The Influence of Ionic Strength on the Kinetics of Selected Enzymes

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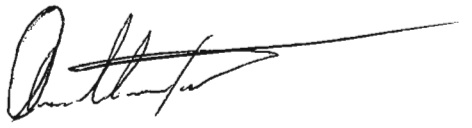
PREFACE

The experimental work described in this thesis was carried out in the School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, from Jan 2003 to April 2005 under the supervision of Professor Clive Dennison.

These studies represent original work by the Author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.



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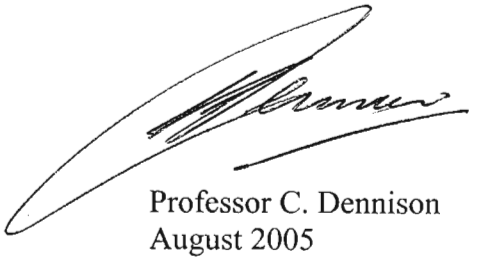

Professor C. Dennison
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ABSTRACT

pH studies are used to gain insight into chemical mechanisms of enzyme catalysed reactions. However, perhaps the most important practical point that is often overlooked in pH studies is control of the ionic strength of reaction mixtures at the various pH values. For example, cathepsins B and L were suspected to be involved in cancer invasion but pH vs activity profiles indicated that they were not active at the extracellular pH (pH 7.2). When these profiles were re-evaluated in buffers of constant ionic strength, as opposed to buffers of constant molarity, it was shown that the enzymes were indeed active at pH 7.2. Other enzymes have also been reported to be sensitive to ionic strength. These include neutrophil elastase, class sigma glutathione S-transferase and penicillin G-acylase amongst others.

The effects of increasing ionic strength on the activity of six enzymes were investigated. α-Glucosidase (from bakers' yeast), elastase (human leukocyte) and trypsin (bovine pancreatic), cathepsin L (sheep liver), cathepsin B (rabbit liver), fruit bromelain (pineapple fruit) were subjected to different ionic strength buffers and their activities and K_m and V_{max} were determined as a function of ionic strength. The influence of ionic strength on K_i values has not been previously reported and was also studied, using the interaction between chicken egg-white cystatin C and cathepsin L as a model.

α-Glucosidase was found to have an ionic strength optimum and elastase showed increasing activity with an increase in ionic strength. Trypsin activity decreased with increasing ionic strength with a substrate containing a positively charged side chain in the P_1 position, and an increase in activity with a substrate containing a hydrophobic group at the P_1 position. Cathepsin B activity increased when acting on the substrate Z-Phe-Arg-NHMec and decreased when acting on Z-Arg-Arg-NHMec, with increasing ionic strength. Bromelain showed an increase in activity with increasing ionic strength. Cathepsin L activity decreased at increasing ionic strength and the K_i values for the cathepsin L-cystatin C interaction increased with increasing ionic strength. The results obtained can be attributed to the nature of the specificity pockets involved in binding the substrate, effects on the catalytic mechanism of the enzyme or structural changes due to increasing ionic strength. These results show that the ionic strength is a significant variable and should be kept constant or at *in vivo* levels when assaying enzymes.

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LIST OF ABBREVIATIONS

A280 nm	Absorbance at 280 nm
bis	*N,N'*-methylenebisacrylamide
CGTase	cyclodextrin glucanotransferase
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
g	Relative centripetal acceleration[1]
h	Hour(s)
k	Pseudo-first order rate constant
k_{ass}	Association constant
k_{cat}	Enzyme turnover number
kDa	Kilodalton(s)
k_{diss}	Dissociation constant
K_i	Inhibition constant
$K_{i(app)}$	Apparent inhibition constant
K_m	Michaelis contant
MEC	Molecular exclusion chromatography
MES	2-[*N*-Morpholino]ethanesulfonic acid
min	Minute(s)
M_r	Relative molecular weight
MW	Molecular weight
NHMec	7-amino-4-methyl coumarin
PAGE	Polyacrylamide gel electrophoresis
PEG	Poly ethylene glycol
RT	Room temperature
s	Second(s)

[1]Sometimes referred to as relative centrifugal force, but centrifugal force is a fictitious force (Dennison, 2003).

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
t-butanol	2-methylpropan-2-ol
TEMED	*N, N, N', N'*-tetramethylethylenediamine
TPP	Three phase partitioning
tricine	N-[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]glycine
V_{max}	Maximum initial velocity
v_0	Initial velocity
v_s	Steady state velocity
v_z	Initial rate velocity
Z	Benzoyloxycarbonyl

CHAPTER 1

INTRODUCTION

The present study has its origins in studies of the role of cathepsins L and B in cancer invasion. Evidence had been accumulating that these enzymes played a role in cancer invasion but, for at least 40 years, it was contended that this could not be an extracellular role as studies on the pH dependence of their activity had revealed that they were not active at extracellular pH.

In 1992, studies in this laboratory revealed that cathepsin L was markedly sensitive to ionic strength, its activity decreasing with increasing ionic strength (Dennison *et al.*, 1992). Consequently, in determining the pH optima of cathepsins L and B it was considered prudent to use buffers of constant ionic strength, as described by Ellis and Morrison (1982), i.e. to use buffers of constant ionic strength but with varying pH. Using this approach, it was found that these enzymes had higher pH optima than had been previously reported and that they were, in fact, active under extracellular conditions of pH and ionic strength (Dehrmann *et al.*, 1995).

The error that had been made in the original determinations of their pH optima was to use buffers of constant molarity with varying pH. In the Henderson-Hasselbalch equation for a buffer made from a weak acid, i.e.

$$pH = pK_a + Log\frac{[salt]}{[acid]}$$

the ionic strength is a function of [*salt*], so, as the pH rises, the ionic strength also rises, if the molarity is kept constant. This effect is shown for phosphate buffers in Figure 1.1 (Dennison, 2003).

For a buffer made from a weak base the relevant form of the Henderson-Hasselbalch equation is:

$$pH = pK_a + Log\frac{[base]}{[salt]}$$

In this case, the ionic strength increases as the pH decreases.

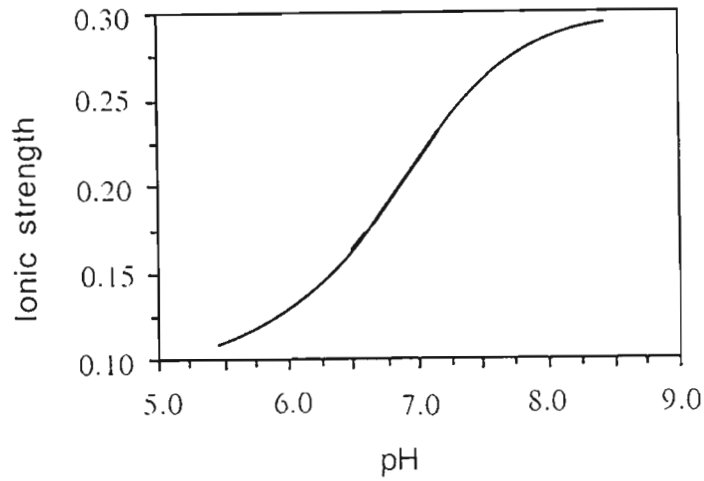


Figure 1.1. The relationship between ionic strength and pH for a 0.1 M phosphate buffer.
(Dennison, 2003).

In determining the pH optimum of cathepsins L and B, using phosphate buffers of constant molarity, the ionic strength would have risen with the pH and, since increasing ionic strength depresses the activity of these enzymes, this would have had the effect of skewing the pH activity profile, giving an erroneously low pH optimum and erroneously suggesting that the enzymes are not active at extracellular pH (Dennison *et al.*, 1992; Dehrmann *et al.*, 1995).

The effects of ionic strength on the activities of cathepsins L and B was thus of key significance in establishing their true potential for extracellular activity in cancer as well as in normal physiological processes such as sperm/egg fusion. Failure to take the effects of ionic strength into account, in this case had led researchers astray for over 40 years.

With the import of ionic strength in this case fully realized, the question arose, "to what extent is ionic strength important in the study of enzymes in general?" To begin to address this question, in the present study the effect of ionic strength on a number of enzymes was explored. Since the central focus of this laboratory is proteinases, the present study also focused on this class of enzymes but was extended to include a glycosidase. All of these enzymes are hydrolases and it was realized that the results may not be applicable to enzymes catalyzing different reactions. Nevertheless it was hoped that some common themes may emerge which might serve to inform researchers working with different enzymes.

1.1 The protein surface

The protein surface is the interface through which an enzyme interacts with the environment, making it the main site at which ionic strength would exert its influence.

The protein surface is composed of charged, polar and hydrophobic residues that are crucial for the stability and activity of the enzyme. Enzymes are amphoteric, containing a number of acidic and basic groups. 95% of all charged/titratable residues are located on the surface of soluble proteins and, in evolutionarily related families of proteins, such residues are particularly prone to substitutions, insertions and deletions (Peterson *et al.*, 1998). When charged residues are buried, they almost always participate in salt bridge formation, or take part in extensive hydrogen bonding (Peterson *et al.*, 1998). The enzyme surface is the point of contact with the solvent, thus the condition of the physical chemical environment affects the surface residues. When a substrate binds to an enzyme it constitutes a special case of changes in the physical chemical environment for the protein (Peterson *et al.*, 1998).

The widespread assumption is that the protein surface consists of hydrophilic residues, and the interior of hydrophobic residues. Three-dimensional structural information indicates that the interior of proteins contain more hydrophobic residues than the protein surface, thus supporting the view that protein folding involves a collapse of a hydrophobic interior, but the protein surface may still contain a large number of hydrophobic residues. From a physical point of view this indicates instability because the protein cannot 'hide' all hydrophobic residues in the protein core. This intrinsic instability might be the underlying mechanism that provides the necessary structural flexibility when a protein adapts its 3D structure to accommodate a ligand (Peterson *et al.*, 1998).

The individual amino acid residues protrude from the protein surface in a complex manner. In the active site region, this may be responsible for the specificity of the enzyme. The local surface residues also influence secondary binding sites on the protein surface (Peterson *et al.*, 1998).

The charges on the titratable groups vary with pH according to their individual pK values. The charge of seven of the 20 naturally occurring amino acid residues is pH dependent, thus choice of pH will determine whether or not a titratable residue carries charge

(Peterson *et al.*, 1998). The electrostatic interactions of the charged groups would be subject to pH and ionic strength effects. However, often only pH effects are considered.

1.2 Electrostatic interactions

Electrostatic interactions make a significant contribution to the stability and function of proteins. This has been shown by the contribution of buried salt bridges to protein stability (Barlow and Thornton, 1983). Studies with ionic denaturants in comparison with non-ionic denaturants indicate that electrostatic interactions are important at the subunit interfaces of multimeric proteins (Gokhale *et al.*, 1999). Site directed mutagenesis of dimeric proteins shows that electrostatic interactions provide the specificity of protein-protein interactions at subunit interfaces (Mainfroid *et al.*, 1996). Studies on the effects of ionic strength have been done on molten globules and denatured states to determine the importance of electrostatic interaction in these conformational states (Goto and Fink, 1989; Stigter *et al.*, 1991; Oliveberg *et al.*, 1994).

1.3 The ionic strength of buffers

Ionic strength is defined as:

$$I = 0.5\sum c_i (z_i)^2$$

Where c_i = concentration of each type of ion (moles/litre), z_i = charge of each type of ion.

The ionic strength of a solution provides a measure of the ionic environment that influences the catalytic activity of enzymes (Ellis and Morrison, 1982). This is because amino acid residues, which undergo protonation–deprotonation reactions, are involved in binding of substrates and conversion to products. Ionic strength effects are also observed with enzymes that utilize charged substrates or that catalyze charge separation. Thus when ionic strength is not controlled in pH-variation studies, a change in enzyme activity attributed to pH might be due to a change in ionic strength. It should also be noted that ionic strength is a measure of the bulk environment and does not necessarily reflect the microenvironment within the active site of an enzyme.

The ionic strength of any single buffer varies significantly over its functional pH range. The extent of the variation depends on the charge on the conjugate base. The ionic strength can be substantially higher than the concentration of the buffer compound. The buffers used for pH studies must not function as substrates or inhibitors of the enzyme. In

addition, they must not act as alternative acceptors for any group involved in a transfer reaction or undergo interaction with an enzyme so as to alter its kinetic properties. The maximum buffering range of a single weak acid or weak base is limited to about one pH unit on either side of the pK_a value (Ellis and Morrison, 1982). The use of buffer mixtures has the following advantages; i) it allows for studies over a wider pH range, ii) if one buffer is a weak base and the other a weak acid, then the amount of acid or alkali that must be added for pH adjustments is reduced, iii) with some buffer mixtures composed of compounds with the correct concentrations and charges on their conjugate bases, there is essentially no change in ionic strength throughout the useful buffer range, thus eliminating the need to add electrolyte (Ellis and Morrison, 1982). The ionic strength of a buffer of fixed molarity can be increased using a neutral non-inhibitory salt such as KCl or NaCl at a defined pH value (Kheirolomoom *et al.*, 1998).

1.4 Previous studies on the effects of ionic strength

The first studies on the effects of ionic strength on proteins were performed by Hofmeister, (1888), who described the effects of different salts on the stability and solubility of proteins.

Lestienne and Bieth, (1980) investigated the effect of ionic strength on the activity of elastase against N-succinyl-(L-alanine)$_3$-***p***-nitroanilide and found that increasing NaCl concentrations led to increasing activity.

Cathepsins L and B were found to have higher pH optima when assayed in buffers of constant ionic strength (Dennison *et al.*, 1992; Dehrmann *et al.*, 1995). As explained above, increasing the pH of constant molarity buffers resulted in an increase in ionic strength, which decreased the activity of the enzyme on the high pH side, resulting in an apparently lower pH optimum (Dennison *et al.*, 1992).

By stability analysis and modeling of pH- and ionic strength inactivation of penicillin G-acylase, Kheirolomoom *et al.* (1998), found that increasing the ionic strength, by the use of non-inhibitory salt, decreased the activity and the inactivation rate constant of this enzyme.

Stevens *et al.* (1998; 2000) studied the effect of ionic strength on the conformation and activity of δ-glutathione S-transferase: in this case increasing ionic strength decreased the activity of the enzyme.

Kitada and Ito (2001) investigated the effects of various salts on mitochondrial processing peptidase and found a decrease in activity and an increasing Michaelis constant (K_m), with increasing ionic strength, indicating decreasing affinity of the enzyme for the substrate.

Bosco *et al.* (2002) found that I values over 0.2 M resulted in a substantial decrease in the activity of lignin peroxidase.

Reeves *et al.* (2002) showed that an increase in ionic strength activated the neutrophil serine proteases cathepsin G and elastase.

1.5 Enzymes and an inhibitor used in the present study

For the purposes of the present study a variety of enzymes were used; α-glucosidase (type I: from bakers' yeast), elastase (human leukocyte), and trypsin (bovine pancreas) which where commercially obtained. Cathepsin B (rabbit liver) was previously isolated in this laboratory, while cathepsin L (sheep liver) and bromelain (pineapple fruit) were isolated as part of the present study. To investigate the effect of ionic strength on K_i the inhibitor chicken egg white cystatin C, was also isolated in the present study.

1.5.1 α-Glucosidase

α-Glucosidase from bakers' yeast (*Sacchoromyces cerevisiae*) is a member of Family 13 of glycosyl hydrolases. The classification scheme for these enzymes is based on amino acid sequence similarities as opposed to substrate specificity. α-Glucosidase is an exo-acting glycoside hydrolase which catalyses the release of α-D-glucose from non-reducing ends of various α-linked substrates (Frandsen *et al.*, 2002). The Family 13 hydrolases are further subclassified into type I – hydrolyzing heterogenous substrates like aryl glucosides and sucrose more efficiently than maltose, type II- being highly active on maltose and isomaltose, but of low activity toward aryl glucosides, and type III – resembling type II, but hydrolyzing di and oligo-saccharides and starch at comparable rates (Chiba, 1997). Yeast α-glucosidases belong to Family 13, type I.

The α-glucosidases [E.C. 3.2.1.20] are a widespread and diverse group of enzymes that serve a variety of functions, depending on the subcellular location and organism in which they are found. Despite their origins in several unrelated gene families, they all share an unusually wide and overlapping substrate specificity making identification, from biochemical properties alone, difficult (Frandsen and Svensson, 1998).

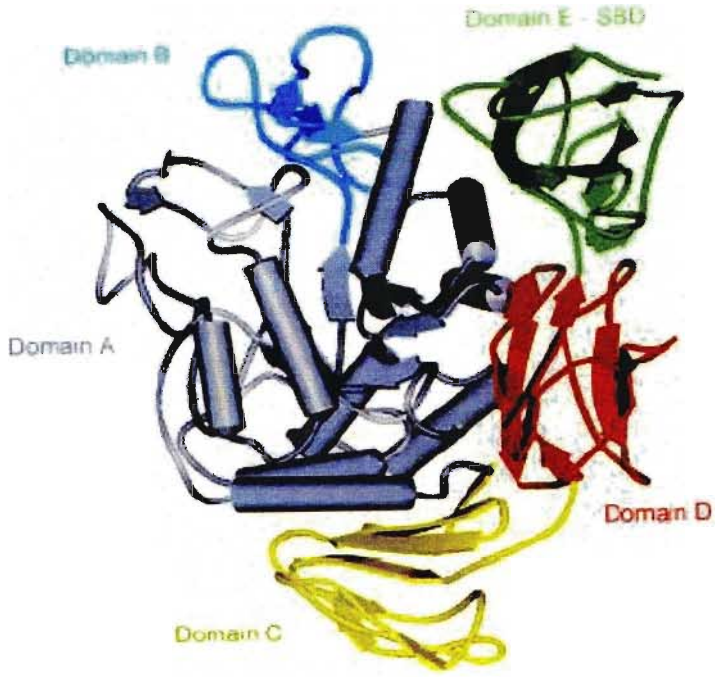


Figure 1.2. Stereo view of CGTase as an example of a five domain member of the α-amylase family.
A is the catalytic (L/K)8 domain, B protruding from that domain. C, D, and E are antiparallel L-strand domains of which E can bind starch. (Janecek *et al.*, 2003)

Glycosyl hydrolases of family 13 include α-amylase, some α-glucosidases and oligo-1,6-glucosidases. α-Amylase is the most widely studied member of Family 13. The covalent participation of a specific active site carboxylate as the nucleophile in the normal catalytic mechanism was confirmed by the identification of Asp-214 as the catalytic nucleophile of *Saccharomyces cerevisiae* α-glucosidase using 5-fluoro glycosyl fluorides (McCarter and Withers, 1996). The Family 13 enzymes are multidomain proteins, but share a common catalytic domain in the form of an $(\alpha/\beta)_8$-barrel, i.e. a barrel of eight parallel β-strands surrounded by eight helices (Figure 1.2). This structure has been demonstrated by X-ray crystallography in several enzymes of the α-amylase family (MacGregor *et al.*, 2001) including cyclodextrin glucanotransferase (CGTase) whose structure is shown in Figure 1.2. Usually the loops that link β-strands to the adjacent helices carry amino acid residues of the active site; some of these loops may be long enough to be considered as domains in their own right. Thus, in most cases where the structure has been determined by crystallography, a large loop between the third β-strand and third helix is discussed as a separate domain (MacGregor *et al.*, 2001). Similarities in domain B amongst members of sub-groups of the α-amylase family have been found (Janecek *et al.*, 1997). The vast

majority of members of the enzyme family have another domain, domain C, following the catalytic β/α-barrel. This domain is made up of β-strands and is thought to stabilise the catalytic domain by shielding hydrophobic residues of domain A from the solvent. It has also been suggested that domain C may aid in substrate binding (Dauter *et al.*, 1999; Lawson *et al.*, 1994). Much of the specificity of each enzyme is thought to be defined by amino acid residues of domains A and B (MacGregor *et al.*, 2001). Domain E is the starch binding domain (SBD) (Figure 1.2) (Janecek *et al.*, 2003)

1.5.2 Binding nomenclature for protease/peptide interaction

The active site grooves of various protease enzymes contain subsites into which substrate can bind (Figure 1.3) (Schechter and Berger, 1967). These subsites are denoted S_1, S_2, S_3, S_1', $S_2'$and S_3' and the corresponding substrate amino acid residues are termed P_1, P_2.....P_3'. The numbering is inverted on either side of the scissile bond.

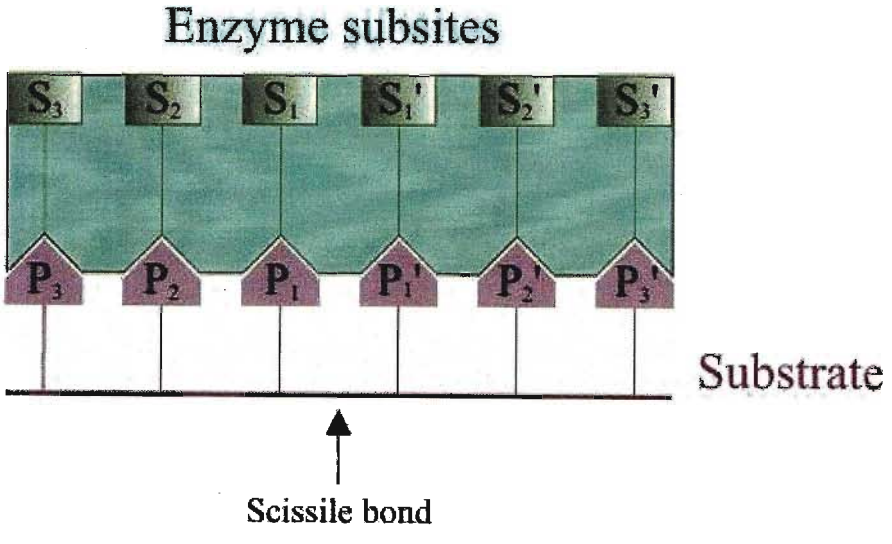
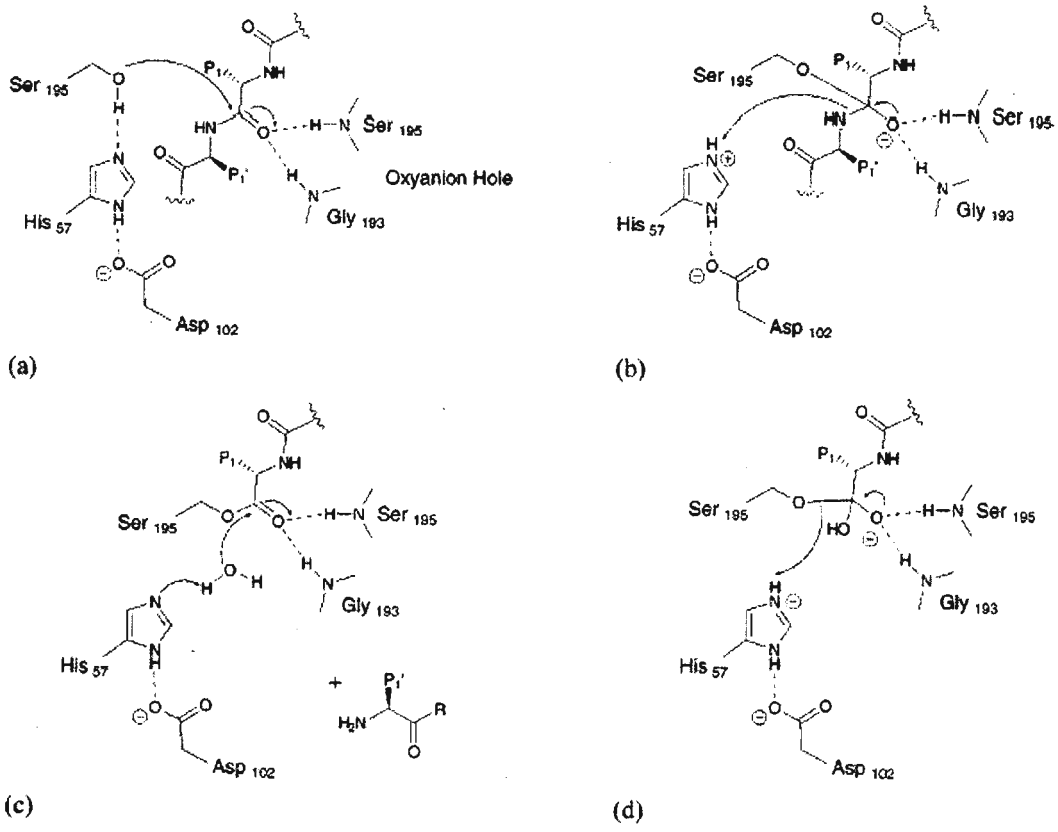


Figure 1.3. The Schechter and Berger nomenclature for binding of a peptide substrate to a peptidase.

1.5.3 Serine proteases

Serine proteases are classified by their substrate specificity, particularly by the type of residue found at P_1. These include trypsin-like (positively charged residues Lys/Arg preferred at P_1), elastase-like (small hydrophobic residues Ala/Val at P_1), or chymotrypsin-like (large hydrophobic residues Phe/Tyr/Leu at P_1). The active site of these enzymes consists of a catalytic triad of Ser-195, His-57 and Asp-102 residues (chymotrypsin numbering system) and an oxyanion hole (Leung *et al.*, 2000).

The general catalytic mechanism that applies to serine proteases (including trypsin and elastase) is show in Scheme 1. Once the substrate binds, hydrogen-bonding interactions with histidine and aspartate residues of the catalytic triad activate the serine hydroxyl for nucleophilic attack on the scissile amide bond, forming the first hemiacetal tetrahedral intermediate (Scheme 1a). Proton transfer from His-57 to the amine of the tetrahedral intermediate facilitates expulsion of the C-terminal fragment of the substrate to give the covalent acyl complex (Scheme 1b). Water attacks the complex to form the second tetrahedral intermediate (Scheme 1c), which collapses via acid-assisted catalysis by His-57 to regenerate Ser-195 and the N-terminal fragment of the cleaved substrate (Scheme 1d).



Scheme 1. General catalytic mechanism for serine proteases.

(Leung *et al.*, 2000).

1.5.3.1 Elastase

Elastase [E.C. 3.4.21.37] is located in the azurophil granules of neutrophils (Ermolieff *et al.*, 1998). Neutrophils play a critical role in the innate immune response against bacteria, fungi, and other pathogens (Mayer-Scholl *et al.*, 2004). They destroy the invader trapped within a phagocytic vacuole. The hypothesis of the killing process that has dominated over the past 40 years is that the lethal agents are the highly reactive superoxide ions and

hydrogen peroxide generated by enzymes in the vacuole (Babior *et al.*, 1975). In addition to acting on the microbe directly, it was thought that hydrogen peroxide also serves as a substrate for destructive halogenation reactions catalysed by myeloperoxidase. Recently it was shown that mice deficient in neutrophil-granule proteases, but normal in respect of superoxide production and halogenating capacity were unable to resist staphylococcal and candidal infections (Reeves *et al.*, 2002). It was shown that activation of the neutrophil provokes the production of a high concentration of reactive oxygen species within the endocytic vacuole. The resulting accumulation of anionic charge is compensated for by a surge of K^+ ions that cross the membrane in a pH-dependent manner (Reeves *et al.*, 2002). These results led to a different hypothesis regarding the killing of endocytosed bacteria. Reeves *et al.* (2002) propose that the O_2^- generating system causes an influx of K^+ into the phagocytic vacuole, resulting in an increase in pH to the optimal for the activity of the granule proteases. Moreover, the proteases become active on release from the anionic proteoglycan matrix at elevated ionic strength and are responsible for killing of bacteria (Reeves *et al.*, 2002). This led to the conclusion that the activity of the enzymes is regulated by the ionic strength of their environment.

Human neutrophil elastase reacts with at least five residues of a substrate or inhibitor (Nakajima *et al.*, 1979). Elastases prefer small or medium-sized hydrophobic residues at the substrate P_1 position. The S_1 pocket of human neutrophil elastase has a hemispheric shape and is hydrophobic, although containing the negative charge of Asp-226. The Asp-226 is not available for the P_1 side chain due to shielding by Val-216 and Val-190 (Czapinska and Otlewski, 1999). The presence of an aliphatic P_1 side chain, which is not fixed in the pocket, permits nonclassical binding of small hydrophobic residues. The pocket accepts branched or long side chains such as Leu, Ile, Val, or Met (Lu *et al.*, 1997). Strong binding of the β-branched side chains of Ile or Val is an unusual feature of elastase, which distinguishes it from virtually all other serine proteases (Gron *et al.*, 1992). Binding of the relatively small Val is possible due to shrinkage of the S_1 pocket and tilting of the valyl side chain, as observed in the methoxysuccinyl (msuc)-Ala-Ala-Pro-Val-cmk/elastase complex (Wei *et al.*, 1988).

The elastase-catalyzed hydrolysis of N-succinyl-(L-alanine)$_3$-***p***-nitroanilide was found to be accelerated by increasing ionic strength, excess substrate and organic solvents, due to an increase in the rate of acyl-enzyme formation (Lestienne and Bieth, 1980). The substrate

activation results from the binding of a second substrate molecule to a regulatory site on the enzyme, thus it can be concluded that this monomeric protease shows allosteric behaviour (Trowbridge *et al.*, 1963; Lestienne and Bieth, 1980). Occupation of the regulatory site is hypothesized to bring about a discrete conformational change of the active site so that the bound substrate molecule is hydrolyzed faster. Lestienne and Bieth (1980), showed that the rate-limiting step in the breakdown of N-succinyl-(L-alanine)$_3$-***p***-nitroanilide is acylation. Lestienne and Bieth, (1980) also found that increasing concentrations of NaCl did not increase K_m significantly and did not prevent binding of the substrate to the regulatory site of elastase. The increase in activity in various organic solvents appeared to be correlated to the hydrophobicity of the solvent. It was speculated that hydrophobic solvents weaken the hydrophobic interactions maintaining the structure of the enzyme: as a result elastase has a more favourable conformation in the presence of these solvents (Maurel, 1978; Lestienne and Bieth, 1980)

1.5.3.2 Trypsin

Trypsinogen is found in the pancreas, and is activated to trypsin in the small intestine. Its pH optimum is approximately pH 8, although this varies slightly with species (Corey *et al.*, 1995). Trypsin folds into a globular molecule with the secondary structure having a predominantly β-structure and few helices (Figure 1.4). The β-structure is organized in two barrels; the interior of each barrel being packed densely with hydrophobic amino acid side chains. There are some hollows filled with water molecules. These water molecules are integral constituents of the molecular structure and have been found in free trypsin (Bode and Schwager, 1975), in trypsin/inhibitor complex (Huber *et al.*, 1974) and in trypsinogen (Bode *et al.*, 1976). Cleaving of the N-terminal activation hexapeptide results in the conversion of pro-enzyme, trypsinogen, to trypsin (Davie and Neurath, 1955; Liu and Wang, 2004).

The "active domain" of trypsin consists of four tightly interdigitating segments, i.e. N-terminus to Gly-19, Gly-142 to Pro-152, Gly-184 to Gly-193 and Gly-216 to Asn-223. In five of the seven hinges, where chains become flexible, a glycine residue is located. Glycine, which has no side chain for interaction with other parts of the molecule, is preferred to mediate flexibility.

The segments around 190-220 are part of the specificity pocket. They are connected by the disulfide 191-220 which can be selectively reduced to make the molecule more flexible

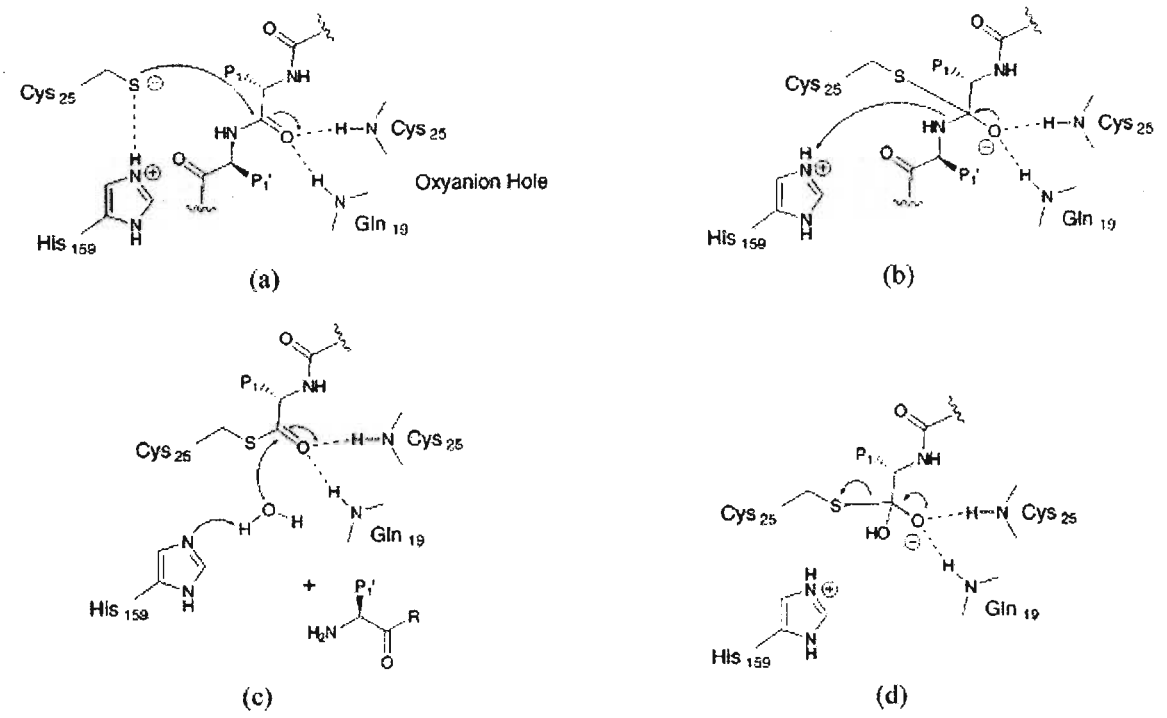
(Knights and Light, 1976). The triggering event that led to the formation of the specificity pocket is the conformational change of Asp-194 which forms a link to His-40 in trypsinogen and an internal salt bridge to Ile-16, the nascent N-terminus of trypsin (Huber and Bode, 1978). Trypsin has a preference at P_1' for Ala and Ser, followed by Arg. Peptides containing residues with large hydrophobic side chains at this position are resistant; this includes Z-Phe-Arg-NHMec since the large and hydrophobic -NHMec group is located at P_1' (Melo *et al.*, 2001).

Figure 1.4. The 3-dimensional structure of trypsin.
The pink ribbons represent the α-helices, arrowed ribbons are β-sheets, grey rope indicates non-secondary structures and the side chains of the active site residues are shown in green (**www.ebi.ac.uk/msd-srv/docs/EMBO/Thornton.ppt**).

1.5.4 Cysteine proteases

Cysteine proteases exist in three structurally distinct classes, papain-like (e.g. cathepsins), ICE-like (caspases), or picorna-viral-like (similar to serine proteases with cysteine replacing serine). The papain group of cysteine proteases has been the most studied until recently. The active site of papain contains a catalytic triad of Cys-25, His-159, and Asn-175. However, it is arguable whether the catalytic dyad of Cys and His is adequate for full catalytic activity. Asn-175 has been proposed to orientate His-159, so that the

imidazole group of His-159 polarizes the thiol group of Cys-25, allowing deprotonation even at neutral to weakly acidic pH (Leung *et al.*, 2000).



Scheme 2. General catalytic mechanism for cysteine proteases.

(Leung *et al.*, 2000)

The thiol group of the active site cysteine and the imidazole ring of histidine are believed to exist as a thiolate/imidazolium ion pair. The nucleophilic thiolate anion readily attacks the scissile amide bond (Scheme 2a). The tetrahedral intermediate produced is stabilized by the oxyanion hole. This intermediate breaks down, via acid-assisted catalysis, to the thioester intermediate (Scheme 2b), releasing the C-terminal substrate fragment (Scheme 2c). Water hydrolysis regenerates active site and the N-terminal substrate fragment (Scheme 2d).

1.5.4.1 Cathepsin L

Cathepsin L [EC 3.4.22.15] is an endolysosomal cysteine proteinase involved in the intracellular degradation of proteins; it is also sometimes secreted in a precursor form known as procathepsin L (Kirschke *et al.*, 1998). Cathepsin L is translated as preprocathepsin L, transferred through the Golgi apparatus as procathepsin L and stored in the lysosomes as mature cathepsin L (Ishidoh and Kominami, 1998). Cathepsin L and procathepsin L are thought to play an active role in cancer progression. An increase in

procathepsin L secretion by human melanoma cells increases their tumorigenicity and switches their phenotype from nonmetastatic to highly metastatic (Rousselet *et al.*, 2004). Anti-cathepsin L antibodies have been shown to reduce tumorigenicity and inhibit formation of the metastatic phenotype of human melanoma (Rousselet *et al.*, 2004).

Although the papain family of cysteine proteases, to which cathepsin L belongs, have been considered primarily as degradative proteases of the endolysosomal system, there are indications that these enzymes have other specific biological functions (Yasothornsrikul *et al.*, 2003). It has been shown that secretory vesicle cathepsin L is responsible for converting proenkephalin to the active enkaphalin peptide neurotransmitter (Yasothornsrikul *et al.*, 2003). This process takes place in the secretory vesicles of neuroendocrine chromaffin cells. Cathepsin L is also believed to play a role in Alzheimer's disease, in which deposition of β-amyloid in the brain is a neuropathological characteristic. Boland and Campbell (2004) demonstrated that β-amyloid increases the activity and concentration of cathepsin L in the cytosol and the tendency of β-amyloid to induce apoptotic changes was prevented by the selective inhibition of cathepsin L.

Cathepsin L has a high sequence identity (41%) and an overall structure similar to that of papain (Fujishima *et al.*, 1997). The S′ region of cathepsin L is similar to that of papain and is a shallow depression rather than a cleft. The indole ring of Trp-189 is located at the centre serving as a flat floor. The floor is surrounded by the side chains of Gln-21, Gly-20, Asn-18, Glu-192, Trp-193 and Leu-144. The S_2 subsite of cathepsin L is deep and hydrophobic and prefers bulky and hydrophobic residues at the P_2 position of the substrate. In the active site cleft Asp-162, Met-161, Asp-160 and Ala-214 form a continuous wall on the right hand side of the S_2 pocket which makes the S_2 pocket narrow. In the S_3 subsite there is an extended structure of Gly-67 and Gly-68 at the centre of this subsite, surrounded by the side chains of Asn-66, Glu-63 and Leu-69 as well as the carbonyl oxygen of Gly-61. Cathepsin L may accommodate a bulky group in this region. The structural information regarding cathepsin L was obtained from the crystal structure of human cathepsin L complexed with E-64 (Fujishima *et al.*, 1997).

The inactivation of cathepsin L was found to be strongly dependent on pH, with the inactivation rate constant increasing exponentially, with decreasing pH, indicating that the pH-induced inactivation of cathepsin L, on the low pH side, is catalyzed by protons (Turk *et al.*, 1999). This finding also suggests that ionic interactions are important for the

stability of cathepsin L and other cysteine proteinases as previously proposed from inactivation studies at neutral pH (Dehrmann *et al.*, 1995; Turk *et al.*, 1995; Dehrmann *et al.*, 1996). In addition, studies on buffer composition have indicated that different ions have different effects on the stability of cysteine proteinases (Dehrmann *et al.*, 1995; Dehrmann *et al.*, 1996). On the basis of these results it has been suggested that the stability of cathepsin L is a result of a delicate balance between hydrophobic and ionic interactions (Turk *et al.*, 1999).

1.5.4.2 Cathepsin B

Cathepsin B is an endolysosomal cysteine proteinase, which plays a role in intracellular protein degradation (Knop *et al.*, 1993; Mort and Buttle, 1997). It has also been implicated in a variety of physiological and pathological processes, such as general intracellular protein turnover (Shaw and Dean, 1980), bone resorption (Delaisse *et al.*, 1991), cartilage proteoglycan breakdown (Buttle and Saklatavala, 1992), antigen processing (Guagliardi *et al.*, 1990) and the malignant progression of tumours (Berquin and Sloane, 1996). Cathepsin B is synthesized as an inactive proenzyme, sorted to the lysosome and is converted into the mature enzyme by limited proteolysis.

Musil *et al.* (1991) determined the structure of human liver cathepsin B. Jia *et al.* (1995) determined the structures of native and benzyloxycarbonyl-Arg-Ser(***O***-Bzl) chloromethylketone complexed recombinant rat cathepsin B. At the protein sequence level, rat cathepsin B is 83.5% identical to the human enzyme with the non-identical residues situated at the surface of the molecule where they do not introduce significant changes in main chain folding.

Cathepsin B has a very similar overall structure to that of papain and can similarly be divided into two domains. The interface region between the two domains forms the active site cleft, with the key catalytic residues located on the upper portion of the V-shaped cleft (Figure 1.5). A long insertion loop (residues 105-125) partially blocks the upper back aspect of the active site cleft. This has been designated as the "occluding loop" (Musil *et al.*, 1991). The partially obstructed active site cleft is not a very rigid "compartment" structure. Toward the end of the long insertion, residues 120-124 form a solvent-exposed segment, of which residues 120-122 create a high wall on the upper left of the active site cleft. Since the bottom floor and the right wall of the active site cleft are

relatively rigid, the flexibility of the high covering loop may be essential for accommodating substrates of various sizes.

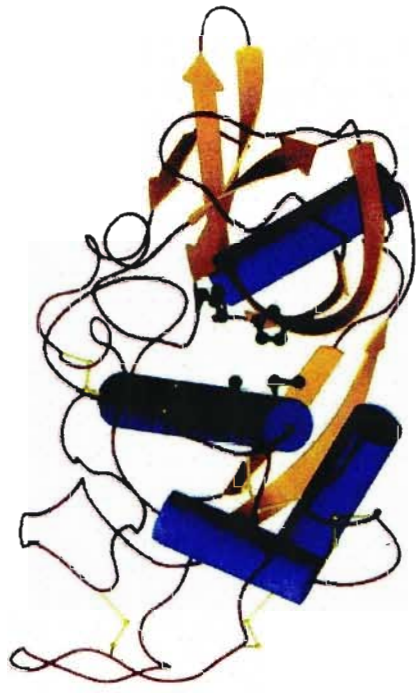


Figure 1.5. Folding of the native structure of rat cathepsin B.
The blue cylinders represent α-helices, arrowed ribbons are β-sheets, purple rope indicates non-secondary structures, disulfide bridges are in yellow and the side chains of the active site residues are shown in green (Jia *et al.*, 1995).

1.5.4.3 Bromelain

"Bromelain" refers to a crude aqueous extract from stems and immature fruits of pineapples (*Ananas comosus*). It contains a complex mixture of different cysteine proteases (stem bromelain, fruit bromelain, ananain and comosain), other enzymes such as phosphatases, glucosidases, peroxidases and cellulases and glycoproteins and carbohydrates among others, which have not yet been completely characterized (Rowan and Buttle, 1994). "Bromelain" also contains cysteine protease inhibitors (Lenarcic *et al.*, 1992). Crude bromelain has many potential clinical applications, including antimetastastic activity (Taussig *et al.*, 1985; Batkin *et al.*, 1988), immune modulation (Engwerda *et al.*, 2001), debridement of wounds (Houck *et al.*, 1983), potentiation of antibiotics (Smyth *et al.*, 1962; Tinozzi and Venegoni, 1978; Neubauer 1961), as a digestive aid (Knill-Jones *et al.* 1970), in surgical procedures and musculoskeletal injuries (Blonstein, 1960; Tassman *et al.*, 1964; Tassman *et al.*, 1965) and for cardiovascular and circulatory applications (Nieper, 1978). These pharmacological

properties may depend not solely on the proteolytic activities, but also on the presence of other factors in crude bromelain. The proteolytic activity does play a role in the inhibition of platelet aggregation and in the anti-inflammatory action. Engwerda *et al.* (2001) showed that stem bromelain modulates T and B cell immune responses *in vitro* and *in vivo*. Stem bromelain has been shown to enhance CD2-mediated T cell activation (Hale and Haynes 1992). Ananain and comosain are used as debriding agents for burn injuries (Houck *et al.*, 1983). The cysteine proteinases in bromelain may be potential anthelmintics (Stepek *et al.*, 2004). A mixture of fruit and stem bromelain was shown to protect piglets from diarrhoea caused by oral challenge with K88-positive enterotoxigenic *E. coli* (Chandler and Mynott, 1998) A fraction of bromelain can cross the intestinal epithelium as a whole, non-degraded protein, under physiological conditions (Castell *et al.*, 1997). The absorbed bromelain can associate with α2 macroglobulin and α1 antichymotrysin (Castell *et al.*, 1997). The hydrolytic activity of the circulating bromelain, for a small substrate, is not blocked by the presence of these plasma antiproteinases or by antibromelain antibodies (Castell *et al.*, 1997).

Synonyms for this enzyme include bromelase, bromelin, ananase, extranase, pinase, traumanase, juice bromelain, pineapple enzyme and fruit bromelain FA2 (www.brenda.uni-koeln.de). Fruit bromelain is a basic protein (Muriachi, 1970) whereas ananain, comosain and stem bromelain are acidic in nature (Harrach *et al.*, 1995; Harrach *et al.*, 1998; Napper *et al.*, 1994; Lee *et al.*, 1997). Its basic nature and relatively lower isoelectric point are the two main features used to distinguish fruit bromelain from the other cysteine proteinases present in pineapple juice. Very little is known about the structural aspects of fruit bromelain because no crystal structure is available to date. Due to its phylogenetic and functional relation to papain it can be speculated to have a general cysteine proteinase mechanism of action. Cysteine proteinases of the papain family generally consist of a globular protein with two domains with a deep cleft between the two domains containing the active site cysteine and histidine residues on either side. These form a thiolate-imidazolium ion pair, which is responsible for catalytic activity (Stepek *et al.*, 2004). The differences between papain and bromelain would be in the amino acids lining the substrate-binding pocket, and would result in binding to different amino acid side chains of substrate (Stepek *et al.*, 2004).

1.5.5 Cystatin C

Cystatin C is a low molecular weight protein isolated from chicken egg white that inhibits the cysteine proteinases ficin, papain, and cathepsin B, cathepsin C and cathepsin L with 1:1 stoichiometry (Fossum and Whitaker, 1968; Sen and Whitaker, 1973; Keilova and Tomasek, 1974; Anastasi *et al.*, 1983; Leonardi *et al.*, 1996). This inhibitor forms complexes with cysteine proteinases even after their catalytic sites have been inactivated by relatively bulky active-site-directed reagents.

The cystatin superfamily is subdivided into three families. Chicken cystatin is a member of the second family which consists of cystatins with a M_r of ~13 000, that contain two intramolecular disulfide bonds but lack carbohydrate groups (Bode *et al.*, 1988). Its amino acid sequence was determined independently by Turk *et al.* (1983) and Schwabe *et al.* (1984). Cystatin C has been resolved into two major fractions with pI values of 6.5 and 5.6 (Turk *et al.*, 1983; Anastasi *et al.*, 1983). Cystatin C from egg-white has similarities to a group of proteins found in mammalian skin (Jarvinen, 1978), squamous cell carcinomata (Rinne, 1980) and rat liver (Kominami *et al.*, 1981).

Abrahamson *et al.* (1987) speculated that cystatin molecules might exhibit two distinct binding sites for their target enzymes: a substrate-like 'primary' binding site, made up of the amino terminus, with the Gly-9I/Ala-10I bond close to the reactive site cysteine upon complex formation, and a 'secondary' site comprising the conserved QVVAG segment in contact with enzyme groups outside the active site.

Bode *et al.* (1988) solved the three dimensional structure of cystatin C and its possible mode of interaction with cysteine proteinases (Figure 1.6). Its structure consists of a straight five-turn α-helix, a five-stranded antiparallel β-pleated sheet that is twisted and wrapped around the α-helix and an appending segment of partially α-helical geometry. The region from Gln-53I to Gly-57I is highly conserved and implicated with binding to cysteine proteinases. It folds into a tight β-hairpin loop which on opposite sides is flanked by the amino-terminal segment and by a second hairpin loop made up of the similarly conserved segment Pro-103I to Trp-104I. These loops and the amino-terminal Gly-9I to Ala-10I form a wedge shaped 'edge' which is complementary to the active site cleft of papain (Figure 1.7). Both hairpin loops of cystatin make major binding interactions with the highly conserved residues Gly-23, Gln-19, Trp-177 and Ala-136 of papain in the neighbourhood of the reactive site Cys-25; the amino-terminal segment Gly-9I/Ala-10I of

bound cystatin is directed towards the substrate subsite S_2, but in an inappropriate conformation and too far away to be attacked by the reactive site Cys-25 (Figure 1.7) (Bode *et al.* 1988).

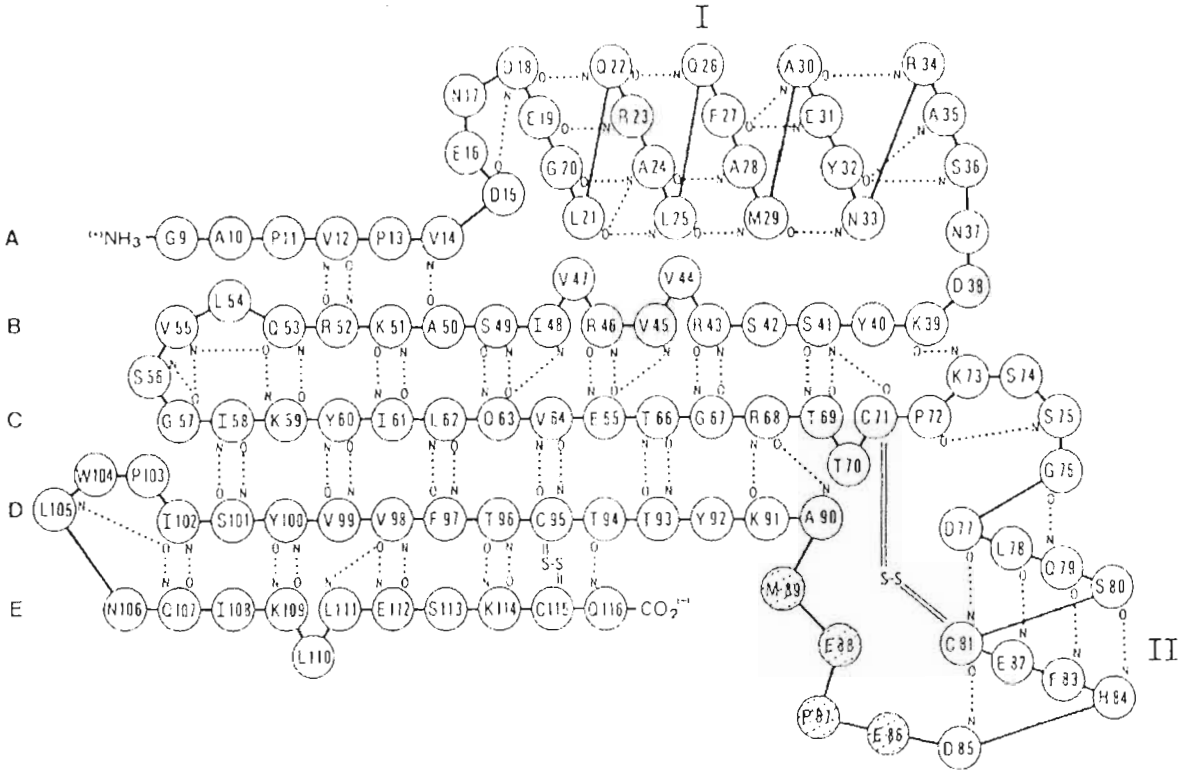


Figure 1.6. Schematic representation of the polypeptide fold arrangement of chicken egg white cystatin.
Shadowed circles represent undefined residues, main chain hydrogen bonds are represented by dashed lines and solid double lines indicate disulfide bridges (Bode *et al.*, 1988).

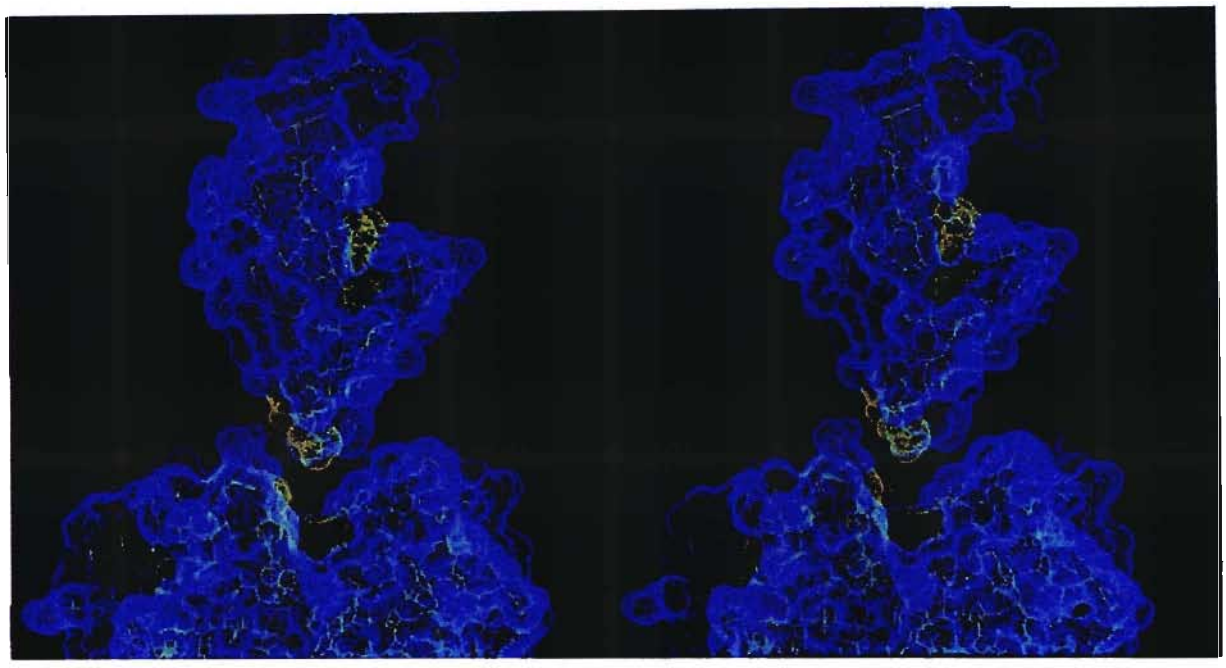


Figure 1.7. Docking of cystatin C and papain.
A 3-D view from the amino-terminal end of the inhibitor along the edge of the cystatin wedge and active site of papain (front view) (Bode *et al.*, 1988). Cystatin C is on top.

1.6 Aims of this study

The aim of this study was to investigate the effect of ionic strength on the activity of various enzymes with results interpreted in terms of the Michaelis constant (K_m) and the turnover. The effect of ionic strength on the K_i of an enzyme interaction with an inhibitor was also investigated.

2.3 Concentration of samples by dialysis against polyethylene glycol

Dilute protein samples were concentrated by dialysis against polyethylene glycol (PEG). Dialysis tubing with a M_r cut-off of 12 000 was used and the M_r of PEG used was 20 000. PEG has a high osmotic pressure when in solution. A concentration gradient is established between the solvent (water) in the dialysis bag and the PEG solution at the exterior surface of the dialysis membrane, which causes water and buffer ions to move out of the bag. Proteins with M_r larger than 12 000 are retained in the bag (Moolman, 2001).

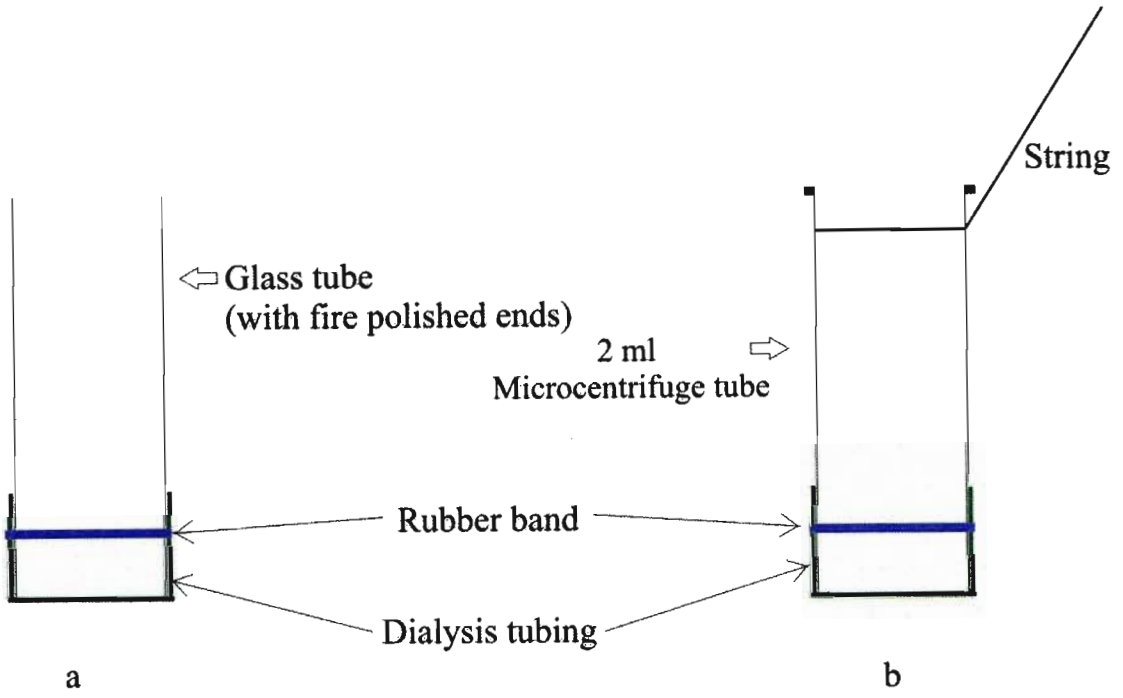


Figure 2.1. Novel apparatus for concentration of small volumes of sample against PEG 20 000.

A low cost, convenient method of concentrating small volumes of protein (< 2 ml) against PEG 20 000, makes use of a simple, novel apparatus (Figure 2.1). It requires a glass tube (0.5 cm diameter, 2 cm length) with both ends open, a single layer of dialysis tubing (M_r cut-off > 12 000) is placed firmly over one end of the tube and secured with a rubber band (Figure 2.1a). Sample is inserted through the open end and the covered surface is placed in contact with the PEG 20 000. The apparatus can be clamped into positions using a retort stand and suitable clamps. A convenient alternative to a glass tube is a 2 ml microcentrifuge tube, the lid and bottom end of the tube being cut off and the bottom covered with a single layer of dialysis tubing secured by a rubber band (Figure 2.1b). By tying a string around the upper open surface, the tube can be stuck to the side of the

container holding the PEG. The container is covered to prevent contaminants from falling into the sample.

This apparatus slows down the rate of concentration by decreasing the surface area for movement of liquid, allowing the process to be carefully monitored. It also makes addition and removal of small volumes convenient and reduces the chances of contamination during the sample removal process. This apparatus can also be used for dialysis of small volumes against other media. In the case of dialysis against buffer/water it is advisable to ensure sufficient space in the tube for an increase in volume of the sample.

The apparatus could be improved by including calibrated markings on the tube surface to give a visual estimate of the volume changes.

2.4. SDS-PAGE

Reducing SDS-PAGE was used to examine the purity of protein samples, and to determine their molecular weights. The inclusion of gelatin in the gels enabled SDS-PAGE to be used to monitor the proteolytic activity of the samples.

Electrophoresis describes the migration of charged particles in an electric field. The most popular medium for electrophoresis is polyacrylamide gel, formed by the polymerization of acrylamide monomer into long chains and the cross-linking of these by bifunctional N,N'-methylene bisacrylamide. The result is a porous gel matrix in which the pore size is of the same order as protein molecules. Proteins migrating through a gel by electrophoreses are subject to molecular sieving which slows down the migration of larger proteins relative to smaller proteins. The Ornstein-Davis (1964) polyacrylamide gel electrophoresis (PAGE) system employs two different buffer systems (and two different gel porosities), to generate a sharp interface between the charged particles in accordance with Kohlrausch's regulatory function. This results in proteins being concentrated into very thin starting bands, which minimizes the effect of diffusion of the protein band in the gel, giving improved resolution and separation according to size, charge and shape (Ornstein, 1964).

In order to determine the molecular weight of the proteins, the charge and shape factors need to be standardized. This is accomplished with the use of the anionic detergent SDS (Shapiro *et al.*, 1967). Molecules of SDS bind to the protein at a constant ratio of 1.4 g to 1 g of protein to produce rod-like complexes (Reynolds and Tanford, 1970). The intrinsic

charges of the polypeptides are insignificant compared with the negative charges provided by the bound detergent, giving all the SDS-polypeptide complexes identical charge densities. Since the distance migrated by an SDS-polypeptide complex is inversely proportional to its size, the molecular weight of individual polypeptides can be determined.

The estimation of the molecular weight of a sample polypeptide, requires a standard curve of $\log_{10}$ (polypeptide molecular weight) versus distance migrated for each of the standard molecular weight markers, relative to the migration of the bromophenol blue dye (Dennison, 2003). From its relative mobility, the molecular weight of an unknown can be read off the curve.

Non-covalently bound subunits are dissociated by boiling in SDS so, a thiol reducing agent, such as 2-mercaptoethanol could be included, to break disulfide bonds. If the sample is boiled in the presence of excess SDS and the reducing agent, the protein will be dissociated into individual polypeptide subunits. This technique allows the molecular weights of subunits to be determined, and the presence of disulfide bridges in proteins may be established.

2.4.1 Tris-tricine SDS-PAGE

The Tris tricine system (Schägger and Von Jagow, 1987) is a modification of the Laemmli (1970) method. The mobility of the protein relative to the trailing ion is decreased, and lower molecular weight proteins may be separated from SDS micelles. In this study a gel system with two porosities was used for the resolution of proteins of 5-100 kDa.

2.4.1.1 Reagents

Gel buffer [3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris (72.7 g) was dissolved in approximately 180 ml of distilled water, SDS [6 ml of a 10% (m/v) solution] was added, and the solution was adjusted to pH 8.45 with HCl and made up to 250 ml.

Monomer solution [49.5% (m/v) acrylamide, 3% (m/v) *N,N'*-methylene-bisacrylamide]. Acrylamide (48 g) and *N,N'*-methylene-bisacrylamide (3 g) were dissolved and made up to 100 ml with distilled water and stored in an amber bottle at 4°C.

10% (m/v) ammonium persulfate. Ammonium persulfate (0.2 g) was made up to 2 ml in distilled water just before use.

Anode buffer [200 mM Tris-HCl, pH 8.9]. Tris (24.22g) was dissolved in approximately 950 ml of distilled water, adjusted to pH 8.9 with HCl and made up to 1 L.

Cathode buffer [100 mM Tris-HCl, 100 mM tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (12.2 g) and tricine (17.9 g) were dissolved in approximately 950 ml of distilled water, SDS [10 ml of a 10% solution] was added, the solution adjusted to pH 8.25 with HCl and made up to 1 L.

10% (m/v) SDS. SDS (10 g) was dissolved in 100 ml distilled water with gentle heating if necessary.

Tris buffer [500 mM Tris-HCl, pH 6.8]. Tris (3 g) was dissolved in 40 ml distilled water, adjusted to pH6.8 with HCl and made up to 50 ml with distilled water. The buffer was made weekly, because of the poor buffering capacity of Tris at 2.1 pH units below its pKa at 4°C.

Reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Tris Buffer (2.5 ml), 10% (m/v) SDS solution (2.5 ml), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with distilled water. The marker dye, 1% bromophenol blue (15 µl) was added before use.

2.4.1.2 Procedure

A Hoefer SE 250 Mighty Small II vertical slab electrophoresis unit was assembled as described in the manufacturer's manual. For each of the two sides of the apparatus, one notched aluminium plate and one glass plate were cleaned with 70% ethanol and clamped with two 1.5 mm polyethylene spacers separating them at the edges. Running gel solution (Table 2.1) was filled into the space between the plates, to a depth of 3 cm from the top of the glass plate. Immediately an overlay of distilled water was applied to allow for even polymerization. The appearance of an interface between gel and overlay indicated that the gel was set (approximately 1 h); the water was removed by inversion. Stacking gel solution (Table 2.1) was poured in, up to the notch on the aluminium plate. A 10 well comb was inserted to form the sample application wells. Once the gel had set (approximately 30 min), the comb was removed and the wells rinsed with distilled water.

Cathode buffer was poured into the top electrode compartment and anode buffer into the bottom compartment. Samples were combined with an equal volume of reducing treatment buffer and incubated in a boiling water bath for 90 s. Bromophenol blue (5 µl)

was added to each sample before loading onto the gels, to visualize migration of the buffer front. Samples were applied into the wells at a concentration of at least 250 ng per band for silver staining. The gel was connected to a power pack and run at 80 V (for two gels), with unlimited mA, until the tracking dye entered the running gel, after which the voltage was increased to 100 V. The gel was run until the tracking dye was about 0.5 cm from the bottom of the running gel, at which point the gel was disconnected from the power supply and the plates, removed. Gels were immediately stained using the silver staining protocol.

Table 2.1. Preparation of the resolving and stacking gels for Tris-tricine SDS-PAGE.

Reagents	Running gel	Stacking gel
Gel buffer	6 ml	1.5 ml
Monomer solution	3.6 ml	0.5 ml
Distilled water	8.4 ml	4 ml
Ammonium persulfate	60 µl	30 µl
TEMED	6 µl	12 µl

2.4.2 Tris-glycine SDS-PAGE

SDS-PAGE was conducted using the method of Ornstein (1964) and Davis (1964) with modifications by Laemmli (1970), who introduced SDS to the protocol.

2.4.2.1 Reagents

Solution A [30% (m/v) acrylamide, 2.7% (m/v) N,N'-methylene-bisacrylamide]. Acrylamide (58.4 g) and N,N'-methylene-bisacrylamide (1.6 g) were dissolved and made up to 200 ml with distilled water. The solution was filtered and stored in an amber bottle at 4°C.

Solution B; 4 x running gel buffer [1.5 M Tris-HCl, pH 8.8]. Tris (36.3 g) was dissolved in about 150 ml distilled water, titrated to pH 8.8 with HCl and made up to 200 ml.

Solution C; 4 x stacking gel buffer [500 mM Tris-HCl, pH 6.8]. Tris (3.0 g) was dissolved in about 40 ml of distilled water, titrated to pH 6.8 with HCl and made up to 50 ml.

Solution D [10% (m/v) sodium dodecyl sulfate]. SDS (10 g) was dissolved in 100 ml distilled water with gentle heating if necessary.

CHAPTER 2

MATERIALS AND METHODS

In this chapter the methods that were used routinely throughout this study are described. Methods considered to be relatively specialized will be described in the relevant chapters. Only reagents that were prepared before use are described, reagents not requiring preparation are referred to at the relevant point in the procedure.

2.1 Materials

α-Glucosidase (type I: from bakers' yeast), papain, 4-methylumbelliferyl α-D-glucoside, N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin, N-methoxy succinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin, N-Benzoyl-Phe-Val-Arg-7-amido-4-methylcoumarin, Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride and Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride were from Sigma. Trypsin (from bovine pancreas) was from Merck. 7-amino-4-methyl coumarin standard was from ICN Biomedicals Inc. Human leukocyte elastase and cathepsin G were from Athens Research and Technology. AminoLink® affinity chromatography resin was from Pierce and SP Sepharose fast flow from Amersham Biosciences. All other reagents were of the highest quality available.

2.2 Bradford dye-binding protein assay

In order to quantitatively evaluate the final product and the various steps in the purification procedure a protein assay is necessary. A rapid method, which is reproducible and uses small amounts of sample, is preferred. One such method is the Bradford dye-binding protein assay (Bradford, 1976); the modified version by Read and Northcote (1981) was used in the present study.

The Bradford dye-binding assay uses Coomassie brilliant blue G-250 to bind protein. Its major advantages are ease of performance, rapidity, relative sensitivity, specificity for proteins and economy (Bradford, 1976). Coomassie brilliant blue dye has three charged forms present in equilibrium at acid pH, the red absorbs at 470 nm, blue at 590 nm and green at 650 nm (Chial and Splittgerber, 1993). The blue form binds the protein forming a complex that absorbs light at 595 nm (Bradford, 1976). Colour development takes less than 5 min, with the protein-dye complex being stable over a period of 1 h. Another

advantage of this assay is its insensitivity to magnesium chloride, potassium chloride, sodium chloride, ethanol and ammonium sulfate. Only detergents, such as SDS, Triton X (1%) and ampholines (1%) interfere with the assay. The interference cannot be compensated for by incorporation in the reagent-blank (Read and Northcote, 1981); the reagents need to be removed prior to assay. Another disadvantage of this procedure is a slight nonlinearity in the response (Bradford, 1976) that stems from an overlap in the spectrum of the two different colour forms of the dye. Over a broad range of protein concentration the degree of curvature is quite large (Zor and Selinger, 1996), making 2-10 μg/ml suitable for assay and construction of a calibration graph. This is a narrow range of relatively high protein concentration. Modifications by Read and Northcote (1981) include increasing the concentration of dye in the assay solution or decreasing the concentration of phosphoric acid. These modifications increase the sensitivity of the assay, permitting accurate determination of 2 μg/ml protein in a sample volume of 50 μl.

2.2.1 Reagents

Distilled water. Prepared using the Millipore, Milli RO system.

Dye reagent. Serva Blue G dye (50 mg) was dissolved in 88% phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). The solution was made up to 500 ml with distilled water and stirred for 30 min. The resulting solution was filtered through Whatman No. 1 filter paper and stored in an amber bottle. Visual checks for precipitation were made prior to use and, if precipitation was observed, the reagent was filtered and re-calibrated or fresh reagent was made.

Standard protein solution. Ovalbumin solution (0.1 mg/ml) was made up in distilled water.

2.2.2 Procedure

Protein samples were diluted, if necessary, to 50 μl with distilled water, dye reagent (950 μl) was added, the solution mixed and allowed to develop for 2 min. The absorbance was read at 595 nm, in plastic microcuvettes (1 ml): five replicates were done for each protein concentration. The blank consisted of distilled water (50 μl) and dye reagent (950 μl). A standard curve, from 0-50 μl (0-5 μg) of the standard protein solution was constructed. Protein concentration was determined using the equation,

$$Absorbance = (slope)(protein\,concentration) + y - intercept$$

generated by linear regression analysis of the standard curve data.

Solution E [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.2 g) was dissolved and made up to 2 ml in distilled water just before use.

Solution F; Tank buffer [25 mM Tris, 192 mM glycine, 0.1% (m/v) SDS, pH8.3]. Tris (12.0 g) and glycine (57.6 g) were dissolved in 4 litres of distilled water. Immediately before use solution D (2.5 ml) was added to 250 ml buffer. The pH is 8.3.

Reducing treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. See Section 2.4.1.1.

2.4.2.2 Procedure

The procedure was the same as in Section 2.4.1.2 with the following modifications. The running and stacking gels were made up as per Table 2.2. Tank buffer containing 0.1% SDS was placed in the top and bottom electrode compartments of the apparatus and the power supply was set at 18 mA per gel, with V on maximum.

Table 2.2. Preparation of the resolving and stacking gels for 12.5% Tris-glycine SDS-PAGE.

Reagents	Running gel (ml)	Stacking gel (ml)
A	6.25	0.94
B	3.75	
C		1.75
D	0.15	0.07
E	0.075	0.035
TEMED	0.0075	0.018
Distilled water	4.75	4.2

2.5 Silver staining

Proteins bands were visualized using the silver stain technique of Blum *et al.* (1987). The sensitivity of this technique allows detection of nanogram amounts of protein. Silver complexes with charged amino acid side chains in proteins, thereby staining protein bands. The process is dependent on formaldehyde being a strong reducing agent under alkaline conditions, which is necessary for visualization of the silver-amino acid complex (Nielsen and Brown, 1984). Image development requires a pH change, which causes the non-specific formation of silver salts on the gel, reducing the contrast. Treating the gel

with thiosulfate, complexes and dissolves these salts, reducing the background (Blum *et al.*, 1987).

2.5.1 Reagents

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.2% (v/v) formaldehyde]. Methanol (100 ml), and glacial acetic acid (24 ml) were made up to 200 ml with distilled water. Just before use, 37% formaldehyde was added (25 µl/50 ml).

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

Pre-treatment solution [0.02% (m/v) $Na_2S_2O_3.5H_2O$]. $Na_2S_2O_3.5H_2O$ (0.1 g) was made up to 500 ml with distilled water.

Impregnation solution [0.2% (m/v) $AgNO_3$, 0.03% (m/v) formaldehyde]. $AgNO_3$ (0.4 g) was made up to 500 ml with distilled water. Just before use, 37% formaldehyde was added (15 µl/50 ml).

Development solution [6% (m/v) Na_2CO_3, 0.0004% (m/v) $Na_2S_2O_3.5H_2O$, 0.02% (v/v) formaldehyde]. Na_2CO_3 (12 g) and pre-treatment solution (4 ml) were made up to 200 ml with distilled water. Just before use, 37% formaldehyde was added (25 µl/50 ml).

Stop solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and glacial acetic acid (12 ml) were made up to 100 ml with distilled water.

2.5.2 Procedure

All steps were carried out at RT in clean glass containers previously rinsed with 70% ethanol. Immediately after electrophoresis, the gel was soaked in fixing solution (1 h or overnight), followed by wash solution (3 x 20 min) to neutralize the gel and allow for subsequent treatment with the acid labile $Na_2S_2O_3$. The gel was treated with pre-treatment solution (1 min), rinsed with distilled water (3 x 20 s), and soaked in impregnation solution (20 min). The gel was rinsed with distilled water (3 x 20 s), to remove excess $AgNO_3$ from the gel surface, and immersed in development solution until bands were evident against a lightly stained background (< 10 min). The gel was rinsed in distilled water (2 x 2 min), treated with stop solution (10 min), and again washed in wash solution (2 x 2 min) before being stored in a sealed plastic bag, in the dark at 4°C.

2.6 Substrate gel electrophoresis

SDS-PAGE conducted on polyacrylamide gels in which gelatin has been co-polymerized allows the separation and visualization of proteolytic enzymes. Proteinases run on the gel, in the presence of SDS, to positions that correspond to their molecular weights. Following this the proteins are renatured by the removal of SDS by incubation in Triton X-100 (Heussen and Dowdle, 1980). The gel is incubated in assay buffer containing any activator(s) required to ensure specific protease activity. Amido black stains a blue background with clear bands indicating gelatin digestion.

2.6.1 Reagents

1% (m/v) gelatin. Gelatin (0.1 g) was dissolved in 10 ml of Solution B (Section 2.4.2.1), with mild heating.

2.5% (v/v) Triton X-100. Triton X-100 (5 ml) was made up to 200 ml with distilled water.

Assay buffer [100 mM sodium acetate, 40 mM cysteine, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (2.86 ml) and Na_2EDTA (0.19 g) were dissolved in 450 ml of distilled water, adjusted to pH 5.5 with NaOH. NaN_3 (0.1 g) was added, the pH checked and readjusted if necessary, and the solution was made up to 500 ml. (NaN_3 is added after titration as it liberates the toxic gas HN_3 upon exposure to acid) Cysteine-HCl (0.70 g) was added to 100 ml buffer immediately before use.

Destaining solution [30% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (300 ml) and acetic acid (100 ml) were made up to 1 L with distilled water.

0.1% (m/v) Amido black. Amido black (0.1 g) was dissolved in 100 ml of destaining solution.

2.6.2 Procedure

The procedure for Tris glycine gels (Section 2.4.2.2) was modified by using 2.25 ml of solution B and 1.5 ml of 1% gelatin solution in the running gel. The sample was combined with an equal volume of reducing treatment buffer, but was not incubated in boiling water. After electrophoresis, the gel was incubated in Triton X-100 (2 x 100 ml, 1 h, RT), followed by incubation in assay buffer, containing 40 mM cysteine (100 ml, 3 h, 37°C). The gel was stained in amido black stain solution (100 ml, 1 h) and destained overnight.

CHAPTER 3

ISOLATION OF CATHEPSIN L, BROMELAIN AND CYSTATIN C

3.1 Introduction

Isolations described in this chapter primarily consisted of two steps, fractionation by salt precipitation and purification by chromatography.

3.1.1 Three-phase partitioning

Combining t-butanol with salt fractionation is the basis of three phase partitioning (TPP). Ammonium sulfate has a polyvalent sulfate group (anion) and a univalent cationic group. Polyvalent anions are more effective at salting out than univalent anions, though having a polyvalent cation would negate the effect. Thus, the polyvalent anion/univalent cation combination of ammonium sulfate makes it an effective salting out agent. Its high solubility, availability and low cost also make it favourable (Dennison, 2003). "Kosmotropes" are agents that stabilize proteins by forcing them into a compact, tight conformation that is stable but less soluble. By contrast, "chaotropes" induce proteins to adopt a loose, open conformation that is more soluble but less stable (Dennison and Lovrien, 1997). The sulfate ion is a kosmotrope that stabilizes protein folding: protein is stabilized but precipitates due to conformational tightening. The sulfate ion works by both a pushing and a pulling mechanism to bring about the "conformational tightening". Once hydrated, the SO_4^{2-} aq ion carries up to 14 water molecules: this large ion crowds the loosely conformed peptide, pushes it and forces conformational tightening. In "pulling", SO_4^{2-} interacts with cationic sites on the protein and draws the protein conformation inward, decreasing protein net hydration. Ionic strength effects, kosmotropy, cavity surface tension enhancement, osmotic stress and exclusion crowding all contribute to the sulfate ion's success as a salting out agent (Dennison and Lovrien, 1997).

Tertiary butanol is a kosmotrope, which is completely miscible with water. Upon the addition of enough salt to a mixture of water and t-butanol there is separation into a lower aqueous phase and upper t-butanol phase. If protein is also present, precipitated protein forms a third phase between the aqueous and t-butanol phases (Pike and Dennison, 1989a). Because of its size and "bushy" structure t-butanol does not easily penetrate folded proteins, acting as a differentiating solvent or cosolvent, and prevents denaturation of

proteins. Tertiary butanol may bind hydrophobic patches on the protein and reversibly inhibits enzyme activity (Dennison and Lovrien, 1997; Dennison *et al.*, 2000). This inhibiting action of t-butanol is not yet full understood, but it has been found that in the presence of t-butanol some proteins undergo conformational changes favouring an increased proportion of α-helices (Pike and Dennison, 1989a).

Some enzymes isolated using TPP have been found to have enhanced activity. Studies in this regard were carried out using the crystal structure of a modified serine proteinase, proteinase K (Singh *et al.*, 2001). It was found that the water structure in the substrate binding site had undergone a rearrangement, with some of the water molecules either displaced or completely absent (Singh *et al.*, 2001). Two acetate ions were identified in the structure. The first, located in the active site, seemed to mimic the role of water in the enzyme activity and stability. The other, located at the surface of the molecule is involved in stabilizing the local structure of the enzyme (Singh *et al.*, 2001). The increased enzymatic activity can be attributed to the presence of an acetate ion in the active site and to a higher overall temperature factor (B-factor). Higher B-factor results in a number of residues, particularly their side chains, adopting more than one conformation. It appears that the protein exists in an excited state, which might help the enzyme to function more rapidly than the original enzyme in aqueous media (Singh *et al.*, 2001).

During conventional salting out the precipitated protein sinks, whereas in the presence of t-butanol, the protein floats upon the aqueous phase (Pike and Dennison, 1989a) (Figure 3.1). Tertiary butanol binding to the protein surface creates an exposed nonpolar character, which helps the protein to float (Dennison and Lovrien, 1997). The t-butanol/protein complex has a lower density than the water/protein complex causing it to float (Pike and Dennison, 1989a). If the hydrophobic character of the protein is increased by t-butanol, it could lead to the protein being more easily salted out, requiring less ammonium sulfate (Pike and Dennison, 1989a). Contaminants that are soluble in organic solvents are removed in the t-butanol layer, including unwanted compounds such as lipids, phenolic compounds, some detergents and tannins (Dennison and Lovrien, 1997). Multimeric proteins in which the subunits are linked by hydrophobic bonds, such as haemoglobin, are denatured by TPP (Jacobs *et al.*, 1989). In the present study, t-butanol itself was found to denature proteins from chicken egg white. The protein precipitated using TPP is concentrated and contains significantly less salt than samples obtained using conventional ammonium sulfate precipitation (Pike and Dennison, 1989b). The addition of

t-butanol prior to homogenization inhibits enzyme activity; this reduces autodigestion, and the formation of artifactual complexes, like that of cathepsin L and stefin B (Pike *et al.*, 1992; Coetzer *et al.*, 1995). Inhibition of protein/protein interactions and proteolysis (autolysis) improves the yields of enzyme obtained (Moolman, 2001). Temperature has little effect on the TPP process, but after TPP at 37°C the protein redissolved slower than after TPP at lower temperatures (Pike and Dennison, 1989a).

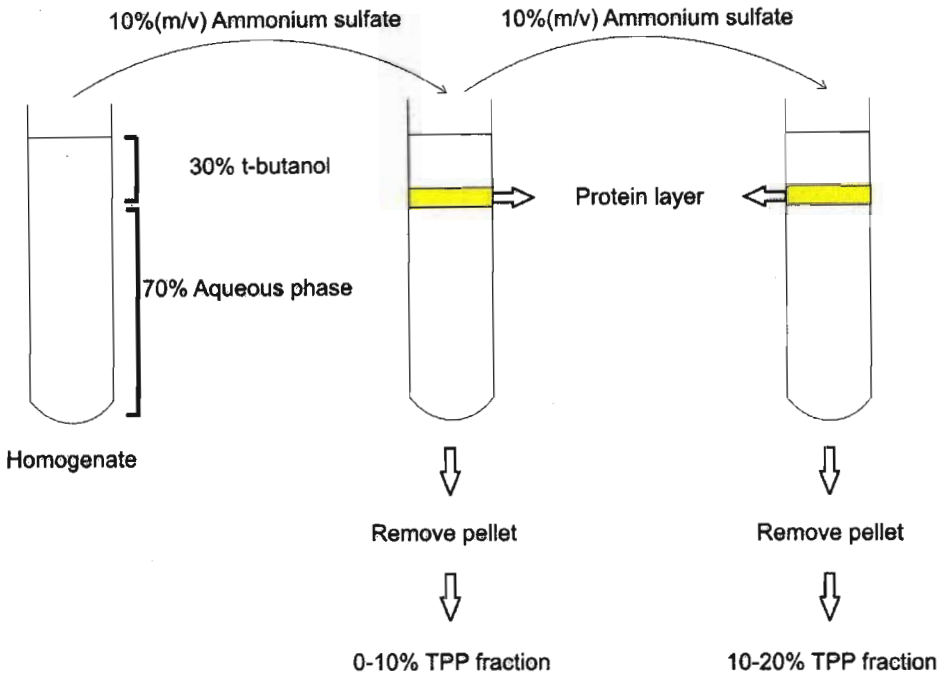


Figure 3.1. The TPP procedure.

A schematic representation of the TPP procedure is shown in Figure 3.1. The homogenate is made up of 70% aqueous phase and 30% t-butanol. Ammonium sulfate is added to the solution and mixed until all the salt is dissolved. After centrifugation, a protein pellet is visible between the aqueous and solvent phases. The pellet is carefully removed and more ammonium sulfate is added to the homogenate. The procedure is repeated until the desired protein precipitates out. A simple equation can be used to calculate the volume of t-butanol to be added before homogenization:

$$\frac{Sample\,Volume + Buffer\,Volume}{70} \times 30 = Volume\,of\,t-butanol$$

3.1.1.1 Previous protein isolations using TPP

TPP has proved to be successful and superior to conventional ammonium sulfate precipitation in the isolation of cathepsin L (Pike and Dennison, 1989b), cathepsin D

(Jacobs *et al.*, 1989), trypanosome proteinases (Troeberg *et al.*, 1996) and cathepsin B (Meinesz, 1996). TPP was used to resolve the thylakoid proteome (Peltier *et al.*, 2004); this included elucidating the likely location of many membrane proteins, including 190 proteins of unknown function. Three phase partitioning with esterified alginate as a macroaffinity ligand led to refolding of urea-denatured pectinase with simultaneous purification (Sharma *et al.*, 2004). The purification of green fluorescent protein was carried out in a single step using TPP (Jain *et al.*, 2004). In the present study TPP was used as a novel method for isolating cystatin C. Several others are listed in Dennison and Lovrien (1997)

3.1.2 Chromatography

For the proteins isolated as described in this chapter, TPP was not sufficient to give a purified product and an additional chromatography step was required. All chromatographic separations depend upon the differential partition of solutes between a mobile phase and a stationary phase (Dennison, 2003). The two types of chromatography used were, ion exchange chromatography, on an SP-Sepharose Fast Flow (Amersham Biosciences) column which was used for all the enzymes, and affinity chromatography on an AminoLink®-papain (Pierce) column used for the inhibitor.

In ion exchange chromatography, charged proteins are separated using a column containing resin with an opposite charge. Upon elution with a salt gradient, proteins are eluted according to their differential affinities for the stationary phase (Dennison, 2003). SP-Sepharose Fast Flow from Amersham Biosciences consists of a highly cross-linked agarose matrix with an anionic group covalently attached.

Affinity chromatography depends on the very specific, complementary steric relationship between the active site and a substrate, or inhibitor as was the case in this study (Dennison, 2003). The desired protein is specifically selected out from a crude mixture essentially in a single step (Dennison, 2003). In order to isolate the inhibitor, cystatin C, papain was immobilized by conjugation to an insoluble matrix. The resin used for the conjugation was AminoLink® (Pierce Biotechnology). The gel support was 4% cross-linked beaded agarose that had been activated to form aldehyde functional groups. The aldehydes react spontaneously with primary amines found on lysine residues and at the amino terminus of a peptide chain. Reductive amination of the resulting Schiff base forms a stable, secondary amine linkage with minimal leakage of the ligand (Gray, 1980). The

reductant used was sodium cyanoborohydride ($NaCNBH_3$). The unreacted aldehyde functional groups on the gel were blocked with the primary amine molecules in Tris-HCl buffer, again in the presence of $NaCNBH_3$ (Figure 3.2).

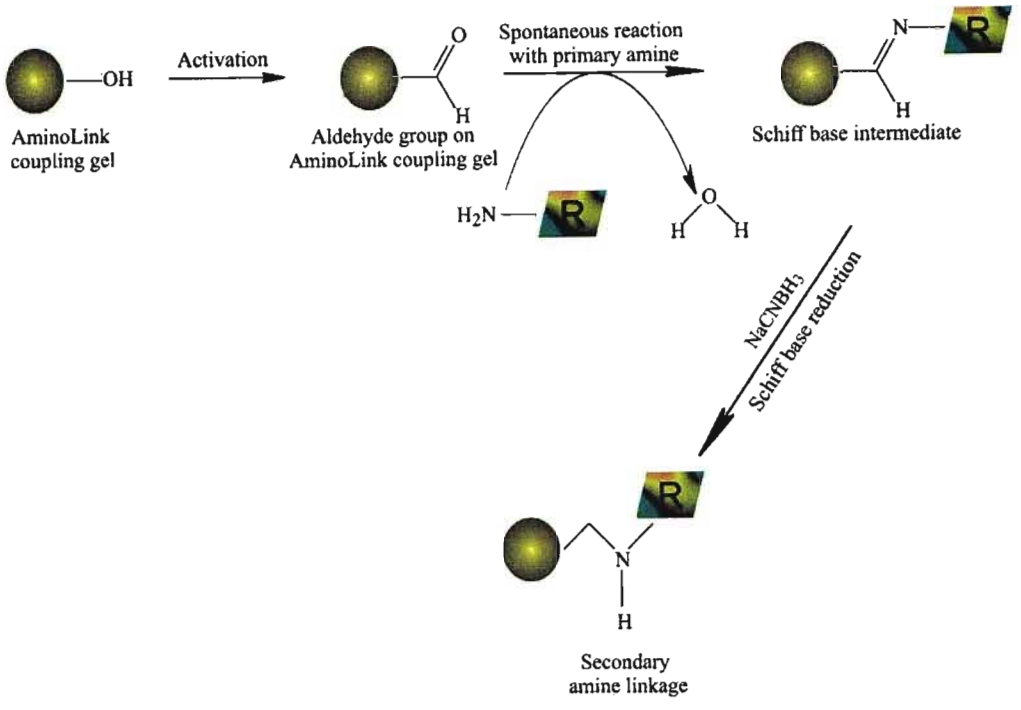


Figure 3.2. Protein coupling to an AminoLink® column.

Using TPP and chromatography as the basis, rapid and cheap purification schemes were employed to isolate cathepsin L, bromelain and cystatin C. These products were used in studies described in Chapter 4.

3.2 Cathepsin L

The various sources of cathepsin L include rat liver, rat kidney, rabbit liver (reviewed by Kirschke *et al.*, 1989), human and sheep liver (Coetzer, 1992), baboon liver (Coetzer 1992; Coetzer *et al.*, 1995) and bovine liver (Mason, 1986), with the highest yield being from sheep liver (Mason, 1986).

Mature cathepsin L exists as a single-chain form, with M_r 28 000 or as a two-chain form consisting of a heavy chain M_r 24 000 and a light chain M_r 4 000 (Kirschke *et al.*, 1998). The occurrence of the two-chain form might be correlated with the length of the isolation procedure (Pike and Dennison, 1989b). Three phase partitioning is a rapid procedure and results in single-chain cathepsin L being isolated (Pike and Dennison, 1989b). The procedure used in the present study was that described by Pike and Dennison (1989b), with

modifications described by Pike *et al.* (1992). A further modification to the protocol was the addition of 30% (v/v) t-butanol prior to homogenization (Dennison *et al.*, 2000; Moolman, 2001). n-Butanol was omitted from the homogenization buffer in the present study.

One of the known artifacts of the isolation of cathepsin L from sheep liver is a proteolytically active, covalent complex with stefin B (Pike, 1990). Stefin B is the cytoplasmic inhibitor of cathepsin L. The complex also occurred after isolations from human and baboon liver (Coetzer *et al.*, 1995). The artifact has been identified after isolation by conventional ammonium sulfate precipitation also and so is not specifically a byproduct of TPP (Pike *et al.*, 1992). However, the complex can be obviated if 30% (v/v) t-butanol is added prior to homogenization (Dennison *et al.*, 2000).

The isoelectric point of mature cathepsin L is in the range of 5.0-6.3 (Kirschke *et al.*, 1998). It is reported to be stable in the pH range 4.5-6.0 and is catalytically active in the presence of thiol compounds and EDTA at pH 3.5-7.0 (Kirschke *et al.*, 1998). Stability of cathepsin L is affected by ionic strength and reductive agents (Dehrmann *et al.*, 1995; 1996).

3.2.1 Cathepsin L assay

The substrate, Z-Phe-Arg-NHMec, is barely fluorescent as an intact molecule but once hydrolyzed the 7-amino-4-methylcoumarin group is liberated which leads to an increase in fluorescence. Over a period of time an increase in hydrolyzation of the substrate may be followed by the increase in fluorescence and v_0 can be obtained from the linear relationship at the origin. This substrate, as well as others suitable for assaying cathepsin L activity, is also susceptible to cathepsin B activity, but is more sensitive to cathepsin L (Barrett and Kirschke, 1981). For samples that may contain a mixture of cathepsins B and L it is necessary to inhibit cathepsin B activity in order to specifically measure cathepsin L activity. A practical method of doing this is to incorporate 3 M urea in the assay buffer; the result is an increase in activity of cathepsin L but the reversible denaturation of cathepsin B (Dehrmann, 1998). An alternative to inhibiting cathepsin B is to correct for its activity, by assaying for cathepsin B using Z-Arg-Arg-NHMec.

Dithiothreitol (DTT) is a protective agent for sulfhydryl groups in proteins and is used to reduce disulfide bonds between cysteine residues (Cleland, 1964)

3.2.1.1 Reagents

Buffer [400 mM Na-acetate, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 9 mM DTT, pH 5.5]. Glacial acetic acid (23 ml) and Na_2EDTA (1.5 g) were dissolved in 950 ml distilled water and adjusted to pH 5.5 with NaOH. NaN_3 (0.2 g) was added, the pH readjusted if necessary and the solution was made up to 1 L with distilled water. DTT was added to a concentration of 9 mM (0.00671 g DTT/5 ml buffer) immediately before use.

1 mM substrate stock solution. Z-Phe-Arg-NHMec (1 mg) was dissolved in DMSO (1.5 ml) and stored at 4°C.

20 µM substrate solution. Substrate stock solution (0.1 ml) was diluted to 5 ml with distilled water.

3.2.1.2 Procedure

Enzyme sample (50 µl), and buffer containing DTT (100 µl), were added to wells of a white FluoroNunc MaxiSorp microtitre plate and pre-incubated for 2 min at 37°C. 20 µM substrate solution (50 µl) was added and the fluorescence of the liberated aminomethylcoumarin was determined in a fluorescence microplate reader (Cambridge Technology Model 7620) with excitation at 360 nm and emission at 460 nm. Fluorescence readings where taken at 1 min intervals over 5 min. v_0 values where determined by plotting fluorescence versus time and determining the slope at the origin.

3.2.2 Cathepsin B assay

Cathepsin B assays were used to distinguish if both enzymes were present in the isolated samples. The cathepsin L assay (Section 3.2.1) is applicable to cathepsins L and B.

3.2.2.1 Reagents

Buffer [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 7 mM DTT, pH 6.0]. NaH_2PO_4 (6.90 g) and Na_2EDTA (0.93 g) were dissolved in 450 ml distilled water and adjusted to pH 6.0 with NaOH. NaN_3 (0.1 g) was added, the pH readjusted if necessary and the solution was made up to 500 ml with distilled water. DTT was added to a concentration of 5 mM (0.0051 g DTT/5 ml buffer) immediately before use.

1 mM substrate stock solution. Z-Arg-Arg-NHMec (1.1 mg) was dissolved in DMSO (1.5 ml) and stored at 4°C.

40 µM substrate solution. Substrate stock solution (0.2 ml) was diluted to 5 ml with distilled water.

3.2.2.2 Procedure

The procedure was the same as described in Section 3.2.1.2 with the buffer and enzyme solutions described in Section 3.2.2.1 being used.

3.2.3 Units of activity

Using a standard 7-aminomethyl coumarin solution, units of activity can be assigned to fluorescence. The fluorescence obtained from a 0.5 μM standard is equal to 1000 arbitrary enzyme fluorescence units, which is equal to 0.1 mU of enzyme activity (Barrett and Kirschke, 1981). The activity can be calculated as total activity or activity per ml, using the following equation:

$$mUnits\ activity = \frac{Fluorescence\ of\ sample \times 0.1\,mU}{Fluorescence\ of\ 0.5\,\mu M\ solution}$$

3.2.3.1 Reagents

1 mM aminomethyl coumarin standard. 7-amino-4-methyl coumarin (1.8 mg) was dissolved in DMSO (10 ml). A 0.5 μM standard was prepared by diluting stock solution (5 μl) with 10 ml of assay buffer.

3.2.3.2 Procedure

0.5 μM 7-amino-4-methyl coumarin (200 μl) was added to each of eight wells of a white FluoroNunc MaxiSorp microtitre plate and incubated for 10 min at 37°C. Thereafter the fluorescence was determined in a fluorescence microplate reader (Cambridge Technology Model 7620) with excitation at 360 nm and emission at 460 nm.

3.2.4 Isolation of bovine liver cathepsin L

3.2.4.1 Reagents

Homogenization buffer [100 mM Na-acetate, 0.1% (m/v) Na_2EDTA, pH 4.0]. Glacial acetic acid (11.44 ml) and Na_2EDTA (2.0 g) were dissolved in 1.9 L distilled water, adjusted to pH 4.0 with NaOH and made up to 2 L with distilled water.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (2.29 ml) and Na_2EDTA (0.74 g) were dissolved in 1.9 L distilled water and adjusted to pH 5.5 with NaOH. NaN_3 (0.4 g) was added, the pH readjusted if necessary and the solution was made up to 2 L with distilled water.

SP-Sepharose Fast Flow. SP-Sepharose (slurry) is mixed with buffer A in a 1:2 ratio, and packed in a glass column. Before use, the column bed was regenerated with two column volumes of 2 M NaCl in buffer A followed by equilibration with 5 column volumes of buffer A.

Bovine liver. Fresh bovine livers were collected from the Cato Ridge abattoir, cut into cubes (1-2 cm^3) and immediately frozen (-70°C) for a minimum of three days before use.

3.2.4.2 Procedure

Cathepsin L was isolated from bovine liver using modifications of the method of Pike *et al.* (1992) used for isolating sheep liver cathepsin L. Frozen bovine liver (100 g) was thawed overnight at 4°C and combined at a ratio of 1:2 (liver mass to homogenization buffer volume) with homogenization buffer, in the presence of 30% t-butanol (v/final volume). Homogenization was done in a Waring blendor (2 min, max speed). The homogenate was centrifuged (6 500 x g, 30 min, 4°C) and the supernatant adjusted to pH 4.2 with 1 M HCl and stirred. The pH 4.2 supernatant was centrifuged (6 000 x g, 20 min, 4°C). The resultant supernatant was collected and used for TPP fractionation.

TPP was effected on the pH 4.2 supernatant by adding crushed, solid $(NH_4)_2SO_4$ to 20% (m/v) [based on the volume of the pH 4.2 supernatant and t-butanol] and dissolving by gentle stirring. The solution was centrifuged (6 000 x g, 15 min, 4°C). The precipitate was discarded and to the collected supernatant and subnatant a further amount of $(NH_4)_2SO_4$ was added to bring the final concentration to 40% (m/v) [based on the volume of the pH 4.2 supernatant and t-butanol]. The supernatant and subnatant were discarded and the precipitate collected and redissolved in buffer A at one fifth of the volume of the pH 4.2 supernatant. The pH was adjusted to pH 5.5 with NaOH and the resuspended material was centrifuged (27 000 x g, 10 min, 4°C) to remove any insoluble material.

The clarified sample was applied onto a column of S-Sepharose (2.5 x 21 cm = 103 ml), pre-equilibrated with buffer A. Unbound material was eluted with 2 column volumes of buffer A. Bound material was eluted with a 0-600 mM NaCl gradient in buffer A in *ca.* 6 column volumes at 50 ml/h. The active fractions corresponding to the active peak were pooled and concentrated by dialysis against PEG 20 000.

3.2.4.3 Results

The purification of bovine liver cathepsin L is summarized in Table 3.1

Table 3.1. The purification of bovine liver cathepsin L, with t-butanol in the homogenization buffer.

	Volume (ml)	Total Protein (mg)	Total Activity (Units)	Specific Activity. (Units/mg)	Purification (fold)	Yield (%)
Homogenate	290	536.5	1.69	3.15×10^{-3}	(1)	(100)
pH 4.2 supernatant	295	348.1	1.03	2.96×10^{-3}	0.9	60.9
TPP cut (20-40%)	59	15.6	0.705	45.19×10^{-3}	14.3	41.7
SP-Sepharose	7	0.742	0.035	47.17×10^{-3}	14.9	2.07

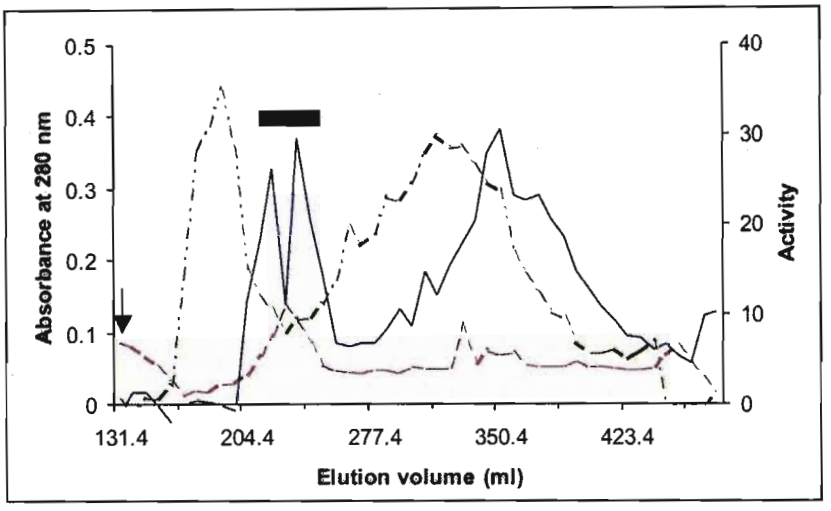


Figure 3.3. Chromatography of the 20-40% pH 4.2 TPP fraction of bovine cathepsin L on S-Sepharose.
Column, 2.5 x 21.0 cm (103 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.5, containing 1 mM Na_2EDTA, 0.02% NaN_3, and eluted with a 0-600 mM NaCl gradient (500 ml) in the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 7.3 ml (8.76 min). (—) Cathepsin L activity against Z-Phe-Arg-NHMec, (▬) pooled fractions, (---) A280 nm, (·---) Cathepsin B activity against Z-Arg-Arg-NHMec

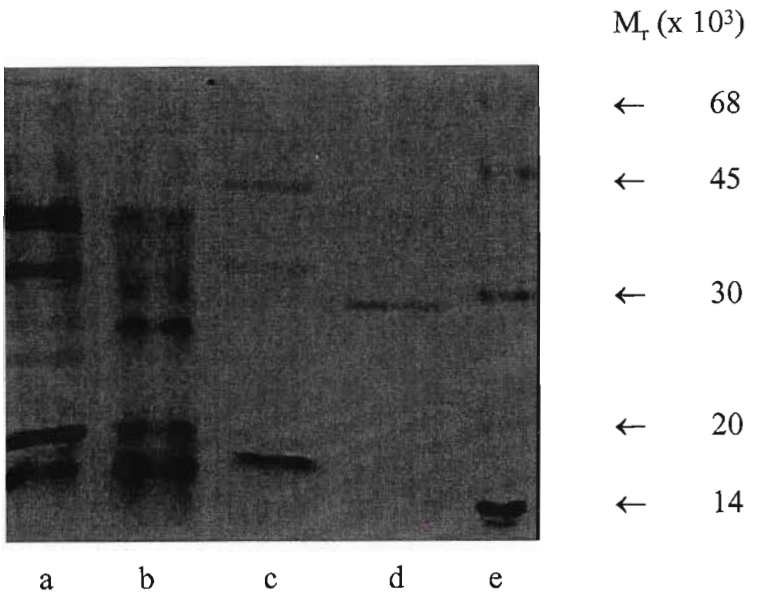


Figure 3.4. Reducing Tris-glycine SDS-PAGE of cathepsin L isolated from bovine liver.
Samples were boiled in reducing treatment buffer and loaded onto a 12% Tris-glycine gel. (a) Liver homogenate fraction; (b) pH 4.2 acid precipitate supernatant; (c) TPP fraction; (d) S-Sepharose fraction; (e) MW markers (BSA, Mr 68 000; ovalbumin, Mr 45 000; carbonic anhydrase, Mr 30 000; trypsin inhibitor, Mr 20 100; lysozyme, Mr 14 000). Visualized using silver staining.

3.2.4.4 Discussion

Kirschke *et al.* (1986) reported isolating 0.8 mg cathepsin L from 1 kg of bovine liver. The method used in their isolation is shown on Table 3.2. The method used in the present study yielded 0.7 mg from 100 g of bovine liver, which is a significant improvement.

Table 3.2. Comparison of procedures used for the isolation of cathepsin L from bovine liver

Step	Procedure	
	(Kirschke *et al.*, 1977;1986)	(Present study)
Starting material	Rat/bovine liver	Bovine liver
1	Homogenisation	Homogenisation (30% t butanol)
2	Osmotic disruption of lysosomes	Acid precipitation
3	Gel filtration	TPP
4	CM-Sephadex chromatography	S-Sepharose cation exchange chromatography

The yield of cathepsin L from bovine liver was nevertheless insufficient for the purposes of this study, so a richer source of enzyme was required. When compared to ox, rat, rabbit and human liver, sheep liver was noted to yield more enzyme than any other species (Mason, 1986). In addition, sheep liver contains cathepsin L with characteristics identical with those of human cathepsin L, which suggests that this tissue can be used as a model for the human enzyme (Mason, 1986).

Cathepsin B is also found in bovine liver and has a similar size and charge as cathepsin L: its activity can be detected using the substrate Z-Arg-Arg-NHMec. Using the present protocol, cathepsin L was precipitated at a higher ammonium sulfate concentration than cathepsin B during TPP, and cathepsin L has a greater affinity for the S-Sepharose so is eluted at a higher salt concentration (Pillay, 1999).

3.2.5 Isolation of cathepsin L from sheep liver

3.2.5.1 Reagents

Homogenization buffer [100 mM Na-acetate, 0.1% (m/v) Na_2EDTA, pH 4.0]. See Section 3.2.4.1.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. See Section 3.2.4.1.

SP-Sepharose Fast Flow. See Section 3.2.4.1.

Sheep liver. Fresh sheep livers were collected from an abattoir, cut into cubes (1-2 cm^3) and immediately frozen (-70°C) for a minimum of three days before use.

3.2.5.2 Procedure

As described in Section 3.2.4.2 sheep liver (150 g) was used.

3.2.5.3 Results

The purification of sheep liver cathepsin L is summarized in Table 3.3.

Table 3.3. The purification of sheep liver cathepsin L, with t-butanol in the homogenization buffer.

	Volume (ml)	Total Protein (mg)	Total Activity (Units)	Specific Activity. (Units/mg)	Purification (fold)	Yield (%)
Homogenate	429	25036	1.559	6.23×10^{-5}	1	100
pH 4.2 supernatant	228	1330	1.057	79.5×10^{-5}	12.8	68
S-Sepharose	5	9.5	0.179	1884×10^{-5}	302	11

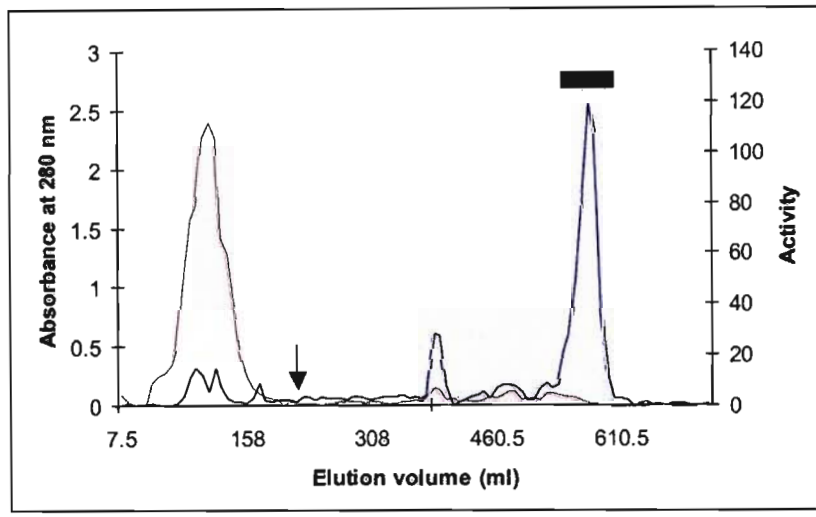


Figure 3.5. Chromatography of the 20-40% pH 4.2 TPP fraction of sheep cathepsin L on S-Sepharose.
Column, 2.5 x 21.0 cm (103 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.5, containing 1 mM Na_2EDTA, 0.02% NaN_3, and eluted with a 0-600 mM NaCl gradient (500 ml) in the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 7.3 ml (8.76 min). (—) Cathepsin L activity against Z-Phe-Arg-NHMec, (▬) pooled fractions, (—) A280 nm.

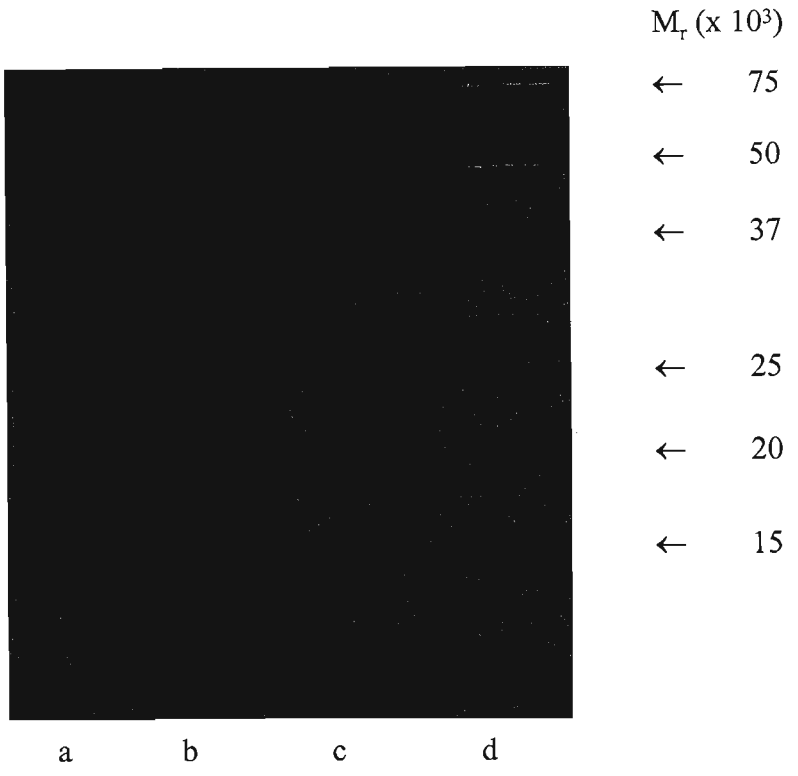


Figure 3.6. Reducing Tris-tricine SDS-PAGE of cathepsin L isolated from sheep liver.
Samples were boiled in reducing treatment buffer and loaded onto a 12% Tris-tricine gel. (a) Liver homogenate fraction, (b) pH 4.2 acid precipitate supernatant, (c) S-Sepharose fraction, (d) MW markers (Bio-Rad Precision Plus Protein™ Standards). Visualized using silver staining.

3.2.5.4 Discussion

Bovine liver gave a lower percentage yield of cathepsin L but the specific activity of the final product was significantly higher than that of sheep cathepsin L. This could imply that the form of cathepsin L present in bovine liver is more active than that in sheep liver; hence there is a smaller amount of the enzyme present in the bovine liver. Mason (1986) reported the yield of sheep cathepsin L to be 1.2 mg per 500 g of wet tissue: the present method gave 32 mg protein per 500 g of wet tissue and the method of Coetzer (1992) resulted in 1.52 mg complexed and 1.52 mg free cathepsin L from approximately 500 g sheep liver.

Mason (1986) carried out "activation" by incubating the pH 4.2 supernatant for 4 h at 37°C followed by 20°C, overnight. In the method of Coetzer (1992), incubation time was greatly reduced, which decreases the length of time cathepsin L was in contact with other proteinases. The crude sample from TPP was relatively salt free and could be applied

directly to the cation exchanger, contributing to a further decrease in contact time with other proteinases and the overall procedure time was reduced.

Table 3.4. Comparison of procedures used for the isolation of cathepsin L from sheep liver

Step	Procedure		
	(Mason, 1986)	(Coetzer (1992)	(Present study)
Starting material	Sheep liver	Sheep liver	Sheep liver
1	Homogenisation	Homogenisation	Homogenisation (30% t-butanol)*
2	Acid precipitation	Acid precipitation	Acid precipitation
3	Sephadex G-25 chromatography	TPP (30% t-butanol)	TPP
4	Sephadex C-50 chromatography	S-Sepharose cation exchange chromatography	S-Sepharose cation exchange chromatography
5	Ammonium sulfate precipitation	Sephadex G-75 chromatography	
6	Phenyl-Sepharose chromatography		
7	Mono S chromatography		
8	Mono S re-chromatography		

* Inhibits artifactual formation of covalent cathepsin L-stefin B complex, obviating necessity for Sephadex G-75 step.

Coetzer (1992) modified the method of Pike and Dennison (1989b) to include MEC on Sephadex G-75 for the removal of the M_r 37 000 proteolytically active cathepsin L/stefin B complex. Dennison *et al.* (2000) found that at 30% t-butanol, cathepsin L is completely inactive and that addition of 30% t-butanol before homogenization prevents formation of the complex. In the present study, therefore, MEC was omitted, making the method quicker and more cost effective and resulted in a much higher yield product of comparable purity.

3.3 Bromelain

Fruit bromelain is a cysteine proteinase found in pineapple (*Ananas comosus*) fruits. It has an isoelectric point of 4.6 and reported molecular masses include 23 kDa (Maurer, 2001), 25 kDa (Rowan *et al.*, 1990), 27 kDa (Ota *et al.*, 1985) and 31 kDa (Yamada *et al.*, 1976). One preparation was reported to contain carbohydrate (Ota *et al.*, 1964), while another reportedly contained neither hexosamine nor neutral sugars (Yamada *et al.*, 1976). Fruit bromelain is a basic protein (Murachi, 1970) whereas ananain, comosain and stem bromelain, the other cysteine proteinases present in pineapple, are acidic in nature (Harrach *et al.*, 1995; Harrach *et al.*, 1998; Napper *et al.*, 1994; Lee *et al.*, 1997). Its basic nature and relatively lower isoelectric point are the two main features used to distinguish fruit bromelain from the other cysteine proteinases present in pineapple juice.

Fruit bromelain has been isolated using acetone precipitation followed by chromatography on DEAE-cellulose (Ota *et al.*, 1972): this can be followed by rechromatography on ECTEOLA-cellulose (Yamada *et al.*, 1976). Rowan and Buttle (1994) retained the acetone precipitation step, with modifications, but substituted cation exchange chromatography on Mono S and affinity chromatography on Sepharose–Ahx-Phe-GlySc. In this laboratory, fruit bromelain has been isolated using a combination of homogenization in 30% t-butanol, fractionation by TPP and chromatography on SP-Sepharose.

3.3.1 Fruit bromelain assay

Fruit bromelain differs in substrate specificity from stem bromelain and comosain, but has a similar specificity to ananain. Ananain and fruit bromelain hydrolyze Z-Phe-Arg-NHMec and Z-Phe-Val-Arg-NHMec but not Z-Arg-Arg-NHMec, whereas stem bromelain and comosain react only with Z-Arg-Arg-NHMec (Rowan *et al.*, 1990). Both fruit bromelain and ananain have a higher affinity for Z-Phe-Val-Arg-NHMec than for Z-Phe-Arg-NHMec (Rowan *et al.*, 1990).

3.3.1.1 Reagents

Buffer [50 mM sodium phosphate, 1 mM Na_2EDTA, 0.02% (w/v) NaN_3, 7 mM DTT, pH 7.2]. NaH_2PO_4 (6.9 g) and Na_2EDTA (0.37 g) were dissolved in 90 ml distilled water and adjusted to pH 7.2 with NaOH. NaN_3 (0.2 g) was added, the pH readjusted if necessary and the solution was made up to 1 L with distilled water. DTT (0.00102 g/ml of buffer) was added just before use.

1 mM Z-Phe-Val-Arg-NHMec substrate stock solution. Z-Phe-Val-Arg-NHMec (3.41 mg) was dissolved in 5 ml DMSO and stored at 4°C.

50 µM Z-Phe-Val-Arg-NHMec working solution. 1 mM stock solution (0.5 ml) was diluted to 10 ml with distilled water.

3.3.1.2 Procedure

The procedure is the same as described in Section 3.2.1.2 with the buffer and enzyme solutions described in Section 3.3.1.1 being used.

3.3.2 Stem bromelain assay

3.3.2.1 Reagents

Buffer [50 mM Na-phosphate, pH 7.2, 0.02% (w/v) NaN_3, 1 mM Na_2EDTA, 7 mM DTT]. See Section 3.3.1.1.

1 mM Z-Arg-Arg-NHMec substrate stock solution. See Section 3.2.2.1.

50 µM Z-Arg-Arg-NHMec working solution. 1 mM stock solution (0.5 ml) was diluted to 10 ml with distilled water.

3.3.2.2 Procedure

The procedure was the same as described in Section 3.2.1.2 with the buffer and enzyme solutions described in Section 3.3.2.1 being used.

3.3.3 Isolation of fruit bromelain

3.3.3.1 Reagents

Homogenization buffer [50 mM, Na-acetate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.0]. Glacial acetic acid (2.86 ml) and Na_2EDTA (0.37 g) were dissolved in 950 ml distilled water and adjusted to pH 5.0 with NaOH. NaN_3 (0.2 g) was added, the pH readjusted if necessary and the solution made up to 1 L with distilled water.

Buffer B [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.0]. Glacial acetic acid (2.29 ml) and Na_2EDTA (0.74 g) were dissolved in 1.9 L distilled water and adjusted to pH 5.0 with NaOH. NaN_3 (0.4 g) was added, the pH readjusted if necessary and the solution was made up to 2 L with distilled water.

SP-Sepharose Fast Flow. See Section 3.2.4.1.

3.3.3.2 Procedure

The leaves, rind and core of a pineapple fruit were removed and the remaining fruit was cut into pieces (1-2 cm^3) and an equal volume of homogenization buffer (pineapple mass to homogenization buffer volume) and 30% t-butanol (v/final volume) was added. The mixture was homogenized in a Waring blendor (max speed, 2 min) and centrifuged (6 500 x g, 30 min, 4°C). The supernatant was retained and 20% (w/v) ammonium sulfate was added (based on the total volume of the solution and t-butanol, before addition of ammonium sulfate) and dissolved. The solution was centrifuged (6 000 x g, 15 min, 4°C) using a swing-out rotor. The precipitate in the middle layer was discarded. To the remaining supernatant and subnatant, a further amount of ammonium sulfate was added to bring the final concentration to 30% ammonium sulfate, based on the volume of t-butanol and homogenate. The salt was dissolved and the solution centrifuged (6 000 g, 15 min, 4°C) using a swing-out rotor. The precipitate was retained and stored in 10 ml buffer B. The resuspended material was centrifuged (27 000 x g, 10 min, 4°C) to remove any insoluble material.

The clarified sample was applied to a column of S-Sepharose (2.5 x 21 cm = 103 ml), pre-equilibrated with buffer B. Unbound material was eluted with 2 column volumes of buffer B. Bound material was eluted with a 0-2 M NaCl gradient in buffer B in *ca.* 5 column volumes at 50 ml/h. The active fractions corresponding to the active peak were pooled and concentrated by dialysis against PEG 20 000.

3.3.3.3 Results

The purification of fruit bromelain from pineapples, using TPP followed by chromatography on S-Sepharose, is summarized in Table 3.5. The purity of the sample; was analyzed by SDS PAGE on a 12% Tris-glycine gel with silver staining

Table 3.5. The purification of fruit bromelain from pineapple, with t-butanol in the homogenization buffer.

	Volume (ml)	Total Protein (mg)	Total Activity (Units)	Specific Activity. (Units/mg) (x 10^3)	Purification (fold)	Yield (%)
Homogenate	4 620	26 745	5.91	0.22	(1)	(100)
TPP cut (20-30%)	126	218	0.69	3.1	14	11.67
S-Sepharose	8.8	33.51	0.56	17	77	9.5

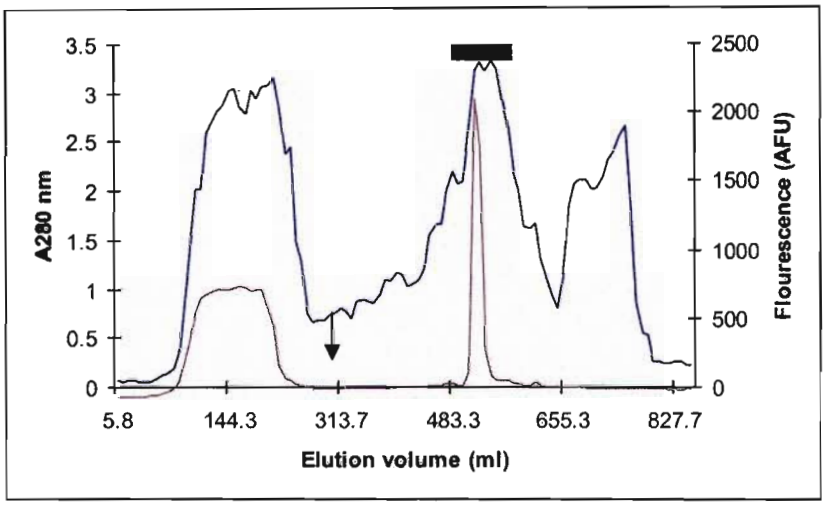


Figure 3.7. Chromatography of the 20-30% TPP fraction of fruit bromelain on S-Sepharose.
Column, 2.5 x 21.0 cm (103 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.0, containing 1 mM Na_2EDTA, 0.02% NaN_3, and eluted with a 0-2 M NaCl gradient (500 ml) in the same buffer applied at ↓; flow rate 50 ml/h (10 cm/h); fractions 7.3 ml (8.76 min). (—) Bromelain activity against Z-Phe-Val-Arg-NHMec, (▬) pooled fractions, (—) A280 nm .

3.3.3.4 Discussion

For this isolation green fruit was used as it has been reported to contain more enzyme than ripe fruit (Ota *et al.*, 1964). The yield from the present study was approximately 33 mg from 1.8 kg of pineapple, Yamada *et al.* (1976) reported 260-300 mg per 100 ml pineapple juice and Ota *et al.*, (1964) 3.3-3.7 g of crude extract per litre of juice.

Table 3.6. Comparison of procedures used for the isolation of fruit bromelain from pineapple.

Step	Procedure			
	Ota *et al.*, 1964	Ota *et al.*, 1972	Yamada *et al.*, 1976	Present study
Starting material	Pineapple fruit juice	Pineapple fruit juice	Pineapple fruit juice	Homogenate (30% t-butanol)
1	Acetone precipitation	Acetone precipitation	Acetone precipitation	TPP
2	DEAE cellulose chromatography	DEAE cellulose chromatography	DEAE cellulose chromatography	S-Sepharose chromatography
3		DEAE cellulose re-chromatography	ECTEOLA cellulose chromatography	
4			ECTEOLA cellulose re-chromatography	

One of the significant problems faced in using previous isolation methods (Ota *et al.*, 1964; Murachi and Neurath, 1960; El-Gharbawi and Whitaker, 1963) was that of autodigestion. This was indicated by analysis of the amino terminal residues. The presence of small but significant amounts of extraneous end groups showed that the preparations were not homogenous. The number of extraneous end groups increases if no attempts are made to minimize autodigestion. The incorporation of t-butanol reduces autodigestion during isolation but once the t-butanol is removed it is possible that autolysis resumes. This is possible because a low rate of activity remains in the absence of the reducing /activating agent DTT. In isoelectric focusing (IEF) (results not shown), several bands were seen very close together around the isoelectric point of fruit bromelain. The even spacing of the bands suggested that they might be due to heterogeneity in glycosylation, rather than autodigestion. IEF confirmed that only the acidic fruit bromelain was present in the final extract. Ota *et al.* (1964), suggested that bromelain isolated in the presence of phenylmercuric acetate does not undergo autodigestion and that the extraneous end groups observed were due to glycosylation. The method of Yamada *et al.* (1976) yielded a 31 kDa enzyme, which was named fruit bromelain FA2 and was reported to be non-glycosylated. The glycosylated nature of the protein cannot be

determined from the present studies, but the method used is relatively quicker, more cost effective and yielded enzyme of sufficient purity for the studies described in Chapter 4.

3.4 Cystatin C

Cystatin C from egg white was originally purified by ovomucin preparation and affinity chromatography on carboxymethylpapain-Sepharose (Anastasi *et al.*, 1983). These steps were followed by chromatofocusing, which separated the two major forms of the protein, with pI 6.5 and pI 5.6 (Anastasi *et al.*, 1983). The two different forms were thought to result from amino-terminal truncation, which may occur during purification (Turk *et al.*, 1983a). However, Laber *et al.* (1989) showed that it was due to phosphorylation; the pI 5.6 form is phosphorylated, whereas the pI 6.5 form is not. Both forms were of MW 12 500-13 100 but ran at higher apparent M_r values on SDS-PAGE (Anastasi *et al.*, 1983; Schwabe *et al.*, 1984).

The original isolation protocol (Anastasi *et al.*, 1983) was modified by Dennehy (1994) by incorporating the alkali treatment of Turk *et al.* (1983b). The method used in the present study was a modified version of that of Dennehy (1994). In the affinity chromatography step, papain was coupled to AminoLink® resin. The protocol was further modified to include TPP, in an attempt to reduce the amount of protein loaded onto the affinity column.

3.4.1 Cystatin C assay

Quantification of cystatin was carried out by measuring inhibition of papain. The procedure using Bz-Arg-$NPhNO_2$ as a substrate (Barrett, 1981) requires large amounts of cystatin, which made it unsuitable for the present study. The micro-scale method uses the fluorometric substrate, Z-Phe-Arg-NHMec (Anastasi *et al.*, 1983), and was modified for use in the present study. The non-ionic detergent, brij 35, was included in the buffer to decrease the non-specific adsorption of many proteins to surfaces (Anastasi *et al.*, 1983).

3.4.1.1 Reagents

Extraction buffer [50 mM NaH_2PO_4, 500 mM NaCl, 0.1% brij 35, 0.02% NaN_3, 9 mM DTT, pH 6.5]. NaH_2PO_4 (7.80 g), NaCl (29.22 g) and brij 35 (1 g) were dissolved in 950 ml distilled water and adjusted to pH 6.5 with NaOH. NaN_3 (0.2 g) was added, the pH readjusted if necessary and the solution was made up to 1 L with distilled water. DTT was added to a concentration of 9 mM (0.00671 g DTT/5 ml buffer) immediately before use.

1 mM substrate stock solution. Z-Phe-Arg-NHMec (1 mg) was dissolved in DMSO (1.5 ml) and stored at 4°C.

20 µM substrate solution. Substrate stock solution (0.1 ml) was diluted to 5 ml with distilled water.

Enzyme [0.1 mg/ml papain, 0.1% brij 35]. 1.0 mg papain was mixed with 0.01g brij 35 and made up to 10 ml with distilled water. Alternatively (10 µl/ml) sheep cathepsin L (isolation procedure given in Chapter 3) was used.

3.4.1.2 Procedure

Enzyme (1 µl), buffer (150 µl) and cystatin C (50 µl) were added to the wells of a white FluoroNunc MaxiSorp microtitre plate and incubated for 10 min at 37°C. 20 µM substrate solution (50 µl) was added and the fluorescence of the liberated aminomethylcoumarin was determined in a fluorescence microplate reader (Cambridge Technology Model 7620) with excitation at 360 nm and emission at 460 nm.

3.4.2 Preparation of AminoLink®-papain-affinity column

3.4.2.1 Reagents

AminoLink® coupling gel. A 2 ml plastic column, pre-filled with 50% slurry in 0.02% NaN_3, was provided in the AminoLink® kit, Pierce Biotechnology.

Coupling buffer [0.1 M sodium phosphate, 0.05% NaN_3, pH 7.0]. Provided in the AminoLink® kit, Pierce Biotechnology.

Quenching buffer [1 M Tris-HCl, 0.05% NaN_3, pH 7.4]. Provided in the AminoLink® kit, Pierce Biotechnology.

Wash solution [1 M NaCl, 0.05% NaN_3]. Provided in the AminoLink® kit, Pierce Biotechnology.

AminoLink® Reductant [sodium cyanoborohydride ($NaCNBH_3$)]. $NaCNBH_3$ (32 mg) was dissolved in 0.5 ml of 10 mM NaOH.

10 mM NaOH. NaOH (0.4 g) was dissolved in 1 L distilled water.

3.4.2.2 Procedure

Papain (18.45 mg) was dissolved in 2 ml coupling buffer and stored at 4°C. 10 mM NaOH (0.5 ml) was added to 32 mg of AminoLink® Reductant, in a fume hood. The column and reagents were incubated at room temperature. Storage buffer present in the column was drained and equilibration was carried out with 5 ml coupling buffer. Throughout the procedure the gel bed was not allowed to run dry. After coupling buffer was drained completely, the bottom cap was replaced on the column and 2 ml of protein solution was added. In a fume hood, 200 µl of the AminoLink® Reductant solution was added to the slurry. The top cap of the column was replaced and the reaction slurry was mixed for 6 h by gentle end-over-end rotation. After mixing, the liquid was drained and the column was

washed with 5 ml coupling buffer. To block the remaining active sites, 2 ml of quenching buffer and 200 µl of AminoLink® Reductant solution where added and mixed for 30 min by gentle end-over-end rotation. The contents were drained and the column was washed with 15 ml of wash solution. Cystatin C extraction buffer (Section 3.4.3.1) was used to wash the column and for storage.

3.4.3 Purification of cystatin C from chicken egg white

3.4.3.1 Reagents

0.25% NaCl. NaCl (1 g) was dissolved in 400 ml distilled water.

3 M NaOH. NaOH (12 g) was dissolved in 100 ml distilled water.

50 mM NaH_2PO_4. NaH_2PO_4 (7.80 g) was dissolved in 1 L distilled water.

1 M HCl. 12 M HCl (8.3 ml) was made up to 100 ml in distilled water

Extraction buffer [50 mM Na-phosphate, 500 mM NaCl, 0.1% brij 35, 0.02% NaN_3, pH 6.5]. NaH_2PO_4 (7.80 g), NaCl (29.22 g) and brij 35 (1 g) were dissolved in 950 ml distilled water and adjusted to pH 6.5 with NaOH. NaN_3 (0.2 g) was added, the pH readjusted if necessary and the solution was made up to 1 L with distilled water.

Elution buffer [50 mM K_2-phosphate, 500 mM NaCl, 0.1% brij 35, 0.02% NaN_3, pH 11.5]. K_2HPO_4 (4.36 g), NaCl (14.61 g) and brij 35 (0.5 g) were dissolved in 450 ml distilled water and adjusted to pH 11.5 with NaOH. NaN_3 (0.1 g) was added, the pH readjusted if necessary and the solution was made up to 500 ml with distilled water.

3.4.3.2 Procedure

The whites from 6 eggs (154 ml) were mixed with an equal volume of 0.25% NaCl (at 4°C), adjusted to pH 11 with 3M NaOH and mixed in a Waring blendor for 2.5 min. The solution was stirred overnight at 4°C, and centrifuged (2 000 x g, 30 min, 4°C). The supernatant was adjusted to pH 6.5 with 1 M HCl and centrifuged (2 000 x g, 30 min, 4°C). The precipitate was discarded and the supernatant retained for affinity chromatography.

Storage buffer was drained from the AminoLink®-papain-affinity column; 2 ml of supernatant was added to the column and mixed by gentle end-over-end rotating at 4°C, overnight. The column was washed with extraction buffer until the A280 nm of the eluent reached approximately zero and remained constant. 10 ml of elution buffer was run through the column, the eluent being collected and concentrated by dialysis against PEG 20 000 to a final volume of approximately 1 ml. The column was equilibrated with 5 ml

extraction buffer and the process was repeated using a further 2 ml of supernatant. The concentrated sample from the first batch was combined with the second and concentrated down to approximately 1 ml, again by dialysis against PEG 20 000.

3.4.3.3 Results

The final product obtained after affinity chromatography was analyzed using Tris-tricine SDS-PAGE. The Chemi Genius 2 Bio Imaging System from Vacutec was used to analyze the Tris-tricine gel. Included in this analysis was the concentration of protein in the cystatin C band in each case. Using these values the concentration of cystatin C in each solution was calculated. For batch 1 (lane b, Figure 3.8) the cystatin C concentration was 42 ng/ml and for batch 2 (lane c, Figure 3.8) the concentration was 55 ng/ml.

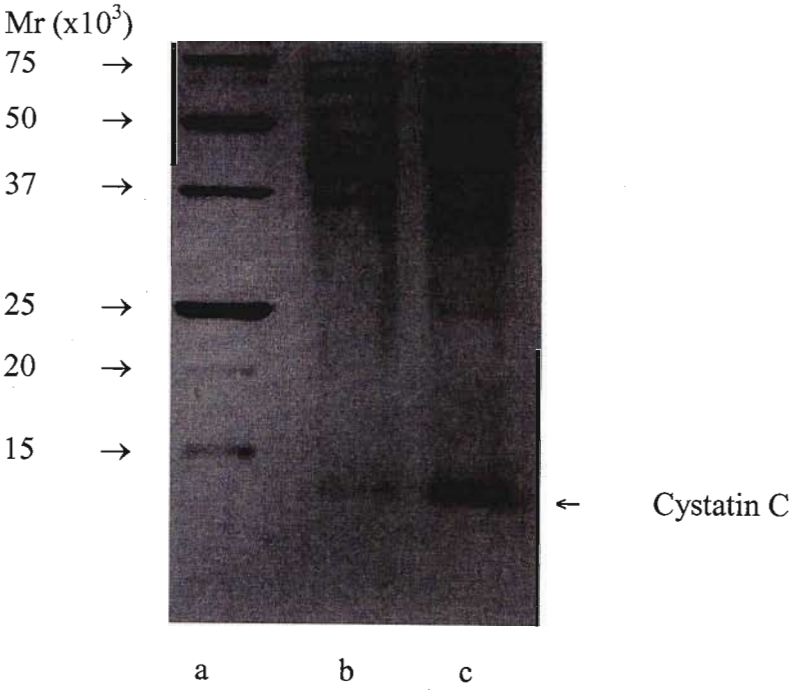


Figure 3.8. Reducing Tris-tricine SDS-PAGE of egg white cystatin from an AminoLink®-papain-affinity column.
Samples were boiled in reducing treatment buffer and loaded onto a 12% Tris-tricine gel. (a) MW markers (Bio-Rad Precision Plus Protein™ Standards). (b) Batch 1 obtained from column. (c) Batch 2 obtained from column. Visualized using silver staining.

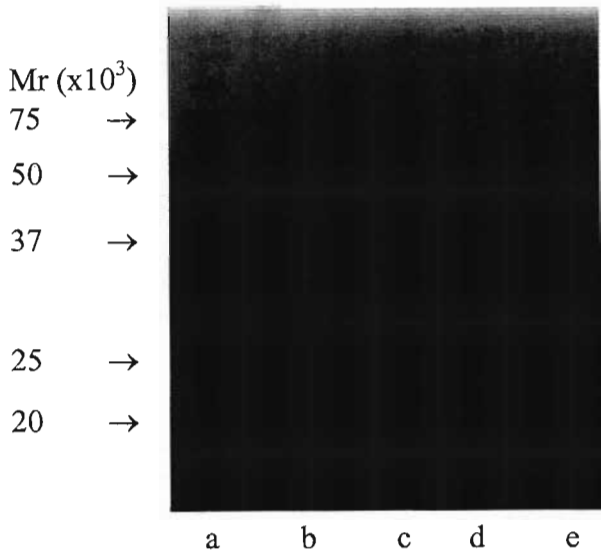


Figure 3.9. Gelatin containing SDS-PAGE of egg white cystatin from AminoLink®-papain-affinity column.
Samples were combined with non-reducing treatment buffer and loaded onto a 12% Tris-glycine gel containing gelatin (a) MW markers (Bio-Rad Precision Plus Protein™ Standards). (b) 5 µl cystatin C sample (c) 10 µl cystatin C sample (d) 20 µl cystatin C sample (e) 30 µl cystatin C sample, visualized using amido black staining.

3.4.3.4 Discussion

The major shortcoming of all these isolation methods (Table 3.7) was that an insubstantial amount of protein was removed in the steps prior to affinity chromatography and that the large amount of protein loaded onto the affinity column resulted in clogging of the column, required numerous washing steps and resulted in relatively more impurities in the final product.

The method of Anastasi *et al.* (1983) yielded 49 µg of cystatin C from 1 egg, whereas the method used in the present study had a significantly lower yield of 2 µg from 1 egg. A further disadvantage of the present method is the presence of a contaminant protease in the final product (Figure 3.9), which causes degradation of the fluorescent substrate Z-Phe-Arg-NHMec (result not shown), used in assaying the cathepsin L/cystatin C interactions. Further investigations are needed to identify this contaminant.

In order to reduce the protein content of the sample before loading onto the column, TPP was explored.

Table 3.7. Comparison of procedures used for the isolation of cystatin C chicken egg white.

Step	Procedure		
	(Anastasi *et al.*, 1983)	(Dennehey, 1994)	(Present study)
Starting material	Chicken egg white	Chicken egg white	Chicken egg white
1	Ovomucin precipitation	Ovomucin precipitation	Ovomucin precipitation
2	CM-papain-Sepharose chromatography	Alkali treatment	Alkali treatment
3	Chromatofocusing	CM-papain-Sepharose chromatography	AminoLink®-papain chromatography
4		Sephadex G-25 chromatography	
5		Q-Sepharose chromatography	

3.4.4 Purification of cystatin C from chicken egg white using TPP.

3.4.4.1 Reagents

0.25% NaCl. See Section 3.4.3.1.

Extraction buffer [50 mM Na-phosphate, 500 mM NaCl, 0.1% brij 35, 0.02% NaN_3, pH 6.5]. See Section 3.4.3.1.

Elution buffer [50 mM K_2-phosphate, 500 mM NaCl, 0.1% brij 35, 0.02% NaN_3, pH 11.5]. See Section 3.4.3.1.

3.4.4.2 Procedure

The white from 1 egg was combined with 2 parts 0.25% NaCl and homogenized in a Waring blendor for 10 s at high speed. 30% t-Butanol (v/final volume) was added, the mixture stirred and centrifuged (2 000 x g, 20 min, 4°C) and the precipitate was discarded. To the supernatant, 10% (w/v) ammonium sulfate was added and dissolved. Centrifugation (2 000 x g, 20 min, 4°C) resulted in a protein pellet, which was removed and mixed with 2-4 ml extraction buffer. The supernatant volume was measured and 10% (w/v) ammonium sulfate was added, based on the new volume, to bring the total ammonium

sulfate volume to 20%. The mixture was centrifuged (2 000 x g, 20 min, 4°C), and the pellet mixed in elution buffer. The procedure was repeated for 30% and 40% ammonium sulfate. The pellets were dialysed overnight against elution buffer to remove the t-butanol. Each of the dialysed samples was centrifuged (48 x g, 2 min, RT). Those samples still containing t-butanol were dialysed for a further 2 h against extraction buffer.

Affinity chromatography was carried out using an AminoLink®-papain-affinity column (Section 3.4.2). The column was washed with elution buffer (2 col. vols.) and equilibrated with extraction buffer (5 col. vol.). The 0-10% TPP cut was applied to the column and mixed by gentle end-over-end rotating for 10 min. The excess liquid was drained from the column and 3 ml extraction buffer was added and the column was washed for 10 min by gentle end-over-end rotation. The A280 nm of the eluent was measured and the wash procedure repeated until the absorbance was approximately zero. Bound proteins were eluted using elution buffer (5 col. vol.) and collected. This protocol was repeated for the 10-20%, 20-30% and 30-40% TPP fractions with the washing and equilibrating steps being carried out prior to each sample application. All the eluted fractions containing cystatin C were combined and concentrated, using PEG 20 000, to give a final volume of approximately 2 ml.

3.4.4.3 Results

The homogenate and TPP fraction before and after affinity chromatography were assayed for inhibition of cathepsin L and for activity against Z-Phe-Arg-NHMec. All samples inhibited cathepsin L and none had activity against Z-Phe-Arg-NHMec.

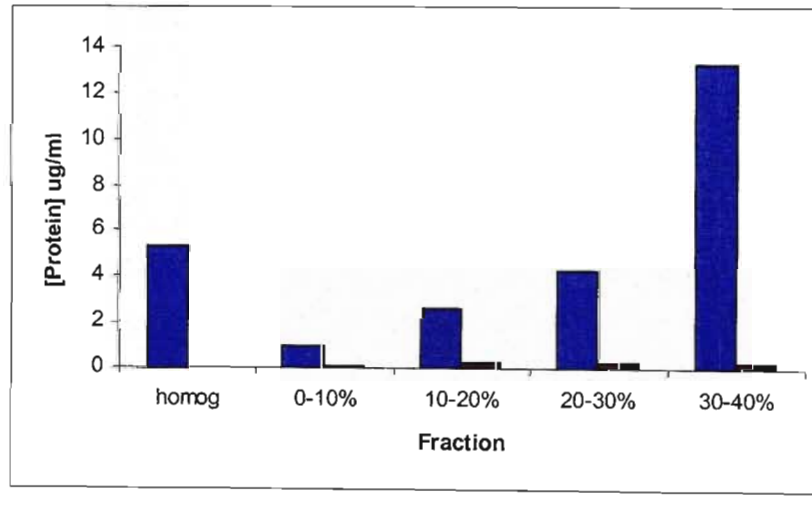


Figure 3.10. Comparison of the protein concentration in each TPP fraction obtained during cystatin C isolation from chicken egg white.
TPP was effected on the homogenate (homog), pellets were removed after 0-10%, 10-20%, 20-30% and 30-40% ammonium sulfate was added. ■TPP cuts prior to affinity chromatography, ■ TPP cuts after affinity chromatography

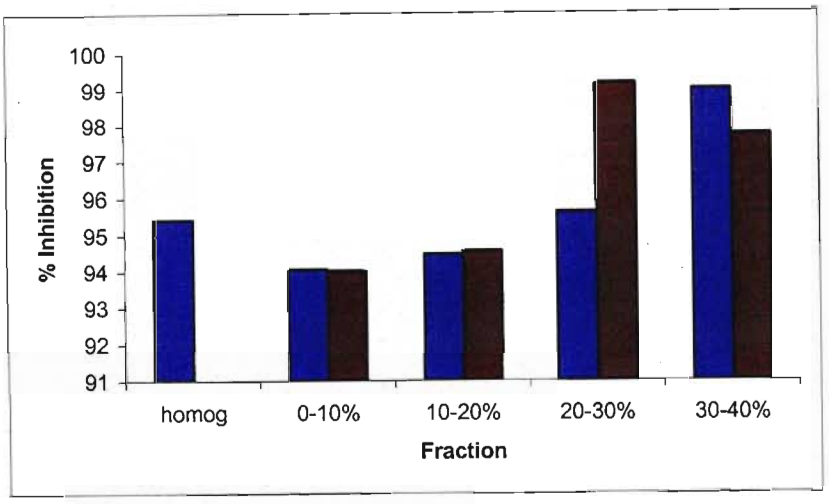


Figure 3.11. Comparison of the % inhibition of cathepsin L by each TPP fraction obtained during cystatin C isolation from chicken egg white.
TPP was effected on the homogenate (homog), pellets were removed after 0-10%, 10-20%, 20-30% and 30-40% ammonium sulfate was added. ■TPP cuts prior to affinity chromatography, ■ TPP cuts after affinity chromatography.

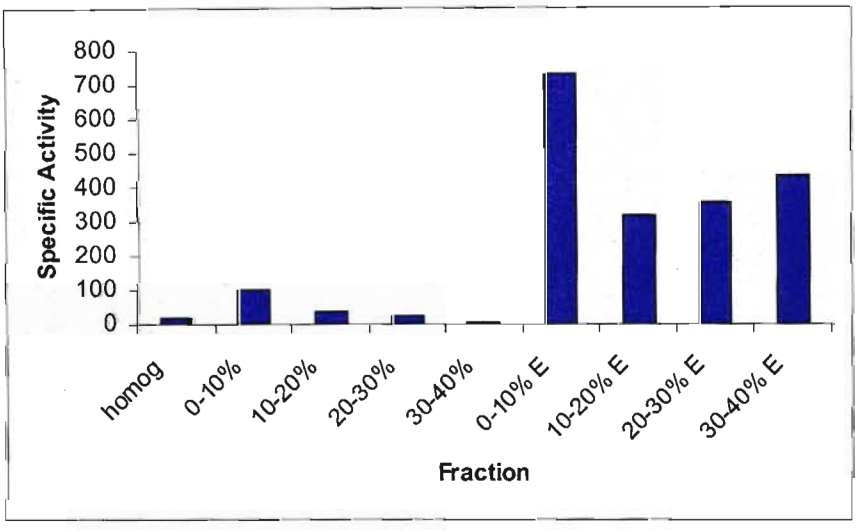


Figure 3.12. Comparison of the % specific activity of each TPP fraction obtained during cystatin C isolation from chicken egg white.
TPP was effected on the homogenate (homog), pellets were removed after 0-10%, 10-20%, 20-30% and 30-40% ammonium sulfate was added prior to affinity chromatography, 0-10%E, 10-20%E, 20-30%E and 30-40%E represents TPP cuts after affinity chromatography.

3.4.4.4 Discussion

The use of t-butanol in the initial steps significantly reduced the protein content, resulting in a more refined sample being loaded onto the column. The column needed fewer washes before elution, contamination of final product was reduced and blockage of the column

was avoided. From Figure 3.12 it is evident that cystatin C precipitated at all ammonium sulfate concentrations, which questions the necessity of the ammonium sulfate precipitation steps. The majority of protein was removed upon addition of 30% t-butanol; this particular value was selected because it is the minimum concentration at which enzymatic activity is inhibited Dennison *et al.* (2000). In future isolations it would be interesting to find out what the effect of increasing the t-butanol concentration might be on the removal of contaminating proteins.

The purity of product obtained after affinity chromatography was sufficient for use in our study. To increase purity, either the chromatofocusing (Anastasi *et al.*, 1983) or Sephadex G-25 chromatography combined with Q-Sepharose chromatography (Dennehey, 1994) could be included. For the studies described in Chapter 4 the TPP fractions were pooled as they all contained cystatin C. The yield was calculated as the sum of protein from the different TPP fractions after they were eluted from the affinity chromatography column and was 0.93 mg from 1 egg.

CHAPTER 4

THE EFFECTS OF IONIC STRENGTH

4.1 Introduction

Enzyme activity is monitored by the appearance of product with time, which may be expressed in the form of a progress curve (Figure 4.1).

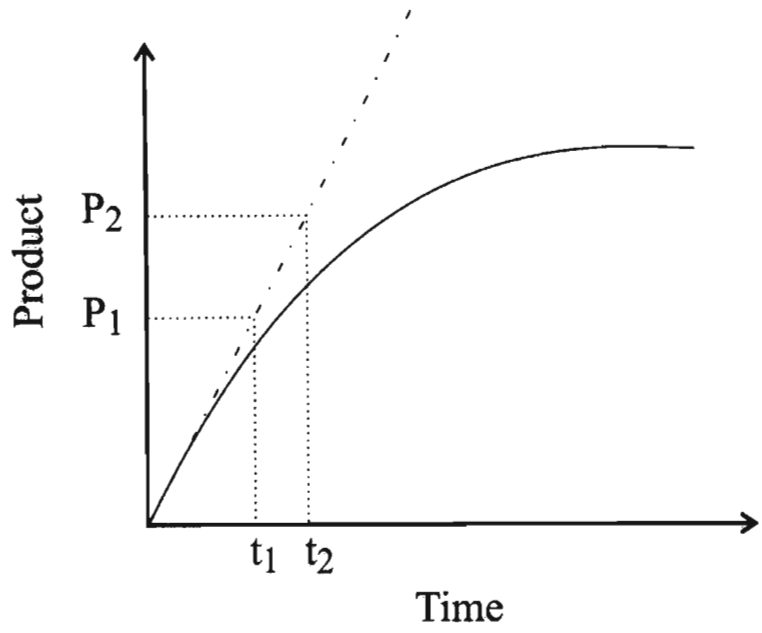


Figure 4.1. Progress curve of an enzyme-catalyzed reaction.

The rate of reaction at any time is given by the slope of the curve at that point. The reaction proceeds initially at a constant rate, which then decreases with the decreasing concentration of the reactants, until the net rate becomes zero. At this point a chemical equilibrium has been reached.

The initial velocity (v_0) of the reaction is the reaction rate at t = 0 and may be determined by drawing a tangent to the curve (Figure 4.1). The units for v_0 are those used for product concentration divided by those used for time. The significance of using v_0 is i) the concentration of each reactant is known from the amounts added; this would not be true at any other point during the reaction, ii) there are no products present at t = 0, so no reverse reactions will be taking place (Palmer, 1981).

Initial velocity is dependent on the initial concentration of the reactants. A graph of initial velocity versus substrate concentration [S_0], at constant total enzyme concentration [E_0] forms a rectangular hyperbola (Figure 4.2).

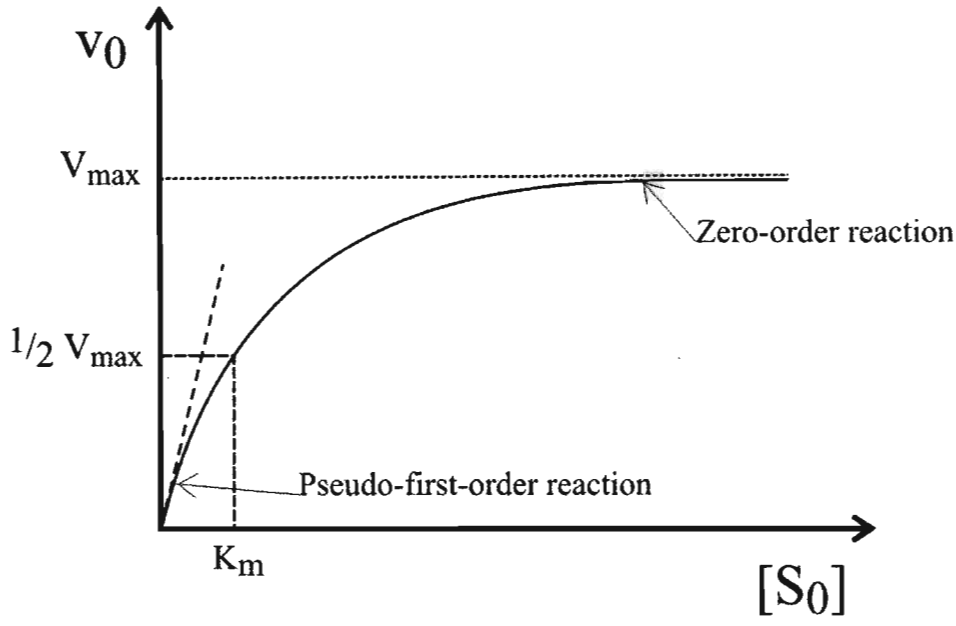


Figure 4.2 Substrate dilution curve for a single-substrate enzyme-catalyzed reaction.

At low substrate concentrations the reaction is pseudo first-order with respect to the substrate, but at high concentrations it becomes zero-order (Brown, 1902).

The relationship between v_0 and [S_0] at steady state is given by the Michaelis–Menten equation (modified by Briggs and Haldane, 1925)

$$v_0 = \frac{V_{max}[S_0]}{[S_0]+K_m} \quad (1)$$

Where K_m is the Michaelis constant and V_{max} the maximum initial velocity at a particular [E_0].

The Michaelis-Menten equation is applicable to many enzyme-catalysed reactions and the constants V_{max} and K_m can be determined. K_m gives an indication of the affinity of the enzyme for the substrate: a low K_m value indicates high affinity of enzyme for substrate, whereas a high K_m value indicates a low affinity.

The Eisenthal and Cornish-Bowden (1974) method uses the reciprocal form of equation 1 at constant [E_0].

$$\frac{1}{v_0} = \frac{K_m + [S_0]}{V_{max}[S_0]} \tag{2}$$

Each v_0, [S_0] pair can be used to generate a line by marking v_0 on the V_{max} axis and $-[S_0]$ on the K_m axis; these two points are then joined up and the line extrapolated. Lines for all v_0, [S_0] pairs at constant [E_0], must pass through the true values of K_m and V_{max}, so these values are given by the point of intersection of all lines (Figure 4.3). Due to experimental error the lines intersect over a range of values, the best estimates of K_m and V_{max} are the medians of the values obtained from the intersections of these lines.

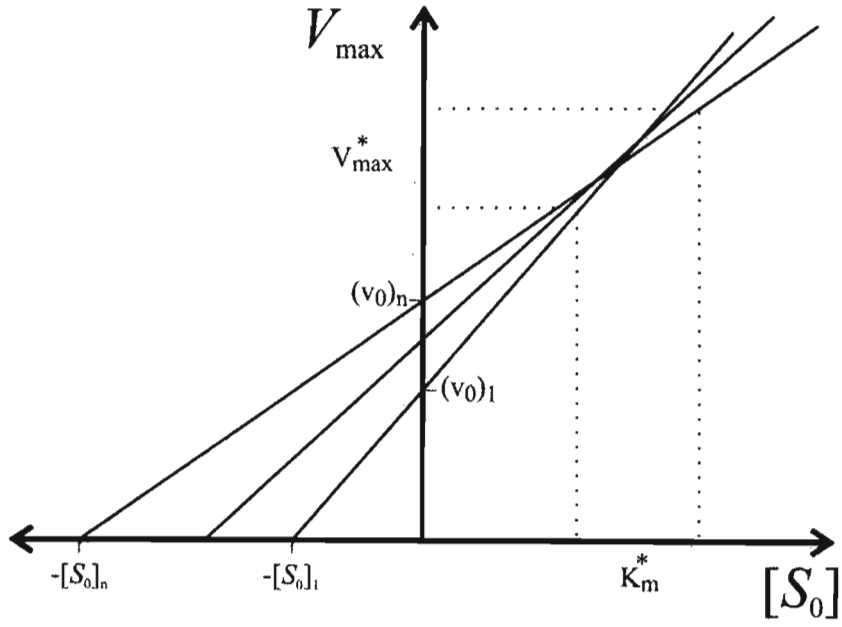


Figure 4.3 Eisenthal Cornish-Bowden plot for an enzyme-catalysed reaction at constant [E_0].
V_{max}* is the best estimate of V_{max}, K_m* is the best estimate of K_m.

In the present study, generation of curves and calculation of the median values was done using the program HYPER (http://www.liv.ac.uk/%7Ejse/software.html).

The formation of a single intermediate complex can include reactions of the type:

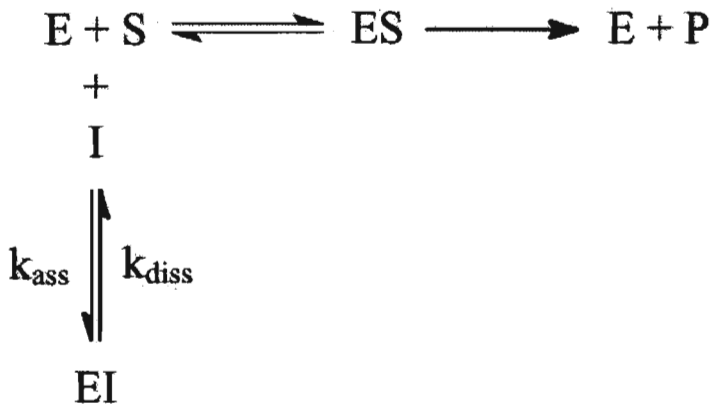
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Reaction 1

Where ES→E + P is the rate limiting step of the overall reaction.

The constant k_{cat}, called the turnover number, is often applied to enzyme catalysed reactions, and is obtained from the general expression $V_{max} = k_{cat}\,[E_0]$, hence at constant $[E_0]$, $V_{max} \propto k_{cat}$. This constant represents the maximum number of substrate molecules that can be converted to products per enzyme molecule per unit time. For reactions which follow reaction 1, $k_{cat} = k_2$.

To study the inhibition of cathepsin L, a simple competitive mechanism of inhibition without a pre-equilibrium step was assumed (Turk *et al.*, 1993).



Reaction 2

The rate constants for complex formation and dissociation are k_{ass} and k_{diss} respectively (Turk *et al.*, 1993). Enzyme is added to a mixture of the inhibitor and fluorogenic substrate and the concentration of [P] is continuously monitored by fluorescence (Turk *et al.*, 1993). The progress curves are fitted by non linear regression to the integrated rate equation (equation 3).

$$[P] = v_s t + (v_z - v_s)(1 - e^{-kt})/k \qquad (3)$$

Where v_s is the steady state velocity, v_z is the initial rate velocity and k is the pseudo-first order rate constant describing the pre steady state of the reaction. k can be determined using equation 4

$$k = k_{ass} \cdot \frac{[I_0]}{(1+[S_0]/K_m)} + k_{diss} \tag{4}$$

for a number of inhibitor concentrations. The linear slope of a plot of k versus $[I_0]$ provides an estimate of $k_{ass}/(1+[S_0]/K_m)$ and the k_{ass} can be calculated using the known values for $[S_0]$ and K_m. Linear regression also provides an estimation of the error in the determination of k_{ass} (Turk *et al.*, 1993). The dissociation constant k_{diss} cannot be accurately determined if the line intercepts the ordinate close to the origin. In such cases k_{diss} is calculated for each inhibitor concentration using equation 5 (Morrison, 1982).

$$k_{diss} = k \cdot v_s / v_z \tag{5}$$

The error is computed from the individual values of k, v_s and v_z at the various inhibitor concentrations. The K_i values are calculated from k_{ass} and k_{diss} according to equation 6

$$K_i = k_{diss}/k_{ass} \tag{6}$$

An alternate method of determining K_i is to use equation 7, which is derived from equations 3 and 4.

$$\frac{v_z - v_s}{v_s} = \frac{[I_0]}{K_i(1+[S_0]/K_m)} \tag{7}$$

Ideally, plots of $(v_z-v_s)/v_s$ versus $[I_0]$, should give straight lines with a low degree of scattering of the data points. In cases of high enzyme-inhibitor affinity (K_i<10 pM), v_s is extremely low compared with v_z and this yields highly scattered data points and unreliable K_i values (Turk, *et al.*, 1993).

Many effective inhibitors have very low K_i values (10^{-8}-10^{-10} M) but the products of most substrates are not detectable at such low concentrations (Salvesen and Nagase, 2001). The method of progress 'curves' (Henderson, 1972) allows for such K_i values to be determined by the addition of inhibitor to a steady-state enzyme-substrate reaction (Nicklin and Barrett, 1984). The advantages of this method are, i) enzyme concentration need not be determined and ii) a reasonable estimate of K_i can be made from a single

assay. This method of Salvesen and Nagase (2001) was used in the present study to determine the K_i for cathepsin L-cystatin C interactions.

Fluorometric assays can be carried out using internally quenched fluorescent substrates for proteases, which consist of peptide chains constructed to match the specificity of the particular enzyme and have suitable chromophores on each side of the cleavable bond (Yaron *et al.*, 1979). One of the chromophores is a fluorescent group and the other is a quencher that causes a reduction of fluorescence intensity of the fluorophore, either by direct intramolecular encounter or by radiationless resonance energy transfer. The interaction between the two groups is efficient even when they are separated by several amino acid residues (Yaron *et al.*, 1979).

7-aminomethylcoumarin amide is a fluorescent tag that has different fluorescent properties when attached to a carboxyl group through an amide bond, compared to the free 7-aminomethyl coumarin (Melo *et al.*, 2001). The almost non-fluorescent substrate is hydrolysed by the protease to liberate the intensely fluorescent 7 amino-4-methylcoumarin (NHMec) (Barrett, 1981b), which is measured with excitation at 360 nm and emission at 460 nm. Most NHMec substrates are water-soluble and show low rates of autolysis upon storage (Sarath *et al.*, 2001). They also have high fluorescence yields, making it possible to assay dilute enzyme preparations at lower substrate concentrations. α-Glucosidase substrate contains a 4-methylumbelliferyl group which, upon hydrolysis, is released as the fluorescent 4-methylumbelliferone anion.

4.1.1 Computation of ionic strength

The desired ionic strength of the buffer solutions was obtained in two ways, the first by adjusting the molarity of the buffer and, second, by the addition of salt.

The following MS BASIC program is an adaptation of that given by Ellis and Morrison (1982). This program calculates the ionic strength of the buffer of a given molarity and then determines what multiple of the answer is required to achieve the target ionic strength. In this approach, only buffer ions contribute to the ionic strength. An alternative is to use sodium chloride to make up the difference between the buffer ionic strength and the target ionic strength (assuming the former is smaller than the latter), as suggested by Ellis and Morrison (1982). The example program, (Dennison, 2003), is for a 0.1 M phosphate buffer, but may be easily adapted for other buffers by appropriate editing.

```
LPRINT "CALCULATION OF THE IONIC STRENGTH OF A BUFFER OF GIVEN MOLARITY"
LPRINT "AND HENCE THE MOLARITY REQUIRED TO GIVE A TARGET IONIC STRENGTH"

REM P1 = pKa of buffer
REM Z = Charge on conjugate base
REM IS = Ionic strength
REM C1 = buffer concentration
REM For phosphate buffer, P1 = 6.84, Z=-2
REM I = Target ionic strength
P1 = 6.84
Z=-2
C1=.1
I=0.1
LPRINT "Buffer is phosphate"
LPRINT "pKa of buffer ="; P1
LPRINT"Charge on conjugate base ="; Z
LPRINT"Concentration of buffer ="; C1
LPRINT"Target ionic strength ="; I
REM H1 is lowest pH
REM H2 is highest pH
REM pH increment = X
H1 = 4!
H2 = 8!
X =.2
LPRINT "pH";CHR$(9) "Ionic strength"; CHR$(9) "Required molarity"
LPRINT
FOR H=H1 TO H2 STEP X
X1=EXP((H-P1)*2.303)
A1=C1/(1+X1)
B1=A1*X1
U=((Z+1)*A1)+(Z*B1)
I1=.5*(((Z+1)*(Z+1))*A1+(Z*Z)*B1+ABS(U))
R=(I/I1)*C1
LPRINT H;CHR$(9)I1; CHR$(9)R
NEXT H
END
```

4.2 α-Glucosidase

4.2.1 α-Glucosidase assay

α-Glucosidase used in this study was from bakers' yeast (*Saccharomyces cerevisiae*) and is classified in the Swiss Prot Database under family 13 of glycosyl hydrolases (www.expasy.org/sprot/). α-Glucosidase activity was assayed using 4-methylumbelliferyl α-glucoside (Meinesz, 1996). This substrate liberates the fluorescent compound

4-methylumbelliferone upon hydrolysis. The amount of substrate cleaved is equivalent to the amount of the highly fluorescent 4-methylumbelliferone anion generated.

4.2.1.1 Reagents

Assay buffer [MES buffers of increasing concentration at pH 6.8]. The concentrations and corresponding mass of MES are given in Table 4.1. The values were computed according to Dennison (2003), (Section 4.1.1). The required mass of MES was dissolved in 95 ml distilled water, adjusted to pH 6.8 and made up to 100 ml with distilled water.

Table 4.1. Molarity and ionic strengths of buffers used in α-glucosidase assays

Buffer No.	[MES] (M)	Mass (g/100 ml)	pH	Ionic strength [a]
1	0.00468	0.09135	6.8	0.004
2	0.0117	0.2284	6.8	0.01
3	0.0468	0.9135	6.8	0.04
4	0.117	2.284	6.8	0.1
5	0.468	9.135	6.8	0.4

[a] The ionic strength given here is the final ionic strength within the assay vessel and not the ionic strength of the corresponding buffer.

20 mM 4-methylumbelliferyl-α-glucoside stock solution. 4-methylumbelliferyl-α-glucoside (0.067 g) was dissolved in 10 ml distilled water, divided into 1 ml aliquots and stored at 4°C.

1 mM 4-methylumbelliferyl-α-glucoside. 20 mM 4-methylumbelliferyl-α-glucoside stock solution (100 µl) was made up to 2 ml with distilled water

Working concentrations of 4-methylumbelliferyl-α-glucoside. The different concentrations of 4-methylumbelliferyl-α-glucoside, used to determine K_m and the corresponding volume of 1 mM stock required to make 1 ml of working solution are given in Table 4.2. The required volume of 1 mM stock was made up to 1 ml with distilled water.

Table 4.2. Working concentrations of 4-methylumbelliferyl-α-glucoside used in α-glucosidase K_m determination.

No.	[Substrate] solution (µM)	Volume of 1 mM stock (µl/ml)	[Substrate] in well (mM)
1	60	60	0.015
2	80	80	0.02
3	100	100	0.025
4	120	120	0.03
5	160	160	0.04
6	200	200	0.05

1 mg/ml α-glucosidase stock solution. α-Glucosidase (1 mg) was made up to 1 ml with distilled water.

1 μg/ml α-glucosidase. Just before use, the α-glucosidase stock solution (10 μl) was made up to 10 ml with distilled water.

4.2.1.2 Procedure

Buffer 1 (Table 4.1) (100 μl) was added to columns 1-6 and rows A-F, of a white 96 well FluoroNunc MaxiSorp microtitre plate. To the buffer-containing wells of row 1, substrate 1 (Table 4.2) (50 μl) was added. The buffer containing wells of the remaining rows contained 50 μl of the corresponding substrate. The assays were carried out on 2 rows at a time starting with rows 1 and 2. 1 μg/ml α-glucosidase (50 μl) was added to each well of the rows containing buffer and substrate and mixed. Enzyme was excluded from row F which served as substrate control wells. This arrangement resulted in 5 replicates of each assay. The fluorescence of the liberated methylumbelliferone was determined in a fluorescence microplate reader (Cambridge Technology Model 7620) with excitation at 360 nm and emission at 460 nm. Fluorescence readings where taken every minute over a period of 5 minutes. v_0 was obtained by plotting fluorescence versus time for each substrate concentration. Hyperbolic regression was effected on the plot of v_0 versus substrate concentration, and the hyperbola was analysed using the program HYPER (http://www.liv.ac.uk/%7Ejse/software.html). The procedure was repeated for each of the remaining buffers. v_0, K_m, V_{max} and V_{max}/K_m were plotted as functions of ionic strength.

4.2.2 Results

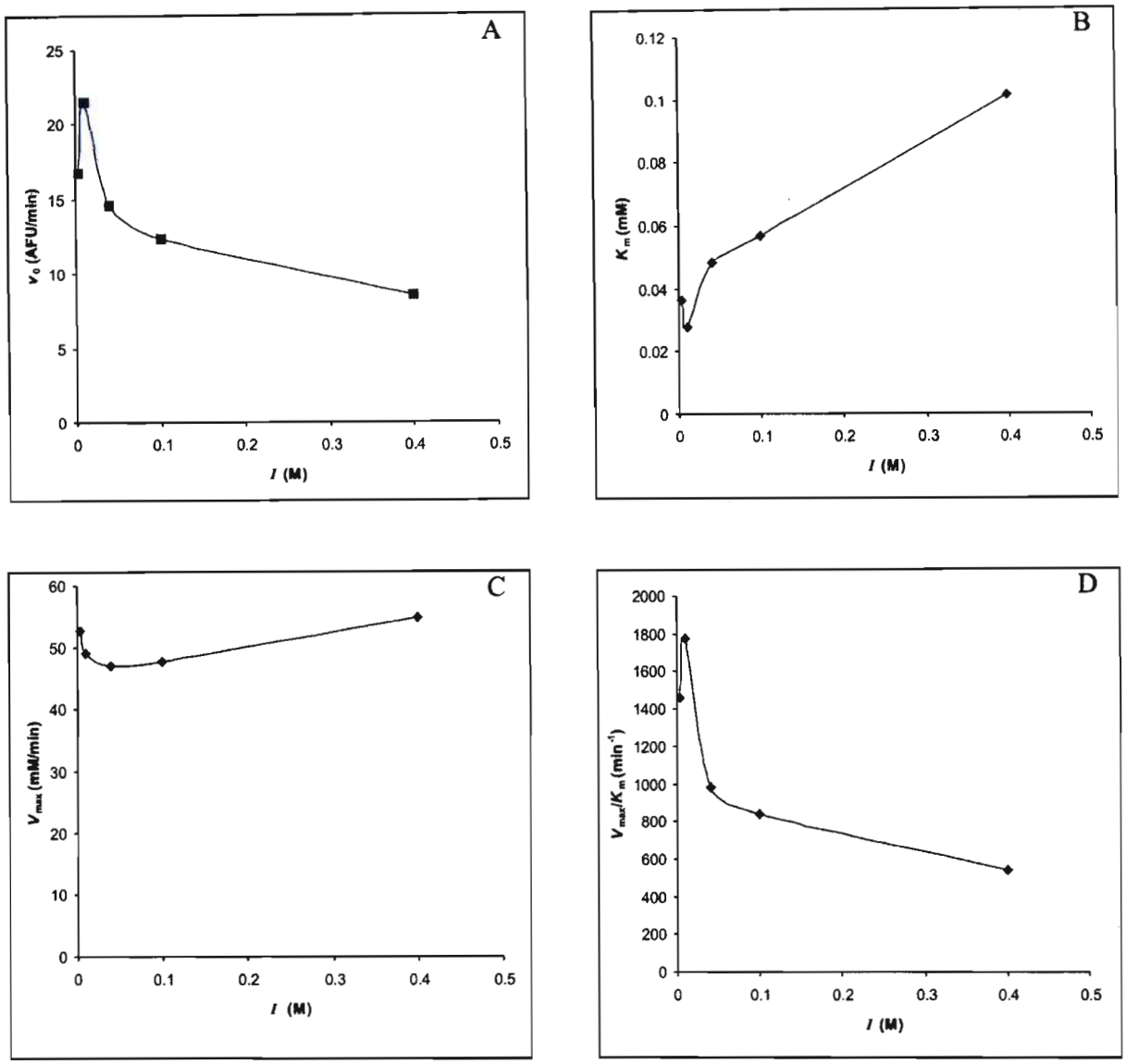


Figure 4.4. The effect of ionic strength on the kinetic constants of α-glucosidase acting on the substrate 4-methylumbelliferyl-α-glucoside.
(A) = Activity, (B) = K_m, (C) = V_{max} and (D) = V_{max}/K_m

A skewed bell-shaped curve was observed for the effect of ionic strength on α-glucosidase (Figure 4.4A). This indicates that, as with pH, α-glucosidase has an ionic strength optimum. It is at this optimum that K_m is at its lowest, indicating a maximal affinity for the substrate (Figure 4.4B). Although there is a decrease in V_{max} at the 'optimum' ionic strength (Figure 4.4C), overall, V_{max}/K_m is maximal at this point (Figure 4.4D).

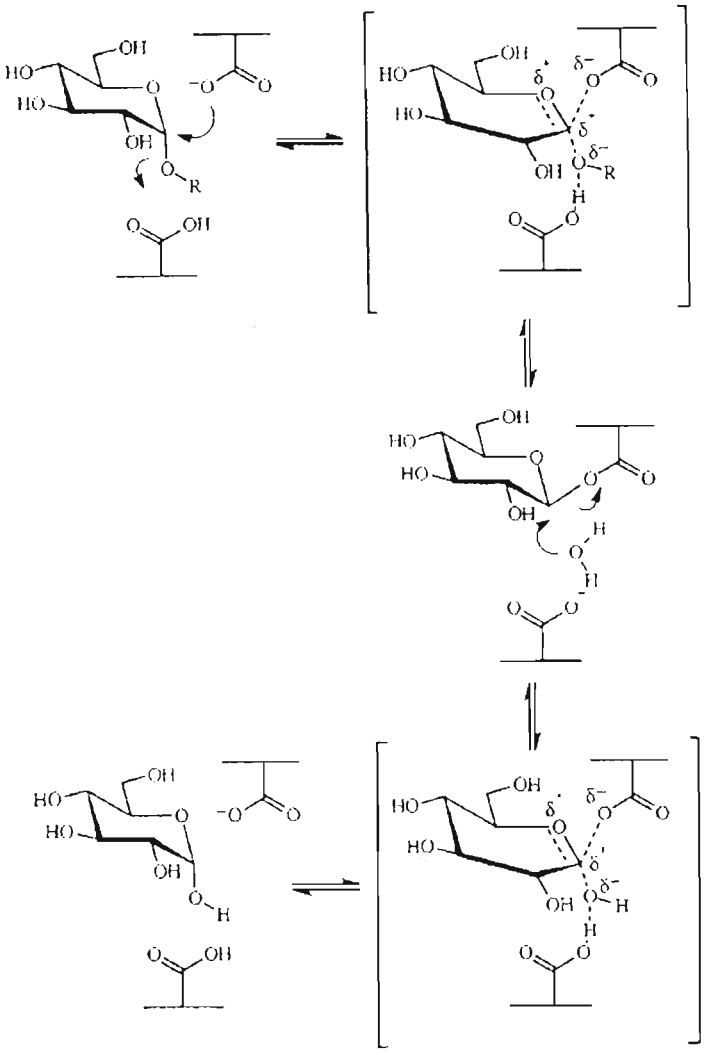
4.2.3 Discussion

α-Glucosidase apparently has an optimum ionic strength. This result bears some similarity with that of Dennison and Marais (1980), where carbohydrases in the rumen fluid of sheep showed a similar bell-shaped activity profile with increasing buffer concentrations. At the 'optimum' ionic strength, K_m is at its lowest which indicates an increased affinity of enzyme for substrate. V_{max} decreases slightly at the 'optimal' ionic strength, indicating a decrease in turnover, but V_{max}/K_m gives an overall increase implying that the effect on affinity is greater than the effect on turnover.

The mechanism by which this enzyme hydrolyses its substrate requires two critical residues, a proton donor (Glu-257) and a proton acceptor (Asp-328) and a third catalytic residue (Asp-229), as is the case of cyclodextrin glycosyltransferase from *Bacillus circulans* (EC 2.4.1.19) (Klein *et al.*, 1992; McCarter and Withers, 1996; MacGregor *et al.*, 2001) (Scheme 3). These glycosyl hydrolases cleave the glycosidic bond with net retention of configuration, thus they are called retaining enzymes and function via a two-step double displacement mechanism (McCarter and Withers, 1996; MacGregor *et al.*, 2001).

The first step involves the attack of an enzymic nucleophile (Glu-257) on the anomeric centre of the sugar, along with general acid catalysis to facilitate loss of the leaving group, leading to the formation of a α-glycosyl enzyme intermediate. This intermediate can then undergo hydrolysis in a second step, with general base catalysis to facilitate attack by water, resulting in cleavage of the glycosidic bond with net retention of anomeric configuration. The active site of these enzymes is known to include a trio of conserved carboxylic acids (Kadziola *et al.*, 1994; Klein *et al.*, 1992; Qian *et al.*, 1994, McCarter and Withers 1994). Presumably, one residue functions as the catalytic nucleophile and one as a general acid/base. The third possibly provides added stabilization of developing positive charge or alters the ionization behaviour of the other catalytic residues (McCarter and Withers, 1996).

The ions in solution would affect the interactions of these residues in different manners and at the 'optimum' ionic strength an optimal balance is struck. Ionic strength could not only affect the interaction of the acid/base groups with the substrate, but may affect the functions of the third catalytic residue.



Scheme 3. Presumed mechanism of a retaining α-glucosidase.

(McCarter and Withers, 1996)

4.3 Elastase

4.3.1 Elastase assay

Leukocyte elastase has a very narrow specificity. It preferentially cleaves Val-X bonds and to a lesser extent Ala-X links, the latter being preferred by pancreatic elastase. It degrades elastin, cartilage, proteoglycans, several collagens and fibronectin (Barrett, 1981b). Spectrophotometric assays of elastase can be carried out with the substrate Boc-Ala-OPhNO_2, which is non specific but sensitive. However, the aryl ester is susceptible to spontaneous hydrolysis above neutral pH (Visser and Blout, 1972). A more stable aryl ester is Z-Ala-ONap. A substrate, which gives greater specificity but less sensitivity, is Suc-Ala-Ala-Ala-NPhNO_2. Sensitivity can be increased by using the fluorogenic substrate MeOSuc-Ala-Ala-Pro-Val-NHMec. The enzyme is stored in the presence of 0.1% brij 35 to prevent loss of enzyme on surfaces (Barrett, 1981b)

4.3.1.1 Reagents

Assay buffers [0.2 M Tris, NaCl of increasing concentrations, pH 8.5]. Tris (2.42 g) was combined with NaCl (the concentrations and corresponding masses of NaCl are given in Table 4.3), dissolved in 95 ml distilled water, adjusted to pH 7.5 with HCl and made up to 100 ml with distilled water.

Table 4.3. Composition of buffers used in elastase assays.

Buffer No	Tris (M)	NaCl (M)	NaCl (g/100 ml)	pH
1	0.2	0	0	7.5
2	0.2	1.0	5.84	7.5
3	0.2	2.0	11.7	7.5
4	0.2	3.0	17.5	7.5

1 mM stock solution of MeOSuc-Ala-Ala-Pro-Val-NHMec. MeOSuc-Ala-Ala-Pro-Val-NHMec (0.6277 mg) was dissolved in DMSO (1 ml).

Working concentrations of MeOSuc-Ala-Ala-Pro-Val-NHMec. The different concentrations of MeOSuc-Ala-Ala-Pro-Val-NHMec used to determine K_m and the corresponding volume of 1 mM stock required to make 1 ml of working solution are given in Table 4.4. The required volume of 1 mM stock was made up to 1 ml with distilled water.

Table 4.4. Working concentrations of MeOSuc-Ala-Ala-Pro-Val-NHMec used in elastase K_m determination.

No.	[Substrate] solution (μM)	Volume of 1 mM stock (μl/ml)	[Substrate] in well (mM)
1	80	80	0.02
2	100	100	0.025
3	200	200	0.05
4	300	300	0.075
5	400	400	0.1
6	500	500	0.125

Elastase storage solution [0.1% (m/v) Brij 35]. Brij 35 (0.1 g) was dissolved in distilled water (100 ml).

1 mg/ml elastase stock solution. Elastase (100 μg) was made up to 100 μl with storage solution

1 μg/ml elastase. Just before use the 1 mg/ml elastase stock solution (10 μl) was made up to 10 ml with storage solution.

4.3.1.2 Procedure

As described in Section 4.2.1.2.

4.3.2 Results

Elastase shows an increase in v_0 with increasing salt concentrations (Figure 4.5A). K_m is decreased, through a minimum at 1 M NaCl, implying an increased affinity of enzyme for the substrate (Figure 4.5B). V_{max} is increased (Figure 4.5C) and V_{max}/K_m shows an overall increase (Figure 4.5D).

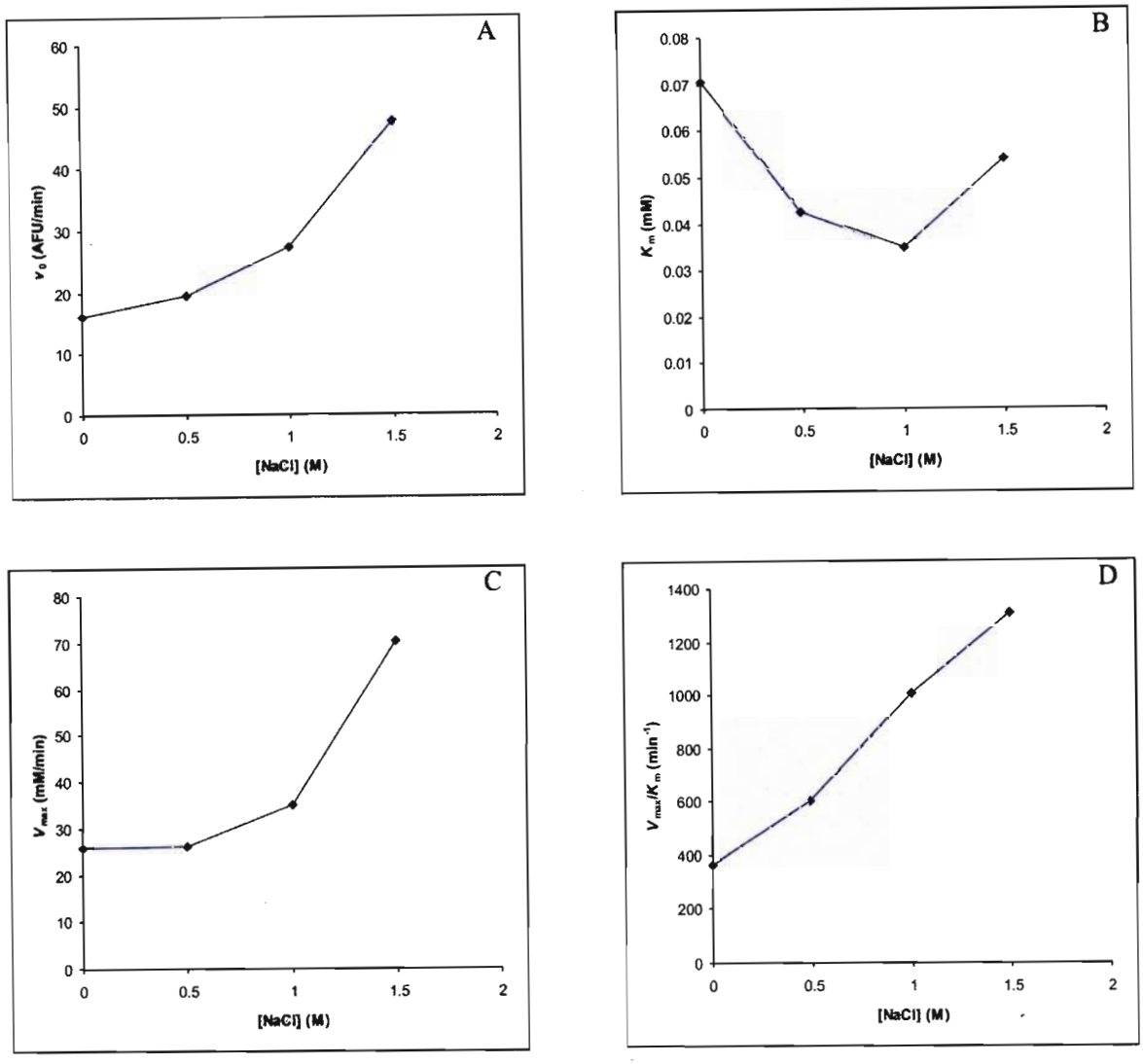


Figure 4.5. The effect of ionic strength on the kinetic constants of elastase acting on the substrate MeOSuc-Ala-Ala-Pro-Val-NHMec
(A) = Activity, (B) = K_m, (C) = V_{max} and (D) = V_{max}/K_m

4.3.3 Discussion

The S_1 pocket of elastase is hydrophobic in character (Czapinska and Otlewski, 1999) and 'prefers' small or medium sized hydrophobic residues at the substrate P_1 position. Increasing ionic strength promotes hydrophobic interactions, which may explain the increases in affinity and turnover. Trowbridge *et al.* (1963) found that a second substrate molecule binds to a regulatory site on the enzyme and brings about a conformational

change of the active site so that the substrate is hydrolysed faster. Increasing NaCl does not seem to interfere with this binding. The results of the present study are consistent with those of Lestienne and Bieth (1980) who showed that elastase activity against N-succinyl-(L-alanine)$_3$-***p***-nitroanilide increased with increasing concentrations of NaCl, and that the rate limiting step is acyl-enzyme formation. The general catalytic mechanism for serine proteases is shown in Scheme 1 (Chapter 1). However, in the present study V_{max} was increased by increasing ionic strength, as in the study of Lestiennne and Bieth (1980). This may indicate that the acylation rate is increased. In the present study an increase in affinity was shown whereas in the previous study affinity was unaffected by ionic strength. This could be due to the different substrates used, with interactions with the more hydrophobic Val in the P_1 position (as used in the present study) perhaps being favoured relative to Ala.

4.4 Trypsin

4.4.1 Trypsin Assay

Trypsin activity was originally determined, spectrophotometrically, using benzoyl arginine ethyl ester (BAEE) as the substrate (Schwert and Takenaka, 1955). Z-Phe-Arg-NHMec is also hydrolysed by trypsin (Melo *et al.*, 2001). In the present study the fluorometric substrates Z-Arg-Arg-NHMec and Z-Ala-Ala-Pro-Phe-NHMec were used. Trypsin was previously reported to not cleave substrates with a Phe group in the P_1 position (Melo *et al.*, 2001). However, in the present study it was shown that trypsin does cleave the substrate Z-Ala-Ala-Pro-Phe-NHMec at sufficient enzyme concentration.

4.4.1.1 Reagents

Assay buffer [0.1 M HEPES, NaCl of increasing concentrations, pH 7.5]. HEPES (2.83 g) was combined with NaCl (the concentrations and corresponding masses of NaCl are given in Table 4.5), dissolved in 95 ml distilled water, adjusted to pH 7.5 and made up to 100 ml with distilled water.

Table 4.5. Composition of buffers used in trypsin assays.

Buffer No	HEPES (M)	NaCl (M)	NaCl (g/100 ml)	pH
1	0.1	0	0	7.5
2	0.1	1.0	5.84	7.5
3	0.1	2.0	11.7	7.5
4	0.1	3.0	17.5	7.5

1 mM stock solution of Suc-Ala-Ala-Pro-Phe-NHMec. Suc-Ala-Ala-Pro-Phe-NHMec (0.6617 mg) was dissolved in DMSO (1 ml).

Working concentrations of Suc-Ala-Ala-Pro-Phe-NHMec. The different concentrations of Suc-Ala-Ala-Pro-Phe-NHMec used to determine K_m and the corresponding volume of 1 mM stock required to make 1 ml of working solution are given in Table 4.6. The required volume of 1 mM stock was made up to 1 ml with distilled water.

Table 4.6. Working concentrations of Suc-Ala-Ala-Pro-Phe-NHMec used in trypsin K_m determination.

No.	[Substrate] solution (μM)	Volume of 1 mM stock (μl/ml)	[Substrate] in well (mM)
1	80	80	0.02
2	100	100	0.025
3	200	200	0.05
4	300	300	0.075
5	400	400	0.1
6	500	500	0.125

1 mM stock solution of Z-Arg-Arg-NHMec. As described in Section 3.2.2.1.

Working concentrations of Z-Arg-Arg-NHMec. The different concentrations of Z-Arg-Arg-NHMec, used to determine K_m and the corresponding volume of 1 mM stock required to make 1 ml of working solution are given in Table 4.7. The required volume of 1 mM stock was made up to 1 ml with distilled water.

Table 4.7. Working concentrations of Z-Arg-Arg-NHMec used in trypsin K_m determination.

No.	[Substrate] solution (μM)	Volume of 1 mM stock (μl/ml)	[Substrate] in well (mM)
1	80	80	0.02
2	100	100	0.025
3	200	200	0.05
4	300	300	0.075
5	400	400	0.1
6	500	500	0.125

Trypsin storage solution [0.001 M HCl, 0.02 M $CaCl_2$]. 0.1 M HCl (1 ml) and $CaCl_2$ (0.29 g) were mixed and made up to 100 ml with distilled water.

1 mg/ml trypsin stock solution. Trypsin (1 mg) was made up to 1 ml with storage solution.

1 μg/ml trypsin. Just before use the trypsin stock solution (10 μl) was made up to 10 ml with storage solution.

4.4.1.2 Procedure

As described in Section 4.2.1.2.

4.4.2 Results

Acting on the substrate Z-Arg-Arg-NHMec, trypsin showed a decrease in activity with increasing ionic strength (Figure 4.6A), K_m (Figure 4.6B) showed a decrease, V_{max} a decrease (Figure 4.6C) and V_{max}/K_m also an overall decrease (Figure 4.6D).

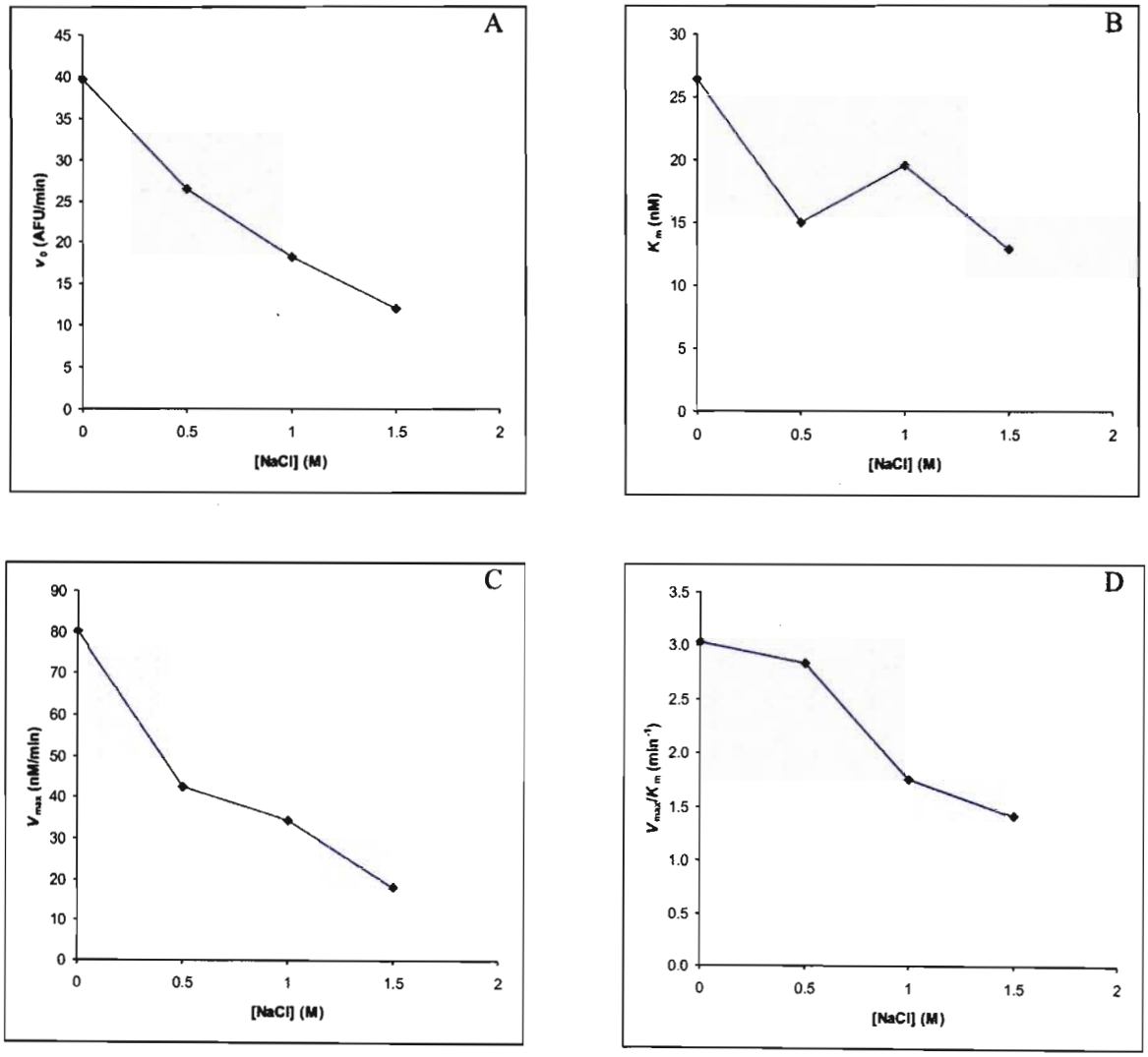


Figure 4.6. The effect of ionic strength on the kinetic constants of trypsin acting on the substrate Z-Arg-Arg-NHMec.
(A) = Activity, (B) = K_m, (C) = V_{max} and (D) = V_{max}/K_m

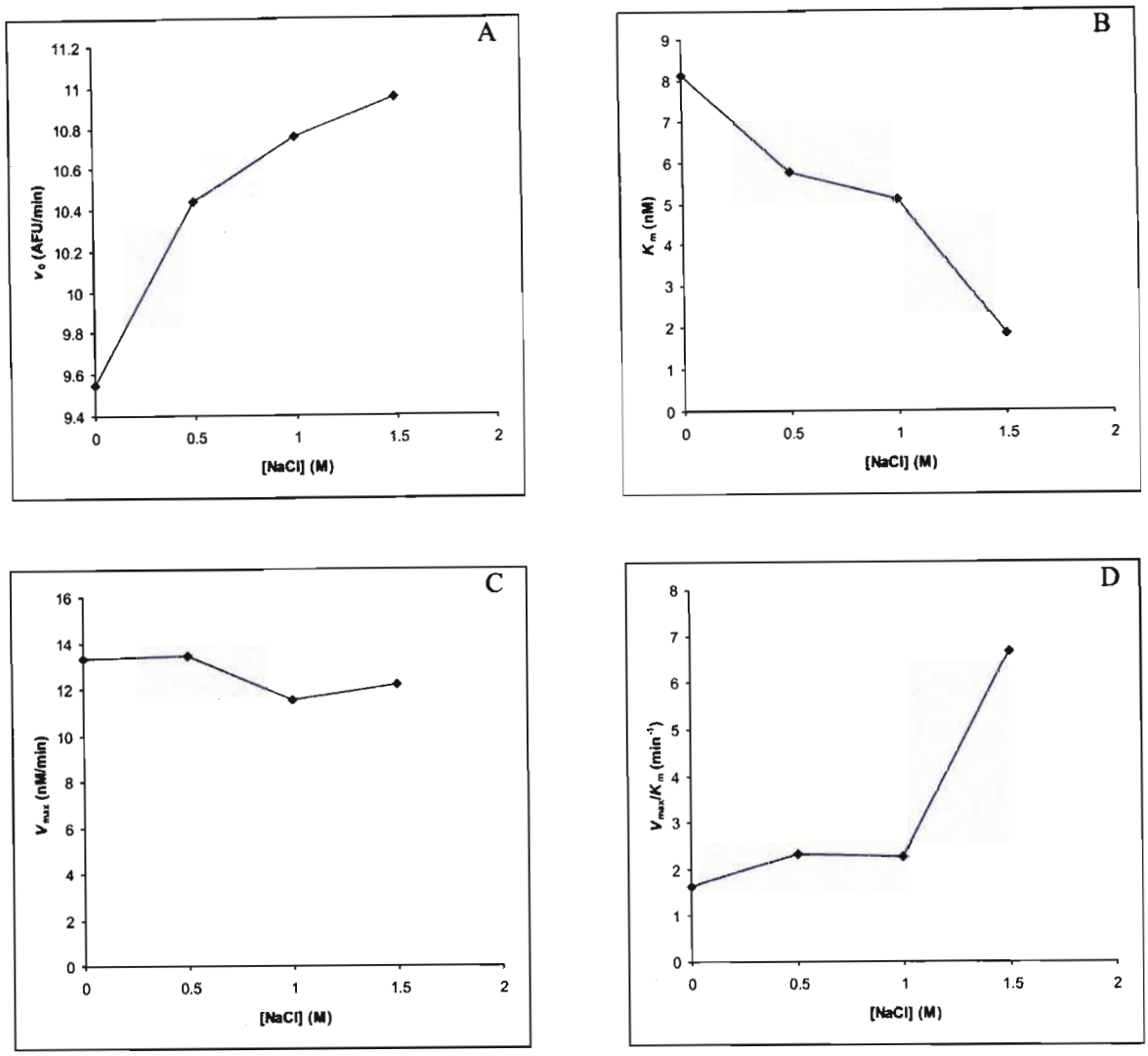


Figure 4.7. The effect of ionic strength on the kinetic constants of trypsin acting on the substrate Suc-Ala-Ala-Pro-Phe-NHMec.
(A) = Activity, (B) = K_m, (C) = V_{max} and (D) = V_{max}/K_m

Acting on the substrate Suc-Ala-Ala-Pro-Phe-NHMec, trypsin showed an increase in activity with increasing ionic strength (Figure 4.7A), K_m decreases (Figure 4.7B), V_{max} remained relatively unchanged (Figure 4.7C) and V_{max}/K_m showed an increase (Figure 4.7D).

4.4.3 Discussion

The specificity pocket of trypsin consists of Gly-226, Gly-219 and Asp-189 (Branden and Tooze, 1991). Due to the hydrophilic nature of this pocket there is an affinity for hydrophilic substrates and, consistent with this, in the presence of increasing ionic strength the activity of trypsin against Z-Arg-Arg-NHMec decreased. However, there was a relatively slight decrease in K_m, which indicates that the affinity of enzyme for substrate is

not significantly affected. There is a steep decrease in turnover, however, which implies that it is the actual hydrolysis process that is affected by ionic strength and not the binding of the enzyme to the substrate.

Trypsin has a significantly lower affinity for hydrophobic substrates such as Z-Ala-Ala-Pro-Phe-NHMec but in the present study, at sufficiently high concentrations of enzyme, activity could be detected. With increasing ionic strength there was an increase in activity against Z-Ala-Ala-Pro-Phe-NHMec. In this case there was a significant increase in affinity of enzyme for substrate, possibly because the charges in the specificity pocket were masked by the ions in solution and/or because the hydrophobic interaction at P_1' was favoured (Melo *et al.*, 2001). The slight decrease in affinity for the Z-Arg-Arg-NHMec with increasing ionic strength would also indicate that the P_1' interaction was being affected. For the reaction involving Z-Ala-Ala-Pro-Phe-NHMec, there was not a significant change in the turnover with increasing ionic strength but V_{max}/K_m showed an overall increase.

4.5 Cathepsin L

4.5.1 Cathepsin L assay

4.5.1.1 Reagents

Buffers [400 mM Na-acetate, 4 mM Na_2EDTA, NaCl of increasing concentrations, 0.02% (m/v) NaN_3, 9 mM DTT, pH 5.5]. Glacial acetic acid (23 ml), Na_2EDTA (1.5 g) and NaCl (Table 4.8) were dissolved in 950 ml distilled water and adjusted to pH 5.5 with NaOH. NaN_3 (0.2 g) was added and made up to 1 L with distilled water. DTT was added to a concentration of 9 mM (0.00671 g DTT/5 ml buffer) immediately before use.

Table 4.8. Concentration of NaCl in each buffer

Buffer No	NaCl (M)	NaCl (g/L)
1	0	0
2	1.0	58.44
3	2.0	116.90
4	3.0	175.30

1 mM substrate stock solution of Z-Phe-Arg-NHMec. As described in Section 3.2.1.1.

Working concentrations of Z-Phe-Arg-NHMec. The different concentrations of Z-Phe-Arg-NHMec used to determine K_m and the corresponding volume of 1 mM stock required to make 1 ml of working solution are given in Table 4.9. The required volume of 1 mM stock was made up to 1 ml with distilled water.

Table 4.9. Working concentrations Z-Phe-Arg-NHMec used in cathepsin L K_m determination.

No.	[Substrate] solution (μM)	Volume of 1 mM stock (μl/ml)	[Substrate] in well (mM)
1	60	60	0.015
2	80	80	0.02
3	100	100	0.025
4	200	200	0.05
5	300	300	0.075
6	400	400	0.1

Cathepsin L solution. Isolated sheep cathepsin L (1.9 mg/ml protein concentration) was used. Cathepsin L (100 μl) was made up to 10 ml in buffer (0 NaCl).

4.5.1.2 Procedure

As described in Section 4.2.1.2.

4.5.2 Results

For cathepsin L, analyzed using Z-Phe-Arg-NHMec, there is a decrease in activity with increasing ionic strength (Figure 4.8A). The increasing K_m (Figure 4.8B) indicates a decreasing affinity between substrate and enzyme. Turnover, given by V_{max} (Figure 4.8C), and V_{max}/K_m (Figure 4.8D) also decrease.

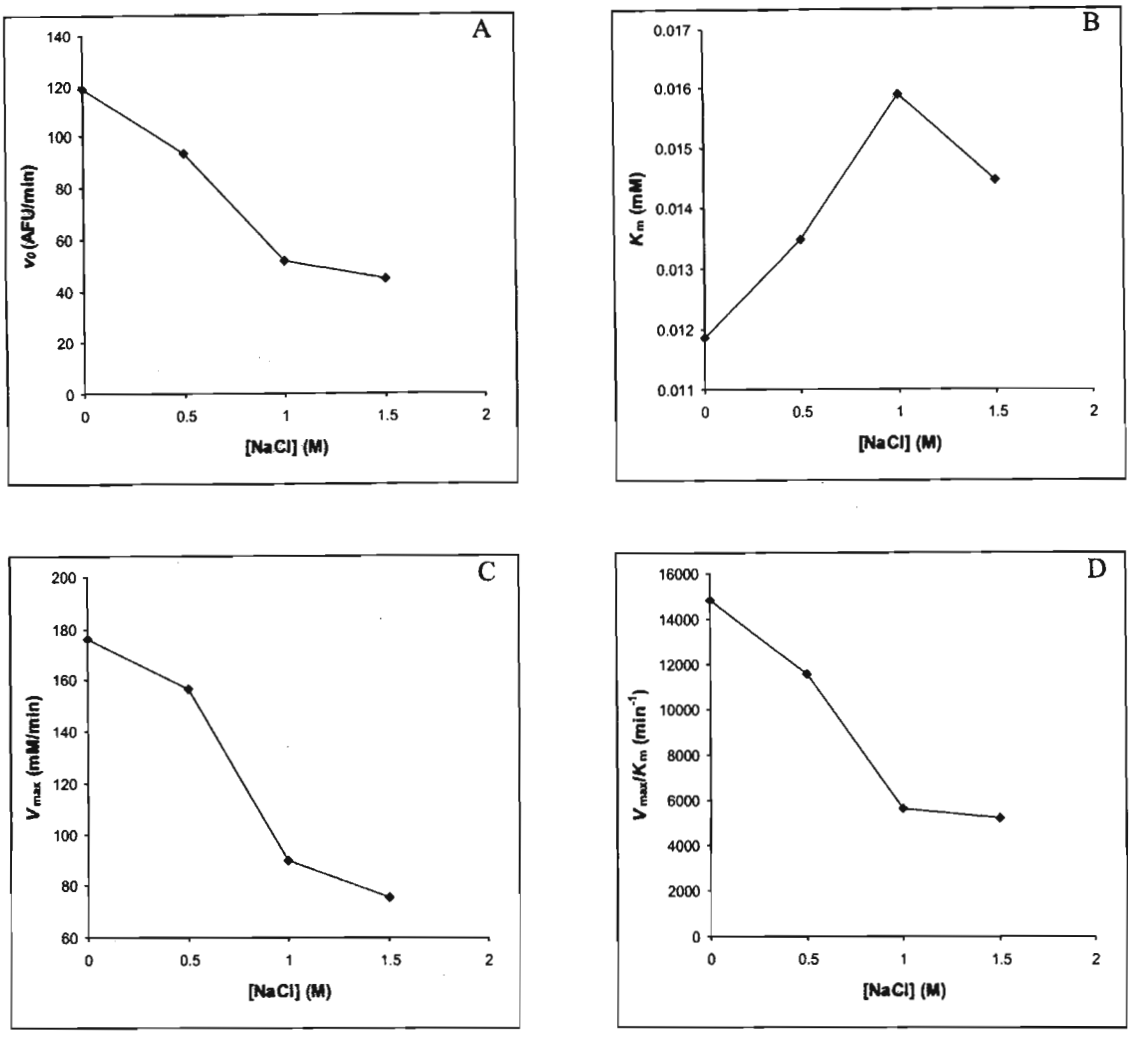


Figure 4.8. The effect of ionic strength on the kinetic constants of cathepsin L acting on the substrate Z-Phe-Arg-NHMec.
(A) = Activity, (B) = K_m, (C) = V_{max} and (D) = V_{max}/K_m

4.5.3 Cathepsin L-cystatin C K_i assays

4.5.3.1 Reagents

Buffer [400 mM Na-acetate, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 9 mM DTT, pH 5.5]. See Section 4.5.1.1.

1 mM substrate stock solution of Z-Phe-Arg-NHMec. See Section 3.2.1.1.

Working concentrations of Z-Phe-Arg-NHMec (100 mM). See Section 4.5.1.1 (Table 4.9).

Cathepsin L solution. Isolated sheep cathepsin L (1.9 mg/ml protein concentration) was used for these assays. Cathepsin L (100 µl) was made up to 10 ml in buffer (0 M NaCl).

Cystatin C solution. Isolated cystatin C (0.116 mg/ml) was used undiluted.

4.5.3.2 Procedure

Buffer 1 (Table 4.8) (100 µl) was added to column 1 and rows A-F, Buffer 2 (Table 4.8) (100 µl) was added to column 2 and rows A-F, Buffer 3 (Table 4.8) (100 µl) was added to column 3 and rows A-F, Buffer 4 (Table 4.8) (100 µl) was added to column 4 and rows A-F. This arrangement resulted in 5 replicates of each assay. 100 µM Z-Phe-Arg-NHMec (50 µl) was added to each of the buffer containing wells. Cathepsin L (40 µl) was added and mixed to all the wells and the fluorescence was measured every minute for 5 minutes in a fluorescence microplate reader (Cambridge Technology Model 7620) with excitation at 360 nm and emission at 460 nm. After 5 minutes, cystatin C (10 µl) was added to rows A-E of the assay wells. Fluorescence was measured every minute for 35 minutes. The replicates were averaged and plotted as a function of time. v_0 was obtained, from the slope of the first set of readings prior to adding cystatin C and v_i was obtained from the slope of the second set of readings after addition of cystatin C.

The enzyme-catalysed hydrolysis of the substrate in the absence of inhibitor was followed to establish v_0, this requires a linear rate and that the enzyme does not inactivate spontaneously under the given assay conditions: both of these conditions were met. Inhibitor (at least 10 fold molar excess over enzyme) was added in no more than 5% of the total assay volume, and the reaction was allowed to proceed until the rate of hydrolysis relaxed to a new steady state, thus establishing the inhibited rate, v_i. The substrate concentration is kept essentially constant by allowing no more than 5% hydrolysis (Salvesen and Nagase, 2001).

Under these conditions $K_{i(app)}$ (i.e. K_i in the presence of substrate) is given by:

$$v_0/v_i = 1 + [I]_0/K_{i(app)} \qquad (8)$$

Where $K_{i(app)}$ was calculated from the known inhibitor concentration and the ratio of the two hydrolysis rates (expressed in arbitrary units).

K_i, the true equilibrium constant, can be calculated from equation (9), (the values for K_m were obtained from Section 4.5.2 for each of the different salt concentrations).

$$K = K_{i(app)}/(1 + [S]_0/K_m) \qquad (9)$$

Therefore, when $[S]_0 << K_m$, $K_i = K_{i(app)}$

4.5.3.3 Results

Both $K_{i(app)}$ and the subsequently calculated K_i show a steep increase with increasing ionic strength (Figure 4.9).

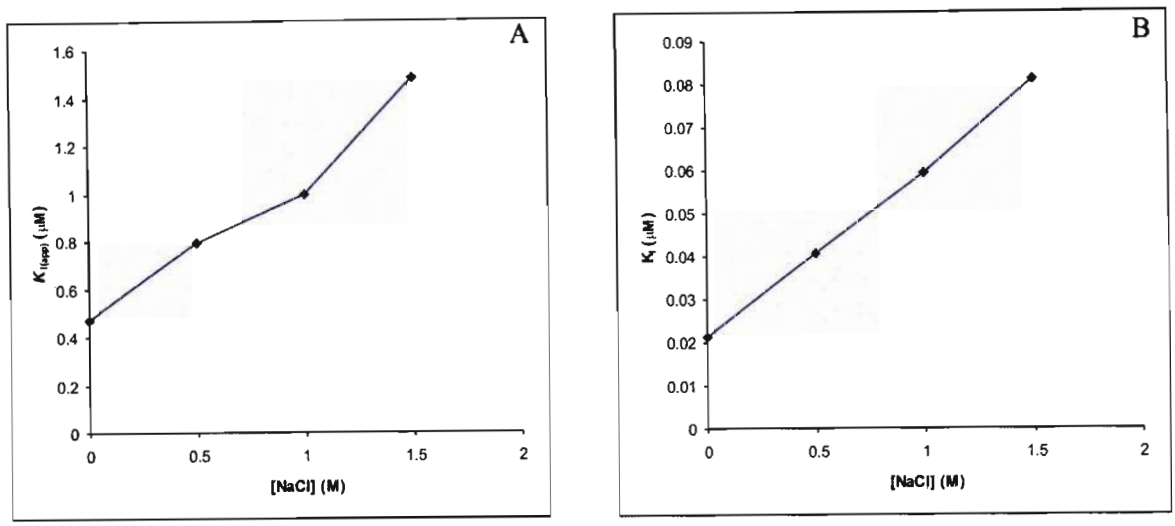


Figure 4.9. The effect of ionic strength on $K_{i(app)}$ and K_i of cathepsin L-cystatin C interaction.
(A) = $K_{i(app)}$; (B) = K_i

4.5.4 Discussion

In the present study cathepsin L showed a decrease in activity on Z-Phe-Arg-NHMec with increasing ionic strength, a result consistent with that of Dennison *et al.* (1992). K_m shows a general increase and V_{max} a general decrease implying a decrease in affinity of enzyme for substrate and decrease in turnover. V_{max}/K_m gives an overall decrease. Cathepsin L cleaves proteins near hydrophobic amino acid residues, which bind to subsites S_2 and S_3 (Kirschke *et al.*, 1988). The S_1 region of the active site is able to accommodate large hydrophobic substituents (Kirschke *et al.*, 1988). Because of the importance placed on the S_2 and S_3 hydrophobic interactions, it would be expected to find an increase in activity at higher salt concentrations, but this is not the case. These results are curious but are consistent with *ionic* bonds between enzyme and substrate, suggesting a role for the Arg residue in the P_1 site. Alternatively, increased ionic strength may change the enzyme's conformation to a form less favourable for binding and catalysis. Cathepsin L stability is a result of a delicate balance between hydrophobic and ionic interactions (Turk *et al.*, 1999). These results imply a greater emphasis on ionic interactions to stabilize cathepsin L.

In addition to reacting with substrates, enzymes also interact with inhibitors and a measure of this interaction is provided by the K_i. K_i values are of particular importance in the use of

inhibitors as therapeutics. However, it has not previously been reported whether K_i values are sensitive to ionic strength. Clearly this is important if extrapolations are to be made to the situation *in vivo*. For the cathepsin L/cystatin C interaction, K_i was found to increase with increasing ionic strength. This implies, i) ionic strength does not only influence substrates but significantly larger inhibitors too, and, ii) ionic strength needs to be a controlled variable when determining K_i. It is thus essential that K_i's of inhibitors intended for therapeutic use are determined at physiological ionic strength and pH.

Papain has a homologous structure to cathepsin L and the interaction between chicken egg white cystatin and papain is documented (Bode *et al.* 1988). Two hairpin loops and the amino-terminal Gly-9I to Ala-10I form a wedge shaped 'edge' which is complementary to the active site cleft of papain (Bode *et al.* 1988). The inserted hairpin loops of cystatin make major binding interactions with the highly conserved residues Gly-23, Gln-19, Trp-177 and Ala-136 of papain in the neighbourhood of the reactive site Cys-25; the amino-terminal (Bode *et al.* 1988). The interacting residues between cystatin C and papain are hydrophobic and such interactions are expected to be favoured by increasing ionic strength. The decrease in inhibition indicates that perhaps it is not these micro interactions that are being affected but rather that the macro structures of the enzyme or inhibitor are adopting conformations that are unsuitable for binding.

4.6 Cathepsin B

4.6.1 Cathepsin B assay

Cathepsin B assays were done using two different substrates Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec.

4.6.1.1 Reagents

Buffer [400 mM Na-acetate, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 9 mM DTT, pH 5.5]. See Section 4.5.1.1.

1 mM substrate stock solution of Z-Phe-Arg-NHMec. See Section 3.2.1.1.

Working concentrations of Z-Phe-Arg-NHMec. See Section 4.5.1.1.

1 mM stock solution of Z-Arg-Arg-NHMec. See Section 3.2.2.1.

Working concentrations of Z-Arg-Arg-NHMec. The different concentrations of Z-Arg-Arg-NHMec, used to determine K_m and the corresponding volume of 1 mM stock required to make 1 ml of working solution are given in Table 4.10. The required volume of 1 mM stock was made up to 1 ml with distilled water.

Table 4.10. Working concentrations of Z-Arg-Arg-NHMec used in cathepsin B K_m determination.

No.	[Substrate] (μM)	Volume of 1 mM stock (μl/ml)	[Substrate] in well (mM)
1	60	60	0.015
2	80	80	0.02
3	100	100	0.025
4	200	200	0.05
5	300	300	0.075
6	400	400	0.1

4.6.1.2 Procedure

As described in Section 4.2.1.2.

4.6.2 Results

Cathepsin B, analyzed using Z-Arg-Arg-NHMec, showed an overall decrease in activity with increasing ionic strength (Figure 4.10A). This is despite the fact that the decrease in K_m (Figure 4.10B) is apparently balanced by decreasing turnover (Figure 4.10C), leaving V_{max}/K_m relatively unaffected by ionic strength (Figure 4.10D).

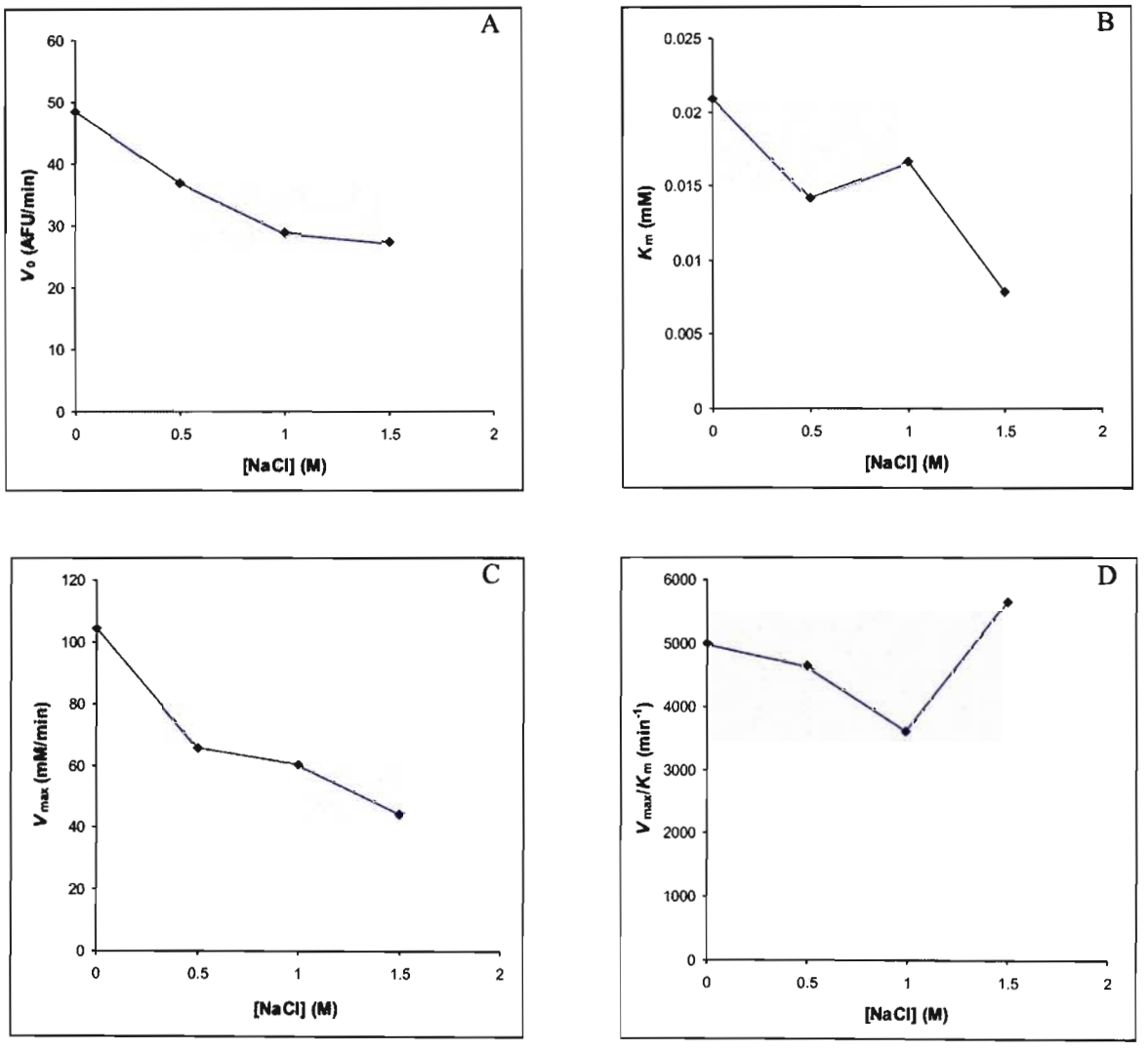


Figure 4.10. The effect of ionic strength on the kinetic constants of cathepsin B acting on the substrate Z-Arg-Arg-NHMec.
(A) = Activity, (B) = K_m, (C) = V_{max} and (D) = V_{max}/K_m

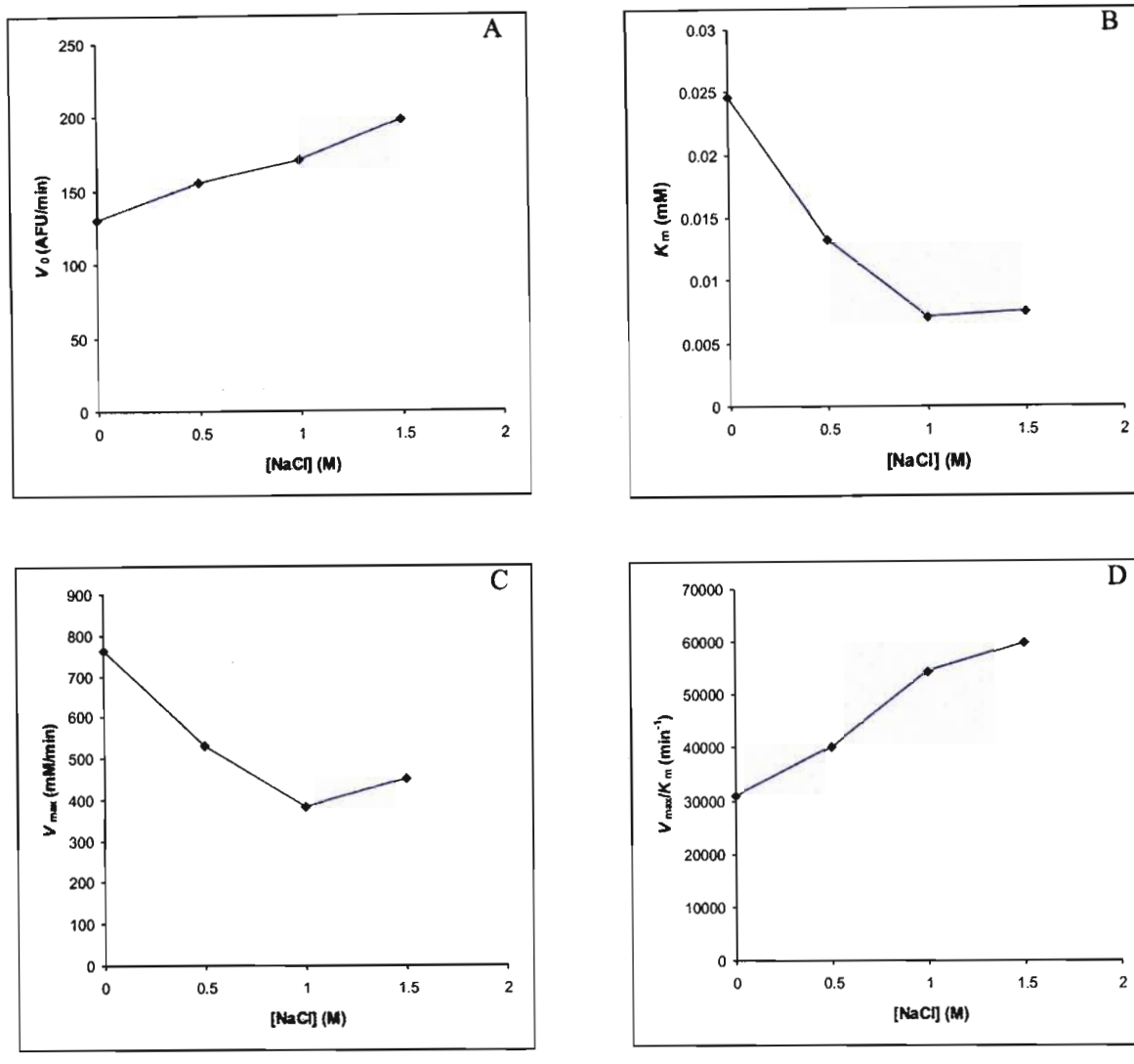


Figure 4.11. The effect of ionic strength on the kinetic constants of cathepsin B acting on the substrate Z-Phe-Arg-NHMec.
(A) = Activity, (B) = K_m, (C) = V_{max} and (D) = V_{max}/K_m)

Cathepsin B was also analyzed using Z-Phe-Arg-NHMec, for which it has a 7-fold higher affinity than for Z-Arg-Arg-NHMec. In this case increasing the ionic strength resulted in increasing activity (Figure 4.11A), which can be attributed largely to the decrease in K_m (Figure 4.11B). Turnover decreased with increasing ionic strength (Figure 4.11C) but the ratio of V_{max}/K_m increased (Figure 4.11D).

4.6.3 Discussion

Cathepsin B, like cathepsin L, has a hydrophobic pocket at S_2 (Kirschke *et al.*, 1988). It is suggested from the interaction with Z-Phe-Arg-NHMec that this pocket plays a significant role in the mechanism of cathepsin B. The interaction with a hydrophobic amino acid residue was favoured by increasing ionic strength. Activity against Z-Arg-Arg-NHMec

decreased indicating that ionic interactions are disrupted by ionic strength. This also shows that for cathepsin B the S_2 binding site plays a greater role than S_1 binding site. The above assumptions are made not on the basis of affinity for either substrate but rather on the higher turnover noted for Z-Phe-Arg-NHMec.

Increasing ionic strength was previously found to inhibit cathepsin B activity (Dehrmann *et al.*, 1995). However, this only applies to interactions of cathepsin B with a hydrophilic residue in the P_2 position of the substrate. The S_2 subsite is a wide pocket extending from the active site cleft toward Glu-245 and is open to solvent. A large P_2 side chain, such as Phe, could be accommodated in the space available. Cathepsin B has a broad S_2 specificity, accepting both Phe and Arg at the P_2 position in substrates but Phe is preferred 7-fold over Arg. In the case of a P_2 Arg residue there is an electrostatic interaction with Glu-245, which is important for substrate binding and contributes to transition state-complex stabilization (Hasnain *et al.*, 1993). The S_1 subsite is relatively large and very hydrophobic but has no strong preference for any particular amino acid residue. The volume enclosed by this pocket would be capable of accommodating a bulky hydrophobic side chain, such as Phe or Trp.

The theory of inactivation is based on the Cys-29 of cathepsin B, which exists as the thiol anion that could form a salt bridge with the imidazolium cation. Oxidation of the active site thiol does not extensively alter the conformation of the catalytic residues but Turk *et al.* (1994) showed that inactivation of cathepsin B, in alkaline medium, is caused by the deprotonation of the imidazole moiety of the Cys-imidazole ion pair. This causes the enzyme to be destabilized and leads to unfolding. Song *et al.* (2000) confirmed that the active-site residue Cys-29 is responsible for the neutral-pH inactivation and refolding barrier of human cathepsin B. Dehrmann *et al.* (1996) showed that by reducing the active site cysteine of cathepsin B and L, the enzymes were not only activated, but their stability around neutral pH was markedly increased. The present results suggest that the enzyme structure is not destabilized because of the increasing activity against Z-Phe-Arg-NHMec at increasing ionic strength. Ionic strength appears to affect the micro interactions between the substrate and enzyme to a small extent as indicated by the decreasing K_m (Figure 4.10B and Figure 4.11B). Depending on the choice of substrate, ionic strength effects on the catalytic mechanism of cathepsin B are more pronounced as indicated by V_{max} values (Figure 4.10C and Figure 4.11C). Activity against Z-Phe-Arg-NHMec, has a significantly larger initial V_{max} also shows in a relatively greater decrease in V_{max}.

4.7 Bromelain

4.7.1 Bromelain assay

4.7.1.1 Reagents

Buffer [400 mM Na-acetate, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 9 mM DTT, pH 5.5]. See Section 4.5.1.1.

1 mM substrate stock solution of Z-Phe-Arg-NHMec. See Section 3.2.1.1.

Working concentrations of Z-Phe-Arg-NHMec. See Section 4.5.1.1.

Bromelain solution. Isolated Bromelain (2 mg/ml protein concentration) was used for these assays. Bromelain (10 µl) was made up to 10 ml in buffer (0 NaCl).

4.7.1.2 Procedure

As described in Section 4.2.1.2.

4.7.2 Results

Analyzed using Z-Phe-Arg-NHMec, bromelain showed increasing activity with increasing ionic strength (Figure 4.12A). Affinity for substrate increased with ionic strength (Figure 4.12B). The increase in activity is limited by decreasing turnover above 2 M NaCl (Figure 4.12C).

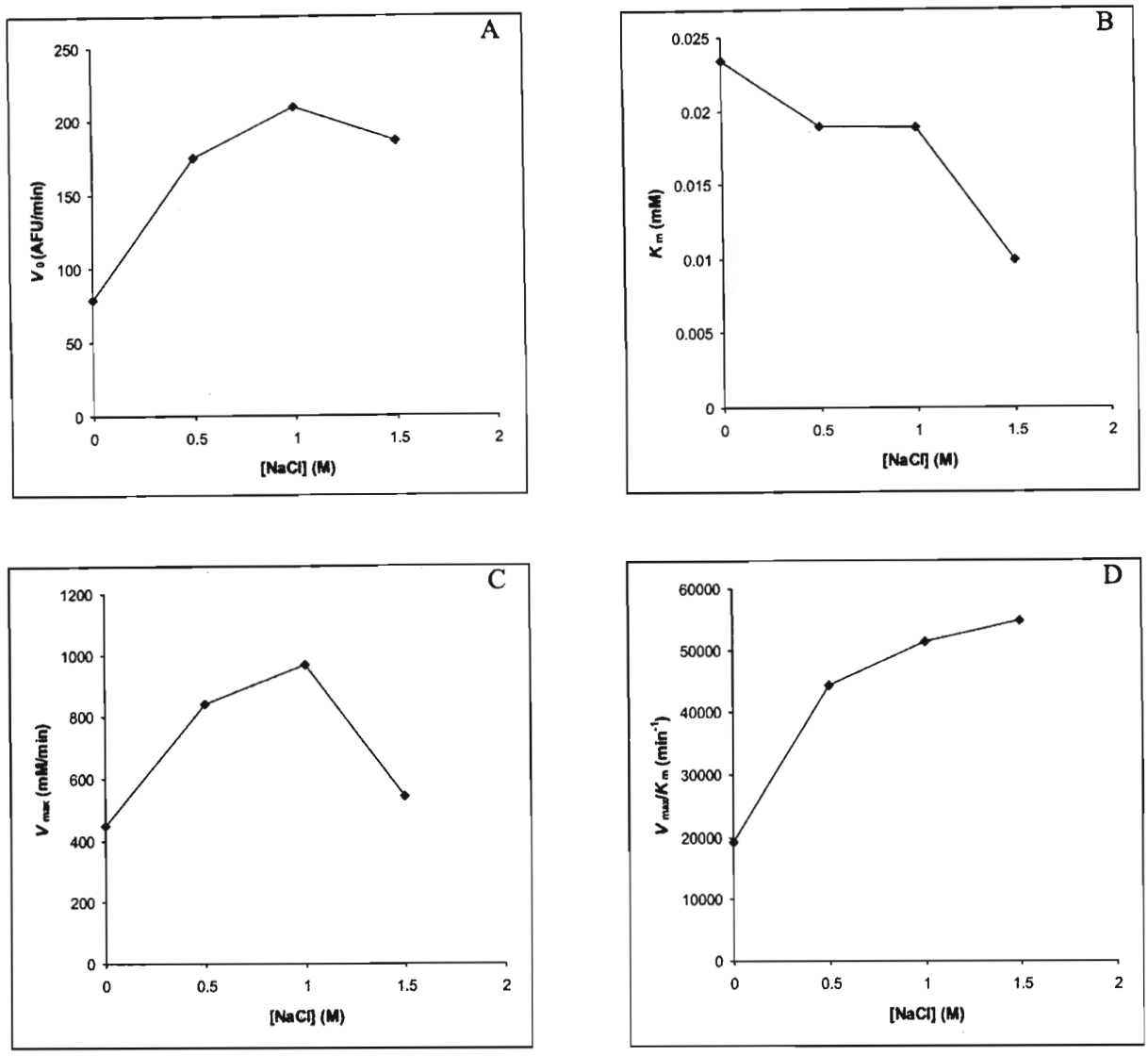


Figure 4.12. The effect of ionic strength on the kinetic constants of bromelain acting on the substrate Z-Phe-Arg-NHMec.
(A) = Activity, (B) = K_m, (C) = V_{max} and (D) = V_{max}/K_m

4.7.3 Discussion

Bromelain showed an increase in v_o with increasing ionic strength, due to a decrease in K_m and increasing V_{max} which resulted in an overall increase of V_{max}/K_m. There are no structural details on bromelain as it has not been crystallized to date. However, it is a cysteine proteinase and is homologous to papain and other cysteine proteinases. The trends observed for bromelain acting on Z-Phe-Arg-NHMec, are similar to those of cathepsin B acting on the same substrate. It can be assumed that the interaction between bromelain and the substrate would be similar to those of cathepsin B.

CHAPTER 5

GENERAL DISCUSSION

The aim of the present study was to determine whether ionic strength is a significant factor in the function of enzymes, in general, as had proven to be the case in determining the pH optima of cathepsins L and B and, also, whether ionic strength needs to be taken into account in the determination of the K_i values of enzyme inhibitors. The latter was considered of potential importance, as extrapolations are often made from K_i values determined *in vitro* to deduce whether inhibitors are significant *in vivo*. Within the limits of the present study, it would appear that from the results a general statement can be made; that ionic strength is indeed a significant factor in both enzyme activity and enzyme inhibition and it would consequently be prudent to always take it into account when working with a new system.

However, ionic strength appears to have different effects on different enzymes and on different enzyme/substrate combinations, which is, perhaps, not surprising. The effects do not necessarily correlate with the enzyme class. For instance, considering the cysteine proteinases, increasing ionic strength decreases the activity of cathepsin L and cathepsin B but increases the activity of bromelain. Similarly, increasing ionic strength decreases the activity of the serine proteinase, trypsin, on the substrate Z-Arg-Arg-NHMec but increases it on the substrate Suc-Ala-Ala-Pro-Phe-NHMec.

To some extent the results can be explained on the basis of known properties of the substrate-binding sub-sites on the enzyme and the residues on the substrate that dock into them. For instance, with increasing ionic strength, a hydrophobic sub-site will generally have an increasing affinity for a hydrophobic residue. However, in addition to these micro-interactions, the overall conformation of the enzyme might be affected by ionic strength and, in turn, this might have an influence on the conformation of the active site. An example is provided by cathepsin B (and, probably cathepsin L, also). Here the enzyme consists of two domains that come together to form the substrate-binding cleft. The domains are held together by a "zipper" of hydrogen bonds, but a key component is the "button" provided by the thiolate-imidazole ionic bond, between the catalytic Cys and His residues (Turk *et al.*, 1994). If the "button" is undone, it appears that the "zipper" will follow. pH is known to affect activity by an effect on the thiolate-imidazole ionic bond

and it is not hard to imagine that ionic strength might have a similar effect. However, with cathepsin B, the increased activity against Z-Phe-Arg-NHMec with increasing ionic strength indicates that ionic strength may not cause a conformational change of this nature. A more specific example of ionic strength affecting structural conformation is provided by class sigma glutathione transferase (Stevens *et al.*, 2000). In this case, increasing ionic strength favoured the monomer over the native dimeric form; concomitantly, the active site was distorted and the enzyme lost 60% of its activity.

The effect of increasing ionic strength on the cathepsin L/cystatin C interaction, i.e. a lowering of the affinity of the interaction, is also most easily explained on the basis of a change in overall conformation of the enzyme and/or the inhibitor. With only one result to hand, however, nothing can be said about the general applicability of this effect: the question thus remains open, but compelling!

In cases where ionic strength affects the affinity of the interaction between the enzyme and the substrate, this would be reflected in K_m values. However, a further effect might be on the reaction mechanism itself. An example is provided by the results of Lestienne and Bieth (1980), which suggested that the increasing activity of elastase with increasing ionic strength (confirmed in the present study) was primarily due to an increase in the rate-limiting step of acyl-enzyme formation.

Cysteine and serine proteinases have a generally common reaction mechanism, involving a nucleophilic Cys or Ser and charge transfer through a His residue (Schemes 1 and 2, Chapter 1). It might be expected that increasing ionic strength might have a generally depressing effect, by masking the charge on the nucleophile and possibly inhibiting charge relay. For example, Lee *et al.* (2002) found that the pKa value of histidine in a staphylococcal nuclease is affected by both salt concentration and the ionic species involved. Bostrom *et al.* (2003) also showed an influence of specific ions upon pKa values. Such effects on the catalytic histidine of proteases, could affect the enzymes' activity. Effects on the reaction mechanism would be expected to be reflected in k_{cat} (V_{max}) values, so that it might be anticipated that an increased ionic strength might generally lead to a reduced V_{max}. Results from the present study show that V_{max} decreases in the case of trypsin, cathepsin L and cathepsin B, but increases in the case of elastase and bromelain, which is not consistent with this simplistic model and suggests that the real situation is more subtle.

The overall effect of ionic strength is reflected in values of V_{max}/K_m (recalling that V_{max} is proportional to k_{cat}). Here, again, there are differences between different enzymes and it might be speculated that these differences reflect an evolutionary selection for a particular function. An example is provided by elastase, where an influx of K^+ ions into the neutrophil granule activates the enzyme and potentiates its bactericidal effects (Reeves *et al.*, 2002). The fact that bromelain is also activated by an increase in ionic strength leads to speculation that this enzyme might have a role in the plant, similar to that of elastase in neutrophils.

The above discussion has largely focused on proteinases. However, the present study has shown that bakers' yeast α-glucosidase is also affected by ionic strength and appears to have an optimal ionic strength, which can be explained on the basis of its reaction mechanism. This result is consistent with an earlier report by Dennison and Marais (1980) that sheep rumen fluid cellulases also have an optimal ionic strength - which corresponds with that of sheep saliva.

5.1 Future studies

The present study has opened windows, but has left many questions unanswered. These include:-

- *Reassessment of enzyme pH-activity profiles in buffers of constant ionic strength.* It has been shown that the assessed pH profiles of cathepsins L and B were inaccurate, as ionic strength was not taken into account (Dehrmann *et al.*, 1995; 1996). The present study has shown that the enzymes tested here were also sensitive to ionic strength, though in different ways. This leaves open the question of the true pH optima of these enzymes and, perhaps, throws doubt on many (almost all?) published values of enzyme pH optima. Clearly a great deal of reassessment is needed.

- *Reassessment of enzyme stabilities as a function of ionic strength.* In previous studies (Dehrmann *et al.*, 1995; 1996) on cathepsins L and B, a close correlation between the effects of ionic strength on activity and its effects on stability of these enzymes was found. The degree to which this applies to other enzymes needs to be established.

- *What is the effect of ionic strength on enzymes of other classes*? In the present study, the enzymes explored were all hydrolases. In future research it will be necessary to establish the effects of ionic strength on other classes of enzymes.

- *The effect of ionic strength on enzyme/inhibitor interactions.* The present study has shown that the interaction of cathepsin L and cystatin C is sensitive to ionic strength. This is not a physiologically relevant interaction but it serves to illustrate that measured K_i values may be sensitive to ionic strength. From this it follows that physiologically relevant K_i values should be measured at physiological pH and ionic strength, if extrapolations are to be made to the *in vivo* situation. Since this has not been common practice, it may be necessary to reassess many measured K_i values and their physiological significance.

From the above a general statement can be made, that in all future measurements aimed at acquiring insight into the physiological situation, physiological pH **and physiological ionic strength and ionic composition** should be used.

Besides this, the present study has raised other questions that might be addressed by further research. These include:-

- *Is there a novel proteinase present in egg white?* Indications that this is the case were obtained in the present study but this will need to be verified and the possibility that it was an artifact, due to contamination, will have to be eliminated.

- *What is the 3D structure of bromelain?* This will require crystallisation of the enzyme which has not yet been reported to date. However, in preliminary studies, that initially formed part of the present study but which are not reported here, bromelain was successfully crystallised, but the crystals were lost *en route* to the X-ray facility in Cape Town. If possible, the crystallisation will have to be repeated and X-ray studies undertaken to establish the 3D structure.

Although it is not a question, as such, the present study has also opened a window to a novel approach to the isolation of cystatin C, and possibly other egg white proteins as well. This could be further explored and optimised in future studies.

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