these serpins regulate a variety of physiological processes including blood coagulation, fibrinolysis and complement activation (Travis *et al.*, 1990). A disturbance of the balance between proteases and their endogenous inhibitors is frequently implicated in the pathogenesis of disease. Such conditions have been described for asthma, inflammation and possibly cancer (Section 1.4.2.4).

The most abundant plasma serpin is α_1-protease inhibitor (α_1PI), also known as α_1-antitrypsin. The most extensively studied target protease of this serpin is the enzyme elastase which is involved in the degradation of the extracellular matrix. Two other serpins, α_2-antiplasmin (α_2AP) and antithrombin III (ATIII) have a similar specificity for trypsin-like proteases. The physiological target of α_2AP is plasmin, and this inhibitor is therefore involved in regulating fibrinolysis (Travis

and Salvesen, 1983). Similarly, ATIII regulates the activity of thrombin, and hence the blood clotting cascade (Travis and Salvesen, 1983). In contrast, α_1-antichymotrypsin (α_1ACT) specifically inhibits proteases with a chymotrypsin-like specificity (Travis and Salvesen, 1983). In spite of its name, the physiological target of α_1ACT is neutrophil cathepsin G, which is released during inflammatory processes (Travis *et al.*, 1990).

Additionally, α_2-macroglobulin (α_2M), which is a general inhibitor of all classes of proteases, occurs in the plasma. Its mechanism of inhibition is distinct from that of typical serpins (Barrett and Starkey, 1973), and it will thus be dealt with separately from them.

An important structural feature of serpins is the presence of a reactive-site loop which is exposed on the surface of these proteins. Serpins react irreversibly with their target enzymes in a 1:1 molar ratio. The formation of enzyme-inhibitor complexes occurs at the reactive-site loop, where the protease hydrolyses, or attempts to hydrolyse, a peptide bond within the reactive-site loop. The specificity of the serpin is determined by the amino acid sequence of the reactive-site loop, as it is this region of the serpin that mimics the substrate. Consequently, it is useful to designate the amino acid sequence of the reactive-site loop in the same way as that of proteinase substrates by the method of Schechter and Berger (1967) (Section 1.2, Fig. 5). The reactive site sequences for a number of serpins and their concentrations in plasma are provided in Table 14.

Interestingly, oligopeptidases are known to catalytically inactivate serpins (Bagarozzi *et al.*, 1996). While it may initially seem counter-intuitive that an oligopeptidase is able to digest part of a large, globular, intact protein, it is believed that the reactive-site loop may protrude far enough from the bulk of the globular protein for the loop to be treated as an isolated peptide. Examination of the three-dimensional structures of α_1ACT and ATIII reveal that the reactive-site loop does indeed protrude from the bulk of the protein (Fig. 37). Bagarozzi *et al.* (1996) reported the characterisation of a chymotrypsin-like serine oligopeptidase which did not display hydrolytic activity against any protein substrates, but catalytically inactivated α_1PI in the reactive-site loop. This raised the possibility that OP-Tb may catalytically inactivate host plasma serpins in a similar manner.

Table 14. Serpins present in human plasma.

Serpin	Plasma concentration (μM)	Reactive-site loop sequence[a]
α_1-Protease inhibitor	25	E-A-I-P-**M**-S-I-P-P-E
α_1-Antichymotrypsin	7	K-I-T-L-**L**-S-A-L-V-E
α_2-Antiplasmin	2	I-A-M-S-**R**-M-S-L-S-S
Antithrombin III	2	V-I-A-G-**R**-S-L-N-P-N
C1q esterase	1	G-G-V-A-**R**-T-L-L-V-P

[a]After Carrell and Stein (1995).

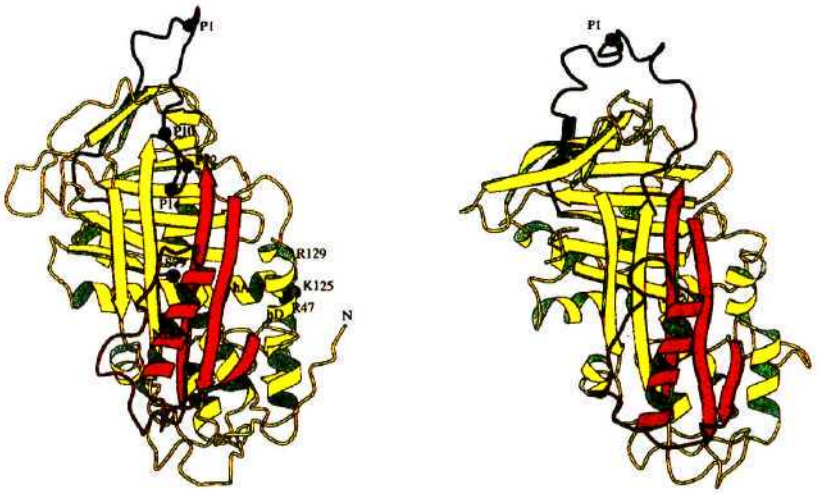


Figure 37. Three-dimensional structures of plasma serpins. Ribbon diagrams of (A) α_1-antichymotrypsin and (B) antithrombin III, illustrating the exposed reactive-site loop containing the P_1 residue (after Carrell and Stein, 1995).

The fact that trypanosome oligopeptidases are without activity against protein substrates suggests that such oligopeptidases cannot contribute to disease pathogenesis through the digestion of host proteins. However, mammalian peptide hormones are distributed throughout the body in the blood plasma and CSF. This raised the possibility that OP-Tb may contribute to disease pathogenesis through the hydrolysis of host peptide hormones, thereby disrupting their hormonal activity. Peptide hormones are a diverse group of biologically active peptides, usually with a M_r of between 1 and 6 kDa and approximately 2-60 amino acid residues in length (Carraway and Reinecke, 1989). The primary function of these peptides is intercellular communication.

Unfortunately, there are few reports of the levels of hormonal regulatory peptides in African trypanosomiasis. The exceptions include adrenocorticotropic hormone (ACTH) in *T. congolense* infection (Mutayoba *et al.*, 1995a, 1995b), and atrial natriuretic factor (ANF) in *T. brucei* infections (Ndung'u *et al.*, 1992). Both are depleted. The biochemical mechanisms promoting these changes have not been elucidated. The detection of an amidolytic activity in infected host sera (Section 6.8.4, Fig. 46) and the demonstration that live parasites hydrolyse Cbz-Arg-Arg-AMC (Section 6.8.5, Fig. 47) raised the possibility that oligopeptidase activity in the host bloodstream could hydrolyse circulating regulatory peptides, resulting the depleted levels of these peptides. The small size of these peptides makes them ideal substrates for oligopeptidases, and the depletion of peptide hormone levels in the host bloodstream and CSF may have serious consequences for the metabolic homeostasis of the host.

It was therefore of interest to determine whether OP-Tb activity is indeed present in the host bloodstream, and whether or not it was regulated by host plasma serpins *in vitro*. This necessitated the purification of two plasma protease inhibitors, α_1-protease inhibitor (α_1PI) (Section 6.2.4) and α_1-antichymotrypsin (α_1ACT) (Section 6.2.3) as the purchase of these compounds was beyond the means of our limited research budget. Furthermore, should plasma protease inhibitors be without activity against OP-Tb (Section 6.3), it would be of further interest to determine whether OP-Tb could proteolytically inactivate host plasma serpins (Section 6.6), and whether OP-Tb has any effect on the peptide hormones that are present in the host bloodstream and CSF (Section 6.7). As no major kinetic differences were observed between OP-Tb and OP-Tc (Section 5.11.1), only OP-Tb was employed in these investigations.

6.2 Serpin Purification

6.2.1 Antichymotrypsin activity assay

Plasma α_1ACT is a serpin which specifically inactivates serine proteases with a chymotrypsin-like specificity. Therefore, the inhibition of α-chymotrypsin was used to follow α_1ACT inhibitory activity throughout the purification. The chymotrypsin-inhibition assay used in the present study is a fluorometric assay, based on the calorimetric assay of Travis and Morii (1981), employing the fluorogenic substrate for chymotrypsin, Suc-Leu-Tyr-AMC.

6.2.1.1 Materials

Assay buffer [50 mM Tris-Cl, pH 8]. Tris (0.3 g) was dissolved in dH_2O (40 ml), titrated with HCl to pH 8, and made up to 50 ml.

Substrate stock solution [1 mM Suc-Leu-Tyr-AMC in DMSO]. Suc-Leu-Tyr-AMC (1 mg) was dissolved in DMSO (1.5 ml), and stored at 4°C.

Substrate working solution [20 µM Suc-Leu-Tyr-AMC in dH_2O]. Substrate stock solution (100 µl) was added to dH_2O (4.9 ml).

Enzyme diluent [1 mM HCl, 50 mM $CaCl_2$]. HCl (32% (v/v), ρ = 1.16 $g.ml^{-1}$) (9.8 µl) and $CaCl_2.2H_2O$ (0.7 g) were dissolved in dH_2O (100 ml).

Chymotrypsin stock solution [1 $mg.ml^{-1}$ in 1 mM HCl, 50 mM $CaCl_2$]. Bovine α-chymotrypsin (1 mg) was dissolved in enzyme diluent (1 ml) and used immediately.

6.2.1.2 Method

To facilitate screening of a large number of fractions for antichymotrypsin activity, activity assays were conducted in 96 well fluorimeter plates. Assays were conducted essentially as described in Section 2.3.3. An appropriate dilution of enzyme (25 µl) was incubated (10 min, 37°C) in assay buffer (50 µl) with the sample of interest (25 µl), after which substrate solution (25 µl) was added, and fluorescence monitored as described in Section 2.3.3.

6.2.2 Antitrypsin activity assay

Inhibition of trypsin was used to follow α_1PI inhibitory activity during the purification. Trypsin was employed as it is available in high purity and is easily detected using synthetic substrates. The trypsin-inhibition assay is a fluorometric assay, based on the calorimetric assay of Travis and Johnson (1981), employing the fluorogenic substrate for trypsin Cbz-Arg-AMC.

6.2.2.1 Materials

Assay buffer [10 mM Tris-Cl, pH 8]. Tris (0.061 g) was dissolved in dH_2O (40 ml), titrated with HCl to pH 8, and made up to 50 ml.

Substrate stock solution [1 mM Cbz-Arg-AMC in DMSO]. Cbz-Arg-AMC (0.9 mg) was dissolved in DMSO (1.5 ml), and stored at 4°C.

Substrate working solution [20 µM Cbz-Arg-AMC in dH_2O]. Substrate stock solution (100 µl) was added to dH_2O (4.9 ml).

Enzyme diluent [2 mM HCl]. HCl (32% (v/v); $\rho = 1.16\ g.ml^{-1}$) (19.6 µl) was dissolved in dH_2O (100 ml).

Trypsin stock solution [0.2 $mg.ml^{-1}$ in 2 mM HCl]. Porcine pancreatic trypsin (1 mg) was dissolved in enzyme diluent (5 ml) and stored at 4°C for not more than 2 weeks.

6.2.2.2 Method

The assay method was exactly the same as that described for α_1ACT activity (Section 5.2.12).

6.2.3 α_1-Antichymotrypsin purification

α_1-Antichymotrypsin (α_1ACT) was purified as described by Travis and Morii (1981) with the exception of an additional molecular exclusion chromatography step on Sephacryl S-100 HR.

6.2.3.1 Materials

10 × Anticoagulant [109 mM trisodium citrate in dH_2O]. Trisodium citrate.$2H_2O$ (6.4 g) was dissolved in dH_2O (200 ml) and used immediately.

Bovine plasma. Bovine plasma was harvested from freshly slaughtered cattle (Abakor abbatoir, Cato Ridge). Plasma (1.8 litres) was harvested into two-litre plastic vessels containing anticoagulant at ten times the nominal concentration (200 ml).

Saturated $(NH_4)_2SO_4$ [4.2 M $(NH_4)_2SO_4$]. $(NH_4)_2SO_4$ (110.88 g) was dissolved in a final volume of 200 ml of dH_2O.

Buffer A [30 mM NaH_2PO_4, 0.02% (m/v) NaN_3, pH 6.8]. NaH_2PO_4 (4.7 g) and NaN_3 (0.2 g) were dissolved in dH_2O (950 ml), adjusted to pH 6.8 with NaOH, and made up to 1 litre.

Buffer B [50 mM Tris-Cl, 0.02% (m/v) NaN_3, pH 8.0]. Tris (6.05 g) and NaN_3 (0.2 g) were dissolved in dH_2O (950 ml) adjusted to pH 8, and made up to 1 litre.

6.2.3.2 Methods

Step 1. Ammonium sulfate fractionation. Pooled plasma (100 ml) was brought to 50% saturation $(NH_4)_2SO_4$ by the addition of an equal volume (100 ml) of saturated (4.2 M) $(NH_4)_2SO_4$, with constant stirring. The mixture was allowed to settle (2 h, 4°C), centrifuged (25 000 × *g*, 30 min, 4°C) and the supernatant was retained and dialysed against buffer A (5 × 2 litres, 4°C).

Step 2. Cibacron Blue-Sepharose fractionation. The dialysed fraction from step 1 was applied to a column of Cibacron Blue F3GA-Sepharose (200 × 25 mm, 4°C, 0.8 $ml.min^{-1}$) equilibrated with buffer A. The column was washed extensively with buffer A and once all unbound contaminants had been eluted, bound protein, including α_1ACT, albumin and other contaminants, was eluted in a single step by application of 100 mM NaCl in buffer A. The flow-through fraction from this step was retained for the purification of α_1PI (Section 5.2.4).

Step 3. Rechromatography on Cibacron Blue Sepharose. Fractions from step 2 exhibiting inhibition of chymotrypsin activity were extensively dialysed against buffer B (5 × 2 litres) and re-chromatographed on the same column of Cibacron Blue F3GA-Sepharose (200 × 25 mm, 4°C, 0.8 $ml.min^{-1}$) equilibrated in buffer B. The column was washed extensively with buffer B. Once all unbound contaminants had been eluted, bound α_1ACT was eluted in a single step by application of 200 mM NaCl in buffer B.

Step 4. Chromatography on Sephacryl S-100 HR. Fractions from step 3 exhibiting chymotrypsin-inhibitory activity were concentrated by ultrafiltration (Section 2.6.1) to 1 ml and applied to a Sephacryl S-100 HR column (900 × 15 mm, 4°C, 0.32 $ml.min^{-1}$), previously equilibrated with buffer B. Protein was eluted with buffer B. Sample purity was evaluated by Tris-Tricine SDS-PAGE (Section 2.7.1), and purified α_1ACT was stored in aliquots at 1 $mg.ml^{-1}$ in buffer B at -75°C.

6.2.4 α_1-Protease inhibitor purification

Bovine α_1PI was purified from the same starting material as α_1ACT exactly as described by Travis and Johnson (1981).

6.2.4.1 Materials

1 M Tris. Tris (12.1 g) was dissolved in dH_2O (100 ml, final volume).

6.2.4.2 Methods

Step 1. Fractionation on DEAE-cellulose. The flow-through fraction from the Cibacron Blue F3GA-Sepharose column (Section 6.2.3.2) was brought to pH 6.8 with 20 mM HCl, and applied to a column of DEAE-cellulose (2.5 × 30 cm, 0.8 $ml.min^{-1}$, 4°C), equilibrated with buffer A. The column was washed with buffer A until protein was no longer evident in the effluent, and bound protein was eluted with a linear gradient of 0 to 0.2 M NaCl in buffer A over 14 column volumes.

Step 2. Fractionation on Sephadex G-75. The bound, active fractions from Step 1 were pooled and adjusted to pH 8 by the addition of 1 M Tris (unbuffered). The sample was concentrated by dialysis to 5 ml, and applied to a Sephadex G-75 column (2.5 × 100 cm, 0.8 $ml.min^{-1}$, 4°C) previously equilibrated with buffer B. Protein was eluted with buffer B. Sample purity was evaluated by Tris-Tricine SDS-PAGE (Section 2.7.1), and α_1PI was stored in aliquots at 1 $mg.ml^{-1}$ in buffer B at -75°C.

6.3 Inhibition of OP-Tb by serpins

6.3.1 Classical serpins

Inhibition of OP-Tb activity by classical serpins (α_1PI, α_1ACT, α_2AP, ATIII) was examined by pre-incubation of OP-Tb (5 min) with a molar excess (at least 50-fold) of each serpin, followed by addition of a fluorogenic substrate. Control experiments in which the inhibitor was omitted were performed in parallel.

6.3.1.1 Method

Enzyme activity was assayed as described in Section 2.3.3, except that enzyme (6.25 nM final concentration, 5 ng, in 25 µl Brij 35), was pre-incubated with assay buffer (50 µl), and either inhibitor solution (10 µl, 1 µM final concentration) or bovine serum or plasma (10 µl) for 10 min at 37°C, after which substrate (25 µl) was added, and the fluorescence recorded after 3 min. Activity was expressed as percentage activity relative to a control, in which the inhibitor solution was replaced with dH_2O.

6.3.2 α_2-Macroglobulin

Inhibition of proteases by α_2M occurs by a different mechanism to that employed by classical serpins. The nett effect is the entrapment of an active protease within the α_2M cage. After entrapment of the protease, the closed α_2M cage remains permeable to peptides and small proteins of molecular masses up to 10 kDa (Barrett, 1981; Barrett and Starkey, 1973). This phenomenon is exploited in testing for protease entrapment by α_2M.

To investigate whether α_2M does indeed entrap a protease, α_2M is incubated with active protease in approximately equimolar active concentrations. After incubation, the protease-α_2M mixture is resolved by MEC (Section 2.5.2). The exclusion limit of the MEC gel is selected so that the gel would include the protease but exclude the α_2M and α_2M-protease complex (Barrett and Starkey, 1973; Mason, 1989). The eluted protein fractions are then examined for protease activity using small fluorogenic peptide substrates, which are small enough to diffuse into the α_2M cage and are acted upon by the entrapped protease. If a protease is entrapped by the α_2M, there would be an apparent shift of enzyme activity on the MEC profile, from a point corresponding to the molecular mass of the native protease to the void volume. While it is conventional to use dithiothreitol in chromatography buffers when working with OP-Tb, these had to be omitted from all investigations involving α_2M, as α_2M is inactivated by free thiols at concentrations above 1 mM (Barrett, 1981).

6.3.2.1 Materials

MEC buffer [50 mM Tris-Cl, 0.02% (m/v) NaN_3, pH 7.4]. Tris (6.05 g) and NaN_3 (0.2 g) were dissolved in dH_2O (950 ml), adjusted to pH 7.4 with HCl, and made up to 1 litre.

6.3.2.2 Method

OP-Tb (0.16 pmoles, 20 µl of an 80 nM solution) was combined with α_2M (0.16 pmoles, 8 µl of a 193 nM solution, active concentration) in MEC buffer (50 µl). The sample was incubated (1 h, 37°C) and applied to a Sephacryl S-200 MEC column (320 × 15 mm, 0.29 $ml.min^{-1}$, 4°C). Fractions (1 ml) were collected and assayed for activity against Cbz-Arg-Arg-AMC as described in Section 2.3.2. At this point, dithiothreitol could be incorporated into the assay buffer as the α_2M-OP-Tb complexes, and native OP-Tb had already been resolved from one another.

6.4 Detection of OP-Tb activity in host plasma

OP-Tb activity was determined in the plasma of infected rats by a method adapted from that of Coleman and Bagdesarian (1976). SBTI, AEBSF and E-64 were used in combinations that permitted discrimination between OP-Tb activity and that of trypanosome cysteine proteases such as trypanopain-Tb, and of rat plasma kallikrein, which also digests the peptide substrates employed.

6.4.1 Materials

Assay buffer [50 mM Tris-Cl, 150 mM NaCl, 10 mM dithiothreitol, 0.02% (m/v) NaN_3]. Tris (0.6 g), NaCl (0.9 g), dithiothreitol (0.154 g) and NaN_3 (0.02 g) were dissolved in dH_2O (80 ml), titrated to pH 8 with HCl, and made up to 100 ml.

SBTI stock solution [120 µM SBTI in dH_2O]. SBTI (3.6 mg) was dissolved in dH_2O (1.5 ml). This yielded a final assay concentration of 20 µM.

AEBSF stock solution [11 mM AEBSF in dH_2O]. AEBSF (4 mg) was dissolved in dH_2O (1.5 ml). This yielded a final assay concentration of 1.85 mM.

E-64 stock solution [10 mM E-64 in dH_2O]. E-64 (5.3 mg) was dissolved in DMSO (50 µl) and made up to 1.5 ml with dH_2O. This yielded a final assay concentration of 1.67 mM.

Substrate stock and working solutions were prepared as described in Section 2.3.1.

6.4.2 Method

The blood of trypanosome-infected rats was harvested at peak parasitaemia by cardiac puncture. Plasma was obtained by centrifugation (3 000 × g, 25 min, 40°C) to pellet red blood cells and trypanosomes. The resultant plasma was examined microscopically to confirm the absence of parasites and red blood cells. Infected blood (25 µl) was preincubated with assay buffer (50 µl) and inhibitor stock solution (25 µl per inhibitor, 10 min). If inhibitors were omitted, dH_2O (25 µl) replaced the inhibitor solution. Final assay volume was 150 µl. Substrate working solution (25 µl) was added after preincubation, and fluorescence determined as described in Section 2.3.2.

6.5 Hydrolysis of Cbz-Arg-Arg-AMC by live trypanosomes

Live trypanosomes hydrolyse Cbz-Phe-Arg-AMC (Lonsdale-Eccles *et al.*, 1995), a substrate that is also hydrolysed by purified OP-Tb (Section 5.11.1, Table 9). Leupeptin, a potent inhibitor of OP-Tb activity (Section 5.11.6, Table 10), completely inhibits this hydrolysis of Cbz-Phe-Arg-AMC by live parasites, while in the presence of E-64 only 50% of the activity is inhibited (Lonsdale-Eccles *et al.*, 1995). This, together with the association of a Cbz-Arg-Arg-AMC hydrolysing activity with the trypanosome cell membrane (Section 3.11.1, Fig. 9) and the partial labelling of the surface of trypanosomes by anti-OP-Tb antibody (Mpimbaza, Lonsdale-Eccles and Wells, unpublished studies) raises the possibility that OP-Tb may also be a membrane-bound surface enzyme. To explore this possibility further, the hydrolysis of Cbz-Arg-Arg-AMC by live trypanosomes in the presence of serine protease inhibitors was investigated.

6.5.1 Materials

As per Sections 2.3.1, 3.2.1. and 6.4.1.

6.5.2 Method

T. b. brucei (8.325×10^6 cells) were resuspended in PSG (75 µl) in a 96-well fluorescent microplate, and pre-incubated (37°C, 10 min) either in the absence of inhibitors, or in the presence of AEBSF (1 mM, final concentration) or inhibitory anti-OP-Tb IgY (1 $mg.ml^{-1}$) (Section 4.9.3). Substrate was then added and fluorescence monitored (Section 2.3.2) at timed intervals for 10 min.

6.6 Inactivation of serpins by OP-Tb

Serpin inactivation by OP-Tb was investigated as described by Mast *et al.* (1991). OP-Tb and serpins were incubated with the serpin in 30-fold molar excess. The mixture was then resolved by Tris-Tricine SDS-PAGE (Section 2.7.1), and the electrophoretic pattern compared with that of a control serpin sample which had not been pre-incubated with OP-Tb. Serpins inactivated by cleavage within their reactive-site loop are characterised by release of a C-terminal peptide of approximately 4 kDa. While this 4 kDa band is often not visible on SDS-PAGE gels, the inactivation of serpins by cleavage in the reactive-site loop induces a conformational change in the serpin, causing it to migrate to a noticeably different position on an SDS-PAGE gel, relative to the intact serpin (Kalsheker *et al.*, 1996; Mast *et al.*, 1991).

6.6.1 Materials

2 × OP-Tb assay buffer [100 mM Tris-Cl, 2 mM dithiothreitol, pH 8]. Tris (1.21 g) and dithiothreitol (0.031 g) were dissolved in dH_2O (90 ml), adjusted to pH 8, and made up to 100 ml .

2 × Papain assay buffer [340 mM sodium acetate, 60 mM acetic acid, 4 mM EDTA, 8 mM dithiothreitol, pH 5.5]. $CH_3COONa.3H_2O$ (4.6 g), glacial acetic acid (34 µl) and $Na_2EDTA.2H_2O$ (0.15 g) were dissolved in dH_2O (90 ml), adjusted to pH 5.5 with NaOH, and made up to 100 ml.

6.6.2 Methods

Serpin digests were performed as described by Mast *et al.* (1991). Serpin (50 µl) and OP-Tb (50 µl) were combined in a 1:30 (enzyme:serpin) molar ratio in assay buffer (100 µl) and incubated at 37°C for 2 h. The reaction was terminated by the addition of non-reducing sample treatment buffer (200 µl) and boiling (10 min). Samples were resolved by non-reducing Tris-Tricine SDS-PAGE (Section 2.7.1) and protein visualised by Coomassie Blue staining (Section 2.7.3).

Positive controls were conducted as follows: bovine α_1PI was cleaved with *S. aureus* V8 proteinase (1:20 molar) ratio in OP-Tb assay buffer, and separately with papain or sheep cathepsin L (1:30 molar ratio) in papain assay buffer. Bovine α_1ACT and human α_2AP and ATIII were incubated with human neutrophil elastase (1:20 molar ratio), and separately papain or sheep cathepsin L (1:30 molar ratio) in papain assay buffer.

6.7 Interaction with regulatory peptides

Reversed-phase high-performance liquid chromatography (RP-HPLC) provides a convenient method for the rapid separation and sensitive resolution of mixtures of low M_r peptides (Krstulovil and Brown, 1982). RP-HPLC was used in this investigation to monitor the hydrolysis of mammalian regulatory peptides by OP-Tb.

6.7.1 Materials

2 × Non-reducing assay buffer [100 mM Tris-Cl, pH 8]. Tris (1.21 g) was dissolved in ddH_2O (90 ml), adjusted to pH 8, and made up to 100 ml.

2 × Reducing assay buffer [100 mM Tris-Cl, 20 mM dithiothreitol, pH 8]. Tris (1.21 g) and dithiothreitol (0.308 g) was dissolved in ddH_2O (90 ml), adjusted to pH 8, and made up to 100 ml .

Peptide substrate solution (1 $\mu g.\mu l^{-1}$). Peptide (100 µg) was dissolved in ddH_2O (100 µl), aliquotted, and stored at -20°C.

Stopping reagent [5% (v/v) TFA in ddH_2O]. TFA (500 µl) was made up to 10 ml with ddH_2O.

Solution A [0.1% (v/v) TFA in ddH_2O]. TFA (1 ml) was made up to 1 litre with ddH_2O. The solution was degassed by sonication prior to use.

Solution B [0.1% (v/v) TFA in acetonitrile]. TFA (1 ml) was made to 1 litre with acetonitrile. The solution was degassed by sonication prior to use.

6.7.2 Methods

HPLC was performed as described by Tetaert *et al.* (1994). Peptide (10 µg, 10 µl of a 1 $\mu g.\mu l^{-1}$ solution) was pre-incubated (10 min, 37°C) in a solution of assay buffer (25 µl) and ddH_2O (10 µl), after which enzyme solution (5 µl) was added, and the reaction mixture incubated at 37°C. Stopping reagent (50 µl) was added at 0, 1, 4 and 16 hours. Products were resolved by RP-HPLC.

HPLC was carried out on a Waters Associated apparatus, equipped with a Waters 994 DAD detector. The acidified reaction mixtures (50 µl) were applied to a Vydac C_{18} protein and peptide column (150 × 3.9 mm, 1 $ml.min^{-1}$), previously equilibrated in solution A. Elution of bound material was achieved with an isocratic gradient of 10-80% solution B over 20 min (3.5% per min, 1 $ml.min^{-1}$). Absorbance was monitored at 214 nm.

6.8 Results and Discussion

6.8.1 Purification of α_1-antichymotrypsin

The chymotrypsin-inhibitory activity present in the ammonium sulfate supernatant was resolved from trypsin-inhibitory activity by chromatography on Cibacron Blue F3GA-Sepharose (Fig. 38). A large peak of chymotrypsin-inhibitory activity was bound by the Cibacron Blue F3GA Sepharose at pH 6.8, whilst trypsin-inhibitory activity and some chymotrypsin-inhibitory

activity were present in the unbound fraction. Chymotrypsin-inhibitory activity present in the flow-through fraction was probably attributable to α_1PI (Travis and Morii, 1981). The trypsin-inhibitory pool was retained for the purification of α_1PI, while α_1ACT was purified from the chymotrypsin-inhibitory fraction that was retained by Cibacron Blue F3GA at pH 8.

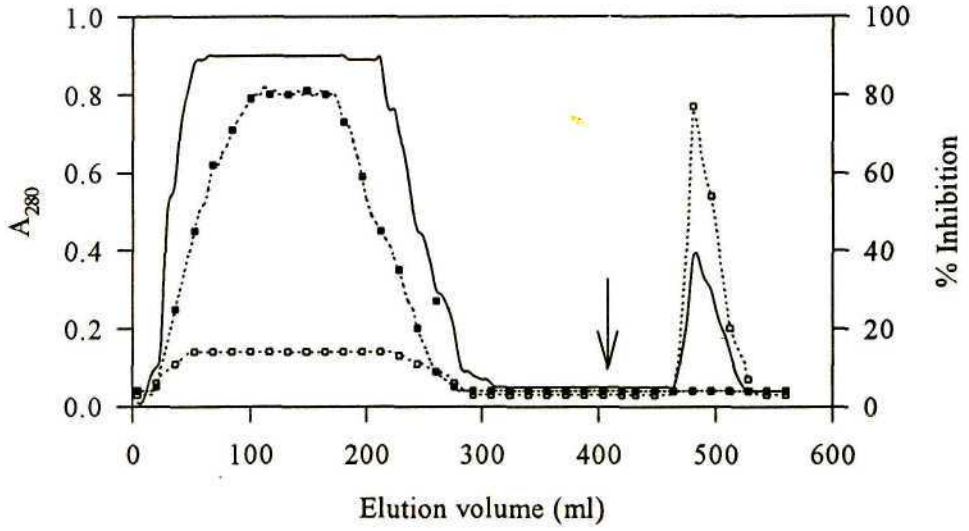


Figure 38. Chromatography of α_1ACT on Cibacron Blue F3GA-Sepharose at pH 6.8. Cibacron Blue F3GA-Sepharose (200 × 25 mm, 0.8 ml.min^{-1}) was equilibrated in buffer A [30 mM NaH_2PO_4, 0.02% (m/v) NaN_3, pH 6.8]. After elution of unbound protein with buffer A, bound protein was eluted in a single step with 100 mM NaCl in buffer A, applied at point ↓. (—) A_{280}; (··□··) % inhibition of chymotrypsin activity; (··■··) % inhibition of trypsin activity.

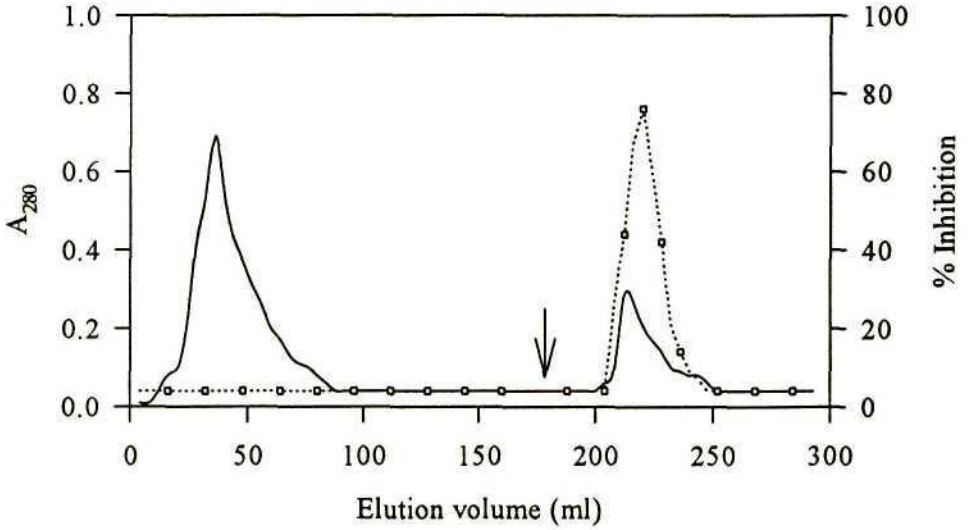


Figure 39. Rechromatography of α_1ACT on Cibacron Blue F3GA-Sepharose at pH 8. Cibacron Blue F3GA-Sepharose (200 × 25 mm, 0.8 ml.min^{-1}) was equilibrated in buffer B [50 mM Tris-Cl, 0.02% (m/v) NaN_3, pH 8.0]. After elution of unbound protein with buffer B, bound protein was eluted in a single step with 100 mM NaCl in buffer B, applied at point ↓. (—) A_{280}, (··□··) % inhibition of chymotrypsin activity.

Rechromatography at pH 8 of the bound, chymotrypsin-inhibitory fraction from Cibacron-Blue F3GA at pH 6.8 caused retention of chymotrypsin-inhibitory activity by the matrix (Fig. 39). The bound, chymotrypsin-inhibitory fraction contained protein contaminants at approximately 27 and 50 kDa (Fig. 41, lane e). These contaminants were successfully removed by MEC on Sephacryl S-100 HR (Fig. 40). The resultant chymotrypsin-inhibitory fraction was homogeneous by Tris-Tricine SDS-PAGE (Fig. 41, lane f), showing a single band at 68 kDa.

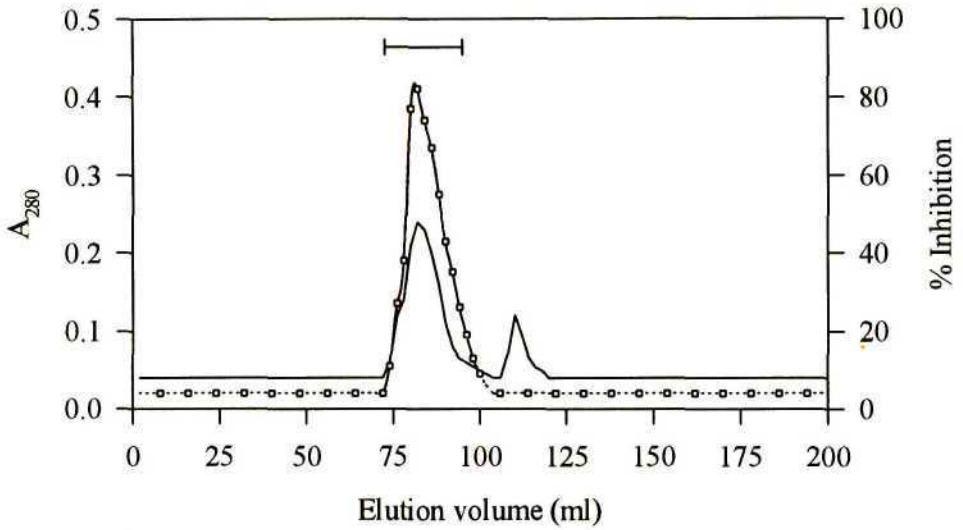


Figure 40. Chromatography of α_1ACT on Sephacryl S-100 HR. Sephacryl S-100 HR (900 × 15 mm, 4°C, 0.32 ml.min^{-1}) was equilibrated in buffer B [50 mM Tris-Cl, 1 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. After loading the column with sample (500 µl), protein was eluted with buffer B. (—) A_{280}, (··□··) % inhibition of chymotrypsin activity, (|——|) fractions pooled.

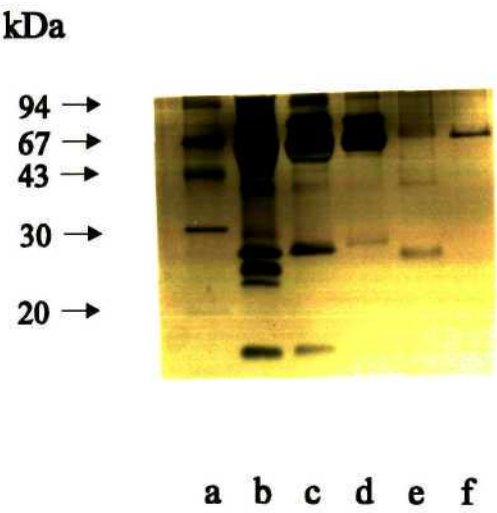


Figure 41. Evaluation of α_1ACT purification by Tris-Tricine SDS-PAGE. (a) M_r markers (as in Section 2.7.1.1); (b) bovine plasma (25 µg); (c) $(NH_4)_2SO_4$ supernatant (25 µg); (d) Cibacron Blue F3GA-Sepharose bound fraction (15 µg); (e) second Cibacron Blue F3GA-Sepharose bound fraction (15 µg); (f) Sephacryl S-100 HR inhibitory fraction (5 µg). Samples were resolved by non-reducing Tris-Tricine SDS-PAGE (Section 2.7.1) and proteins visualised with silver staining (Section 2.7.4).

6.8.2 Purification of α_1-protease inhibitor

The unbound peak from Cibacron Blue F3GA-Sepharose chromatography at pH 6.8 exhibiting trypsin-inhibitory activity was applied to a DEAE-cellulose resin, and bound proteins eluted with a gradient of 0-0.2 M NaCl (Fig. 42). The α_1PI eluted at approximately 40 mM NaCl, and the active fractions were pooled and further resolved by molecular exclusion chromatography on Sephadex G-75 Fig. 43). The resultant inhibitory fraction was homogeneous by Tris-Tricine SDS-PAGE (Fig. 44), giving a single band at about 68 kDa..

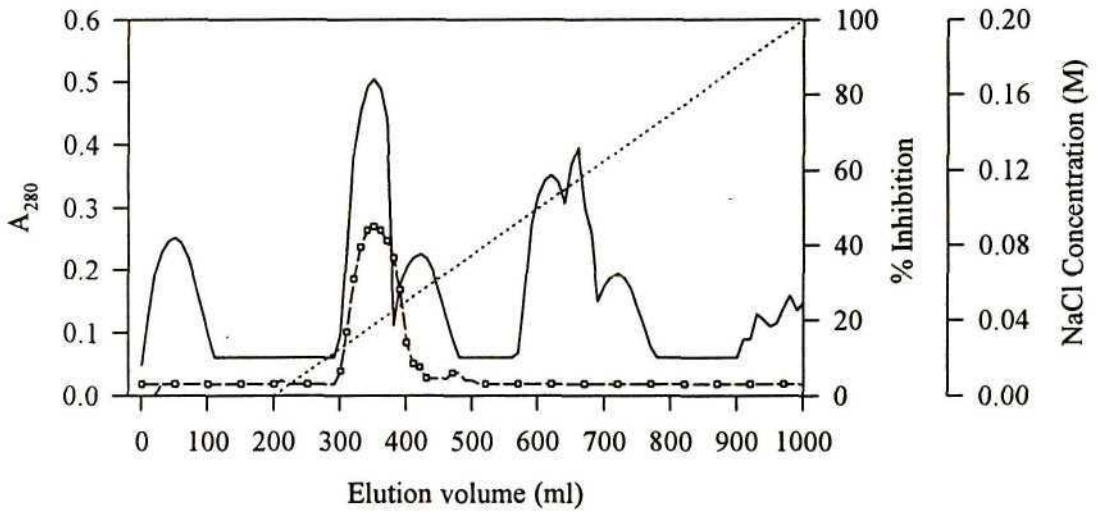


Figure 42. Chromatography of α_1PI on DEAE-cellulose. DEAE-cellulose (300 × 25 mm, 4°C, 0.49 ml.min^{-1}) was equilibrated in buffer B [50 mM Tris-Cl, 1 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. After loading the column with sample (500 µl), protein was eluted with buffer B. (—) A_{280}, (··□··) % inhibition of trypsin activity.

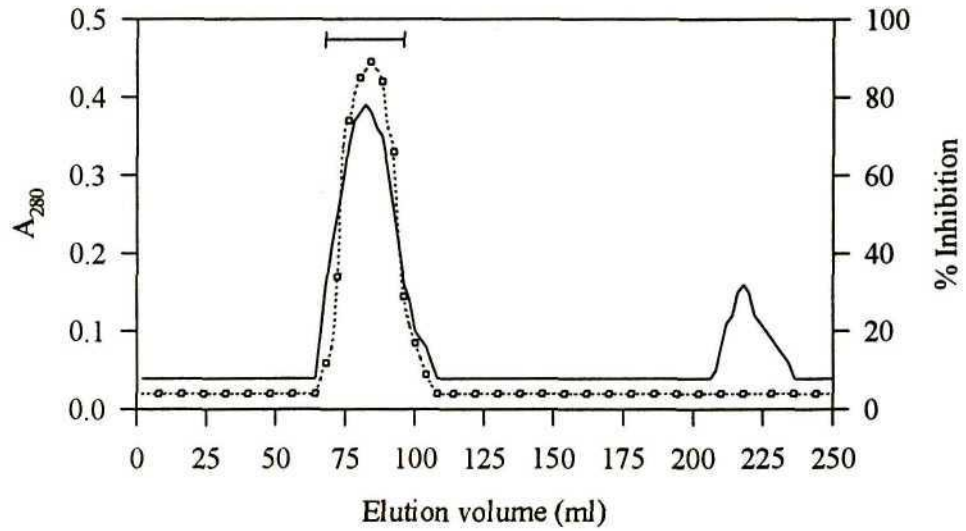


Figure 43. Chromatography of α_1PI on Sephadex G-75. Sephadex G-75 (1000 × 25 mm, 4°C, 0.49 ml.min^{-1}) was equilibrated in buffer B [50 mM Tris-Cl, 1 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8]. After loading the column with sample (500 µl), protein was eluted with buffer B. (—) A_{280}, (··□··) % inhibition of trypsin activity, (|——|) fractions pooled.

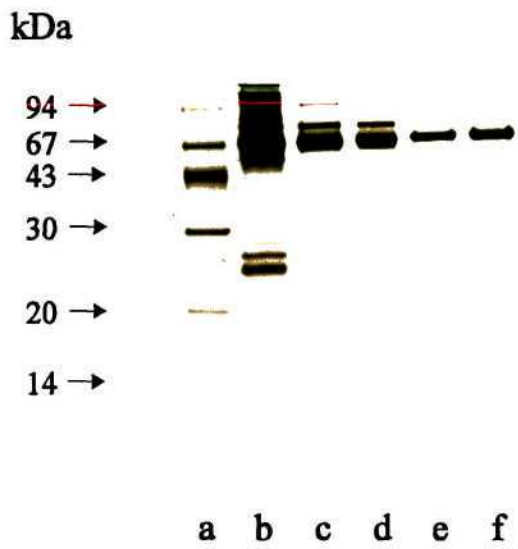


Figure 44. Evaluation of α_1PI purification by Tris-Tricine SDS-PAGE. (a) M_r markers (as in Section 2.7.1.1); (b) bovine plasma (25 μg); (c) $(NH_4)_2SO_4$ supernatant (25 μg); (d) Cibacron Blue F3GA-Sepharose unbound fraction (15 μg); (e) DEAE-cellulose bound fraction (10 μg); (f) Sephadex G-75 inhibitory fraction (7.5 μg). Samples were resolved by non-reducing Tris-Tricine SDS-PAGE (Section 2.7.1) and proteins visualised with silver staining (Section 2.7.4).

6.8.3 Inhibition of OP-Tb by host protease inhibitors

OP-Tb activity was not inhibited by any host plasma protease inhibitors, including general serine protease inhibitors (α_1-protease inhibitor, α_1-antichymotrypsin, α_2-antiplasmin and antithrombin III) and the two major cysteine protease inhibitors of mammalian plasma and cerebrospinal fluid, kininogen and cystatin C (Abrahamson *et al.*, 1986; Barrett *et al.*, 1984; Table 15) . No inhibition was observed by either bovine serum or plasma (results not shown). OP-Tb was incubated with bovine plasma and bovine serum in case these fluids contained an as-yet-undescribed protease inhibitor that may have inhibitory activity against OP-Tb.

No shift in the elution profile of OP-Tb and OP-Tb-α_2M mixtures was observed, and it was therefore concluded that no complex formation occurred between α_2M and OP-Tb (Fig. 45). This suggests that α_2M is not effective in controlling OP-Tb activity in the host bloodstream, as was observed for the other classical host plasma serpins (Section Table 15). There are a number of potential reasons for this. It is possible that OP-Tb is incapable of cleaving the α_2M bait region. This is plausible as it has been demonstrated that OP-Tb is incapable of cleaving any of the protein substrates tested (Section 5.11.9, Fig. 35 and Section 6.8.6, Fig. 48). Additionally, Barrett (1981) suggested that α_2M does not entrap proteases larger than 75 kDa. As native OP-Tb has a M_r of approximately 80 kDa, it is possible that it is simply too large to fit into the α_2M trap.

Table 15. Inhibition of OP-Tb activity by host protease inhibitors.

Inhibitor[a]	Activity relative to uninhibited control (%)[b]			
	OP-Tb activity[c] against Cbz-Arg-Arg-AMC	Trypsin activity[d] against Cbz-Arg-AMC	Chymotrypsin activity[e] against Suc-Leu-Tyr-AMC	Cathepsin L activity[f] against Cbz-Phe-Arg-AMC
none	100	100	100	100
α_1-Protease inhibitor	98 ± 4	0	n.d.	n.d.
α_1-Antichymotrypsin	99 ± 7	n.d.[g]	0	n.d.
α_2-Antiplasmin	100 ± 2	0	n.d.	n.d.
Antithrombin III	96 ± 6	0	n.d.	n.d.
L-Kininogen	107 ± 8	n.d.	n.d.	16 ± 8
Cystatin C	99 ± 9	n.d.	n.d.	10 ± 1

[a]Inhibitors at 1 μM final concentration.
[b]Data reflect mean % ± S.D. (n=3).
[c]In 50 mM Tris-Cl, 1 mM dithiothreitol, pH 8.
[d]In 50 mM Tris-Cl, pH 7.5.
[e]In 50 mM Tris-Cl, 1 mM $CaCl_2$, pH 7.5.
[f]In 270 mM sodium acetate, 30 mM acetic acid, 2 mM EDTA, 4 mM dithiothreitol, pH 5.5.
[g]n.d., not determined.

These data together suggest that OP-Tb, if released into the host bloodstream, would not be regulated by any of the host plasma protease inhibitors. The enzyme would therefore remain active in the host bloodstream, where it could contribute to disease pathogenesis through the digestion of host peptides. With this in mind, acutely infected murine plasma and chronically infected bovine plasma were examined for OP-Tb activity.

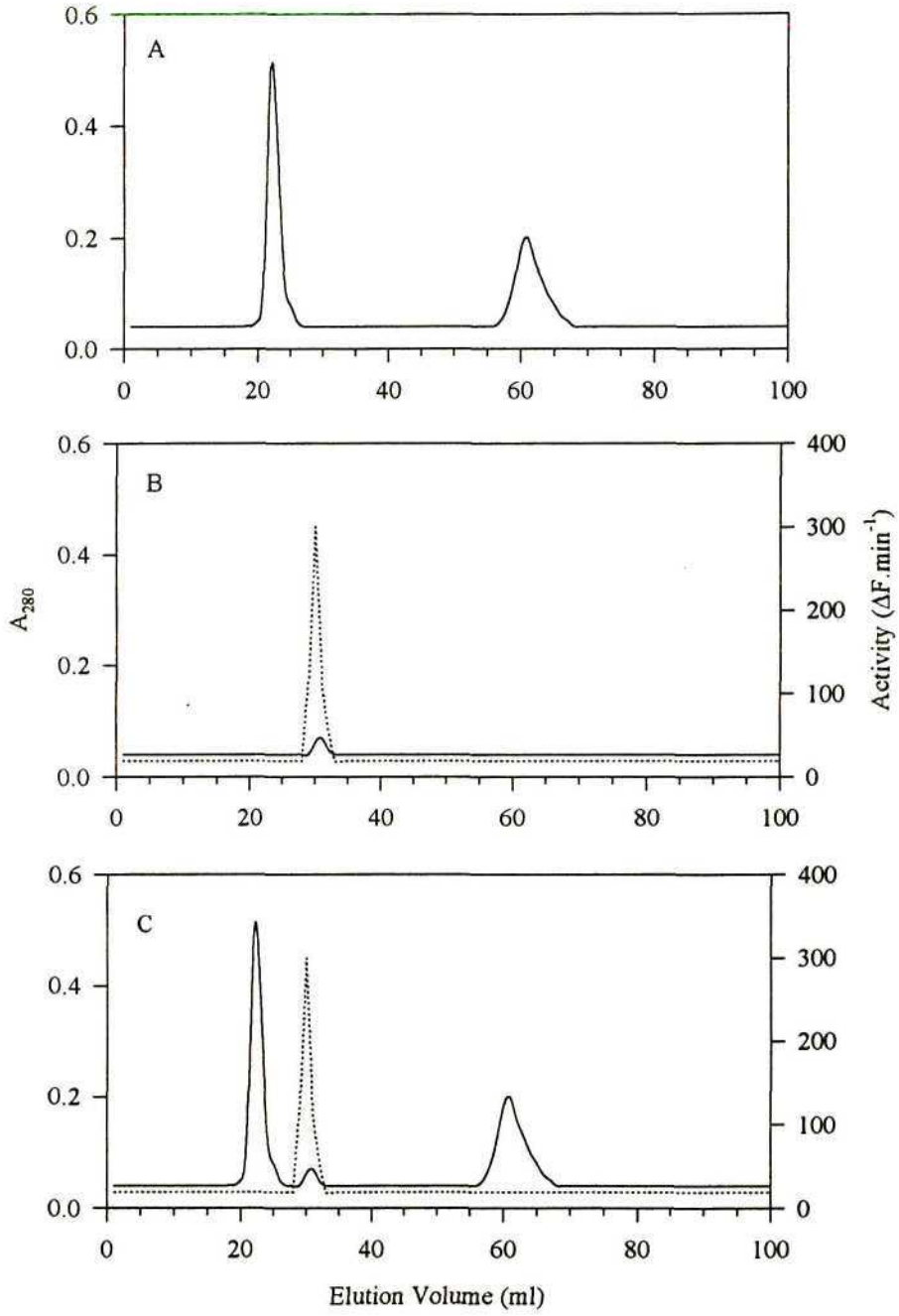


Figure 45. Interaction of OP-Tb with α_2M. α_2M, OP-Tb and potential complexes of α_2M and OP-Tb were resolved by MEC in equilibration buffer [50 mM Tris-Cl, 1 mM dithiothreitol, 0.02% (m/v) NaN_3, pH 8] on a Sephacryl S-200 column (320 × 15 mm, 0.32 $ml.min^{-1}$). (A) Elution profile for α_2M alone, (B) elution profile for OP-Tb alone, (C) elution profile for α_2M after incubation with OP-Tb. (–) A_{280}, (···) activity against Cbz-Arg-Arg-AMC.

6.8.4 Detection of activity in host plasma

Activity against Cbz-Arg-Arg-AMC was detected in the plasma of infected rodents. This activity was not inhibited by E-64 suggesting that it was not attributable to host or trypanosome cysteine proteases such as cathepsin L or trypanopain-Tb. Inhibition of the activity by AEBSF suggested that a trypsin-like serine protease was responsible for the observed activity, whilst a lack of inhibition by SBTI suggested that this protease was in fact a serine oligopeptidase rather than a blood-derived protease such as kallikrein or thrombin. The activity was not detected in the plasma of uninfected rodents. The same AEBSF-sensitive, SBTI-insensitive activity against Cbz-Arg-Arg-AMC was also observed in chronically infected bovine serum, albeit to a lesser degree (approximately 500 $\Delta F.min^{-1}.25$ µl $serum^{-1}$). Again, this activity was not observed in the sera of uninfected cattle (results not shown).

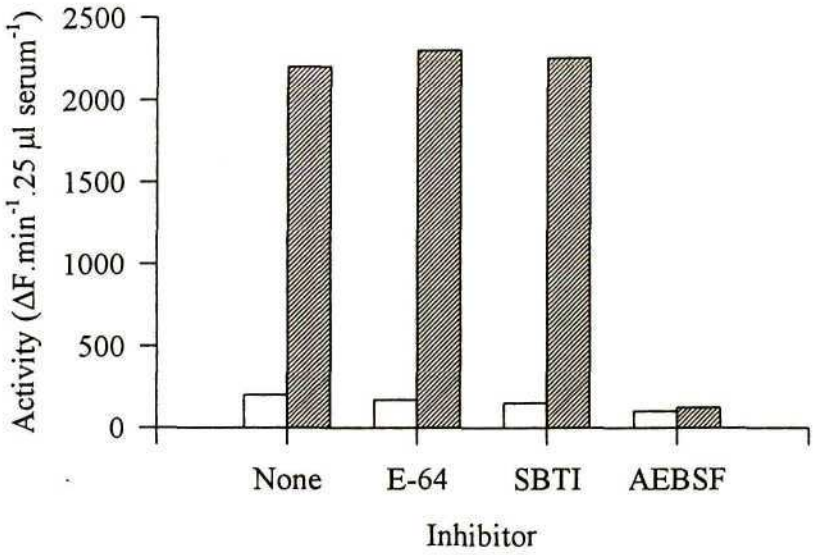


Figure 46. Detection of serine oligopeptidase activity in infected rat plasma. Enzyme assays were conducted exactly as described in Section 5.4.2 on the plasma of infected (shaded bars) and uninfected (unshaded bars) rat plasma. The inhibitors E-64, SBTI and AEBSF were used to discriminated between serine and cysteine protease activity, and between protease and oligopeptidase activity.

Peptidase activity has been described in the plasma of mice infected with *T. b. brucei* (Knowles *et al.*, 1987), and Tetaert *et al.* (1993) reported the unusual cleavage of peptide hormones in the serum of rats infected with *T. b. brucei*, concluding that the activity was attributable to a serine endopeptidase. The present study demonstrated a serine oligopeptidase activity in the plasma of rats infected with *T. b. brucei*, and it is quite likely that this is the same activity identified by Tetaert *et al.* (1993). However, Tetaert *et al.* (1993) also reported that the activity observed in infected rat serum was inhibited by 5 mM DFMO and 3.5 mM EDTA. OP-Tb is not inhibited by either DFMO (Section 7.6.1, Table 16) or EDTA (Section 5.11.6, Table 10), and it is possible that Tataert's

group was working with more than one protease, although exactly how DFMO, a highly specific suicide inhibitor of ornithine decarboxylase, could inhibit a proteolytic enzyme is not obvious.

6.8.5 Cbz-Arg-Arg-AMC hydrolysis by live trypanosomes

The hydrolysis of Cbz-Arg-Arg-AMC by live trypanosomes (Fig. 47) was almost completely abolished in the presence of AEBSF, a potent inhibitor of serine proteases, including OP-Tb. Furthermore, this activity was inhibited by approximately 50% in the presence of inhibitory anti-OP-Tb antibodies (Section 4.9.3, Figs 18 and 19). Taken together, these data suggest that OP-Tb may be present on the trypanosome cell-surface or may be released by a type III secretion mechanism (Burleigh *et al.*, 1997) or by rupturing of the parasites.

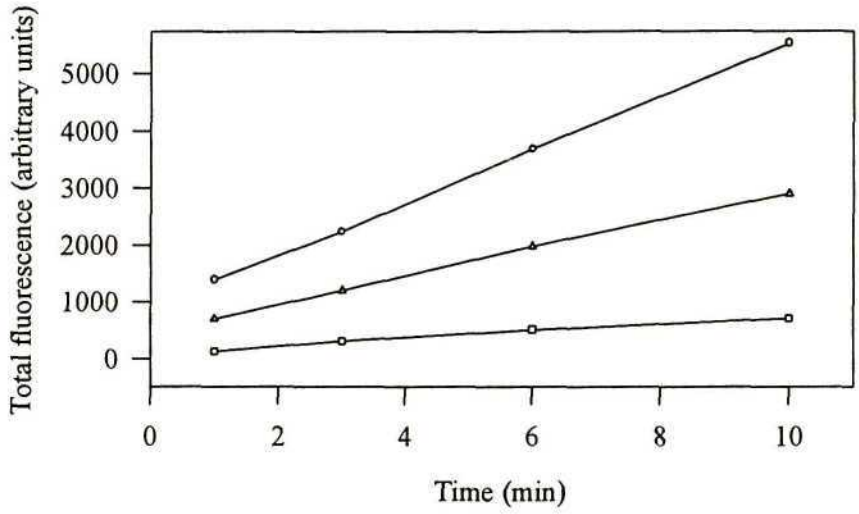


Figure 47. Hydrolysis of Cbz-Arg-Arg-AMC by live trypanosomes. Live *T. b. brucei* (8.325×10^6 cells) were resuspended in PSG (75 µl) containing no inhibitors (–○–), AEBSF (1 mM, –□–) or anti-OP-Tb IgY (1 $mg.ml^{-1}$, –Δ–). After pre-equilibration (10 min), activity against Cbz-Arg-Arg-AMC was determined as described in Section 2.3.3. The S.D. for each data point was less than ±10%.

Metalloproteinases are known to be present on the cell surface of related *Leishmania* parasites (Etges and Bouvier 1993), and the gene encoding the *T. brucei* homologue of this enzyme has been isolated (El-Sayed & Donelson, 1997). However, the metalloproteinase does not have activity against Cbz-Arg-Arg-AMC (Bouvier *et al.*, 1990). Furthermore, although the Cbz-Arg Arg-AMC hydrolysing activity was only inhibited by 50% in the presence of inhibitory antibodies, AEBSF caused almost 100% inhibition, suggesting that this activity is entirely attributable to a serine protease. Thus, either an additional surface-bound serine protease is responsible for the remaining 50% of the activity, or the antibodies are not able to inhibit all of the surface-bound enzyme. The trypanosome cell-surface is known to be coated

with variable surface glycoproteins (VSG; Donelson and Turner, 1985), and it is possible that such antibodies may experience some steric hindrance during antibody-antigen interaction at the cell surface, accounting for this partial inhibition.

6.8.6 Inactivation of serpins by OP-Tb

No inactivation of α_2AP, α_1PI, α_1ACT or ATIII by OP-Tb was observed. However, all these serpins were cleaved by other cysteine or serine proteinases, serving as positive controls (Fig. 48).

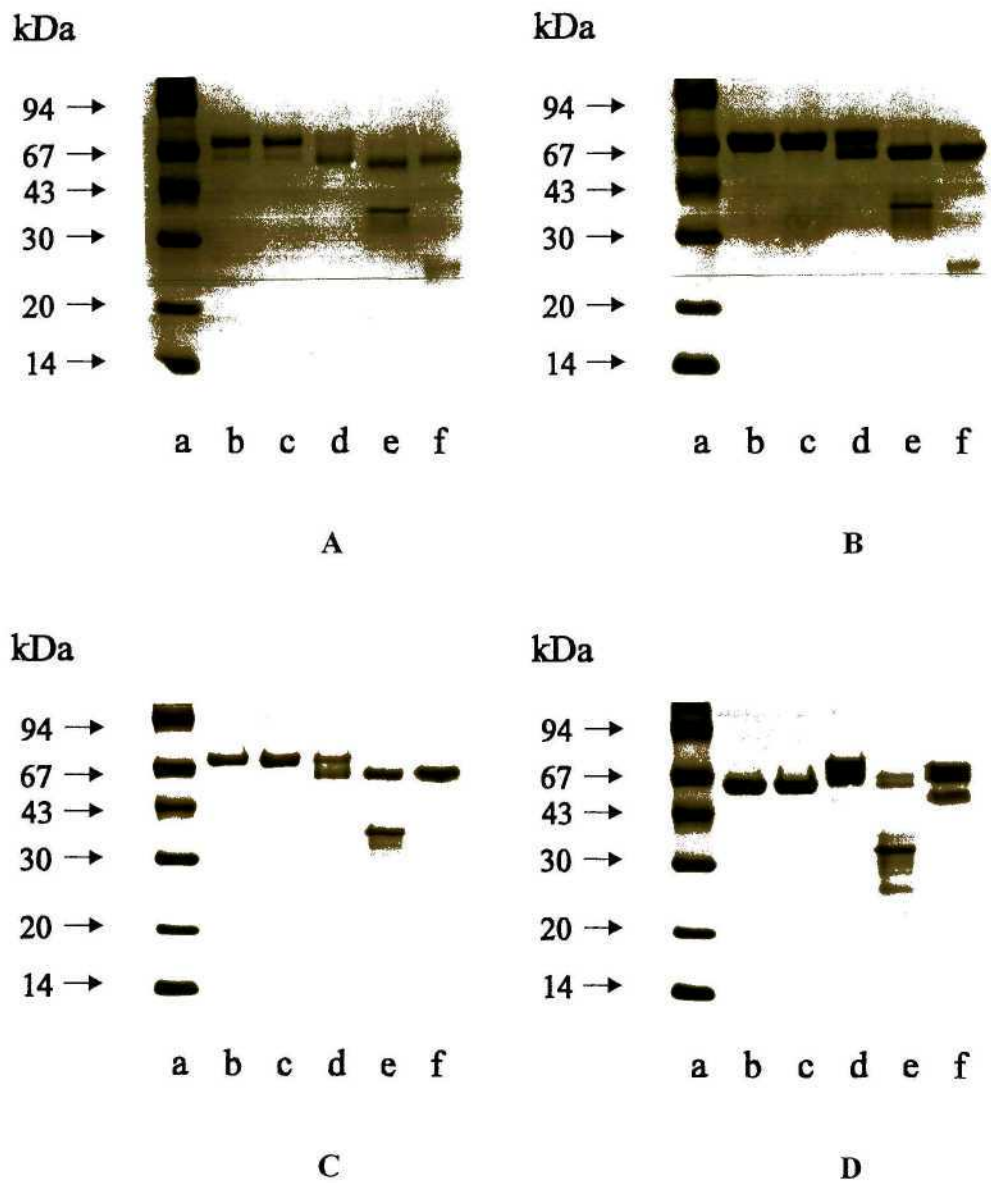


Figure 48. Proteolytic digestion of serpins. Digestion of (A) α_2AP, (B) α_1PI, (C) α_1ACT, (D) ATIII by proteolytic enzymes. Serpins were incubated with proteases for 2 h in assay buffer (Section 5.5). Lane (a) M_r markers (Section 2.7.1.1); (b) intact serpin; (c) serpin incubated with OP-Tb; (d) serpin incubated with either *S. aureus* V8 proteinase (B) or human neutrophil elastase (HNE) (A, C, D); (e) serpin incubated with ovine cathepsin L; (f) serpin incubated with papain. Reaction mixtures were resolved by non-reducing Tris-Tricine SDS-PAGE (Section 2.7.1).

Native α_2AP, and α_2AP incubated in the presence of OP-Tb exhibited the same electrophoretic mobility (Fig. 48), suggesting that OP-Tb did not hydrolyse this serpin. However, α_2AP incubated in the presence of human neutrophil elastase resulted in a mixture of cleaved and uncleaved α_2AP, producing bands at approximately 60 and 70 kDa. Human α_2AP incubated in the presence of cathepsin L and papain was completely cleaved, producing a major band at 60 kDa (Fig. 48A). Similar results were obtained for α_1PI (Fig. 48B) and α_1ACT (Fig. 48C).

Similarly, native ATIII and ATIII incubated in the presence of OP-Tb did not exhibit any shift in electrophoretic mobility, suggesting that ATIII is not hydrolysed by OP-Tb (Fig. 48D). In contrast to α_2AP, α_1PI and α_1ACT, incubation of ATIII with human neutrophil elastase, cathepsin L or papain resulted in a band at a slightly higher molecular mass than the native enzyme. This phenomenon has also been observed for cleaved ATIII by Mast *et al.* (1991). The cleavage points for these serpins are illustrated in Fig. 49.

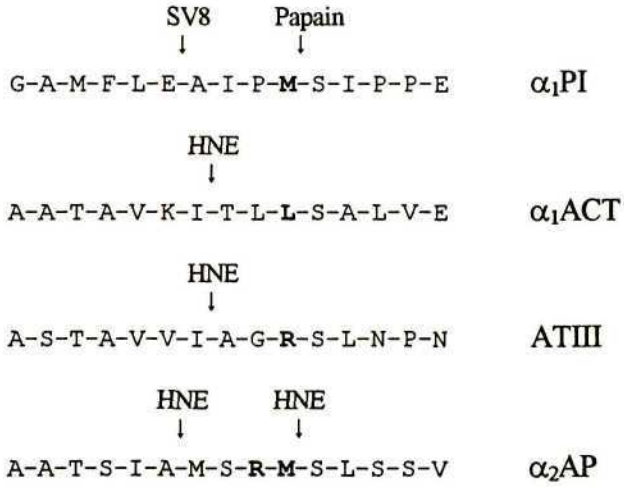


Figure 49. Reactive-site loop cleavage sites of serpins. Some reactive-site loop cleavage sites on serpins by papain, *S. aureus* V8 proteinase (SV8) and human neutrophil elastase (HNE) have been determined (after Mast *et al.*, 1991). P_1 residues are given in bold type. In the case of α_2AP, P_1 for plasmin is Arg, while P_1 for chymotrypsin is Met. Cathepsin L cleavage sites have not been determined for these serpins.

Pathogen-derived proteases are known to catalytically inactivate host protease inhibitors, and disturbances in the protease-inhibitor levels in the bloodstream and CSF may have serious implications for the host (section 1.3.2.4). Bagarozzi *et al.* (1996) described the inactivation of α_1PI in the reactive-site loop by a an oligopeptidase which otherwise had no activity against any other protein substrates. Therefore, OP-Tb was examined for activity against mammalian serpins.

None of the plasma serpins tested, including α_1PI, α_1ACT, α_2AP and ATIII were hydrolysed by OP-Tb. However, all serpins were cleaved by cathepsin L, papain, and either human neutrophil elastase or *S. aureus* V8 proteinase. This suggests that OP-Tb present in the host bloodstream does not contribute to pathogenesis through the digestion of host plasma serpins.

6.8.7 Interaction with regulatory peptides

Hydrolysis of peptide hormones was monitored by RP-HPLC. OP-Tb degraded neurotensin, reduced [Arg^8]vasopressin (AVP), ANF and glucagon. The degradation of neurotensin by OP-Tb is illustrated in Fig. 50. The gradual disappearance of the intact neurotensin peak at approximately 14 min is evident, as is the gradual appearance of peaks at 9 min and 13 min, attributable to neurotensin degradation products. The hydrolysis of AVP, ANF and glucagon was monitored in a similar manner.

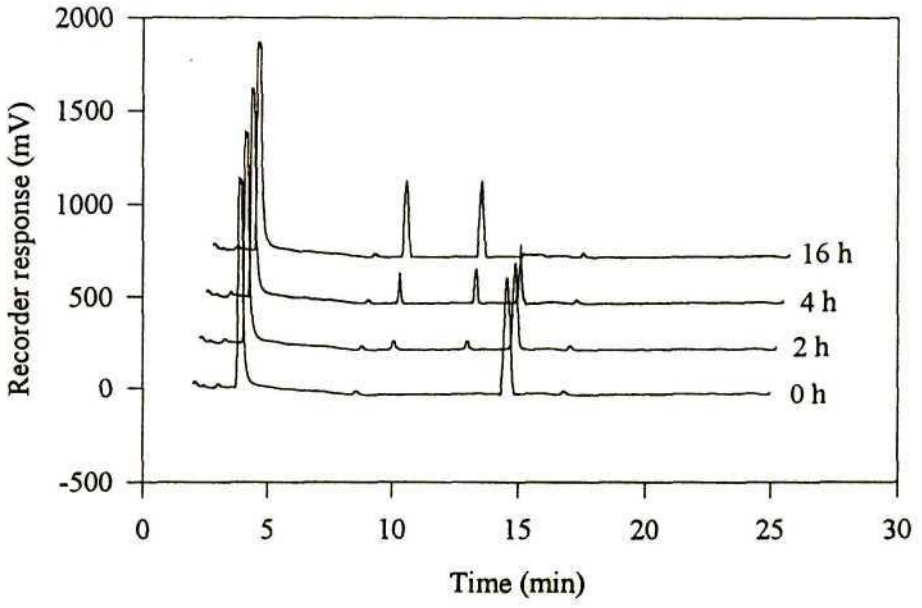


Figure 50. Degradation of neurotensin by OP-Tb. Neurotensin (10 µg) was incubated with OP-Tb (10 ng) for various time intervals and the reaction mixtures resolved by RP-HPLC as described in Section 6.7.1.

The amino acid sequences and putative cleavage sites of the peptide hormones hydrolysed by OP-Tb are illustrated in Fig. 51. Similar data for peptides not degraded by OP-Tb are illustrated in Fig. 52. The cleavage of mammalian peptide hormones was undertaken for two reasons, (1) to determine the possible substrate size-exclusion limit for OP-Tb, and (2) to determine whether OP-Tb was able to degrade host peptide hormones, thereby suggesting a potential role for OP-Tb in the pathogenesis of African trypanosomiasis.

Preliminary studies with a variety of peptides containing suitable cleavage sites suggested that molecules that were larger than 4 kDa were not cleaved by OP-Tb, indicating that this may represent an approximate substrate-size exclusion limit.

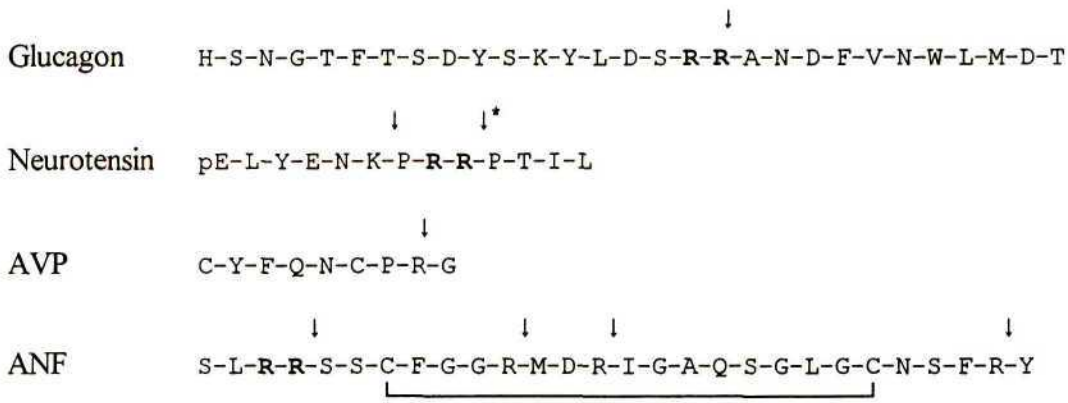


Figure 51. Peptide hormones cleaved by OP-Tb. Potential cleavage sites are indicated by arrows (↓), paired basic residues are indicated in bold type. Disulfide bridges are indicated by a solid line. *This putative cleavage point in neurotensin (1673 Da) has been confirmed by amino acid analysis. Abbreviations: AVP ([Arg^8]vasopressin, 1084 Da), ANF (atrial natriuretic factor, 3080 Da).

Neurotensin is a peptide hormone present in the blood and CSF of mammals, where it regulates a number of metabolic processes by up-regulating the secretion of a number of protein hormones. Neurotensin enhances the secretion of growth hormone, prolactin and thyrotropin (Aronin *et al.*, 1986). Rivier *et al.* (1977) demonstrated that while the integrity of the eight N-terminal residues of neurotensin was unrelated to its biological activity, any cleavage in the six C-terminal residues abolished its biological activity. As OP-Tb would most likely cleave neurotensin in this region, it is reasonable to assume that neurotensin cleavage by OP-Tb would destroy its biological activity. OP-Tb may therefore be responsible for the depletion of neurotensin in infected hosts. The nett effect would be a reduction in the secretion of growth hormone, prolactin and thyrotropin. The general state of emaciation, compromised immune system and thyroid dysfunction observed in trypanosome-infected animals (Section 1.2.1) may be attributable, at least in part, to the depletion of host neurotensin, and the down-stream effects that such cleavage would have.

Intact ANF was cleaved into multiple peptides by OP-Tb, as revealed by RP-HPLC (result not shown). This suggests that OP-Tb may also cleave ANF in the host bloodstream, and depleted levels of this hormone have been reported by Ndung'u *et al.* (1992). ANF plays an important role in the regulation of blood volume, blood pressure, diuresis and natriuresis (deBold, 1985). Cleavage of ANF, particularly in the five residue C-terminal region, is known to abolish the

biological activity of ANF (Kangawa *et al.*, 1984). As OP-Tb can cleave ANF *in vitro*, possible at the putative cleavage site in the five residue C-terminal region, it is possible that OP-Tb may reduce circulating levels of ANF in the host bloodstream thereby effecting alterations in blood pressure and blood volume. This is consistent with the symptoms of African trypanosomiasis (Section 1.2.1).

Glucagon (3483 Da), which is also cleaved by OP-Tb *in vitro* (Fig. 51), plays an important role in glucose homeostasis, where it mobilises glucose from carbohydrate stores in response to hypoglycaemia. The fact that OP-Tb is active in the bloodstream, and that OP-Tb hydrolysed glucagon *in vitro* suggests that OP-Tb may degrade circulating glucagon in the bloodstream of infected hosts, thereby reducing blood-glucagon levels. Clinical manifestation of depressed glucagon levels would include hypoglycaemia and anaemia (Walsh, 1987), and both symptoms are not inconsistent with those observed in African trypanosomiasis (Section 1.2.1).

[Arg^8]vasopressin was only cleaved by OP-Tb after reduction of the disulfide bridge (Fig. 51), and intact vasopressin was not cleaved (Fig. 52). As only intact vasopressin has biological activity, the degradation of the reduced form is probably not of *in vivo* significance.

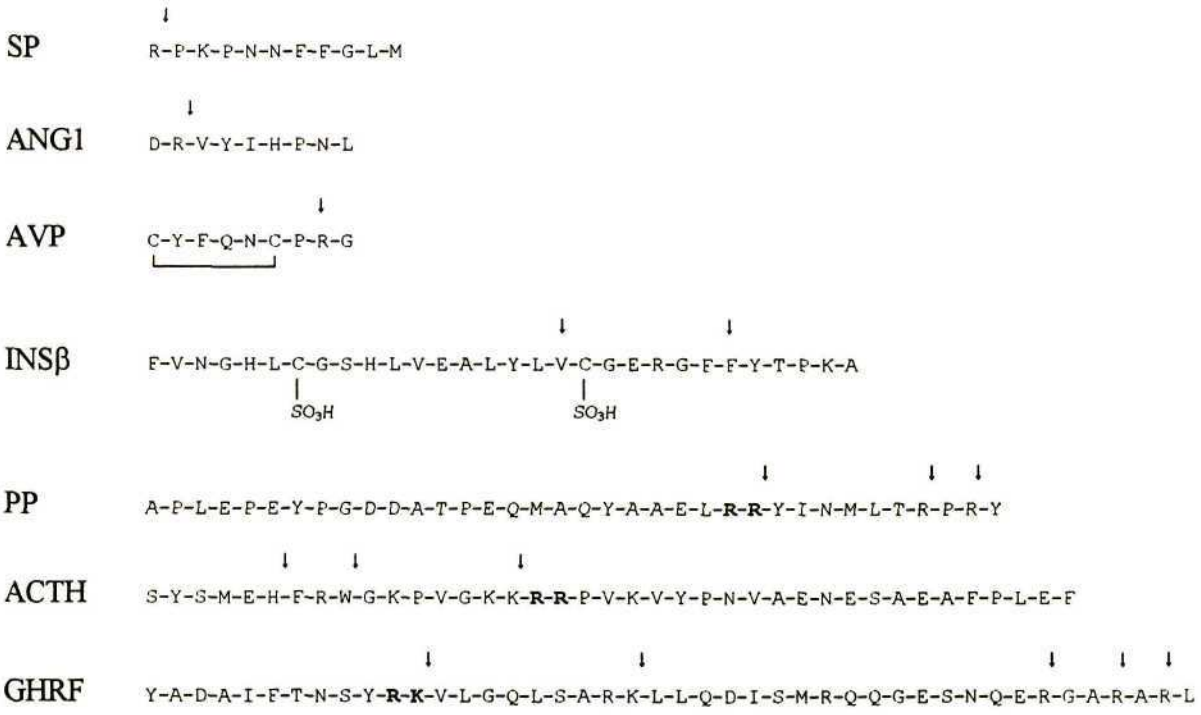


Figure 52. Peptide hormones not cleaved by OP-Tb. Potential cleavage sites are indicated by arrows (↓), paired basic residues are indicated in bold type. Disulfide bridges are indicated by a solid line. Abbreviations: SP (substance P, 1348 Da), ANG1 (angiotensin 1, 1296 Da), INSβ (insulin oxidised β chain, 3496 Da), PP (pancreatic polypeptide, 4182 Da), ACTH (adrenocorticotrophic hormone, 4541 Da) and GHRF (growth-hormone releasing factor, 5040 Da).

6.9 Concluding remarks

OP-Tb is not effectively inhibited in the mammalian host bloodstream and OP-Tb activity was not inhibited in the presence of host plasma protease inhibitors of cysteine and serine proteases, or the general protease inhibitor α_2M. Furthermore, an enzyme activity attributable to OP-Tb was detected in the plasma of infected rodents and cattle, and live parasites were able to hydrolyse Cbz-Arg-Arg-AMC. The inhibition of this hydrolysis by anti-OP-Tb antibodies suggested the presence of OP-Tb on the cell surface. It would therefore appear that OP-Tb is exposed to the extracellular milieu of the host bloodstream, either bound to the cell-surface of the trypanosome, or released by the parasite into the host bloodstream.

OP-Tb was unable to catalytically inactivate host plasma serpins, although this phenomenon has been reported for a pollen-derived oligopeptidase (Bagarozzi *et al.*, 1996). However, OP-Tb was found to degrade a number of host peptide hormones, and it is suggested that OP-Tb may contribute to the pathogenesis of African trypanosomiasis through the digestion of peptide hormones in the host bloodstream, thereby interfering with the normal metabolic homeostasis of the host animal.

Chapter 7

OP-Tb as a therapeutic target

7.1 Introduction

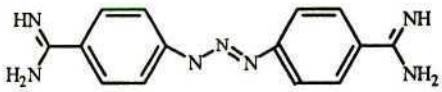
The chemotherapy of African trypanosomiasis currently involves five major trypanocidal drugs (Pentacarinate®, Berenil®, Germanin®, Arsobal® and Ornidyl™), the chemical structures of which are illustrated in Fig. 53. However, currently-employed chemotherapeutic strategies are plagued by several problems, including toxic side-effects, increasing drug resistance, and high cost.

Pentamidine. Pentamidine is an aromatic diamidine, most commonly marketed as the isethionate salt (M_r 593), known as Pentacarinate® (May & Baker, United Kingdom). While the biochemical mechanism of the anti-trypanosome activity of pentamidine is not understood (Pépin & Milford, 1994), pentamidine has been shown to be a reversible inhibitor of trypanosome S-adenosyl-L-methionine decarboxylase, and thus it has been suggested that it may down-regulate the synthesis of trypanosome polyamines (Bitonti *et al.* 1986). Pentamidine has also been proposed as an inhibitor of glycolysis, DNA and RNA synthesis, and amino acid transport within trypanosomes (Sands *et al.*, 1985). During therapeutic regimens, pentamidine typically reaches concentrations of up to 13 μM in the host bloodstream (Sands *et al.*, 1985), and is actively concentrated by bloodstream-form trypanosomes which possess pentamidine transporters, and is present at intracellular concentrations of approximately 80 μM (Damper and Patton, 1976).

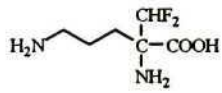
Pentamidine administration has frequent and severe side-effects, including thrombocytopaenia, arrhythmias and chronic liver and renal failure, which are exacerbated in HIV-co-infection, a common complication of human African trypanosomiasis (Goa and Campoli-Richards, 1987). The new-found application for pentamidine in the treatment of *Pneumocystis carinii* pneumonia in AIDS patients has created a new and very lucrative market for pentamidine, and has resulted in an increase in the cost of the drug, from US$1 to US$30 per 300 mg vial (Pépin and Milford, 1994).

Diminazene. Diminazene, marketed in the form of diminazene aceturate (M_r 587) as Berenil® (Hoechst Veterinär, Munich, Germany) is only employed in veterinary chemotherapy. The mode of action of diminazene is not understood but it is known to irreversibly inhibit trypanosome S-adenosyl-L-methionine decarboxylase (Bitonti *et al.*, 1986) and to selectively block kinetoplast DNA (kDNA) synthesis by binding to the minor groove of DNA (Newton and LePage, 1967).

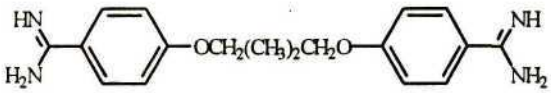
Diminazene



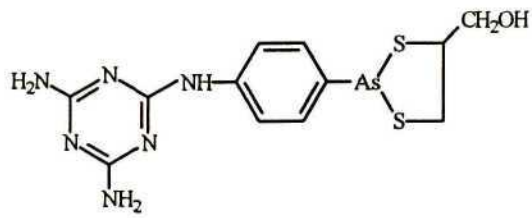
DL-α-difluoromethylornithine



Pentamidine



Melarsoprol



Suramin

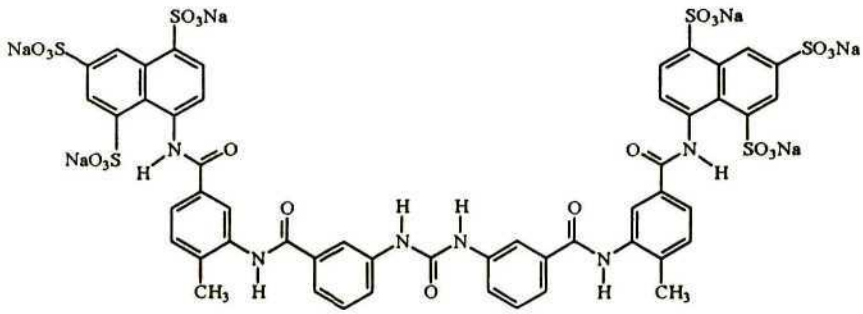


Figure 53. Chemical structures of trypanocidal drugs (after Wang, 1995).

Hoechst has never been interested in marketing diminazene for human trypanosomiasis because the limited market does not justify the high cost of the required toxicological studies. Thus, while Berenil® costs only US$1.50 per treatment, this drug is restricted to veterinary use (Pépin and Milford, 1994). Nevertheless, due to its effectiveness and low cost, it has been

used effectively in endemic countries to treat human trypanosomiasis, where it has a low (typically 5-15%) relapse rate (Abaru and Matova, 1984). With the exception of two cases of fatal reactive encephalopathy (De Raadt, 1967) there seem to be few toxic side-effects.

Suramin. Suramin has been used since 1923 for the chemotherapy of human trypanosomiasis (Kleine and Fischer, 1923). Suramin, the symmetrical urea of the sodium salt of *m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonic acid, is a sulfated naphthylamine (M_r 1429), usually marketed as Germanin® or Bayer 205® (Bayer, Leverkusen, Germany). The biochemical mechanism of the anti-trypanosome activity of suramin is not known. However, it is known to inhibit numerous enzymes *in vitro*, including L-α-glycerophosphate oxidase (Fairlamb and Bowman, 1977), RNA polymerase, fumarase, hexokinase, urease and trypsin (Hawking, 1978). Suramin reaches a plasma concentration of 100 μM (Collins *et al.*, 1986), and similar intracellular concentrations within trypanosomes (Wang, 1995). In addition to its trypanocidal activity, it is also known to be an inhibitor of viral reverse transcriptase (De Clercq, 1979), and has been investigated as a potential lead-compound for the design of new anti-viral (De Clercq, 1987), anti-tumour (Le Rocca *et al.*, 1990) and anti-fertility (Jones *et al.*, 1996) agents.

Suramin, which costs approximately US$35 per treatment, is regarded as a most effective drug for the treatment of early-stage trypanosomiasis (Apted, 1980). While few side effects apart from moderate renal toxicity have been documented, patients who are moribund on admission are reported to die immediately after the administration of suramin (Pépin *et al.*, 1989). Concomitant onchocerciasis, another common co-infection with African trypanosomiasis, introduces the risk of hypersensitivity reactions, and the use of suramin is not recommended in these cases (Pépin and Milford, 1994). Due to the poor CNS penetration of pentamidine, diminazene and suramin, these drugs are not effective in treating late-stage trypanosomiasis, and melarsoprol and DFMO are generally used in this instance.

Melarsoprol and melarsen oxide. Melarsoprol is a trivalent arsenical (M_r 398) marketed as Arsobal® by Rhône Poulenc (Paris, France). The cellular target of melarsoprol is generally regarded as trypanothione (Fairlamb *et al.*, 1989) but this compound is also known to be an inhibitor of trypanosome phosphofructokinase (K_i = 1 μM) and fructose-2,6-bisphosphatase (K_i = 2 μM) (Flynn and Bowman, 1969). Trypanothione [N^1,N^8-bis(glutathionyl)spermidine] is a conjugate of

glutathione and spermidine which is believed to be a major factor involved in the thiol-disulfide redox balance of trypanosomes. Fairlamb *et al.* (1987) showed that melarsoprol and melarsen oxide forms stable adducts with trypanothione. This melarsen-trypanothione adduct inhibits *T. b. brucei* trypanothione reductase (K_i = 18 μM) which is thought to be a key enzyme in the regulation of the thiol-disulfide state of trypanothione.

Common and severe adverse effects of melarsoprol therapy are reactive and haemorrhagic encephalopathy, tremors and polyneuropathy (Pepin *et al.*, 1989). These are probably direct toxic effects of arsenic deposition in the spinal cord (Gherardi *et al.*, 1990). In spite of being the most effective drug on the market, it is also one of the more expensive drugs, costing around US$45 per course.

Difluoromethylornithine. DL-α-difluoromethylornithine (DFMO) is an ornithine analogue marketed as Ornidyl™ by Marion-Merrell-Dow (Cincinnati, USA). Unlike most other trypanocidal drugs, DFMO crosses the blood-brain barrier, and has an excellent CSF penetration. A mean CSF/plasma ratio of 0.91 has been reported for adults, with a CSF drug concentration of 50 nM (Milord *et al.*, 1993). Melarsoprol and DFMO are therefore the only means of treating trypanosomiasis once it has reached the stage of CNS involvement. DFMO has the added advantage of oral administration, which is useful as other treatments are usually administered by intramuscular injection, and the injection sites are known to be extremely sensitive (Pépin and Milford, 1994). DFMO is a selective and irreversible inhibitor of ornithine decarboxylase (McCann *et al.*, 1986), for which it has a K_i of 130 μM. Ornithine decarboxylase catalyses the conversion of ornithine to putrescine, the first and rate-limiting step in the synthesis of putrescine and the polyamines spermine and spermidine (Bacchi *et al.*, 1980). Polyamines are essential for the growth and multiplication of all eukaryotic cells, where they contribute to the regulation of protein and nucleic acid synthesis (Pegg and McCann, 1982). DFMO is thus the only trypanocidal drug whose mechanism of action is understood (Pépin and Milford, 1994).

The only alternative to melarsoprol for late-stage infections is DFMO. However, serious toxic side-effects have been reported for DFMO, and some researchers have reported that between 5 and 20 % of patients die during therapy (Pépin *et al.*, 1987; Milford *et al.*, 1992). This appears particularly common in children under two years of age, and is highly problematic in

HIV co-infections (Pépin *et al.*, 1987; Milford *et al.*, 1992). Side-effects include bone marrow suppression, convulsions, abortion and leucopenia (Milford *et al.*, 1992, 1993). Furthermore, this is the most expensive treatment available, costing US$150 per course, well beyond the means of most people likely to be afflicted. In 1993, Marion-Merrell-Dow ceased production of DFMO (Pépin and Milford, 1994).

Thus, with the single exception of DFMO, the biochemical basis for the trypanocidal action of the remaining drugs is unknown. Suramin is known to inhibit a number of trypsin-like serine proteases (Fong and Good, 1972; Eisen and Loveday, 1973), and the two diamidines (pentamidine and diminazene) have structural similarities to the side-chains of basic amino acids, suggesting that these compounds may have inhibitory activity against trypanosome oligopeptidases, which are trypsin-like serine proteases. Furthermore, melarsoprol is known to react with free thiols, and given the sensitivity of trypanosome serine oligopeptidases to thiol-reactive agents (Section 5.11.6, Table 11), investigation of the reaction between these oligopeptidases and melarsoprol was also warranted. For these reasons, the effects of these trypanocidal agents on OP-Tb activity was investigated (Section 7.2), in an attempt to elucidate potential trypanocidal mechanisms involving the trypanosome serine oligopeptidases.

These serious side-effects of the currently used therapeutic agents, their high costs and the increasing incidence of drug-resistance exhibited by African trypanosomes (Pépin and Milford, 1994), strongly suggests that alternative, more effective chemotherapeutic strategies need to be developed. While the use of protease inhibitors in the treatment of protozoan diseases is not without precedent, previous efforts have generally targeted the lysosomal cysteine proteases of parasitic protozoans. Therefore, one of the aims of the present study was to explore the potential of the recently identified serine oligopeptidases of African trypanosomes as therapeutic targets. This chapter reports the effects of general protease inhibitors on trypanosomes in culture (Section 7.3), and extends these findings to more specific inhibitors of OP-Tb, including peptidyl chloromethylketones and diazomethylketones (Section 7.4) and peptidyl phosphonate diphenyl esters (Section 7.5).

7.2 Interaction of trypanocidal drugs with OP-Tb

As OP-Tb may have a regulatory role within trypanosomes, and since the mechanism of anti-trypanosome action is unknown for many trypanocidal drugs, the effect of the five major

trypanocidal drugs on OP-Tb activity was investigated. The interaction of suramin with OP-Tb was further investigated with a series of suramin analogues which were custom synthesised (these are illustrated in Figs 59 and 60), in which various moieties, such as the length of the spacer-arm connecting the sulfonated naphthylamine groups, and the number of sulfonic acid groups, were varied. These analogues were then examined for their ability to inhibit OP-Tb, and for their trypanocidal activity. The structure of suramin is illustrated in Fig. 54, and follows the nomenclature devised by Jentsch *et al.* (1987). Suramin is a symmetrical molecule that can be sub-divided into three regions: an acidic naphthalene residue; an aminobenzoyl residue; and a central urea group. For compounds with symmetrical structures it is sufficient to describe only half the molecule, thus the code for suramin is Aa-Bb-Ba-Cc (as opposed to Aa-Bb-Ba-Cc-Ba-Bb-Aa). This system describes the different structural elements of the synthesised analogues listed in Table 17. Further derivatives are found as footnotes to Table 17.

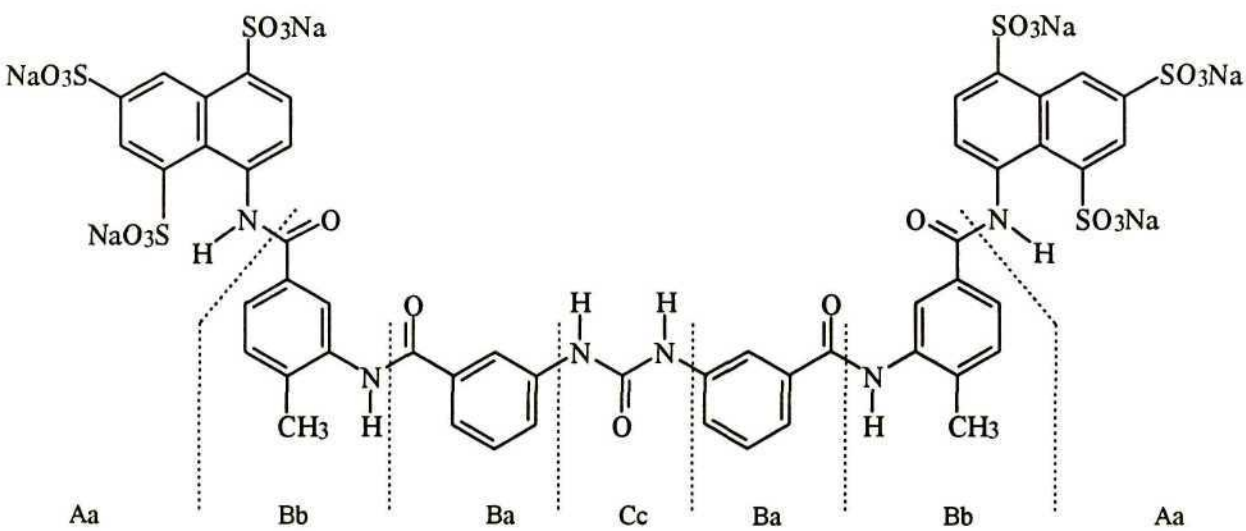


Figure 54. Chemical formula of suramin and designated nomenclature for suramin analogues. Analogue nomenclature follows that of Jentsch *et al.* (1987).

7.2.1 Materials

10 mM Stock drug solutions. Suramin hexasodium salt (0.142 g), Berenil® (58.2 mg), pentamidine isethionate (59.3 mg) and DFMO (16.8 mg) were dissolved in dH_2O (10 ml). Melarsoprol® (3.9 mg) and melarsen oxide (2.4 mg) were dissolved in DMF (1 ml).

Suramin analogues. NF-037 (2.80 mg), NF-031 (2.80 mg), NF-065 (2.26 mg), NF-013 (2.32 mg), NF-058 (2.38 mg), NF-023 (2.32 mg), NF-078 (0.92 mg), NF-035 (1.2 mg), NF-036 (1.2 mg), NF-064 (3.08 mg) and NF-226 (1.2 mg) were each dissolved separately in dH_2O (100 µl).

7.2.2 Method

V_{max} and K_m were determined in the absence and presence of trypanocidal drugs exactly as described in Section 5.1. If altered values for these factors [denoted V_{max} apparent (V'_{max}) and K_m apparent (K'_m), respectively] were obtained in the presence of trypanocidal drugs, these data were used to diagnose the mechanisms of inhibition. For competitive inhibitors, The K_i was determined as described in Section 5.6.2. For non-competitive inhibition, the K_i was determined from the relationship

$$\frac{1}{V'_{max}} = \left(1 + \frac{[I]}{K_i}\right)\frac{1}{V_{max}}$$ (Michal, 1983).

In the case of the arsenicals, melarsoprol and melarsen oxide, an uninhibited K_m was determined in the presence of an appropriate concentration of DMF, in order to negate the effect of this solvent on the K_m and V_{max}. In the case of hyperbolic non-competitive inhibition, the K_i was determined from the complex steady-state rate equation described by Baici (1987) as modified by Szedlacsek *et al.* (1988) and Cadène *et al.* (1997),

$$\frac{v_i}{v_0} = \frac{v_0 - v_\infty}{2v_0}\sqrt{\left(\frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0} + \frac{[I]}{[E]_0} - 1\right)^2 + 4\frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0}} + \frac{v_0 + v_\infty}{v_0 - v_\infty} - \frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0} - \frac{[I]}{[E]_0}$$

where $\sigma = [S]/K_m$ and $v_\infty = \beta\, k_{cat}\,[E]_0[S]/[S]+\alpha K_m$. The parameters α and β are dimensionless factors representing the change in the K_m and k_{cat} respectively in the presence of an inhibitor and v_∞ is the rate extrapolated at infinite [I]. Once K_m, k_{cat}, α and β had been determined separately, the K_i was calculated by non-linear regression analysis, fitting (v_i, [I]) pairs to the above equation in which all other parameters were fixed.

7.3 Trypanocidal activity of general protease inhibitors

The trypanocidal activity of a variety of general protease inhibitors was explored to determine whether exposure of *T. b. brucei* to protease inhibitors in culture had any affect on cell viability and growth.

7.3.1 Materials

Stock inhibitor solutions. Inhibitors (required mass to prepare 100 mM solutions) were dissolved in the specified volume of DMSO: antipain (6.8 mg, 100 µl); iodoacetate (10 mg, 500 µl); DCI (5.3 mg, 250 µl); Iodoacetamide (18 mg, 1 ml); leupeptin (5.8 mg, 120 µl); E-64 (8 mg, 122 µl); Pepstatin A (3.4 mg, 50 µl); 1,10-phenanthroline (19.8 mg, 1 ml); PMSF (17.4 mg, 1 ml). Similarly, the following masses of inhibitor were dissolved in the corresponding volume of dH_2O: AEBSF (100 mM, 12 mg, 500 µl); aprotinin (13.6 mM, 8.8 mg, 100 µl); SBTI (20 mM, 43 mg, 100 µl).

7.3.2 Method

Cell culture was undertaken exactly as described in Section 2.8. *T. b. brucei* was cultured in MEM (2.5 ml) to which inhibitor solutions (25 µl, 100 mM) had been added to give a final inhibitor concentration of 1 mM. Final concentrations of SBTI and aprotonin were 200 µM and 140 µM respectively. Control experiments were performed in parallel, which contained either 25 µl of DMSO or dH_2O only, without inhibitors. The number of motile trypanosomes was determined after 24 h, using a haemocytometer.

7.4 Trypanocidal activity of peptidyl methylketones

Peptidyl chloromethylketones (peptidyl-CH_2Cl) and diazomethylketones (peptidyl-CHN_2) are irreversible inhibitors of cysteine and serine proteases. These inhibitors are widely used as irreversible inhibitors of cysteine proteinases such as cathepsin L and cathepsin B (Kirschke and Shaw, 1981) where they bind covalently to the catalytic cysteine residue. They are also employed as inhibitors of serine proteases, where they bind to the active-site serine and histidine residues (Fig. 55).

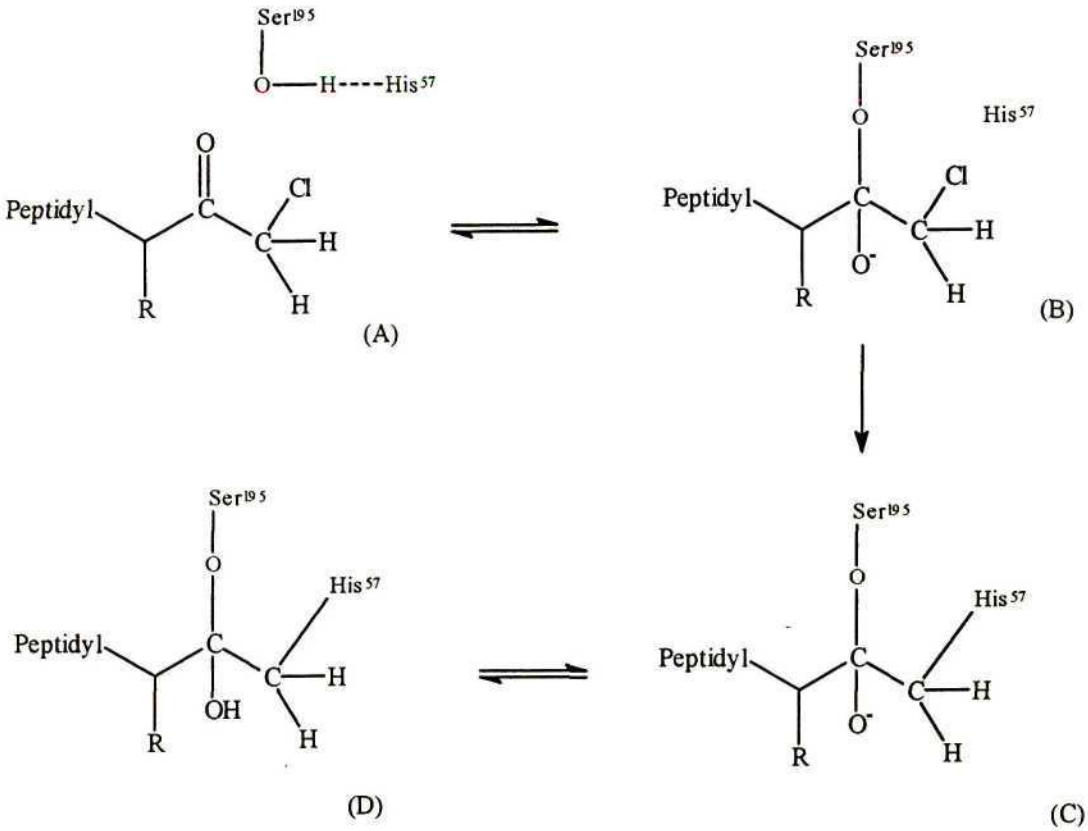


Figure 55. Mechanism of inhibition of a serine protease by a peptidyl chloromethylketone. An enzyme-inhibitor complex (A) is formed when the inhibitor binds to the enzyme active-site. The active-site serine then reacts with the carbonyl of the chloromethylketone (B) to give a tetrahedral structure, after which the N^{ε} nitrogen of the active-site histidine imidazole ring is alkylated (C, D) (after Powers and Harper, 1986).

In the present study, various peptidyl chloromethylketones and diazomethylketones, containing an arginine residue in the P_1 site, were tested for their ability to inactivate OP-Tb, the only serine protease described from *T. b. brucei* to date. These inhibitors were also tested for their ability of kill trypanosomes in culture.

In addition, a biotinylated chloromethylketone analogue in combination with protein blotting ("active-site blotting") was employed in an effort to determine which trypanosome proteases are targeted by these inhibitors in live trypanosomes. The synthesis and characterisation of biotinylated affinity labels for trypsin-like (biotin-ArgCH_2Cl) and chymotrypsin-like (biotin-PheCH_2Cl) enzymes have been described by Kay *et al.* (1992). Enzymes which have been covalently labelled with biotin by such probes are denatured by boiling and/or reduction and subjected to SDS-PAGE and electroblotted to nitrocellulose membranes. The labelled protein is then detected using an avidin-reporter-enzyme conjugate.

7.4.1 Materials

10 mM Synthetic inhibitor stock solutions. D-Val-Phe-LysCH_2Cl (0.7 mg), Leu-Glu-Gly-ArgCH_2Cl (0.5 mg), Ac-Ala-Ala-Ala-AlaCH_2Cl (0.4 mg), MeSuc-Ala-Ala-Pro-ValCH_2Cl (0.5 mg), D-Phe-Pro-ArgCH_2Cl (0.5 mg), Cbz-Phe-PheCH_2Cl (0.5 mg), Cbz-Gly-Gly-PheCH_2Cl (0.5 mg), Cbz-Gly-Leu-PheCH_2Cl (0.5 mg), D-Val-Leu-LysCH_2Cl (0.6 mg), Cbz-Phe-LysCH_2Cl (0.6 mg), Cbz-Phe-PheCHN_2 (0.5 mg), Cbz-Phe-AlaCHN_2 (0.4 mg), Cbz-Phe-Gly-TyrCHN_2 (0.5 mg), Cbz-Ala-AlaCHN_2 (0.3 mg), Cbz-Phe-Tyr(OBut)CHN_2 (0.5 mg), Cbz-Ile-LeuCHN_2 (0.4 mg), Boc-Val-Leu-Gly-LysCHN_2 (0.6 mg) and Z-Phe-AlaCHN_2 (0.5 mg) were each dissolved separately in DMSO (100 µl).

Stock biotin-Arg-CH_2Cl (100 µM). Biotin-ArgCH_2Cl (10 mg) was dissolved in DMSO (230 µl) and stored at -20°C.

All other reagents as per Sections 2.7.1.1., 2.8.1 and 3.2.

7.4.2 Method

The ability of peptidyl chloromethylketones and diazomethylketones to inactivate OP-Tb was investigated through the determination of the k_{ass}, exactly as described in Section 5.6.2, and the EC_{50} for these inhibitors, acting on *T. b. brucei* in culture, were determined exactly as described in Section 2.8.2.

For active-site labelling, OP-Tb (1 µg, 12.5 pmol, 20 µl) or *T. b. brucei* lysates (100 µg, 20 µl) were incubated (1 h, 37°C) in assay buffer (20 µl) with a 20-fold molar excess of biotin-ArgCH_2Cl (0.25 nmol, 2.5 µl of stock solution), after which non-reducing sample treatment buffer (20 µl) was added, and samples boiled for 10 min. Additionally, *T. b. brucei* was cultured (5×10^6 cells.ml^{-1}) in the presence of biotin-ArgCH_2Cl (100 µM) for 24 h as described in Section 5.8.2. Cells were then pelleted by centrifugation (3000 × g, 10 min, 4°C) and resuspended in PSG. Non-covalently-bound probe was removed by three successive cycles of pelleting by centrifugation followed by resuspension in PSG. Cells were then lysed by the addition of non-reducing sample treatment buffer (20 µl) and boiled for 10 min. Samples were subjected to Tris-Tricine SDS-PAGE (Section 2.7.1) and electroblotted onto

nitrocellulose (Section 2.7.5). Blots were then developed with an avidin-AP conjugate as described for the monoclonal bovine anti-IgM-biotin conjugate (Section 4.4).

7.5 Trypanocidal activity of peptidyl phosphonate diphenyl esters

Peptidyl α-aminoalkyl phosphonate diphenyl esters [peptidyl^P(OPh)$_2$] represent a further class of irreversible protease inhibitors, which are specific for serine proteases, without documented activity against cysteine proteases (reviewed by Oleksyszyn and Powers, 1994). The mechanism of inhibition of serine proteases by peptidyl^P(OPh)$_2$ inhibitors is illustrated in Fig. 56.

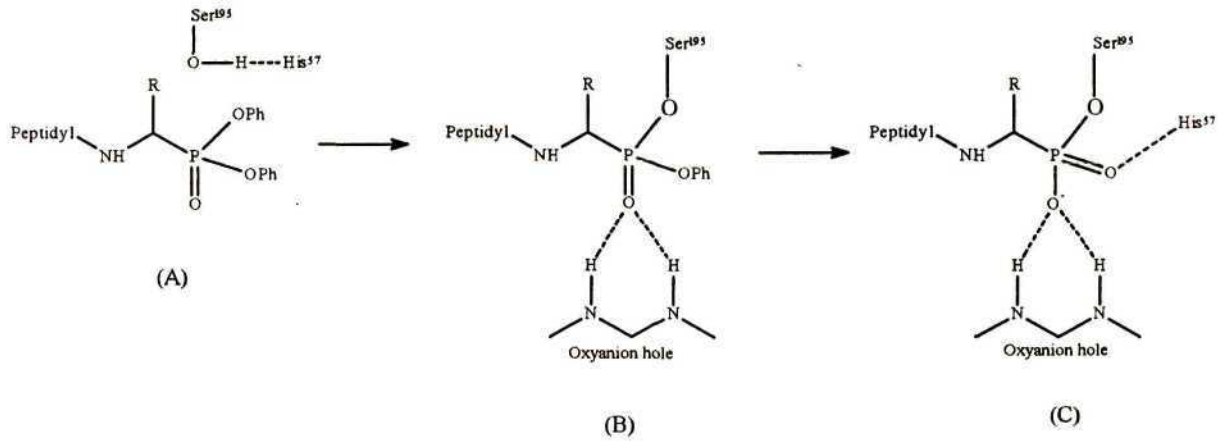


Figure 56. Mechanism of inhibition of a serine protease by a peptidyl α-aminoalkyl phosphonate diphenyl ester derivative. An enzyme-inhibitor complex (A) is formed when the inhibitor binds to the enzyme active-site. Phosphonylation of the active-site serine residue occurs (B) to form a covalent phosphonate diester, which then ages (C) to a monoester (after Oleksyszyn and Powers, 1994).

After initial complex formation, the active-site serine of the protease attacks the phosphorus atom of the peptidyl^P(OPh)$_2$ and phosphonylation of the serine proceeds via a pentacoordinate intermediate, forming a tetrahedral inhibition product, with the loss of one of the phenoxy groups from the peptidyl^P(OPh)$_2$. This complex subsequently ages to a mature complex with the loss of the second phenoxy group from the inhibitor (Oleksyszyn and Powers, 1994).

While the peptidyl^P(OPh)$_2$ inhibitors are generally much slower inactivators of serine proteases than are, for example peptidyl-CH_2Cl inhibitors, the peptidyl^P(OPh)$_2$ inhibitors exhibit remarkable stability under physiological conditions. The $t_{½} > 4$ days at pH 7.5 and $t_{½} > 24$ h in plasma (Oleksyszyn and Powers, 1994). As with the peptidyl methylketones, inhibitor specificity towards a particular protease can be enhanced by changing the amino acid sequence

of the peptidyl portion to suit the P_4-P_1 substrate specificity of the inhibitor of interest. Thus, peptidyl$^P(OPh)_2$ inhibitors may represent an excellent compromise between chemical stability and inhibitory potency.

Oleksyszyn *et al.* (1994) reported the synthesis of novel amidine-containing peptidyl$^P(OPh)_2$ inhibitors, in which the arginine phosphonate derivatives have been replaced with 4-amidinophenylglycine (4AmPhGly). These 4AmPhGly derivatives are more easily synthesised and are more potent inhibitors than their arginine phosphonate counterparts. Furthermore, these inhibitors are extremely stable under physiological conditions and form extremely stable enzyme-inhibitor complexes, making them suitable for *in vivo* studies.

In the present study, peptidyl$^P(OPh)_2$ inhibitors containing a 4AmPhGly analogue in the P_1 position were evaluated for inhibitory potency against OP-Tb, and for trypanocidal activity against trypanosomes in culture. An attempt was also made to identify the proteases targeted in living trypanosomes. Finally, the effect of peptidyl$^P(OPh)_2$ administration on disease progression in infected mice was evaluated.

7.5.1 Materials

Stock inhibitor solutions. Suc-(4AmPhGly)$^P(OPh)_2$ (6.58 mg; 127 mM), Cbz-Phe-(4AmPhGly)$^P(OPh)_2$ (2.8 mg, 40.1 mM), Cbz-Ala-(4AmPhGly)$^P(OPh)_2$ (5.8 mg, 93 mM), Cbz-Pro-(4AmPhGly)$^P(OPh)_2$ (9.3, 143 mg), Cbz-Lys-(4AmPhGly)$^P(OPh)_2$ (14.1 mg, 207 mM), Cbz-Gly-(4AmPhGly)$^P(OPh)_2$ (3.83 mg, 60.6 mM), Cbz Glu-Gly-(4AmPhGly)$^P(OPh)_2$ (2.16 mg, 27.8 mM) were dissolved in DMSO (100 µl).

1 mM Fla-Adp-Lys-(4AmPhGly)$^P(OPh)_2$ stock solution. Fla-Adp-Lys-(4AmPhGly)$^P(OPh)_2$ (1.11 mg) was dissolved in DMSO (1 ml).

All other reagents as per Sections 2.7.1.1., 2.8.1 and 3.2.

7.5.2 Method

The ability of peptidyl$^P(OPh)_2$ inhibitors to inactivate OP-Tb was investigated through the determination of the k_{ass}, as described in Section 5.6.2. The EC_{50} for these inhibitors acting on *T. b. brucei* in culture, were determined exactly as described in Section 2.8.2.

For active-site labelling, a fluorescent-labelled probe, [Fla-Adp-Lys-(4AmPhGly)^{P}(OPh)$_2$] was employed (Abulyeman *et al.*, 1997). OP-Tb (100 ng, ±1.25 pmol, 20 µl) was incubated (1 h, 37°C) in assay buffer (20 µl) with a vast molar excess of probe (58 µM final concentration, 2.5 µl of stock solution), after which non-reducing sample treatment buffer (20 µl) was added, and samples boiled for 10 min. Additionally, *T. b. brucei* (5×10^6 cells.ml^{-1}) were cultured in the presence of this inhibitor (100 µM, 2 h) and then prepared for analysis by Tris-Tricine SDS-PAGE as described in Section 2.7.1.1. Fluorescent-labelled proteins were visualised on a Fotodyne Foto UV transilluminator (Fotodyne, New Berlin, USA).

The administration of peptidyl^{P}(OPh)$_2$ inhibitors to infected mice, and the effect on disease progression was investigated in two ways. Due to the limited amount of material available, only Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ was tested in mice.

The effect of Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ on experimental *T. b. brucei* infection was initially examined by injecting BALB/c mice intraperitoneally with *T. b. brucei* (6×10^3 cells per mouse, in 100 µl PSG) and Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ (either 1 mg, 0.1 mg or 0.01 mg per mouse, in 25 µl DMSO). Two groups of control mice were set up. One group received *T. b. brucei* (6×10^3 cells per mouse, in 100 µl PSG) and DMSO (25 µl) alone (i.e. only trypanosomes and no inhibitors). The second control group received PSG (100 µl) and DMSO (25 µl) alone (i.e. no trypanosomes or inhibitors).

The effect of delayed, repeated administration on Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ on disease progression in mice was also examined. Again, BALB/c mice were injected intraperitoneally with *T. b. brucei* (6×10^3 cells per mouse, in 100 µl PSG). Three hours later, Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ in DMSO (25 µl) was administered at three different concentrations (0.25, 0.1 or 0.005 mg per mouse). Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ in DMSO (25 µl) was thereafter administered daily at these three concentrations. Two groups of control mice were set up. One group received *T. b. brucei* (6×10^3 cells per mouse, in 100 µl PSG), and DMSO (25 µl) alone three hours later (i.e. only trypanosomes and no inhibitors). The second control group received PSG (100 µl) and DMSO (25 µl) alone three hours later (i.e. no trypanosomes or inhibitors). DMSO (25 µl) was administered to both control groups daily.

7.6 Results and discussion

7.6.1 Interactions with trypanocidal drugs

No inhibition of OP-Tb activity against Cbz-Arg-Arg-AMC was observed in the case of DFMO, melarsoprol or melarsen oxide (Table 16). However, pentamidine, diminazene and suramin all inhibited OP-Tb activity against Cbz-Arg-Arg-AMC. The various K_i values for the inhibition of OP-Tb by trypanocidal drugs, and the diagnosed mechanisms of inhibition are illustrated in Table 16.

Table 16. Inhibition of OP-Tb by trypanocidal drugs[a].

Drug		Inhibition	K_i[b]
Trade-name	active ingredient	mechanism	(μM)
Pentacarinate®	pentamidine isethionate	competitive	3.4
Berenil®	diminazene aceturate	mixed	n.d.
Arsobal®	melarsoprol	none	n.d.
Melarsen oxide	melarsen oxide	none	n.d.
Ornidyl™	α-difluoromethylornithine	none	n.d.

[a]Data for suramin and its analogues are presented in Table 17.
[b]n.d., not determined

Pentamidine isethionate is the active principle of the trypanocidal drug Pentacarinate®. The mechanism of action of this drug is not understood (Pépin and Milford, 1994), although it has been proposed that it intercalates with trypanosome DNA, obstructing transcription. The pentamidine molecule represents two benzamidine groups joined together (Fig. 53), and therefore resembles the paired basic cleavage sites in peptide substrates of OP-Tb. OP-Tb was competitively inhibited by pentamidine with a K_i of 3.4 μM (Table 16; Fig. 57A), which compares well with the K_i for bovine β-trypsin (2.3 μM) and bovine mast-cell tryptase (1.2 μM) (Fiorucci *et al.*, 1997). During therapeutic regimens, pentamidine typically reaches concentrations of up to 13 μM in the host bloodstream (Sands *et al.*, 1985), and is actively concentrated by bloodstream-form trypanosomes which possess pentamidine transporters, where it is present at intracellular concentrations of approximately 80 μM (Damper and Patton, 1976). For an inhibitor to be considered physiologically relevant, its *in vivo* concentration must be $>10K_i$ (Bieth, 1980). As the intracellular concentration of pentamidine in bloodstream-form

trypanosomes is approximately $24K_i$, this introduces the possibility that pentamidine may exert part of its trypanocidal activity through the inhibition of OP-Tb.

Berenil® exhibited mixed inhibition of OP-Tb (Fig. 57B). The active component of this trypanocidal drug is diminazene aceturate, which, like pentamidine, resembles two benzamidine groups joined together, although with a shorter spacer-region (Fig. 53). Thus diminazene also mimics the paired basic cleavage site of OP-Tb substrates. The commercially available Berenil® preparations contain 45% (m/m) diminazene aceturate, and the remaining mass is made up of phenyldimethyl pyrazolone, (Leach and Roberts, 1981). This raises the possibility that the diminazene aceturate competitively inhibits OP-Tb activity, while the phenyldimethyl pyrazolone contributes to the inhibition of OP-Tb by Berenil in a non-competitive manner. Purified diminazene aceturate is not commercially available, therefore this hypothesis could not be investigated further, and the K_i was not determined. Bloodstream-form trypanosomes also posses transporters which concentrate diminazene, raising the intracellular concentration of the drug relative to the concentration in the bloodstream (Pépin and Milford, 1994), and the possibility of diminazene aceturate inhibiting OP-Tb with serious physiological consequences for the trypanosome, can not be ruled out.

Suramin is known to inhibit a number of proteolytic enzymes with a trypsin-like specificity, including C1 esterase (Fong and Good, 1972), trypsin (Hawking, 1978) and kallikrein, thrombin and plasmin (Eisen and Loveday, 1973). The mechanism of this inhibition is by an unknown mechanism. Kinetic analysis of kallikrein inhibition by suramin revealed that it was not of a competitive nature (Eisen and Loveday, 1973). More recently, Cadène *et al.* (1997) reported the inhibition of neutrophil elastase, cathepsin G and proteinase 3 by suramin, by a hyperbolic non-competitive mechanism. Suramin was shown to inhibit OP-Tb activity in a non-competitive manner (Fig. 57C), and the clustering of the V_{max} data points at high [I] in Fig. 54C suggested that a hyperbolic situation may also apply to the suramin inhibition of OP-Tb.

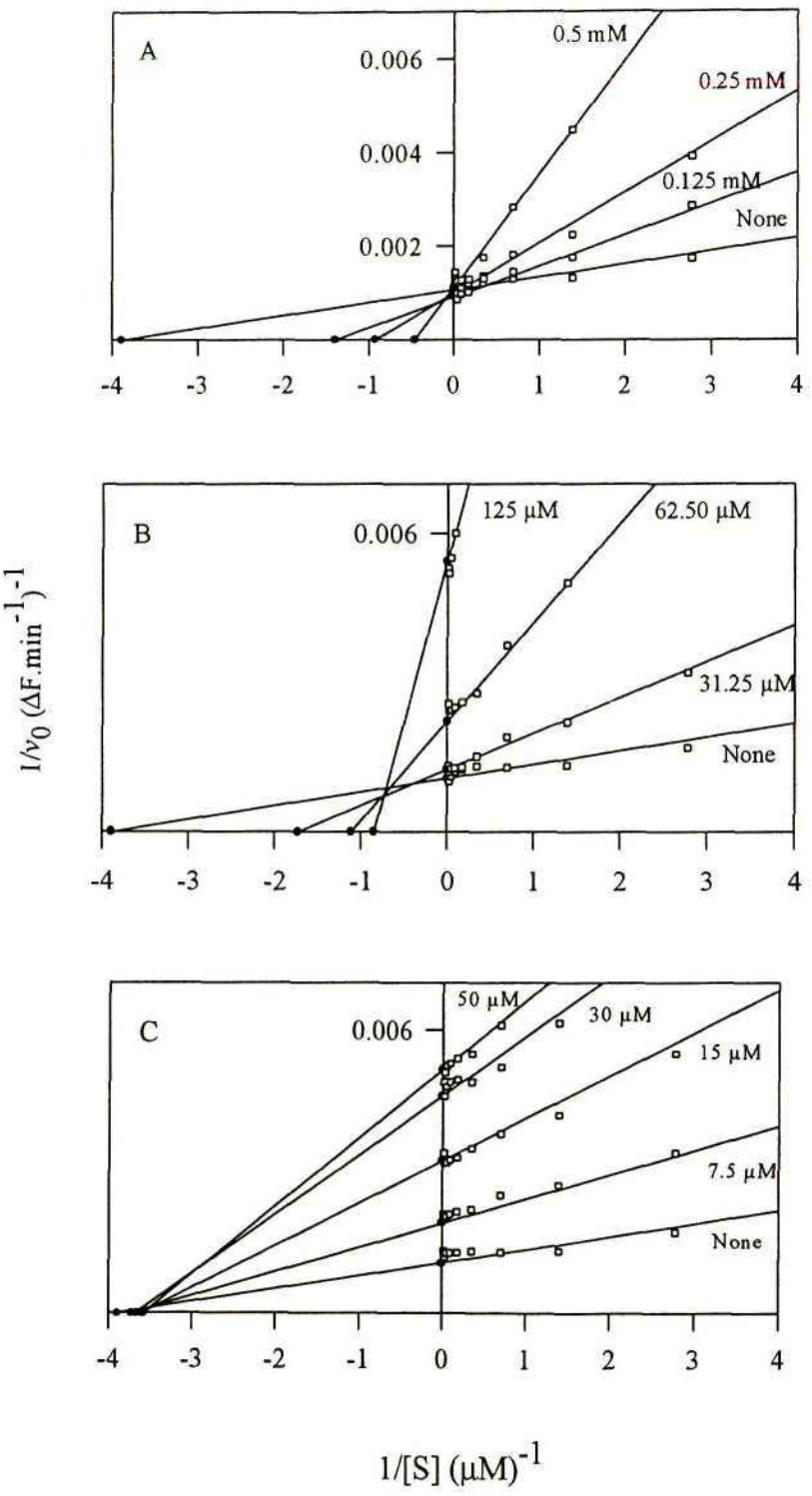


Figure 57. Diagnosis of inhibition mechanisms by trypanocidal drugs. Lineweaver-Burk plots of kinetic data obtained for the inhibition of Cbz-Arg-Arg-AMC hydrolysis by OP-Tb over a range of inhibitor concentrations for (A) pentamidine, (B) Berenil® and (C) suramin.

As is evident from Fig. 58A, the inhibition of OP-Tb activity against Cbz-Arg-Arg-AMC by suramin was maximal at 7.5 μM suramin. At this concentration, OP-Tb retained about 25% of its hydrolytic activity. Doubling the suramin concentration to 15 μM did not result in any further increase in inhibitory activity (Fig 58A), suggesting that the inhibition was indeed of a hyperbolic nature, with a K_i of 6.7 μM (Table 17). Furthermore, the $[E]_0/K_i$ ratio was <0.01, indicating that there was no tight binding of inhibitor to enzyme (Bieth, 1995). This parallels the situation observed for the inhibition of neutrophil proteinase 3 by suramin, which also exhibited non-tight-binding hyperbolic inhibition by suramin (Cadène *et al.* 1997).

There is generally a good relationship between the basicity of a protease and its affinity for suramin. For example, the basic neutrophil proteases (pI > 9.1) have much higher affinities for suramin than do less basic proteases such as trypsin. In contrast to these findings, OP-Tb has a pI of 5.1 (Kornblatt *et al.*, 1992). In order to further explore the interaction of suramin with OP-Tb, The inhibition of OP-Tb activity by suramin was investigated in the presence of NaCl. The presence of NaCl abolished the inhibition of OP-Tb by suramin, suggesting that electrostatic effects were involved in the OP-Tb-suramin interaction (Fig. 58B). Despite its low pI, OP-Tb has 24 lysine residues and 59 arginine residues (Table 7), and we propose that the sulfonic acid groups of suramin interact with these basic residues of OP-Tb. However, additional specific interactions appear to also be involved, as polysulfated glycosaminoglycans such as heparin do not inhibit OP-Tb (Table 12), suggesting that the inhibition is not due to a non-specific electrostatic interaction with polysulfated compounds. As shown later, the interactions appear to be quite specific in nature.

During trypanosomiasis treatment regimens suramin reaches concentrations of approximately 100 μM within the host bloodstream (Fairlamb and Bowman, 1980). Suramin is not actively concentrated in the parasite where it also reaches intracellular concentrations of approximately 100 μM (Wang, 1995). As these concentrations are approximately $15K_i$, the inhibition of OP-Tb by suramin may be physiologically relevant, and this may explain at least in part, the trypanocidal action of suramin which, to date, remains unelucidated (Wang, 1995). Despite being in use for over 70 years, trypanosome resistance to suramin has not been reported, indirectly suggesting that suramin may act on multiple targets within trypanosomes. Given the low K_i of suramin for OP-Tb , and its cytosolic location, it is likely that OP-Tb is one of these

targets. Because we suspect that OP-Tb released into the host bloodstream may to contribute to disease pathogenesis through the digestion of host peptide hormones (Section 6.8.7), the administration of suramin to infected hosts may help to abrogate the effects of the enzyme in the host bloodstream. Indeed therapeutic concentrations of suramin abolished over 80% of the OP-Tb activity in the blood of infected rats (results not shown), thereby lending credence to this idea.

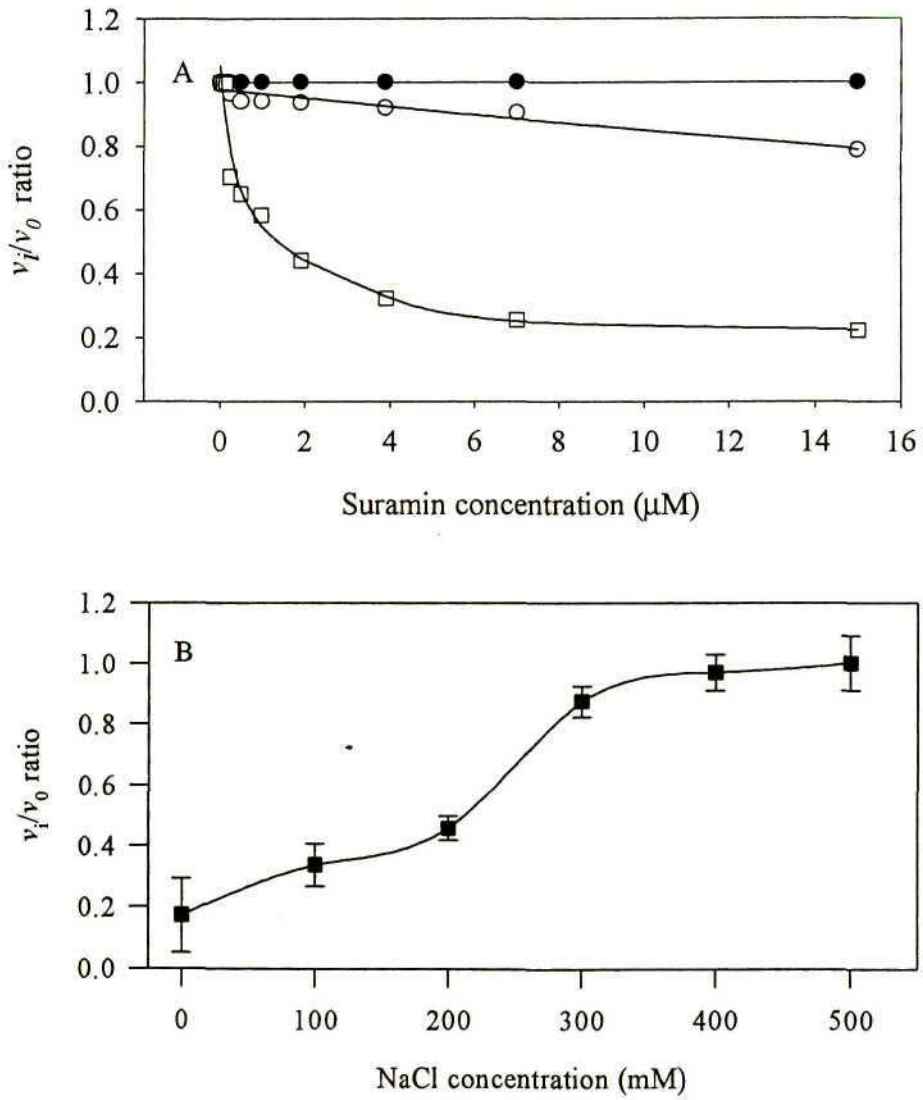


Figure 58. Effect of suramin on substrate hydrolysis by serine proteases. (A) Effect of increasing concentrations of suramin on the activity of OP-Tb (20 nM) against Cbz-Arg-Arg-AMC (□), chymotrypsin (25 nM) against Suc-Leu-Tyr-AMC (●) and trypsin (25 nM) against Cbz-Arg-AMC (○). Ordinates (v_i/v_0), initial rate in the presence of suramin/initial rate in the absence of suramin. (B) Effect of NaCl on the inhibition of OP-Tb (20 nM) activity against Cbz-Arg-Arg-AMC by suramin (■). Error bars represent the mean v_i/v_0 ± SD (n = 3). All substrates at 5 μM.

It is evident from Table 17 that both suramin, and its demethylated analogue (NF-037; Fig. 59), in which the methyl groups of the methylbenzoyl (Bb) moiety have been removed, were potent inhibitors of OP-Tb activity, and were highly trypanocidal when compared with the other analogues. This suggests that these methyl groups do not contribute to either the inhibition of OP-Tb activity or the trypanocidal activity. In fact, the absence of these groups is desirable, as it resulted in a 19% decrease in the K_i, and a 16% increase in the EC_{50}.

These findings contrast with those of Fourneau *et al.* (1924) who reported that demethylation of suramin resulted in a complete loss of its trypanocidal activity. It is significant, however, that the EC_{50} values determined here were determined *in vitro*, whereas Forneau *et al.* (1924) investigated the ability of suramin and its analogues to cure trypanosome infection *in vivo*, in mice. Taken together, these data suggest that, while the methyl groups are not important for the inhibition of OP-Tb activity or for the trypanocidal activity of suramin, they may be extremely important for the metabolism and/or transport of suramin in mice.

Table 17. Inhibition constants and EC_{50} values for suramin analogues.

Compound[a]	Chemical code[b]	K_i[c] (μM)	EC_{50} (μM)
NF-037	Aa-Ba-Ba-Cc	5.4	74.5
NF-171 (Suramin)	Aa-Bb-Ba-Cc	6.7	88.2
NF-031	Ab-Bk-Bk-Cc	62.2	96.7
NF-065	Ae-Bb-Ba-Cc	77.3	104.3
NF-013	Ab-Bk-Cc	127.7	128.7
NF-058	Aa-Bb-Cc	159.2	167.4
NF-023	Aa-Ba-Cc	161.0	172.2
NF-078	Aa-Ba-Bb-Cc	167.2	>220.0
NF-035	Ab-Cl	n.d.[c]	>220.0
NF-036	Aa-Cl	n.d.	>220.0
NF-064	Aa-Bb-Ba-Cf	n.d	>220.0
NF-226	Aa-Ba-Bb-Cc	n.d.	>220.0

[a]Analogue nomenclature is detailed in Jentsch *et al.* (1987).

[b]Additional structural elements are: Ab, 1-aminonaphthalene-4,6,8-trisulfonic acid; Ac, aniline-3-sulfonic acid; Ae, aniline-2,4-disulfonic acid; Bk, 4-C-benzoyl; Cf, -NH-CO-1,4-C_6H_6-CO-NH; Cl, -NH-(7-chloro-4-quinolinyl); Cm, -NH-CO-(5-nitro-2-furyl).

[c]n.d., not determined. In these cases, no inhibition of OP-Tb activity against Cbz-Arg-Arg-AMC was detected.

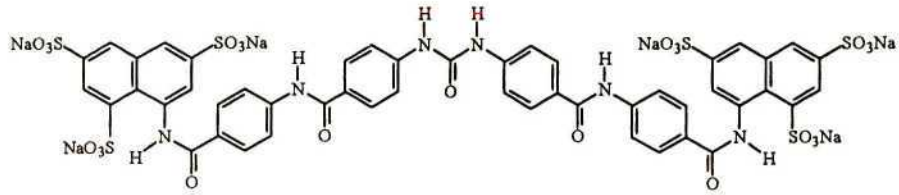
Both the number and spatial arrangement of the sulfonate groups on the aminonaphthyl (Aa) moiety were important for inhibitory and trypanocidal activity. Replacing each of the 1-naphthylamino-4,6,8-trisulfonic acid (Aa) moieties of suramin with aniline-2,4-disulfonic acid (to generate NF-065; Fig. 59) resulted in a 12-fold increase in K_i, but only a 15% increase in EC_{50}. Removal of a second sulfonate group from each of the aniline moieties in NF-065 (i.e. replacement of both the aniline-2,4-disulfonic acid moeities of NF-064 with aniline-3-sulfonic acid to give NF-078; Fig. 59) further elevated the K_i 25-fold when compared with suramin, and this analogue possessed no trypanocidal activity at the concentrations tested.

It is immediately apparent from Table 17 that the length of the molecule is also very important for both inhibitory potency and trypanocidal activity. Removal of the aminobenzoyl (Ba) group of suramin (to generate NF-058; Fig. 59) resulted in a 24-fold increase in K_i, and a two-fold increase in EC_{50}. Alternatively, removal of the aminomethylbenzoyl (Bb) groups from suramin (to generate NF-023; Fig. 59) had little effect on K_i or EC_{50} when compared with NF-058; which is in agreement with our previous findings (compare NF-037 and suramin) that these methyl groups are not important for inhibitory or trypanocidal activity.

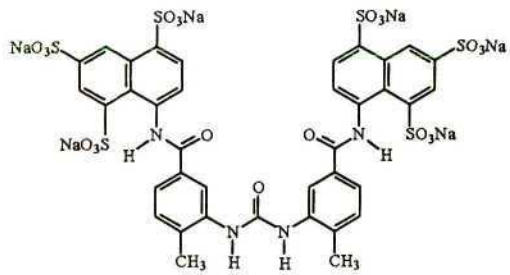
Replacement of both the aminobenzoyl (Ba) and aminomethylbenzoyl (Bb) groups of suramin with 4-C-benzoyl groups (to give NF-031; Fig. 59) elevated the K_i nine-fold, with a small (10%) increase in EC_{50}. Removal of two of these 4-C-benzoyl groups (one from each half of the molecule, to give NF-013; Fig. 59) further elevated the K_i to 19-fold when compared with suramin, and increased the EC_{50} by 31% when compared to that of suramin. This indicated that a reduction in the length of the molecule was accompanied by a reduction in inhibitory and trypanocidal activity.

The importance of the length and symmetry of the molecule is also illustrated by the lack of inhibitory or trypanocidal activity of the "half-suramin" analogue, NF-036 (Fig. 60). Rearrangement of one of the sulfonate groups on the naphthylene ring (NF-035; Fig. 60), or attachment of the 4,6,8-substituted aminonaphthylsulfonate to a 5-nitro-2-furyl group (NF-226; Fig. 60) did not improve the inhibitory or trypanocidal activity.

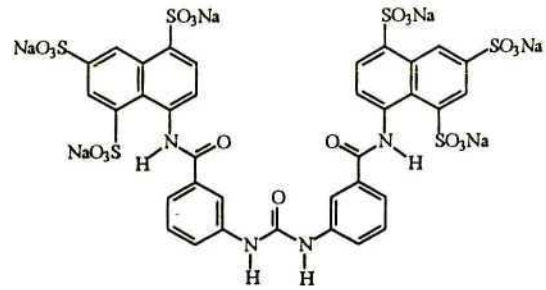
NF031 (M_r 1401.10)



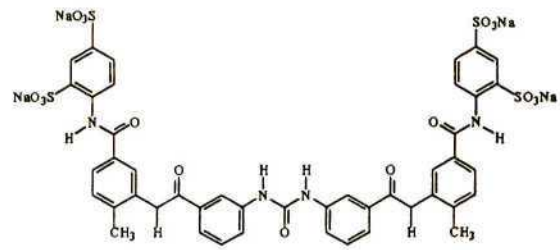
NF058 (M_r 1190.91)



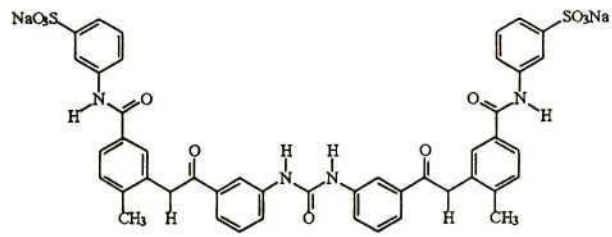
NF023 (M_r 1162.85)



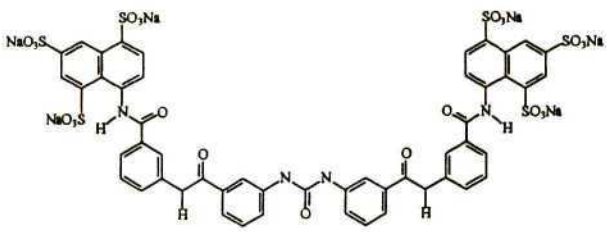
NF 065 (M_r 1124.95)



NF078 (M_r 920.87)



NF 037 (demethylated suramin; M_r 1401.1)



NF 013 (M_r 1162.85)

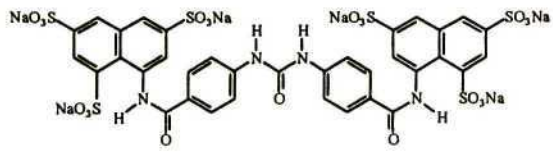


Figure 59. Symmetrical analogues of suramin (P.D. Nickel, unpublished).

No inhibitory activity was seen when the molecule was lengthened by the introduction of an additional benzene ring into the urea linker-region, and when one the of the aminonaphthyltrisulfonic acid groups was rotated 180% with respect to the same group on the

other half of the molecule (NF-064; Fig. 60). This once again points to the importance of the spatial arrangement of the various substituents.

There was a significant correlation ($P = 0.03$; by paired student's t-test) between the inhibitory potency and trypanocidal activity of suramin analogues, which strongly suggests that OP-Tb represents an important intracellular target for suramin. In the absence of a three-dimensional structure of OP-Tb, it is difficult to predict how suramin may interact, in such a specific fashion, with OP-Tb. While the OP-Tb-suramin interaction appears to be electrostatic, due to the reversing effect of NaCl, it is unlikely that it represents a non-specific electrostatic association between OP-Tb and the negatively charged sulfonic acid groups of suramin, as polysulfated glycosaminoglycans like heparin do not inhibit OP-Tb activity (Table 13).

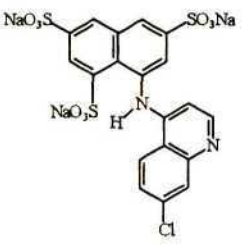
Hart *et al.* (1989) examined the interactions of suramin and trypanosome phosphoglycerate kinase (PGK). A striking feature of this enzyme is the presence of two clusters of positively-charged amino acids on the surface, separated by a distance of 4 nm, one on each of the two domains of PGK. Individual charges within each cluster are separated by a distance of 0.7 nm. This disposition of charges suggested to Hart *et al.* (1989) that there was a possible complementarity between PGK and the negative charges of suramin. Computer modelling of a putative PGK-suramin complex confirmed that electrostatic interactions between PGK and suramin would bridge the PGK active-site and block the mechanism of action of the enzyme.

With the PGK-suramin model in mind, if the active site of OP-Tb is buried in an active-site "pit" as has been suggested for oligopeptidases (Section 1.22), it is possible that a suramin molecule spanning the entrance to such a pit, may block access of substrate to such a pit. OP-Tb contains a total of 83 basic amino acid residues (Table 7). Whether or not some of these residues are clustered together to form suramin-binding sites will only be established once a three-dimensional structure of the molecule is available.

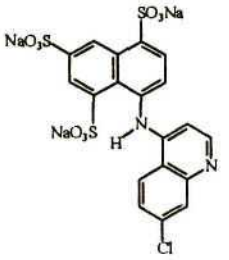
Both of the trivalent arsenicals (melarsoprol and melarsen oxide) and DFMO were without effect on OP-Tb activity. The sensitivity of OP-Tb to reducing agents (Fig. 31), and its inactivation by thiol-reactive agents (Table 11) and heavy-metal ions (Table 13) suggested that trivalent arsenicals may inactivate OP-Tb by covalent attachment to an essential cysteine

residue located at the catalytic site, as is the case with iodoacetate, iodoacetamide, *p*CMB and *N*-ethylmaleimide. The lack of inhibitory activity may be explained by the observations of Cunningham *et al.* (1994) that melarsoprol and melarsen oxide generally form adducts with dithiols, and not free thiols. Both compounds, however, form stable adducts with a unique trypanosome metabolite, trypanothione, effectively reducing trypanothione concentrations within the parasite during therapy with these drugs (Fairlamb *et al.*, 1992). Trypanothione is thought to maintain the correct intracellular redox state (Fairlamb *et al.*, 1987).

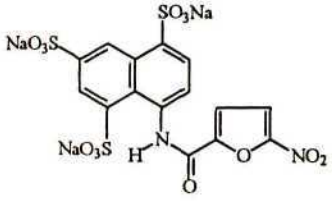
NF 035 (M_r 610.89)



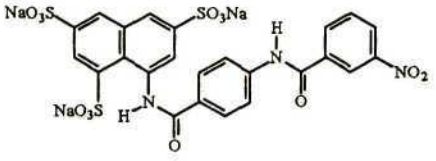
NF036 (Mr 610.89)



NF 226 (M_r 588.37)



NF 033 (M_r 717.53)



NF064 (M_r 1124.95)

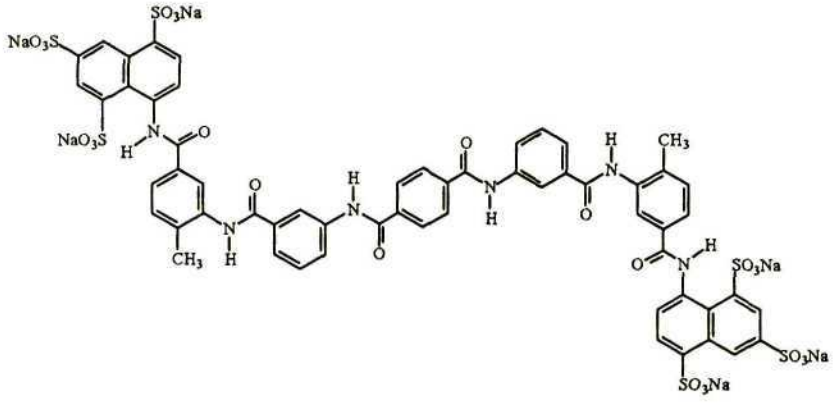


Fig 60. Asymmetrical analogues of suramin (P.D. Nickel, unpublished).

Considering that OP-Tb is activated by reducing agents, which may serve as *in vivo* regulators of OP-Tb activity, the depletion of intracellular trypanothione concentrations that result from melarsoprol and melarsen oxide therapy may down-regulate OP-Tb activity indirectly.

DFMO is an irreversible inhibitor of trypanosome ornithine decarboxylase, an enzyme which catalyses the rate-limiting step of polyamine biosynthesis. While no direct effect was observed on OP-Tb activity, therapeutic levels of DFMO are reported to decrease trypanosome intracellular spermidine levels by 76% after 48 h (Fairlamb *et al.*, 1987). The present study revealed that OP-Tb activity is enhanced in the presence of spermidine (Table 13), suggesting that OP-Tb activity is possibly regulated by polyamines *in vivo*. Depletion of intracellular polyamine levels by DFMO therapy may down-regulate OP-Tb activity within the trypanosome, possibly contributing to the trypanocidal action of DFMO. Furthermore, trypanothione is a glutathione-spermidine conjugate. DFMO, which reduces intracellular spermidine concentrations, also decreases intracellular trypanothione levels by up to 50% (Fairlamb *et al.*, 1987). Thus, DFMO may promote the down-regulation of OP-Tb activity not only directly through the reduction of intracellular polyamine levels, but also through the depletion of intracellular trypanothione, which would activate OP-Tb.

7.6.2 Trypanocidal activity of general protease inhibitors

With the exception of the proteinacious protease inhibitors (SBTI and aprotinin), all classes of protease inhibitors were trypanocidal to varying degrees (Fig. 61). However, there was no correlation between the trypanocidal activity of reversible inhibitors (E-64, leupeptin and antipain) and their K_i for OP-Tb. Nor was there a correlation between the trypanocidal activity of irreversible inhibitors (AEBSF, PMSF and DCI) and their k_{ass} for OP-Tb. Thus it seems likely that inhibitors acted on multiple targets within the trypanosomes. Alternatively, while such compounds may be powerful inhibitors of the purified enzyme, they may have variable abilities to actually enter the cell.

Proteinacious inhibitors of trypsin-like serine proteases (SBTI and aprotinin) were without trypanocidal activity. As it is unlikely that these inhibitors would enter trypanosomes, other than by internalisation into lysosomes, which have not been reported to contain trypsin-like serine proteases, their lack of trypanocidal activity is not surprising. In contrast, low molecular mass serine protease inhibitors were trypanocidal (DCI > AEBSF > PMSF). As these

inhibitors are specific inhibitors of serine proteases, it appears that the inhibition of parasite serine proteases is indeed detrimental to parasite viability.

The peptide aldehydes, antipain and leupeptin, which are non-class specific inhibitors of trypsin-like proteases, were both trypanocidal. However, peptide aldehyde data must be treated with caution. Wilcox and Mason (1992) demonstrated that leupeptin has very poor cell-penetration properties and does not enter the lysosomes of mammalian cells in culture. This was attributed to the inability of this inhibitor to diffuse across membranes, probably due to the charged guanido group in the molecule. Antipain, which contains two guanido groups (Umezawa *et al.* 1976), is probably even less likely to diffuse across the plasma membrane for the same reason. Furthermore, as the argininals probably exist primarily as inactive cyclic carbinolamines in aqueous solution (Schultz *et al.*, 1989), the actual [I] is probably below the predicted [I] in the cultures.

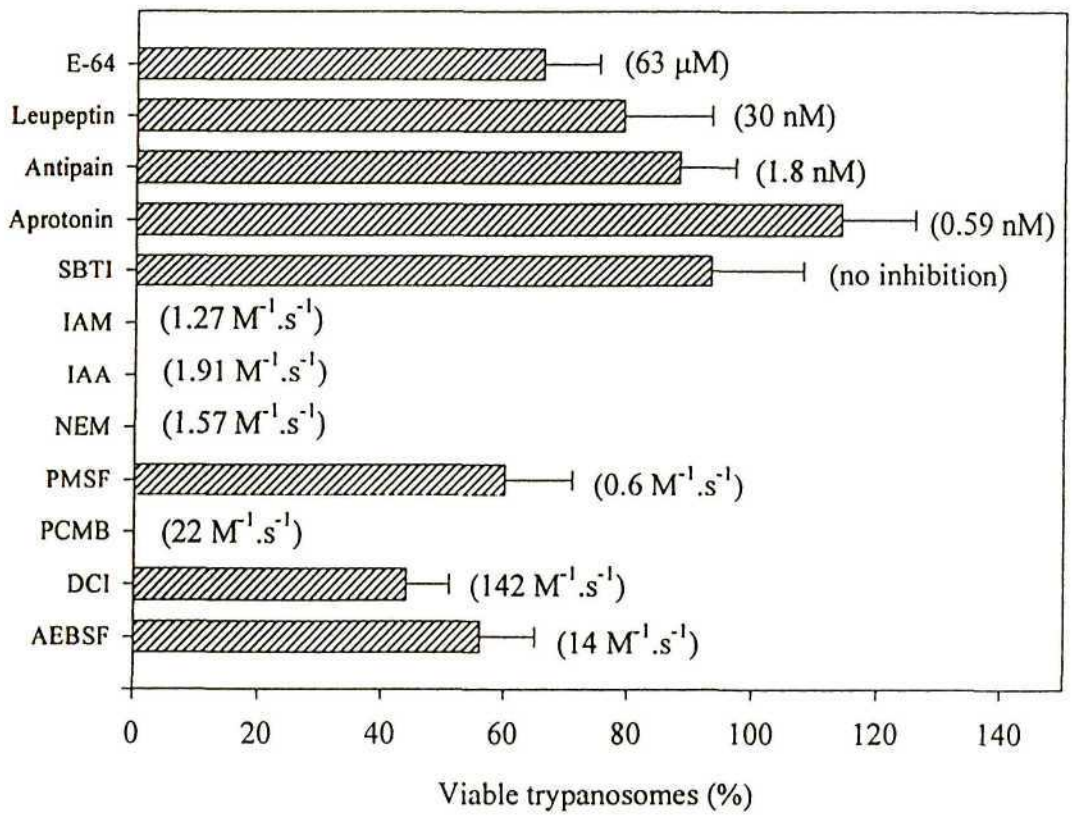


Figure 61. Trypanocidal activity of protease inhibitors. Bars indicate the fraction of viable cells after 24 h relative to uninhibited controls. Numbers in parentheses indicate the K_i of reversible inhibitors for OP-Tb inhibition (units of concentration; Table 10) or the k_{ass} of irreversible inhibitors for OP-Tb inhibition ($M^{-1}.s^{-1}$; Table 11). All inhibitors were at 1 mM, with the exception of aprotinin (140 μM) SBTI (200 μM).

The metal chelators, 1,10-phenanthroline and EDTA were weakly trypanocidal. Jones *et al.* (1996) reported the inhibition of growth and multiplication of *T. cruzi* epimastigotes *in vitro* by iron chelating agents, although this was not attributed to the inhibition of parasite proteases, but rather the chelation of iron where it interfered with the iron requirements of the parasites. African trypanosomes contain a gene encoding a putative membrane-bound metalloproteinase (El-Sayed and Donelson, 1997), and a phenanthroline-sensitive membrane-associated protease has been observed on gelatin zymograms (Section 1.3.1). However, it is clearly not possible to say yet whether the inhibition of this protease by metal-ion chelators is responsible for this growth inhibition. Additionally, Kawabata and Ichishima (1997) reported the inhibition of miltpain, a cysteine protease from salmon milt, by 1,10-phenanthroline, raising the possibility that trypanosome cysteine proteases may also be targets for the metal-ion chelators. However, neither phenanthroline nor EDTA have activity against trypanopain-Tb, the major lysosomal cysteine protease of *T. b. brucei* (Troeberg *et al.*, 1996).

E-64 appeared to have some trypanocidal properties, although it also exhibits poor cell penetration in mammalian cell-lines (Wilcox and Mason, 1992). E-64 is a weak reversible inhibitor of OP-Tb (K_i 63 μM) and is also known to inhibit trypanopain-Tb (Troeberg *et al.*, 1996). Whether the inhibition of either of these enzymes by E-64 is of significance to parasite viability remains to be determined.

Thiol-reactive agents, including *p*CMB, iodoacetamide, iodoacetate and *N*-ethylmaleimide were all strongly trypanocidal, killing 100% of the parasites in culture at an inhibitor concentration of 1 mM. These compounds non-specifically alkylate both thiol, and to a lesser degree, amino groups on proteins (Barron, 1955). Their inhibitory activity therefore cannot be attributed exclusively to the inhibition of parasite proteases, although both trypanopain-Tb (Troeberg *et al.*, 1996) and OP-Tb (Section 5.11.6, Table 11) are known to be inhibited by these compounds.

Thus, protease inhibitors, including serine protease inhibitors, have trypanocidal properties. It was therefore of interest to further explore the therapeutic potential of serine protease inhibitors. As a starting point, the trypanocidal activity of a variety of chloromethylketones and diazomethylketones was investigated. While these compounds are known to inhibit both

cysteine and serine proteases, the incorporation of the peptide portion permits a measure of selectivity, based upon the P_3-P_1 specificities of the proteases under investigation.

7.6.3 Trypanocidal activity of peptidyl methylketones

Chloromethylketones and diazomethylketones are irreversible inhibitors of cysteine and serine proteases, generally binding covalently to the catalytic cysteine and histidine residues respectively (Shaw 1994). The effect of peptidyl-CH_2Cl and peptidyl-CHN_2 inhibitors on OP-Tb activity against Cbz-Arg-Arg-AMC is illustrated in Table 18. OP-Tb was rapidly inhibited by synthetic covalent inhibitors that had basic residues (arginine and lysine) in P_1. Both hydrophobic (phenylalanine) and small, uncharged (glycine) residues, as well as basic residues, were acceptable in P_2, which is consistent with the trends observed for the hydrolysis of fluorogenic peptide substrates by OP-Tb (Section 5.11.1, Table 9).

Table 18. Peptidyl methylketone inhibitors of OP-Tb[a].

Inhibitor	k_{ass} ($\times 10^5$ $M^{-1}.s^{-1}$)	$t_{½}$ (s)	"delay time" (min)	EC_{50} (μM)
Tos-LysCH_2Cl	5.23 ± 0.23	7.97	1.33	27 ± 9
biotin-ArgCH_2Cl	4.45 ± 0.49	9.35	1.56	42 ± 7
Boc-Val-Leu-Gly-LysCHN_2	3.26 ± 0.36	12.83	2.14	38 ± 15
Leu-Glu-Gly-ArgCH_2Cl	3.04 ± 0.27	13.59	2.26	>100
Cbz-Phe-LysCH_2Cl	2.67 ± 0.08	15.57	2.59	32 ± 14
Asp-Val-Phe-LysCH_2Cl	2.47 ± 0.16	16.82	2.80	>100
Asp-Val-Leu-LysCH_2Cl	2.07 ± 0.44	20.08	3.35	>100
Asp-Phe-Pro-ArgCH_2Cl	1.27 ± 0.39	32.69	5.44	67 ± 27

[a]No inhibition was detected after 30 min pre-incubation with Ac-Ala-Ala-Ala-AlaCH_2Cl; Cbz-Gly-Gly-PheCH_2Cl; Cbz-Gly-Leu-PheCH_2Cl; Cbz-Leu-Leu-MetCHN_2; biotin-PheCH_2Cl; Tos-PheCH_2Cl; Cbz-Ala-AlaCHN_2; Cbz-Ile-LeuCHN_2; Cbz-Phe-AlaCH_2Cl; Cbz-Phe-AlaCHN_2; Cbz-Phe-Gly-TyrCHN_2; Cbz-Phe-PheCH_2Cl; Cbz-Phe-PheCHN_2; Cbz-Phe-Tyr(OBut)CHN_2 or MeoSuc-Ala-Ala-Pro-ValCH_2Cl.
[b]Data reflect the mean k_{ass} ± SD (n = 3)
[c]$t_{½}$ and "delay time" at 10 μM inhibitor concentration

The rate of inactivation of OP-Tb by peptidyl-CH_2Cl inhibitors was comparable to that observed for a variety of other trypsin-like proteases, which also had k_{ass} values in the order of 10^5 $M^{-1}.s^{-1}$ (Kettner and Shaw, 1981). The inhibition of members of the prolyl oligopeptidase

family of serine proteases (the "S9" family of Barrett and Rawlings, 1995) by peptidyl-CH_2Cl inhibitors has also been described (Stone *et al.*, 1991). It was found that [^{3}H]Ac-Ala-Ala-ProCH_2Cl irreversibly inactivated prolyl oligopeptidase with covalent modification apparent in four cysteine residues and the active-site histidine residue.

Eukaryotic serine proteinases are generally not inhibited by diazomethylketones, which are widely regarded as specific inhibitors of cysteine proteinases (Green and Shaw, 1980). However, OP-Tb was rapidly inactivated by Boc-Val-Leu-Gly-LysCHN_2 at a rate that was comparable (k_{ass} = 3.26 × 10^5 $M^{-1}.s^{-1}$) to that with which some lysosomal cysteine proteinases are inhibited by peptidyl-CHN_2 inhibitors (Crawford *et al.*, 1988; Angliker *et al.*, 1991). This acute sensitivity to peptidyl-CHN_2 inhibitors has also been observed for members of the prolyl oligopeptidase family of serine proteinases. Stone *et al.* (1992) described the inhibition of prolyl oligopeptidase by Cbz-Ala-Ala-PheCHN_2. However, this inhibitor acted as a competitive reversible inhibitor of prolyl oligopeptidase with a K_i of 16 nM. Covalent modification of the enzyme, employing [^{3}H]Ac-Ala-Ala-ProCHN_2, was only demonstrable after denaturation of the enzyme, and the tritiated inhibitor was found on the active-site serine residue. In contrast, Cbz-Ala-Ala-PheCHN_2 inactivates prokaryotic chymotrypsin-like serine proteinases including subtilisin (k_{ass} = 714 $M^{-1}.s^{-1}$) and thermitase (k_{ass} > 2.6 × 10^5 $M^{-1}.s^{-1}$) by alkylation of the active-site histidine (reviewed in Shaw, 1994). The mechanism by which Boc-Val-Leu-Gly-LysCHN_2 inhibits OP-Tb remains to be established.

There was no correlation between the k_{ass} of the inhibitors for OP-Tb and the EC_{50} for trypanosomes in culture (Table 18). This suggested either that the trypanocidal activity of these inhibitors was not related to the ability of the inhibitors to inactivate OP-Tb within the parasites, or that the different inhibitors had different cell permeability properties. Generally those inhibitors that contained more than one charged amino acid residue (for example, those containing aspartic or glutamic acid, in addition to a basic residue in P_1) were poorly trypanocidal, or not trypanocidal at all at the concentrations tested. This probably reflects the poor membrane-permeability of these highly charged peptides. It would be interesting to test these peptides after which the acidic group has been either esterified or amidated.

Peptidyl-CHN_2 inhibitors, including Cbz-[^{125}I]iodo-Tyr-AlaCHN_2 and Cbz-Phe-AlaCHN_2 are known to penetrate into mammalian cells in culture (Wilcox and Mason, 1992) and Cbz-Ala-Ala-ProCHN_2 is known to inactivate prolyl oligopeptidase in intact macrophages (reviewed in Shaw, 1994). Furthermore, the trypanocidal properties of Cbz-Ala-PheCHN_2 and Cbz-Phe-ImNvaCHN_2 have been documented, where they were shown to promote the lysis of *T. b. brucei* in culture at inhibitor concentrations of 100 μM. Indeed, incubation of live trypanosomes in the presence of the affinity label Cbz-Ala-[^{3}H]PheCH_2F resulted in the radiolabelling of a 68 kDa trypanosome protein (Ashall *et al.*, 1990b). This suggested to these investigators that the parasites contained a 68 kDa protease which was essential for parasite viability. Using the affinity label biotin-ArgCH_2Cl, three bands at approximately 68, 80 and 100 kDa were targeted in *T. b. brucei* lysates (Fig. 62, lane a), and a single band at 80 kDa was evident in the purified OP-Tb lane (Fig. 62, lane b). Live trypanosomes incubated in the presence of biotin-ArgCH_2Cl gave rise to a major band at 80 kDa, and a very faint band at about 68 kDa (Fig 62, lane c)

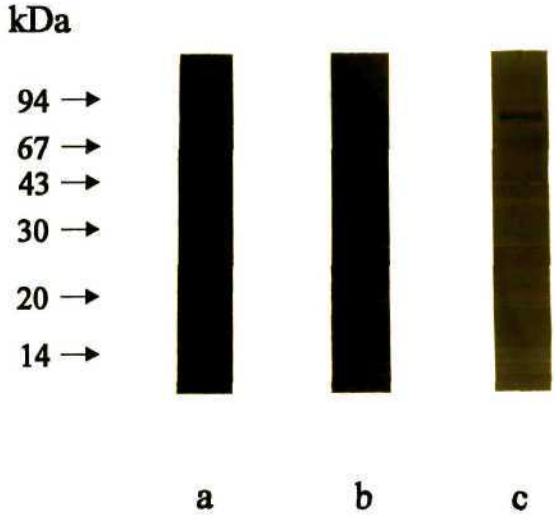


Figure 62. Active-site blots of *T. b. brucei* lysates and purified OP-Tb. Labelling of (a) *T. b. brucei* lysates (100 μg); (b) purified OP-Tb (1 μg) and (c) live trypanosomes with biotin-ArgCH_2Cl as described in Section 7.4.2.

The 80 kDa band evident in all three samples is probably attributable to OP-Tb. The low molecular mass band at 68 kDa may be due either to an active, processed form of OP-Tb, or to another cysteine or serine protease with a preference for basic residues in P_1. The pro-hormone convertases

(Lazure *et al.*, 1983) and the catalytic subunits of the proteasome (Hua *et al.*, 1996; Lomo *et al.*, 1997) are potential candidates. Albumin is known to be an extremely "sticky" protein as it is a carrier of a large number of non-proteinaceous compounds in the bloodstream. This raises the possibility that biotin-ArgCH_2Cl reacted in a non-specific fashion with residual rat serum albumin in the trypanosome lysates, generating a band at 68 kDa. Trypanopain-Tb, and its complexes with L-kininogen which are evident on substrate SDS-PAGE gels (Section 5.11.10; Fig. 36), also have a preference for basic residues in P_1. The 105 kDa trypanopain-Tb-L-kininogen complex may be responsible for the band evident at about 100 kD on the active-site blot, despite the absence of a 30 kDa band due to trypanopain-Tb alone. Alternatively this band may be due to OP-Tb in a complex with a regulatory protein, although such complexes are usually non-covalent and would not normally be expected to be stable to boiling in the presence of SDS. Alternatively, this band may be attributable to any one of a number of other proteases still awaiting discovery.

While both peptidyl-CH_2Cl and peptidyl-CHN_2 were potent inhibitors of OP-Tb and were also strongly trypanocidal, these inhibitors are not class-specific; they inactivate both cysteine and serine proteases at comparable rates. Therefore, the trypanocidal properties of a second class of inhibitors, the peptidyl phosphonate diphenyl esters, which are class-specific inhibitors of serine proteases, were also investigated (Section 7.6.4).

7.6.4 Trypanocidal activity of peptidyl phosphonate diphenyl esters

The peptidyl-(4AmPhGly)$^P(OPh)_2$ inhibitors were considerably slower irreversible inhibitors of OP-Tb activity when compared to peptidyl-CH_2Cl and peptidyl-CHN_2 inhibitors. This is consistent with the slower inhibition of other enzymes (Oleksyszyn and Powers, 1994). It is possible that 4AmPhGly is not a good substitute for arginine or lysine in the P_1 position for OP-Tb. While the series of inhibitors tested all contained the arginine analogue 4AmPhGly in the P_1 position, a variety of residues were present in the P_2 positions. There was little difference between the k_{ass} values obtained for the dipeptide inhibitors, irrespective of the residue in the P_2 position. This is consistent with the observations made using fluorogenic peptide substrates (Section 5.11.1, Table 9) which suggested that OP-Tb has little P_2 specificity, although the inclusion of a succinyl blocking group in the P_2 region appeared unfavourable. Lengthening of the peptide from a dipeptide to a tripeptide elevated the k_{ass} when compared with all the dipeptide inhibitors, perhaps suggesting that a contact between the enzyme and peptide portion of the inhibitor over the P_3-P_1 region is preferable to only P_2-P_1

interaction, and may help to "dock" the inhibitor for a longer period of time, or in a better position, for phosphonylation of the active-site serine residue.

In spite of the considerably lower k_{ass} values for the peptidyl-(4AmPhGly)^P(OPh)$_2$ inhibitors when compared with the efficacy of OP-Tb inhibition by peptidyl-CH_2Cl and peptidyl-CHN_2 inhibitors, the peptidyl-(4AmPhGly)^P(OPh)$_2$ inhibitors had comparable trypanocidal properties. This is possibly attributed to the considerable stability of the peptidyl-(4AmPhGly)^P(OPh)$_2$ inhibitors at physiological pH ($t_{½}$ > 4 days; Oleksyszyn and Powers, 1994) when compared with peptidyl-CH_2Cl inhibitors, which are unstable above pH 6 (Shaw, 1965). There was no direct correlation between OP-Tb inhibition and EC_{50}, again suggesting the possibility of multiple target proteases within the trypanosome, or that the inhibitors were not all equally membrane-permeable.

In support of the latter, the highly charged peptides like Suc-(4AmPhGly)^P(OPh)$_2$ and Cbz-Glu-Gly-(4AmPhGly)^P(OPh)$_2$ did not exhibit any trypanocidal activity at the concentrations tested, perhaps indicating that these inhibitors could not diffuse well across the cell membrane.

Table 19. Peptidyl diphenyl phosphonate ester inhibitors of OP-Tb[a].

Inhibitor	k_{ass}[b] ($M^{-1}.s^{-1}$)	$t_{½}$ (s)	[I][c] (μM)	EC_{50} (μM)
Suc-(4AmPhGly)^P(OPh)$_2$	12 ± 4	249	232	>100
Cbz-Phe-(4AmPhGly)^P(OPh)$_2$	102 ± 12	31	221	42 ± 6
Cbz-Ala-(4AmPhGly)^P(OPh)$_2$	130 ± 19	23	230	51 ± 23
Cbz-Pro-(4AmPhGly)^P(OPh)$_2$	106 ± 27	29	229	72 ± 22
Cbz-Lys-(4AmPhGly)^P(OPh)$_2$	109 ± 8	45	141	62 ± 18
Cbz-Gly-(4AmPhGly)^P(OPh)$_2$	164 ± 27	30	140	47 ± 11
Cbz-Glu-Gly-(4AmPhGly)^P(OPh)$_2$	442 ± 43	11	140	>100

[a]No kinetic data were obtained for Fla-Adp-Lys-(4AmPhGly)^P(OPh)$_2$ due to the intense fluorescence of this compound at the Cbz-Arg-Arg-AMC excitation and emission wavelengths.
[b]Data reflect the mean k_{ass} ± SD (n = 3).
[c][I] at which $t_{½}$ was calculated.

To determine which trypanosome proteases were targeted in live trypanosomes by the peptidyl-(4AmPhGly)^{P}(OPh)$_2$ inhibitors, a fluorescein-derivatised inhibitor was employed in a similar manner to the biotinylated peptidyl-CH_2Cl in Section 7.5.2.

T. b. brucei cultured in the presence of Fla-Adp-Lys-(4AmPhGly)^{P}(OPh)$_2$ yielded two fluorescein-labelled proteins (Fig. 63). One of these proteins migrated on SDS-PAGE gels to a position corresponding to that of purified OP-Tb incubated with the same affinity label, suggesting that this band also represents OP-Tb. A second trypanosome protein of approximately 30 kDa was also labelled by Fla-Adp-Lys-(4AmPhGly)^{P}(OPh)$_2$. The identity of this protein remains unknown but may be trypanopain which would cast some doubt upon the claimed group specificity of this inhibitor series. Alternatively, there may be at least two trypanosome proteases that are targeted by peptidyl-(4AmPhGly)^{P}(OPh)$_2$ inhibitors, and the inhibition of either or both of these proteases may be responsible for the trypanocidal activity of peptidyl-(4AmPhGly)^{P}(OPh)$_2$ inhibitors.

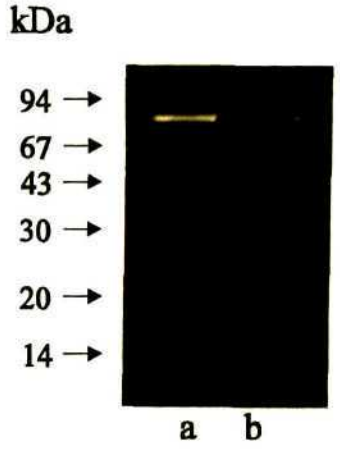


Fig 63. Active-site labelling with fluorescent peptidyl phosphonate diphenyl ester derivatives. OP-Tb (A) and live *T. b. brucei* (B) and were labelled with Fla-Adp-Lys-(4AmPhGly)^{P}(OPh)$_2$ as described in Section 7.5.2.

In order to examine the effect of administration of peptidyl-(4AmPhGly)^{P}(OPh)$_2$ inhibitors on disease progression in mice, these inhibitors were administered in two different regimens to experimentally-infected BALB/c mice.

Studies in mice suggested that Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ had trypanocidal properties *in vivo* as this compound was able to completely clear parasites from infected mice when administered daily at a dose of 5 mg per kilogram body mass (Table 20). All the surviving animals completely cleared the infection, as determined by thick blood-films taken from the tail vein, and were still alive 20 days post-infection. Daily administration of higher quantities (12.5 mg per kilogram body mass) did not improve the proportion of mice that cleared infection, but rather prematurely led to the death of 66% of the experimental group. This is possibly attributed to the toxicity of the reagent. Due to paucity of material, the toxicity of these compounds in mice could not be investigated further. However, it is known that peptidyl-(4AmPhGly)^{P}(OPh)$_2$ prolong the prothrombin time and activated partial thromboplastin time (Oleksyszyn *et al.*, 1994), and it is possible that these compounds interfered with the haematological homeostasis of the mice with lethal consequences. At 50-fold lower concentrations, administration of Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ had no observable effect on disease progression, and all experimental animals died at a similar time to control animals that did not receive the inhibitor.

Table 20. Effect of administration of Cbz-Gly-(4AmPhGly)^{P}(OPh)$_2$ on the progression of trypanosomiasis in BALB/c mice.

Treatment regimen and dose	Survival up to day 6 (number mice surviving / number of total mice treated)
Control 1 (+ parasites - inhibitor)	0/6
Control 2 (- parasites - inhibitor)	6/6
Single administration of inhibitor	
1 mg[a]	4/6
0.1 mg[a]	5/6
0.01 mg	0/6
Daily administration of inhibitor	
12.5 mg/kg/day	2/6
5 mg/kg/day[a]	5/6
0.1 mg/kg/day	0/6

[a]All the surviving animals were still alive, and apparently free of parasites (as determined from a thick blood-film prepared from a tail-vein blood sample), twenty days post infection.

7.7 Concluding statements

The biochemical mechanisms by which trypanocidal drugs exert their cytotoxic effects is not understood. Inhibition data indicate that pentamidine and suramin both inhibit OP-Tb with a K_i low enough to make such inhibition of potential *in vivo* significance, and it is possible that the trypanocidal activity of both drugs is due, at least in part, to the inhibition of OP-Tb within the trypanosome. The correlation between the *in vivo* trypanocidal properties of various suramin analogues and the K_i of such analogues for OP-Tb, suggested that OP-Tb may be essential for parasite viability. Should this be the case, OP-Tb may provide a potentially interesting target for therapeutic intervention.

A variety of general protease inhibitors exhibited either trypanocidal or trypanostatic properties when tested against *T. b. brucei* in culture. Such compounds included a number of specific inhibitors of serine proteases, suggesting that these enzymes were potential targets for drug design. To explore this possibility further, a variety of serine protease inhibitors were tested for (a) inhibitory activity against OP-Tb, the only serine protease isolated from African trypanosomes to date, and (b) for trypanocidal activity against *T. b. brucei* in culture.

Peptidyl-CH_2Cl were very fast ($>10^5$ $M^{-1}.s^{-1}$), irreversible inhibitors of OP-Tb, and demonstrated potent trypanocidal activity, with EC_{50} values in the low millimolar range. A biotinylated peptidyl-CH_2Cl labelled three bands in trypanosome lysates, one of which appeared to be OP-Tb, based on identical migration on SDS-PAGE. A band corresponding to the molecular mass of OP-Tb was also labelled in live trypanosomes incubated in the presence of this biotinylated inhibitor. These data suggested that OP-Tb was indeed a target for these inhibitors in live cells, and that the trypanocidal activity may be due, at least in part, to the inhibition of this enzyme within live parasites.

A peptidyl-CHN_2 inhibitor also inhibited OP-Tb at a rate comparable to the peptidyl-CH_2Cl inhibitors. Considering the observations of Stone *et al.* (1992), it remains to be determined whether peptidyl-CHN_2 inhibitors are reversible inhibitors of OP-Tb, as described for members of the prolyl oligopeptidase (S9) family of serine proteases, or irreversible inhibitors of OP-Tb, as has been described for prokaryotic chymotrypsin-like serine proteases (reviewed in Shaw,

1994). Whatever the case may be, Boc-Val-Leu-Gly-LysCHN$_2$ had comparable trypanocidal properties to peptidyl-CH_2Cl inhibitors.

As peptidyl-CH_2Cl and peptidyl-CHN_2 inhibitors will inhibit both serine and cysteine proteases, another group of inhibitors, peptidyl-(4AmPhGly)$^P(OPh)_2$ inhibitors were examined for the inhibitory and trypanocidal efficacy, as this group of inhibitors is claimed to be class-specific for serine proteases. Despite being slower inhibitors of OP-Tb when compared with peptidyl-CH_2Cl and peptidyl-CHN_2 inhibitors, a variety of peptidyl-(4AmPhGly)$^P(OPh)_2$ inhibitors exhibited comparable activity against *T. b. brucei* in culture. Active-site labelling revealed that a protein with a molecular mass comparable to that of OP-Tb was the major protein labelled in live trypanosomes, although a second, unidentified protein was also labelled, suggesting that OP-Tb was indeed a cellular target of these inhibitors, and that their trypanocidal activity may be mediated, at least in part, through the inhibition of OP-Tb. The observation that the only common band labelled by the different trypanocidal reagents had a molecule mass of 80 kDa suggests that the enzyme OP-Tb may be the trypanocidal target of the inhibitors.

Even if OP-Tb is not the target, because daily administration of one of these inhibitors, Cbz-Gly-(4AmPhGly)$^P(OPh)_2$, effected complete clearance of parasites from experimentally infected animals in 83% of the experimental group, it is clear that trypanosome serine proteases are exciting potential targets for therapeutic intervention and so warrant further study.

Chapter 8

General Discussion

It has been proposed that proteases may be important mediators of pathogenesis in trypanosomiasis (McKerrow *et al.*, 1993), but to date, the lysosomal cysteine proteases are the only proteolytic enzymes from African trypanosomes that have been extensively characterised (reviewed by Lonsdale-Eccles, 1991; Coombs and Mottram, 1997). Upon release into the host, however, these trypanosome-derived cysteine proteases are rapidly inactivated by endogenous host cysteine protease inhibitors, in particular, the kininogens and cystatins of the host serum (Troeberg *et al.*, 1996). This suggests that these enzymes are not major contributors to the disease pathology (although they remain essential for parasite viability). For this reason, other classes of parasite proteases are now under investigation as potential contributors to pathogenic mechanisms. The serine proteases of African trypanosomes are one such group of proteases, and while serine protease activity has been identified in African trypanosomes (Kornblatt *et al.*, 1992) the enzymes responsible for this activity have not been purified or extensively characterised. Thus, one of the aims of this investigation was to purify, to electrophoretic homogeneity, the serine protease from African trypanosomes responsible for this activity.

There are two major groups of African trypanosomes that are pathogenic for mammals, and members of the two groups of parasites cause very different disease in their host animals. It was therefore important to purify serine proteases from a representative member of each of these two groups of African trypanosomes, in order to investigate whether these enzymes differed in their enzymatic properties, which would perhaps account for the different pathologies observed (Boreham, 1979). For these reasons, enzyme purification was attempted from *T. b. brucei*, a representative of the "humoral" sub-group of African trypanosomes, and from *T. congolense*, a representative of the "haematic" sub-group of African trypanosomes.

Once purified, a comprehensive enzymatic characterisation of these two enzymes was undertaken, to gain information about the substrate specificity, the mechanisms of regulation and the optimum conditions for activity of these enzymes. These investigations served the

important dual function of providing some much-needed basic kinetic information about a member of the prolyl oligopeptidase family of serine proteases, which currently represents one of the less well understood groups of proteolytic enzymes. Having characterised the enzymatic properties of these trypanosome oligopeptidases, the potential role that these oligopeptidases may play in disease pathogenesis, and in the immune response of host animals to these enzymes was investigated. Additionally, the potential therapeutic value of inhibiting trypanosome oligopeptidases was investigated.

8.1 The structure and enzymatic properties of trypanosome oligopeptidases

A serine protease, designated OP-Tb, was isolated from *T. b. brucei* lysates by a combination of precipitation and column chromatography. This is the first time that this enzyme has been purified to electrophoretic homogeneity. A particularly useful step in the isolation of OP-Tb was the crude fractionation of trypanosome lysates by three-phase partitioning, a relatively new protein fractionation technique developed in our department (Jacobs *et al.*, 1989). OP-Tb activity co-precipitated with that of the major cysteine protease of *T. b. brucei*, trypanopain-Tb. This was fortuitous as it permitted the simultaneous purification of the two enzymes studied in our laboratory from the same, highly limited, starting material. These two proteases were easily resolved from one another by anion-exchange chromatography. Antibodies generated against OP-Tb cross-reacted with a related enzyme in *T. congolense* lysates, and these antibodies provided a useful tool for the immunoaffinity isolation of a related oligopeptidase, designated OP-Tc, from *T. congolense*.

These trypanosome oligopeptidases rapidly (k_{cat} >100 s^{-1}) hydrolysed a number of low-molecular mass fluorogenic peptide substrates which contained the basic residues arginine and lysine in the P_1 position, indicating a trypsin-like specificity. Hydrophobic and small uncharged residues were tolerated in P_2, although the enzyme exhibited the lowest K_m (260 nM) for substrates in which basic residues occupied both the P_1 and P_2 positions, and there appeared to be little specificity in the P_3 region. However, OP-Tb did not hydrolyse any protein substrates, and this information, together with the apparent lack of inhibition by high molecular-mass protease inhibitors, suggested that OP-Tb was an oligopeptidase rather than a proteinase.

It remains desirable to active-site map the P′ regions of the enzyme to obtain information on the amino-acid residue specificity, if any, in this region. For this purpose, an internally

quenched octapeptide, blocked at the N-terminus with an *o*-aminobenzoyl (Abz) group and at the C-terminus with a *N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp) group was designed as described by Del Nery *et al.* (1997). The peptide, Abz-Ala-Gly-Gly-Arg-Gly-Ala-Gly-Gln-EDDnp, contains a single arginine residue, Arg^4, which acts as the P_1 residue. Syntheses of peptides containing a single amino acid substitution at Gly^5, holding all other residues constant, is now underway. A comparison of the k_{cat}/K_m ratios for the OP-Tb catalysed hydrolysis of each of these peptides will give an indication of the P_1' specificity of the enzyme, and similar technology can be extended to exploring preference at other P′ sites.

The inhibition of OP-Tb by low molecular mass, irreversible inhibitors of serine proteases, in particular the mechanism-based 3,4-dichloroisocoumarin, suggested that the enzyme is a serine protease. However, the apparent stimulation of OP-Tb activity by reducing agents, and its inhibition by thiol-reactive agents have resulted in investigators mistakenly attributing such activity to a cysteine protease (Ashall, 1990; Ashall *et al.*, 1990b).

The non-essential activation of serine proteases by reducing agents, and their inhibition by thiol-reactive agents is not without precedent. A number of proteases belonging to the subtilisin family (the "S8" family in the nomenclature of Barrett and Rawlings, 1995) of serine proteases exhibit sensitivity to reducing agents, including the pro-hormone convertases kexin and furin (Van den Ouweland *et al.*, 1991), as well as cerevisin (Moehle *et al.*, 1987), thermitase (Kominami *et al.*, 1986) and protease K (Jany *et al.*, 1986). Although all of these enzymes are inhibited by bulky thiol-reactive agents, none display thiol-activatable activity. However, other serine proteases that also belong to the same subtilisin family of serine proteases do not demonstrate any sensitivity to thiol-reactive agents. These include subtilisin BPN′, an artificially mutated subtilisin from *B. amyloliquefascians* (Wells *et al.*, 1983) and subtilisin derived from *B. subtilis* Carlsberg (Smith *et al.*, 1986). Examination of the amino acid sequences surrounding the catalytic histidine, serine and aspartic acid residues of these proteases reveals why this may be the case (Fig. 64). All serine proteases of the subtilisin family that are inhibited by thiol-reactive agents possess a conserved cysteine residue four residues C-terminal to the catalytic histidine residue. This cysteine residue is replaced by a valine residue in proteases of the same family that do not demonstrate this sensitivity to thiol-reactive agents.

-SYVIDTGVNI-DGNGHGTHCAGTI-LSGTSMASPHVAGL-	cerevisin
-VYVIDTGIEA-DGNGHGTHCAGTL-ISGTSMATPHVAGL-	protease K
-IAIVDTGVQS-DGNGHGTHCAGTA-LSGTSMATPHVAGL-	thermitase
-AAIVDDGLDY-SDDYHGTRCAGEI-HGGTSAAAPLAAGV-	kexin
-VSILDDGIEK-MDNRHGTRCAGEV-HGTGSASAPLAAGI-	furin
-VAVLDTGIQA-DGNGHGTHVAGTL-LNGTSMASPHVAGA-	subtilisin Carlsberg
-VAVIDTGIAA-DNNSHGTHVAGTL-YNGTSMASPHVAGA-	subtilisin BPN′

Figure 64. Alignment of sequences surrounding the catalytic triad of members of the subtilisin family of serine proteases. Aspartic acid (**D**), histidine (**H**) and serine (**S**) residues of the catalytic triad are presented in bold type. Conserved cysteine residues are underlined (C). A dash (-) indicates a break in the primary sequence. Refer to text for information sources.

It was therefore of interest to determine whether a parallel situation existed for members of the prolyl oligopeptidase (S9) family of serine proteases, as porcine prolyl oligopeptidase (EC 3.4.21.26) displays acute sensitivity to thiol-blocking reagents, and is activated in the presence of reducing agents (Moriyama *et al.*, 1988). Three analogous enzymes from microbial sources, including *E. coli* (Kanatani *et al.*, 1991), *F. meningosepticum* (Yoshimoto *et al.* 1991) and *M. lacunata* (Yoshimoto *et al.*, 1995) demonstrate similar sensitivity, albeit to a lesser degree. While it is not known if the analogous enzyme from *T. cruzi* is activated by reducing agents, *p*CMB is known to inhibit the enzyme at nanomolar concentrations (Burleigh and Andrews, 1997). The amino acid sequences surrounding the catalytic triad of these proteases are illustrated in Fig. 65.

-QNCFDDFQCAAEY-LTINGGSNGGLL-KAGHGAGK-	prolyl oligopeptidase
-KNTFNDYLDACDA-CYAMGGSAGGML-DSGHGGKS-	*E. coli* oligopeptidase B
-KNVFNDFIAAGEY-MALSGRSNGGLL-NAGHGAGR-	*F. meningosepticum* oligopeptidase B
-RNTFTDFIAAAKH-MAARGGSAGGLL-GAGHFGKS-	*M. lacunata* oligopeptidase B
-QAGLHDPRVAYWE-LACEGRSAGGLL-ESGHFSPS-	*T. cruzi* oligopeptidase B

Figure 65. Alignment of sequences surrounding the catalytic triad of members of the prolyl oligopeptidase subclass of serine proteases. Aspartic acid (**D**), histidine (**H**) and serine (**S**) residues of the catalytic triad are presented in bold type. Unconserved cysteine residues are double-underlined (C). A dash (-) indicates a break in the primary sequence. Refer to text for information sources.

All members of the prolyl oligopeptidase family of serine proteases which display sensitivity to thiol-reactive agents contain at least one cysteine residue in close proximity to at least one of the three catalytic residues. Covalent attachment of bulky thiol-reactive groups to these cysteine residues may cause steric hindrance that interferes either with substrate binding or with the charge-relay system of the catalytic residues (Craik *et al.*, 1987). It is also possible that essential cysteine residues that are far removed from the catalytic apparatus in the primary

structure of the enzyme are brought into close proximity to the catalytic site by the folding (tertiary structure) of the enzyme. This situation will only be clarified once X-ray crystallographic data reveal the precise position of all the residues in the intact enzyme. Thus, the presence of reducing agents may ensure that these cysteine residues are maintained in a reduced state, which perhaps maintains an ideal charge environment for catalysis, and this may explain, at least in part, the activating effects of reducing agents on enzyme activity.

An interesting mechanism of thiol-activation has recently been proposed for the metallo-oligopeptidase called "Endopeptidase 3.4.24.15" (EC 3.4.24.15), a thermolysin-like oligopeptidase (Shrimpton *et al.*, 1997). This protease forms an inactive homodimer under non-reducing conditions. The covalent dimer is formed by any one of three cysteine residues on one molecule forming a disulfide bridge with any one of these residues on another molecule. The disulfide bridge is broken by low concentrations (up to 100 μM) of dithiothreitol, with concomitant regain of activity. It has been hypothesised that access to the catalytic site is blocked in the dimer, preventing substrate hydrolysis (Shrimpton *et al.* 1997). Whether a similar mechanism is in operation for OP-Tb and OP-Tc remains to be determined.

The molecular mass, sensitivity to reducing agents and substrate specificity strongly suggest that OP-Tb and OP-Tc belong to the prolyl oligopeptidase (S9) family of serine proteases (Barrett and Rawlings, 1995). This hypothesis is supported by the homology observed between the primary OP-Tb sequence (as determined from OP-Tb-derived peptides) and *T. cruzi* oligopeptidase B, which is a member of this family of serine proteases - as determined from the primary sequence of the gene encoding this enzyme, which has been cloned (Burleigh *et al.*, 1997). This hypothesis can only be confirmed once the *T. b. brucei* and *T. congolense* enzymes have been cloned, and their gene sequences known.

8.2 The physiology and pathophysiology of trypanosome oligopeptidases

The *in vivo* role of trypanosome oligopeptidases within the trypanosome is not known. Protease variations among different life-cycle stages of African trypanosomes have been observed (Mbawa *et al.*, 1991), possibly implicating these proteases in regulatory roles within the trypanosome. Soluble Cbz-Gly-Gly-Arg-AMC hydrolysing activity of *T. congolense* strain IL 3000 was considerably elevated in epimastigote forms of the parasite (specific activity 156 $pmol^{-1}.s^{-1}.mg^{-1}$) relative to metacyclic forms of the parasite where the specific activity was

reduced by 50% (70 $pmol^{-1}.s^{-1}.mg^{-1}$; Mbawa *et al.*, 1991). Activity was further reduced five-fold in bloodstream forms of the parasite (specific activity of 24 $pmol^{-1}.s^{-1}.mg^{-1}$). Similar trends in soluble Cbz-Gly-Gly-Arg-AMC activity were also observed in *T. vivax* (Mbawa *et al.*, 1997). While the enzyme responsible for this activity was not identified, its activity against Cbz-Gly-Gly-Arg-AMC, soluble nature, inhibition by both DFP and organomercurial compounds and its alkaline pH optimum (Lonsdale-Eccles and Grab, 1987) strongly suggest that it may be OP-Tc, and a related enzyme in *T. vivax*. The differential expression of this activity suggests that the enzyme may play a regulatory role in the parasite life cycle, such as the differentiation into different life-cycle stages.

This is supported further by the observations of Kato *et al.* (1992), who reported the purification of an oligopeptidase, designated "protease In", with a trypsin-like specificity, from *E. coli.* This protease momentarily appears immediately before DNA synthesis in the cell cycle of *E. coli*. This suggests that such a cytosolic oligopeptidase may have a regulatory function within the cell, perhaps through the degradation of intracellular peptide hormones.

The *in vitro* degradation of peptide hormones by OP-Tb and OP-Tc, demonstrated in the present study, raises the possibility that these enzymes may function to process or degrade peptide hormones in the trypanosomes themselves. While peptide hormones were initially believed to be unique to vertebrate organisms, subsequent research has revealed that similar molecules exist in a number of invertebrates, including insects, helminths, coelenterates, molluscs, as well as bacteria, yeasts and protozoans (reviewed in LeRoith *et al.*, 1986). Of particular interest to this investigation was the detection of such peptide hormones in protozoans. Molecules resembling peptide hormones including insulin (LeRoith *et al.*, 1980), ACTH, β-endorphin, calcitonin (Deftos *et al.*, 1985) and neurotensin (Bhatnager and Carraway, 1981) have been identified in unicellular organisms.

It appears therefore that unicellular protozoans and other invertebrates also possess peptide hormones, and it seems reasonable that they would also possess enzymes capable of metabolising such hormones. OP-Tb may be one such enzyme. The identification of peptide hormone substrates within trypanosomes, and the design of trypanosomes in which the oligopeptidase gene has been "knocked out", may provide keys to the physiological function of

this enzyme. Targeted gene disruption technology, while still in its infancy, has been performed in *L. mexicana* (Mottram *et al.*, 1997) and in *T. cruzi* (Norma Andrews, Department of Cell Biology, Yale Univesrity School of Medicine, New Haven, U.S.A., *pers. comm.*) and so opens up the potential for exploring these aspects further.

The biochemical mechanisms of the pathogenesis of African trypanosomiasis are poorly understood. An understanding of the mechanisms of the disease processes could lead to better methods of treatment, possibly by inhibiting or reversing the important pathological changes, or by alleviating some of the symptoms of the disease. It was shown in the present study that OP-Tb was highly active and stable at physiological pH and ionic strength. Further to this, OP-Tb was not inhibited by any high molecular mass inhibitors of serine or cysteine proteases in the mammalian host bloodstream, including cystatins, kininogens, serpins and α_2M. Neither was OP-Tb activity inhibited in the presence of bovine serum or plasma. This raised the possibility that should OP-Tb enter the host bloodstream, it would retain its activity and may contribute to disease pathogenesis through the digestion of host proteins.

The presence of peptidase activity in infected murine plasma (Knowles *et al.*, 1987), and the unusual cleavage of peptide hormones in the blood of infected rats (Tetaert *et al.*, 1993) strongly suggests that a peptide-degrading enzyme is present and active in the bloodstream of infected animals. As OP-Tb was shown to hydrolyse neurotensin, ANF and glucagon *in vitro*, it is not unreasonable to assume that a similar situation may exist *in vivo*, in the bloodstream of infected hosts. The degradation of these peptides in the bloodstream of the host would have profound implications in terms of disease pathogenesis.

While some peptide hormones have been shown in the present study to be degraded by OP-Tb *in vitro*, most still remain to be tested. An indication of some potential peptide hormone substrates for OP-Tb present in the mammalian bloodstream and CSF are presented in Table 21. This list is by no means comprehensive.

To illustrate how the anomalous degradation of these hormones could play a role in the pathogenesis of trypanosomiasis, the example will be taken of β-melanoctye stimulating hormone (β-MSH). β-MSH acts directly on the thyroid gland in mice to release the thyroid

hormones 3,5,3′,5′-tetraiodo-L-thyronine (thyroxine, T_4) and 3,5,3′-triiodo-L-thyronine (T_3) (Schally *et al.*, 1967). Depressed levels of both T_3 and T_4 have been observed in humans infected with *T. brucei gambiense* (Boersma *et al.*, 1989) and in goats infected with *T. congolense* (Mutayoba *et al.*, 1988a, 1988b; Section 1.2.1). As we have demonstrated that OP-Tb is active the host bloodstream, which also serves as the vehicle for β-MSH distribution in the body, it is quite possible that β-MSH is degraded by OP-Tb in the host bloodstream. The nett effect of this would be, *inter alia*, depleted levels of T_3 and T_4 in infected animals.

Table 21. Potential peptide hormone substrates for OP-Tb in the mammalian bloodstream[a].

Peptide Hormone	Sequence
Neuropeptide K	DADLYGHGQISH**KR**HKTASFVGLM
β-Endorphin	YGGFMTELVTLFKNAIIKNAY**KK**GE
Dynorphin A	YGGFL**RR**IRPKLKWDNQ
Dynorphin B	YGGFL**RR**QFKVVT
VIP	HSDADNYTRL**RK**QMAV**KK**YLNSILN
Adrenal Peptide E	MDYQ**KR**TGGFL
β-MSH	AE**KK**DGPTRMEHFRWGSPPKD
Neuromedin U-25	FKVDEEFQGPIVSEN**RR**YFLFRPRN
Kinetensin	IA**RR**HPYFL
BNP	SPKGFG**RK**MDRISSSSGLGCKVL**RR**H

[a]All peptides have a molecular mass of less than 4 kDa, contain paired basic residues, and lack disulfide bridges. Paired basic residues are indicated in bold-type.

The gastrointestinal tract peptide hormones (neuropeptide K and kinetensin; Walsh, 1987) and dynorphins A and B (Koob *et al.*, 1982) have important functions in gut motility, behaviour and appetite-modulating functions; and neuromedin U-25 is a potent hypertensive polypeptide of the central nervous system (Minamino *et al.*, 1985). In a similar manner described for β-MSH, the anomalous degradation of any or all of the peptide hormones presented in Table 20 could give rise to the confusing clinical picture presented by trypanosome-infected hosts.

The cytokines are known to function as important effector molecules against parasitic infections (Liew *et al.*, 1997). OP-Tb may also function in trypanosome infections to

inactivate such cytokines, e.g. β-endorphin. β-Endorphins are peptide hormones that elicit several biological reactions including analgesia, behavioural changes and growth-hormone release. β-Endorphins are known to modulate T-lymphocyte activation, with the N- and C-terminal domains having antagonistic effects. C-terminal peptides (β-endorphin$_{18-31}$) are involved in both $CD4^+$ and $CD8^+$ T-cell activation, whilst the N-terminal peptides (β-endorphin$_{1-16}$) abrogate this activity (van den Berg *et al.*, 1993). T-lymphocytes secrete a metalloprotease (T-cell peptidase) involved in the cleavage of β-endorphin into these discrete peptides between Leu^{17} and Phe^{18}, and this phenomenon is thought to have important immunoregulatory activity (Miller *et al.*, 1996). The activating C-terminal peptide possesses a putative cleavage site for OP-Tb (between Lys^{29} and Glu^{30}) in the intact β-endorphin peptide and in the β-endorphin$_{18-31}$ peptide that is generated by T-cell peptidase. This introduces the possibility that OP-Tb may degrade T-cell activating peptides in the host bloodstream. Such effects are consistent with the reports of depression of T-cell responses in African trypanosomiasis for both rodents infected with *T. b. brucei* (Pearson *et al.*, 1978), and *T. congolense* infection in cattle (Flynn and Sileghem, 1993).

It therefore appears highly probable that OP-Tb, which is released into the host bloodstream, and possibly the CSF, remains unregulated in host body fluids. It may then interfere with metabolic homeostasis through the inactivation of host peptide hormones, contributing significantly to the pathogenesis of African trypanosomiasis. This possibility needs to be explored further. The determination of the K_m and k_{cat} for the degradation of peptide hormones by OP-Tb would indicate whether the reaction is of *in vivo* significance, and such data may be supported by the demonstration that the levels of prospective peptide-hormone substrates is diminished in infected hosts. Furthermore, should trypanosomes in which the OP-Tb gene has been eliminated, or knocked out, remain viable, it would be interesting to observe whether there was any effect on the pathogenesis and disease progression caused by such genetically altered parasites.

8.3 Are trypanosome oligopeptidases potential compounds for vaccine development?

The production of anti-oligopeptidase antibodies by infected hosts would be desirable for two reasons. Oligopeptidase activity released into the host bloodstream may be abolished should the host generate antibodies to oligopeptidases, and this would abrogate any contribution of

this released oligopeptidase to disease pathology. Secondly, these antibodies would promote complement-mediated trypanosome-lysis by binding to any oligopeptidase on the surface of the parasites. The latter seems particularly promising since the present study has shown some evidence for the presence of surface-bound OP-Tb.

Inhibitory anti-OP-Tb antibodies were produced in chickens in the present study and Moriyama *et al.* (1988) reported the generation of inhibitory anti-porcine prolyl endopeptidase antibodies in rabbits. This suggests that infected mammalian hosts may generate their own antibodies, perhaps even inhibitory antibodies, against trypanosome oligopeptidases, which would be extremely desirable in terms of host defence. In spite of this, both chronically infected bovine serum and acutely infected murine serum exhibited OP-Tb-like activity, suggesting that if such antibodies were produced, they were ineffective at abolishing this activity in the bloodstream of infected animals.

The generation of antibodies to parasite invariant antigens is known to provide a measure of protective immunity. The immunoprotective potential of antibody generation to trypanosome cysteine proteases has been reported (reviewed by Authié, 1994). Such immunprotective phenomena have also been described for antibodies generated against the surface-bound metalloprotease of *Leishmania* (Russell and Alexander, 1988; Jardin *et al.*, 1990) and a serine protease from *Schistosoma* (Darani *et al.*, 1997).

Preliminary investigations in the present study, employing sera from experimentally infected trypanotolerant N'Dama and trypanosusceptible Boran cattle suggest that antibodies are not generated to OP-Tc during chronic infection, and there is therefore no correlation between trypanotolerance and anti-OP-Tc antibody production. Taken together, these data suggest that neither OP-Tb nor OP-Tb-derived synthetic peptides are useful antigens for vaccine development. However, active immunisation with purified OP-Tb or OP-Tb, followed by a challenge infection will be required to conclusively establish this.

8.4 Are trypanosome oligopeptidases potential chemotherapeutic targets?

Current therapeutic strategies employed for the treatment of African trypanosomiasis are plagued by high costs, ever-increasing drug resistance by the parasites, and unpleasant (and often fatal) side-effects. Furthermore, the biochemical basis of the trypanocidal activity of

these drugs is largely not understood. The present study thus addressed two questions. (1) To determine whether trypanosome oligopeptidases may be targets for the currently employed drugs, which would improve our understanding of the biochemical mechanisms of how these drugs work; and (2) to determine whether serine protease inhibitors may serve as useful lead-compounds for the development of novel therapeutic strategies.

The trypanocidal drugs suramin, pentamidine isethionate and Berenil® were shown in the present study to inhibit OP-Tb activity *in vitro*. Suramin, a sulfated naphthylamine was a non-competitive inhibitor of OP-Tb activity. Additionally, structure-function studies with suramin analogues, in particular the correlation between inhibitory potency and trypanocidal efficacy, strongly suggest that OP-Tb is a target for this drug. Pentamidine, the active principle of Pentacarinate® acted as a competitive inhibitor of OP-Tb, and again, the kinetics of the reaction suggested that the inhibition of OP-Tb by pentamidine was of *in vivo* significance. Both of these findings introduce a hitherto undescribed potential mechanism of action of these drugs.

Other trypanocidal drugs, including DFMO, melarsoprol and melarsen oxide did not inhibit OP-Tb activity *in vitro*. However, it remains possible that they do indirectly down-regulate OP-Tb activity within the trypanosome. DFMO is an irreversible inhibitor of trypanosome ornithine decarboxylase, and therapeutic levels of DFMO typically decrease trypanosome intracellular spermidine levels by 76% after 48 h (Fairlamb *et al.*, 1987). The present study revealed that OP-Tb activity is enhanced in the presence of spermidine suggesting that OP-Tb activity is possibly regulated by polyamines *in vivo*. The depletion of polyamine levels by DFMO may therefore down-regulate OP-Tb activity within the trypanosome, possibly contributing to the trypanocidal action of DFMO.

OP-Tb is also sensitive to the presence of reducing agents, and the thiol-disulfide redox balance within trypanosomes may also regulate OP-Tb activity within the parasites. Melarsoprol and melarsen oxide effectively reduce trypanothione concentrations within the parasite during therapy with these drugs. Trypanothione is thought to maintain the correct intracellular redox state (Fairlamb *et al.*, 1987). This suggests that the depletion of intracellular trypanothione concentrations that result from melarsoprol and melarsen oxide

therapy may down-regulate OP-Tb activity. Furthermore, trypanothione is a glutathione-spermidine conjugate. DFMO, which reduces intracellular spermidine concentrations also decreases intracellular trypanothione levels by up to 50% during treatment regimens (Fairlamb *et al.*, 1987). Thus, DFMO may promote the down-regulation of OP-Tb activity not only directly through the reduction of intracellular polyamine levels, but also through the depletion of intracellular trypanothione.

To date, the possibility of exploiting parasite proteases in anti-parasite chemotherapy has been restricted to the cysteine proteases of parasite protozoa (reviewed by Coombs and Mottram, 1997). The trypanocidal activity of the OP-Tb inhibitors, including peptidyl methylketones and phosphonate diphenyl esters, demonstrated for the first time in the present study, suggest that these compounds have exciting potential as lead-structures for the development of novel drugs. For example, administration of peptidyl phosphonate diphenyl esters resulted in an 83% survival of experimentally infected mice. In contrast, no untreated animals survived. Regardless of the mode of action, these results point to a new and exciting potential series of trypanocidal agents.

There is currently considerable interest in the development of novel serine protease inhibitors, and it would be prudent to examine a variety of classes of these inhibitors for trypanocidal activity. Alternative inhibitors include novel thiazolidine derivatives (Sudo *et al.*, 1997), including those incorporating a novel amino-acid-derived heterocyclic scaffold (Groutas *et al.*, 1997). Additionally, the synthesis and anti-metastatic activity of boro-amino acid analogues has been described (Kinder *et al.*, 1992), as well as anticoagulant candidates such as novel P_1-argininal derivatives featuring a conformationally constrained P_2-P_3 bicyclic lactam moiety (Tamura *et al.*, 1997); and P_2-P_4 azapeptidomimetic P_1-argininal and P_1-ketoargininamide derivatives (Semple *et al.*, 1997). This list is by no means comprehensive. Also, older inhibitors, such as fluoromethylketones have recently been modified to enhance their oral bioavailability (Veale *et al.*, 1997).

The most immediate problem likely to be encountered in targeting trypsin-like serine proteases of blood parasites is that the major proteolytic activity in the bloodstream is also attributable to trypsin-like serine proteases such as plasmin, thrombin, kallikrein and complement proteases.

There are, however, two possible ways around this. Active site mapping of these proteases has already revealed some differences in the sub-site specificities of these proteases. The incorporation of a peptide moiety into inhibitors may enhance the specificity of the inhibitor for the trypanosome enzymes, without interfering with normal plasma processes such as coagulation and complement activation. A subtle but very significant difference between the trypanosome oligopeptidases and plasma trypsin-like proteases is the sensitivity of the trypanosome enzymes to thiol-reactive agents, and it would be very useful to exploit this difference in the design of novel inhibitors which react with the trypanosome enzyme.

The identification of a peptidolytic activity in *P. berghei* (Schrével *et al.*, 1984) that closely resembles OP-Tb suggests that novel inhibitors directed at OP-Tb may also inactivate a related enzyme in the malaria parasite, opening up an opportunity to extend this work to the development of novel antimalarial strategies. Similarly, an enzyme similar to the oligopeptidases of African trypanosomes has been described from the South American trypanosome, *T. cruzi* (Burleigh and Andrews, 1996; Burleigh *et al.*, 1997). The reported resistance of South American trypanosomes to drugs currently employed to treat Chagas' disease (Filardi and Brener, 1987) underscores the necessity of developing novel drugs for the treatment of this disease as well. Again, the trypanocidal activity of oligopeptidase inhibitors may introduce exciting possibilities here.

8.5 Cloning and hyperexpression of OP-Tb

The cloning of trypanosome oligopeptidases is the next logical step in the characterisation of this enzyme, the determination of its role in disease pathogenesis and to aid drug design research. Current studies are hindered by the extremely small yields of enzyme (approximately 70 μg from 10^{10} parasites), and the difficulty in producing adequate quantities of source material. Cloning of potentially similar enzymes from bacterial sources has already been achieved, suggesting that the necessary oligonucleotide primers and cell lines are available. Furthermore, a number of *T. b. brucei* gene libraries already exist, and enzyme assays for screening colonies have already been developed. The cloning of the oligopeptidase gene would conclusively illustrate to which class of serine proteases this enzyme belongs. Furthermore, obtaining a gene sequence would lay necessary groundwork for the generation of a null-mutant, in which the oligopeptidase gene was inactivated. Such a mutant would be invaluable in elucidating the pathological and pathophysiological roles of these enzymes.

Placing the gene under the control of a high-expression vector would generate sufficient material for further study, including crystallisation of the enzyme. Despite considerable effort, the crystallisation of *E. coli* oligopeptidase B has met with no success (Yoshimoto *et al.*, 1991). To date, no three-dimensional structure of any oligopeptidase is available. Knowledge of the three-dimensional structure obtained from X-ray crystallography may answer a number of questions regarding the sensitivity of OP-Tb to reducing agents, and the physical mechanism by which the enzyme restricts the size of substrates. Furthermore, computer-modelling of inhibitor structures to the oligopeptidase active site, in conjunction with active-site mapping of the P′ region of the oligopeptidases, may facilitate the design of extremely specific inhibitors of this enzyme for therapeutic use.

8.6 Conclusion

The objectives of the present study were to characterise a recently identified oligopeptidase from African trypanosomes and to ascertain whether these oligopeptidases may contribute to the pathogenesis of African trypanosomiasis. In the present study, oligopeptidases were isolated from representative members of two distinct groups of African trypanosomes, namely *T. b. brucei* and *T. congolense*. This represents the first time that these oligopeptidases have been purified to electrophoretic homogeneity, permitting the first enzymatic characterisation of these enzymes. While there were no differences in the specificity trends of these two enzymes, the K_m values for the hydrolysis of peptides by OP-Tb were consistently lower than those observed for OP-Tc.

The present study also contributed to the understanding of the potential mechanisms of pathogenesis by demonstrating that OP-Tb was active in the bloodstream of infected hosts, where it was not regulated by any endogenous inhibitory molecules. The demonstration that OP-Tb degrades host peptide hormones *in vitro* suggests that it may also do so in the host bloodstream. This opens up a multitude of potential mechanisms by which circulating OP-Tb, released by trypanosomes into the bloodstream, may contribute significantly to the disruption of the metabolic homeostasis of the host animals. Preliminary data also suggest that these intriguing enzymes may be exciting new targets for the development of novel, more effective chemotherapeutic strategies.

References

Abaru, D.E. and Matova, F.S. (1984) Berenil in the treatment of early-stage human trypanosomiasis cases. *Bull. Trim. Inf. Gloss. Trypanosom.* **7:** 150-151.

Abulyaman, A.S., Jackson, D.S., Hudig, D., Woodard, S.L. and Powers, J.C. (1997) Synthesis and kinetic studies of diphenyl 1-(*N*-peptidylamino)alkanephosphonate esters and their biotinylated derivatives as inhibitors of serine proteases and probes for lymphocyte granzymes. *Arch. Biochem. Biophys.* **344:** 271-280.

Abrahamson, M., Salvesen, G., Barrett, A.J. and Grubb, A. (1986) Isolation of six cysteine protease inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids. *J. Biol. Chem.* **261:** 11282-11289.

Ackers, G.K. (1970) Analytical gel chromatography of proteins. *Adv. Prot. Chem.* **24:** 343-446.

Alexander, J.W. and Good, R.A. (1977) *Fundamentals of clinical immunology*. W.B. Anders, Philadelphia, pp. 151-157.

Allen, R.C. and Budowle, B. (1994) *Gel electrophoresis of proteins and nucleic acids.* Walter de Gruyter, Berlin. 352 pp.

Altman, P.L. and Dittmer, D.S. (1961) *Blood and other body fluids*. Federation of American Societies for Experimental Biology, Bethesda, pp. 20-22.

Andrews, P. (1965) The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.* **96:** 595-606.

Angliker, H., Zumbrunn, A. and Shaw, E. (1991) Synthesis of histidine-containing dipeptide affinity-labelling reagents. *Int. J. Peptide Protein Res.* **38:** 346-349.

Apted, F.I.C. (1980) Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the eastern hemisphere. *Pharmac. Therap.* **11:** 391-413.

Aronin, N., Coslovsky, R. and Leeman, S.T. (1986) Substance P and neurotensin: their roles in the regulation of anterior pituitary function. *Annu. Rev. Physiol.* **48:** 537-549.

Arroyo, R. and Alderete, J.F. (1989) *Trichomonas vaginalis* surface protease activity is necessary for parasite adherence to epithelial cells. *Infect. Immun.* **57:** 2291-2297.

Ashall, F. (1990) Characterisation of an alkaline peptidase of *Trypanosoma cruzi* and other trypanostomatids. *Mol. Biochem. Parasitol.* **38:** 77-88.

Ashall, F., Harris, D., Roberts, H., Healy, N. and Shaw, E. (1990a) Substrate specificity and inhibitor sensitivity of a trypanosomatid alkaline peptidase. *Biochim. Biophys. Acta* **1035:** 293-299.

Ashall, F., Angliker, H. and Shaw, E. (1990b) Lysis of trypanosomes by peptidyl fluoromethyl ketones. *Biochem. Biophys. Res. Comm.* **170:** 923-929.

Askonas, B.A. (1951) The use of organic solvents at low temperature for the separation of enzymes: application to aqueous rabbit-muscle extract. *Biochem. J.* **48:** 42-48.

Authié, E. (1994) Trypanosomiasis and trypanotolerance in cattle: a role for congopain? *Parasitol. Today* **10:** 360-364.

Authié, E., Muteti, D.K., Mbawa, Z., Lonsdale-Eccles, J., Webster, P. and Wells, C. (1992) Identification of a major antigen of *Trypanosoma congolense* as a cysteine proteinase. *Mol. Biochem. Parasitol.* **56:** 103-116.

Authié, E., Duvallet, G., Robertson, C. and Williams, D.J.L. (1993a) Antibody response to a 33 kDa cysteine proteinase of *Trypanosoma congolense*: relationship to 'trypanotolerance' in cattle. *Parasite Immunol.* **15:** 465-474.

Authié, E., Muteti, D.K. and Williams, D.J.L. (1993b) Antibody response to invariant antigens of *Trypanosoma congolense* in cattle of differing susceptibility to trypanosomiasis. *Parasite Immunol.* **15:** 101-111.

Authié, E., Boulangé, A., Muteti, D., Taylor, K. and Williams, D.J.L. (1994) Antibody responses to invariant antigens of *Trypanosoma comgolense*, **in:** *Towards increased use of trypanotolerance: current research and future directions. Proceedings of a workshop jointly organised by the International Laboratory for Research on Animal Diseases and the International Livestock Centre for Africa, held at ILRAD, Nairobi, Kenya, 26-29 April 1993 (G.J. Rowlands and A.J. Teale, Eds.).* ILRAD, Nairobi, pp. 55-60.

Bacchi, C.J., Nathan, H.C., Hunter, S.H., McCann, P.P. and Sjoesdma, A. (1980) Polyamine metabolism: a potential therapeutic target in trypanosomes. *Science* **210:** 332-334.

Bagarozzi, D.A., Pike, R.N., Potempa, J. and Travis, J. (1996) Purification and characterisation of a novel endopeptidase in ragweed (*Ambrosia artemisiifolia*) pollen. *J. Biol. Chem.* **271:** 26227-26232.

Baici, A. (1987) Graphical and statistical analysis of hyperbolic tight-binding inhibition. *Biochem. J.* **244:** 793-796.

Balber, A.E., Bangs, J.D., Jones, S.M. and Proia, R.L. (1979) Inactivation or elimination of potentially trypanolytic, complement-activating immune complexes by pathogenic trypanosomes. *Infect. Immun.* **24:** 617-627.

Baltz, T., Baltz, D., Giroud, Ch. and Crockett, J. (1985) Cultivation in semi-defined medium of animal infective forms of *Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense* and *T. gambiense*. *EMBO J.* **4:** 1273-1277.

Barrett, A.J. (1981) α_2-Macroglobulin; **in:** *Methods in enzymology. Volume 80. Proteolytic enzymes. Part C (Lorand, L., Ed.).* Academic Press, New York, pp. 647-663.

Barrett, A.J. (1994) Classification of peptidases, **in:** *Methods in enzymology. Volume 244. Proteolytic enzymes: serine and cysteine proteases (A.J. Barrett, Ed.).* Academic Press, San Diego, pp. 1-15.

Barrett, A.J. and Starkey, P.M. (1973) The interaction of α_2-macroglobulin with proteases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem. J.* **133:** 709-724.

Barrett, A.J. and Kirschke, H. (1981) Cathepsin B, Cathepsin H, Cathepsin L, **in:** *Methods in enzymology. Volume 80. Proteolytic enzymes. Part C. (L. Lorand, Ed.).* Academic Press, San Diego, pp. 535-560.

Barrett, A.J., Kambhavi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M. and Hanada, K. (1982) L-*trans*-epoxysuccinyl-leucylamido(4-guanido)butane (E-64) and its analogues as inhibitors of cysteine proteases, including cathepsins B, H and L. *Biochem. J.*, **201:** 189-198.

Barrett, A.J., Davies, M.E. and Grubb, A. (1984) The place of human γ-trace (cystatin C) amongst cysteine protease inhibitors. *Biochem. Biophys. Res. Comm.* **120:** 631-636.

Barrett, A.J. and Brown, M.A. (1990) Chicken liver Pz-peptidase, a thiol-dependent metallo-endopeptidase. *Biochem. J.* **271:** 701-706.

Barrett, A.J. and Rawlings, N.D. (1992) Oligopeptidases, and the emergence of the prolyl oligopeptidase family. *Biol. Chem. Hoppe-Seyler* **373:** 353-360.

Barrett, A.J. and Rawlings, N.D. (1995) Families and clans of serine peptidases. *Arch. Biochem. Biophys.* **318:** 247-250.

Barrett-Connor, E., Ugoretz, R.J. and Braude, A.I. (1973) Disseminated intravascular coagulation syndrome in trypanosomiasis. *Arch. Int. Med.* **131:** 574-577.

Barron, E.S. (1951) Thiol groups of biological importance. *Adv. Enzymol.* **11:** 201-253.

Basson, W., Page, M.L. and Myburg, D.P. (1977) Human trypanosomiasis in southern Africa. *S. Afr. Med. J.* **51:** 453-457.

Beith, J.G. (1980) Pathophysiological interpretation of kinetic constants of protease inhibitors. *Bull. Eur. Physiopath. Resp.* **16(Suppl.):** 183-195.

Beith, J.G. (1995) Theoretical and practical aspects of proteinase inhibition kinetics; **in:** *Methods in Enzymology. Vol. 248. Proteolytic enzymes: aspartic and metallo peptidases (A.J. Barrett, Ed.).* Academic Press, San Diego, pp. 59-84.

Belew, M., Peterson, E.A. and Porath, J. (1985) A high-capacity hydrophobic adsorbent for human serum albumin. *Anal. Biochem.* **151:** 438-441.

Benesch, R. and Benesch, R.E. (1962) Determination of -SH groups in proteins. *Meth. Biochem. Anal.* **10:** 43-70.

Bhatnagar, Y.M. and Carraway, R. (1981) Polypeptides related to mammalian bioactive peptides in unicellular protozoa. *Peptides* **2:** 51-29.

Bhoola, K.D., Figuera, C.D. and Worthy, K. (1992) Bioregulation of kinins: kallikreins, kininogens and kininases. *Pharm. Rev.* **44:** 1-80.

Birk, Y. (1976a) Lima bean trypsin inhibitor, **in:** *Methods in enzymology. Volume 45. Proteolytic Enzymes. Part B. (L. Lorand, Ed.).* Academic Press, San Diego, pp. 707-709.

Birk, Y. (1976b) Trypsin and chymotrypsin inhibitors from soybeans, **in:** *Methods in enzymology. Volume 45. Proteolytic Enzymes. Part B. (L. Lorand, Ed.).* Academic Press, San Diego, pp. 700-707.

Bitonti, A.J., Dumont, J.A. and McCann, P.P. (1986) Characterisation of *Trypanosoma brucei brucei* S-adenosyl-L-methionine decarboxylase and its inhibition by Berenil, pentamidine and methylglyoxal bis(guanylhydrazone). *Biochem. J.* **237:** 518-521.

Blum, H., Beier, H. and Gross, H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8:** 93-99.

Boersma, A., Hublart, M., Boutignon, F., Noireau, F., Lemesre, J.L., O'Herbomez, M. and Degand, P. (1989) Alterations in thyroid function in patients with *Trypanosoma brucei gambiense* infection. *Trans. Roy. Soc. Trop. Med. Hyg.* **83:** 208-209.

Bontempi, E., Franke de Cazzulo, B.M., Ruiz, A.M., Cazzulo, J.J. (1984) Purification and some properties of an acidic protease from epimastigotes of *Trypanosoma cruzi*. *Comp. Biochem. Physiol.* **77B:** 599-604.

Boreham, P.F.L. (1979) The pathogenesis of African and American trypanosomiasis, **in:** *Biochemistry and physiology of protozoa. Volume 2 (2nd edition) (M. Levandowsky and S.H. Hutner, Eds.).* Academic Press, New York, pp. 429-455.

Boreham, P.F.L. and Wright, I.G. (1976) Hypotension in rabbits infected with *Trypanosoma brucei*. *Br. J. Pharmacol.* **58:** 137-139.

Boulange, A. and Authié, E. (1994) A 69-kDa immunodominant antigen of *Trypanosoma (Nannomas) congolense* is homologous to immunoglobulin heavy chain binding protein (BiP). *Parasitology,* **109:** 163-173.

Bouvier, J., Schneider, P., Etges, R. and Bordier, C. (1990) Peptide substrate specificity of the membrane-bound metalloprotease of *Leishmania*. *Biochemistry* **29:** 10113-10119.

Bowman, I.B.R. and Flynn, I.W. (1976) Oxidative metabolism of trypanosomes, **in:** *Biology of the Kinetoplastida (W.H.R. Lumsden and D.A. Evans, Eds.).* Academic Press, London, pp. 376-435.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Branquinha, M.H., Vermello, A.B., Goldenberg, S. and Bonaldo, M.C. (1996) Ubiquity of cysteine- and metalloproteinase activities in a wide range of trypanostomatids. *J. Euk. Microbiol.* **43:** 131-135.

Briggs, G.E. and Haldane, J.B.S. (1925) A note on the kinetics of enzyme action. *Biochem. J.* **19:** 338-339.

Broden, A. (1904) Les infectious á trypanosomes au Congo chez l'homme et les animaux. *Bull. Soc. Etudes Colon.* February 1904, pp. 2-3.

Bronner, U., Doua, F., Ericsson, O., Gustafsson, L.L., Miézan, T.W., Rais, M. and Rombo, L. (1991) Pentamidine concentrations in plasma, whole blood and cerebrospinal fluid during treatment of *Trypanosoma gambiense* infection in Côte d'Ivoire. *Trans. Roy. Soc. Trop. Med. Hyg.* **85:** 608-611.

Brown, J.L. and Roberts, W.K. (1976) Evidence that approximately eighty percent of the soluble proteins from Ehrlich ascites cells are amino-terminally acetylated. *J. Biol. Chem.* **251:** 1009-1013.

Brown, R.E., Jarvis, K.L., Hyland, K.J. (1989) Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* **180:** 136-139.

Bruce, D. (1895) *Preliminary report on the tsetse fly disease or nagana in Zululand.* Ubombo, Zululand. December 1895. 26 pp.

Brun, R. and Schonenberger, M. (1979) Cultivation and *in vitro* cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Trop.* **36:** 289-294.

Budavari, S., O'Niel, M.J., Smith, A. and Heckelman, P.E. (1987) *The Merck index: an encyclopaedia of chemicals, drugs and biologicals (11th edition).* Merck, Rahway p. 1204.

Burleigh, B.A. and Andrews, N.W. (1995) A 120 kDa alkaline peptidase from *Trypanosoma cruzi* is involved in the generation of a novel Ca^{2+}-signalling factor for mammalian cell. *J. Biol. Chem.* **270:** 5172-5180.

Burleigh, B.A., Caler, E.V., Webster, P. and Andrews, N.W. (1997) A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca^{2+} signalling in mammalian cells. *J. Cell Biol.* **136:** 609-620.

Butler, J.E. (1980) Antibody-antigen and antibody-hapten reactions, **in:** *Enzyme immunoassay (E.T. Maggio, Ed.).* CRC Press, Boca Raton, pp. 5-52.

Cadène, M., Duranton, J., North, A., Si-Tahar, M., Chignard, M. and Bieth, J.G. (1997) Inhibition of neutrophil serine proteinases by suramin. *J. Biol. Chem.* **272:** 9950-9955.

Carraway, S. and Reinecke, H.T. (1989) Regulatory peptides of mammals, **in:** *Comparative physiology of the regulatory peptides (S.V. Holmgren, Ed.).* John Wiley, Chichester, pp. 3-17.

Carrell, R.W. and Stein, P. (1995) The biostructural pathology of the serpins: critical function of sheet opening mechanisms. *Biol. Chem. Hoppe-Seyler* **377:** 1-17.

Carrington, M. (1993) Culturing and biological cloning of *Trypanosoma brucei*, **in:** *Methods in molecular biology. Volume 21. Protocols in molecular parasitology (J.E. Hyde, Ed.).* Humana Press, Totowa, pp. 1-13.

Catty, D. and Raykundalia, C. (1988) Production and quality control of polyclonal antibodies, **in:** *Antibodies: a practical approach. Vol. I. (D. Catty, Ed.).* IRL Press, Eynsham, pp. 10-53.

Caughey, G.H., Raymond, W.W., Bacci, E., Lombardy, R.J. and Tidwell, R.R. (1993) Bis(5-amidino-2-benzamizazolyl)methane and related amidines are potent, reversible inhibitors of mast cell tryptases. *J. Pharm. Exp. Therap.* **264(2):** 676-682.

Cazullo, J.-J., Couso, R., Raimondi, A., Wernstedt, C. and Hellman, U. (1989) Further characterisation and partial amino acid sequence of a cysteine protease from *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* **33:** 33-41.

Cazzulo, J.-J., Martínez, J., Franke de Cazzulo, B.M. (1990) Some kinetic properties of a cysteine protease (cruzipain) from *Trypanosoma cruzi*. *Biochim. Biophys. Acta* **1037:** 186-191.

Chandler A.C. and Read, C.P. (1961) Introduction to parasitology with special reference to the parasites of man. Wiley, London, pp. 131-160.

Chasan, R. and Anderson, K.V. (1989) The role of Easter, an apparent serine protease, in organising the dorsal-ventral pattern of *Drosophila* embryo. *Cell* **56:** 391-400.

Chase, T. and Shaw, E. (1970) Titration of trypsin, plasmin and thromobin with para-nitrophyl-p-guanidobenzoate, **in:** *Methods in enzymology. Proteolytic enzymes. Part A. (G.E. Perlman and L. Lorand, Eds.).* Academic Press, San Diego, pp. 20-30.

Clarke, B. and Engvall, E. (1981) Enzyme-linked immunosorbent assay (ELISA): theoretical and practical aspects; **in:** *Enzyme-immunoassay (E.T. Maggio, Ed.)*. CRC Press, Boca Raton, pp. 167-196.

Clarkson, M.J. and Penhale, W.J. (1973) Serum protein changes in trypanosomiasis in cattle. *Trans. Roy. Soc. Trop. Med. Hyg.* **67:** 273-282.

Cleary, P.P., Prahbu, U., Dale, J.B., Wexler, D.E. and Handly, J. (1992) Streptococcal C_{5a} peptidase is a highly specific endopeptidase. *Infect. Immun.* **60:** 5219-5223.

Clonis, Y.D. (1987) Dye-ligand chromatography; **in:** *Reactive dyes in protein and enzyme technology (Y.D. Clonis, A. Atkinson, C.J. Bruton and C.R. Lowe, Eds.)*. Stockton Press, New York, pp. 33-49.

Coetzer, T.H.T. (1985) *Preparation and characterisation of antibodies against mouse Ig (all classes)*. Internal Report, Bioclones (Pty) Ltd, Stellenbosch, South Africa. 22 pp.

Coetzer, T.H.T. (1992) *Type IV collagenase and cathepsins L and H: proteases involved in tumour invasion*. PhD thesis, University of Natal, Pietermaritzburg, South Africa. 219 pp.

Coetzer, T.H.T., Elliott, E., Fortgens, P.H., Pike, R.N. and Dennison, C. (1991) Anti-peptide antibodies to cathepsins B, L and D and type IV collagenase: specific recognition and inhibition of the enzymes. *J. Immunol. Meth.* **136:** 199-210.

Coleman, R.W. and Bagdasarian, A. (1976) Human kallikrein and prekallikrein; **in:** *Methods in enzymology. Volume 45. Proteolytic enzymes. Part B. (L. Lorand, Ed.)*. Academic Press, New York, pp. 303-322.

Collins, J.M., Klecker, R.W., Yarchoan, R., Lane, H.C., Fauci, A.S., Redfield, R.R., Broder, S. and Myers, C.E. (1986) Clinical pharmokinetics of suramin in patients with HTLV-III/LAV infection. *J. Clin. Pharmacol.* **26:** 22-26.

Compton, S.J. and Jones, C.G. (1985) Mechanism of dye response and interference in the Bradford protein assay. *Anal. Biochem.* **151:** 269-274.

Coombes, G.H. and Mottram, J.C. (1997) Parasite proteinases and amino acid metabolism: possibilities for chemotherapeutic exploitation. *Parasitology*, **114:** Suppl. S61-S80.

Cooper, T.G. (1977) *The tools of biochemistry*. John Wiley and Sons, New York, pp. 137-168.

Craik, C.S., Roczniak, S., Largman, C. and Rutter, W.J. (1987) The catalytic role of the active site aspartic acid in serine proteases. *Science* **228:** 291-297.

Crawford, C., Mason, R.W., Wikstrom, P. and Shaw, E. (1988) The design of peptidyldiazomethane inhibitors to distinguish between the cysteine proteinases calpain II, cathepsin L and cathepsin B. *Biochem. J.* **253:** 751-758.

Cunningham, M.L., Zvelebil, M.J. and Fairlamb, A.H. (1994) Mechanisms of inhibition of trypanothione reductase by trivalent organic arsenicals. *Eur. J. Biochem.* **221:** 285-295.

Damper, D. and Patton, C.L. (1976) Pentamidine transport in *Trypanosoma brucei* - kinetics and specificity. *Biochem. Pharmacol.* **25:** 271-276.

Daniel-Ribiero, C., Tirard, S., Monjour, L., Homberg, J.-C. and Gentiline, M. (1983) Relevance of autoantigens to autoimmunity in African trypanosomiasis: study of DNA and thyroglobulin antibodies. *Acta Trop.* **40:** 321-329.

Darani, H.Y., Curtis, R.H.C., McNeice, C., Price, H.B., Sayers, J.R. and Doesnhoff, M.J. (1997) *Schistosoma mansoni*: anomalous immunogenic properties of a 27 kDa larval serine protease associated with protective immunity. *Parasitology* **115:** 237-247.

Davie, E.W., Fujikawa, K. and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance and regulation. *Biochemistry* **30:** 10363-10370.

Davis, C.E. (1980) Thrombocytopenia: a uniform complication of African trypanosomiasis. *Acta Trop.* **39:** 123-133.

Davis, V.J. (1964) Disc electrophoresis - II. Methods and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121:** 404-427.

Dawson, R.M.C., Elliot, D.C., Elltoi, W.H. and Jones, K.M. (1968) *Data for biochemical research.* Clarendon Press, Oxford, p. 628.

De Clercq, E. (1979) Suramin: a potent inhibitor of the reverse transcriptase of RNA tumour viruses. *Cancer Lett.* **8:** 9-22.

De Clercq, E. (1987) Suramin in the teatment of AIDS: mechanism of action. *Antiviral Res.* **7:** 1-10.

De Raadt, P. (1967) Reactive encephalopathy occurring as a complication during treatment of *T. rhodesiense* with non-arsenical drugs. *East Afr. Tryp. Res. Org. Ann. Rep. 1966*, pp. 85-87.

deBold, A.J. (1985) Atrial natriuretic factor: a hormone produced by the heart. *Science*, **230:** 767-770.

Deftos, L., DeRoith, D., Shiloach, J. and Roth., J. (1985) Neurotensin analogues of *Tetrahymena*. *Horm. Metab. Res.* **17:** 82-85.

Dehrmann, F.M., Coetzer, T.H.T., Pike, R.N. and Dennison, C. (1995) Mature cathepsin L is substantially active in the ionic milieu of the extracellular medium. *Arch. Biochem. Biophys.* **324:** 93-98.

Dehrmann, F.M., Elliott, E. and Dennison, C. (1996) Reductive activation markedly increases the stability of cathepsins B and L to extracellular ionic conditions. *Biol. Chem. Hoppe-Seyler* **377:** 391-394.

Deisenhofer, J. and Steigmann, W. (1974) The model of the basic pancreatic trypsin inhibitor refined at 1.5 Å resolution, **in:** *Proteinase inhibitors: Bayer symposium V (H. Fritz, H. Tschesche, L.J. Greene and T. Truscheit, Eds.)*. Springer Verlag, Berlin, pp. 484-496.

Del Nery, E., Juliano, M.A., Meldal, M., Svendsen, I., Scharfstein, J., Walmsley, A. and Juliano, L. (1997) Characterisation of the substrate specificity of the major cysteine protease (cruzipain) from *Trypanosoma cruzi* using a portion-mixing combinatorial library and fluorogenic peptides. *Biochem. J.* **323:** 427-433.

Dennison, C., Pike, R.N., Coetzer, T.H.T. and Kirk, K.C. (1992) Characterisation of the activity and stability of single-chain cathepsin L and of proteolytically active cathepsin L/cystatin complexes. *Biol. Chem. Hoppe-Seyler* **373:** 419-425.

Du Toit, R. (1946) The tsetse-fly problem and its control in South Africa. *Presidential address delivered to the South African Biological Society on 19th September 1946.*

Dunn, B.M. (1989) Determination of protease mechanism, **in:** *Proteolytic enzymes: a practical approach (R.J. Benyon and J.S. Bond, Eds.).* Oxford University Press, Oxford, pp. 57-81.

Duszenko, M., Ferguson, M.A.J., Lamont, G.S., Rifkin, M.B. & Cross, G.A.M. (1985) Cysteine eliminates the feeder cell requirement for cultivation of *Trypanosoma brucei* bloodstream forms *in vitro*. *J. Exp. Med.* **162:** 1256-1263.

Duszenko, M., Mühlstädt, K. & Broder, A. (1992) Cysteine is an essential growth factor for *Trypanosoma brucei* bloodstream forms. *Mol. Biochem. Parasit.* **50:** 269-274.

Dutton, J.E. (1902) Preliminary note upon a trypanosome ocurring in the blood of man. *Thompson Yates Lab. Repts.* **4:** 455.

Eadie, G.S. (1942) The inhibition of cholinesterase by physostigmine and prostigmine. *J. Biol. Chem.* **146:** 85-93.

Eisen, V. and Loveday, C. (1973) Effects of suramin on complement, blood clotting, fibrinolysis and kinin formation. *Br. J. Pharmacol.* **49:** 678-687.

Eisenthal, R. and Cornish-Bowden, A. (1974) The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. *Biochem. J.* **139:** 715-720.

El-Sayed, N.M.A. and Donelson, J.E. (1997) African trypanosomes have differentially expressed genes encoding homologues of the *Leishmania* GP63 surface protease. *J. Biol. Chem.* **272:** 26742-26748.

El-Sayed, N.M.A., Alarcon, C.M., Beck, J.C., Sheffield, V.C. and Donelson, J.E. (1995) cDNA expressed sequence tags of *Trypanosoma brucei rhodiense* provide new insights into the biology of the parasite. *Mol. Biochem. Parasitol.* **73:** 75-90.

Ellis, K.J. and Morrison, J.F. (1982) Buffers of constant ionic strength for studying pH-dependent processes, **in:** *Methods in enzymology. Volume 87. Enzyme kinetics and mechanism. Part C. (D.L. Purich, Ed.).* Academic Press, San Diego, pp. 405-426.

Engvall, E. and Perlmann, P. (1971) Enzyme-linked immunosorbent assay (ELISA): Quantitative assay of immunoglobulin G. *Immunochemistry* **8**, 871-874.

Etges, R. and Bouvier, J. (1993) The metalloproteinase of *Leishmania*: leishmanolysin. *Ciência e Cultura* **45:** 330-338.

Facer, C.A., Crossley, J.M., Clarkson, M.J. and Jenkins, G.C. (1982) Haemolytic anaemia in bovine trypanosomiasis. *J. Comp. Pathol.*, **92:** 393-401.

Fairlamb, A.H. and Bowman, I.B.R. (1977) *Trypanosoma brucei*: suramin and other trypanocidal compounds' effects on glycerol-3-phosphate oxidase. *Exp. Parasitol.* **43:** 353-361.

Fairlamb, A.H., Henderson, G.B., Bacchi, C.J. and Cerami, A. (1987) *In vivo* effects of difluormethylornithine on trypanothione and polyamine levels in bloodstream forms of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **53:** 213-222.

Fairlamb, A.H., Henderson, G.H. and Cerami, A. (1989) Trypanothione is the primary target for arsenical drugs against African trypanosomes. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 2607-2611.

Fairlamb, A.H. and Cerami, A. (1992) Metabolism and functions of trypanothione in the kinetoplastida. *Annu. Rev. Microbiol.* **46:** 695-729.

Filardi, L.S. and Brener, Z. (1987) Suscpetibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas' disease. *Trans. Roy. Soc. Trop. Med. Hyg.* **81:** 755-759.

Fink, A.L. (1987) Acyl group transfer - the serine proteinases; **in:** *Enzyme mechanisms (M.I. Page and A. Williams, Eds.)*. Royal Society of Chemistry, London, pp. 159-177.

Fiorucci, L., Erba, F., Bolognesi, M., Coletta, M. and Ascoli, F. (1997) pH dependence of bovine mast cell tryptase catalytic activity and of its inhibition by 4',6-diamidine-2-phenylindole. *FEBS Lett.* **408:** 85-88.

Flynn I.W. and Bowman, I.B.R. (1969) Further studies on the mode of action of arsenicals on trypanosome pyruvate kinase. *Trans. Roy. Soc. Trop. Med. Hyg.* **63:** 121.

Flynn, J.N. and Sileghem, M. (1993) Immunosuppression in trypanotolerant N'Dama cattle following *Trypanosoma congolense* infection. *Parasite Immunol.* **15:** 547-552.

Fong, J.S.C. and Good, R.A. (1972) Suramin - a potent reversible and competitive inhibitor of complement systems. *Clin. Exp. Immunol.*, **10:** 127-138.

Forde, R.M. (1902) Some clinical notes on a European patient in whose blood a trypanosome was observed. *J. Trop. Med. Hyg.* **5:** 261-262.

Forneau, E., Tréfouel, J. and Vallée, J. (1924) Recherches de chimothérapie dans la série du 205 Bayer: urées des acids aminobenzoylaminonaphthaléniques. *Ann. Inst. Pasteur* **38:** 81-114.

Franke de Cazullo, B.M.F., Martínez, J., North, M.J., Coombs, G.H. and Cazullo, J.-J. (1994) Effects of protease inhibitors on the growth and differentiation of *Trypanosoma cruzi*. *FEMS Microbiol. Lett.* **124:** 81-86.

Friderich, G., Klumpp, S., Russell, C.B., Hindrichsen, R.D., Kellner, R. and Schultz, J.E. (1992) Purification, characterisation and structure of protein phosphatase 1 from the cilia of *Paramecium tetraurelia*. *Eur. J. Biochem.* **209:** 43-49.

Friedenauer, S. and Berlet, H.H. (1989) Sensitivity and variability of the Bradford protein assay in the presence of detergents. *Anal. Biochem.* **178:** 263-268.

Fripp, P.J. (1983) *An introduction to human parasitology with reference to southern Africa.* Macmillan, Johannesburg, pp. 23-29.

Fritz, H. and Wunderer, G. (1983) Biochemistry and applications of aprotonin, the kallikrein inhibitor from bovine organs. *Arzneim. Forsch.* **33:** 479-494.

Fuller, R.W., Brake, F. and Thorner, J. (1986) Yeast prohormone processing enzyme (*KEX2* gene product) is a Ca^{2+}-dependent serine protease. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 1434-1438.

Gallais, P., Cros, R., Pruvost, A. (1953) Etude clinique biologique électroencéphalique, parasitologique de la trypanosomiase d'inoculation. *Méd. Trop.* **13:** 807-843.

Geratz, J.D. (1973) Structure-activity relationships for the inhibition of plasmin and plasminogen activation by aromatic diamidines and a study of the effect of plasma proteins on the inhibition process. *Thrombos. Diathes. Haemorrh.*, **29:** 154-167.

Gherardi, R.K., Chariot, P., Vanderstigel, M., Malapert, D., Verroust, J., Astier, A., Brun-Baisson, C. and Schaeffer, A. (1990) Organic arsenic-induced Guillian-Barré-like syndrome due to melarsoprol: a clinical electrophysiological and pathological study. *Muscle Nerve* **13:** 637-645.

Goa, K.L. and Campoli-Richards, D.M. (1987) Pentamidine isethionate: a review of its antiprotozoal activity, pharmacokinetic properties and therapeutic use in *Pneumocystis carinii* pneumonia. *Drugs* **33:** 242-258.

Gottesman, S. and Maurizi, M.R. (1992) Regulation by proteolysis: energy-dependent proteases and their targets. *Microbiol. Rev.* **56:** 592-621.

Gould, S.S. amd Castro, G.A. (1994) Suppression by *Trypanosoma brucei* of anaphylaxis-mediated ion transport in the small intestine of rats. *Immunology* **81:** 468-474.

Grab, D.J. and Bwayo, J.J. (1982) Isopycnic isolation of African trypanosomes on Percoll gradients formed *in situ*. *Acta Trop.* **39:** 363-366.

Gratecos, D., Detwiler, T.C., Hurd, S. and Fischer, E. (1977) Rabbit muscle phosphorylase phosphatase. 1. Purification and chemical properties. *Biochemistry* **16:** 4812-4817.

Green, G.D.J. and Shaw, E. (1980) Peptidyl diazomethyl ketones are specific inactivators of thiol proteinases. *J. Biol. Chem.* **256:** 1923-1928.

Greenwood, B.M. and Whittle, H.C. (1980) The pathogenesis of sleeping sickness. *Trans. Roy. Soc. Trop. Med. Hyg.* **74:** 716-725.

Grellier, P., Picard, I., Bernard, F., Mayer, R. and Heidrich, H.-G. (1989) Purification and identification of a neutral endopeptidase in *Plasmodium falciparum* schizonts and merozoites. *Parisitol. Res.* **75:** 455-460.

Grogan, J., Rotmans, P., Ghoneim, H., Deelder, A., Yazdanbakhsh, M. and Klinkert, M. (1997) Recognition of *Schistoma mansoni* cathepsin B and L by humman IgG1 and IgG4 antibodies. *Parasite Immunol.* **19:** 215-220.

Groutas, W.C., Kuang, R., Venkataraman, R., Epp, J.B., Ruan, S. and Prakash, O. (1997) Structure-based design of a general class of mechanism-based inhibitors of the serine proteinases employing a novel amino-acid-derived heterocyclic scaffold. *Biochemistry* **36:** 4739-4750.

Hall, H.T.B. (1977) *Disease and parasites of livestock of the tropics*. Longman, London, pp. 163-171.

Hanes, C.S. (1932) Studies on plant amylases. I. The effects of starch concentration upon the velocity of hydrolysis by amines of germinated barley. *Biochem. J.* **26:** 1406-1421.

Harper, W.J., Hemmi, K., Powers, J.C. (1985) Reaction of serine proteases with substituted isocoumarins: discovery of 3,4-dichloroisocoumarin, a new general mechanism-based serine protease inhibitor. *Biochemistry*, **24:** 1831-1841.

Hart, D., Langridge, A., Barlow, D. and Sutton, B. (1989) Antiparasitic drug design. *Parasitol. Today* **5:** 117-122.

Harth, G., Andrews, N., Mills, A.A., Engel, J.C., Smith, R. and McKerrow, J.H. (1993) Peptide-fluoromethyl ketones arrest intracellular replication and intercellular transmission of *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* **58:** 17-24.

Hawking, F. (1978) Suramin: with special reference to onchocerciasis. *Adv. Pharmacol. Chemother.* **15:** 289-322.

Hermanson, G.T., Mallia, A.K. and Smith, P.K. (1992) *Immobilised affinity ligand techniques*. Academic Press, San Diego. 454 pp.

Hesse, F., Selzer, P.M., Mühlstädt, K. & Duszenko, M. (1995) A novel cultivation technique for long-term maintenance of bloodstream-form trypanosomes *in vitro*. *Mol. Biochem. Parasit.* **70:** 157-166.

Heussen, C. and Dowdle, E.B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerised substrates. *Anal. Biochem.* **102:** 196-202.

Hirumi, H., Doyle, J. J. and Hirumi, K. (1977) African trypanosomes: cultivation of animal infective *Trypanosoma brucei in vitro*. *Science* **196:** 992-996.

Hoffmeister, H. (1974) Potential applications of polyacrylamide-gel electrophoresis for clinical diagnosis, **in:** *Electrophoresis and isoelectric focusing in polyacrylamide gels: advances of methods and theories, biochemical and clinical applications (R.C. Allen and H.R. Maurer, Eds.)*. Walter de Gruyter, Berlin, pp. 266-283.

Hofstee, B.H.J. (1952) Specificity of esterases. I. Identification of two pancreatic aliesterases. *J. Biol. Chem.* **199:** 357-365.

Holder, I.A. and Haidaris, C.G. (1979) Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: extracellular protease and elastase as *in vivo* virulence factors. *Can. J. Microbiol.* **25:** 593-599.

Höyhtyä, M., Tupeenniemi-Hujanen, T., Stetlet-Stevenson, W., Krutsch, H., Tryggvason, K. and Liotta, L. (1988) Monoclonal antibodies to type IV collagenase recognise a protein with limited sequence homology to interstitial collagenase and stromelysin. *FEBS Lett.* **233:** 109-113.

Hua, S.-B., To, W.-Y., Nguyen, T.T., Wong, M.-L., Wang, C.C. (1996) Purification and characterisation of proteasomes from *Trypanosoma brucei*. *Mol. Biochem. Parasit.*, **78:** 33-46.

Huber, R., Kukla, D., Rühlmann, A. and Steigmann, W. (1971) The atomic structure of the basic trypsin inhibitor of bovine organs (kallikrein inhibitor), **in:** *Proceedings of an international research conference on proteinase inhibitors, Munich, November 1970 (H. Fritz and H. Tschesche, Eds.)*. de Gruyter, Berlin, pp. 56-64.

Hublart, M., Tetaert, D., Croix, D., Boutignon, F., Degand, P and Boersma, A. (1990) Gonadotrophic dysfunction produced by *Trypanosoma brucei* in the rat. *Acta Trop.* **47:** 177-184.

Huet, G., Richet, C., Demeyer, D., Bisiau, H., Soudan, B., Tetaert, D., Han, K.K. and Degand, P. (1992) Characterisation of different proteolytic activities in *Trypanosoma brucei brucei*. *Biochim. Biophys. Acta* **1138:** 213-221.

Hulmes, J.D., Miedel, M.C. and Pan, Y.-C.E. (1989) Strategies for microcharacterisation of proteins using direct chemistry on sequencer supports, **in:** *Techniques in protein chemistry (T.E. Hugli, Ed.)*. Academic Press, San Diego, pp. 7-16.

Ijiri, Y., Matsumoto, K., Kamata, R., Nishino, N., Okamura, R., Kambara, T. and Yamamoto, T. (1994) Suppression of polymorphonuclear leukocyte chemotaxis by *Pseudomonas aeruginosa* elastase *in vitro*: a study of the mechanism and the correlation with ring abscess in pseudomonal keratitis. *Int. J. Exp. Path.* **75:** 441-451.

International Laboratory for Research on Animal Diseases (1991) *ILRAD 1990: Annual report of the International laboratory for Research on Animals Diseases.* International Laboratory for Research on Animal Diseases, Nairobi. 119 pp.

International Laboratory for Research on Animal Diseases (1994) *ILRAD 1993: Annual report of the International laboratory for Research on Animals Diseases.* International Laboratory for Research on Animal Diseases, Nairobi. 137 pp.

Ishikawa, E., Miyai, K. and Imagawa, M. (1986) Thyroid-stimulating hormone, TSA, human thyrotropin; **in:** *Methods of enzymatic analysis. Volume IX. Proteins and Peptides (H.U. Bergenmeyer, J. Bergenmeyer and Graßl, M., Eds.)*. Verlag Chemie, Weinheim, pp. 405-419.

Jacobs, G.R., Pike, R.N. and Dennison, C. (1989) Isolation of cathepsin D using three-phase partitioning in *t*-butanol/water/ammonium sulfate. *Anal. Biochem.* **180:** 169-171.

James, A.T. and Morris, L.J. (1964) *New biochemistry separations*. D. van Nostrand, London, pp. 94-96, 113.

Jameson, G.W., Roberts, D.V., Adams, R.W., Kyle, W.S. and Elmore, D.T. (1973) Determination of the operational molarity of solutions of bovine alpha-chymotrypsin, trypsin, thrombin and factor Xa by spectrofluorometric titration. *Biochem. J.* **131:** 107-117.

Jany, K.D., Lederer, G. and Mayer, B. (1986) Amino acid sequence of proteinase K from the mould *Tritirachium album* Limber. Proteinase K - a subtilisin related enzyme with disulfide bonds. *FEBS Lett.* **199:** 139-144.

Jardin, A., Alexander, J., The, H.S., Ou, D. and Olafson, R. (1990) Immunoprotective *Leishmania major* synthetic T-cell epitopes. *J. Exp. Parasitol.* **172:** 645-648.

John, R.A. (1992) Photometric assays, **in:** *Enzyme assays: a practical approach (R. Eisenthal and M.J. Danson, Eds.)*. Oxford University Press, Oxford, pp. 59-92.

Joiner, K.A., Brown, E.J. and Frank, M.M. (1984) Complement and bacteria: chemistry and biology in host defence. *Annu. Rev. Immunol.* **2:** 461-491.

Jones, M.M., Singh, P.K., Lanem J.E., Ridrigues, R.R., Nesset, A., Saurez, C.C., Bogitsh, B.J. and Carter, C.E. (1996) Inhibition of *Trypanosoma cruzi* epimastigotes *in vitro* by iron chelating agents. *Arzneim. Forsch.* **46:** 1158-1162.

Jones, R., Parry, R., Lo Leggio, L. and Nickel, P. (1996) Inhibition of sperm-zona binding by suramin, a potential 'lead' compound for design of new anti-fertility agents. *Mol. Hum. Reprod.* **2:** 597-605.

Kalsheker, N.A., Deam, S., Chambers, L., Sreedham, S., Brocklehurst, K. and Lomas, D.A. (1996) The house dust mite allergen *Der p*1 catalytically inactivates α_1-antitrypsin by specific reactive centre loop cleavage: a mechanism that promotes airway inflammation and asthma. *Biochem. Biophys. Res. Comm.* **221:** 59-61.

Kaminishi, H., Hamatake, T., Cho, T., Tamaki, T., Suenaga, N., Fujii, T., Hagihara, Y. and Maeda, H. (1994) Activation of the blood clotting factors by microbial proteases. *FEMS Microbiol. Lett.* **121:** 327-332.

Kanatani, A., Masuda, T., Shimoda, T., Misoka, F., Lin, S., Yoshimoto, T. and Tsuru, D. (1991) Protease II from *Escherichia coli*: sequencing and expression of the enzyme gene and characterisation of the expressed enzyme. *J. Biochem.* **110:** 315-320.

Kanatani, A., Yoshimoto, T., Kitazono, A., Kokubo, T., Tsuru, D. (1993) Prolyl endopeptidase from *Aeromonas hydrophila*: cloning, expression and sequencing of the enzyme gene and characterisation of the expressed enzyme. *J. Biochem.* **113:** 790-796.

Kangawa, K. and Matsuo, H. (1984) Purification and complete amino acid sequence of α-human atrial natriuretic polypeptide (α-hANP). *Biochem. Biophys. Res. Comm.*, **118(1):** 131-139.

Kato, M., Irasawa, T., Ohtani, M., Muramatu, M. (1992) Purification and characterisation of proteinase In, a trypsin-like protease, in *Escherichia coli*. *Eur. J. Biochem.* **210:** 1007-1014.

Kawabata, C. and Ichishima, E. (1997) Unique inhibition of miltpain, a new cysteine proteinase from the milt of chum salmon, by *o*-phenanthroline, phenanthrenequinone, phenazine, and acridine. *Biosci. Biotech. Biochem.* **61:** 1405-1407.

Kay, G., Bailie, J.R., Halliday, I.M., Nelson, J. and Walker, B. (1992) The synthesis, kinetic characterisation and application of biotinylated aminoacylchloromethanes for the detection of chymotrypsin and trypsin-like serine proteinases. *Biochem. J.* **283:** 455-459.

Kemeny, D.M. and Chantler, S. (1988) An introduction to ELISA; **in:** *ELISA and other solid-phase immunoassays: theoretical and practical aspects (D.M. Kemeny and S.J. Challacombe, Eds.)*. John Wiley and Sons, Chichester, pp. 1-29.

Kettner, C. and Shaw, E. (1980) Inactivation of trypsin-like enzymes with peptides of arginine chloromethyl ketone, **in:** *Methods in Enzymology. Volume 80. Proteolytic Enzymes. Part C (Lorand, L., Ed.)*. Academic Press, New York, p. 754-764.

Kézdy, F.J. and Kaiser, E.T. (1981) Active-site titration of cysteine proteinases, **in:** *Methods in enzymology. Vol. 80. Proteolytic enzymes. Part C. (L. Lorand, Ed.)*. Academic Press, New York, pp. 3-11.

Kierszenbaum, F. and Weinman, D. (1977) Antibody-independant activation of the alternative complement pathway in human serum by parasitic cells. *Immunology* **32:** 245-249.

Kilian, M., Reinholdt, J., Lomholt, H., Poulsen, K. and Frandsen, E.V.G. (1996) Biological significance of IgA1 proteases in bacterial colonisation and pathogenesis: critical evaluation of experimental evidence. *Acta Pathol. Microbiol. Scand* **104:** 321-338.

Kinder, D.H., Elstad, C.A., Meadows, G.G. and Ames, M.M. (1992) Antimetastatic activity of boro-amino acid analogue protease inhibitors against B16BL6 melanoma *in vivo*. *Invasion Metastasis* **12:** 309-319.

Kirschke, H. and Shaw, E. (1981) Rapid inactivation of cathepsin L by Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2. *Biochem. Biophys. Res. Comm.* **101:** 454-458.

Kishimoto, T. and Hirano, T. (1988) Molecular regulation of B lymphocyte response. *Annu. Rev. Immunol.* **6:** 485-544.

Kleine, F.K. (1909) Positive Infektionversuche mit *Trypanosoma brucei* durch *Glossina palpalis*. *D. Med. Woch.* **15:** 460-470.

Kleine, F.K. and Fischer, W. (1923) Bericht über die Prüfung von "Bayer 205" in Afrika. *D. Med. Woch.* **32:** 1039-1041

Knight, C.G. (1995) Fluorimetric assays of proteolytic enzymes; **in:** *Methods in Enzymology. Vol. 248. Proteolytic enzymes: aspartic and metallo peptidases (A.J. Barrett, Ed.)*. Academic Press, San Diego, pp. 18-34.

Knowles, G., Black, S.J. and Whitlaw, D.D. (1987) Peptidase in the plasma of mice infected with *Trypanosoma brucei brucei*. *Parasitology*, **95:** 291-300.

Kominami, F., Hottschultze, H., Leuschel, L., Maier, K. and Holzer, H. (1986) The substrate specificity of protease B from bakers yeast. *Biochim. Biophys. Acta* **661:** 136-141.

Koob, G.F. and Bloom, F.E. (1982) Behavioral effects of neuropeptides: endorphins and vasopressin. *Annu. Rev. Physiol.* **44:** 571-582.

Kornblatt, J.D., Mpimbaza, G.W.N. and Lonsdale-Eccles, J.D. (1992) Characterisation of an endopeptidase of *Trypanosoma brucei brucei*. *Arch. Biochem. Biophys.*, **293:** 25-31.

Krstulovil, A.M. and Brown, P.R. (1982) *Reversed-phase high-performance liquid chromatography: theory, practice and biomedical applications.* John Wiley & Sons, New York. 296 pp.

Kuzoe, F.A.S. (1993) Current situation of African trypanosomiasis. *Acta Trop.* **54:** 153-162.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **227:** 680-685.

La Rocca, R.V., Stein, C.A. and Danesi, R. (1990) Suramin, a novel anti-tumor compound. *J. Steroid Biochem. Mol. Biol.* **37:** 893-898.

Laderach, D., Cerban, F., Motran, C., Vottero, de Cima, E.V. and Gea, S. (1996) *Trypanosoma cruzi*: the major cysteinyl proteinase (cruzipain) is a relevant immunogen of parasite acid antigens (FIII). *Int. J. Parasitol.* **26:** 1249-1254.

Lanham, S.M. (1968) Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. *Nature* **218:** 1273-1274.

Lanham, S.M. and Godfrey, D.G. (1970) Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Exp. Parasitol.* **28:** 521-534

Laveran, A. and Mesnil, F. (1904) *Trypanosomes et trypanosomiasis*. Masson, Paris. p. 410.

Lazure, C., Seidah, N.G., Pelaprat, D. and Chretien, M. (1983) Proteases and post-translational processing of prohormones: a review. *Can. J. Biochem. Cell Biol.*, **61:** 501-515.

Leach, T.M. and Roberts, C.J. (1981) Present status of chemotherapy and chemoprophylaxis of animal trypanosomiasis in the eastern hemisphere. *Pharmacol. Therap.* **13:** 91-147.

LeGendre, N., Mansfield, M., Weiss, A. and Matsudaira, P. (1993) Purification of proteins and peptides by SDS-PAGE, **in:** *A practical guide to protein and peptide purification for microsequencing (2nd edition) (P. Matsudaira, Ed.)*. Academic Press, San Diego, pp. 71-101.

Lenarčič, B., Križaj, I., Žunec, P. and Turk, V. (1996) Differences in the specificity for the interactions of stefins A, B and D with cysteine proteinases. *FEBS Lett.* **395:** 113-118.

LeRoith, D., Shiloach, J., Roth, J. and Lesniak, M.A. (1980) Evolutionary origins of vertebrate hormones: substances similar to mammalian insulins are native to unicellular eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* **77:** 6184-6188.

LeRoith, D., Delahunty, G., Wilson, G.L., Roberts, C.T., Shemer, J., Hart, C., Lesniak, M.A., Shiloach, J. (1986) Evolutionary aspects of the endocrine and nervous systems. *Rec. Prog. Horm. Res.* **42:** 549-587.

Li, R., Kenyon, G.L., Cohen, F.E., Chen, X., Gong, B., Dominguez, J.N., Davidson, E., Kurzban, G., Miller, R.E., Nuzum, E.O., Rosenthal, P.J. and McKerrow, J.H. (1995) *In vitro* antimalarial activity of chalcones and their derivatives. *J. Med. Chem.* **38:** 5031-5037.

Lim Tung, H.Y., Pelech, S., Fisher, M.J., Pogson, C.I. and Cohen, P. (1985) The protein phosphatases involved in cellular regulation: influence of polyamines on the activities of protein phosphatase-1 and protein phosphatase-2A. *Eur. J. Biochem.* **149:** 305-313.

Liew, F.Y., Wei, X.Q. and Proudfoot, L. (1997) Cytokines and nitric oxide as effector molecules against parasitic infections. *Phil. Trans. Roy. Soc. Lond.* **B352:** 1311-1315.

Lineweaver, H. and Burk, D. (1934) Determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56:** 658-666.

Lomo, P.O., Coetzer, T.H.T. and Lonsdale-Eccles, J.D. (1997) Purification and characterisation of the proteasome from *Trypanosoma brucei brucei*. *Immunopharm.* **36:** 27-39.

Lonsdale-Eccles, J.D. (1991) Proteases of African Trypanosomes, **in:** *Biochemical Protozoology (G. Coombs and M.J. North, Eds.)*. Taylor and Francis, London. pp. 200-207.

Lonsdale-Eccles, J.D. and Mpimbaza, G.W.N. (1986) Thiol-dependent proteases of African trypanosomes. *Eur. J. Biochem.* **155:** 469-473.

Lonsdale-Eccles, J.D. and Grab, D.J. (1987) Lysosomal and non-lysosomal peptide hydrolases of the bloodstream forms of *Trypanosoma brucei brucei*. *Eur. J. Biochem.*, **169:** 467-475.

Lonsdale-Eccles, J.D., Mpimbaza, G.W.N., Nkhungulu, Z.R., Olobo, J., Smith, L., Tosomba, O.M. and Grab, D.J. (1995) Trypanostomatid cysteine protease activity may be enhanced by a kininogen-like moiety from host serum. *Biochem. J.* **305:** 549-556.

Losos, G.J. and Ikede, B.O. (1972) Pathology of the disease in sheep produced experimentally by *Trypanosoma brucei*. *Vet. Pathol.* **9:** 278-289.

Lottspeich, F. (1994) *Microcharacterisation of proteins; in: Microcharacterisation of proteins (R. Kellner, F. Lottspeich and H.E. Meyer, Eds.)*. Verlag Chemie, Weinheim, pp. 3-10.

Lushbaugh, W.B. (1988) Proteases of *Entamoeba histolytica*; **in:** *Amebiasis: human infection by Entamoeba histolytica*. Wiley and Sons, New York, pp. 219-231.

Maeda, H., Molla, A., Oda, T. and Katsuki, T. (1987) Internalisation of serratial protease into cells as an enzyme inhibitor complex with α_2-macroglobulin and regeneration of protease activity and cytotoxicity. *J. Biol. Chem.* **262:** 10946-10950.

Maeda, H. and Molla, A. (1989) Pathogenic potentials of bacterial proteases. *Clin. Chem. Acta* **185:** 357-368.

Maeda, H. and Yamamoto, T. (1996) Pathogenic mechanisms induced by microbial proteases in microbial infections. *Biol. Chem. Hoppe-Seyler* **377:** 217-226.

Mäkinen, P.-L., Mäkinen, K.K. and Syed, S.A. (1994) An endo-acting proline-specific oligopeptidase from *Treponema denticola* ATCC 35405: evidence of hydrolysis of human bioactive peptides. *Inf. Imm.* **62:** 4938-4947.

Mäkinen, K.K., Mäkinen, P.-L., Loesche, W.J. and Syed, S.A. (1995) Purification and general properties of an oligopeptidase from *Treponema denticola* ATCC 35405 - a human oral spirochaete. *Arch. Biochem. Biophys.* **316:** 689-698.

Mansfield, J.M. (1990) Immunology of African trypanosomiasis; **in:** *Modern parasite biology. Cellular, immunological and molecular aspects (D.J. Wyler, Ed.)*. W.H. Freeman, New York, pp. 222-249.

Markwardt, F., Drawert, J. and Walsmann, P. (1974) Synthetic low molecular weight inhibitors of serum kallikrein. *Biochem. Pharmacol.* **23:** 2247-2256.

Martínez, J., Campetella, O., Frasch, A.C.C. and Cazullo J.-J. (1991) The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is antigenic in human infections. *Infect. Immun.* **59:** 4275-4277.

Maruo, K., Akaike, T., Matsushima, Y., Kohmoto, S., Inada, Y., Ono, T., Arao, T. and Maeda, H. (1991) Triggering of the vascular permeability reaction by activation of the Hagemann factor-prekallikrein system by house-dust mite protease. *Biochim. Biophys. Acta* **1074:** 62-68.

Mason, R.W. (1989) Interaction of lysosomal cysteine proteases with α_2-macroglobulin: conclusive evidence for the endopeptidase activities of cathepsins B and H. *Arch. Biochem. Biophys.* **273(2):** 367-374.

Mast, A.E., Enghild, J.J., Pizzo, S.V. and Salvesen, G. (1991) Analysis of the plasma elimination kinetics and conformational stabilities of native, protease-complexed, and reactive site cleaved serpins: comparison of α_1-protease inhibitor, α_1-antichymotrypsin, antithrombin III, α_2-antiplasmin, angiotensinogen and ovalbumin. *Biochemistry* **30:** 1723-1730.

Matheson, N., Schmidt, J. and Travis, J. (1995) Isolation and properties of an angiotensin II-cleaving peptidase from mesquite pollen. *Am. J. Respir. Cell Mol. Biol.* **12:** 441-448.

Matrisian, L.M. (1992) The matrix-degrading metalloproteases. *BioEssays* **14:** 455-463.

Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylene difluoride membranes. *J. Biol. Chem.* **262:** 10035-10038.

Matsudaira, P. (1993) Introduction, **in:** *A practical guide to protein and peptide purification for microsequencing (2nd edition) (P. Matsudaira, Ed.)*. Academic Press, San Diego, pp. 3-16.

Mbawa, Z.R., Gumm, I.D., Fish, W.R. and Lonsdale-Eccles, J.D. (1991) Endopeptidase variations among different life-cycle stages of African trypanosomes. *Eur. J. Biochem.* **195:** 183-190.

Mbawa, Z.R., Gumm, I.D., Shaw, E. and Lonsdale-Eccles, J.D. (1992) Characterisation of a cysteine protease from bloodstream forms of *Trypanosoma congolense*. *Eur. J. Biochem.* **204:** 371-379.

McCann, P.P., Bacchi, C.J., Clarkson, A.B., Bey, P., Sjoerdsma, A., Schechter, P.J., Walzer, P.D. and Barlow, J.L.R. (1986) Inhibition of polyamine biosynthesis by alpha-difluoromethylornithine in African trypanosomes and *Pneumocystis carinii* as a basis of chemotherapy: biochemical and clinical aspects. *Am. J. Trop. Med. Hyg.* **35:** 1153-1156.

McKerrow, J.H., McGrath, M.E. and Engel, J.C. (1991) The cysteine protease of *Trypanosoma cruzi* as a model for antiparasite drug design. *Parasitol. Today* **11(8):** 279-282.

McKerrow, J.H., Sun, E., Rosenthal, P.J. and Bouvier, J. (1993) The proteases and pathogenicity of parasitic protozoa. *Annu. Rev. Microbiol.* **47:** 821-853.

McKie, N., Dando, P.M., Brown, M.A. and Barrett, A.J. (1995) Rat thimet oligopeptidase: large scale expression in *Escherichia coli* and charcterisation of the recombinant enzyme. *Biochem. J.* **309:** 203-207.

Michal, G. (1983) Determination of Michaelis constants and inhibitor constants; **in:** *Methods of enzymatic analysis. Volume I. Fundamentals. (H.U. Bergenmeyer, J. Bergenmeyer and M. Graßl, Eds.)*. Verlag Chemie, Weinheim, pp. 86-97.

Miller, B.C., Thiele, D., Hersh, L.B. and Cottan, G.L. (1996) A secreted peptidase involved in T-cell β-endorphin metabolism. *Immunopharm.* **31:** 151-161.

Milford, F., Pépin, J., Loko, L., Ethier, L. and Mpia, B. (1992) Efficacy and toxicity of eflornithine for treatment of *Trypanosoma brucei gambiense* sleeping sickness. *Lancet* **340:** 652-655.

Milord, F., Loko, L., Ethier, L., Mpia, B. and Pépin, J. (1993) Eflornithine concentrations in serum and cerebrospinal fluid of 63 patients treated for *Trypanosoma brucei gambiense* sleeping sickness. *Trans. Roy. Soc. Trop. Med. Hyg.* **87:** 473-477.

Minamino, N., Kangawa, K. and Matsuo, H. (1985) Neuromedin U-8 and U-25: novel uterine stimulators and hypertension peptides identified in the porcine spinal cord. *Biochem. Biophys. Res. Comm.* **130:** 1078-1091.

Miura, M., Zhu, H., Rotello, R., Hartweig, E.A. and Yuan, J. (1993) Induction of apoptosis in fibroblasts by interleukin-1β-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced*-3. *Cell* **75:** 653-660.

Moehle, C.M., Tizard, R., Lennon, S.K., Smart, J. and Jones, E.W. (1987) Protease B of the yeast *Saccharomyces cerevisiae* is homologous to the subtilisin-like family of serine proteases. *Mol. Cell. Biol.* **12:** 4390-4399.

Moriyama, A., Nakanishi, M. and Sasaki, M. (1988) Porcine muscle prolyl endopeptidase and its endogenous substrates. *J. Biochem.* **104:** 112-117.

Morrison, W.I., Murray, M., Sayer, P.D. and Preston, J.M. (1981a) The pathogenesis of experimentally induced *Trypanosoma brucei* infection in the dog. I. Tissue and organ damage. *Am. J. Pathol.* **102:** 168-181.

Morrison, W.I., Murray, M., Sayer, P.D. and Preston, J.M. (1981b) The pathogenesis of experimentally induced *Trypanosoma brucei* infection in the dog. II. Changes in the lymphoid organs. *Am. J. Pathol.* **102:** 182-194.

Mottram, J.C., Souza, A.E., Hutchinson, J.E., Carter, R., Frame, M.J. and Coombs, G.H. (1997) Evidence from disruption of the lmcpb gene array of *Leishmania mexicana* that cysteine proteinases are virulence factors. *Proc. Natl. Acad. Sci. U.S.A.* **93:** 6008-6013.

Müller, N., Imboden, M., Detmet, E., Mansfield, J.M. and Seebeck, T. (1993) Cytoskeleton-associated antigens from African trypanosomes are recognised by self-reactive antibodies of uninfected mice. *Parasitology*, **107:** 411-417.

Murray, M. and Dexter, T.M. (1988) Anaemia in bovine African trypanosomiasis. *Acta Trop.* **45:** 389-342.

Musuoke, A.J., Nantulya, V.M., Barbet, A.F., Kironde, F. and McGuire, T.C. (1981) Bovine immune response to African trypanosomes: specific antibodies to variable surface glycoproteins of *Trypanosoma brucei*. *Parasite Immunol.* **3:** 97-107.

Mutayoba, B.M., Gombe, S., Waindi, E.N. and Kaaya, G.P. (1988a) Depression of ovarian function and plasma progesterone and estradiol-17β in female goats chronically infected with *Trypanosoma congolense*. *Acta Endocrin.* **117:** 477-484.

Mutayoba, B.M., O'Hara, T. and Gombe, S. (1988b) Trypanosome-induced depression of plasma thyroxine levels in prepubertal and adult female goats. *Acta Endocrin.* **119:** 21-26.

Mutayoba, B.M., Eckersall, P.D., Cestnik, V., Jeffcoate, I.A., Gray, C.E. and Holmes, P.H. (1995a) Effects of *Trypanosoma congolense* on pituitary and adrenocortical function in sheep: changes in the adrenal glands and corticol secretion. *Res. Vet. Sci.* **58:** 174-179.

Mutayoba, B.M., Eckersall, P.D., Seely, C., Gray, C.E., Cestnik, V., Jeffcoate, I.A. and Holmes, P.H. (1995b) Effects of *Trypanosoma congolense* on pituitary and adrenocortical function in sheep: responses to exogenous corticotrophin-releasing hormone. *Res. Vet. Sci.* **58:** 180-185.

Nagle, R.B., Dong, S., Guillot, J.M., McDaniel, K.M., Lindsley, N.R. (1980) Pathology of experimental African trypanosomiasis in rabbits infected with *Trypanosoma congolense*. *Am. J. Trop. Med. Hyg.* **29:** 1187-1195.

Naval, J., Calvo. M., Fermín, L. and Piñeiro, A. (1982) Interactions of different albumins and animal sera with insolubilised Cibacron Blue. Evaluation of apparent affinity constants. *Comp. Biochem. Physiol.* **71B(3):** 403-407.

Ndung'u, J.M., Wirght, N.G., Jennings, F.W. and Murray, M. (1992) Changes in atrial natriuretic factor and plasma renin activity in dogs infected with *Trypanosoma brucei*. *Parasitol. Res.* **78:** 553-558.

Nelson, D.J., LaFon, S.W., Tuttle, J.V., Miller, W.H., Miller, R.L. (1979) Allopurinol ribonucleoside as an antileishmanial agent. *J. Biol. Chem.*, **254:** 11544-11549.

Neugebauer, J.M. (1990) Detergents: an overview, **in:** *Methods in enzymology. Volume 182. Guide to protein purification. (M.P. Deutscher, Ed.)*. Academic Press, San Diego, pp. 239-253.

Neville, D.M. (1971) Molecular weight determinations of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* **246:** 6328-6334.

Newton, B.A. and LePage, R.W.F. (1967) Preferential inhibition of deoxyribonucleic acid synthesis by the trypanocide Berenil. *Biochem. J.*, **105:** 50-51.

Nicklin, M.J.H. and Barrett, A.J. (1984) Inhibition of cysteine proteinases and dipeptidyl peptidase I by egg-white cystatin. *Biochem. J.* **245:** 245-253.

Nielsen, K., Sheppard, J., Holmes, W. and Tizard, I. (1978) Experimental bovine trypanosomiasis: changes in serum immunoglobulins, complement and complement components in infected cattle. *Immunology* **35:** 817-824.

Nielsen, M.H. and Nielsen, R. (1975) Electron microscopy of *Trichomonas vaginalis* Donné. Interaction with vaginal epithelium in human trichomoniasis. *Acta Pathol. Microbiol. Scand.* **83:** 305.

Nishikata, M. (1984) Trypsin-like protease from soybean seeds. Purification and some properties. *J. Biochem.* **95:** 1169-1177.

O'Leary, R.M., Gallagher, S.P. and O'Connor, B. (1996) Purification and characterisation of a novel membrane-bound form of prolyl endopeptidase from bovine brain. *Int. J. Biochem. Cell Biol.* **28:** 441-449.

Odegaard, B.H., Anderson, P.C. and Lovrien, R. (1984) Resolution of the multienzyme cellulase complex of *T. reesei* QM9414. *J. Appl. Biochem.* **6:** 156-183.

Oleksyszyn, J., Boduzek, B., Kam, C.-M. and Powers, J.C. (1994) Novel amidine-containing peptidyl phosphonates as irreversible inhibitors for blood coagulation and related serine proteases. *J. Med. Chem.* **37:** 226-231.

Oleksyszyn, J. and Powers, J.C. (1994) Amino acid and peptide phosphonate derivatives as specific inhibitors of serine peptidases; in: *Methods in enzymology. Volume 244. Proteolytic enzymes: serine and cysteine proteases (A.J. Barrett, Ed.)*. Academic Press, San Diego, pp. 423-441.

Ornstein, L. (1964) Disc electrophoresis - I. Background and theory. *Ann. N. Y. Acad. Sci.* **121:** 321-349.

Pearson, T.W., Roelants, G.E., Lundin, L.B. and Mayor-Withey, K.S. (1978) Immune depression in trypanosome-infected mice. I. Depressed T-lymphocyte responses. *Eur. J. Immunol.* **8:** 723-727.

Pegg, A.E. and McCann, P.P. (1982) Polyamine metabolism and function. *Am. J. Physiol.* **243:** C212-C221.

Pépin, J., Guern, C., Milford, F. and Schecter, P.J. (1987) Difluoromethylornithine for arseno-resistant *T. b. gambiense* sleeping sickness. *Lancet* **330:** 1431-1433.

Pépin, J., Milford, F., Guern, C., Mpia, B., Ethier, L. and Mansinse, D. (1989) Trial of prednisolone for prevention of melarsoprol-induced encephalopathy in gambiense sleeping sickness. *Lancet* **333:** 1246-1250.

Pépin, J. and Milford, F. (1994) The treatment of human African trypanosomiasis. *Adv. Parasitol.* **33:** 1-47.

Perona, J.J. and Craik, C.S. (1995) Structural basis of substrate specificity in the serine proteases. *Prot. Sci.* **4:** 337-360.

Peterson, E.A. (1970) Cellulosic ion-exchangers, **in:** *Laboratory techniques in biochemistry and molecular biology. Volume 2. Part II. (T.S. Work and E. Work, Eds.).* North-Holland, London, pp. 223-397.

Pike, R.N. and Dennison, C. (1989) Protein fractionation by three-phase partitioning (TPP) in aqueous/*t*-butanol mixtures. *Biotechnol. Bioeng.* **33:** 221-228.

Pike, R.N., Coetzer, T.H.T. and Dennison, C. (1992) Proteolytically active complexes of cathepsin L and a cysteine proteinase inhibitor; purification and demonstration of their formation *in vitro*. *Arch. Biochem. Biophys.* **294:** 623-629.

Playfair, J.H.L., Taverne, J., Bate, C.A.W. and De Souza, J.B. (1990) The malaria vaccine: anti-parasite or anti-disease. *Immunol. Today* **11:** 25-29.

Plimmer, H.G. and Bradford, J.R.C. (1908) A preliminary note on the morphology and distribution of the organism found in tsetse fly disease. *Proc. Roy. Soc. Lond.* **B65:** 274-282.

Polgár, L. (1991) pH-dependant mechanism in the catalysis of prolyl oligopeptidase from pig muscle. *Eur. J. Biochem.* **197:** 441-447.

Polson, A., Coetzer, T.H.T., Kruger, J., von Maltzahn, E. and van der Merwe, K.J. (1985) Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol. Invest.* **14:** 323-327.

Polson, A., von Wechmar, M.B. and van Regenmortel, M.V.H. (1980a) Isolation of viral IgY antibodies from yolks of immunized hens. *Immunol. Comm.* **9:** 475-493.

Polson, A., von Wechmar, B. and Fazakerley, G. (1980b) Antibodies to proteins from yolk of immunized hens. *Immunol. Comm.* **9:** 495-514.

Poltera, A.A. (1985) Pathology of human African trypanosomiasis with reference to experimental African trypanosomiasis and infections of the central nervous system. *Brit. Med. Bull.* **41:** 169-174.

Powers, J.C. and Harper, J.W. (1986) Inhibitors of serine proteinases, **in:** *Proteinase inhibitors (A.J. Barrett and G. Salvesen, Eds.).* Elsevier, Amsterdam, pp. 55-152.

Rauber, P., Angliker, H., Wallace, B. and Shaw, E. (1986) The synthesis of peptidyl-fluoromethylketones and their properties as inhibitors of serine and cysteine proteases. *Biochem. J.* **239:** 633-640.

Read, C.P. (1972) *Animal parasitism.* Prentice Hall, Englewood Cliffs. 192 pp.

Read, S.M. and Northcote, D.H. (1981) Minimisation of variation in the response to different proteins of the Coomassie Blue dye-binding assay for protein. *Anal. Biochem.* **116:** 53-64

Reed, S., Keene, W.E. and McKerrow, J.H. (1989) Thiol protease expression correlates with pathogenicity of *Entamoeba histolytica. J. Clin. Microbiol.* **27:** 2772-2777.

Reed, S.L., Ember, J.A., Herdman, D.S., DiScipio, R.G., Hugli, T.E. and Gigli, I. (1995) The extracellular neutral cysteine protease of *Entamoeba histolytica* degrades anaphylatoxins C3a and C5a. *J. Immunol.* **155:** 266-274.

Rees, L.H. and Lowry, P.J. (1979) Adrenocorticotrophin and lipotrophin, **in:** *Hormones in blood (3rd edition) (C.H. Gray and V.H.T. James, Eds).* Academic Press, New York, pp. 129-178.

Rivier, J.E., Lazarus, L.H., Perrin, M.H. and Brown, M.R. (1977) Naurotensin analogues. Structure-activity relationships. *J. Med. Chem.* **20:** 1409-1412.

Rennex, D., Hemmings, B.A., Hofsteenge, J. and Stone S.R. (1991) cDNA cloning of porcine brain prolyl oligopeptidase and identification of the active-site seryl residue. *Biochemistry*, **30:** 2195-2203.

Reynolds, J.A. and Tanford, C. (1970) The gross conformation of protein-sodium dodecyl sulfate complexes. *J. Biol. Chem.* **19:** 5161-5165.

Robertson, C.D., North, M.J., Lockwood, B.C., Coombs, G.H. (1990) Analysis of the proteases of *Trypanosoma brucei. J. Gen. Microbiol.*, **136:** 921-925.

Rockett, K.A., Playfair, J.H.L., Ashall, F., Targett, G.A.T., Angliker, H. and Shaw, E. (1990) Inhibition of intraerythrocytic development of *Plasmodium falciparum* by proteinase inhibitors. *FEBS Lett.* **259:** 257-259.

Roelants, G.E. (1986) Natural resistance to African trypanosomiasis. A viewpoint. *Parasite Immunol.* **8:** 1-10.

Roelants, G.E., Pearson, T.W., Morrison, W.I. and Mayor-Withey, K.S. (1979) Immune depression in trypanosome-infected mice. IV. Kinetics of suppression and alleviation by the trypanocidal drug Berenil. *Clin. Exp. Immunol.* **37:** 457-469.

Roelants, G.E. and Pinder, M. (1984) The immunobiology of African trypanosomes. *Contemp. Topics Immunobiol.* **12:** 225-238.

Roitt, I.M. (1991) *Essential immunology (7th edition).* Blackwell, Oxford, p. 168.

Roitt, I.M. (1994) *Essential immunology (8th edition).* Blackwell, Oxford, pp. 188-191

Rosenthal, P.J., Kim, K., McKerrow, J.H. and Leech, J.H. (1987) Identification of three stage-specific proteases of *Plasmodium falciparum. J. Exp. Med.* **166:** 816-821.

Rosenthal, P.J., Wollish, W.S., Palmer, J.T. and Rasnick, D. (1991) Antimalarial effects of peptide inhibitors of a *Plasmodium falciparum* cysteine protease. *J. Clin. Invest.* **88:** 1467-1472.

Rosenthal, P.J., Olson, J.E., Lee, G.K., Palmer, J.T., Klaus, J.L. and Rasnick, D. (1996) Antimalarial effects of vinyl sulfone proteinase inhibitors. *Antimicrob. Agents Chemother.* **40:** 1600-1603.

Rossomando, E.F. (1990) Ion-exchange chromatography, **in:** *Methods in enzymology. Volume 182. Protein purification (M.P. Deutscher, Ed.).* Academic Press, San Diego, pp. 309-316.

Rudin, W., Poltera, A.A. and Jenni, L. (1983) An EM study on cerebral trypanosomiasis in rodents and primates. *Contrib. Microbiol. Immunol.* **7:** 165-172.

Russo, D.C.W., Grab, D.J., Lonsdale-Eccles, J.D., Shaw, M.K. and Williams, D.J.L. (1993) Directional movement of variable surface glycoprotein-antibody complexes in *Trypanosoma brucei*. *Eur. J. Cell Biol.* **62:** 432-441.

Russell, D.G. and Alexander, J. (1988) Effective immunisation against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. *J. Immunol.* **140:** 1274-1279.

Sakanari, J.A., Staunton, C.E., Eakin, A.E., Craik, C.S. and McKerrow, J.H. (1989) Serine proteases from nematode and protozoan parasites: isolation of sequence homologues using generic molecular probes. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 4863-4867.

Salvesen, G. and Nagase, H. (1989) Inhibition of proteolytic enzymes, **in:** *Proteolytic enzymes: a practical approach (R.J. Benyon and J.S. Bonds, Eds.).* Oxford University Press, Oxford, pp. 83-104.

Sands, M., Kron, M.A. and Brown, R.B. (1985) Pentamidine: a review. *Rev. Inf. Dis.* **7:** 625-634.

Santana, J.M., Grellier, P., Schrével, J. and Teixeira, A.R.L. (1997) A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV. *Biochem. J.* **324:** 129-137.

Sarath, G., de la Motte, R.S. and Wagner, F.W. (1989) Protease assay methods, **in:** *Proteolytic enzymes: a practical approach (R.J. Benyon and J.S. Bonds, Eds.).* Oxford University Press, Oxford, pp. 25-55.

Schägger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166:** 368-379.

Schally, A.V., Kastin, A.J., Locke, W. and Bowers, C.Y. (1967) Hypothalamic releasing and inhibiting factors; **in:** *Hormones in blood. Volume 1. (2nd edition). (C.H. Gray and A.L. Bacharach, Eds.).* Academic Press, London, pp. 491-525.

Scharpe, S., De Meester, I., Hendriks, D., Vanhoof, G., van Sande, M. Vriend, G. (1991) Proteases and their inhibitors: today and tomorrow. *Biochimie* **73:** 121-126.

Schechter, I. and Berger, A. (1967) On the size of the active-site in proteases. I. Papain. *Biochem. Biophys. Res. Comm.* **27:** 157-162.

Schiavo, G., Benfenati, F., Poulais, B., Rossetto, O., deLaurento, P.P., Das Gupta, B.R. and Montecucco, C. (1992) Tetanus and botulinum B neurotoxins block neurotransmitter released by proteolytic cleavage of synaptobrevin. *Nature* **359:** 832-835.

Schmidt, G.D. and Roberts, L.S. (1989) *Foundations of parasitology*. Times Mirror, St. Louis. 750 pp.

Schrével, J., Bernard, F., Maintier, C., Mayer, R. and Monsigny, M. (1984) Detection and characterisation of a selective endopeptidase from *Plasmodium berghei* by using fluorogenic peptidyl substrates. *Biochem. Biophys. Res. Comm.* **124:** 703-710.

Schultz, R.M., Varma-Nelson, P., Ortiz, R., Kozlowski, K.A., Orawski, A.T., Pagart, P. and Frankfater, A. (1989) Active and inactive forms of the transition state analogue protease inhibitor leupeptin: explanation of the observed slow binding of leupeptin to cathepsin B and papain. *J. Biol. Chem.* **264:** 1497-1507.

Schultze, H.E. and Heremans, J.F. (1966) *Molecular biology of human proteins with special reference to plasma proteins. Volume I. Nature and metabolism of extracellular proteins*. Elsevier, Amsterdam, pp. 173-223.

Scott, C.F., Whitaker, E.J., Hammond, F. and Coleman, R. (1993) Purification and characterisation of a potent 70 kDa thiol protease from *Porphyromonas gingivalis* that cleaves kininogen and fibrinogen. *J. Biol. Chem.* **268:** 7935-7942.

Semple, J.E., Rowley, D.C., Brunck, T.K. and Ripka, W.C. (1997) Synthesis and biological activity of P_2-P_4 azapeptidomimetic P_1-argininal and P_1-ketoargininamide derivatives: a novel class of serine protease inhibitors. *Bioorg. Med. Chem. Lett.* **7:** 315-320.

Shaw, E. (1994) Peptidyl diazomethanes as inhibitors of cysteine and serine proteinases; **in:** *Methods in enzymology. Volume 244. Proteolytic enzymes: serine and cysteine proteases (A.J. Barrett, Ed.)*. Academic Press, San Diego, pp. 649-656.

Shaw, E. and Green, G.D.J. (1981) Inactivation of thiol proteases with peptidyl diazomethyl ketones, **in:** *Methods in Enzymology. Volume 80. Proteolytic Enzymes. Part C (Lorand, L., Ed.)*. Academic Press, New York, p. 820-832.

Shrimpton, C.N., Glucksman, M.J., Lew, R.A., Tullai, J.W., Margulies, E.H., Roberts, J.L. and Smith, A. I. (1997) Thiol activation of endopeptidase EC 3.4.24.15: a novel mechanism for the regulation of catalytic activity. *J. Biol. Chem.* **272:** 17395-17399.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76-85.

Smith, E., Delange, R., Evans, W., Landau, M., Marland, F. (1986) Subtilisin Carlsberg. V. The complete sequence compared with subtilisin BPN'. *J. Biol. Chem.* **243:** 2184-2191.

Smith, F.G., Sato, T., Varille, V.A. and Robillard, J.E. (1989) Atrial natriuretic factor during fetal and postnatal life: a review. *J. Dev. Physiol.*, **12:** 55-62.

Sottrup-Jensen, L. (1989) α-Macroglobulins: structure, shape, and mechanism of proteinase complex formation. *J. Biol. Chem.* **264:** 11539-11542.

Sreedharan, S.K., Verma, C., Vaces, L.S.D., Brocklehurst, S.M., Gharbia, S.E., Shah, H.N. and Brocklehurst, K. (1996) Demonstration that 1-*trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E-64) is oneof the most effective low M_r inhibitors of trypsin-catalysed hydrolysis. Characterisation by kinetic analysis and by energy minimisation and molecular dynamics stimulation of the E-64-β-trypsin complex. *Biochem. J.* **316:** 777-786.

Stephens, J.W.W. and Fantham, H.B. (1910) On the perculiar morphology of a trypanosome from a case of African sleeping sickness amd the possibility of its being a new species (*T. rhodesiense*). *Proc. Roy. Soc. Lond.* **B83:** 28-33.

Stone, S.R., Rennex, D., Wikstrom, P., Shaw, E. and Hofsteenge, J. (1991) Inactivation of prolyl oligopeptidase by a peptidylchloromethane: kinetics of inactivation and identification of the sites of modiciation. *Biochem. J.* **276:** 837-840.

Stone, S.R., Rennex, D., Wikstrom, P., Shaw, E. and Hofsteenge, J. (1992) Peptidyldiazomethanes: a novel mechanism of interaction with prolyl endopeptidase. *Biochem. J.* **283:** 871-876.

Subbayya, I.N.S., Ray, S.S., Balaram, P. and Balaram, H. (1997) Metabolic enzymes as potential drug targets in *Plasmodium falciparum*. *Ind. J. Med. Res.* **106:** 79-94.

Sweet, R.M., Wright, H.T., Janin, J., Chothia, C.H. and Blow, D.M. (1974) Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (kunitz) at 2.6-Å resolution. *Biochemistry*, **13:** 4212-4228.

Swellengrebel, N.H. and Sterman, M.M. (1961) Hemoflagellates, **in:** *Animal parasites in man (N.H. Swellengrebel, Ed.)*. Van Nostrand, Princeton, pp. 49-71.

Syrovy, L. and Hodny, Z. (1991) Staining and quantification of proteins separated by polyacrylamide gel electrophoresis. *J. Chromatog.* **569:** 175-196.

Szedlacsek, S.E., Ostafe, V., Serban, M. and Vlad, O. (1987) A re-evaluation of the kinetic equations for hyperbolic tight-binding inhibition. *Biochem. J.* **254:** 311-312.

Tamura, S.Y., Goldman, E.A., Brunck, T.K., Ripka, W.C. and Semple, J.E. (1997) Rational design, synthesis and serine protease inhibitory activity of a novel P_1-argininal derivative featuring a conformationally constrained P_2-P_3 bicyclic lactam moeity. *Bioorg. Med. Chem. Lett.* **7:** 331-226.

Tetaert, D., Soudan, B., Huet-Duvillier, G., Degand, P. and Boersma, A. (1993) Unusual cleavage of peptidic hormones generated by trypanosome enzymes released in infested rat serum. *Int. J. Peptide Protein Res.* **41:** 147-152.

Tetaert, D., Soudan, B., Lo-Guidice, J.-M., Richet, C., Degard, P., Boussard, G., Mariller, Spik, G. (1994) Combination of high-performance anion-exchange chromatography and electrospray mass spectrometry for analysis of the *in vitro* O-glycosylated mucin motif peptide. *J. Chrom.* **B(658):** 31-38.

Tizard, I., Nielsen, K.H., Seed, J.R. and Hall, J.E. (1978) Biologically active products from African trypanosomes. *Microbiol. Rev.* **42:** 661-681.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose cheets: procedure and applications. *Proc. Natl. Acad. Sci. U.S.A.* **76:** 4350-4354.

Trail, J.C.M., d'Ieteren, G.D.M. and Teale, A.J. (1989) Trypanotolerance and the value of conserving livestock genetic resources. *Genome* **31:** 805-812.

Travis, J. and Johnson, D. (1981) Human α_1-antitrypsin; **in:** *Methods in Enzymology. Volume 80. Proteolytic Enzymes. Part C (Lorand, L., Ed.)*. Academic Press, New York, p. 754-764.

Travis, J. and Morii, M. (1981) Human α_1-antichymotrypsin; **in:** *Methods in Enzymology. Volume 80. Proteolytic Enzymes. Part C (Lorand, L., Ed.)*. Academic Press, New York, p. 765-771.

Travis, J. and Salvesen, G.S. (1983) Human plasma protease inhibitors. *Annu. Rev. Biochem.* **52:** 655-709.

Travis, J., Bowen, J., Tewksbury, D. Johnson, D and Panell, R. (1976) Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. *Biochem. J.* **157:** 301-306.

Travis, J., Guzdek, A., Potempa, J and Watorek, W. (1990) Serpins: structure and mechanisms of action. *Biol. Chem. Hoppe-Seyler* **371:** 3-11.

Troeberg, L., Pike, R.N., Morty, R.E., Berry, R.K., Coetzer, T.H.T. and Lonsdale-Eccles, J.D. (1996) Proteases from *Trypanosoma brucei brucei*: purification, characterisation and interactions with host regulatory molecules. *Eur. J. Biochem.* **238:** 728-736.

Ukoli, F.M.A. (1984) *Introduction to parasitology in tropical Africa*. John Wiley, Chichester. 464 pp.

Umezawa, H. (1976) Structures and activities of protease inhibitors of microbial origin; **in:** *Methods in enzymology. Volume 45. Proteolytic Enzymes. Part B. (L. Lorand, Ed.)*. Academic Press, San Diego, pp. 678-694.

van den Berg, P., Rozing, J. and Nagelkerken, L. (1993) Identification of two moeities of β-endorphin with opposing effects on rat T-cell proliferation. *Immunology*, **79:** 18-23.

van den Ouweland, A.M.W., Van Duijnhoven, H.L.P., Keizer, G.D., Dorssors, L.C.J. and Van de Ven, W.J.M. (1990) Structural homology between the human *fur* gene product and the subtilisin-like protease encoded by yeast KEX2. *Nucl. Acids Res.* **18:** 664-674.

Van der Jagt, D.L., Hunsaker, L.A. and Campos, N.M. (1986) Characterisation of a hemoglobin-degrading, low molecular weight protease from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **18:** 389-400.

Veale, C.A., Bernstein, P.R., Bohnert, C.M., Brown, F.J., Bryant, C., Damewood, J.R., Earley, R., Feeney, S.W., Edwards, P.D., Gomes, B., Hulsizer, J.M., Kosmider, B.J., Krell, R.D., Moore, G., Salcedo, T.W., Shaw, A., Silberstein, D.S., Steelman, G.B., Stein, M., Strimpler, A., Thomas, R.M., Vacek, E.P., Williams, J.C., Wolanin, D.J. and Woolson, S. (1997) Orally active trifluormethyl ketone inhibitors of human leukocyte elastase. *J. Med. Chem.* **40:** 3173-3181.

Vickerman, K. (1976) The diversity of kinetoplastid flagellates; **in:** *Biology of the Kinetoplastida (W.H.R. Lumsden and D.A. Evans, Eds.)*. Academic Press, London, pp. 1-34.

Vickerman, K., Myler, P.J. and Stuart, K.D. (1991) African trypanosomiasis, **in:** *Immunology and pathology of parasites (3rd edition) (K.S. Warren, Ed.)*. Blackwell, Boston, pp. 170-212.

Walgate, R. (1994) Sleeping sickness on the boil in Zaïre. *TDR News*, **46:** 6.

Wang, C.C. (1995) Molecular mechanisms and theurapeutic approaches to the treatment of African trypanosomiasis. *Annu. Rev. Pharmacol. Toxicol.* **35:** 93-127.

Ward, K. and Seib, P.A. (1970) Cellulose, lichenan and chitin, **in:** *The carbohydrates, chemistry and biochemistry (W. Pigman and D. Horton, Eds.)*. Academic Press, New York, pp. 413-415.

Weber, K. and Osborne, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406-4412.

Wells, J.T., Ferrari, E., Hemer, D., Estell, D. and Chen, E. (1983) Cloning, sequencing and expression of subtilisin from *B. subtilis*. *Nucl. Acids. Res.* **11:** 7911-7925.

Wilcox, D. and Mason, R.W. (1992) Inhibition of cysteine proteinases in lysosomes and whole cells. *Biochem. J.* **285:** 495-502.

Wilson, K. and Goulding, K.H. (1986) *A biologists guide to the principles and techniques of practical biochemistry*. Edward Arnold, Suffolk, pp. 127-133.

Wiser, M.F., Lonsdale-Eccles, J.D., D'Alessandro, A. and Grab, D.J. (1997) A cryptic protease activity from *Trypanosoma cruzi* revealed by preincubation with kininogen at low temperature. *Biochem. Biophys. Res. Comm.* 240: 540-544.

Wolga, J.I., Daniel-Ribeiro, C., Giallat, J., Stahl, J.P., Micoud, M. and Gentilini, M. (1981) Autoanticorps dans les trypanosomiases humaines africaines. Anticorps anti-muscle lisse au cours d'une maladie a *Trypanosoma gambiense*. *Bull. Soc. Path. Exotique* **74:** 676-684.

Yoshimoto, T., Kanatani, A., Shimoda, T., Inaoka, T., Kokubo, T. and Tsuru, D. (1991) Prolyl endopeptidase from *Flavobacterium meningosepticum*: cloning and sequencing of the enzyme gene. *J. Biochem.* **110:** 873-878.

Yoshimoto, T., Tabira, J., Kabashima, T., Inoue, S. and Ito, K. (1995) Protease II from *Moraxella lacunata*: cloning, sequencing, and expression of the enzyme gene, and crystallization of the expressed enzyme. *J. Biochem.* **117:** 654-660.

Yoshimoto, T., Miyazaki, K., Haraguchi, N., Kitazono, A., Kabashima, T. and Ito, K. (1997) Cloning and expression of the cDNA encoding prolyl oligopeptidase (prolyl endopeptidase) from bovine brain. *Biol. Pharm. Bull.* **20:** 1047-1050.

Youan, B.B.C., Coulibaly, S., Miezan, T.B., Doua, F. and Bamba, M. (1997) *In vivo* evaluation of sixteen plant extracts on mice innoculated with *Trypanosoma brucei gambiense*. *Bull. W.H.O.* **75:** 343-348.

Young, J.R. (1985) *The molecular genetics of antigenic variation in* Trypanosoma brucei. PhD thesis, Cambridge University, United Kingdom. 214 pp.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M. and Horvitz, H.R. (1993) The *C. elegans* cell death gene *ced*-3 encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell* **75:** 641-652.

Zak, B. (1958) Simple procedure for the single sample determination of serum copper and iron. *Clin. Chem. Acta* **3:** 328-334.

Ziemann, H. (1905) Beitrage zur trypanosomenfrage. *Zbl. Bakt. I. Orig.* **38:** 307 and 429.

Appendix 1

Triple and single-letter amino acid codes

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Homophenylalanine	hPhe	hF
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Pyroglutamic Acid	pGlu	pG
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix 2

Publications

Eur. J. Biochem. 238, 728–736 (1996)

Proteases from *Trypanosoma brucei brucei*

Purification, characterisation and interactions with host regulatory molecules

Linda TROEBERG, Robert N. PIKE, Rory E. MORTY, Ronald K. BERRY, Theresa H. T. COETZER and John D. LONSDALE-ECCLES

Department of Biochemistry, University of Natal, Scottsville, South Africa

(Received 16 February 1996) – EJB 96 0225/4

African trypanosomes contain proteases that may be released into the bloodstream of their infected hosts. This paper describes a novel, combined isolation of a cysteine proteinase (called trypanopain-Tb) and a serine oligopeptidase (which we call oligopeptidase-Tb) from *Trypanosoma brucei brucei*, as well as a comparison of the activities of these two enzymes against several host regulatory molecules.

The enzymes differed in various respects. Firstly, purified trypanopain-Tb was shown to readily cleave proteins such as gelatin maximally at acidic pH. In contrast, oligopeptidase-Tb, which is optimally active at alkaline pH, did not hydrolyse proteins larger than 4 kDa. However, it readily hydrolysed various polypeptides, including neurotensin and atrial natriuretic factor.

The interaction of the two enzymes with mammalian protease inhibitors also differed. Cystatins and α_2-macroglobulin effectively inhibited trypanopain-Tb, with the K_i values for cystatin C and low-molecular-mass kininogen ($\approx 10^{-11}$ M) predicting that trypanopain-Tb is likely to be effectively controlled by these inhibitors if released into the host bloodstream. In contrast, oligopeptidase-Tb was not inhibited by serpins or α_2-macroglobulin, suggesting that it may remain active if released into the host bloodstream. In support of these *in vitro* results, the blood of trypanosome-infected rats displayed no trypanopain-Tb-like activity, but exhibited high oligopeptidase-Tb-like activity. Thus, while trypanopain-Tb seems likely to be confined to an intracellular role within the parasite, oligopeptidase-Tb has the potential to remain active in the host bloodstream and so contribute directly to pathogenesis.

Keywords: *Trypanosoma brucei*; cysteine proteinase; oligopeptidase; cystatin.

African trypanosomes are protozoan parasites which cause the diseases nagana and sleeping sickness in cattle and humans, respectively. These diseases are characterised by intermittent fever, progressive anaemia, general loss of condition and, ultimately, the death of the infected host. Nagana is still of great economic importance in Africa, where it prevents livestock farming in many areas, and certain parts of Africa are experiencing a large resurgence in human trypanosomiasis (Walgate, 1994). While the parasite's major lysosomal cysteine proteinase (trypanopain) is considered a potentially important factor in the development of the disease (Authié et al., 1993; Russo et al., 1994), the role of a cytoplasmic serine oligopeptidase (which we call oligopeptidase-Tb, OP-Tb) has not yet been explored.

Trypanopain is lysosomally located (Mbawa et al., 1991) and, as such, is likely to be centrally involved in intracellular digestive and catabolic proteolysis. Additionally, the enzyme is proposed to help the parasite escape opsonisation by degrading internalised antibody-variant surface glycoprotein complexes (Russo et al., 1994). Enzyme released into the host bloodstream has also been proposed to contribute to pathogenesis more directly by degrading various host proteins. Also, since trypanotolerant cattle infected with *Trypanosoma congolense* produce antibodies to trypanopain-Tc, while susceptible cattle do not, immune targeting of the enzyme may help protect infected hosts (Authié et al., 1993). While the cDNA of trypanopain-Tb from *Trypanosoma brucei brucei* has been sequenced (Mottram et al., 1989), no purification of the enzyme to electrophoretic homogeneity, or characterisation of its basic enzymatic properties, has been reported. The interactions of trypanopain-Tb with mammalian cysteine proteinase inhibitors and other host regulatory molecules also have not been reported before.

While OP-Tb has previously been partially purified from *T. b. brucei* (Huet et al., 1992; Kornblatt et al., 1992) and *Trypanosoma cruzi* (Ashall, 1990), the role of this enzyme in pathogenesis has not been investigated. Previous data indicates that the enzyme is a serine protease, but its sensitivity to reagents which modify cysteine residues suggests that reduction of a cysteine residue is required for full enzyme activity (Kornblatt et al., 1992). The enzyme also appears to be restricted in its action, cleaving only small peptide substrates. Both these facets of the enzyme's activity require further investigation.

Here we report the joint purification of trypanopain-Tb and OP-Tb from *T. b. brucei*, the effects of various potential *in vivo* inhibitors on the enzymes and the action of the enzymes on potentially relevant host peptide and protein substrates.

Correspondence to T. H. T. Coetzer, Department of Biochemistry, University of Natal, Private Bag X01, Scottsville, South Africa 3209

Fax: +27 331 260 5462.

Phone: +27 331 260 5467.

Abbreviations. α_2-M, α_2-macroglobulin; Boc, butoxycarbonyl; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; $H_2NEtPhSO_2F$, 4-(2-aminoethyl)-benzenesulfonyl fluoride; iPr_2P-F, di-isopropyl fluorophosphate; L-kininogen, low-molecular-mass kininogen; MeO-Suc, methoxysuccinyl; NHMec, aminomethyl coumarin; OP-Tb, trypanosomal oligopeptidase; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; rTM, rat trypanopain modulator; Suc, succinyl; SBTI, soybean trypsin inhibitor; $TosLysCH_2Cl$, tosyllysylchloromethane; $TosPheCH_2Cl$, tosylphenylalanylchloromethane; Z, benzyloxycarbonyl.

MATERIALS AND METHODS

Materials. HiLoad™ Q-Sepharose and HiTrap™ Blue Sepharose were from Pharmacia. DEAE-cellulose was from What-

man. 4-(2-Aminoethyl)-benzenesulfonyl fluoride ($H_2NEtPhSO_2F$), BSA and α_2-macroglobulin (α_2-M) were from Boehringer Mannheim. Chymostatin, leupeptin and antipain were from Cambridge Research Chemicals (UK) and di-isopropyl fluorophosphate (iPr_2P-F) was from Fluka. [1-^{14}C]Acetic anhydride was from Amersham. All other inhibitors, benzyloxycarbonyl phenylanlanyl-arginyl-aminomethyl coumarin (Z-Phe-Arg-NHMec), Z-Arg-Arg-NHMec, pepstatin-A−Sepharose, poly-(L-lysine)-Sepharose, fibrinogen, rabbit IgG, Percoll™ and Sephacryl S-100 HR were from Sigma. The C18 HPLC column was from Vydac. Sheep stefin B was purified as described by Pike et al. (1992). Human recombinant stefin A was from CalBiochem and human low-molecular-mass kininogen (L-kininogen) was purified from human plasma as described by Gounaris et al. (1984). Recombinant human L-kininogen domain 3 was a gift from Dr Ennes Auerswald (Ludwig-Maximilians-Universität München) human cystatin C was a gift from Dr Magnus Abrahamson (University of Lund, Malmö General Hospital) and serpins were a gift from Dr Jan Potempa (Jagiellonian University, Cracow).

Growth and purification of trypanosomes. *T. b. brucei* (clone ILTat 1.1) was grown in rats and purified from the infected blood by a combination of Percoll™ gradient centrifugation (Grab and Bwayo, 1982) and anion-exchange chromatography on DEAE-cellulose (Lanham and Godfrey, 1970). Purified trypanosomes were stored at −70°C until used.

Enzyme and protein assays. Trypanopain-Tb activity against 5 μM Z-Phe-Arg-NHMec at 37°C was initially measured as described by Barrett and Kirschke (1981) using an assay buffer of 170 mM sodium acetate, 30 mM acetic acid, 2 mM Na_2EDTA, 3 mM dithiothreitol, pH 5.5. Following enzyme characterisation, the assay buffer was amended to 200 mM sodium phosphate pH 7.0 containing 30 mM cysteine and 2 mM Na_2EDTA. OP-Tb was similarly assayed against 5 μM Z-Arg-Arg-NHMec in 100 mM Tris/HCl, 10 mM dithiothreitol, pH 8.0 (Kornblatt et al., 1992).

Protein assays were conducted according to the methods of Bradford as modified by Read and Northcote (1981) and Smith et al. (1985).

Isolation of trypanopain-Tb and OP-Tb. Trypanosomes (≈5.5×10^9 parasites) were thawed and lysed by addition of 0.1% (by vol.) Triton X-100 (final concentration). Three-phase partitioning (Pike and Dennison, 1989) was performed on the lysate, with all enzyme activity precipitating between 10−25% (mass/vol. of the total volume) ammonium sulfate (results not shown). The pellet was redissolved in buffer A (20 mM sodium acetate, 1 mM Na_2EDTA, pH 5.5) and loaded onto HiLoad™ Q-Sepharose (26×110 mm, flow rate 1 ml · min^{-1}) equilibrated in buffer A with 100 mM NaCl. After elution of the unbound material (which contained OP-Tb and was retained for OP-Tb purification), a linear gradient of 0.1−1 M NaCl in buffer A was applied over five column volumes. Fractions active against Z-Phe-Arg-NHMec, but inactive against Z-Arg-Arg-NHMec and therefore identified as containing trypanopain-Tb, were pooled and dialysed against buffer A. Since preliminary studies had identified pepstatin A as a weak inhibitor of trypanopain-Tb, this inhibitor was considered to be a potential affinity chromatography ligand. Active samples were therefore loaded onto pepstatin-A−Sepharose (13×10 mm, flow rate 0.26 ml · min^{-1}) equilibrated in buffer A containing 500 mM NaCl. Following elution of the unbound material, trypanopain-Tb was eluted with 5% (mass/vol.) Brij-35 in buffer A with 500 mM NaCl. Fractions active against Z-Phe-Arg-NHMec were applied to HiTrap™ Blue Sepharose (9×23 mm, flow rate 1 ml · min^{-1}) equilibrated in 50 mM Tris/HCl pH 8.0 to remove suspected albumin contamination. Active fractions were concentrated and stored at −20°C mixed with glycerol (1:1). The purity of the enzyme was analysed by tricine/SDS/PAGE (Shägger and von Jagow, 1987).

The unbound material from HiLoad™ Q-Sepharose was active against Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec, but insensitive to L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64), indicating that it contained OP-Tb (Kornblatt et al., 1992). This fraction was dialysed against buffer B (50 mM Tris/HCl, 1 mM dithiothreitol, pH 8.0) and applied to *p*-aminobenzamidine−Sepharose (15×50 mm, flow rate 0.3 ml · min^{-1}). After the elution of unbound material, bound OP-Tb was eluted with a step gradient of 250 mM NaCl in buffer B. Following dialysis against buffer B, the sample was applied to HiLoad™ Q-Sepharose equilibrated in buffer B containing 100 mM NaCl, and a five-column-volume gradient of 0.1−1 M NaCl applied. The active fractions were diluted four-fold in buffer B and applied to poly-(L-lysine)−Sepharose (55×15 mm, flow rate 1 ml · min^{-1}) equilibrated in buffer B containing 100 mM NaCl. A 10-column-volume gradient of 0.1−1.5 M NaCl in buffer B was applied to elute the bound material. Active fractions were concentrated and applied in buffer B to a Sephacryl S-100 HR gel filtration column (15×850 mm, flow rate 10 ml · h^{-1}). Following analysis of enzyme purity by tricine/SDS/PAGE, the purified enzyme was concentrated and stored at −20°C mixed with glycerol (1:1).

Characterisation of enzymes. The activity of trypanopain-Tb and OP-Tb in the presence of various inhibitors was investigated by incubating the enzymes with each inhibitor for 15 min at 37°C before assaying residual activity against Z-Phe-Arg-NHMec or Z-Arg-Arg-NHMec, respectively, as described above. OP-Tb experiments were conducted in the presence of either 10 mM dithiothreitol (indicated as + dithiothreitol in Table 2) or in residual dithiothreitol remaining from purification (≈40 μM dithiothreitol, indicated as − dithiothreitol in Table 2). Since chloromethanes and alkylating agents are inactivated by reducing agents, care was taken to minimise the relative amount of reducing agents used with these inhibitors. Concentrated enzymes were thus activated (10 min at 37°C in 30 mM cysteine for trypanopain-Tb; 10 mM dithiothreitol for OP-Tb), before being diluted to the required working concentration in assay buffer containing inhibitor (final reducing agent concentrations of 3 mM cysteine for trypanopain-Tb, 1 mM dithiothreitol for OP-Tb).

The effects of various reducing agents on trypanopain-Tb and OP-Tb activity were investigated by adding dithiothreitol, cysteine · HCl, reduced glutathione or 2-mercaptoethanol (1−100 mM for trypanopain-Tb and 1−25 mM for OP-Tb) to the relevant assay buffer. The pH profile of each enzyme against synthetic substrates was investigated by substituting assay buffer with constant-ionic-strength acetate/Mes/Tris buffers (100 mM acetate, 200 mM Tris, 100 mM Mes, 4 mM Na_2EDTA) of pH 4.0−9.0 (Ellis and Morrison, 1982). Trypanopain-Tb hydrolysis of [^{14}C]gelatin at various pH values was assessed using acetate/Mes/Tris buffers as described above, with the [^{14}C]gelatin prepared using [1-^{14}C]acetic anhydride as described by Cawston and Barrett (1979). The pH stability of the enzymes was determined by incubating the enzymes in acetate/Mes/Tris buffers (25 mM acetate, 50 mM Tris, 25 mM Mes, 1 mM Na_2EDTA) for 1 h at 37°C before addition of a pH-7.0 assay buffer and Z-Phe-Arg-NHMec for trypanopain-Tb, or pH-8.0 assay buffer and Z-Arg-Arg-NHMec for OP-Tb. The initial incubation was done either in the presence or absence of reducing agents (30 mM cysteine for trypanopain-Tb and 10 mM dithiothreitol for OP-Tb).

Following determination of the concentration of active trypanopain-Tb by titration with E-64 (Barrett and Kirschke, 1981),

trypanopain-Tb (0.05 pmol) was incubated with readily hydrolysed synthetic substrates (25–100 μM) for 10 min at 37°C in continuous assays, or with poorly hydrolysed synthetic substrates for 4 h at 37°C in stopped time assays. K_m, V_{max} and k_{cat} were determined from the direct linear plot (Eisenthal and Cornish-Bowden, 1974) using the software package Hyper 1.01 (© 1992-1993, J. S. Easterby, UK).

Digestion of protein and peptide substrates by trypanopain-Tb and OP-Tb. Fibrinogen, BSA and rabbit IgG were digested with trypanopain-Tb at 37°C over 1 h at various molar ratios of enzyme/substrate in 100 mM Tris/HCl, 30 mM cysteine, pH 7.4. After the reactions were stopped by the addition of 1 mM E-64, samples were boiled in reducing treatment buffer and analysed by electrophoresis on 10% tricine gels (Shägger and von Jagow, 1987). Trypanopain-Tb digestion of serpins was analysed at 37°C at 1:100 and 1:10 molar ratios of enzyme/inhibitor in 25 mM Tris, 10 mM cysteine, pH 7.4. Reactions were stopped by the addition of 20 μM E-64. To determine the extent of serpin degradation, remaining inhibition of trypsin or chymotrypsin was determined (Potempa et al., 1986) and the degradation products analysed by tricine/SDS/PAGE.

OP-Tb was incubated with oligopeptide substrates at various molar ratios of enzyme/substrate in 0.1 M Tris/HCl, 10 mM dithiothreitol, pH 8.0 for 1–16 h at 37°C, after which the reaction was stopped by acidification with an equal volume of 5% (by vol.) trifluoroacetic acid. The samples were placed on ice until analysed by HPLC on a C18 column (flow rate 1 ml · min^{-1}) using a gradient of 10–90% (by vol.) acetonitrile in water containing 0.1% (by vol.) trifluoroacetic acid.

Association with cystatins and α_2-M. The kinetics of trypanopain-Tb inhibition by various cystatins were determined as described by Salvesen and Nagase (1992) with data treatment as described by Henderson (1972). Trypanopain-Tb and OP-Tb association with α_2-M was assessed by comparing the elution volumes from Sephacryl S-100 (for trypanopain-Tb) and Sephacryl S-200 (for OP-Tb) (both 15×300 mm, flow rate 10 cm · h^{-1}, equilibrated with 50 mM Tris/HCl pH 8.0 containing 1 mM dithiothreitol for OP-Tb only) of each enzyme alone with that for each enzyme previously incubated with bovine α_2-M (≈ 1:1 molar ratio, Mason, 1989).

Measurement of enzyme activity in the blood of infected rats. The blood of trypanosome-infected rats was harvested by cardiac puncture at peak parasitaemia, and centrifuged (3000×*g*, 25 min, 4°C). The supernatant was microscopically confirmed to be free of both parasites and blood cells, and then assayed against Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec in the presence of various inhibitors. Since OP-Tb, rat plasma kallikrein and trypanopain-Tb all hydrolyse Z-Phe-Arg-NHMec, soybean trypsin inhibitor (SBTI, 20 μM), $H_2NEtPhSO_2F$ (1.85 mM) and E-64 (1.7 mM) were used to discriminate between the activities of the three enzymes. SBTI inhibits blood plasma kallikrein (Coleman and Bagdasarian, 1976) but not OP-Tb, while $H_2NEtPhSO_2F$ inhibits both kallikrein and OP-Tb, so these inhibitors were used to discriminate between the two activities. Hydrolysis of Z-Arg-Arg-NHMec in the presence of these inhibitors was additionally investigated to confirm the presence of OP-Tb.

RESULTS

Proteinase isolation. As far as we are aware, this is the first time trypanopain-Tb and OP-Tb have been completely and demonstrably purified from *T. b. brucei* (Fig. 1). Three-phase partitioning proved to be a very efficient crude purification method, removing large quantities of extraneous trypanosomal proteins

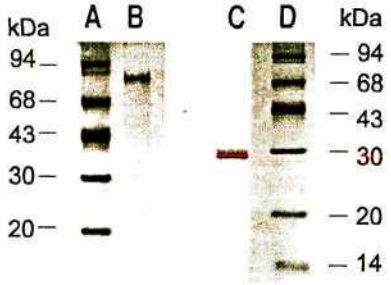


Fig. 1. Silver-stained tricine/SDS/PAGE of trypanopain-Tb and OP-Tb. OP-Tb (lane B, 250 ng) and trypanopain-Tb (lane C, 250 ng) were treated with non-reducing buffer and electrophoresed on a 10% tricine/SDS/PAGE gel prior to silver staining (Blum et al., 1987). Molecular mass markers, shown in lanes A and D, are phosphorylase *b* (94 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), SBTI (20 kDa) and α-lactalbumin (14.4 kDa).

(Table 1). Additionally, the salt remaining from this procedure reduced the amount of protein binding to HiLoad™ Q-Sepharose at pH 5.5, and thus increased the efficiency of this step for trypanopain-Tb purification. This initial chromatography step on HiLoad™ Q-Sepharose also separated OP-Tb from trypanopain-Tb (Fig. 2), taking advantage of the unusual affinity of trypanopain-Tb for this anion exchanger at pH 5.5 and making it possible for the two enzymes to be purified from the same trypanosome preparation. The current method is thus more economical and convenient than previously reported approaches.

Various affinity chromatography resins were tested for the purification of trypanopain-Tb. The high affinity with which inhibitors, such as stefin B and chicken egg white cystatin, bound trypanopain-Tb rendered these ligands unsuitable for affinity chromatography purposes. While pepstatin A typically inhibits aspartic proteinases, it surprisingly also inhibits trypanopain-Tb weakly (Pamer et al., 1989) and was thus considered a potentially suitable affinity chromatography ligand. Pepstatin-A–Sepharose chromatography proved to be a very successful purification step, with electrophoresis of the column eluate showing that trypanopain-Tb was contaminated only by a 68-kDa protein, suspected to be rat serum albumin. This contaminant was successfully removed by HiTrap™ Blue Sepharose. The speed of the trypanopain-Tb purification procedure was found to be critical for maintenance of enzyme activity, with activity irreversibly lost if more than one day was taken for purification.

Two affinity chromatography steps were used in the purification of OP-Tb, namely *p*-aminobenzamidine–Sepharose and poly-(L-lysine)–Sepharose chromatography. Since benzamidine is a fairly weak inhibitor of OP-Tb (62% inhibition, Table 2), OP-Tb could be eluted from this column using fairly low salt concentrations (250 mM NaCl). Poly-(L-lysine)-Sepharose, on the other hand, bound OP-Tb more strongly, with approximately 450 mM NaCl required to elute the enzyme. While *p*-aminobenzamidine–Sepharose was particularly effective in removing large quantities of contaminating protein (increasing the specific activity 30-fold, Table 1), poly-(L-lysine)–Sepharose was essential for removing persistent contamination by a 68-kDa protein, shown by amino acid analysis to be rat albumin, as well as other minor contaminants.

Trypanopain-Tb characterisation. Trypanopain-Tb activity against Z-Phe-Arg-NHMec was optimal across a plateau between pH 5.5 and 8.0 (Fig. 3). The enzyme was optimally stable between pH 4.0 and 7.0 in the presence of cysteine, and between pH 4.0 and 8.0 in the absence of cysteine. At all pH values tested, more trypanopain-Tb activity was maintained after a 1-h

Table 1. Purification table for the isolation of trypanopain-Tb and OP-Tb from *T. b. brucei*

Enzyme	Fraction	Total protein	Total activity	Specific activity	Purification	Yield
		mg	pmols · s^{-1}	pmol · s^{-1} · mg^{-1}	-fold	%
Trypanopain-Tb	Lysate	213	4562	22	1	100
	Three-phase partitioning	9	1144	127	6	25
	HiLoad™ Q-Sepharose	0.2	397	1 985	90	9
	Pepstatin-A−Sepharose	0.04	252	6 300	286	5.5
	HiTrap™ Blue Sepharose	0.01	245	24 500	1114	5.3
OP-Tb	Lysate	213	3245	15	1	100
	Three-phase partitioning	9	2044	227	15	63
	HiLoad™ Q-Sepharose (pH 5.5)	8.6	1947	226	15	60
	p-Aminobenzamidine−Sepharose	0.211	1429	6 773	452	44
	HiLoad™ Q-Sepharose (pH 8)	0.134	1428	10 657	710	44
	Poly(L-lysine)−Sepharose	0.104	941	9 048	603	29
	Sephacryl S-100	0.068	909	13 368	891	28

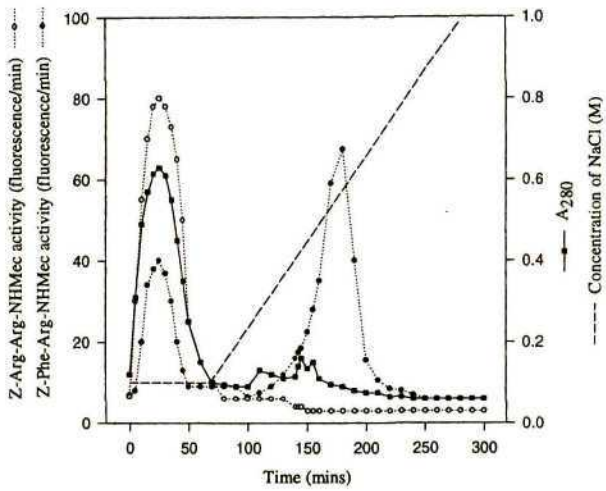


Fig. 2. Elution profile of HiLoad™ Q-Sepharose showing separation of trypanopain-Tb and OP-Tb. HiLoad™ Q-Sepharose (26×110 mm, flow rate 1 ml · min^{-1}) was equilibrated in loading buffer (100 mM NaCl in 20 mM sodium acetate, 1 mM Na_2EDTA, 100 mM NaCl, 0.02% (mass/vol.) NaN_3, pH 5.5). The 10−25% $(NH_4)_2SO_4$ fraction of the three-phase partitioning was loaded onto the column and the bound material eluted with a five-column-volume gradient of 0.1−1 M NaCl. While OP-Tb fractions (unbound) were active against both Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec, trypanopain-Tb-containing fractions (bound) were active against only Z-Phe-Arg-NHMec.

incubation in the absence of cysteine than in its presence, indicating that the active enzyme undergoes some autocatalysis. Trypanopain-Tb was active against [^{14}C]gelatin over a far narrower and more acidic pH range than against Z-Phe-Arg-NHMec, with hydrolysis being optimal at pH 5.7 (Fig. 3). While activity decreased sharply on either side of this pH, trypanopain-Tb was still 44% active at pH 7.4, suggesting that it would be active under physiological pH conditions.

Trypanopain-Tb was only active in the presence of reducing agents, thus displaying typical cysteine proteinase latency. Cysteine · HCl was the most effective of the reducing agents tested, with an optimal final concentration of 30 mM. Dithiothreitol was maximally effective at 20 mM, with 2-mercaptoethanol reaching optimum efficiency at much higher concentrations (250 mM). Reduced glutathione was a comparatively poor activator. All subsequent trypanopain-Tb assays against Z-Phe-Arg-NHMec were thus performed at pH 7.0 (in the middle of the optimal pH range) using 200 mM sodium phosphate containing 30 mM cysteine and 2 mM Na_2EDTA (final concentrations).

Table 2. Effects of various inhibitors on trypanopain-Tb activity against Z-Phe-Arg-NHMec and OP-Tb activity against Z-Arg-Arg-NHMec at 37°C. Enzymes were incubated with each inhibitor for 15 min at 37°C and then assayed as usual. OP-Tb experiments were conducted in the presence of either 10 mM dithiothreitol (indicated as + dithiothreitol) or in residual dithiothreitol remaining from purification (≈ 40 μM dithiothreitol, indicated as − dithiothreitol). Where different concentrations of inhibitor were used for OP-Tb and trypanopain-Tb, the concentrations for trypanopain-Tb are given first. IAA, iodoacetate; IAN, iodoacetamide; NEM, *N*-ethylmaleimide; nd, not determined.

Inhibitor	Concentration	Activity of		
		Trypanopain-Tb	OP-Tb activity	
			− dithiothreitol	+ dithiothreitol
	mM	% of control		
E-64	0.001/0.2	0	70	85
IAA	0.1/10	0	8	59
NEM	10	0	21	16
IAN	10	0	90	67
Oxidised glutathione	10	100	182	100
$H_2NEtPhSO_2F$	0.5	100	0	3
iPr_2P-F	10	100	14	9
$PhMeSO_2F$	10	100	100	95
$TosLysCH_2Cl$	1	0	5	5
$TosPheCH_2Cl$	1	0	78	89
Leupeptin	0.1	0	3	2
Antipain	0.1	0	8	8
Chymostatin	0.1	1	35	37
Benzamidine	10	nd	33	38
Aprotinin	0.03	nd	56	36
SBTI	0.01	100	100	100
EDTA	10	100	154	103
Pepstatin A	0.1	78	100	100

Typical cysteine proteinase inhibitors (including E-64 and iodoacetate) inhibited trypanopain-Tb completely, while typical serine and metalloproteinases inhibitors such as iPr_2P-F and EDTA, respectively, had no effect on enzyme activity (Table 2). Leupeptin, antipain and chymostatin were all effective inhibitors of the enzyme (99−100% inhibition). Interestingly, pepstatin A (22% inhibition) also inhibited trypanopain-Tb.

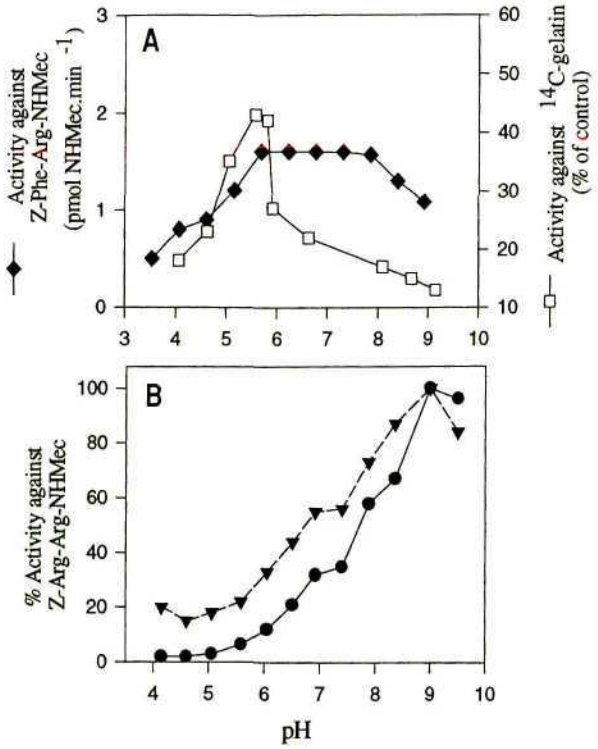


Fig. 3. pH profile of trypanopain-Tb and OP-Tb activity at 37°C. Enzyme activity at various pH values was assessed using constant-ionic-strength acetate/Mes/Tris buffers (Ellis and Morrison, 1982) of pH 4.0–9.0. (**A**) Trypanopain-Tb (1.5 ng) activity against 5 μM Z-Phe-Arg-NHMec (–◆–) and [^{14}C]-gelatin (–□–); (**B**) OP-Tb (2 ng) activity against 5 μM Z-Arg-Arg-NHMec in the presence (●) or absence (▼) of 10 mM dithiothreitol.

Table 3. Kinetic constants for trypanopain-Tb hydrolysis of various NHMec substrates. Values are calculated from 10-min or 4-h stopped-time assays. Constants were calculated at 37°C using the direct linear plot (Eisenthal and Cornish-Bowden, 1974). No hydrolysis occurred in 4 h with Suc-Ala-Ala-Phe-NHMec, Glu-Gly-Gly-Phe-NHMec, Z-Gly-Pro-NHMec, Gly-NHMec, Leu-NHMec, Ac-Ala-Ala-Tyr-NHMec, Ac-Ala-Ala-Pro-Ala-NHMec, Z-Pro-Arg-NHMec, Glu-Gly-Arg-NHMec, Z-Gly-Gly-Arg-NHMec, Boc-Val-Gly-Arg-NHMec, Boc-Leu-Gly-Arg-NHMec, Boc-Ile-Glu-Gly-Arg-NHMec, Z-Arg-NHMec, Z-Ala-Arg-Arg-NHMec or Arg-NHMec.

Substrate	Time	K_m	k_{cat}	k_{cat}/K_m
		μM	s^{-1}	$s^{-1}\,\mu M^{-1}$
Z-Arg-Arg-NHMec	4 h	12	0.126	0.01
Z-Phe-Arg-NHMec	10 min	1.2	13.4	11.3
Pro-Phe-Arg-NHMec	10 min	4.2	4.2	1
Val-Leu-Lys-NHMec	10 min	25	8.2	0.3
Ala-Leu-Lys-NHMec	10 min	27	6.5	0.24
Suc-Ala-Phe-Lys-NHMec	10 min	42	0.006	0.002
Ala-Phe-Lys-NHMec	10 min	28	5.2	0.19
Suc-Leu-Tyr-NHMec	4 h	54	0.05	0.001
MeOSuc-Asp-Tyr-Met-NHMec	4 h	68	0.09	0.001
MeOSuc-Gly-Trp-Met-NHMec	4 h	4	0.04	0.011

Trypanopain-Tb degradation of synthetic substrates and proteins. Of the synthetic substrates tested, Z-Phe-Arg-NHMec was most readily cleaved by trypanopain-Tb, with a K_m of 1.2 μM and k_{cat} of 13.4 s^{-1} (Table 3). Pro-Phe-Arg-NHMec was also readily hydrolysed. All other substrates tested, including Z-Arg-Arg-NHMec used to monitor OP-Tb isolation, were comparatively poorly hydrolysed.

Trypanopain-Tb hydrolysed fibrinogen, BSA and rabbit IgG at physiological pH. At a 1:100 molar ratio of enzyme/substrate, both the α and β chains of fibrinogen were substantially degraded within 15 min at pH 7.4, while the γ chain remained resistant over the test period (1 h). However, at a 1:1000 molar ratio of enzyme/substrate, no degradation occurred. Trypanopain-Tb also readily degraded BSA (1:500 and 1:1000 molar ratio) and IgG (1:100 and 1:1 000 molar ratio) within 15 min, hydrolysing the latter predominantly in the heavy-chain region.

Characterisation of OP-Tb. OP-Tb activity was substantially increased by the presence of reducing agents; dithiothreitol was the most effective, increasing activity 10-fold at 25 mM and 6-fold at 1 mM. Cysteine was almost as effective as dithiothreitol at lower concentrations, but activity was lowered at concentrations above 5 mM. Glutathione was about half as effective as dithiothreitol.

Irrespective of the presence or absence of reducing agents, OP-Tb was found to be most active between pH 8 and 9.5 at 25°C (Fig. 3). In contrast, the pH stability of the purified enzyme was slightly affected by the presence of a reducing agent, with the enzyme being most stable in the absence of dithiothreitol in the pH range 6–7.5 and in the presence of dithiothreitol at pH 8–9. These differences, however, were all ranged between 80–100% activity and it was only below pH 6 and above pH 9 that stability decreased significantly.

The serine proteinase inhibitors iPr_2P-F and $H_2NEtPhSO_2F$ completely inhibited OP-Tb, supporting the hypothesis that OP-Tb is a serine protease (Table 2). Leupeptin and antipain were very effective inhibitors of OP-Tb, as was tosyllysylchloromethane ($TosLysCH_2Cl$). However, a basic residue was required for the chloromethane to be active, as shown by the lack of inhibition tosylphenylalanylchloromethane ($TosPheCH_2Cl$). The enzyme was not inhibited by phenylmethylsulfonyl fluoride ($PhMeSO_2F$) or Z-Phe-Phe-CHN_2. Benzamidine was found to be a weak inhibitor of the enzyme, possibly explaining the success of *p*-aminobenzamidine–Sepharose affinity chromatography as a step in the isolation of OP-Tb. EDTA increased OP-Tb activity, indicating that the enzyme is not stabilised by divalent cations. While E-64 had no effect on activity, iodoacetate, iodoacetamide and *N*-ethylmaleimide, which modify cysteine residues, all reduced OP-Tb activity to various degrees (Table 2).

Cleavage of protein and peptide substrates by OP-Tb. OP-Tb did not hydrolyse fibrinogen, BSA, ovalbumin, carbonic anhydrase, SBTI, lysozyme, cystatin C or cytochrome *c* at pH 8.0. Therefore, as speculated by Kornblatt et al. (1992), the enzyme appears to be an oligopeptidase rather than a proteinase. We thus examined a range of substrates of increasing molecular mass in order to characterise the minimum size of substrates excluded from the active site of the enzyme. Since OP-Tb cleaves most efficiently after arginine residues (Kornblatt et al., 1992), only peptides containing such residues were tested. Where possible, peptides containing two consecutive basic residues were chosen to increase the likelihood of hydrolysis. Additionally, peptides with regulatory roles in the host system were chosen in order to shed light on the potential physiological relevance of OP-Tb.

OP-Tb was found to completely cleave reduced [Arg^8]vasopressin (1084.2 Da, CYFQNCPRG) and [Lys^8]vasopressin (1056.2 Da; CYFQNCPKG; 1:100 molar ratios) in 4 h, while the oxidised forms of these peptides remained uncleaved after 16 h. Neurotensin (1672.9 Da, ELYENKPRRPYIL) was also completely hydrolysed in 4 h at a 1:500 molar ratio of enzyme/

Table 4. Equilibrium constant for inhibition, association and dissociation rate constants for cystatin inhibition of trypanopain-Tb (0.05 nM) at 30C. Note that the associations of cystatin C and L-kininogen domain 3 with trypanopain-Tb was too rapid for accurate measurement and the value given is an estimate.

Inhibitor	K_i	k_{on}	Predicted k_{off}
	nM	$M^{-1} \cdot s^{-1}$	s^{-1}
Human stefin A	0.045	2.13×10^7	9.6×10^{-4}
Sheep stefin B	0.004	6.9×10^7	2.8×10^{-4}
Human cystatin C	0.001	$\approx 1.7 \times 10^8$	1.7×10^{-4}
Human L-kininogen	0.0035	2.5×10^7	8.8×10^{-5}
Human L-kininogen domain 3	0.0044	$\approx 1.1 \times 10^8$	4.8×10^{-4}

Table 5. Effect of inhibitors on Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec activity in the blood of rats infected with *T. b. brucei*. The blood of infected rats was assayed against synthetic substrates in the absence or presence of various inhibitors for 30 min at 37°C. Values are in arbitrary units.

Substrate	Rats	Total activity with			
		no inhibitors	E-64 (1.67 mM)	SBTI (20 μM)	$H_2NEtPhSO_2F$ (1.85 mM)
Z-Phe-Arg-NHMec	infected	1521	1585	1573	297
	uninfected	234	177	193	193
Z-Arg-Arg-NHMec	infected	2244	2372	2284	97
	uninfected	104	97	74	55

peptide. The point of cleavage in this instance was determined to be after the second arginine residue in the sequence, reinforcing the specificity of OP-Tb for two consecutive basic residues in a given sequence. Atrial natriuretic factor (3080.5 Da, SLRRSSCFGGRMDRIGAQSGLGCNSFRY) was effectively cleaved at a 1:100 molar ratio, with the reduced form of the peptide undergoing more cleavage than the oxidised form. The importance of the state of substrate reduction suggests that substrate conformation is critical for OP-Tb activity. Peptides such as angiotensin I (1296.5 Da, DRVYIHPFHL), substance P (1347.6 Da, RPKPQQFFGLM) and oxidised insulin B chain (3495.9 Da, FVNQHLCGSHLVEALYLVCGERGFFYTPKA; 1:100 molar ratios) were not cleaved after 16 h, possibly due to the particular amino acid residues surrounding the basic residues present in these peptides (Kornblatt et al., 1992), although it is possible that a size restriction was the reason for non-cleavage of the insulin B chain. Pancreatic polypeptide (4181.7 Da) and growth-hormone-releasing factor (5039.7 Da) were also not cleaved, possibly due to the sizes of these peptides. OP-Tb thus does not appear to cleave peptides larger than 4 kDa. Further investigation of peptides between 3–4 kDa which contain suitable consecutive basic residues is needed to clarify the exclusion limit more finely.

Interaction of trypanopain-Tb and OP-Tb with host proteinase inhibitors. k_{on} and predicted k_{off} values for trypanopain-Tb association with various cystatins were in the $10^7-10^8\,M^{-1} \cdot s^{-1}$ and 10^{-4} s^{-1} range, respectively, while K_i values were in the 10^{-11} M range (Table 4). Cystatin C and L-kininogen were the most effective inhibitors. Interestingly, very little difference was observed between the inhibition by whole L-kininogen and by domain 3 of the molecule, suggesting that this domain is likely to be predominantly responsible for the inhibition observed with the whole molecule. Assuming a plasma concentration of 7.5 μM for L-kininogen and 80 nM for cystatin C (Abrahamson, 1993), trypanopain-Tb would be expected to associate very rapidly with these inhibitors: the half-life (Bieth, 1980) of the enzyme in the presence of L-kininogen can be calculated as 0.006 s and with cystatin C, 0.08 s. The inhibitor-enzyme complexes can also be predicted to dissociate slowly, with a half-life of 131 min for L-kininogen and 67 min for cystatin C. Trypanopain-Tb is thus likely to be inhibited effectively by cystatin C and (especially) L-kininogen *in vivo*. Additionally, trypanopain-Tb bound to α_2-M (≈ 1:1 molar ratio) and is thus also likely to be effectively controlled by this inhibitor *in vivo*.

Trypanopain-Tb was found to cleave the serpin inhibitor antithrombin III, but only at enzyme/inhibitor ratios of 1:10, so it is unlikely that such hydrolysis would occur *in vivo*. Trypanopain-Tb did not cleave other serpins, such as α_1-proteinase inhibitor, α_1-antichymotrypsin and α_2-antiplasmin.

OP-Tb did not interact with any of the tested host system inhibitors, including serpins, cystatins and α_2-M, presumably due to the limited interaction of the active site with higher-molecular-mass proteins in general.

Measurement of enzyme activity in infected rat blood. High levels of activity against both Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were detected in the blood of infected rats, while little activity was detectable in the blood of control, uninfected animals (Table 5). This activity was insensitive to E-64 and SBTI, but entirely eliminated by $H_2NEtPhSO_2F$. OP-Tb is thus likely to be predominantly responsible for the hydrolysis observed, suggesting that OP-Tb may be released into the bloodstream of infected rats. Secondly, this study supports the conclusion arrived at from *in vitro* studies with the purified enzymes, namely that while trypanopain-Tb is likely to be effectively controlled in the bloodstream, OP-Tb seems to remain active.

DISCUSSION

Isolation of trypanopain-Tb and OP-Tb. A cysteine proteinase has previously been isolated from *T. congolense* (Mbawa et al., 1992) but we have now purified one from *T. b. brucei*. OP-Tb from *T. b. brucei* has been partially purified and characterised (Kornblatt et al., 1992), but here we report the purification of the enzyme to homogeneity as well as a more complete characterisation of certain aspects of its activity. The linked isolation of these proteinases as reported here has the added advantage of maximising the yield of both enzymes from a single parasite preparation.

Characterisation of trypanopain-Tb. Trypanopain-Tb was shown to be a fairly typical cysteine proteinase, similar to trypanopain-Tc from *T. congolense* (Mbawa et al., 1992) and cruzipain from *T. cruzi* (Cazzulo et al., 1989). Interestingly, the aspartic proteinase inhibitor pepstatin A inhibits trypanopain-Tb slightly (Table 2; Pamer et al., 1989). In contrast, pepstatin A inhibits trypanopain-Tc by only 9.7% (Mbawa et al., 1992) and does not affect cruzipain (Murta et al., 1990; Cazzulo et al., 1989). Pepstatin A appears to be a competitive inhibitor of trypanopain-Tb (data not shown), and may act as a substrate analogue. This unusual inhibition was successfully exploited in the purification of trypanopain-Tb, using pepstatin-A–Sepharose.

Trypanopain-Tb was only active in the presence of reducing agents, and appeared to be quite similar to cathepsin L (Dennison et al., 1992) in its requirement of fairly high concentra-

tions of reducing agent for optimal activity. It was shown to have cathepsin-L-like specificity for synthetic substrates, hydrolysing Z-Phe-Arg-NHMec and Pro-Phe-Arg-NHMec most rapidly of the substrates tested. Generally, only substrates with basic residues such as Arg or Lys in P_1 and hydrophobic residues such as Phe or Leu in P_2 were hydrolysed (Table 3). The presence of an acidic residue in P_4 appeared to be unacceptable. Similar specificity has been reported for trypanopain-Tc (Mbawa et al., 1992), cruzipain (Cazzulo et al., 1990) and mammalian cathepsin L (Barrett and Kirschke, 1981; Dufour and Ribadeau-Dumas, 1988). Minimal hydrolysis of Z-Arg-Arg-NHMec was observed in the current study (4-h incubation required to demonstrate hydrolysis), allowing this substrate to be used for monitoring OP-Tb isolation. This contradicts a previous report by Pamer et al. (1991) that trypanopain-Tb cleaves Z-Arg-Arg-NHMec readily, with a K_m of 3.2 μM, although it must be noted that only partially purified trypanopain-Tb was used in their study. Trypanopain-Tc does not hydrolyse Z-Arg-Arg-NHMec (Mbawa et al., 1992), while cruzipain cleaves it poorly (K_m 41 μM; Lima et al., 1992).

Of the serpins tested, only antithrombin III was cleaved by trypanopain-Tb. Interestingly, cathepsin L, which has been found to be one of the closest mammalian analogues to trypanopain-Tb (Mottram et al., 1989), cleaves α_1-proteinase inhibitor very effectively (Johnson et al., 1986). Cathepsin L also cleaved antithrombin III and α_2-antiplasmin very effectively at much lower enzyme/substrate ratios than did trypanopain-Tb (data not shown). This indicates that these two enzymes, while very similar in substrate specificity, are not always comparable.

Effect of host proteinase inhibitors on trypanopain-Tb. It has been postulated that if released into the host bloodstream, trypanopain-Tb could contribute to pathogenesis by degrading various host proteins. For this to occur, the enzyme would have to remain active in the presence of host proteinase inhibitors such as cystatins, kininogens and α_2-M. Trypanopain-Tb is, however, effectively inhibited by kininogens, various cystatins and α_2-M *in vitro* (Table 4). The physiological concentrations of cystatin C and L-kininogen are more than 10 times K_i, so these inhibitors are likely to inhibit any trypanopain-Tb secreted or released by trypanosomes *in vivo* (Bieth, 1980). Interestingly, the most abundant cysteine proteinase inhibitors in blood are also the most effective *in vitro* inhibitors of the purified enzyme. Since cystatins are relatively conserved between species (Barrett et al., 1986) and the trypanosomal cysteine proteinases sequenced to date are highly similar to each other (Mottram et al., 1989; Pamer et al., 1990; Eakin et al., 1992; Fish et al., 1995), this *in vitro* approach implies that trypanopains from various trypanosome species are likely to be effectively inhibited by cystatins in the bloodstream of a variety of mammalian hosts. This conclusion is supported by Nwagwu et al. (1988), who showed that addition of rat serum to isolated parasites inhibited a trypanopain-Tb-like activity. Additionally, the Z-Phe-Arg-NHMec activity detected in the blood of infected rats in the present study was not E-64-inhibitable (Table 5), suggesting that there are not significant concentrations of active trypanopain-Tb in the blood of infected animals. Thus while trypanopain-Tb readily degrades IgG and BSA *in vitro*, it appears that this is unlikely to occur in the host bloodstream.

In apparent conflict with this conclusion, Lonsdale-Eccles et al. (1995) reported that a kininogen-like molecule from rat serum enhances trypanopain-Tb activity in fibrinogen zymograms of *T. b. brucei* lysates. This molecule (called rat trypanopain modulator, rTM) cross-reacts to some extent with anti-(human kininogen) antibodies and has many other features in common with kininogens, such as its size (68 kDa), its acid and heat stability, and the presence of 15-kDa proteinase-sensitive domains. Additionally, L-kininogen was shown to enhance trypanopain activity on fibrinogen zymograms in a similar manner to rTM. The current study, however, clearly indicates that purified human L-kininogen is an effective inhibitor of purified trypanopain-Tb with a K_i of 0.0035 nM. Pike et al. (1992) reported a similar apparent contradiction in the interaction between a cysteine proteinase and its putative inhibitor. While purified stefin B inhibited purified cathepsin L as expected, active covalent complexes of the enzyme and inhibitor were shown to form under certain circumstances. An unidentified factor, which may be absent *in vitro* studies using purified components, may contribute to the formation of these active complexes *in vivo*. It is possible that this putative factor modifies the interaction between trypanopain-Tb and L-kininogen *in vivo*, resulting in the formation of active complexes as reported by Lonsdale-Eccles et al. (1995). Such an interaction may therefore interfere with effective control of trypanopain in the host bloodstream.

Effect of reducing agents and cysteine proteinase inhibitors on OP-Tb. Because OP-Tb is inhibited by iPr_2P-F and $H_2NEtPhSO_2F$, it is likely to be a serine protease. However, the enzyme is not inhibited by some other serine protease inhibitors, namely $PhMeSO_2F$ and SBTI. The lack of inhibition by $PhMeSO_2F$ is not entirely unexpected in view of the subsite specificity of the enzyme as discussed by Kornblatt et al. (1992). SBTI (20.1 kDa), on the other hand, is probably ineffective because it is too large to gain complete access to the active site of the enzyme.

Additionally, previous work on this enzyme (Kornblatt et al., 1992) and a similar enzyme from *T. cruzi* (Ashall, 1990; Ashall et al., 1990) indicated that some compounds which are generally considered to be cysteine protease inhibitors reduce OP-Tb activity. Here we have been able to delineate more clearly the effect of various cysteine protease inhibitors on this enzyme. While OP-Tb is unaffected by the common cysteine protease inhibitor E-64, the enzyme is inactivated by compounds such as iodoacetate, iodoacetamide and *N*-ethylmaleimide, that covalently modify cysteine residues. It is interesting to note that these three compounds act in rather contrasting manners, depending on the presence or absence of dithiothreitol, possibly reflecting the particular micro-environment of the cysteine residue involved (Table 2). This data, together with the fact that OP-Tb is activated by reducing agents, suggests that the enzyme contains a cysteine residue which must be reduced and available for maximum activity and thus involved in the control of OP-Tb activity. This may provide an alternative means of controlling this enzyme *in vivo*, which would be of particular importance in light of the insensitivity of OP-Tb to more typical means of control by host inhibitors, including serpins and α_2-M.

Hydrolysis of peptides by OP-Tb. OP-Tb successfully cleaved reduced [Arg^8]vasopressin, [Lys^8]vasopressin, neurotensin and atrial natriuretic factor, but was found to be inactive against substrates of more than 4 kDa. The enzyme is likely to cleave only small peptides *in vivo*. The fact that atrial natriuretic factor, [Arg^8]vasopressin and [Lys^8]vasopressin were more readily cleaved when reduced suggests that access to the OP-Tb active site is probably dependent on conformation as well as size. A similar enzyme from *T. cruzi* was found to be vital for the infectivity of this organism, since it cleaves and activates a cytoplasmic factor involved in a calcium signalling mechanism which mediates the entry of trypanosomes into cells (Burleigh and Andrews, 1995). While a similar function for OP-Tb is unlikely since African trypanosomes are not intracellular parasites,

OP-Tb may play a role in the activation of other hormone-like peptides in *T. b. brucei*.

OP-Tb was not inhibited by any mammalian protein proteinase inhibitor used in this study. In view of the restricted access to the active site of the proteinase, this is not entirely unexpected. Upon release of this enzyme from the parasite, as we have shown is likely to occur during infections, the enzyme will thus be free to act upon small regulatory molecules in its immediate environment. The demonstration here that the enzyme is able to cleave native neurotensin and atrial natriuretic factor, which have diverse roles in the complex mechanisms of control over bodily functions, means that this enzyme has the potential to seriously disrupt control mechanisms of the host system. Symptoms of sleeping sickness are difficult to define in general terms, but altered blood volume, and eventual disruption of neurological systems, is not inconsistent with the general symptoms found (Tizard et al., 1978); thus inappropriate cleavage of regulatory molecules by OP-Tb may have profound implications for the host. It will be of interest to determine the levels of various hormones in hosts infected by *T. b. brucei* in order to investigate this possibility more directly.

In conclusion, this study suggests that OP-Tb is more likely than trypanopain-Tb to have an extracellular, pathological role in African trypanosomiasis, making OP-Tb an attractive target for future studies in drug design.

This work was supported by grants from the Foundation for Research Development and the University of Natal Research Fund. The authors are particularly grateful to Dr Ennes Auerswald for human L-kininogen domain 3, Dr Magnus Abrahamson for recombinant human cystatin C and to Dr Jan Potempa for serpins.

REFERENCES

Abrahamson, M. (1993) Cystatins-protein inhibitors of papain-like cysteine proteinases, *Cienc. Cult. Soc. Bras. Progr. Cienc. 445*, 299–304.

Ashall, F. (1990) Characterisation of an alkaline peptidase of *Trypanosoma cruzi* and other trypanosomatids, *Mol. Biochem. Parasitol. 38*, 77–88.

Ashall, F., Harris, D., Roberts, H., Healy, N. & Shaw, E. (1990) Substrate specificity and inhibitor sensitivity of a trypanosomatid alkaline peptidase, *Biochim. Biophys. Acta 1035*, 293–299.

Authié, E., Duvallet, G., Robertson, C. & Williams, D. J. L. (1993) Antibody response to a 33 kDa cysteine protease of *Trypanosoma congolense*: relationship to 'trypanotolerance' in cattle, *Parasite Immunol. 15*, 465-474.

Barrett, A. J. & Kirschke, H. (1981) Cathepsin B, cathepsin H and cathepsin L, *Methods Enzymol. 80C*, 535–561.

Barrett, A. J., Rawlings, N. D., Davies, M. E., Machleidt, W., Salvesen, G. & Turk, V. (1986) Cysteine proteinase inhibitors of the cystatin superfamily, in *Proteinase inhibitors* (Barrett, A. J. & Salvesen, G., eds) pp. 515–569, Elsevier, Amsterdam.

Bieth, J. G. (1980) Pathophysiological interpretation of kinetic constants of protease inhibitors, *Bull. Eur. Physiopathol. Respir. 16*, 183–195.

Blum, H., Beier, H. & Gross, H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels, *Electrophoresis 8*, 93–99.

Burleigh, B. A. & Andrews, N. W. (1995) A 120-kDa alkaline peptidase from *Trypanosoma cruzi* is involved in the generation of a novel Ca^{2+}-signalling factor for mammalian cells, *J. Biol. Chem. 270*, 5172–5180.

Cawston, T. E. & Barrett, A. J. (1979) A rapid and reproducible assay for collagenase using [1-^{14}C]acetylated collagen, *Anal. Biochem. 99*, 340–345.

Cazzulo, J. J., Couso, R., Raimondi, A., Wernstedt, C. & Hellman, U. (1989) Further characterization and partial amino acid sequence of a cysteine proteinase from *Trypanosoma cruzi*, *Mol. Biochem. Parasitol. 33*, 33–42.

Cazzulo, J. J., Cazzulo Franke, M. C., Martínez, J. & Franke de Cazzulo, B. M. (1990) Some kinetic properties of a cysteine proteinase (cruzipain) from *Trypanosoma cruzi*, *Biochim. Biophys. Acta 1037*, 186–191.

Coleman, R. W. & Bagdasarian, A. (1976) Human kallikrein and prekallikrein, in *Methods Enzymol. 45B*, 303–322.

Dennison, C., Pike, R., Coetzer, T. & Kirk, K. (1992) Characterisation of the activity and stability of single-chain cathepsin L and of proteolytically active cathepsin L/cystatin complexes, *Biol. Chem. Hoppe-Seyler 373*, 419–425.

Dufour, E. & Ribadeau-Dumas, B. (1988) Proteolytic specificity of chicken cathepsin L on bovine β-casein, *Biosci. Rep. 8*, 185–191.

Eakin, A. E., Mills, A. A., Harth, G., McKerrow, J. H. & Craik, C. S. (1992) The sequence, organisation and expression of the major cysteine protease (cruzipain) from *Trypanosoma cruzi*, *J. Biol. Chem. 267*, 7411–7420.

Eisenthal, R. & Cornish-Bowden, A. (1974) The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters, *Biochem. J. 139*, 715–720.

Ellis, K. J. & Morrison, J. F. (1982) Buffers of constant ionic strength for studying pH-dependent processes, *Methods Enzymol. 87C*, 405–426.

Fish, W. R., Nkhungulu, Z. M., Muriuki, C. W., Ndegwa, D. M., Lonsdale-Eccles, J. D. & Steyaert, J. (1995) The cDNA and deduced amino acid sequence of a cysteine protease from *Trypanosoma (Nannomonas) congolense* metacyclic forms, *Gene 161*, 125–128.

Gounaris, A. D., Brown, M. A. & Barrett, A. J. (1984) Human plasma α-cysteine proteinase inhibitor, *Biochem. J. 221*, 445–452.

Grab, D. J. & Bwayo, J. J. (1982) Isopycnic isolation of African trypanosomes on Percoll gradients formed *in situ*, *Acta Trop. 39*, 363–366.

Henderson, P. J. F. (1972) A linear equation that describes the steady-state kinetics of enzymes and subcellular particles interacting with tightly bound inhibitors, *Biochem. J. 102*, 193–202.

Huet, G., Richet, C., Demeyer, D., Bisiau, H., Soudan, B., Tetaert, D., Ki Han, K. & Degand, P. (1992) Characterisation of different proteolytic activities in *Trypanosoma brucei brucei*, *Biochim. Biophys. Acta 1138*, 213–221.

Johnson, D. A., Barrett, A. J. & Mason, R. W. (1986) Cathepsin L inactivates -proteinase inhibitor by cleavage in the active site region, *J. Biol. Chem. 261*, 14748–14751.

Kornblatt, M. J., Mpimbaza, G. W. N. & Lonsdale-Eccles, J. D. (1992) Characterisation of an endopeptidase of *Trypanosoma brucei brucei*, *Arch. Biochem. Biophys. 293*, 25–31.

Lanham, S. M. & Godfrey, D. G (1970) Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose, *Exp. Parasitol. 25*, 521–534.

Lima, A. P. C. A., Scharfstein, J., Storer, A. C. & Ménard, R. (1992) Temperature-dependent substrate inhibition of the cysteine proteinase (GP57/51) from *Trypanosoma cruzi*, *Mol. Biochem. Parasitol. 56*, 335–338.

Lonsdale-Eccles, J. D., Mpimbaza, G. W. N., Nkhungulu, Z. R. M., Olobo, J., Smith, L., Tosomba, O. M. & Grab, D. J. (1995) Trypanosomatid cysteine protease activity may be enhanced by a kininogen-like moeity from host serum, *Biochem. J. 305*, 549–556.

Mason, R. W. (1989) Interaction of lysosomal cysteine proteinases with α_2-macroglobulin: conclusive evidence for the endopeptidase activities of cathepsins B and H, *Arch. Biochem. Biophys. 273*, 367–374.

Mbawa, Z. R., Webster, P. & Lonsdale-Eccles, J. D. (1991) Immunolocalisation of a cysteine protease within the lysosomal system of *Trypanosoma congolense*, *Eur. J. Cell Biol. 56*, 243–250.

Mbawa, Z. R., Gumm, I. D., Shaw, E. & Lonsdale-Eccles, J. D. (1992) Characterisation of a cysteine protease from bloodstream forms of *Trypanosoma congolense*, *Eur. J. Biochem. 204*, 371–379.

Mottram, J. C., North, M. J., Barry, J. D. & Coombs, G. H. (1989) A cysteine protease cDNA from *Trypanosoma brucei* predicts an enzyme with an unusual C-terminal extension, *FEBS Lett. 258*, 211–215.

Murta, A. C. M., Persechini, P. M., de Souto Padron, T., de Souza, W., Guimarães, J. A. & Scharfstein, J. (1990) Structural and functional identification of GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase, *Mol. Biochem. Parasitol. 43*, 27–38.

Nwagwu, M., Okenu, D. M. N., Olusi, T. A. & Molokwu, R. I. (1988) *Trypanosoma brucei* releases proteases extracellularly, *Trans. R. Soc. Trop. Med. Hyg. 82*, 577.

Pamer, E. G., So, M. & Davis, C. E. (1989) Identification of a developmentally regulated cysteine protease of *Trypanosoma brucei*, *Mol. Biochem. Parasitol. 33*, 27–32.

Pamer, E. G., Davis, C. E., Eakin, A. & So, M. (1990) Cloning and sequencing of the cysteine protease cDNA from *Trypanosoma brucei rhodesiense*, *Nucleic Acids Res. 18*, 6164.

Pamer, E. G., Davis, C. E. & So, M. (1991) Expression and deletion analysis of the *Trypanosoma brucei rhodesiense* cysteine proteinase in *Escherichia coli*, *Infect. Immun. 59*, 1074–1078.

Pike, R. N. & Dennison, C. (1989) Protein fractionation by three-phase partitioning (TPP) in aqueous/*t*-butanol mixtures, *Biotech. Bioeng. 33*, 221–228.

Pike, R. N., Coetzer, T. H. T. & Dennison, C. (1992) Proteolytically active complexes of cathepsin L and a cysteine proteinase inhibitor; purification and demonstration of their formation *in vitro*, *Arch. Biochem. Biophys. 294*, 623–629.

Potempa, J., Watorek, W. & Travis, J. (1986) The inactivation of human plasma alpha-1-proteinase inhibitor by proteinases from *Staphylococcus aureus*, *J. Biol. Chem. 261*, 14330–14334.

Read, S. M. & Northcote, D. H. (1981) Minimization of variation in the response to different proteins of the Coomassie Blue dye-binding assay for protein, *Anal. Biochem. 116*, 53–64.

Russo, D. C. W., Williams, D. J. L. & Grab, D. J. (1994) Directional movement of variable surface glycoprotein-antibody complexes in *Trypanosoma brucei*, *Parasitol. Res. 80*, 487–492.

Salvesen, G. & Nagase, H. (1992) Inhibition of proteolytic enzymes, in *Proteolytic enzymes: a practical approach* (Benyon, R. J. & Bond, J. S., eds) pp. 83–104, IRL Press, Oxford.

Shägger, H. & von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal. Biochem. 166*, 368–379.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Measurement of protein using bicinchononic acid, *Anal. Biochem. 150*, 76–85.

Tizard, I., Nielsen, K. H., Seed, J. R. & Hall, J. E. (1978) Biologically active products from African trypanosomes, *Microbiol. Rev. 42*, 661–681.

Walgate, R. (1994) Sleeping sickness on the boil in Zaire, *TDR News 46*, 6.

A trypanosome oligopeptidase as a target for the trypanocidal agents pentamidine, diminazene and suramin

Rory E. Morty[a], Linda Troeberg[a], Robert N. Pike[b], Roy Jones[c], Peter Nickel[d], John D. Lonsdale-Eccles[e], Theresa H.T. Coetzer[a,*]

[a] *Department of Biochemistry, University of Natal, Private Bag X01, Scottsville 3209, South Africa*
[b] *Department of Biochemistry and Molecular Biology, Monash University, Clayton, Vic. 3168, Australia*
[c] *Department of Development and Genetics, The Babraham Institute, Cambridge CB2 4AT, UK*
[d] *Pharmazeutisches Institut, Universität Bonn, D-53121 Bonn, Germany*
[e] *Department of Biochemistry and Molecular Genetics, University of Alabama, Birmingham, AL, USA*

Received 27 April 1998; revised version received 13 July 1998

Abstract African trypanosomes contain a cytosolic serine oligopeptidase, called OP-Tb, that is reversibly inhibited by the active principles of three of the five most commonly used trypanocidal drugs: pentamidine, diminazene and suramin. OP-Tb was inhibited by pentamidine in a competitive manner, and by suramin in a partial, non-competitive manner. The inhibition of OP-Tb by a variety of suramin analogues correlated with the trypanocidal efficacy of these analogues ($P = 0.03$; by paired Student's *t*-test). Since intracellular (therapeutic) concentrations of pentamidine and suramin are reported to reach approximately $206K_i$ and $15K_i$ respectively, we suggest that these drugs may exert part of their trypanocidal activity through the inhibition of OP-Tb.

Key words: Protease; Oligopeptidase; Suramin; Pentamidine; Diminazene; *Trypanosoma brucei*

1. Introduction

African trypanosomes are protozoan parasites that cause widespread disease in livestock (e.g. cattle) as well as in humans. Collectively, the diseases are referred to as African trypanosomiasis. A number of drugs are available for the chemotherapy of African trypanosomiasis, including sulfonated naphthylamines such as suramin [1]; aromatic diamidines, including pentamidine [2] and diminazene [3]; the trivalent arsenical, melarsoprol [4]; and α-DL-difluoromethylornithine (DFMO), a suicide inhibitor of ornithine decarboxylase [5]. With the exception of DFMO, the in vivo targets of the drugs have not been unequivocally identified. Consequently, the biochemical basis of their trypanocidal action is unclear [6]. During the course of our studies on the peptidases of African trypanosomes, we have identified and purified a cytosolic [7] oligopeptidase from the African trypanosome *Trypanosoma brucei brucei*, which we called OP-Tb [8,9]. The substrate specificity of OP-Tb, which preferentially cleaves peptides after pairs of basic amino acid residues [8,9], suggested that the aromatic diamidines (pentamidine and diminazene), would act as competitive inhibitors of OP-Tb. Similarly, previous reports of the inhibition of trypsin-like serine peptidases by suramin [10–12] raised the possibility that suramin might inhibit OP-Tb. Furthermore, OP-Tb activity is abolished in the presence of thiol-reactive compounds [9], and since melarsoprol forms adducts with thiol-containing proteins via disulfide bonds [13,14], it was also possible that melarsoprol could inactivate OP-Tb. Here we report that pentamidine, diminazene and suramin are indeed inhibitors of OP-Tb, and that, in the case of pentamidine and suramin, the inhibition of OP-Tb by these inhibitors is likely to be of in vivo significance. This represents the first report where peptidases have been identified as possible targets of trypanocidal drugs and we suggest that serine peptidase inhibitors may represent a class of lead compounds for the development of new chemotherapeutic agents that are active against OP-Tb. Recent reports of drug resistance by African trypanosomes [15–17] underscore the importance of developing new and improved therapeutic strategies.

2. Materials and methods

2.1. Materials

Melarsoprol and melarsen oxide were from Dr. Alan Fairlamb, Biomedical Sciences Institute, University of Dundee. DFMO was from Dr. Andrew Peregrine, International Livestock Research Institute, Nairobi, Kenya. Suramin was from Bayer (Leverkusen, Germany). Berenil was from Hoechst Veterinär (Munich, Germany). Pentamidine and diminazene aceturate (minimum 90% purity) were from Sigma (St. Louis, MO, USA). The structure of suramin (Fig. 1) illustrates that it is a symmetrical molecule and that each half, connected by a central urea group, can be sub-divided into three regions: an acidic naphthalene moiety (R_1), a methylbenzoyl moiety (R_2) and a benzoyl (R_3) moiety. Analogues were synthesized as described previously [18] with various substitutions at R_1, R_2 and R_3 (Tables 1 and 2). Analogue nomenclature is adapted from that described in [19].

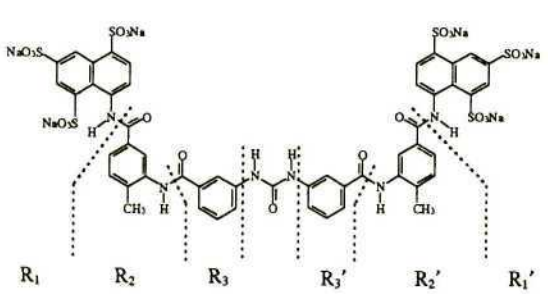


Fig. 1. Chemical formula of suramin and designated nomenclature for suramin analogues (modified from [18,19]).

*Corresponding author. Fax: (27) (331) 260 5462.
E-mail: coetzer@unpsun1.cc.unp.ac.za

Abbreviations: AMC, 7-amino-4-methylcoumarin; Cbz, carbobenzoxy; DFMO, α-DL-difluoromethylornithine; PGK, phosphoglycerate kinase; Suc, succinyl

PII: S0014-5793(98)00914-4

2.2. Isolation of OP-Tb

OP-Tb was purified from *T. b. brucei* clone ILTat 1.1 [9] and active enzyme concentration determined using 4-methylumbelliferyl-*p*-guanidobenzoate as described [20].

2.3. Diagnosis of inhibition mechanism

Inhibition mechanisms were diagnosed from the effect of a drug on the K_m and V_{max}. OP-Tb (1.5 ng, 18.75 fmol active concentration) was pre-incubated in assay buffer (50 mM Tris-HCl, 10 mM dithiothreitol, pH 8) for 10 min at 37°C, with or without drug, followed by the addition of substrate. The initial steady-state velocity was determined by continuous assay for a range of substrate concentrations (45 nM–75 μM). While the kinetic data are represented graphically as Lineweaver-Burk plots (Fig. 2), due to the inherent error in the determination of these parameters graphically [21], actual values for K_m and V_{max} were determined by hyperbolic regression using the software package Hyper 1.01 (obtained from Dr. J.S. Easterby, University of Liverpool, UK). The k_{cat} was determined from $k_{cat} = V_{max}/[E]_0$, where $[E]_0$ represents the active enzyme concentration. Dithiothreitol was omitted from assays involving organo-arsenicals to prevent potential complex formation of arsenic with free thiols [22]. Organo-arsenicals were made up as stock solutions in dimethylformamide, and diluted with assay buffer, while the other inhibitors were made up as aqueous stock solutions. In the case of melarsoprol and melarsen oxide, the K_m and V_{max} values were determined in the presence of residual concentrations of dimethylformamide, in order to negate the effect of this solvent on these parameters. Non-competitive inhibition was distinguished from irreversible inhibition by diluting out the inhibitory effect of compounds exhibiting either mode of inhibition. This was achieved with a succession of four dilution/concentration cycles where enzyme-inhibitor samples were diluted with assay buffer and concentrated to the original volume in 3 ml polysulfone concentrators (7000×*g*, 10°C). Trypsin and chymotrypsin were assayed as described previously [12].

2.4. Calculation of inhibition constant

For competitive inhibitors, the apparent K_i ($K_{i(app)}$) was determined in the presence of Cbz-Arg-Arg-AMC as previously described [23], and corrected for the presence of substrate (K_m=245 nM) by dividing the $K_{i(app)}$ by the factor $1+[S]/K_m$ to give the true K_i [23]. For partial non-competitive inhibitors, the K_i was determined from the complex steady-state rate equation of Baici [24] as modified by Szedlacsek et al. [25],

$$\frac{v_i}{v_o} = \frac{v_o - v_\infty}{2v_o}\sqrt{\left(\frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0} + \frac{[I]}{[E]_0} - 1\right)^2 + 4\frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0}} + \frac{v_o + v_\infty}{v_o - v_\infty} - \frac{1+\sigma}{\alpha+\sigma}\cdot\frac{\alpha K_i}{[E]_0} - \frac{[I]}{[E]_0} \quad (1)$$

where $\sigma = [S]/K_m$, $v_\infty = \beta\, k_{cat}\,[E]_0[S]/[S]+\alpha K_m$. The parameters α and β are dimensionless factors representing the change in the K_m and k_{cat}, respectively, in the presence of an inhibitor and v_∞ is the rate extrapolated at infinite inhibitor concentration, [I]. Once K_m, k_{cat}, α and β had been determined separately, the K_i was calculated by non-linear regression analysis, fitting (v_i, [I]) pairs to Eq. 1, in which all other parameters were fixed.

2.5. Cytotoxicity assays

Trypanosomes were cultured [26] and cytotoxicity assays performed as previously described [27]. Cultures were maintained in 24-well cell-culture plates (1 ml culture volume). Drugs were dissolved in dimethylsulfoxide, and added at various concentrations to trypanosome cultures. The final dimethylsulfoxide concentration was maintained at 1% (v/v). Control cultures (without drugs, but containing 1% (v/v) dimethylsulfoxide) were incubated under the same conditions. Cell numbers were determined (in triplicate) with a hemocytometer, and data analyzed graphically, plotting growth inhibition versus drug concentration, from which the effective concentration inhibiting the growth of trypanosome populations by 50% (EC_{50}) was calculated.

3. Results and discussion

The trivalent arsenicals, melarsoprol or melarsen oxide, and the ornithine analogue DFMO had no effect on the hydrolysis of Cbz-Arg-Arg-AMC by OP-Tb relative to uninhibited controls (data not shown). However, OP-Tb activity is enhanced in the presence of reducing agents and polyamines [8]. Since melarsoprol therapy depletes intracellular reducing agents (namely trypanothione) and since DFMO therapy depletes intracellular polyamine levels [28] either drug may down-regulate OP-Tb activity within trypanosomes through the depletion of intracellular OP-Tb activators.

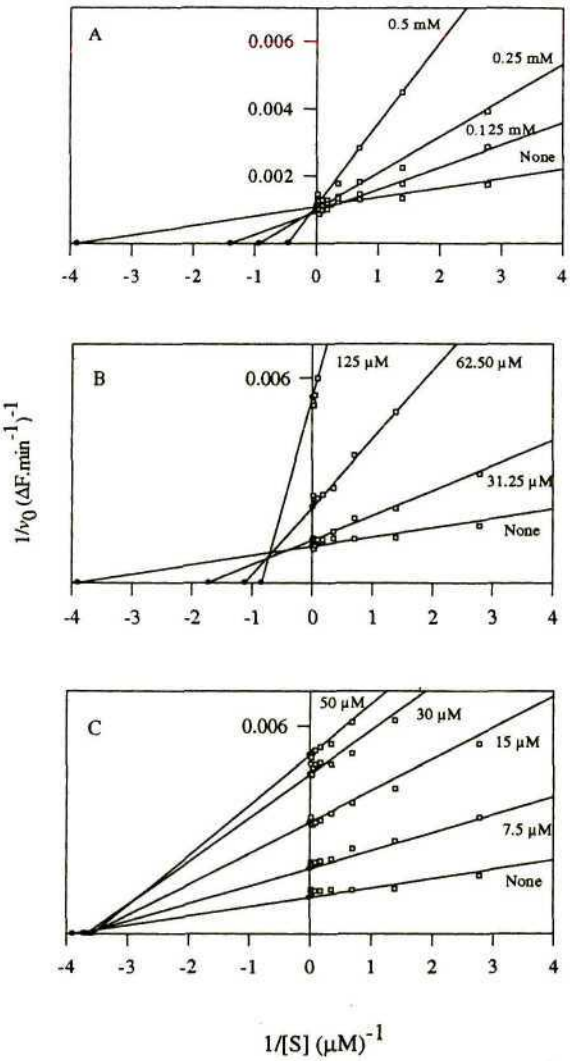


Fig. 2. Diagnosis of inhibition mechanisms by trypanocidal drugs. Lineweaver-Burk plots of kinetic data obtained for the inhibition of Cbz-Arg-Arg-AMC hydrolysis by OP-Tb over a range of inhibitor concentrations for (A) pentamidine, (B) Berenil and (C) suramin.

OP-Tb was competitively inhibited by pentamidine with a K_i of 3.4 μM (Fig. 2A), which compares well with the K_i observed for bovine β-trypsin (2.3 μM) [29], bovine mast-cell tryptase (1.2 μM) [30] and human plasmin (3.3 μM) [31]. Pentamidine isethionate is the active principle of the trypanocidal drug Pentacarinate. The mechanism of its trypanocidal activity is not understood, although it has previously been attributed to inhibition of trypanosome *S*-adenosyl-L-methionine decarboxylase [32] or to the intercalation with trypanosome DNA [33]. Structurally, the pentamidine molecule represents two benzamidine molecules joined together, resembling a pair of basic amino acid (arginine) residues in

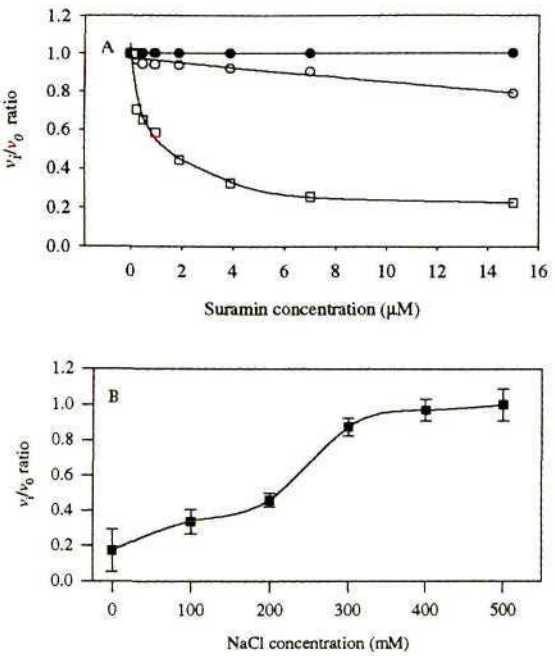


Fig. 3. Effect of suramin on substrate hydrolysis by serine proteases. A: Effect of increasing concentrations of suramin on the activity of OP-Tb (20 nM) against Cbz-Arg-Arg-AMC (□), chymotrypsin (25 nM) against Suc-Leu-Tyr-AMC (●) and trypsin (25 nM) against Cbz-Arg-AMC (○). Ordinates (v_i/v_o), initial rate in the presence of suramin/initial rate in the absence of suramin. B: Effect of NaCl on the inhibition of OP-Tb (20 nM) activity against Cbz-Arg-Arg-AMC by suramin (■). Error bars represent the mean $v_i/v_o \pm$ S.D. ($n = 3$). All substrates at 5 μM.

a dipeptide. During therapeutic regimens, pentamidine typically reaches concentrations of up to 13 μM in the host bloodstream [34] where it has a plasma half-life of 47 h [35]. Furthermore, pentamidine is actively concentrated by trypanosomes which possess pentamidine transporters [36], reaching intracellular concentrations of between 700 μM and 1 mM in 3 h [37]. For an inhibitor to be considered physiologically relevant, its in vivo concentration must be $> 10K_i$ [38]. As the intracellular concentration of pentamidine within trypanosomes is likely to reach at least 700 μM ($= 206K_i$) during therapeutic regimes, it is possible that pentamidine may exert its trypanocidal activity through the inhibition of OP-Tb, which is a cytosolic enzyme.

Commercially available preparations of the trypanocidal drug Berenil contain 45% (m/m) diminazene aceturate, and 55% (m/m) phenyldimethyl pyrazolone [39]. Diminazene aceturate is the active principle of this drug and is known to inhibit *S*-adenosyl-L-methionine decarboxylase [32] and extranuclear DNA synthesis in trypanosomes [40]. However, the mechanism of the trypanocidal activity of diminazene has not been unequivocally determined. Diminazene is like pentamidine in that it resembles two benzamidine molecules joined together, albeit with a shorter spacer region. Thus, like pentamidine, it also mimics potential substrates for OP-Tb. This structural similarity to pentamidine suggests that diminazene molecules may behave similarly with respect to OP-Tb, within the parasites. Berenil exhibited mixed inhibition of OP-Tb (Fig. 2B). This raises the possibility that the diminazene aceturate competitively inhibits OP-Tb activity, while the phenyldimethyl pyrazolone contributes to the inhibition of OP-Tb by Berenil in a non-competitive manner. However, similar data were obtained using a 90% pure preparation of diminanizene aceturate (results not shown), suggesting that diminazene aceturate itself is entirely responsible for the mixed inhibition observed.

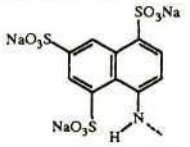
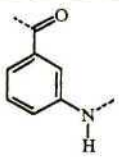
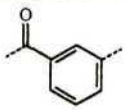
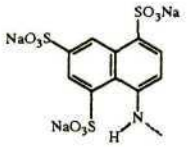
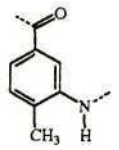
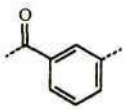
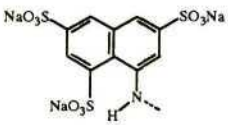
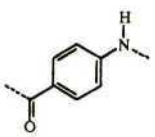
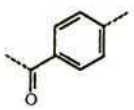
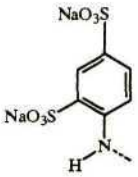
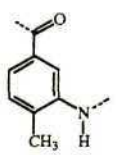
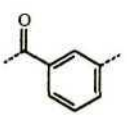
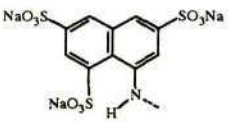
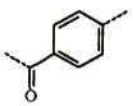
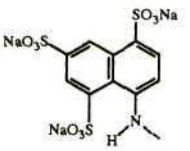
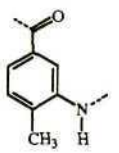
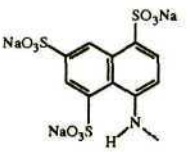
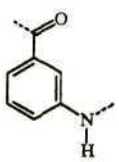
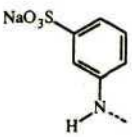
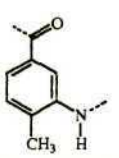
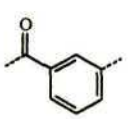
Suramin is known to inhibit a number of serine peptidases. These include kallikrein, thrombin and plasmin [10], C1 esterase [11] and trypsin [41]. However, the mechanism is unknown. Kinetic analysis of kallikrein inhibition by suramin revealed that it was not competitive. More detailed analysis of the suramin interaction with neutrophil elastase, cathepsin G and proteinase 3 indicated that suramin was a non-competitive inhibitor of these enzymes [12]. Here we show that the suramin inhibition of OP-Tb activity against Cbz-Arg-Arg-AMC resulted in a 4.5-fold reduction in V_{max} with no apparent effect on K_m (Fig. 2C). As it was possible to dilute out the effect of suramin with a series of four dilution/concentration cycles (results not shown), the inhibition by suramin was reversible, and therefore non-competitive. The clustering of the V_{max} values at high suramin concentrations (ordinate, Fig. 2C) suggested that a partial inhibition mechanism was operating [12,24,25], and this did indeed prove to be the case (Fig. 3A). The $[E]_0/K_i$ ratio for the inhibition of OP-Tb by suramin was < 0.01, indicating that there was no tight binding of inhibitor to enzyme [38]. A similar situation was observed for the inhibition of neutrophil proteinase 3 by suramin, which also exhibited partial, non-competitive inhibition by suramin with an $[E]_0/K_i$ ratio < 0.01 [12].

In general there is a good relationship between the basicity of an enzyme and its affinity for suramin [12], but this rule appears to break down with OP-Tb which has a p*I* of 5.1 [7]. However, despite its low p*I*, OP-Tb has 24 lysine residues and 59 arginine residues (unpublished data). We suspect that the sulfonic acid groups of suramin electrostatically interact with these basic residues, a conclusion supported by the observation that NaCl abolished the inhibition of OP-Tb by suramin (Fig. 3B). However, additional specific interactions appear to be involved, as polysulfonated glycosaminoglycans such as heparin do not inhibit OP-Tb (unpublished data), suggesting that the inhibition is not due to a non-specific electrostatic effect with polysulfonated compounds.

Both suramin and its demethylated analogue (NF037, Table 1) were potent inhibitors of OP-Tb activity, and were equally trypanocidal. These findings contrast with those of Forneau et al. [42], who reported that demethylation of suramin resulted in complete loss of trypanocidal activity. However, the EC_{50} values reported here were determined in vitro, whereas Forneau et al. [42] investigated the ability of suramin analogues to cure trypanosome infection in vivo, in mice. Taken together, these data suggest that, while the methyl groups are unimportant for OP-Tb-inhibitory activity and trypanocidal efficacy, they may be essential for the metabolism and/or transport of suramin in mice.

Both the number and spatial arrangement of the sulfonic acid groups on the aminonaphthyl (R_1) moiety were important for inhibitory and trypanocidal activity. Replacing each of the 1-naphthylamino-4,6,8-trisulfonic acid moieties of suramin with aniline-2,4-disulfonic acid (NF065; Table 1) resulted in a 12-fold increase in K_i, but only a 15% increase in EC_{50}. Removal of a second sulfonate group from each of the aniline moieties in NF065 (NF078; Table 1) elevated the K_i 25-fold when compared with suramin. This weak inhibitor

Table 1
Inhibition constants and EC_{50} values for symmetrical suramin analogues

Analogue	Structural substituents			K_i	EC_{50}
	$R_1 = R_1'$	$R_2 = R_2'$	$R_3 = R_3'$	(μM)	(μM)
NF037				5.4	74.5
Suramin				6.7	88.2
NF031				62.2	96.7
NF065				77.3	104.3
NF013			absent	127.7	128.7
NF058			absent	159.2	167.4
NF023			absent	161.0	172.2
NF078				167.2	>220.0

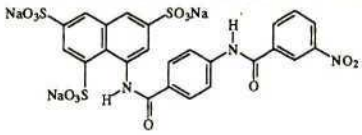
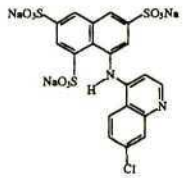
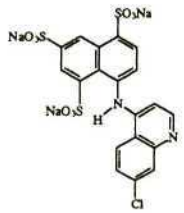
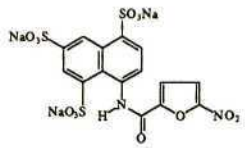
Analogue nomenclature is detailed in [19].

possessed no trypanocidal activity at the concentrations tested.

It is apparent from Tables 1 and 2 that the length of the molecule is also important for both inhibitory potency and trypanocidal activity. Removal of the benzoyl (R_3) group of suramin (NF058; Table 1) increased the K_i 24-fold and the EC_{50} two-fold. Demethylation of the methylbenzoyl (R_2) moiety of this shortened molecule (to generate NF023 from NF058; Table 1) had little effect on K_i or EC_{50} when compared with NF058. This is in agreement with our findings that these methyl groups are not important for inhibitory or trypanocidal activity.

Replacement of both the methylbenzoyl (R_2) and benzoyl (R_3) groups of suramin with 4-C-benzoyl groups (NF031; Table 1) elevated the K_i nine-fold, with a 10% increase in EC_{50}. Subsequent removal of two of these 4-C-benzoyl groups

Table 2
Inhibition constants and EC_{50} values for asymmetrical and half-suramin analogues

Analogue	Chemical structure	K_i (μM)	EC_{50} (μM)
NF033		308.8	>220.0
NF035		n.m.	>220.0
NF036		n.m.	>220.0
NF226		n.m.	>220.0

n.m. denotes that no inhibitory activity was observed at the concentrations tested. Analogue nomenclature is detailed in [19].

(NF013; Table 1) increased the K_i 19-fold and the EC_{50} by 31% when compared with suramin. This indicated that a reduction in the length of the molecule was accompanied by a reduction in both inhibitory and trypanocidal activity.

The importance of the length and symmetry of the molecule is also illustrated by the lack of inhibitory or trypanocidal activity of the 'half-suramin' analogue NF036 (Table 2). Neither the rearrangement of one of the sulfonate groups on the naphthylene ring (NF035; Table 2), nor the attachment of the 4,6,8-substituted aminonaphthylsulfonate to a 5-nitro-2-furyl group (NF226; Table 2) improved the inhibitory or trypanocidal activity.

In the absence of a three-dimensional structure of OP-Tb, it is difficult to predict how suramin may interact, in such a specific fashion, with OP-Tb. Hart et al. [43] examined the interactions of suramin and trypanosome phosphoglycerate kinase (PGK). Computer modelling of a putative PGK-suramin complex illustrated that electrostatic interactions between PGK and suramin would bridge the PGK active site and block the mechanism of action of the enzyme. However, it is premature to speculate whether or not suramin may somehow occlude the active-site 'pit' proposed to exist in oligopeptidases [44].

There was a significant correlation ($P = 0.03$; by paired Student's t-test; Table 1) between the inhibitory potency and trypanocidal efficacy of suramin analogues, which strongly suggests that OP-Tb represents an important intracellular target for suramin. During treatment regimens, suramin reaches concentrations of approximately 100 μM within the host bloodstream [45] and, although not actively concentrated in the parasite, it reaches intracellular concentrations of approximately 100 μM [6]. Since suramin has a K_i of 6.7 μM for OP-Tb, the inhibition of OP-Tb by suramin may be physiologically relevant and this may explain, at least in part, the trypanocidal action of suramin, which remains unelucidated [6].

The trypanocidal action of suramin has previously been attributed to the inhibition of trypanosome glycolytic enzymes, and while K_i values for the inhibition of these enzymes by suramin have not been determined, IC_{50} values of 10–100 μM have been reported [46]. Additionally, a K_i of 15 μM has been reported for the inhibition of trypanosome 6-phosphogluconate dehydrogenase by suramin [47]. However, Wang [6] pointed out that all nine glycolytic enzymes are protected from cytosolic suramin by compartmentalization in a glycosome, which is unlikely to take up suramin by diffusion or endocytosis. Suramin has been used for over 70 years [6] and appears still to be effective against the parasite as there are few reports of *T. b. brucei* resistance [48]. As trypanosomes do not readily develop resistance to suramin, it seems likely that the drug may act on multiple targets in the parasite. Given that the K_i for the inhibition of OP-Tb by suramin is lower than that reported for other enzymes, as well as the significant correlation between trypanocidal efficacy and OP-Tb inhibition, and the cytosolic location of OP-Tb, we propose that OP-Tb is one of these targets.

Acknowledgements: This investigation received financial support from the South African Foundation for Research Development and the

University of Natal Research Fund. The authors thank Dr. Michael P. Barrett for providing pre-publication copies of manuscripts, Dr. Omalokoho M. Tosomba and Adam S. Dawe for the translation of [42], Ronald K. Berry for performing the hyperbolic regression, and Dr. Andrew Peregrine for helpful discussions.

References

[1] Kleine, P.K. and Fischer, W. (1923) Deutsch. Med. Wochenschr. 32, 1039–1041.
[2] Lourie, E.M. and Yorke, W. (1939) Ann. Trop. Med. Parasitol. 33, 289–304.
[3] Jensch, H. (1937) Angew. Chem. 50, 891–895.
[4] Friedham, E.A.H. (1949) Am. J. Trop. Med. Hyg. 29, 173–180.
[5] McCann, P.P., Bacchi, C.J., Hanson, W.L., Cain, G.D., Nathan, H.C., Hutner, S.H. and Sjoerdsma, A. (1981) Adv. Polyamine Res. 3, 97–110.
[6] Wang, C.C. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 93–127.
[7] Kornblatt, J.D., Mpimbaza, G.W.N. and Lonsdale-Eccles, J.D. (1992) Arch. Biochem. Biophys. 293, 25–31.
[8] Troeberg, L., Pike, R.N., Morty, R., Berry, R.K., Coetzer, T.H.T. and Lonsdale-Eccles, J.D. (1996) Eur. J. Biochem. 238, 728–736.
[9] Lonsdale-Eccles, J.D. and Grab, D.J. (1987) Eur. J. Biochem. 169, 467–475.
[10] Eisen, V. and Loveday, C. (1973) Br. J. Pharmacol. 49, 678–687.
[11] Fong, J.S.C. and Good, R.A. (1972) Clin. Exp. Immunol. 10, 127–138.
[12] Cadène, M., Duranton, J., North, A., Si-Tahar, M., Chignard, M. and Bieth, J.G. (1997) J. Biol. Chem. 272, 9950–9955.
[13] Cunningham, M.L., Zvelebil, M.J. and Fairlamb, A.H. (1994) Eur. J. Biochem. 221, 285–295.
[14] Johnson, R.M. (1963) in: Metabolic Inhibitors (Hochster, R.M. and Quastel, J.H., Eds.), pp. 99–118, Academic Press, London.
[15] Pinder, M. and Authié, E. (1984) Acta Trop. 41, 247–252.
[16] Schönefeld, A., Röttcher, D. and Moloo, S.K. (1987) Tropen. Med. Parasitol. 38, 177–180.
[17] Sutherland, I.A. and Holmes, P.H. (1993) Acta Trop. 54, 271–278.
[18] Nickel, P., Haack, H.-J., Widjaja, H., Ardanuy, U., Gurgel, C., Düwel, D., Loewe, H. and Raether, W. (1986) Arzneim.-Forsch./Drug Res. 36, 1153–1157.
[19] Jentsch, K.D., Hunsman, G., Hartmann, H. and Nickel, P. (1987) J. Gen. Virol. 68, 2183–2192.
[20] Coleman, P.L., Latham, H.G. and Shaw, E. (1981) Methods Enzymol. 80, 12–25.
[21] Dixon, M. and Webb, E.C. (1979) Enzymes, Longman, London.
[22] Barron, E.S.G. (1951) Adv. Enzymol. 11, 201–253.
[23] Salvesen, G. and Nagase, H. (1989) in: Proteolytic Enzymes: A Practical Approach (Benyon, R.J. and Bond, J.S., Eds.), pp. 83–104, IRL Press, Oxford.
[24] Baici, A. (1987) Biochem. J. 244, 793–796.
[25] Szedlacsek, S.E., Ostafe, V., Serban, M. and Vlad, M.O. (1988) Biochem. J. 254, 311–312.
[26] Hesse, F., Selzer, P.M., Mühlstädt, K. and Duszenko, M. (1995) Mol. Biochem. Parasitol. 70, 157–166.
[27] Kaminsky, R. and Zweygarth, E. (1989) Antimicrob. Agents Chemother. 33, 881–885.
[28] Fairlamb, A.H., Henderson, G.B., Bacchi, C.J. and Cerami, A. (1987) Mol. Biochem. Parasitol. 24, 185–191.
[29] Caughey, G.H., Raymond, W.W., Bacci, E., Lombardy, R.J. and Tidwell, R.R. (1993) J. Pharmacol. Exp. Ther. 264, 676–682.
[30] Fiorucci, L., Erba, F., Bolognesi, M. and Ascoli, F. (1997) FEBS Lett. 408, 85–88.
[31] Geratz, J.D. (1973) Thromb. Diathes. Haemorrh. 29, 154–167.
[32] Bitonti, A.J., Dumont, J.A. and McCann, P.P. (1986) Biochem. J. 237, 685–689.
[33] Hajduck, S.L. (1978) Prog. Mol. Subcell. Biol. 6, 158–200.
[34] Sands, M., Kron, M.A. and Brown, R.B. (1985) Rev. Infect. Dis. 7, 625–634.
[35] Bronner, U., Doua, F., Ericsson, Ö., Gustaffson, L.L., Miézan, T.W., Rais, M. and Rombo, L. (1991) Trans. R. Soc. Trop. Med. Hyg. 85, 608–611.
[36] Damper, D. and Patton, C.L. (1976) Biochem. Pharmacol. 25, 271–276.
[37] Berger, B.J., Carter, N.S. and Fairlamb, A.H. (1995) Mol. Biochem. Parasitol. 69, 289–298.
[38] Beith, J.G. (1995) Methods Enzymol. 248, 59–84.
[39] Leach, T.M. and Roberts, C.J. (1981) Pharmacol. Ther. 13, 91–147.
[40] Newton, B.A. and Le Page, R.W.F. (1967) Biochem. J. 105, 50–51.
[41] Hawking, F. (1978) Adv. Pharmacol. Chemother. 15, 289–322.
[42] Forneau, E., Tréfouel, J., Tréfouel, M. and Vallée, J. (1924) Ann. Inst. Pasteur 38, 81–112.
[43] Hart, D., Langridge, A., Barlow, D. and Sutton, B. (1989) Parasitol. Today 5, 117–122.
[44] Barrett, A.J. and Rawlings, N.D. (1992) Biol. Chem. Hoppe-Seyler 373, 353–360.
[45] Fairlamb, A.H. and Bowman, I.B.R. (1980) Mol. Biochem. Parasitol. 1, 315–333.
[46] Wilson, M., Callens, M., Kunz, D.A., Pieré, J. and Opperdoes, F.R. (1993) Mol. Biochem. Parasitol. 59, 201–210.
[47] Hanau, S., Rippa, M., Bertelli, M., Dallocchio, F. and Barrett, M.P. (1996) Eur. J. Biochem. 240, 592–599.
[48] Scott, A.G., Tait, A. and Turner, M.R. (1996) Acta Trop. 60, 251–262.