A STUDY OF THE PROTEINASE, CATHEPSIN L, IN THE CONTEXT OF TUMOUR INVASION

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This thesis is dedicated to the memory of my mother,

Elizabeth Pike.

PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg, from January 1987 to December 1990, under the supervision of Dr. 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.

Robert Neil Pike
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ABSTRACT

The proteinase, cathepsin L, has been strongly implicated in the processes of tumour invasion and metastasis. A new purification method, three-phase partitioning, characterised in terms of the parameters which affected its fractionation of proteins, was found to simplify the purification of cathepsin L from sheep liver. This method, together with a novel cation-exchange step on S-Sepharose and molecular exclusion chromatography, enabled the enzyme to be purified to homogeneity, in a single-chain form.

A further enzyme fraction was isolated as a proteolytically active complex with the endogenous inhibitor of cysteine proteinases, cystatin. Studies on the proteolytically active complex revealed that approximately 60% of it was covalently bound and proteolytically active, while the other 40% was non-covalently bound and proteolytically inactive, in the manner normally found for the binding of cystatin to cysteine proteinases.

A cystatin fraction from sheep liver containing variants of cystatin β, was shown to be able to form complexes with free cathepsin L *in vitro* in a pH-dependent, rapid process, which was mildly stimulated by a reducing agent. Cathepsin L was also isolated from human spleen, but only as a proteolytically inactive complex, presumably also with cystatin(s). The complexed and free cathepsin L from sheep liver were analysed for their pH-dependent characteristics, and it was found that both forms of the enzyme were more active and stable at, or near, neutral pH, than would have been expected from published values.

Specific polyclonal antibodies to pure sheep cathepsin L were raised in rabbits and chickens. The chicken egg yolk antibodies were of a much higher titre and were immunoinhibitory towards the enzyme, which the rabbit antibodies were not. Anti-peptide antibodies, raised in rabbits against a peptide sequence selected from the active site of human cathepsin L, were highly specific for cathepsin L and immunoinhibitory towards the enzyme. Together with the polyclonal anti-cathepsin L antibodies, they show promise for immunoinhibitory and immunocytochemical studies on the enzyme, and as potential anti-tumour drugs.

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ABBREVIATIONS

ABTS, 2,2-Azino-di-[3-ethylbenzthiazoline sulphonate (6)]

AMT, acetate-MES-Tris buffer

BM, basement membrane

BSA, bovine serum albumin

CHO, chinese hamster ovary

CM, carboxymethyl

CD, circular dichroism

CAMOR, carrier agent-modified residues

Dip-F, di-isopropyl fluoride

DTT, dithiothreitol

DMSO, dimethyl sulphoxide

dist.H_2O, distilled water

ECM, extracellular matrix

EDTA, ethylene diamine tetra-acetic acid

EGF, epidermal growth factor

ELISA, enzyme linked immunosorbent assay

E-64, L-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)butane

FPLC, fast protein liquid chromatography

HIV, human immunodeficiency virus

HRPO, horse radish peroxidase

IgG, immunoglobulin G

IgY, immunoglobulin Y

IGSS, immunogold labelling with silver amplification

[I], inhibitor concentration

KLH, keyhole limpet haemocyanin

k_{obs}, observed equilibrium co-efficient

K_i, inhibition constant

K_{av}, availability constant

MEP, major excreted protein

MCP, mouse cysteine proteinase

M-6-P, mannose-6-phosphate

mRNA, messenger ribonucleic acid

MW, molecular weight

M_r, relative molecular weight

MEC, molecular exclusion chromatography

MES, 2(n-morpholino)ethanesulphonic acid

NHMec, 7-amino-4-methyl coumarin

PDGF, platelet-derived growth factor

PAGE, polyacrylamide gel electrophoresis

PEG, polyethylene glycol

PBS, phosphate-buffered saline

pI, isoelectric point

PMSF, phenyl methyl sulphonyl fluoride

p21, protein of M_r 21 000

Q-Sepharose, quaternary amine-Sepharose

RT, room temperature

®, registered trademark

R-value, correlation coefficient

SBTI, soya bean trypsin inhibitor

SDS, sodium dodecyl sulphate

SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

S-Sepharose, Sulphomethyl-Sepharose

TPP, three-phase partitioning

TEMED, N,N,N',N', tetramethyl ethylene diamine

TBS, tris-buffered saline

TEA, triethylamine

TPI, tissue proteinase inhibitor

$t_{1/2}$, half-life

Z, benzoylcarboxy

CHAPTER 1

INTRODUCTION

Cancer, and in particular malignant cancer, is one of the most prevalent and lethal of the diseases afflicting man and his animals today. Benign tumours with their inherent characteristic of localised growth only, are usually successfully dealt with surgically. Malignant tumours, however, with their ability to metastasize and invade into healthy tissues, thus spreading faster than surgical methods can cope with, can not be treated effectively yet.

Present therapy for tumours usually consists of chemotherapy and/or radiotherapy, targeted, in both instances, at the more rapidly dividing cells of the tumour population. There are extremely adverse side effects to these treatments, however, since the cell division necessary for the maintenance of normal bodily functions is affected simultaneously with the cancer cell growth. Since the processes of invasion and metastasis are fundamental to the condition of tumour malignancy, but not to normal body functions, blocking of these processes should prove to be more effective therapeutically, with minimal harmful side effects.

Malignant tumours originate from a cell whose genetic material is transformed so that it gains the characteristics of uncontrolled growth and division, and the ability to invade tissue. The primary tumour mass, originating from such a cell, induces a process known as angiogenesis, whereby it secretes factors which induce the host tissue to vascularize the tumour mass, allowing it access to blood-borne nutrients and factors which enable it to continue growth and development (Schirrmacher, 1985, Blood and Zetter, 1990).

The characteristically less adhesive, malignant, tumour cells are later shed from the primary tumour, and initiate the process of metastasis by invading the surrounding tissue, thereby gaining access to either the lymph or blood circulatory systems. Once inside one of these systems they are particularly vulnerable to the host's immune system and further progress of metastasis depends on their ability to evade or overcome the host's defenses. If they do so successfully, they will continue to circulate until they become lodged in the small blood or lymph vessels of a secondary organ. Upon lodging they invade out of the circulatory system and into the tissue of the secondary organ. Colonisation of this tissue then occurs and the cycle repeats itself (Goldfarb, 1982). Inhibition of any of the metastatic stages will halt the entire process, with concomitant therapeutic value. Due to the role they play in allowing the cell to move into and out of the circulatory system, the invasive processes in metastasis are vital to its success, and are thus of particular interest.

In order to invade host tissue, tumour cells have to be able to penetrate and thereby destroy the integrity of host tissue components. The host tissue surrounding the tumour mass could either be composed of the cells of the connective tissue, embedded in an extracellular matrix (ECM) of proteins such as collagen, proteoglycans, glycoproteins and elastin, or organ parenchymal cells, including epithelium, endothelium and mesothelium (Jones and De Clerck, 1982). The connective tissue, within its ECM, is founded on, and separated from parenchymal tissue, by the basement membrane (BM). Apart from the separative function of the BM, it provides a base for the orderly growth of cells and thus maintains tissue architecture. The BM is composed of the proteins collagen, laminin, heparin sulphate, chondroitin sulphate and entactin, and is a highly crosslinked structure which is impermeable to cells and even some macromolecules (Jones and De Clerck, 1982). These are therefore the barriers which invading tumour cells have to overcome.

Three possible mechanisms for invasion have been proposed (Fidler, 1978):

(i) Mechanical pressure created by the uncontrolled growth of the tumour could simply force cells through the tissue;

(ii) The cells shed from the tumour mass have motility and could thus move through the host tissue and around obstacles;

(iii) Tissue is degraded by enzymes released from the tumour, allowing motile cancer cells to penetrate the tissue.

Although it has been postulated (Blood and Zetter, 1990) that initial access to newly formed, and therefore more permeable, blood vessels, induced by angiogenesis, may be by means of the second method, a number of factors support the third method of invasion. Generally BM is impermeable to cells and therefore tumour cells will not be able to penetrate it without dissolution of the tissue. Dissolution of the BM has previously been noticed around invasive tumour cells in microscopic studies (Goldfarb, 1982). Furthermore, tumour cells *in vitro* can actively degrade isolated BM and ECM; purified tumour cell-derived proteases degrade BM, and protease inhibitors block penetration of the ECM by tumour cells *in vitro* (Liotta *et al.*, 1982). BM and ECM are mainly comprised of protein, and so proteolytic enzymes, as putative invasive agents, have been a particular focus of attention.

Tryggvason *et al.* (1987), point out that proteinases involved in tumour invasion could be secreted in excess by the tumour itself, or could be host derived enzymes, stimulated to excess secretion by tumour produced factors. Alternatively, the excess proteinase activity could be due to the absence of proteinase inhibitors normally present to control the activity of proteinases. Whichever of the three mechanisms is operative, it is still of interest to discover which, if any,

of the proteinases found in the normal body is responsible for the tumour invasion. It is to this question that the present study is addressed.

Several classes of proteinases have been implicated in the process of tumour invasion, including serine proteinases, such as plasminogen activator (Dano *et al.*, 1985), metalloproteinases, such as collagenase (Liotta *et al.*, 1982), aspartic proteinases, such as cathepsin D (Spyratos *et al.*, 1989) and cysteine proteinases, such as cathepsin B (Sloane and Honn, 1984). The general characteristics of the different classes of proteinases are summarised in Table 1. Most studies carried out to date have been on the serine and metalloproteinases but, although there is a lot of evidence for their involvement in tumour invasion, no general mechanism for tumour invasion has emerged from these studies. There is the possibility that another group of enzymes, such as the cysteine proteinases, may contribute to this process, and that studies on these enzymes may shed new light on a general mechanism for tumour invasion. There is also a strong possibility that all the classes of proteinase may be involved through a cascade mechanism, and/or that different types of tumours, due to the well known heterogeneity of tumours, may use different proteinases in tumour invasion.

Table 1: The general characteristics of the major classes of proteinases (adapted from Neurath, 1989; Barrett, 1980)

Class	Representative proteinases	Characteristic active site residues	pH range for activity	Typical inhibitors
Serine	**Chymotrypsin** Trypsin Plasminogen activator	Asp^{102}, Ser^{195}, His^{57}	7-9	DipF
Cysteine	**Papain** Cathepsins B, H, L	Cys^{25}, His^{159}, Asp^{15}	3-8	Iodo-acetate
Aspartic	**Penicillopepsin** Cathepsin D	Asp^{33}, Asp^{213}	2-7	Pepstatin
Metallo	**Thermolysin** Collagenases	Zn, Glu^{143}, His^{231}	7-9	EDTA 1,10-phenan-throline

[a] Residue numbering refers to that used in the representative enzyme in bold letters

The general characteristics of the various cysteine proteinases, and the differences between them, are summarised in Table 2. Cathepsin B was the first member of the cysteine proteinase group to be implicated in invasion by malignant breast tumours (Poole *et al.*, 1978). Various researchers later reported more incidences of its involvement, for instance in metastatic mouse melanoma cells (Sloane and Honn, 1984) and in the ascites fluid of patients with malignant tumours (Mort *et al.*, 1983). Cathepsin L, first isolated by Kirschke *et al.*, (1977), is another cysteine proteinase which has the potential to play a role in tumour invasion, due to its ability to degrade a wide range of protein substrates at much faster rates than cathepsin B. Cathepsin L is normally a lysosomal proteinase with a M_r of approx. 30 000 (Table 2).

Table 2: General characteristics of the different lysosomal cysteine proteinases (from Kirschke *et al.*, 1980)

Proteinase	M_r	pI
Cathepsin B	24-30 000	5-6
Cathepsin H	28 000	6-7
Cathepsin L	21-29 000	5,5-6
Cathepsin N	20-35 000	5,1-6,5
Cathepsin S	19-25 000	6,3-6,9

From the above information, it is obvious that in order to be an agent of tumour invasion, a proteinase must have the capability of degrading the proteins constituting the BM and ECM. Cathepsin L has the ability to degrade many of the proteins found in the ECM, including collagen (Kirchke *et al.*, 1982, Maciewicz, *et al.*, 1990), elastin (Mason *et al.*, 1986a) and proteoglycan aggregates (Nguyen *et al.*, 1990). Human kidney cathepsin L was found to be able to degrade the glomerular BM of the kidney at much higher rates than cathepsin B, and it was postulated that cathepsin L was the primary enzyme responsible for the recycling of this membrane in normal tissue (Baricos *et al.*, 1988). This evidence, in particular the latter, shows that, under the right conditions, cathepsin L has the ability to degrade the proteins relevant in tumour invasion. The degradation of these substrates by cathepsin L, however, has mostly been reported to occur optimally at pH 5-6, and not at all at the physiological pH of 7,2, since the human enzyme is postulated to be inactivated at neutral pH (Mason *et al.*, 1985). This problem may possibly be overcome by various mechanisms which will be discussed later.

Besides the above, indirect evidence, there is direct evidence of the involvement of cathepsin L with tumours. Gottesman (1978), first noted the excess secretion of a protein by transformed murine fibroblast cells in contrast to the normal cells. This protein was named the Major Excreted Protein (MEP). Later work (Gottesman and Sobel, 1980) showed that excess MEP production was effected at the pre–translational level by both a transforming virus and a tumour promoter. Subsequently MEP was purified and found to contain mannose–6–phosphate [M-6-P] (Sahagian and Gottesman, 1982).

Lysosomal enzymes are synthesized in precursor form in the rough endoplasmic reticulum and are trafficked via the Golgi apparatus to the lysosomes. This trafficking is mediated by a mannose-6-phosphate marker, which, when added in the cis-Golgi, allows recognition by receptors in the trans-Golgi, and in turn directs the enzymes to the lysosome (Stein *et al.*, 1987). The M-6-P marker therefore identifies MEP as a prospective lysosomal enzyme.

Possession of the M-6-P marker also allows internalisation of lysosomal enzymes when they are secreted (secretion and internalisation is a lesser-used alternative to the intracellular processing route in normal cells), but in the study of Sahagian and Gottesman (1982) it was found that, even though it contained this marker, MEP was not as efficiently internalized after secretion as was another lysosomal enzyme, β-galactosidase. As a result, most of the MEP remained in the extracellular environment. Gal *et al.* (1985) used an antiserum to MEP and pulse chase experiments to show that most of the protein existed in a high molecular weight (M_r 39 000) form and was secreted in this form by transformed cells, in contrast to normal cells where most of the higher MW form was processed to M_r 29 000 and 20 000 forms, which were retained in the cell to a large extent. The possible reasons for the excess secretion of MEP are discussed later.

It was later found that MEP was an acid activable proteinase with activity against the ECM components, fibronectin, collagen and laminin (Gal and Gottesman, 1986a), and that it cleaved protein bonds with a specificity similar to that of cathepsin L (Gal and Gottesman, 1986b). Several workers (Mason *et al.*, 1986b; Troen *et al.*, 1987; Denhardt *et al.*, 1986) then showed that the sequence of cathepsin L was very similar to MEP (greater than 80% sequence homology). Portnoy *et al.* (1986) independently found that inflamed murine macrophages secreted very high levels of a protein they named mouse cysteine proteinase (MCP). They also found, by its high sequence homology and catalytic similarities, that this protein was, in fact, the mouse macrophage version of MEP. Mason *et al.* (1987) proved conclusively that MEP is sequentially and catalytically similar to cathepsin L. MEP, in contrast with cathepsin L, is alkaline stable, and the activity of its higher MW form is confined to a narrow range between pH 5-6, while the normal enzyme has a broad range of activity from pH 4-6,5. Within its

optimal range it does, however, catalytically closely resemble cathepsin L. When exposed to pH 3 (originally thought to be its optimal pH for activity), MEP autocatalytically converts from its M_r 39 000 form to the M_r 29 000 form which is similar in every way to cathepsin L. In a similar discovery, Recklies and Mort (1985) reported the secretion of a high molecular weight version of cathepsin L (M_r 35 000) from mouse mammary gland in culture, with similar catalytic properties to MEP.

To summarise the above evidence thus far, it would appear that in normal cells, MEP is the precursor of cathepsin L. The presence of an N-terminal extension peptide serves to stabilise the enzyme in the higher pH environment of the Golgi apparatus, where it would be unstable in its mature form. This molecule is then processed to its mature M_r 29 000 form *en route* to its lysosomal destination (Nishimura *et al.*, 1988a).

Recent studies have shed some light on the mechanism for the specific secretion of MEP in large amounts by transformed cells, in contrast to other lysosomal enzymes which are processed normally in the transformed cells. These studies initially revealed that secretion of the enzyme could be due to both its increased synthesis in the transformed cells (25-fold increase), and the fact that it had a 10-fold lower affinity for the M-6-P receptor (Dong *et al.*, 1989).

The basis for the low affinity of MEP for the M-6-P receptor has been the subject of two recent, independent studies, which provided conflicting results. Dong and Sahagian (1990) found that MEP from the NIH 3T3 cell line contained one oligosaccharide, phosphorylated in two positions by phosphomonoester moieties. As they had previously found, the MEP molecule had a low affinity for M-6-P receptor in comparison to other lysosomal hydrolases (Dong *et al.*, 1989). The oligosaccharides isolated from MEP, and from lysosomal hydrolases which were normal in their (high) affinity for the receptor, bound to the receptor with the same affinity as each other and the MEP molecule. This showed that the MEP oligosaccharides, as <u>individual</u> units, were normal, and that the MEP protein structure had no effect on the binding of the oligosaccharides to the receptor. MEP from the Chinese Hamster Ovary (CHO) cell line had a normal, high affinity for the M-6-P receptor, in contrast to MEP from NIH 3T3 cells. This higher affinity was attributed to the presence of <u>two</u> phosphorylated oligosaccharides on the CHO protein in contrast to the one found on NIH 3T3 MEP. Removal of one oligosaccharide from the CHO MEP converted it to a similar low affinity ligand to NIH 3T3 MEP. The low affinity of the NIH 3T3 MEP molecule for the receptor was therefore attributed to the lack of a second phosphorylated oligosaccharide.

Lazzarino and Gabel (1990), agree with Dong and Sahagian (1990) on the actual stucture of the oligosaccharide, and the fact that these oligosaccharides show a similar affinity for M-6-P receptor compared to oligosaccharides from normal lysosomal hydrolases. A crucial difference in their experimental findings, however, was that the oligosaccharides from MEP were not similar in their binding to the receptor compared to MEP, which they found had much lower affinity, whereas Dong and Sahagian (1990) found that the oligosaccharides and MEP were very similar in their binding affinity for the receptor. MEP bound to the receptor heterogenously, in that there were three positions of elution from a M-6-P column: most of the MEP did not bind at all, with some intermediate species and a high affinity fraction. These results are also in contrast to those of Dong and Sahagian (1990), where MEP eluted as one homogenous species from a M-6-P column. Lazzarino and Gabel (1990) therefore decided that protein determinants on the MEP molecule were causing the difference between the binding of the individual oligosaccharides and the binding of the whole protein, possibly by binding to the oligosaccharides in some way. They confirmed this hypothesis by examining the behaviour of tryptic glycopeptides on a receptor column. They found that the glycopeptides and the individual oligosaccharide units had a similar affinity for the receptor, thus confirming, in their view, the theory that it was protein determinants on the MEP molecule which were interfering with the interaction of the otherwise normal oligosaccharide units with the receptor.

Obviously there are fundamental differences in the findings of the two groups in this regard, which need to be rationalised in order to find a common mechanism to explain the above phenomena. The crucial difference in the two sets of results obtained, is the similarity, or otherwise, of the MEP molecule and its individual oligosaccharides with respect to their binding to the receptor. In this regard the researchers used different methods, with Dong and Sahagian (1990) employing M-6-P gradient elution of ligands from a receptor column, while Lazzarino and Gabel (1990) used a step gradient of M-6-P to elute ligands from a similar column. In the author's view the approach of Dong and Sahagian (1990), in this vital experiment, was possibly the more valid of the two, and may have given a more reliable indication of the elution position of the various ligands, than could be achieved using a simple step elution protocol. The two groups used different cell lines, Lazzarino and Gabel (1990) using the J-774 mouse macrophage cell line in comparison to the NIH3T3 and CHO cell lines used by Dong and Sahagian (1990). The use of these different cell lines could also have given rise to several of the differences between the two groups, and could explain their different findings. Clarification of the conflicts in the findings of the two investigations is needed before the mechanism of MEP's lowered interaction with its receptor can be adequately explained.

Mouse NIH-3T3 or Balb/c-3T3 cells treated with Platelet Derived Growth Factor (PDGF) (Frick *et al.*, 1985; Scher *et al.*, 1983), were also found to increase both the synthesis and secretion of MEP. Later work on this phenomenon has now shown even more about the mechanism for MEP secretion. Treatment with PDGF between 2 and 14h caused both elevated synthesis and secretion of MEP, but secretion returned to low levels after 18h, despite the fact that synthesis continued at an elevated level (Prence *et al.*, 1990). This suggested that another factor was also involved in the process, since the MEP was still being synthesized at high levels, but was not being secreted as would be expected in the theory of Dong *et al.* (1989), whereby increased synthesis would cause the MEP to be secreted due to its low affinity for the receptor. Prence *et al.* (1990) postulated, due to the finding that M-6-P receptor-mediated-endocytosis increased in PDGF treated cells, that the distribution of the receptors may also be changed, depleting the amount of receptor in the Golgi apparatus in favour of the endocytic vesicles nearer the cell membrane, thus giving an additional mechanism for the increased secretion of MEP.

Enhanced MEP secretion could therefore be due to an interaction of the three mechanisms of increased synthesis, low affinity for M-6-P receptors and redistribution of these receptors. The reduction in secretion after 18 h could be due to a removal of the effects of PDGF on the secretion process, possibly by cycling the receptors back to the golgi apparatus. Further work is, however, needed to finally elucidate the reasons for the aberrant behaviour of this molecule in transformed cells, which in turn could reveal much about the role of cathepsin L in the transformed phenotype.

Further evidence for the involvement of MEP/cathepsin L in tumour invasion and metastasis is provided by its relationship to the *ras* oncogene. The *ras* oncogene is recognised as one of the major causative agents of cancer (Barbacid, 1987). MEP was identified as the major *ras* induced protein in Kirsten virus transformed murine fibroblasts (Joseph *et al.*, 1987). Denhardt *et al.* (1987), found that MEP expression, by *ras* oncogene transformed mouse cell lines, correlated with their metastatic ability. They also found that increased *ras* expression correlated with increased expression of MEP mRNA, suggesting that the MEP gene is under the control of the *ras* gene. Taniguchi *et al.* (1990) found that the increased invasive and metastatic capability of a rat fibroblast cell, brought about by the transfer of *v-fos* into the cell line, correlated with increased levels of secretion of pro-cathepsin L, showing that MEP/pro-cathepsin L could correlate with the presence of other oncogenes, and its secretion could thus be some common mechanism for tumour invasion.

Some seemingly contradictory evidence was supplied by Hiwasa *et al.* (1987), when they claimed that the direct product of the *ras* oncogene, the so–called p21 protein (M_r 21 000), had an inhibitory effect on cathepsins B and L, the effect on cathepsin L being much greater. In a later paper it was shown that p21 caused an inhibition of cathepsin L-induced degradation of the epidermal growth factor (EGF) receptor (Hiwasa *et al.*, 1988). EGF is a growth factor which causes cells to divide and grow, and mediation of this replication is controlled by catabolism of the EGF receptor, following its internalisation by endocytosis, so that the cell does not divide endlessly. The authors note that there is evidence for the association of p21 molecules with various receptor molecules, and there is, therefore, a possibility that it could be co-internalised with the EGF receptor. They also note that EGF is preferentially cleaved by cathepsin L in relation to other molecules in a cell homogenate, making it likely that the proteinase, effecting the *in vivo* catabolism of the receptor, is cathepsin L. Inhibition of this process by p21 following its co-internalisation with EGF, might cause the cell to continue to divide uncontrollably, as in tumours. Under *ras* oncogene control, a cell could therefore gain the characteristics of uncontrolled growth and division, combined with the ability to carry out proteolytically mediated invasion: the very characteristics of the malignant tumour cell. It should be noted, however, that the extrapolation of the above results, most of which were obtained under *in vitro* conditions, is highly speculative and their confirmation would require similar results from *in vivo* experiments.

More recently, work has been carried out on the involvement and location of cathepsin L in a variety of tumour cells. Studies on the expression of mRNA for five proteinases, cathepsins B, D, H, L and S in murine melanomas of varying metastatic potential relative to normal tissues, revealed that only levels of cathepsin B mRNA correlated with increased levels of metastasis of the melanomas (Qian *et al.*, 1989). In contrast to this result, both cathepsins B and L were detected in pre-malignant and malignant human colorectal tumour cell lines by immunological probes (Maciewicz *et al.*, 1989). Precursors of both were secreted chiefly in the pre-malignant cell lines, but mature forms of the enzymes were only evident in the malignant cell lines, suggesting that the invasiveness of the malignant cell lines was due more to their ability to produce mature forms of the enzymes than their ability to produce large amounts of precursors. Cathepsin B was found to be mainly lysosomal in its distribution within the cells while cathepsin L was found to be membrane associated. Both enzymes were found to be capable of solubilising isolated BM. Cathepsin L was also found to be membrane associated in murine and human melanomas, and the relative specific activity of cathepsin L was found to be 7-fold higher in cell lines of high metastatic potential, indicating a correlation with the metastatic potential of the cells (Rozhin *et al.*, 1989).

Yagel *et al.* (1989) showed that inhibitors selective for cathepsin L inhibited the invasion of human amnion membranes *in vitro* by murine melanoma and mammary carcinoma cells. Metalloproteinase inhibitors were found to be more inhibitory, however, and the authors therefore postulated that there was some interaction, possibly an activation of the metalloproteinase by cathepsin L, to bring about the full invasiveness of the cells. Cathepsin L has also been isolated from a metastatic human pancreatic carcinoma cell line in the form of a complex between pro-enzyme and mature enzyme, with increased neutral pH and temperature stability (Yamaguchi *et al.*, 1990).

Although cathepsin L was mainly studied here in terms of its involvement in tumour invasion, it must be mentioned that the enzyme has been implicated in other disease states. Cathepsin L was shown to cleave, and thus inactivate, α1-proteinase inhibitor (Johnson *et al.*, 1986). This proteinase inhibitor has several important functions in the body, amongst these being the control of the proteinase, elastase. Without this control, elastase can damage the structural protein, elastin, thus causing pathological conditions such as emphysema. Cathepsin L has also been shown to be involved in arthritis (Etherington *et al.*, 1988) and muscular dystrophy (Kominami *et al.*, 1987).

From the aforegoing evidence it may be seen that cathepsin L has the ability, in terms of its activity against protein substrates, to be a mediator of tumour invasion and for this reason, together with observations on its presence in tumour tissue, it has been implicated in various ways in tumour invasion. The biggest problem with this hypothesis, however, is that the common forms of the enzyme, isolated to date, appear to have very little, if any, activity at the neutral pH of the extracellular environment.

The pH of the extracellular space around tumours has been shown to fall to about pH 6,8 (Gullino, *et al.*, 1965), but this is still not low enough for the activity of MEP, and the forms of cathepsin L isolated to date are both unstable and barely active at this pH. One solution to this problem has been proposed by Silver *et al.* (1988), from their work using microelectrodes to study the microenvironment beneath adherent macrophages and osteoclasts. Osteoclasts are involved in the destruction of bone, and macrophages in the destruction of collagen, both while they are in contact with their substrates. Microelectrode studies of the pH in these attachment zones, revealed that the cells decreased the pH to values of about 3 in these zones. The fall in pH has been proposed to be due to the sealing off of these zones of attachment by the cells and the use of proton pumps to acidify them. There is, therefore, the possibility that tumour cells may work through a similar mechanism which would allow the use of cysteine and/or aspartic proteinases in tumour invasion. This mechanism has not yet been demonstrated to occur *in vivo*, however.

Another way in which cathepsin L could be active in tumour invasion, is by virtue of a changed pH optimum, either through some as yet undefined mechanism, or through attachment to a membrane, as has been demonstrated by Rozhin *et al.* (1989) and Maciewicz *et al.* (1989) for cathepsin L in tumour cells. Although changes in the catalytic behaviour of cathepsin L induced by membrane attachment have yet to be demonstrated, the binding of the cysteine proteinase, cathepsin M, to the lysosomal membrane was found to change its pH optimum quite markedly (Pontremoli *et al.*, 1982), and this may also be true for cathepsin L.

Many questions still remain unanswered about the involvement of cathepsin L in tumours and tumour invasion. Most of the work carried out in this direction has been on transformed cells in culture, with only a few of the studies revealing any information about the behaviour of the cells *in vivo*, in terms of their metastatic potential. Studies *in vitro*, however, have definite limitations in their relevance to the more complex situation of tumours *in vivo*.

The ultimate aim of the study reported here was to elucidate the role of cathepsin L in cancer *in vivo*, and in particular in tumour invasion. To this end a strategy was devised, to isolate cathepsin L from a suitable source and to raise antibodies to the purified product, for use in immunocytochemical studies. In this way it should be possible to locate the enzyme in actual tumour biopsy material, and thus possibly determine the function of the enzyme in the tumours. A further aim was to produce inhibitory antibodies to cathepsin L which may be useful research tools in the study of the role of cathepsin L in cancer and which could possibly also have therapeutic applications.

In order to study the role of cathepsin L in human tumour invasion, antibodies which could target the human enzyme were necessary. The development of methods for the purification of the enzyme from a human source, which must obviously precede the production of polyclonal antibodies, are, however, fraught with difficulties. Human tissue, suitable for the isolation of enzymes, is very difficult to obtain due to ethical constraints, and when it is obtained, this is usually fairly long after the time of death. Similarly, a regular supply, especially necessary in the development stages of a new purification procedure, would be difficult to ensure. Animal tissue is an obvious alternative in this respect, particularly those from the larger domestic animals, since these animals are commercially slaughtered on a regular basis, and each animal will provide a large amount of tissue. This makes them preferable (on ethical grounds) to experimental animals, such as mice and rats, which would have to be sacrificed in large numbers to provide the same amount of tissue, solely for the envisaged experiment. Sheep livers were chosen for this study due to the observation of Mason (1986), that this organ provided the largest yield of cathepsin L (relative to mass) compared to rabbit, rat, bovine and human livers. In addition to this, Mason (1986) found that antibodies raised to sheep cathepsin L cross-react

with human cathepsin L, making the sheep liver a suitable source for the isolation of cathepsin L to be used as an immunogen for raising antibodies which would recognise human cathepsin L.

In order to achieve the first aim of the study, i.e. to purify cathepsin L, a new method was devised for its isolation, employing a novel method called three-phase partitioning (TPP). Preliminary studies had suggested that TPP might be usefully applied to the isolation of cathepsins and so a systematic study of the method was undertaken (Chapter 3). This systematic study provided a sound basis for the subsequent application of the method in the isolation of cathepsin L and it is consequently also reported here. In addition to the TPP step, novel cation exchange chromatography steps were also used in this purification procedure (Chapter 4).

Tests on the antibodies produced against cathepsin L, isolated by this procedure, revealed the presence of a contaminant which later proved to be the natural proteinase inhibitor, cystatin, complexed to a proportion of the isolated cathepsin L. Characterisation of the complexed enzyme revealed that it was surprisingly active against both the sensitive fluorogenic peptide substrate and a less sensitive protein substrate, implying that cystatin was not acting as a normal inhibitor in this context. The complex was characterised on SDS-PAGE and it was found that the cystatin was covalently bound to a proportion of the cathepsin L in the complex fraction, showing that the inhibitor was not binding in a normal manner to the enzyme, a result which was confirmed by active site-titration of the complex. The formation of the proteolytically active, covalent complex could be mimicked *in vitro*, by incubation of free cathepsin L with isolated cystatin fractions, in a pH-dependent manner. In consequence, the isolation procedure was revised to separate the free enzyme from complexed cathepsin L, and so allow the production of specific polyclonal antibodies for use in future immunocytochemical studies on tumours, beyond the scope of this thesis.

The complexed and free forms of cathepsin L were characterised in terms of their pH-dependent characteristics, using established methods, and a new method developed in this study, and both the free and complexed enzyme were found to be more active and stable in the neutral region than was predicted from published values. The complexed enzyme was slightly more stable in the neutral region than the free enzyme. These results suggest that cathepsin L has properties consistent with a possible role in tumour invasion.

Polyclonal antibodies produced to cathepsin L in both chickens and rabbits were characterised, whereupon it was found that chicken antibodies had a decisive advantage over rabbit antibodies, in that they were inhibitory in contrast to the rabbit antibodies (Chapter 5). In addition to these polyclonal antibodies, anti-peptide antibodies against a peptide sequence in the active site region of human cathepsin L were made. These antibodies proved effective in targeting cathepsin L specifically in western blots and ELISA techniques, as well as being immunoinhibitory towards the enzyme. This peptide antibody may be useful as a therapeutic agent in pathologies occasioned by excessive extracellular cathepsin L activity (Dennison and Pike, 1990a and b).

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

The techniques involved in this study were mainly associated with column chromatography, the analysis of fractions derived therefrom, and immunological techniques used to analyse the antibodies, either anti-peptide or polyclonal, produced against cathepsin L. The techniques described in this chapter could be described as basic to the study, i.e. they are well characterised techniques which were vital to the studies undertaken, but were not easily fitted into the pattern of later chapters. Some techniques, on the other hand, chromatography being an example, are more easily described in the context of their specific applications and are thus included in the relevant chapters. Most of the techniques will be described here in general terms, with specific details of the conditions pertaining to individual experiments being described in the relevant sections of the text.

2.2 Materials

For convenience, the source of specialised products used in this study will be described here. Most of the common chemicals used in the study were from BDH or Merck, and were of the highest purity available. Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were from Cambridge Research Chemicals, UK. Z-Phe-Phe-CHN_2 was from Enzyme System Products, Livermore, Ca., USA. Z-Phe-Tyr-(O-t-Bu)-CHN_2 was a gift from Dr E Shaw of the Friederich Meischer Institute, Basel, Switzerland. Pepstatin, cysteine.HCl, azocasein, DTT, 7-amino-4-methyl coumarin, all standard proteins, papain (2 x crystallised), Sepharose-4B, S-Sepharose, Q-Sepharose, Sephadex G-75, E-64, leupeptin SBTI and keyhole limpet haemocyanin (KLH) were from Sigma Chemical Co.. ABTS and PMSF were from Boehringer Mannheim, SA. Horse radish peroxidase (HRPO) was from Seravac. Human Cathepsin B was a gift from Dr D Buttle of the Strangeways Research Laboratories, Cambridge, UK, and human kidney cathepsin L was from Novabiochem, UK. Serva blue G dye was from Serva. The ELISA plates used in this study were Nunc-Immuno Maxisorp F96 plates supplied by the Weil Organisation, R. S. A..

2.3 Protein Assay

The Bradford dye-binding assay (Bradford, 1976) was introduced as a rapid, but sensitive protein determination technique, which is free of interference from most substances except detergents and phenolics. The method involves the binding of protein to the Coomassie Blue

G-250 dye molecule, converting it from an orange to a blue colour, stable after 2 min and up to 1h. The Bradford assay has been widely adopted, but there have been criticisms that its response to various proteins differs widely, making the extrapolation of results from standard proteins to sample proteins inaccurate (Pierce and Suelter, 1977; Van Kley and Hale, 1977). The assay was therefore modified by Read and Northcote (1981) to minimise the variation and increase the sensitivity of the assay. The major modifications were a change to Serva blue G dye and changes in the concentration of the dye and acid/alcohol components of the dye reagent.

The assay used in this study followed the original Bradford method, but using the Serva blue G dye. This was found to effectively eliminate the variations in response between different standard proteins. Modifications to the dye concentration and acid/alcohol ratios according to Read and Northcote (1981) were, however, not made since these seemed to promote precipitation of the dye, resulting in a short reagent shelf life. In contrast, the dye reagent described below could be stored for months, and the assay gave similar linear responses to proteins such as ovalbumin, lysozyme, BSA and γ-globulin; proteins which had been found to vary considerably in their response to the Coomassie blue G-250 reagent. Ovalbumin was generally used as the standard protein in this assay since it had a response to the dye reagent comparable to the average of the other three proteins.

The only drawback to the method was found to be the decreased range of protein concentration over which it could be used, in comparison to the Read and Northcote modified method. This was presumably due to the lower amounts of dye used, but the assay was still useful for the amounts of protein measured in this study. A micro-protein assay was also utilised, using the dye reagent in the quantities suggested by Read and Northcote (1981). This method proved very useful in the direct measurement of more dilute fractions from column chromatographic procedures.

2.3.1 Reagents

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 dist.H_2O and stirred for 30 min on a magnetic stirrer. The resulting solution was filtered through Whatman No. 1 filter paper and stored in a brown bottle. The solution could be stored for up to 6 months, although visual checks for precipitation were made before use. If precipitation was visible, the reagent was filtered and re-calibrated before use.

Standard protein solution. A 1 mg/ml ovalbumin solution was made up in dist.H_2O. This was diluted to 0,1 mg/ml for the micro-assay.

2.3.2 Procedure

Macro-assay. Standard ovalbumin solution (0-25 µl), or sample protein, was diluted to a final volume of 100 µl with dist.H_2O to give the desired protein concentration (0-25 µg). Dye reagent (5 ml) was added and the mixture was vortexed. The colour was allowed to develop for 2 min and the A_{595} of the solution was read in 3 ml plastic cuvettes, against a blank of dist.H_2O for the standard proteins or buffer for sample proteins. Assays were usually carried out in triplicate at 5 concentrations of ovalbumin.

Micro-assay. Protein standard (0-50 µl of the 0,1 mg/ml solution, i.e. 1-5 µg) or sample was diluted to 50 µl with dist.H_2O, or buffer, respectively, in 1,5 ml eppendorf tubes. Dye reagent (950 µl) was added, and the mixture was mixed by inversion of the tube. The colour was allowed to develop for 2 min after mixing, and the A_{595} was read, in 1 ml plastic micro-cuvettes, as above. Assays were usually carried out in triplicate at 5 concentrations of ovalbumin.

Results for the above assays were calculated from equations generated by linear regression analysis of the standard curves, for each assay type, developed for each batch of dye reagent made up.

2.4 Enzyme Assays

Cathepsin L is a strongly proteolytic cysteine endopeptidase, i.e. it digests many proteins at a much higher rate than related proteinases, such as cathepsin B and H, and is activated by thiol compounds (Barrett and Kirschke, 1981). The azocasein assay method has proved very useful in studies on cathepsin L, as the enzyme's high activity against this substrate can distinguish it from related proteinases, especially upon addition of 3M urea, which enhances the activity of cathepsin L, but reduces that of cathepsins B and H, and pepstatin, which inhibits cathepsin D (Wiederanders *et al.*, 1986). No specific synthetic substrate has yet been found for cathepsin L, but it does have high activity against the synthetic substrate, Z-Phe-Arg-NHMec. Both substrates were used in this study, the azocasein assay on a routine basis, and the synthetic substrate for more specialised uses.

2.4.1 Azocasein assay

Azocasein has been used as a substrate for lysosomal proteinases since Langner *et al.* (1973) first characterised it for this purpose. Azocasein is derived from casein by coupling diazotized sulphanilic acid or sulphanilimide to its histidine or tyrosine side chains. Cleavage of the substrate releases smaller peptides, with their attached yellow coloured azo groups, which are soluble in trichloroacetic acid (TCA), in contrast to the larger protein remnants. Removal of TCA-precipitated protein from the digests therefore leaves a yellow coloured supernatant, the intensity of which can be measured at A_{366}. The A_{366} value is thus a measure of the quantity of small peptides released by proteolysis, thereby quantifying the amount of proteinase present. Curiously, the absorption maximum of azocasein-derived TCA-soluble peptides is not at 366 nm. This was the wavelength setting used by Langner *et al.* (1973), due to the limitations of their spectrophotometer. Nevertheless, 366 nm has become universally used for azocasein assays. The method followed here is almost exactly as described by Barrett and Kirschke (1981).

2.4.1.1 Reagents

Assay buffer [100mM Na-acetate, 1mM Na_2EDTA, 0,02% (w/v) NaN_3 and 1 µg/ml pepstatin]. Glacial acetic acid (2,9 ml), $Na_2EDTA.2H_2O$ (0,19 g) and NaN_3 (0,1 g) were dissolved in 450 ml of dist.H_2O. The pH was titrated to 5,0 with NaOH, pepstatin (500 µg) was added and the volume was made up to 500 ml with dist.H_2O. Shortly before the assay, cysteine.HCl (0,04 g) was added to 5 ml of the buffer.

6% (w/v) Azocasein. Azocasein (3 g) was weighed into a glass beaker and dissolved with gentle magnetic stirring at room temperature for about 1 h.

Azocasein/3M urea solution. The 6% azocasein solution (50 ml) was mixed with urea (54 g), and the urea was dissolved by stirring on a magnetic heater stirrer at about 30°C. The volume was made up to 150 ml with the assay buffer (not containing cysteine).

5% (w/v) TCA. TCA (25 g) was dissolved in 500 ml of dist.H_2O.

2.4.1.2 Procedure

Sample (0,2 ml) was mixed with assay buffer containing cysteine (0,2 ml). After activation, for 5 min at 37°C, azocasein/urea solution (0,4 ml) was added. A sample (0,2 ml) of the mixture was immediately withdrawn and mixed with 5% TCA (1 ml) in an eppendorf centrifuge tube. This served as the blank for the reaction. After a specified period of time (usually 30 min), a further sample (0,2 ml) was withdrawn and mixed with TCA (1 ml). The samples in the 1,5 ml centrifuge tubes were centrifuged in a Sigma mini-centrifuge (12 000 x g, 5 min, RT). The A_{366} values of the supernatants were read using glass micro-cuvettes with blacked-out sides, which prevented transmission of light around the sample. Transmission of light around the sample was found to cause a non-linear response in the assay.

Standard curves, to calculate the units of activity, were prepared by incubating the azocasein solution for several days with a proteinase (in early studies, trypsin was used, but later cathepsin L was used to enhance the standardisation) to allow complete digestion of the substrate. The resulting mixture was precipitated in 5% (w/v) TCA in the same proportions as the procedure above, and was treated as a 100% hydrolysate, i.e. the A_{366} of this supernatant was the value obtained when all the azocasein was proteolytically cleaved at all possible points.

Dilutions of this mixture in 5% (w/v) TCA gave further points on the curve of % hydrolysis of azocasein versus A_{366}. Linear regression analysis of this curve gave an equation which was used to calculate % hydrolysis from any A_{366}:

$$\% \text{ Hydrolysis } (\% H) = (A_{366} - 0{,}0055) / 0{,}0203$$

The units of activity have been defined by Schwartz and Barrett (1980) as 1 unit = 1 µg of azocasein digested in 1 min, and therefore to calculate the number of units given by a %H-value, the following equation was used:

$$\text{Units} = (\% H / 100 \times 2000 \text{ µg azocasein}) / \text{time.}$$

Hence

$$\text{Units/ ml} = 5 \times \text{units.}$$

2.4.2 Synthetic substrate assay

Z-Phe-Arg-NHMec, a substrate which releases its intensely fluorescent 7-amino-4-methyl coumarin group upon cleavage, is the most commonly used synthetic substrate for the assay of cathepsin L (Barrett and Kirschke, 1981). This substrate is also cleaved by other enzymes such as tissue kallikrein, and by cathepsin B, and therefore cannot be classed as a specific substrate for cathepsin L. Cathepsin B activity can be accounted for, however, by using the substrate, Z-Arg-Arg-NHMec, which is specifically cleaved by this enzyme, and any activity, suspected to be due to cathepsin L, can be confirmed by the use of suitable inhibitors. The methods followed here were as described by Barrett and Kirschke (1981); the procedure for the assay of cathepsin B with Z-Arg-Arg-NHMec, is outlined where it is different from the Z-Phe-Arg-NHMec procedure.

2.4.2.1 Reagents

Z-Phe-Arg-NHMec assay buffer [340mM Na-acetate, 60mM acetic acid, 4mM Na_2EDTA, 0,02% (w/v) NaN_3, 8mM DTT, pH 5,5]. Na-acetate.$3H_2O$ (23,13 g), glacial acetic acid (1,72 ml), $Na_2EDTA.2H_2O$ (0,75 g) and NaN_3 (0,1 g) were dissolved in 450 ml of dist.H_2O, and the pH was titrated to 5,5 with NaOH. The volume was made up to 500 ml and DTT (0,0062 g) was added to 5 ml of the buffer just before the assay.

Z-Arg-Arg-NHMec assay buffer [352mM KH_2PO_4, 48mM Na_2HPO_4, 4mM Na_2EDTA, 0,02% (w/v) NaN_3, 2,7mM Cysteine.HCl, pH 6,0]. KH_2PO_4 (23,95 g), $Na_2HPO_4.2H_2O$ (4,27 g), $Na_2EDTA.2H_2O$ (0,75 g) and NaN_3 (0,1 g) were dissolved in 450 ml of dist.H_2O, titrated to pH 6,0 with NaOH and made up to 500 ml. Prior to the assay cysteine.HCl (0,0024 g) was added to 5 ml of buffer.

1mM substrate stock solutions. Z-Phe-Arg-NHMec (1 mg) and Z-Arg-Arg- NHMec (1,1 mg) were dissolved in separate volumes of DMSO (1,5 ml) and stored at 4°C.

20µM substrate solutions. Substrate stock solution (0,1 ml) was diluted to 5 ml with dist.H_2O.

Diluent. 0,1% (w/v) Brij 35 was used to dilute enzymes to the correct concentration for the assay.

Stopping reagent (100mM monochloroacetate, 30mM Na-acetate, 70mM acetic acid, pH 4,3). Monochloroacetate (9,45 g), Na-acetate.$3H_2O$ (4,08 g) and glacial acetic acid (4 ml) were dissolved in 950 ml of dist.H_2O, titrated to pH 4,3 with NaOH and made up to 1 litre. Great care was taken with the highly corrosive monochloroacetate.

1mM aminomethyl coumarin standard. 7-amino-4-methyl coumarin (1,8 mg) was dissolved in DMSO (10 ml). The standard was used as a 0,5µM solution by diluting stock solution (5 µl) in a 1:1 mixture of the assay buffer and stopping reagent (10 ml).

2.4.2.2 Procedure

Usually 1-5 ng of cathepsin L was used and 25-250 ng of cathepsin B. The appropriate volume of enzyme solution was diluted to 500µl with diluent, followed by the addition of assay buffer (250 µl). After a 1 min activation period at 30°C, substrate (250 µl) was added and incubated for 10 min at 30°C. Stopping reagent (1 ml) was added and the solution was briefly vortexed. A blank was prepared by adding stopping reagent before the enzyme. Fluorescence was read on a Hitachi F-2000 spectrofluorimeter with excitation at 370 nm and emission at 460 nm. Continuous assays could also be carried out in much the same way (i.e. by eliminating the addition of the stopping reagent) to assess the linearity of the reactions, but were hampered, initially, by the lack of a temperature controlled cell on the instrument.

According to Barrett and Kirschke (1981), the reading given by the 0,5µM standard can be taken to be equal to 1000 arbitrary enzyme units, which in turn are equal to 0,1 mU of actual enzyme activity. The reading given by a 0,25µM standard was 5000 and thus milliunits of activity in a sample could be calculated as follows:

$$\text{mUnits activity} = \text{Fluorescent intensity}/5000 \times 0{,}05 \text{ mU}$$

According to the volume of the sample used, the total activity or activity per ml was calculated.

2.5 Concentration of Samples

Samples from column chromatography were often too dilute to be directly visualised on SDS-PAGE, or to be used for innoculations into experimental animals. Ultrafiltration, a commonly used technique for the concentration of proteins, was not used as it has been reported that rabbit cathepsin L is denatured by its use (Mason *et al.*, 1984). Simple techniques were therefore used for the concentration of large amounts of sample, such as the dialysis of fractions against commercial sucrose or polyethylene glycol (PEG) (M_r 20 000). Dialysis against PEG was

much more expensive and was only used when it was judged to be undesirable to have sucrose in the samples; the advantage of dialysing against the high MW PEG being that it didn't move into the dialysis bag. The principle involved in this kind of concentration, is the movement of water out of the samples due to the concentration gradient set up between the sample and sugar or PEG on the outside of the dialysis bag. This technique proved to be simple and effective, but was difficult to apply to large numbers of samples, e.g. in the analysis of a chromatography run by SDS-PAGE. In these instances a quick, small-scale method, whereby protein-SDS complexes are precipitated by KCl, was used. In conjunction with the dialysis concentration techniques a 50-fold concentration could be achieved.

2.5.1 Dialysis against sucrose or PEG

The protein sample was sealed into a dialysis bag (M_r 12 000 cut-off), which was placed into a bed of commercial white sugar or PEG (M_r 20 000) in a plastic tray at 4°C. Once the sample had been sufficiently concentrated (usually 5 to 10-fold in 2-4 hrs) the bag was briefly rinsed in water and the sample was squeezed out. During the preparation of samples for SDS-PAGE, and throughout the entire procedure, gloves were worn to prevent contamination of the samples by keratins. These proteins are troublesome contaminants which cross-react with most antibodies non-specifically and appear as contaminants at a M_r of 68 000 on SDS-PAGE (Ochs, 1983; Shapiro, 1987).

2.5.2 SDS/KCl Precipitation

2.5.2.1 Reagents

5% (w/v) SDS. SDS (0,5 g) was dissolved in 10 ml of dist.H_2O.

3M KCl. KCl (2,24 g) was dissolved in 10 ml of dist.H_2O.

2.5.2.2 Procedure

5% SDS (50 µl) was added to the sample (500 µl) in a 1,5 ml eppendorf centrifuge tube. The solution was mixed by inverting the tube and 3M KCl (50 µl) was added. The mixture was again inverted and centrifuged (12 000 x g, 2 min, RT), the supernatant was discarded and the precipitate was dissolved in stacking gel buffer (50 µl) and treatment buffer (50 µl).

2.6 SDS-PAGE

SDS-PAGE of protein fractions was used extensively in this study to analyse the purity of chromatography fractions, and as a prelude to western blotting of proteins for immunological analysis. Non-denaturing PAGE was not used at all for the analysis of cathepsin L fractions, since the most often used PAGE system, i.e. anionic PAGE, involves the use of high pH buffers which results in the denaturation of the cathepsins, most of which are unstable above pH 7. Cationic PAGE was attempted but results were unsatisfactory due to the lack of a tracking dye and other problems with this generally less well characterized system. Non-denaturing PAGE was used for the analysis of cystatin fractions, which are stable at alkaline pH, and in this case the procedure outlined below was followed in the same way, except that SDS was not included in the solutions.

The non-denaturing PAGE system separates proteins on the basis of their charge-to-mass ratio. The separation of proteins by SDS-PAGE is usually entirely dependent on differences in their size, since SDS binds strongly to most proteins (Reynolds and Tanford, 1970), converting them from globular native proteins to highly negatively charged rods, the length of which is dependent on their MW. The negative charges of the SDS effectively mask the charges of the proteins, giving them all similar charge-to-mass ratios. The mobility of a protein on SDS-PAGE therefore depends on its size only. Its mobility can be plotted against the log of its MW and, when this has been done for several known proteins, a calibration curve may be constructed and used to estimate the MW of an unknown protein from its mobility. For this study the discontinuous PAGE and SDS-PAGE system of Laemmli (1970) was used on the Hoefer SE250 Mighty Small slab gel unit.

2.6.1 Reagents

Solution **A**: Monomer Solution [30% (w/v) acrylamide, 2,7% (w/v) Bis-acrylamide]. Acrylamide (58,4 g) and Bis-acrylamide (1,6 g) were dissolved and made up to 200 ml with dist.H_2O.

Solution **B**: 4 x Running Gel Buffer (1,5M Tris-HCl, pH 8,8). Tris (36,3 g) was dissolved in approximately 150 ml of dist.H_2O, titrated to pH 8,8 with HCl and made up to 200 ml.

Solution **C**: 4 x Stacking Gel Buffer (500mM Tris-HCl, pH 6,8). Tris (12 g) was dissolved in 150 ml dist.H_2O, titrated with HCl to pH 6.8 and made up to 200 ml.

Solutions A, B, and C were filtered through Whatman No. 1 filter paper before use.

Solution D: 10% (w/v) SDS. SDS (10 g) was dissolved in 100 ml dist.H_2O with gentle heating if necessary.

Solution E: Initiator [10% (w/v) Ammonium persulphate]. Ammonium persulphate (0,5 g) was made up to 5 ml just before use.

Solution F: Tank Buffer [250mM Tris-HCl, 192mM Glycine, 0,1% (w/v) SDS, pH 8,3]. Tris (12 g) and glycine (57,6 g) were dissolved and made up to 4 litres with dist.H_2O. Prior to use, 2,5 ml of SDS stock (solution E) was added to 250 ml for use in the Mighty Small apparatus.

Solution G: Treatment Buffer [125mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6,8]. Buffer C (2,5 ml), 10% SDS (4 ml) (solution E), glycerol (2 ml) and β–mercaptoethanol (1 ml) were made up to 10 ml with dist.H_2O.

Stain stock solution [1% (w/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in 100 ml of dist.H_2O by magnetic stirring for 1 h at room temperature. The solution was filtered through Whatman No. 1 filter paper.

Staining solution [0,125% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid). Stain stock (62,5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml), and the volume was brought to 500 ml with dist.H_2O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid] Methanol (500 ml) was mixed with acetic acid (100 ml) and the volume was made up to 1 litre with dist.H_2O.

Destaining solution II [7% (v/v) acetic acid, 5% (v/v) methanol] Acetic acid (70 ml) was mixed with methanol (50 ml), and the volume was made up to 1 litre with dist.H_2O.

12,5% Running gel solution.	6,25 ml	A
	3,75 ml	B
	0,15 ml	D
	0,075 ml	E
	0,01 ml TEMED	
	4,75 ml dist.H_2O	

Stacking gel solution.	0,95 ml	A
	1,75 ml	C
	0,07 ml	D
	0,035 ml	E
	0,012 ml TEMED	
	4,20 ml dist.H_2O	

2.6.2 Procedure

For SDS-PAGE, the Hoefer SE250 apparatus was assembled as described in the manufacturer's manual. This involved cleaning one notched aluminium plate and one glass plate with ethanol for each of the two sides of the apparatus, and clamping these with two 1,5 mm plastic spacers separating them at the edges. The bottom space was filled with molten 1% agarose and this was allowed to solidify. The running gel solution was run into the space between the plates, to a depth 3,5 cm from the top of the glass plate, and overlayered with dist.H_2O to allow for even polymerisation. Once the gel had set (evidenced by the appearance of the interface between gel solution and water, usually about 1 h), the water was shaken out. Stacking gel solution was poured in, up to the notch of the aluminium plate, and a 10- or 15-well comb was inserted to form the sample application wells. Once this gel had set (about 30 min) the comb was removed and the wells were rinsed with dist.H_2O.

Tank buffer containing SDS (no SDS was added to the tank buffer for PAGE) was poured into the upper and lower electrode compartments. Suitable amounts of samples (at least 1 µg of protein per band for the Coomassie blue R-250 staining procedure) were applied into the wells using a Hamilton microsyringe. The gel unit was connected to a power pack and run at 18 mA per gel until the bromophenol blue tracker dye was about 0,5 cm from the bottom of the running gel. At this point, the apparatus was disconnected from the power supply, the plates were removed, and levered apart using a plastic spacer. The gel was removed using gloves and placed into Coomassie blue R-250 staining solution for 4 h. The staining solution was removed at the end of this time and the gel was placed into destain I, overnight, and into destain II to effect complete destaining. Gels were stored in polythene zip-seal bags and kept well hydrated until photographed. They were stable in this form for long periods.

2.6.3 Combined Serva blue G/ silver stain procedure

Following electrophoresis, the protein bands were visualised using various staining procedures. For most purposes the commonly used Coomassie blue R-250 staining procedure was used, but if more sensitive staining was needed, the combined Serva blue G/silver stain technique

outlined by De Moreno *et al.* (1985) was used. The first part of this procedure, the staining with Serva blue G, was also very useful for the rapid visualisation of protein bands if immediate results were required, since it only took 3 hours.

2.6.3.1 Reagents

Serva blue G dye reagent [0,25% (w/v) Serva blue G, 50% (v/v) methanol, 12,5% (w/v) TCA]. Serva blue G (1,25 g) was dissolved in methanol (250 ml), following which dist.H_2O (125 ml) and 50% (w/v) TCA (125 ml) were added with continuous stirring.

40% (v/v) methanol, 10% (v/v) acetic acid. Methanol (400 ml) and acetic acid (100 ml) were diluted to 1 litre with dist.H_2O.

5% (w/v) TCA. TCA (50 g) was dissolved in 1 litre of dist.H_2O.

10% (v/v) ethanol, 5% (v/v) acetic acid. Absolute ethanol (100 ml) was mixed with acetic acid (50 ml) and diluted to 1 litre with dist.H_2O.

3,4mM $K_2Cr_2O_7$ - 3,2mM HNO_3. $K_2Cr_2O_7$ (0,25 g) and 55% HNO_3 (0,068 ml) were dissolved in 250 ml of dist.H_2O.

12mM $AgNO_3$. $AgNO_3$ (0,51 g) was dissolved in 250 ml of dist.H_2O.

280mM Na_2CO_3. Na_2CO_3 (14,84 g) was dissolved in 500 ml of dist.H_2O and 35% formaldehyde (0,5 ml) was added to every litre.

5% (v/v) acetic acid. Acetic acid (5 ml) was diluted to 100 ml with dist.H_2O.

2.6.3.2 Procedure

All steps were carried out in a shaker incubator at 100 rpm and room temperature (RT). The electrophoresis gel was soaked in 40% methanol-10% acetic acid (100 ml, 3 x 20 min), and stained in Serva blue G dye reagent (50 ml, 30 min.). Destaining was effected in 5% TCA (100 ml, 3 x 10 min), 40% methanol-10% acetic acid (100 ml, 2 x 10 min) and 10% ethanol-5% acetic acid (100 ml, 2 x 10 min). Following destaining, the gel was incubated in 3,4mM K_2CrO_7 - 32mM HNO_3 (50 ml, 10 min), washed in dist.H_2O (100 ml, 6 x 10 min), soaked in 12mM silver nitrate (50 ml, 30 min) and washed in dist.H_2O (100 ml, 2 min). Silver stained

bands were developed by three changes of the 0,28M Na_2CO_3 buffer (50 ml), the solution being removed whenever too much precipitate formed in the solution. Development was stopped by immersing the gel in 5% acetic acid (50 ml, 5 min), following which it was placed in dist.H_2O and stored in polythene zip-seal bags.

2.7 Isolation of Antibodies

Antibodies were isolated using PEG precipitation methods which proved to be very simple and efficient in the purification of both IgG and IgY to near homogeneity. IgY was isolated from egg yolks using the method of Polson *et al.* (1985), while IgG from serum was isolated using a method derived from findings about the precipitation of serum components using PEG (Polson *et al.*, 1964).

2.7.1 Reagents

10mM Borate buffer, pH 8,6. A sodium borate solution was prepared by dissolving boric acid (2,16 g), NaCl (2,19 g), NaOH (0,7 g) and 37% HCl (0,62 ml) in 950 ml of dist.H_2O, checking and adjusting the pH if necessary, and making the solution up to 1 litre.

100mM Na-Phosphate buffer, pH 7,6. $NaH_2PO_4.H_2O$ (13,8 g) and NaN_3 (0,2 g) were dissolved in 950 ml of dist.H_20, the pH was titrated to 7,6 using NaOH, and the volume was made up to 1 litre.

2.7.2 Procedure for the isolation of IgG from rabbit serum

One volume of rabbit serum was mixed with two volumes of borate buffer. Solid polyethylene glycol (M_r 6 000) was added to 14% (w/v), and dissolved with constant gentle stirring, after which the mixture was centrifuged (12 000 x g, 10 min, RT). The pellet was re-dissolved in the original serum volume, using 100mM Na-phosphate buffer, pH 7,6. PEG was again added to 14% (w/v), dissolved with stirring, and the solution was centrifuged (12 000 x g, 10 min, RT). The pellet was redissolved in half the original serum volume, using 100mM Na-phosphate buffer, pH 7,6.

2.7.3 Procedure for the isolation of IgY from chicken egg yolks

Egg yolks were separated from the egg white and carefully washed under running water to remove all traces of albumin. The yolk sac was punctured and the yolk volume determined in a measuring cylinder. Two volumes of 100mM Na-phosphate buffer, pH 7,6 were added and mixed thoroughly. Solid PEG (M_r 6 000) was added to 3,5% (w/v) and dissolved by gentle stirring. The precipitated vitellin fraction was removed by centrifugation (4 420 x g, 30 min, RT), and the supernatant fluid was filtered through absorbent cotton wool to remove the lipid fraction. The PEG concentration was increased to 12% [i.e. 8,5% (w/v) was added], the solution was mixed thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant was discarded and the pellet was dissolved in 100mM Na-phosphate buffer, pH 7,6, in a volume equal to the volume obtained after filtration. The final concentration of PEG was brought to 12% (w/v), the solution was stirred thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant fluid was discarded and the final antibody pellet was dissolved in 1/6 of the original egg yolk volume, using 100mM Na-phosphate buffer, pH 7,6.

2.7.4 Determination of [IgY] and [IgG]

The A_{280} of a 1 in 40 dilution of IgY and IgG solutions in 100mM phosphate buffer was read and the concentration of IgY and IgG in the undiluted solution was calculated [extinction coefficient of IgY = 1,25 (T. Coetzer, personal communication) and that of IgG = 1,43 (Hudson and Hay, 1980)].

2.8 Western Blotting

This technique is a useful extension of SDS-PAGE, allowing qualitative evaluation of antibodies produced to a particular antigen. The evaluation of the cross reactions of antibodies of known origin with unknown components of a mixture is also possible. The technique involves the electrophoretic transfer of proteins, separated by SDS-PAGE, to paper with a high protein binding capacity, such as nitrocellulose. In this study the technique of Towbin *et al.* (1979) was followed with minor modifications. The separated proteins so bound are reacted with primary antibodies and a secondary detection system. The secondary detection system usually consists of antibodies, reactive toward the primary antibody, and conjugated to an enzyme (such as HRPO), the reaction products of which can form a visible precipitate. The more sensitive Protein A-gold, with silver amplification, staining technique of Moeremans *et al.* (1984) was used in the cases where the HRPO system was not sensitive enough.

2.8.1 Reagents

Blotting buffer. Tris (27,23 g) and glycine (64,8 g) were dissolved in 3,5 litres of dist.H_2O, and methanol (900 ml) was added. The volume was made up to 4,5 litres in a large beaker, the exact volume not being critical. Prior to use, 4,5 ml of 10% SDS was added.

Tris buffered saline (20mM Tris, 200mM NaCl, pH 7,4). Tris (2,42 g) and NaCl (11,69 g) were dissolved in 950 ml of dist.H_2O, titrated to pH 7,4 with HCl, and made up to 1 litre.

100mM Na-borate buffer, pH 7,4. Na-borate (6,18 g) was dissolved in 950ml of dist.H_2O, titrated to pH 7,4 with NaOH, and made up to 1 litre.

HRPO linked secondary antibodies. The conjugation of HRPO to immunoglobulin was carried out according to Hudson and Hay (1980). HRPO (4 mg) was dissolved in 1 ml of dist.H_2O and a freshly prepared 100mM sodium periodate solution (200 µl) was added. The mixture was stirred for 20 min at RT (the mixture usually turned a greenish-brown colour at this stage). This mixture was dialysed against a 1mM Na-acetate buffer, pH 4,4, overnight at 4°C. The pH was raised to 9-9,5 by the addition of 200mM Na_2CO_3 buffer, pH 9,5 (20 µl), and 1 ml of an 8 mg/ml IgG fraction was immediately added. This solution was left at RT for 2 h. Freshly prepared 4 mg/ml Na-borohydride solution (100 µl) was added and the solution was left at 4°C for 2 h to reduce any free enzyme. This mixture was dialysed against 100mM Na-borate buffer, pH 7,4 overnight at 4°C, an equal volume of 60% glycerol in 100mM Na-borate buffer, pH 7,4 was added, and the conjugate was stored at 4°C. The dilution of conjugate to be used was established in a checkerboard ELISA. The conjugate dilution used was that which gave the most even titration over a serially diluted primary antibody range.

4-chloro-1-napthol substrate solution [0,06% (w/v) 4-chloro-1-napthol, 0,0015% (v/v) H_2O_2]. 4-chloro-1-napthol (0,03 g) was dissolved in methanol (10 ml). 2 ml of this solution was diluted to 10 ml with TBS, with the addition of 35% peroxide (4 µl).

2.8.2 Procedure

Following SDS-PAGE, usually on duplicate gels, one gel was stained to show the total protein pattern, while the other was used for blotting. Nitrocellulose was cut to a suitable size and, to avoid entrapment of air, carefully floated onto blotting buffer, before being totally immersed. The immersed nitrocellulose was sandwiched, with the gel lying squarely on top of it, between 3 pieces of Whatman No. 4 filter paper, also totally immersed in blotting buffer, and

was positioned in the sandwich of the western blotting apparatus. The sandwich was placed into the apparatus, filled with blotting buffer. The whole apparatus was situated in a further tank of cold water, kept at 8°C by a refrigerated circulator. The apparatus was connected to a power pack so that the nitrocellulose was on the anode side of the gel, and blotting was effected for 16 h at 200 mA. The buffer was stirred by a magnetic stirrer throughout the process. After 16 h, the sandwich was removed and the filter paper was peeled off the gel. The gel was carefully removed, following which it was stained to assess the efficiency of the blotting, which was usually very good.

The nitrocellulose sheet was removed from the filter paper and air dried for about 1 h. The following steps were followed for staining with HRPO linked secondary antibodies, all steps being carried out at room temperature. The nitrocellulose strip was blocked for 1 h with 5% low fat milk powder in TBS, washed in TBS (2 x 5 min) and incubated for 2 h with primary antibody in 0,5% BSA-TBS. Following washing in TBS (2 x 5 min), it was incubated in HRPO-linked secondary antibody in 0,5% BSA-TBS for 1 hour, and again washed in TBS (2 x 5 min). It was immersed in substrate solution and reacted in the dark until bands were clearly evident against a lightly-stained background. Finally, the strip was removed from the substrate solution, and washed in 0,1% (w/v) NaN_3 in TBS, further washed in TBS, and dried between filter paper. This last step ensured good preservation of the bands before photography.

2.8.3 Protein A Gold Labelling with Silver Amplification

2.8.3.1 Reagents

Protein A gold was prepared by Edith Elliott of the Department of Biochemistry.

Immuno-Gold labelling with Silver Amplification (IGSS) buffer II. Tris (7,9 g) and NaCl (9 g) were dissolved in 950 ml of dist.H_2O, titrated to pH 8,2 with 1M HCl, and made up to 1 litre with dist.H_2O.

Tris buffer [20mM Tris, 150mM NaCl, 0,02% (w/v) NaN_3, pH 8,2]. Tris (0,24 g), NaCl (0,88 g) and NaN_3 (0,01 g) were dissolved in 95ml of dist.H_2O, titrated to pH 8,2 with 1M HCl, and made up to 100 ml.

Citrate buffer. Citric acid.H_2O (0,75 g) and Na_3-citrate.$2H_2O$ (0,69 g) were dissolved in 25 ml of dist.H_2O.

Silver Development Solution A. Hydroquinone (170 mg) was dissolved in 17 ml of the citrate buffer.

Silver Development Solution B. Silver lactate (17 mg) was dissolved in 3 ml of dist.H_2O.

5% (w/v) thiosulphate. Sodium thiosulphate (5 g) was dissolved in 100 ml of dist.H_2O.

2.8.3.2 Procedure.

The HRPO procedure (see 2.7.2) was followed up to, and including the incubation with primary antibody. Thereafter, the nitrocellulose strip was washed with TBS (1 x 5 min), and IGSS II (2 x 5 min). It was then incubated with Protein A-gold at a suitable dilution (dependent on the probe's A_{520}, but usually 1:20) in 1% BSA in pH 8,2 Tris buffer. It was washed with IGSS buffer II (2 x 5 min) and dist.H_2O (3 x 5 min). Washing in dist.H_2O was not continued if stripping of the faintly-visible pink bands, showing binding by gold probes, was observed. Silver development solutions A and B were mixed, immediately prior to use, and placed in a petri dish in the semi-dark. The nitrocellulose strip was incubated in this mixture till sufficient amplification of the bands was observed, after which it was washed in the 5% thiosulphate solution for 5 min. It was then washed thoroughly in dist.H_2O and dried between sheets of filter paper, before being photographed.

2.9 Enzyme Linked Immunosorbent Assay (ELISA)

This technique is useful for the quantitative evaluation of antibodies produced against a particular antigen, and for evaluating the extent to which these antibodies react with other antigens, related and unrelated. It merges well with western blotting for the total evaluation of antibodies since, while western blotting will give information about the specificity of the antibodies and the types of molecules with which they cross-react, ELISAs will give quantitative data about these reactions.

2.9.1 Reagents

Phosphate buffered saline (PBS), pH 7,2. NaCl (8 g), KCl (0,2 g), $Na_2HPO_4.2H_2O$ (1,15 g) and KH_2PO_4 (0,2 g) were dissolved in 1 litre of dist.H_2O.

0,5% (w/v) Bovine serum albumin-PBS (BSA-PBS). BSA (0,5 g) was dissolved in 100 ml of PBS.

0,1% PBS-Tween. Tween 20® (1 ml) was made up to 1 litre in PBS.

0,15M citrate–phosphate buffer, pH 5,0. A solution of citric acid.H_2O (21,0 g/l) was titrated with a solution of $Na_2HPO_4.2H_2O$ (35,6 g/l) to pH 5,0.

Substrate solution [0,05% (w/v) ABTS and 0,0015% (v/v) H_2O_2 in citrate-phosphate buffer]. ABTS (7,5 mg) and H_2O_2 (7,5 µl) were dissolved in citrate–phosphate buffer, pH 5,0 (15 ml) for one ELISA plate.

Stopping buffer (citrate-phosphate-0.1% (w/v) NaN_3). NaN_3 (0,1 g) was made up to 100 ml in citrate–phosphate buffer.

Carbonate coating buffer. $NaHCO_3$ (0,21 g) was dissolved in 45 ml of dist.H_2O, titrated to pH 6,0 with HCl, and made up to 50 ml.

2.9.2 Procedure

Cathepsin L was coated at a concentration of 1 µg/ml in coating buffer (150 µl, 3 h at 37°C, followed by 17 h at 4°C), while cathepsin L peptide was coated in PBS at a concentration of 5 µg/ml (150 µl, 16 h). Both coating concentrations were determined using a checkerboard ELISA, the coating concentration chosen being that which gave the most even titration over a range of serially diluted antibody. Non–specific binding of antibody was prevented by blocking the wells with 0,5% BSA-PBS (200 µl, 1 h at 37°C), and the plates were washed three times with PBS-Tween.

A serial two-fold dilution, starting from 1/10 serum dilution (or 2mg/ml IgG) was prepared on the plate in 0,5% BSA-PBS and incubated (100 µl, 2 h at 37°C), the plates were washed three times with PBS-Tween, the HRPO-linked secondary antibody, at a suitable dilution in 0,5% BSA-PBS, was added to each well and incubated (120 µl, 1 h at 37°C), and the plates were washed three times with PBS–Tween. Substrate solution (150 µl) was added to each

well and colour was allowed to develop in the dark against the the background of the controls (usually 10-20 min). The enzyme reaction was stopped by addition of citrate–phosphate–NaN_3 buffer (50 µl) and the A_{405} of each well was measured on a Titertek ELISA plate reader.

2.10 Preparation of carboxymethyl-papain-Sepharose

This affinity matrix, for the isolation of cystatins from sheep liver, was prepared by activating Sepharose-4B with cyanogen bromide (CNBr) by the method of Kohn and Wilchek (1982). Carboxymethyl(CM)-papain was prepared and coupled to the activated Sepharose according to Anastasi *et al.* (1983).

2.10.1 Reagents

1M CNBr in acetone. CNBr (25 g) was dissolved in acetone (236 ml) and stored at -20°C.

1,5M triethylamine (TEA) in 60% acetone. TEA (20,9 ml) was dissolved in 60% acetone (100 ml).

Washing medium. 100mM HCl was mixed 1 : 1 with acetone.

Coupling medium. 100mM $NaHCO_3$ adjusted to pH 9,0 with NaOH.

CM-papain. The papain preparation contained 83% protein, and 60 mg was therefore weighed out to give 50 mg of protein. This was activated in 7,5 ml of 2mM cysteine.HCl, 1mM Na_2EDTA, 100mM NaH_2PO_4, pH 6,0 for 10 min at RT. Following this 10mM iodoacetate was added and allowed to react with the activated papain for 15 min to form CM-papain. This solution was dialysed overnight against 10 volumes of coupling medium.

2.10.2 Procedure

Wet Sepharose-4B (25 g) was weighed out and washed on a buchner funnel with ice-cold 30% acetone, followed by 60% acetone, and resuspended in 60% acetone. This suspension was cooled to -20°C in the freezer. 1M CNBr (3,5 ml) was added to the Sepharose in an ice-bath, with stirring, to bring the Sepharose to 15 mg CNBr/g. Following this, 1,5M TEA (3,5 ml) was added dropwise over 2-3 min, with stirring. The Sepharose was poured into 125 ml of ice cold washing medium, and washed with 60% acetone, 30% acetone, dist.H_2O and coupling medium (all ice cold).

CM-Papain in 25 ml of coupling medium was added to the activated Sepharose, also in 25 ml of coupling buffer. The mixture was shaken on a Heidolph "end-over-end" stirrer overnight at 4°C. The gel was treated for 1 h with 100mM glycine, as above, and washed with 2 volumes of cold 100mM citrate, pH 3,0 and with 2 volumes of 100mM phosphate, pH 11,0. Monitoring the A_{280} of the CM-Papain before and after the conjugation, allowed conjugation efficiency to be estimated at about 94%.

CHAPTER 3

THREE-PHASE PARTITIONING

3.1 Introduction

Three-phase partitioning (TPP) is a method discovered serendipitously in the course of studies on the effects of water–miscible organic solvents on the activity of enzymes (Tan and Lovrien, 1972). The potential application of the method to the isolation of proteins was described at a conference by workers from Lovrien's laboratory (Anderson *et al.*, 1981). They documented the method in detail, when describing its use in the purification of cellulases from *Trichoderma reesei* (Odegaard *et al.*, 1984). They found that t-butanol, normally completely miscible with water, separated from the aqueous phase on the addition of a salt such as ammonium sulphate. Any protein present in the initial mixture was precipitated into a third phase between the t-butanol and aqueous phases. An advantage of the technique was that it removed tannins, phenolics, lipids and enzyme inhibitors from protein mixtures, thus improving the activity of enzymes, while simultaneously concentrating the proteins, more or less specifically. Further advantages were that only low speed centrifugation was necessary to resolve the phases, and that the process could be carried out at room temperature, unlike many other protein fractionation procedures involving organic solvents. An advantage, only really appreciated later, is that after TPP, the precipitated fractions are relatively salt free, unlike the equivalent fractions from ammonium sulphate precipitation.

Their early results led Lovrien *et al.* (1987) to make a further study of the effect of TPP on the fractionation of various crude enzyme preparations, mostly of plant and microbial origin. In all cases large increases in the total and specific activities of the enzymes were reported, emphasising the potential of the technique for protein purification. TPP was also used by Niehaus and Dilts (1982) for the purification of mannitol dehydrogenase enzymes. A procedure similar to TPP, using n-butanol in place of t-butanol, was used by Verger *et al.* (1969) to isolate the lipase enzyme from the pancreas. In addition to the work emanating from this laboratory (Jacobs *et al.*, 1989; Pike and Dennison, 1989a), Pol *et al.* (1990) have recently used TPP to simplify the purification of several red blood cell proteins, using the selective denaturation of haemoglobin by TPP, enhanced by the additional use of a small proportion of chloroform.

TPP seemed to be a potentially useful technique for the purification of proteolytic enzymes, but since it had only been used empirically, there were few systematic guidelines to its use. A study was therefore made of the technique in order to gain insight for its use in the isolation of proteases. A group of standard proteins of known physico-chemical properties, chosen for their

range in molecular weights and isoelectric points (pI), rather than their enzymatic activity, was used for this study, since the effect of TPP on enzymatic activities had been covered in the study by Lovrien *et al.* (1987). The selected proteins were subjected to TPP under various conditions to monitor the effect of the conditions on the precipitation of the proteins, and their solubility after TPP.

Since the pH of aqueous solutions, relative to the pI of proteins, has a strong effect on their solubility, the effect of pH on the TPP process was investigated. The effect of MW was also monitored since this property has been known to affect the partitioning of proteins, although no conclusive explanation of this phenomenon exists. Salting out, another frequently used technique for the crude fractionation of proteins, is known to be affected by the protein concentration and temperature of protein mixtures (Dixon and Webb, 1961), and the effect of these two parameters on TPP was therefore tested. The effect of varying the ratio of t-butanol and ammonium sulphate was tested to determine the working range of the TPP system in both the presence and absence of protein. Since the TPP mixture contains both a non–polar and a polar component (t-butanol and water respectively), the effect of the surface hydrophobicity was of interest. This was investigated by ranking the proteins in terms of their hydrophobicity, using their elution order from a hydrophobic interaction chromatography column, and trying to correlate their subsequent behaviour in TPP with this ranking.

3.2 Materials and Methods

3.2.1 Standard proteins. The standards used were: Bovine serum albumin (M_r 66 500, pI 4,8), cytochrome C (M_r 12 400, pI 10,0), γ-globulin (M_r 160 000, pI ca. 6,0), haemoglobin (M_r 68 000, pI 7,0), lysozyme (M_r 14 300, pI 11,1), myoglobin (M_r 17 000, pI 7,0) and ovalbumin (M_r 45 000, pI 4,6). All standards were obtained from Sigma Chemical Co., St Louis, Mo..

3.2.2 Three-phase partitioning. The protein sample was dissolved in buffer (10mM), of the desired pH, at a concentration of 0,5 mg/ml (except where stated otherwise). The A_{280} of the solution was measured, and designated "A". t-Butanol was added to constitute 30% (v/v) of the total mixture volume, and thoroughly mixed in. Ammonium sulphate crystals were added to the mixture to the desired concentration, expressed as a percentage (w/v) of the total mixture volume, and the solution was mixed vigorously to dissolve the salt (the presence of t-butanol alters the physical properties of the solution so that frothing does not occur and there is no apparent denaturation of proteins, or loss of activity of the enzymes tested, before and after mixing). During these operations the solution was maintained at 25°C, except where the effect of temperature was being investigated. The mixture was centrifuged (3 000 x g, 10 min, RT), and the three phases were collected separately.

The A_{280} of the aqueous phase of the partitioned mixture was measured against a blank, treated identically, but with no protein present, and this value was designated "B". The interphase (third phase) layer was dissolved (as far as possible) in a volume of 10mM phosphate buffer, pH 7,0, equal to that of the original buffer. Material which would not redissolve was removed by centrifugation and the A_{280} of the resulting supernatant was measured, and designated "C".

The percentage of the original protein which was extracted into the third phase, was calculated as follows:

$$\% \text{ protein} = \frac{A - [B \times F/I]}{A} \times 100$$

Where, F = final volume, and I = original volume of the aqueous phase

(It should be noted that no protein enters the t-butanol phase)

The apparent percentage solubility of the protein after TPP was calculated as follows:

$$\% \text{ solubility of protein} = \frac{C}{A - B} \times 100$$

3.2.3 Density measurements. Solution densities at 25°C were measured in a Parr DMA 10 precision density meter.

3.2.4 Circular dichroism (CD) spectra. CD-spectra were measured using a Jasco JA-20 spectro-polarimeter. The proteins were dissolved in a 10mM buffer, with a pH equal to the pI of the test protein, or in a mixture of buffer and t-butanol, up to 50% t-butanol (v/v). The interpretation of CD spectra has been discussed by Tinoco and Cantor (1970) and by Johnson (1985).

3.2.5 Hydrophobicity ranking. Test proteins were ranked for hydrophobicity by their elution order from a phenyl-Sepharose column. The test proteins ovalbumin, cytochrome C, γ-globulin, BSA and lysozyme were dissolved in 1 ml of loading buffer (20mM NaH_2PO_4, 800mM $(NH_4)_2SO_4$, pH 6,8) at a concentration of 1 mg/ml. The proteins were loaded onto phenyl-Sepharose, equilibrated in the same buffer, and eluted with a gradient of decreasing $[(NH_4)_2SO_4]$. Following this, a step gradient of 50% (v/v) ethylene glycol in 20mM NaH_2PO_4, pH 6,8, was applied to the column. Eluted fractions were analysed by SDS-PAGE.

3.3 Results

3.3.1 The effect of different proportions of ammonium sulphate and t-butanol. This experiment was initially carried out in the absence of protein to determine the amount of salt necessary to partition a homogenous mixture of t-butanol/dist.H_2O into two phases. Since an earlier experiment had determined that t-butanol/dist.H_2O mixtures only remained homogenous up to 50% t-butanol, in the absence of salt, this was used as the upper limit of t-butanol in this experiment. Salt was added in small increments to mixtures of water and t-butanol until two phases formed. This information formed the basis of the lower curve in Fig. 1, which in turn was used as a basis for the determination of the amount of salt required, in the presence of differing proportions of solvents, to form a well resolved third phase when the proteins ovalbumin and BSA were present. This information was used as a basis for all further work on the characterisation of TPP.

The results in Fig. 1 show that there was a reciprocal relationship between the amounts of ammonium sulphate and t-butanol needed to form a two-phase system. The curves for the proteins BSA and ovalbumin showed that more salt is required in the TPP system in the presence of protein in order to obtain a well resolved third phase. The solubility of the test proteins was lower at t-butanol concentrations below 30% (v/v), while higher t-butanol concentrations offered no further advantage, so 30% t-butanol was chosen as the optimum t-butanol concentration for further experiments.

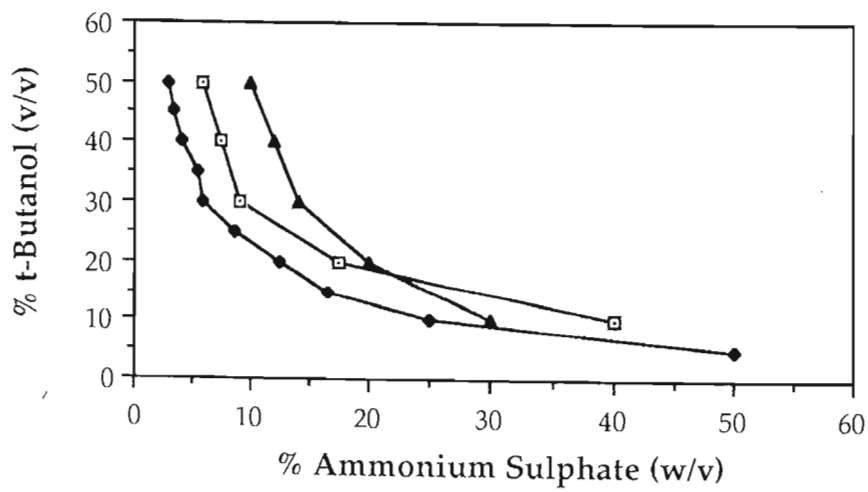


Figure 1. The proportions of ammonium sulphate and t-butanol required to form distinct phases.

Two phase system in the absence of protein (◆), three phase system in the presence of ovalbumin at pH 4,6 (❑) and three phase system in the presence of BSA at pH 4,8 (▲).

3.3.2 The effect of the density of the phases. In carrying out the above experiment, the question of the mechanism of TPP arose, particularly with regard to the way in which proteins float in TPP in contrast to their behaviour in conventional salting out, where they sink. The one answer postulated for this question was that the difference could be due to a density effect, i.e. t-butanol might alter the density of the lower phase and/or of the proteins so that the proteins float instead of sinking. Measurement of the solution densities revealed that, in the TPP process (at a constant nominal t-butanol concentration of 30%), the densities of the phases are complex functions of the nominal ammonium sulphate concentration, i.e. the density did not vary linearly with the ammonium sulphate concentration, as was the case for simple ammonium sulphate solutions, but varied according to a more complex set of factors not yet elucidated (Fig. 2).

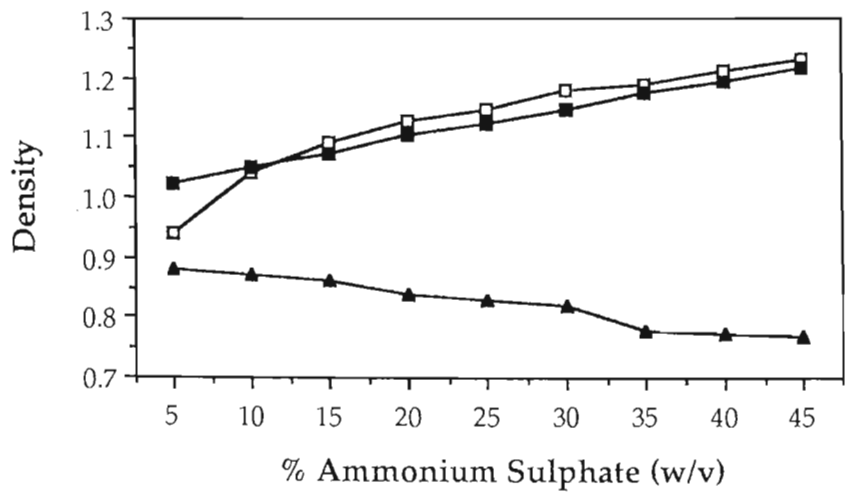


Figure 2. The effect of ammonium sulphate concentration on the density of the aqueous (lower) and t-butanol (upper) phases of two phase systems comprised of aqueous 10mM citrate buffer (pH 4.8), t-butanol (nominally 30%) and ammonium sulphate, and the density of single phase aqueous ammonium sulphate solutions in the same buffer. The aqueous phase of the TPP systems (❑), the t-butanol phase of the TPP systems (▲) and aqueous ammonium sulphate solutions (■).

To ensure that the statement that proteins float in TPP and not in salting out, was indeed correct, some confirmatory tests were made. Using BSA (2mg/ml), it was confirmed that the protein was precipitated and sank in 30% ammonium sulphate in a pH 4,8 buffer, but floated on the aqueous phase of TPP carried out at a nominal ammonium sulphate concentration of 25%, having the same density as a 30% ammonium sulphate solution. Similarly, ovalbumin (2 mg/ml) salted out and sank in 60% ammonium sulphate but floated when precipitated by 30% ammonium sulphate in TPP. In the latter example, the TPP aqueous phase was actually less

dense than the simple 60% ammonium sulphate (Fig. 2). The density of the aqueous phase, by itself, would therefore not seem to provide a simple solution to the mechanism of TPP.

3.3.3 The effects of pH. In order to determine the effect of pH, TPP was carried out at a series of different pH values for each of the standard proteins, except myoglobin which was totally denatured by TPP. In each case the standard protein was dissolved in a 10mM buffer (citrate, glycinate or phosphate) at the required pH, and TPP was effected using a salt concentration sufficient to extract at least 90% of the protein at the optimum pH. This gave an indication of the effect of pH on the extraction at uniform salt concentration, since the amount of protein extracted at other pH values was compared to the optimum. The solubility of the proteins after TPP at the various pH values was also tested and expressed as a percentage of that extracted.

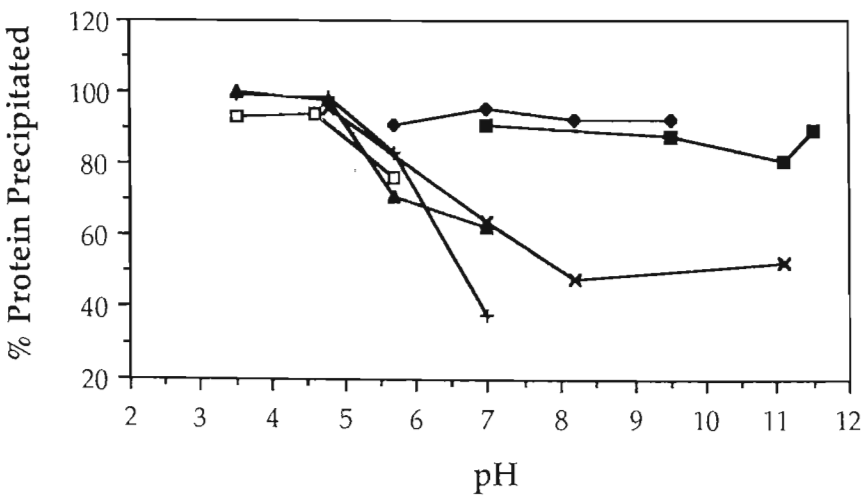


Figure 3. The effect of pH on the precipitation of proteins into the third phase in TPP. The percentage of BSA (+), ovalbumin (□), lysozyme (■), cytochrome C (×), γ-globulin (▲) and haemoglobin (◆) precipitated.

From Fig. 3 it may be seen that, with the exception of lysozyme and haemoglobin, the proteins were maximally extracted at lower pH values, usually below their pI, where they have a net positive charge. From Fig. 4, however, it may be seen that maximal solubility after TPP was obtained at higher pH values, usually above or equal to the pI of the protein. This implies that the protein is less likely to be denatured in the TPP process if it has an overall neutral charge during the process. Haemoglobin, the only test protein with a quaternary structure, was completely denatured by TPP, being completely insoluble after the process. Myoglobin was similarly denatured with the loss of its non-covalently bound haem group into the t-butanol layer.

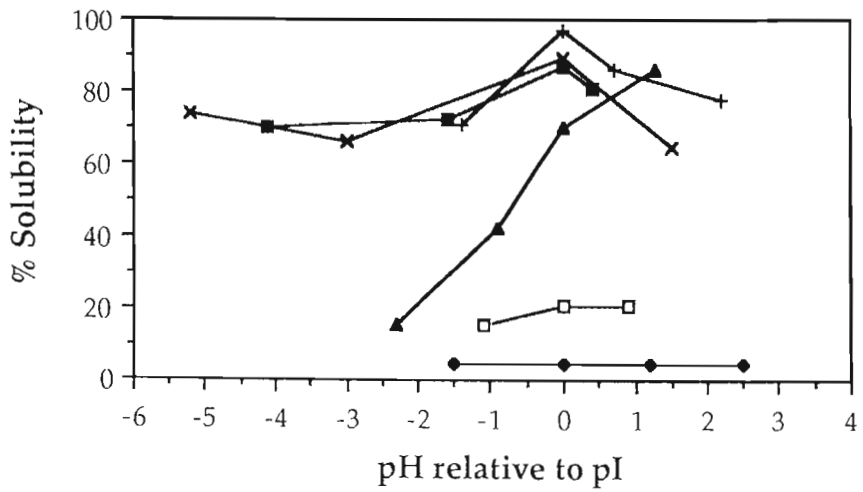


Figure 4: The effect of the pH at which TPP is conducted on the solubility of proteins after TPP.

The percentage solubility of BSA (+), ovalbumin (❑), lysozyme (■), cytochrome C (×), γ-globulin (▲) and haemoglobin (◆).

3.3.4 The effect of molecular weight. The effect of molecular weight on TPP was determined by the amount of ammonium sulphate required to precipitate at least 90% of the test protein at the optimal pH for the precipitation of the protein concerned. The results (Fig. 5) show that there is a reciprocal relationship between the amount of salt required and the molecular weight of the protein. The effect is apparently enhanced in the lower molecular weight ranges, where proportionately more salt is required for precipitation of the protein. The hydrophobicity of the protein may also play a part here, however, since cytochrome C, which is a hydrophilic protein (section 3.3.8), has a disproportionately high salt requirement for precipitation.

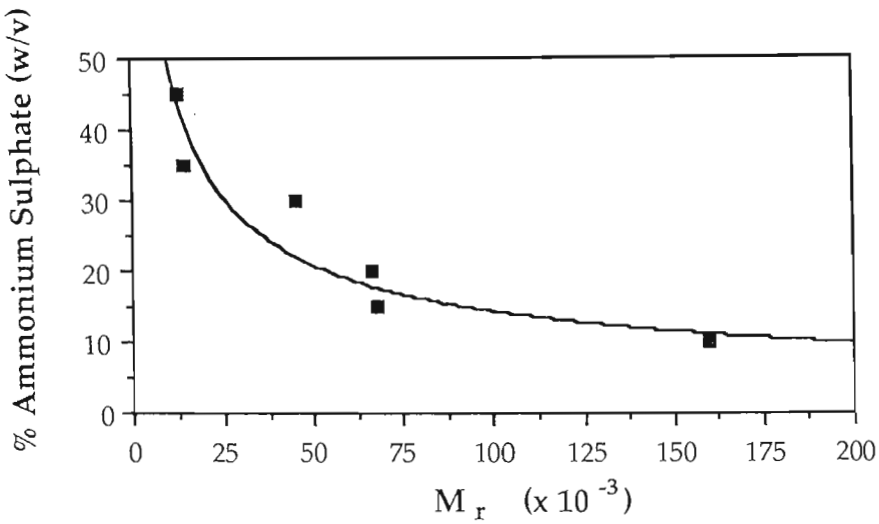


Figure 5. The effect of the molecular weight of the test protein upon the concentration of ammonium sulphate required to precipitate 90% of the protein into the third phase in TPP.

3.3.5 The effect of protein concentration. The effect of protein concentration on TPP was determined using γ-globulin, BSA and ovalbumin as test proteins, at pH 5,7, 4,8 and 4,6 respectively, and at different initial concentrations. A fixed concentration of t-butanol [30% (v/v)] was used and the concentration of protein remaining in solution was measured, after TPP had been effected by the addition of different amounts of ammonium sulphate to replicate samples. The results, in Fig. 6, suggest that protein has an effect on the TPP process different from its effect on conventional salting out (Dixon and Webb, 1961), as a given protein gives rise to a family of precipitation curves, each determined by the initial concentration of the protein. Practically, this implies that the initial concentration of a protein in a solution will have a very marked effect on the precipitation of the protein, and thus the protein concentration of solutions being subjected to TPP needs to very carefully monitored in order to obtain reproducible results with any given protein.

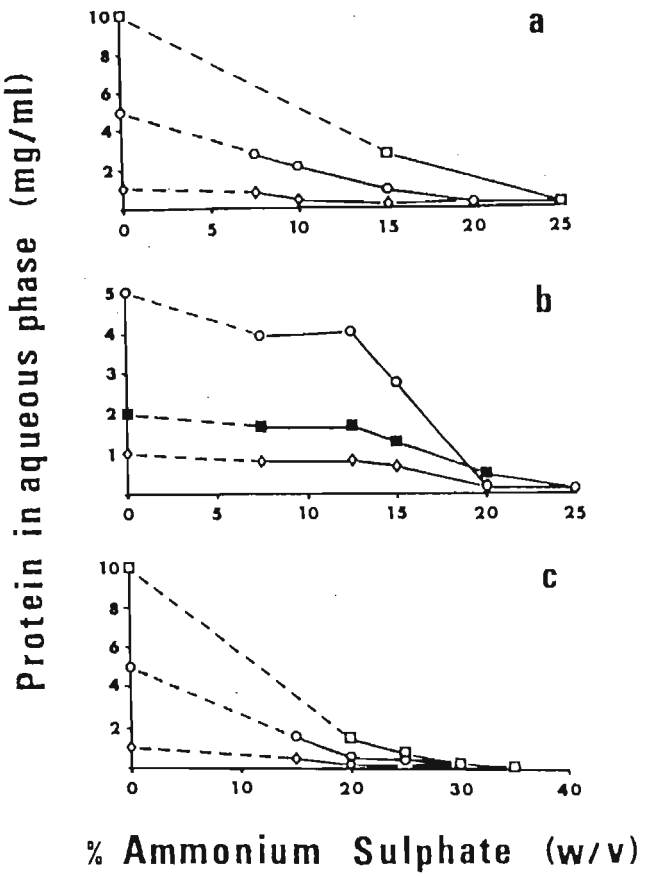


Figure 6. The effect of protein concentration on the TPP process.
a = bovine γ-globulin, b = BSA, c = ovalbumin; the initial concentration in each case is indicated by the intercept on the y-axis. The dashed line joins successive points but the protein concentration could not be measured in this region as milky solutions were formed.

At low ammonium sulphate concentrations, before a well resolved third phase is obtained, milky solutions are formed at higher protein concentrations. It was subsequently found that addition of 10% sucrose (added to increase the density of the aqueous phase) clarifies these solutions but does not result in phase separation.

3.3.6 The effect of temperature. This was measured at pH 4,8 and at an ammonium sulphate concentration of 30% (w/v), using BSA as the test protein. Temperature has little effect on the TPP process, as neither the amount of protein precipitated into the third phase, nor the subsequent solubility of this protein was markedly affected (Fig. 7). Similar results were found for ovalbumin in terms of solubility, where, in an attempt to increase the protein's solubility, TPP was carried out at 4°C instead of the usual 25°C with no apparent increase in solubility. The only result of interest was that TPP on BSA at 37°C increased the amount of time necessary for the dissolution of the protein after TPP.

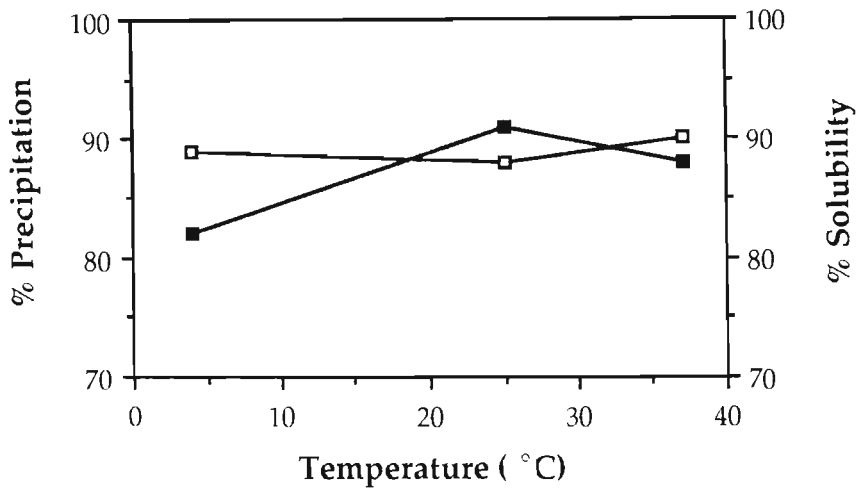


Figure 7. The effect of temperature on protein precipitation and solubility after TPP. Percentage protein precipitated into the third phase (■) and percentage solubility after TPP (□).

3.3.7 The effect of t-butanol upon the conformation of proteins in aqueous solution. The addition of t-butanol, up to 50% (v/v), to proteins in solution, at their pI and at 25°C, had little effect upon the conformation of the proteins tested, i.e. BSA, lysozyme, cytochrome C, γ-globulin, and ovalbumin. In no case was there a shift to a random coil structure; the only obvious change was a slight increase in the α-helical content (The CD spectra of BSA, lysozyme and cytochrome C are shown in Fig. 8.). This suggests that, at least for the proteins tested, t-butanol *per se* is not a denaturing agent.

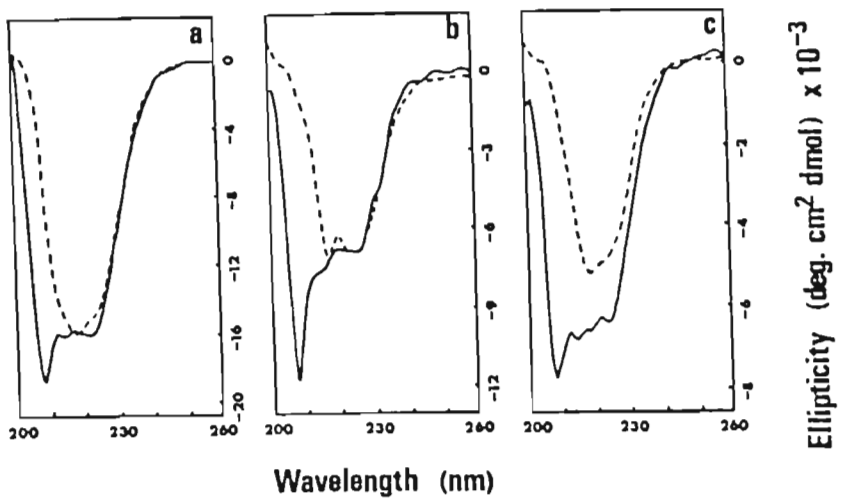


Figure 8. Circular dichroism spectra of, (a) bovine serum albumin, (b) lysozyme and (c) cytochrome C.
The solid line represents the spectrum of sample in buffer containing 50% t-butanol while the dotted line represents the spectrum of sample in buffer alone.

3.3.8 Hydrophobicity ranking. The elution order of the proteins from phenyl-Sepharose, as analysed by SDS-PAGE, appeared to be cytochrome C, γ-globulin, ovalbumin/lysozyme, and BSA. Since the phenyl-Sepharose separates proteins on the basis of their surface hydrophobocity, this may be regarded as a tentative ranking order for this parameter.

3.4 Discussion

The objective of the studies reported here was to determine how the physico-chemical properties and concentration of a protein affect its behaviour in the TPP process, as this knowledge would be useful in using the method to its best advantage. The results obtained indicate that proteins precipitate (into the third phase) most readily at or below their pI, but that proteins were most soluble after TPP when this was carried out above the pI of the protein. This finding, that TPP generally works best above the pI of a protein, has been confirmed by Pol *et al.* (1990). The amount of salt required to precipitate a protein was found to vary inversely with the protein molecular weight, although the hydrophilicity of the protein may also have an effect here. Salt and t-butanol appear to have a similar effect in that more of one could compensate for less of the other. Protein concentration affects the precipitation of protein in TPP in a manner different from its effect in conventional salting out (Dixon and Webb, 1961). In conventional salting out a given protein has a single solubility curve, which is a function of the precipitating salt concentration, such that there exists a reciprocal relationship between the amount of protein in solution and the salt concentration required to initiate its precipitation. In the case of TPP, however, this does not seem to be the case, in that each protein appears to yield a family of solubility curves, each curve being a function of the initial protein concentration.

The solubility of proteins after TPP provides some measure of the extent of their denaturation. Evidence provided by the measurement of CD-spectra suggests that simple exposure of the protein to t-butanol apparently does not denature single-chain proteins. However, the subsequent salting out with $(NH_4)_2SO_4$, the step required to form the three phases, is apparently much more damaging to proteins in the presence of t-butanol than in its absence. Non-covalently bonded oligomeric proteins such as haemoglobin or myoglobin (in which the haem group is non-covalently attached), are completely denatured by the TPP process, as evidenced by their subsequent complete lack of solubility. Single-chain proteins are denatured to a variable extent which appears to depend partly upon the pH at which the TPP process is carried out, relative to the pI of the protein. TPP therefore appears to be a method whereby stable single chain proteins might be separated from oligomers, by denaturation of the latter. It must, however, be pointed out that recent work on the enzymes catalase and superoxide dismutase, which are oligomeric proteins, showed that these proteins survive fractionation by TPP (Deutsch, Pol and Visser, personal communication). These are unusually robust oligomers however (Schonbaum and Chance, 1976, Malmström *et al.*, 1975), and their behaviour in TPP may not be typical. A general effect of TPP on oligomers, therefore, remains to be established.

In general, proteins are denatured to the least extent at their pI. It is difficult to assign a physical significance to this, although the pI might be the pH at which the protein is hydrated to the least extent. Individual structural features of the proteins might also have some bearing on their susceptibility to denaturation, for example the extent to which the protein structure is stabilised by disulphide bridges. A factor can be derived by dividing the MW of the protein by the number of disulphide bridges and for the proteins examined in this study it appears that, if this factor is less than 10 000, the protein will largely escape denaturation.

The results of the experiments reported here permit the construction of a tentative model to explain the TPP process, in comparison to ammonium sulphate precipitation. In a purely aqueous system, the addition of water to a protein results in a hydrated, soluble protein. The addition of ammonium sulphate forms hydrated ammonium sulphate, water is abstracted from the protein and the protein may precipitate as a consequence. This is a postulated mechanism for the process of ammonium sulphate precipitation, but TPP is obviously different from this in that the protein floats after precipitation instead of sinking as it does in ammonium sulphate precipitation. Since a change of density in the aqueous phase is not responsible for this phenomenon, some other process must be at work. Addition of t-butanol to water [to 50% (v/v)] results in a single phase system of hydrated t-butanol, in which proteins may be soluble. Dissolved proteins will equilibrate with the solvent and the co-solvent. The addition of ammonium sulphate abstracts water, leaving t-butanol to separate as a distinct phase. The

protein, while equilibrating with the new proportions of available solvent and co-solvent, may precipitate out of solution. This protein, having an increased proportion of adsorbed t-butanol, may have a lower density and, due to this, will float on the aqueous layer.

The above study established guidelines for the use of TPP in the isolation of proteins. As was pointed out by Lovrien *et al.* (1987), however, the study of a technique for the purification of crude proteins, by using standard pre-purified proteins has its limitations. The contaminants in crude extracts, such as lipids, carbohydrates and smaller organic molecules can interfere and thus negate the theoretical guidelines laid down by such studies. While this limitation is appreciated, this study complements Lovrien's study on enzymes in the context of the crude extract, and together the two studies may provide fairly comprehensive guidelines for the use of the technique in both contexts. The guidelines laid down by this study have been published (Pike and Dennison, 1989) and were subsequently used in the isolation of cathepsin L from sheep liver, and from human spleen, as described in the following chapters, as well as for the isolation of cathepsin D, in further studies (Jacobs *et al.*, 1989; Coetzer *et al.*, 1990).

CHAPTER 4

THE ISOLATION AND CHARACTERISATION OF CATHEPSIN L FROM SHEEP LIVER AND HUMAN SPLEEN: STUDIES ON THE FORMATION OF A PROTEOLYTICALLY ACTIVE COMPLEX WITH CYSTATIN

4.1 Introduction

The aim of this study was to purify cathepsin L from a suitable source, and, following this, to produce specific polyclonal antibodies which could be used in the immunocytochemistry of tumours. The isolation of cathepsin L proved to be a major part of this study, as it was more difficult than at first anticipated, necessitating the development of new techniques. The production of polyclonal antibodies to the enzyme is obviously interactive with the purification procedure, since the antibodies produced to a preparation of the enzyme will in turn convey information about the preparation, and therefore about the purification procedure. This proved to be so in this study where two purification procedures which at first seemed to be suitable, were found to be unsuccessful when the purification procedure was repeated many times in order to produce sufficient material for the production of antibodies.

Cathepsin L has been purified from various sources, including rat liver (Kirschke *et al.*, 1977), rat kidney (Bando *et al*, 1986), rabbit liver (Mason *et al.*, 1984), human liver (Mason *et al.*, 1985), sheep and bovine liver (Mason, 1986) and rabbit spleen (Maciewicz *et al.*, 1988). For the reasons given in chapter 1, sheep liver was decided upon as first choice for an organ from which to isolate the enzyme.

Freezing of tissue results in rupture of the lysosomes and, upon subsequent thawing and homogenisation, cytosolic inhibitors are able to complex with enzymes released from the ruptured lysosomes. In the purification procedures of Kirschke *et al.* (1977) and Bando *et al.* (1986), lysosomes were first isolated to partially purify cathepsin L, and to keep the enzyme separate from inhibitors in the cytosol. Isolation of lysosomes is quite inconvenient, however, in that fresh tissue has to be used and the isolation procedures are fairly demanding. The discovery by Mason *et al.* (1985) that cathepsin L from human liver could be activated after homogenisation of frozen liver, by a process known as "autolysis", was therefore of considerable interest.

Autolysis, consisting of the incubation of the homogenate at a low pH and elevated temperature, was thought to effect the disruption of enzyme-inhibitor complexes and/or the activation of latent pro-enzymes. Both Mason *et al.* (1985) and Matsumoto *et al.* (1983) have

speculated that "autolysis" may be effected by an uncharacterised proteinase in crude extracts, which digests away the inhibitor complexed with the cathepsin L. However, neither Mason *et al.* (1985) nor Matsumoto *et al.* (1983) were able to inhibit the activation process using inhibitors of most known classes of proteinases. The use of this process, even though its mechanism remains obscure, obviates the need to isolate lysosomes, and has the further advantage that stored, frozen, livers can be used. For these reasons the procedure of Mason (1986), for the initial extraction and activation of cathepsin L, was used throughout the present study.

Cathepsin L is known to bind strongly to cation exchangers, even at pH values close to its pI. This seemingly anomalous situation may be due to the charge distribution of the positive amino acids in the molecule, rather than the overall ionisation state of the molecule relative to its pI, as has been noticed previously for other proteins in their interaction with ion-exchangers (Kopaciewicz *et al.*, 1983). Various cation exchangers have therefore been employed in previous purifications of cathepsin L, including CM–Sephadex (Kirschke *et al.*, 1977, Bando *et al.*, 1986, Mason *et al.*, 1984, Mason *et al.*, 1985, Mason, 1986) and the FPLC Mono S system (Mason *et al.*, 1985, Mason, 1986).

Initially the purification procedure of Mason *et al.* (1984), for the purification of cathepsin L from rabbit liver, was used in an attempt to purify the enzyme from sheep liver. This involved the use of salting-out as an initial crude purification step, followed by chromatography on CM-Sephadex at pH 5,5, molecular exclusion chromatography on Sephadex G-75 and re-chromatography on CM-Sephadex at pH 5,5. This method did not result in the purification of cathepsin L, however, and a new method was devised, involving the use of TPP, in an attempt to obtain greater purification in the initial stages.

Using TPP according to the guidelines laid down in the previous chapter, it was found that this method was far superior to ammonium sulphate precipitation for the crude purification of cathepsin L from sheep liver. When applied to bovine and rabbit livers the same was found to be true, showing that TPP may be generally useful for the crude purification of cathepsin L from different species.

Following these discoveries, a new purification procedure was devised using cation-exchange chromatography on CM-Accell, a silica based cation-exchanger with high resolution properties (Strickler and Gemski, 1986), followed by hydrophobic chromatography on phenyl-Sepharose. This procedure was successfully used for the purification of cathepsin L from sheep liver, but the purity of the final preparation was found to be dependent on the resolution between two closely eluting peaks on phenyl-Sepharose, which varied from one purification to

the next. Due to this inconsistency, the repeated purification necessary to provide immunogen for an immunisation regimen was not possible.

This problem was solved by the use of S-Sepharose fast flow, which is a low pressure equivalent of the Mono-S FPLC cation-exchanger, used by Mason (1986) for the purification of sheep liver cathepsin L. Initial studies established that a pure preparation of the enzyme was obtained when this gel was used after phenyl-Sepharose in the purification procedure described above. Later it was found, however, that S-Sepharose could be used directly after crude purification by TPP, without preliminary desalting on Sephadex G-25, as samples from TPP are relatively salt-free. This procedure gave a large initial purification of cathepsin L, and a single further step on S-Sepharose, at a lower pH, was sufficient to purify the enzyme to apparent homogeneity on SDS-PAGE.

The characterisation of rabbit polyclonal antibodies in a western blot, against the apparently pure preparation produced above, showed the presence of a contaminant of M_r 14 000, however, suggesting that the preparation was not, in fact, pure. Analysis of the S-Sepharose pH 4,5 fraction by MEC revealed the presence, in addition to free cathepsin L, of a M_r 37 000 complex between cathepsin L and the M_r 14 000 component, which was later found to be the cysteine proteinase inhibitor, cystatin β. The purification procedure was revised to replace the pH 4,5 S-Sepharose step with an MEC step on Sephadex G-75, which resulted in the purification of the enzyme/inhibitor complex and the free, single-chain enzyme. SDS-PAGE studies on the enzyme/inhibitor complex revealed that a significant proportion of the complex did not dissociate without reduction, indicating the presence of a covalent bond between enzyme and inhibitor, not usually found in interactions of cystatin with cysteine proteinases (Nicklin and Barrett, 1984).

The formation of the complex, and its nature, was investigated using cystatin isolated from sheep liver, and sub-typed by anion exchange chromatography. The interaction of the isolated inhibitor and cathepsin L, over time, was found to mimic that of the isolated complex, allowing generation of this complex *in vitro*, in a pH-dependent manner.

The availability of human spleen as a fairly convenient source for human cathepsin L became apparent during this study, and use was therefore made of this opportunity to isolate cathepsin L from this source. Cathepsin L has not been purified from human spleen previously, although its presence in this organ has been demonstrated by active site probes (Mason *et al.*, 1989). Cathepsin L could only be isolated in the form of a proteolytically inactive complex from this source, however, limiting its usefulness. The demonstration of the presence of a similar complex in sheep spleen to that in the human spleen, indicated that an organ difference was

responsible for the discrepancy between the results for human spleen and sheep liver, rather than a species difference.

The complexed and free forms of cathepsin L were characterised in terms of their pH optima and stability since both were new forms of cathepsin L. Differences were found between published and measured values of the pH optimum and stability of the single-chain enzyme. Very little difference was found between the complexed and free forms of the enzyme in their pH optima, but differences were found in their pH stability, when a new method for the analysis of this parameter, based on the half-life of the enzyme at various pH values, was applied. The forms of the enzyme were also tested in terms of their interaction with various inhibitors and activators to confirm the identity of the enzyme, and to characterise the accessibility of the active site in the enzyme/inhibitor complex.

4.2 Reagents

Buffer A [20mM Na-acetate, 1mM Na_2EDTA, 0,02% (w/v) NaN_3, pH 5,5]. Glacial acetic acid (2,29 ml), Na_2EDTA (0,75 g) and NaN_3 (0,4 g) were dissolved in 1,9 litres of dist.H_2O, the pH was adjusted to 5,5 with NaOH, and the volume was made up to 2 litres with dist.H_2O.

Buffer B [50mM $NaH_2PO_4.H_2O$, 0,5M NaCl, 0,1% (w/v) Brij 35, 0,02% (w/v) NaN_3, pH 6,5]. $NaH_2PO_4.H_2O$ (6,9 g), NaCl (29,22 g), NaN_3 (0,2 g) and Brij 35 (1 g) were dissolved in 950 ml of dist.H_2O, the pH was adjusted to 6,5 with NaOH, and the volume was made up to 1 litre.

Buffer C [50mM $K_2HPO_4.3H_2O$, 0,5M NaCl, 0,1% (w/v) Brij 35, 0,02% (w/v) NaN_3, pH 11,5]. $K_2HPO_4.3H_2O$ (5,71 g), NaCl (14,61 g), NaN_3 (0,1 g) and Brij 35 (0,5 g) were dissolved in 450 ml of dist.H_2O, the pH was adjusted to 6,5 with NaOH, and the volume was made up to 500 ml.

20mM Ethanolamine, 0,02% NaN_3 (w/v), pH 9,5. Ethanolamine (1,2 ml of a 99% solution, d = 1,016) and NaN_3 (0,2 g) were dissolved in 950 ml of dist.H_2O, the pH was adjusted to 9,5 with HCl, and the volume made up to 1 litre.

Acetate-MES-Tris (AMT) buffer for pH optimum and stability measurements (50mM acetate, 50mM MES and 100mM Tris). Glacial acetic acid (1,43 ml), MES (4,88 g) and Tris (6,06 g) were dissolved in 400 ml of dist.H_2O. This solution was split into 10 aliquots (40 ml), each of which was titrated to pH values in the range of 4-8,5 using HCl or NaOH, and diluted to 50 ml with dist.H_2O.

1% (w/v) gelatin in running gel buffer. Gelatin (0,1 g) was dissolved in running gel buffer (10 ml) for SDS-PAGE (see section 2.6.1), with mild heating.

Overlay buffer for substrate gels [0,05% (w/v) gelatin]. Gelatin (0,005 g) was dissolved in running gel buffer (2 ml) and diluted to 10 ml with dist.H_2O, with mild heating.

0,1% (w/v) amido black. Amido black (0,1 g) was dissolved in methanol: acetic acid: dist.H_2O in the proportions 30:10:60 (100 ml), and filtered through Whatman No. 1 filter paper before use.

S-Sepharose fast flow. S-Sepharose was prepared by diluting 70 ml of the supplied hydrated gel in 140 ml of buffer A, and packing the resulting slurry into a glass column under gravity. The column bed was sealed by a plunger, and initially regenerated with two column volumes of 1M NaCl in buffer A. The gel was equilibrated with four column volumes of 200mM NaCl in buffer A, before use. In between purification procedures the column was routinely regenerated with one column volume of 1M NaCl in buffer A.

Q-Sepharose fast flow. Q-Sepharose (25 ml) was prepared in the same way as the S-Sepharose, except that all preparation steps were carried out in 20mM ethanolamine buffer, pH 9,5.

Sheep liver. Fresh sheep livers were obtained from Cato Ridge abattoir and frozen at -70°C for at least 3 days before use. Livers were not generally frozen for longer than 3 weeks before use.

Human spleen. This was obtained from the Dept. of Surgery, Medical School, University of Natal, immediately following surgery, and frozen at -70°C. The spleen obtained for this study was removed from a patient suffering from congestive splenomagaly and portal hyperplasia, probably as a secondary effect of bilharzia infection. The spleen was screened by the Natal Institute of Immunology and was found to be negative for both HIV or hepatitis B infection.

4.3 Procedures

4.3.1 Purification of cathepsin L from sheep liver

4.3.1.1 Preparation of autolysed pH 4,2 supernatant. Livers were treated as described by Mason (1986). Frozen liver was thawed, minced and mixed 1:2 with a solution of 2% (v/v) n –Butanol/1% (w/v) NaCl/0.1% (w/v) EDTA, and homogenized in a Waring blendor for 2,5 min. The resulting homogenate was centrifuged (6 500 x g, 30 min, 4°C). The supernatant was adjusted to pH 4,2 with HCl, and stirred for 16 h at 20°C, to effect activation of the enzyme. The mixture was centrifuged (6 000 x g, 20 min, 4°C) and the supernatant was used for ammonium sulphate and TPP fractionation.

4.3.1.2 Optimisation and comparison of ammonium sulphate precipitation and TPP. Ammonium sulphate precipitation and TPP were tested for their relative effectiveness in the purification of cathepsin L from bovine, sheep and rabbit livers. The fractionation of the pH 4,2 extract, prepared as described above, in each case, was optimised as follows:

1) Ammonium sulphate precipitation was optimised in an experiment in which fractions, obtained by 10% increments in $(NH_4)_2SO_4$ saturation, were assayed for protein content and cathepsin L activity. A fraction, obtained by a cut from 30–65% saturation, at pH 4,2 and 4°C, was found to be optimal, for the livers of all three species.
2) TPP was optimised in a similar manner, except that 5% increments in $(NH_4)_2SO_4$ were used. An optimal fraction was reproducibly obtained from the pH 4,2 supernatant, from the livers of all three species, by cutting between 20% and 30% $(NH_4)_2SO_4$, at pH 4,2, in the presence of 30% t–butanol.

4.3.1.3 Purification procedure. For the purification of cathepsin L from sheep liver, the liver was treated as outlined above. TPP was effected on the pH 4,2 supernatant by adding and mixing in 30% (v/v) of t-butanol (in this step, initially, the supernatant was at 4°C and the t-butanol was at 25°C; subsequent steps were done at 4°C). Ammonium sulphate (20% w/v) (based on the original volume of supernatant plus t-butanol) was added and dissolved by gentle stirring. The resulting mixture was centrifuged (6 000 x g, 10 min, 4°C) in a swing-out rotor. The TPP supernatant and subnatant were poured away from the precipitate, which was discarded. A further amount of $(NH_4)_2SO_4$ was added, to bring the solution to 30% (w/v) $(NH_4)_2SO_4$, and well stirred. Upon complete dissolution of the salt, the mixture was again centrifuged (6 000 x g, 10 min, 4°C), and the interfacial precipitate was collected for further study.

The precipitate from TPP was redissolved, in one fifth of the pH 4,2 supernatant volume, in buffer A. The pH was readjusted to 5,5, and the resuspended material

was centrifuged (27 000 x g, 10 min, 4°C) to remove any insoluble material. The supernatant was loaded directly onto an S–Sepharose column (2,5 x 14 cm = 70 ml), equilibrated with buffer A, containing 200mM NaCl, and eluted with a 200-600mM NaCl gradient in buffer A. Fractions corresponding to the peak of activity, eluted at about 400mM NaCl, were pooled and dialysed against 10 volumes of buffer A, pH 4,5, containing 200mM NaCl, for 10 h. The dialysed solution was re-applied to the S–Sepharose column, equilibrated with buffer A, pH 4,5, containing 200mM NaCl, and eluted with a 200-600mM NaCl gradient. The active fraction was pooled, concentrated by dialysis against sucrose and analysed by SDS–PAGE.

The purification procedure was later revised to include chromatography on a calibrated column of Sephadex G-75 (2,5 x 90 cm = 450 ml) at a flow rate of 25 ml/h, in place of the pH 4,5 S-Sepharose chromatography. Peaks with activity against azocasein or Z-Phe-Arg-NHMec (K_{av} = 0,27 and 0,43) were collected for further study.

4.3.2 The purification of cathepsin L from human and sheep spleen. The purification of human and sheep spleen cathepsin L was carried out as described for the sheep liver cathepsin L.

4.3.3 Characterisation of free and cystatin-complexed cathepsin L from sheep liver, human spleen and a commercial source

4.3.3.1 Measurement of inhibition characteristics. Inhibition by iodoacetate, pepstatin and PMSF and activation by DTT were measured at the concentrations described by Kirschke *et al.* (1977). Inhibition by leupeptin, Z-Phe-Tyr-(O-t-Bu)-CHN_2 and Z-Phe-Phe-CHN_2, was tested at a range of concentrations from 10^{-5} to 10^{-9}M. Inhibitors were incubated together with the enzymes for 15 min at 30°C before the enzymes were assayed at pH 5,5 against Z-Phe-Arg-NHMec, as described previously (section 2.4.2).

The inhibitor E-64 was used for active site titration of the different forms of the sheep liver cathepsin L, as described by Barrett and Kirschke (1981). Enzyme (25 µl) was incubated together with the inhibitor (25 µl of a 1-10µM solution) for 30 min at 30°C in the presence of the pH 5,5 assay buffer, containing activator (50 µl). After 30 min the mixture was diluted to 1 ml by the addition of 0,1% Brij 35, and aliquots (25 µl) were assayed for activity as described previously. For the titration of complex against azocasein using E-64, the above procedure was followed but the incubation mixture with E-64 was not diluted for the assay.

4.3.3.2 Measurement of pH-dependent characteristics. To determine their pH optima, the enzymes were assayed in a buffer of uniform ionic strength across the pH range. This

was to avoid any effects due to the buffer or differing ionic strength, and the AMT buffer described by Ellis and Morrison (1982) (see section 4.2) was used for this purpose.

The pH optimum was tested in a standard 10 min stopped time assay against Z-Phe-Arg-NHMec at 30°C, in the buffers at pH 4-8,5, each containing 8mM DTT. The pH stability of the enzymes was tested according to Mason (1986), by incubating the enzyme in diluent (250 µl) and the AMT buffers (250 µl) at various pHs (containing no DTT) for 1 h at 37°C, following which pH 5,5 assay buffer (250 µl), containing 8mM DTT, was added and a 10 min stopped time assay against Z-Phe-Arg-NHMec was carried out at 30°C. For the pH stability test as described by Kirschke *et al.* (1989), the enzyme was incubated in diluent (500 µl) and AMT buffers (250 µl), in the presence of 2mM DTT, for 1 h at 37°C, the Z-Phe-Arg-NHMec substrate (250 µl) was added, and a 10 min stopped time assay was carried out at 37°C. All the above tests were carried out in quintuplicate for each pH.

The half-lives of the free and cystatin-complexed cathepsin L from sheep liver, were measured at the various pHs by a modification of the method of Kirschke *et al.* (1989). The enzymes were incubated in the diluent (500 µl) and AMT buffers (250 µl) at 37°C, in the presence of 2mM DTT, and, at 5, 10, 20, 30, 45 and 60 min intervals, Z-Phe-Arg-NHMec substrate (250 µl) was added, and an assay was carried out at 37°C for 10 min. The effect of each pH, on each form of enzyme, was tested in triplicate for each time interval. At each pH, the logarithms of the mean activity values were plotted against time, and the lines generated were analysed by linear regression, and the slopes of the lines were used to calculate k_{obs} and $t_{½}$ values, using the equations (Segel, 1976):

$$k_{obs} = \text{slope} \times 2,3$$

and

$$t_{½} = 0,693/k_{obs}$$

4.3.4 Purification and sub-fractionation of cystatin from sheep liver

A cystatin fraction was isolated from sheep liver as described by Green *et al.* (1984), for the isolation of the inhibitor from human liver. Frozen sheep liver (300 g) was thawed, minced and mixed 1:2 with 1% (w/v) NaCl/2% (v/v) n-butanol/3mM disodium EDTA for 2,5 min, at 4°C, in a Waring blendor. This homogenate was centrifuged (2 000 x g, 30 min, 4°C) and the resulting supernatant was filtered through glass wool under vacuum. The supernatant was adjusted to pH 11,0 by the addition of 3M NaOH, with stirring. This solution was left to stir for 2 h at 4°C, following which the pH was adjusted to 6,5 by the gradual addition of 2M HCl. Precipitated protein was removed by centrifugation (2 000 x g, 30 min, 4°C).

The supernatant (at 4°C) was placed in an ice/NaCl bath and an equal volume of acetone at -20°C was run in, with stirring, over a period of 10 min. The mixture was centrifuged

(2 000 x g, 30 min, 4°C) and the precipitate was discarded. Acetone was added to the supernatant, in the same volume as before, with stirring, over 10 min. The mixture was centrifuged as before (2 000 x g, 30 min, 4°C) and the precipitate was collected. The pellet was extracted overnight at 4°C, with stirring, in 50 ml of Buffer B. The undissolved material was removed by centrifugation (10 000 x g, 30 min, 4°C).

The redissolved protein was applied to a CM-papain Sepharose column (2,5 x 5 cm = 25 ml) equilibrated in buffer B. The column was washed till the A_{280} reached baseline, whereupon the bound protein was eluted using Buffer C. The single peak of protein collected was adjusted to pH 6,5, and concentrated against sucrose in M_r 12 000 cut-off dialysis tubing (Sigma).

The cystatin fraction from CM-Papain (18 ml) was applied to a Sephadex G-25 column (2,5 x 40 cm = 400 ml), previously equilibrated in 20mM ethanolamine, pH 9,5, and eluted at a flow rate of 50 ml/hr. The desalted cystatin fraction was applied to a Q-Sepharose column (1 x 25 cm = 20 ml), equilibrated in the same buffer. Bound cystatin fractions were eluted at 10 ml/hr with two gradients: one from 0-200mM NaCl, followed by another from 200-1000mM NaCl, both in 6 column volumes of ethanolamine buffer. The fractions collected were assayed for their inhibitory activity against 10μM papain, and active fractions were pooled and concentrated against sucrose in M_r 12 000 cut-off dialysis tubing.

4.3.5 Substrate SDS-PAGE

The procedure for SDS-PAGE was modified from that described in section 2.6.2, in that 0,1% (w/v) gelatin was incorporated into the gel, to allow the detection of proteinases (Heussen and Dowdle, 1980). This was carried out by adding 1% (w/v) gelatin in running gel buffer (1,5 ml), to running gel buffer (2,25 ml) and the rest of the solution for casting a 12,5% gel (section 2.6), all at 37°C, and pouring the gel as quickly as possible. The gel was overlaid with the overlay solution for substrate gels, and allowed to set. The SDS-PAGE was carried out as normal.

Once the electrophoresis was finished, the running gel was soaked in two changes of 2,5% (w/v) Triton X-100 (50 ml) over 1 h at RT. Following this, the gel was incubated in the pH 5,0 assay buffer, containing 40mM cysteine (50 ml), described for azocasein assays (section 2.4.1), for 3 h at 37°C. It was stained in 0,1% (w/v) amido black solution for 1 h, and then destained in several changes of methanol: acetic acid: dist.H_2O (30: 10: 60). The presence of proteolytically active components in the gel was indicated by clear bands on the gel after staining due to the digestion of the gelatin.

4.4 Results

4.4.1 Optimisation and comparison of ammonium sulphate precipitation and TPP for the fractionation of pH 4,2 supernatants from sheep, bovine and rabbit livers. The results obtained from a comparative study of the relative merits of TPP and ammonium sulphate precipitation, applied to the crude fractionation of cathepsin L from bovine, sheep and rabbit livers, are presented in Table 3.

Table 3: A comparison of TPP and ammonium sulphate precipitation for the crude fractionation of cathepsin L from sheep, rabbit and bovine livers.

Step	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Sheep:			
pH 4,2 Supernatant	5	1	100
$(NH_4)_2SO_4$ precipitation	7	1.3	58
TPP	18	3.5	60
Rabbit:			
pH 4,2 Supernatant	11.6	1	100
$(NH_4)_2SO_4$ precipitation	5.8	0.5	29
TPP	16.7	1.44	11
Bovine:			
pH 4,2 Supernatant	0.86	1	100
$(NH_4)_2SO_4$ Precipitation	7.6	8.8	95
TPP	9	10.5	48

A comparison of the specific activities obtained suggested that TPP was superior to ammonium sulphate precipitation in the case of rabbit and sheep livers, but was only marginally superior in the case of bovine livers. The results support the findings of Mason (1986), in that the yield of cathepsin L–like activity from sheep liver was much higher than that from the livers of the other two species. The electrophoretic patterns of the various crude fractions (Fig. 9) also demonstrate the above findings, in that the TPP samples for rabbit and bovine cathepsin L, are much purer than the ammonium sulphate sample.

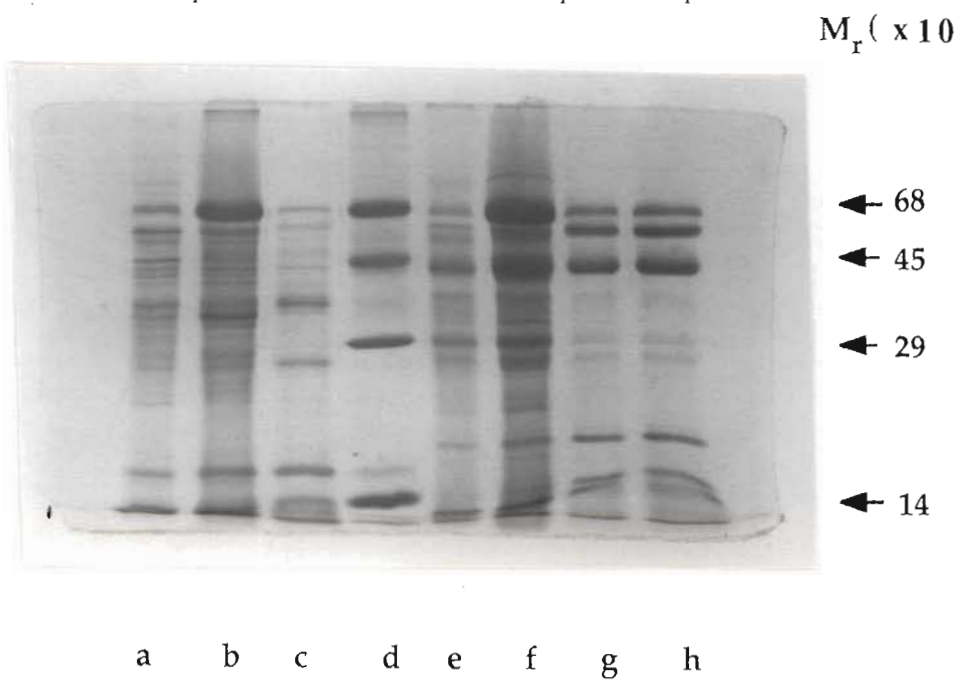


Figure 9: SDS-PAGE of TPP and ammonium sulphate precipitation fractions of the pH 4,2 supernatants from bovine and rabbit livers.
a) pH 4,2 supernatant; b) ammonium sulphate precipitation fraction; and c) TPP fraction from bovine liver. d) MW standards, BSA (M_r 68 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 29 000) and lysozyme (M_r 14 000). e) pH 4,2 supernatant; f) ammonium sulphate precipitation; and g) and h) TPP fractions from rabbit liver.

4.4.2 Purification of sheep liver cathepsin L. The results above suggest that TPP may be superior to ammonium sulphate precipitation for the crude fractionation of cathepsin L from livers of various species. Three-phase partitioning, however, must also be considered in relation to the chromatography steps subsequently needed to finally purify the enzyme. After TPP, the sample had a much lower salt concentration, than after ammonium sulphate precipitation, and could therefore be applied directly to the S–Sepharose column, without prior desalting.

Equilibration of the column in relatively high salt concentrations (200mM), resulted in a larger unbound peak and a lesser amount of protein being bound to the column. Resolution of the cathepsin L peak from other bound proteins was thus simplified (Fig. 10), and a large increase in specific activity was achieved in the first chromatography step (Table 4). In spite of this, and the fact that cathepsin L activity eluted in a seemingly homogenous peak, the active fraction was not pure when analysed by SDS–PAGE (Fig. 12). Rechromatography at pH 4,5 removed the remaining contaminants (Fig. 11), resulting in an apparently pure fraction, as judged by SDS–PAGE (Fig. 12).

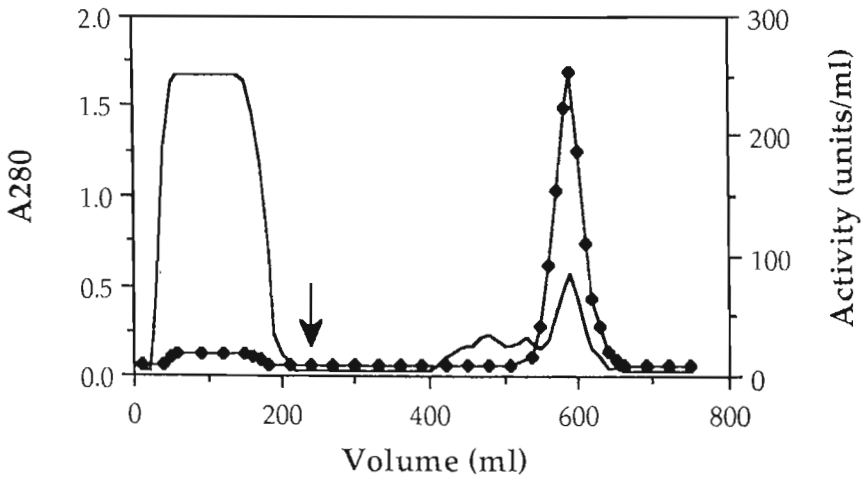


Figure 10. S–Sepharose chromatography, at pH 5,5, of TPP fraction containing sheep liver cathepsin L.

Column, (2.5 x 14.5 cm = 70 ml bed volume); Buffer, buffer A with 200mM NaCl, followed by 200–600mM NaCl gradient, in 5 column volumes, in buffer A, applied at point ↓, followed by 2 column volumes of buffer A containing 600mM NaCl. Flow rate, 50 ml/h (10 cm/h); Fractions: 10 ml (12 min.). (–), A_{280} and (◆), enzyme activity (units/ml) from the azocasein assay.

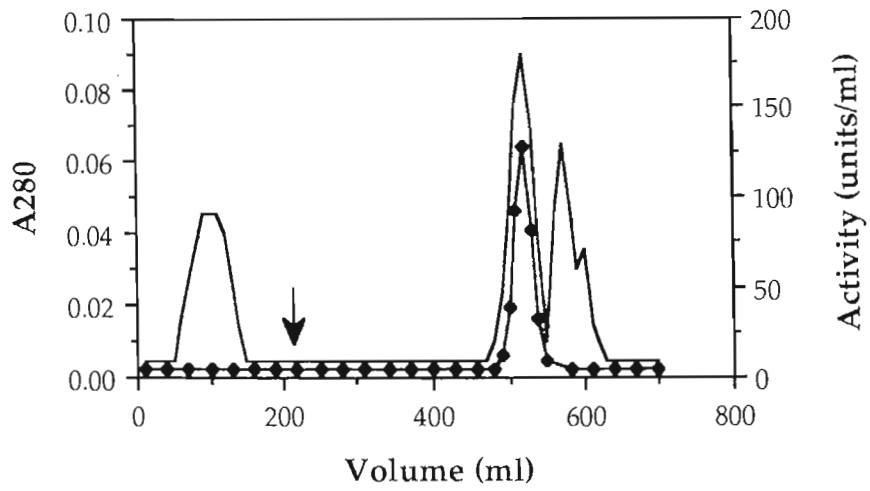


Figure 11. Rechromatography on S–Sepharose, at pH 4,5, of fraction containing cathepsin L. As for Fig. 10, except that all buffers were at pH 4.5. (–), A_{280} and (◆), enzyme activity (units/ml) from the azocasein assay.

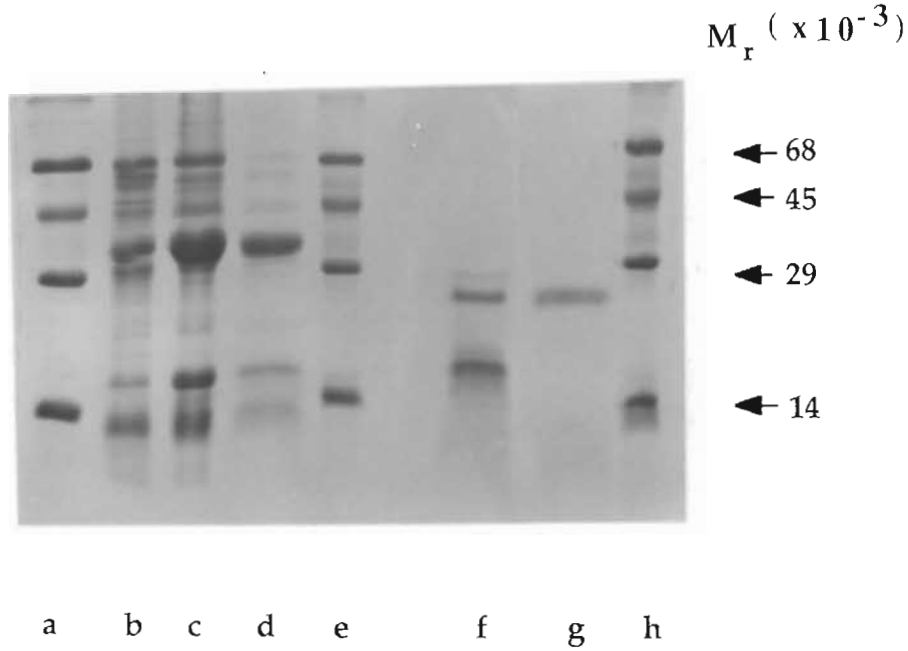


Figure 12. Reducing SDS–PAGE of fractions obtained during isolation and purification of cathepsin L from sheep liver.
a) Molecular weight standards, BSA (M_r 68 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 29 000) and lysozyme (M_r 14 000); b) supernatant after pH 4.2 precipitation; c) TPP cut - 20–30% ammonium sulphate; d) unbound material from S-Sepharose, pH 5.5; e) MW standards; f) S-Sepharose pH 5.5 fraction; g) S-Sepharose, pH 4.5 fraction; h) MW standards.

Immunoblotting analysis of antibodies produced against this apparently pure fraction revealed that, in addition to cathepsin L, they strongly targeted a M_r 14 000 band in crude and isolated cathepsin L fractions (see Fig. 16). The presence of this contaminant was confirmed by SDS-PAGE analysis of fifty times concentrated samples of this fraction, as opposed to the five times concentrated samples used previously.

Sephadex G-75 chromatography (Fig. 13) revealed that the active peak from ion-exchange chromatography could be resolved into two peaks, with M_r 37 000 and 26 000. In the first few isolations, the M_r 37 000 peak was not active against azocasein, while the M_r 26 000 peak was. Later the situation changed, for no discernible reason, to both peaks having activity against azocasein. None of the conditions under which cathepsin L was isolated were changed and thus reasons for this change are not known. The two peaks both specifically manifested cathepsin L activity in being active against Z-Phe-Arg-NHMec, but not against Z-Arg-Arg-NHMec, the synthetic substrate for cathepsin B.

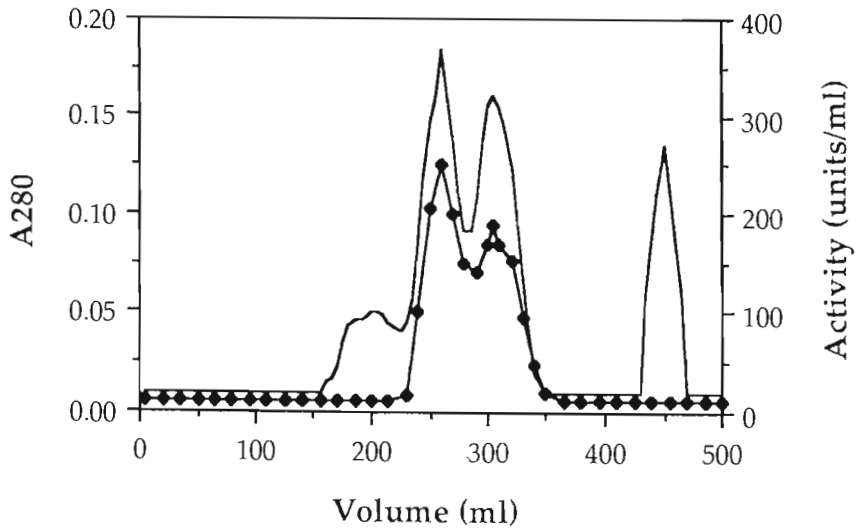


Figure 13. Molecular exclusion chromatography of pH 5,5 S-Sepharose fraction on Sephadex G-75.

Column, 2.5 x 87.5 cm (430 ml bed volume); Buffer, buffer A with 200mM NaCl; Flow rate, 25 ml/h (5 cm/h); Fractions, 5 ml (12 min). Void volume was 150 ml. (–), A_{280}; (◆), enzyme activity (units/ml) from the azocasein assay.

Table 4: Purification of sheep liver cathepsin L.

Step	Vol. (ml)	Total Protein (mg)	Total Activity (mU)	Specific Activity (mU/mg)	Purification (fold)	Yield (%)
Homogenate pH 4,2	970	18965	62434	3,3	1	100
supernatant	670	2401	36760	15,3	4,6	59
TPP	160	1249	24810	20	6	40
S-Sepharose pH 5,5	40	4,37	7312	1673	507	12
MEC (complex)	13,5	0,49	1462	2984	904	2,3
MEC (free)	18	0,55	1232	2240	679	2

The final isolation procedure for cathepsin L was successful in the purification of highly pure cathepsin L (Table 4). The yield was approximately 0,55 mg from 400 g of sheep liver which was lower than that reported previously for sheep liver (Mason, 1986), possibly due to the isolation, in this study, of a portion of the enzyme complexed to inhibitor.

As shown in Fig. 14, the purified cathepsin L exhibits only one band, with and without reduction. This indicates that cathepsin L was isolated as a single-chain form by this procedure, since no change in MW was observed with or without reduction. This is in contrast to the previous isolation procedure for sheep liver cathepsin L by (Mason, 1986), where a two-chain form of cathepsin L was isolated. The purified cathepsin L was used to immunise both rabbits and chickens, the former as described previously (Mason, 1986), and the latter as part of a novel study on the differences which may occur in antibodies produced against cathepsin L in the different experimental animals.

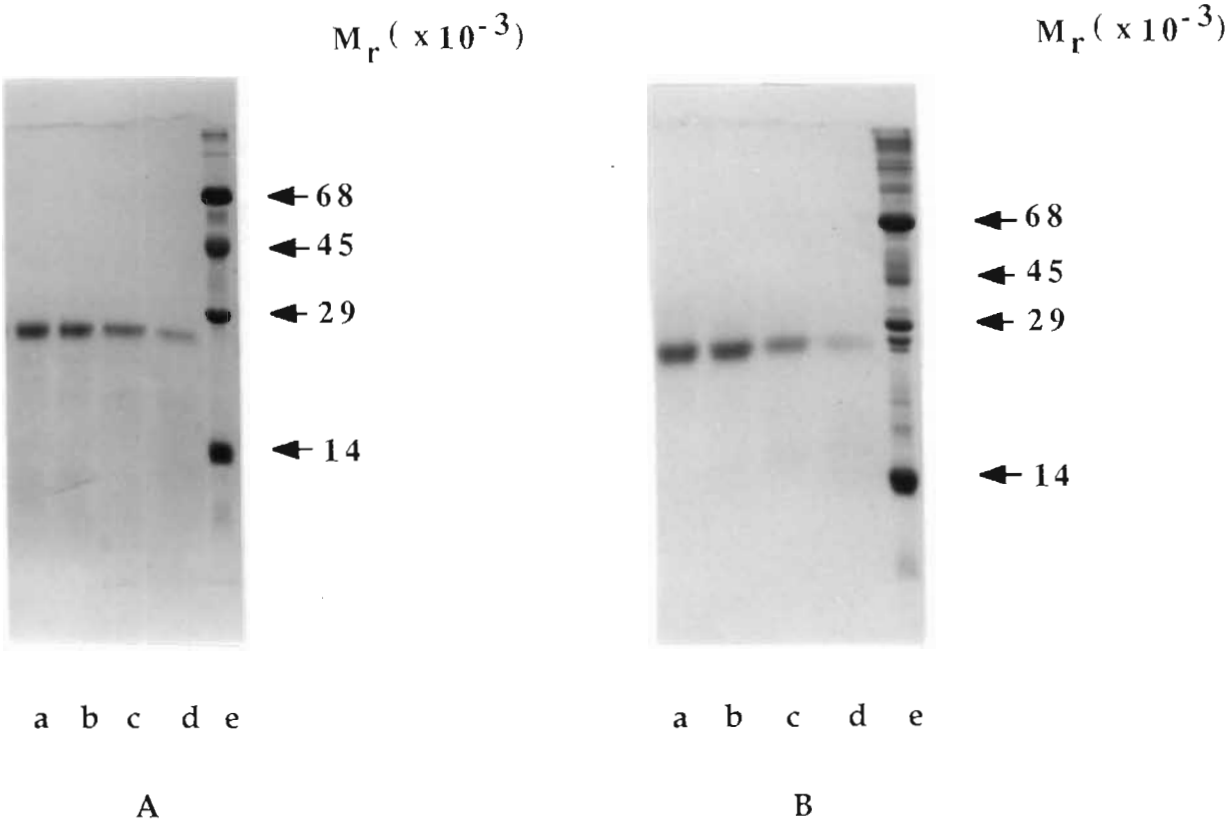


Figure 14: SDS-PAGE of reduced and non-reduced single-chain cathepsin L.
A) reduced free cathepsin L, a) 5 µg; b) 2,5 µg; c) 1 µg; d) 0,5 µg; e) reduced MW standards (BSA, M_r 68 000; Ovalbumin, M_r 45 000; Carbonic Anhydrase, M_r 29 000; Lysozyme, M_r 14 000). B) free cathepsin L without reduction, a) 5 µg; b) 2,5 µg; c) 1 µg; d) 0,5 µg; e) non-reduced MW standards (BSA, M_r 68 000; Ovalbumin, M_r 45 000; Carbonic Anhydrase, M_r 29 000; Lysozyme, M_r 14 000)

On SDS-PAGE with reduction, the M_r 37 000 peak separated into two components, of M_r 26 000 and M_r 14 000, while the M_r 26 000 peak consisted of a single protein (Fig. 15). The molecular weight of the smaller component of the M_r 37 000 peak (M_r 14 000, on SDS-PAGE) is very similar to that reported for the natural inhibitor of cysteine proteinases, cystatin (Anastasi *et al.*, 1983) and this fact, together with the initial inactivity of the M_r 37 000 peak against azocasein, led to the tentative hypothesis that the higher MW peak might consist of cathepsin L complexed to cystatin. The appearance of the complex on SDS-PAGE, without reduction, will be described below in section 4.4.4.

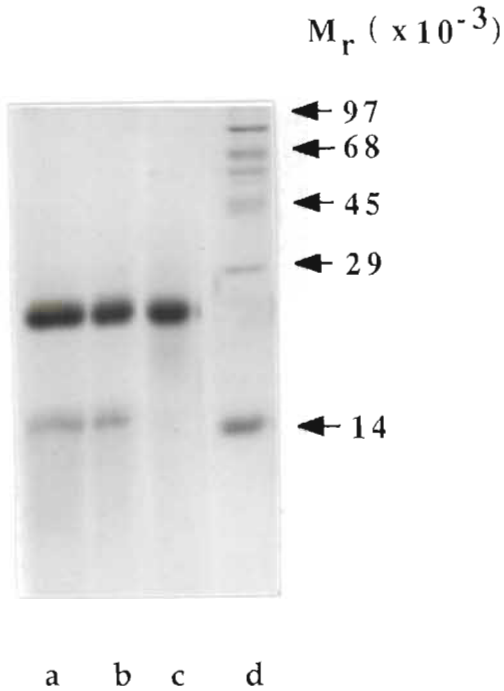
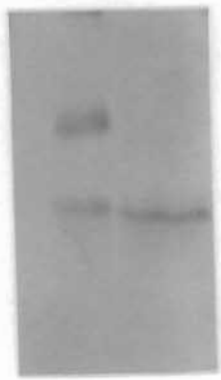


Figure 15: SDS-PAGE of reduced free and cystatin-complexed sheep liver cathepsin L. 10 μg (protein) of each sample was loaded onto 12,5% gels. a) and b), sheep liver cathepsin L complexed with cystatin reduced; c) free sheep liver cathepsin L; d) M.W. markers (Phosphorylase b, M_r 97 000; BSA, M_r 68 000; Ovalbumin, M_r 45 000; Carbonic anhydrase, M_r 29 000; Lysozyme, M_r 14 000)

In order to test the above hypothesis, a cystatin fraction was isolated from sheep liver by the method of Green *et al.* (1984), and the immune cross-reactivity of the isolated cystatin was compared with components of the complex. This was done using antibodies raised to the complex at a stage when it was mistakenly thought to be pure cathepsin L. Immuno-blotting analysis confirmed the hypothesis, since the anti-complex antibodies cross-reacted specifically with the M_r 14 000 component (Fig. 16).



a b

Figure 16: Western blot of anti-cystatin-complexed cathepsin L antibodies against the complex and a sheep liver cystatin fraction.
a), cathepsin L complexed to cystatin (10 µg), and b) the sheep liver cystatin fraction (10 µg) were electrophoresed on 12,5% SDS-PAGE, transferred to nitrocellulose, reacted with 0.5 mg/ml anti-complex IgG and the reaction was visualised using the sheep anti-rabbit-HRPO system as described in section 2.8.2.

4.4.3 Sub-fractionation of a cystatin fraction from sheep liver. The isolation method of Green *et al.* (1984) [see 4.3.4], for the purification of a cystatin fraction from sheep liver, proved to be very simple to follow, and a single peak was eluted from the CM-papain Sepharose affinity column. This peak was found to be inhibitory towards papain (the method for this assay is not shown, but it is the same as that for cathepsin L, except that papain was used and the inhibitor fractions [50 µl] were incorporated into the assay), confirming that it was an active cystatin fraction. This cystatin fraction was sub-fractionated on a Q-Sepharose anion-exchange column, as described by Abrahamson *et al.* (1986), for cystatins from human urine. The results (Fig. 17) show that very little unbound material was eluted, while the first gradient eluted three inhibitory sub-fractions, and the second gradient eluted another inhibitory peak, not completely resolved from a non-inhibitory protein peak.

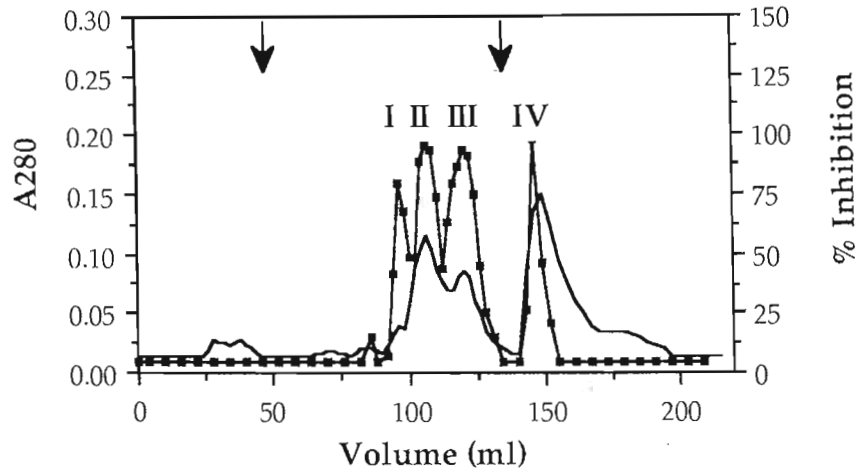


Figure 17: Anion exchange chromatography of sheep liver cystatin fraction on Q-Sepharose. Column, 1 x 24.5 cm (20 ml bed volume); Buffer, 20mM ethanolamine, pH 9,5, followed by a 0-200mM NaCl gradient, in 5 column volumes, in the same buffer, followed by a 5 column-volume gradient (200-1000mM NaCl).The buffer changes are indicated by ↓. Flow rate, 10 ml/h (13 cm/h); Fractions, 2 ml (12 min.). (–), A_{280} and (■), inhibitor activity, expressed as the percentage inhibition of 10µM papain activity against Z-Phe-Arg-NHMec.

SDS-PAGE analysis of the cystatin sub-fractions (Fig. 18 A and B) showed that they were all reduced to a common size of M_r 14 000 by reduction, but without reduction several differences were noticed. Inhibitory sub-fraction I still ran as a M_r 14 000 band, while sub-fraction II seemed to run at a slightly higher MW. Sub-fraction III appeared to be a dimer with M_r 28 000. Sub-fraction IV ran as a M_r 14 000 band contaminated by several proteins. These contaminants did not seem to be inhibitors, as they were more visible in a later fraction, which had much less inhibitory activity than sub-fraction IV. The inhibitory activity in sub-fraction IV therefore seems to correlate with the M_r 14 000 band.

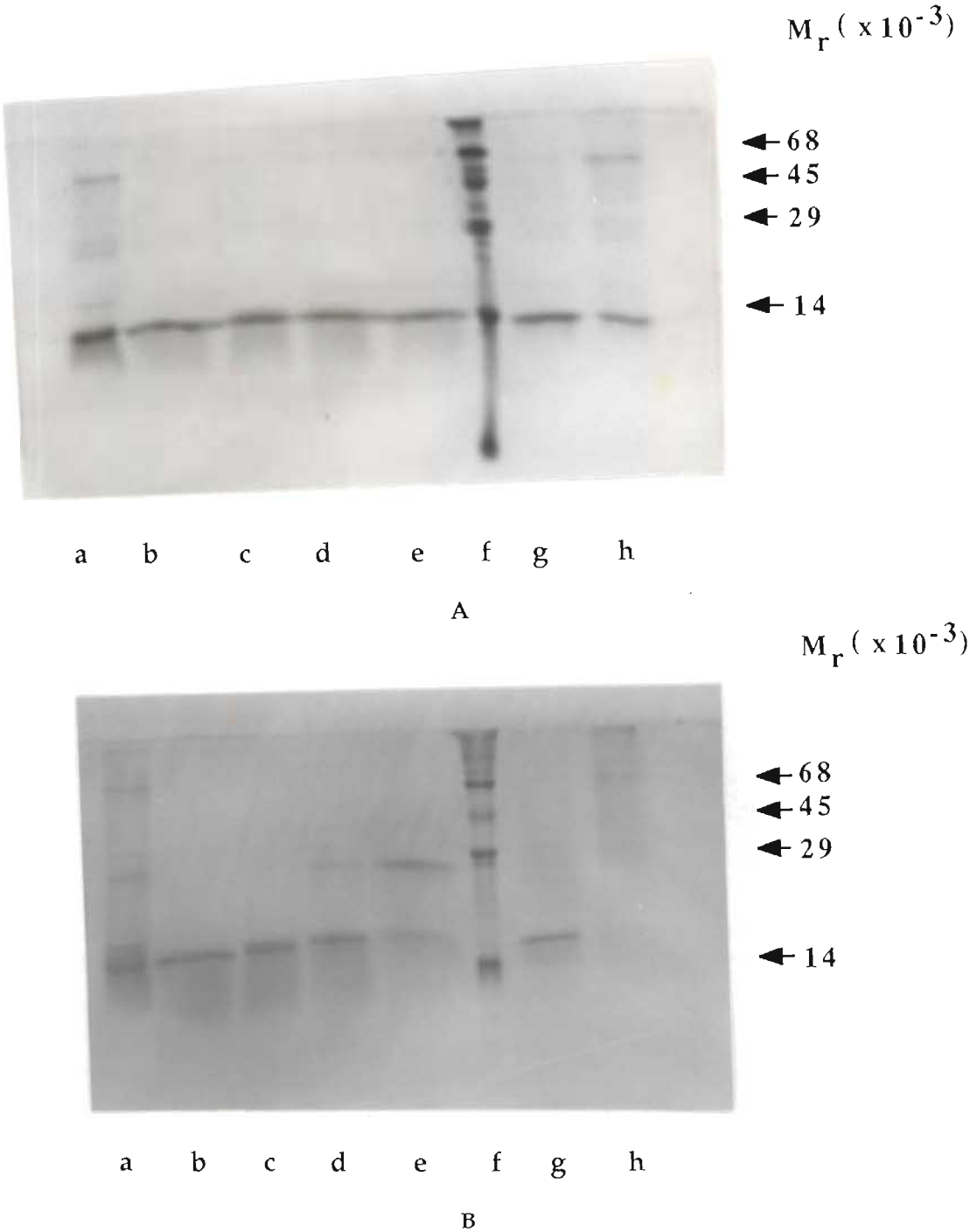


Figure 18: SDS-PAGE of sub-fractions from anion exchange chromatography of sheep liver cystatin fraction.

A, reduced sub-fractions and B, non-reduced sub-fractions on 15% SDS-PAGE. a) sheep liver cystatin fraction; b) sub-fraction I; c) sub-fraction II; d) sub-fraction intermediate to sub-fractions II and III; e) sub-fraction III; f) MW markers (BSA, M_r 68 000; Ovalbumin, M_r 45 000; Carbonic Anhydrase, M_r 29 000; Lysozyme, M_r 14 000); g) sub-fraction IV; h) fractions after sub-fraction IV on the salt gradient.

The different inhibitory sub-fractions were analysed on a continuous PAGE system, devised by using the running gel buffer in the stacking gel as well, which ensured that the pH of

the electrophoresis system was above 8,3 at all times. This was done in order that cystatin B, which has a neutral pI in the region of 6-7, would run towards the positive pole, which it might not in the stacking gel buffer, of pH 6,8. The inhibitors showed a PAGE pattern (Fig. 19) that one might expect from fractions separated on an anion-exchanger i.e. sub-fraction I ran slower than sub-fraction II, while sub-fraction IV ran relatively far down the electrophoretogram. Sub-fraction III ran intermediate to sub-fractions I and II, a result which may be explained by its being a dimer, and therefore running more slowly due to its higher MW, in spite of having a higher negative charge, and therefore eluting later on Q-Sepharose.

Figure 19: Continuous PAGE of sheep liver cystatin sub-fractions.

2 µg of each sub-fraction was applied to the continuous 7,5% PAGE. Each sub-fraction was also treated with 1mM DTT for 10 min at RT and loaded in alternate lanes (b,d,f,h and j)

a) and b) sub-fraction I; c) and d) sub-fraction II; e) and f) sub-fraction intermediate to sub-fractions II and III; g) and h) sub-fraction III; i) and j) sub-fraction IV;.

Two sub-fractionations of cystatin fractions from liver have been reported previously. Wakamatsu *et al.* (1984) fractionated rat liver cystatin into three sub-fractions: TPI-1, TPI-2 and TPI-D. The TPI-D fraction was a dimer form of the inhibitor, while TPI-1 and 2 were both M_r 14 000 forms on SDS-PAGE. On PAGE, TPI-2 migrated faster than TPI-1, while TPI-D migrated intermediate to these two. When the inhibitors were compared in terms of their inhibitory capabilities in the absence of a reducing agent, it was found that only TPI-1 was active, but the addition of 1mM DTT activated TPI-2 and TPI-D. The mobility of TPI-1 on PAGE was unaffected by treatment with DTT, while similar treatment of TPI-2 and TPI-D resulted in their mobility being changed to the same as that of TPI-1. Thus the removal of a negative substituent from TPI-2 by reduction, converted the inhibitor to an active form, while reduction of the TPI-D dimer also activated it. The component attached to TPI-2 was identified as glutathione, attached to the cysteine residue at position 3, in cystatin β. The attachment of

differentially charged groups to TPI-2 showed that negatively charged substituents on Cys 3 inactivated the inhibitor, while positive or neutral substituents had no effect. The TPI-1 form was also shown to have a free cysteine residue at position 3, but it was not as reactive as that in TPI-2, for an unknown reason. Thus all the forms of inhibitor from rat liver were shown to be cystatin β, a result confirmed by later immunoassay results on the distribution of the different types of cystatins in the various rat tissues, where it was shown that the rat liver contained only cystatin β in significant amounts (Kominami *et al.*,1984).

Green *et al.* (1984) fractionated human liver cystatins on a chromatofocusing system, on the basis of their pI values. They found that the cystatins could be separated into several basic sub-fractions and one acidic sub-fraction. The acidic sub-fractions were immunologically distinct from the basic sub-fraction and characterisation of the sub-fractions, by sequence analysis, showed that they were cystatins A and B respectively. Cystatin A did not have a free cysteine in its structure, while cystatin B had a free cysteine at position 3, and in this respect, and others, was very similar to cystatin β from rat liver.

At first it seemed that the situation in sheep liver was a mixture of the above situations for rat and human liver, with sub-fraction I being similar to TPI-1, sub-fraction II being similar to TPI-2, sub-fraction III being similar to TPI-D and sub-fraction IV most resembling cystatin A from human liver, due to its high mobility on PAGE, indicating an acidic pI. As a final characterisation, the sub-fractions were run on PAGE, with and without DTT treatment (Fig. 19). Sub-fractions I-III fell within predictions, based on the results of Wakamatsu *et al.* (1984), since DTT treatment caused them to run with the same mobility, while without this treatment, sub-fractions I-III had differently mobilities. The result for sub-fraction IV was surprising, however, in that it was also converted to run similarly to sub-fraction I. This indicated that all the forms of cystatin in the sheep liver were β-type cystatins, since all of them seem to contain a reactive cysteine residue, which is affected by treatment with a reducing agent. Thus the situation in sheep liver seems to parallel that of rat liver quite closely, although an additional form of the cystatin β-type of inhibitor, presumably with a very strongly negatively-charged substituent attached to it, was found in the sheep liver. The different forms of cystatins were used to investigate if any one cystatin sub-fraction was more active in the formation of active complex with cathepsin L, thereby giving some information about the mechanism for the formation of the complex.

4.4.4 Characterisation of the cystatin/cathepsin L complex and studies on the formation of the complex *in vitro* with cystatin β-type inhibitor sub-fractions. The enzyme/inhibitor complex was split into M_r 26 000 and 14 000 components on SDS-PAGE with reduction (Fig. 15). Anastasi *et al.* (1983) and Nicklin and Barrett (1984), showed that complexes of chicken egg

white (CEW)- cystatin with papain dissociate on SDS-PAGE with or without reduction. This was not found to be the case with the complex isolated here, however, since M_r 68 000, 42 000, 37 000, 26 000 and 14 000 components were found on SDS-PAGE without reduction (Fig. 20), indicating that a proportion of the complex does not dissociate without reduction. This in turn indicates that a proportion of the inhibitor is covalently attached to the enzyme, possibly represented by the M_r 68 000, 42 000, 37 000 forms, while a further proportion is non-covalently bound in a more normal manner, possibly represented by the M_r 26 000 and 14 000 components.

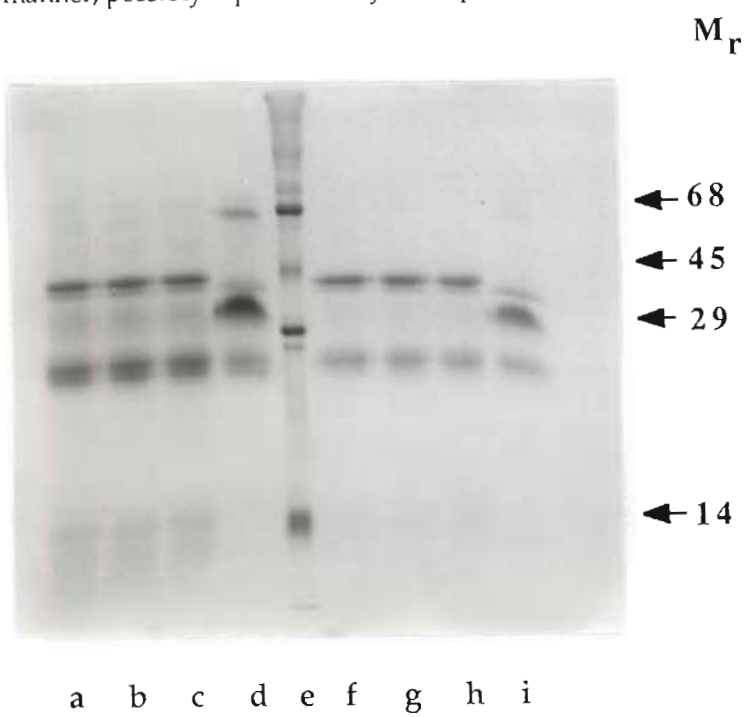


Figure 20: SDS-PAGE, without reduction, of the isolated cystatin complexed sheep liver cathepsin L , treated at pH 4,2.
The isolated cystatin-complexed cathepsin L (10µg) was incubated at pH 4,2 at 37°C, for a) 5 min; b) 30 min; c) 60 min; or d) untreated. e) MW markers (BSA, M_r 68 000; Ovalbumin, M_r 45 000; Carbonic Anhydrase, M_r 29 000; Lysozyme, M_r 14 000) and f), g), h) and i) are 5 µg of the complex treated in the same order.

Incubation of the complex at pH 4,2 was thought to have the potential to disrupt the complex, and remove the cystatin from cathepsin L, as it was at this pH that the activation of cathepsin L (by removal of bound inhibitors) was found to be maximal (Mason *et al.*, 1985). Incubation at pH 4,2, before SDS-PAGE without reduction, had the effect of converting the covalently-bound complex at M_r 37 000 to a M_r 45 000 form, the M_r 68 000 form was lost and the M_r 42 000 and 26 000 forms remained as they were (Fig. 20). This difference was noted even after very short incubation times, of the order of 5 min at pH 4,2.

The fact that the cathepsin L/cystatin complex, isolated by a purification procedure involving autolysis (see section 4.3.1.1), was proteolytically active, led to concern that autolysis

might be playing some part in producing this seemingly anomalous activity. Purification procedures with and without autolysis were therefore undertaken, on portions of the same liver, to investigate the effect of autolysis. The activities against azocasein and Z-Phe-Arg-NHMec of the isolated free and complexed cathepsin L, from each procedure, are shown in Table 5. Autolysis had no effect on the relative amounts of complexed and free cathepsin L on MEC, which were as shown in Fig. 14. This suggested that autolysis was not really necessary or effective in the isolation of free cathepsin L from sheep liver. The only real effect was activation of the complex by autolysis, so that it became more active against azocasein. It should be noted that the complexed enzyme, after autolysis, was more active than the free enzyme in tests against both azocasein and the synthetic substrate, indicating that the bound cystatin was apparently acting as an activator of cathepsin L. This may indicate that autolysis may be incomplete as carried out in this procedure.

Table 5. Activity of free and cystatin-complexed forms of cathepsin L, from autolysed and non-autolysed sheep liver, against azocasein and Z-Phe-Arg-NHMec.

Sample	Activity (units)	
	Azocasein[a]	Z-Phe-Arg-NHMec[b]
Free enzyme, after autolysis	30	1.91×10^{-6}
Complexed enzyme, after autolysis	40	2.26×10^{-6}
Free enzyme, before autolysis	23	1.71×10^{-6}
Complexed enzyme, before autolysis	15	1.36×10^{-6}

[a]For azocasein tests, 10 µg of free enzyme and 15 µg of complex (0,384 nmoles of enzyme in each case, assuming a 1:1 molar ratio of enzyme to inhibitor, and using 26:14 as a mass ratio) was incubated for 2 h at 37°C in pH 5.0 buffer containing 40 mM cysteine.

[b]For Z-Phe-Arg-NHMec tests, 4.0 ng and 6,15 ng of free and complexed enzyme, respectively (0,154 pmoles of enzyme in each case), were used in 10 min stopped time assays.

Initially the M_r 26 000 component, found in the complex electrophoretogram, without reduction, was suspected to be free cathepsin L contaminating the complex fraction. Re-chromatography of the complex peak on MEC failed to separate any free cathepsin L, however, showing that the "free" cathepsin L was probably cathepsin L complexed to cystatin in the normal non-covalent manner, which dissociated on SDS-PAGE.

Another concern was that the active, covalent complex might be an artifact of the TPP process used in the purification procedure, since it had not been reported previously from any

other purification procedure, and the TPP step was the only really unique step in the purification procedure developed in this study. To test this hypothesis, ammonium sulphate fractionation was substituted for the TPP step in the purification procedure. This substitution was found to have no effect on the complex, however, since active complex was isolated in the same proportion with free enzyme, compared to a parallel TPP isolation, and the complex had exactly the same characteristics. Thus it was established that the active, covalent complex was probably not an artifact of the unique purification procedures employed in this study.

Isolated cystatin and cathepsin L fractions from sheep liver, could also form covalent complexes when they were incubated together for 1 h at 37°C at pH 5,5. As shown in Fig. 21, all cystatin sub-fractions, except sub-fraction I, were able to form visible amounts of the covalent complex with cathepsin L. At pH 4,2, however, the situation reversed to that where only sub-fraction I formed visible amounts of covalent complex with cathepsin L (Fig. 22).

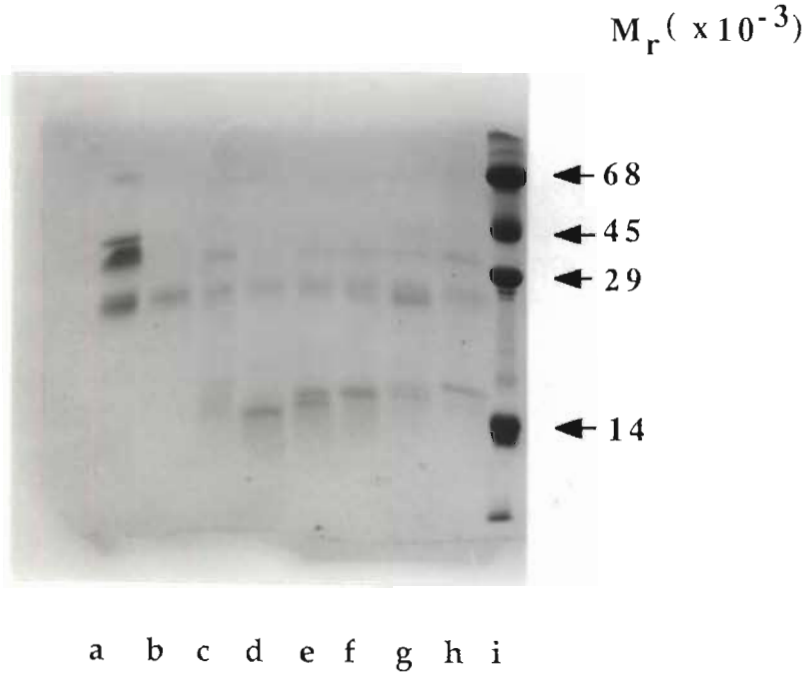


Figure 21: SDS-PAGE, without reduction, of complexes formed *in vitro*, at pH 5,5, between sheep liver cystatin β sub-fractions and cathepsin L.

Free cathepsin L (10 µg) was incubated with cystatin sub-fractions (10 µg) for 1 h at 37 °C (2:1 molar ratio cystatin: cathepsin L). 2,5 µg (protein) was loaded onto 12,5% non-reducing SDS-PAGE after treatment with non-reducing treatment buffer. a) isolated complex (10 µg); b) free cathepsin L (5 µg); free cathepsin L treated with:- c) whole cystatin fraction; d) cystatin sub-fraction I; e) cystatin sub-fraction II; f) cystatin sub-fraction intermediate to sub-fractions II and III; g) cystatin sub-fraction III; h) cystatin sub-fraction IV; i) MW standards (as before).

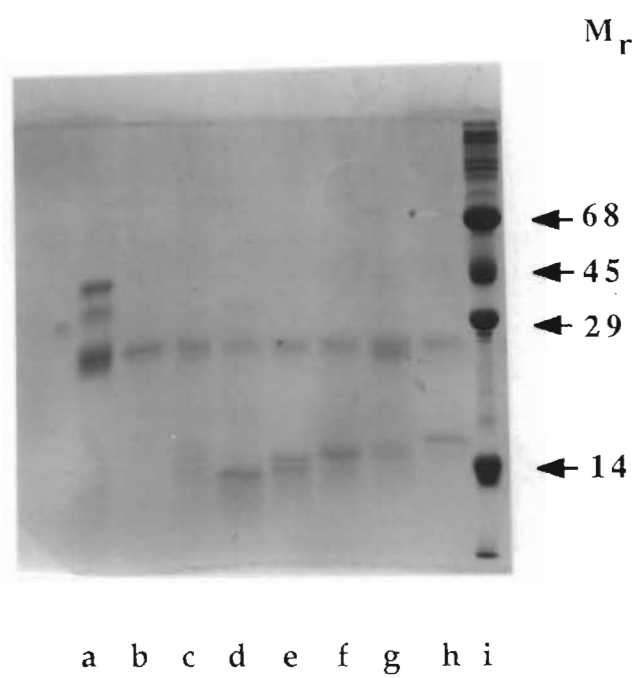


Figure 22: SDS-PAGE, without reduction, of complexes formed *in vitro*, at pH 4,2, between sheep liver cystatin β sub-fractions and cathepsin L.
As for Figure 21 above except all fractions were treated at pH 4,2.

In order to ascertain which of the different forms of the complex revealed by SDS-PAGE, without reduction, were proteolytically active, the fractions were examined by substrate gel electrophoresis. Substrate SDS-PAGE of the complex, showed that the M_r 68 000 and 42 000/37 000 components of the complex were proteolytically active, while the dissociated M_r 26 000 form was inactive (Fig. 23). This shows that the covalent proportion of the complex is proteolytically active, demonstrating that the covalent enzyme-inhibitor interaction has not inactivated the enzyme, and that any disulphide linkages with the cystatin are therefore not formed with the active site cysteine of cathepsin L. The non-activity of the M_r 26 000 component was surprising as the enzyme should be free at this stage, and therefore should show proteolytic activity in common with free enzyme run on the substrate gel (Fig. 23, lane c).

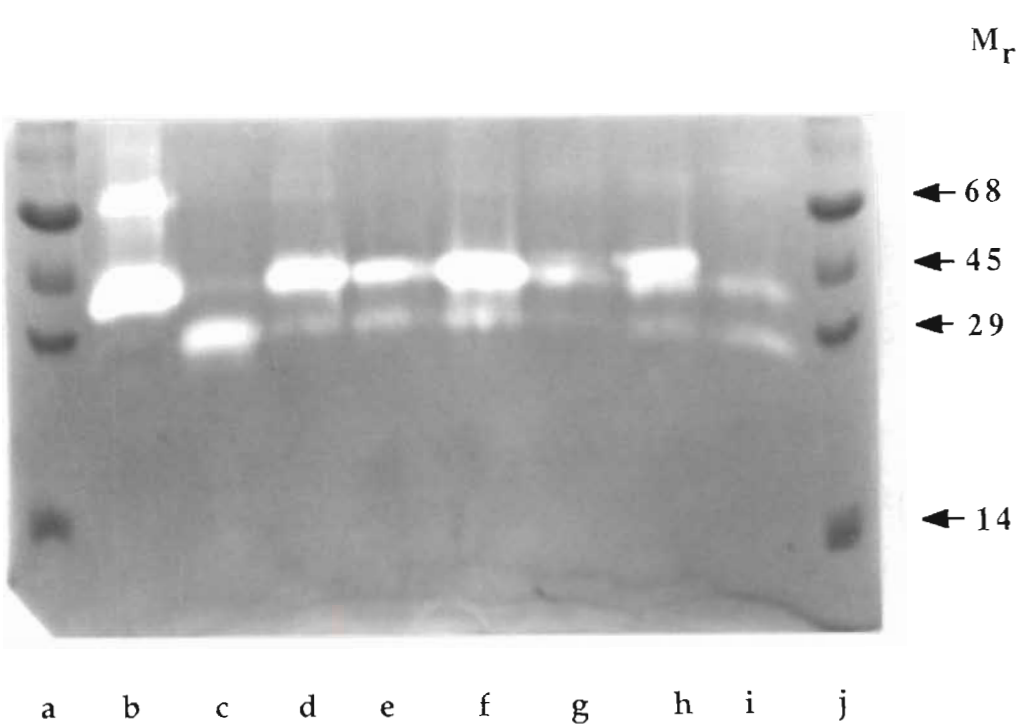


Figure 23: Substrate SDS-PAGE of the isolated cystatin-complexed cathepsin L and the complexes formed *in vitro*, at pH 5,5, between sheep liver cystatin β sub-fractions and cathepsin L.

Treatment of samples was as described in Figure 21 and the procedure for the substrate gel was carried out as described in section 4.3.5. a) MW standards (as described previously); b) isolated cystatin-complexed cathepsin L (10 µg); c) free cathepsin L (5 µg); free cathepsin L treated with d) whole cystatin fraction; e) cystatin sub-fraction I; f) cystatin sub-fraction II; g) cystatin sub-fraction intermediate to sub-fractions II and III; h) cystatin sub-fraction III; i) cystatin sub-fraction IV; j) MW standards.

Substrate SDS-PAGE of the covalent complexes formed *in vitro* between isolated cathepsin L and cystatin sub-fractions, demonstrated that they were also active, as was the M_r 26 000 component in these fractions. The covalent complexes were surprisingly active in these fractions, since they showed more activity than the dissociated cathepsin L present, despite the fact that they apparently contained less protein, based on the intensity of staining with Coomassie blue shown in the parallel study on normal SDS-PAGE shown in Figure 21. This result might indicate that the covalent complexes were more stable to the SDS-PAGE procedure than the free enzyme, and therefore were more active, or it might be a further indication of the activating effect of the bound cystatin in this situation. This might also explain why the dissociated cathepsin L in the isolated complex fraction was not active.

Differences were noted between the reactions of the various inhibitor sub-fractions in their binding to cathepsin L (Fig. 23). The unfractionated cystatin and sub-fraction II formed

similarly large amounts of active, covalent complex. Sub-fraction I did form active complex with cathepsin L in spite of the lack of visible complex in Figure 21, but this complex was less than that for sub-fraction II and unfractionated cystatin. Sub-fraction III formed fairly large amounts of active complex, but there seemed to be two bands of activity in the covalent complex region, suggesting some sort of heterogeneity in its interaction with the enzyme. Sub-fraction IV formed very little active complex, in spite of forming visible amounts of the complex on a normal SDS-PAGE gel (Fig. 21).

Substrate SDS-PAGE of samples after incubation at pH 4,2 (Fig. 24), revealed that the complex was inactivated by this treatment. The M_r 45 000 component of the complex, formed by treatment at pH 4,2, was not active, indicating that the change in the form of the complex was linked to its inactivation. As noted above (Fig. 22), only sub-fraction I and, to a lesser extent, sub-fraction II formed a covalent complex with cathepsin L at pH 4,2. The covalent complexes in these fractions retained their activity after formation at pH 4,2 as shown in the substrate gel (Fig. 24). Free cathepsin L also retained its activity after treatment at pH 4,2, as did the dissociated cathepsin L of the complexes formed *in vitro* .

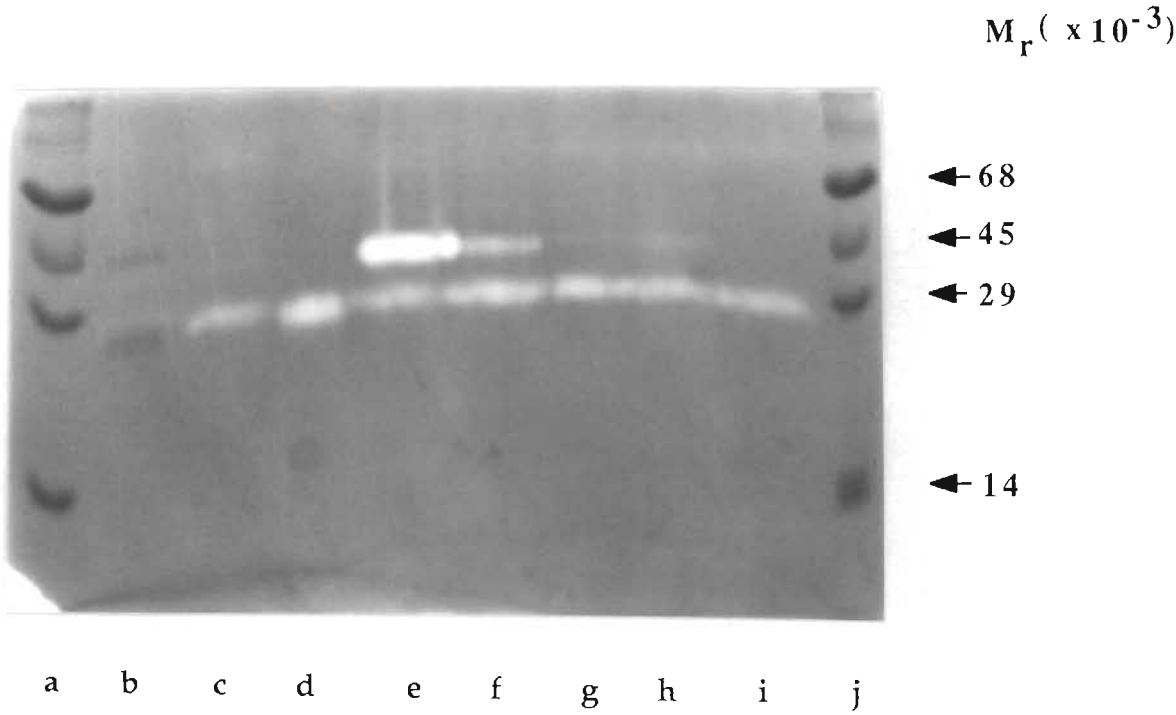


Figure 24: Substrate SDS-PAGE of the isolated cystatin-complexed cathepsin L and the complexes formed *in vitro*, at pH 4,2, between sheep liver cystatin β sub-fractions and cathepsin L.

Experimental details were as described for Fig. 23, except that all incubations were at pH 4,2.

The differential result observed for the formation of the covalent complex at pH 5,5 and pH 4,2, led to further investigation of the effect of pH. The effect of DTT was also investigated, as it was thought that a reducing agent may inhibit formation of the covalent (disulphide) bond between cystatin and cathepsin L. The results (Fig. 25) show that pH had a very definite effect on the formation of the complex since the complex was formed at pH 5,5 and above, but not below this value. This pH therefore seems to be pivotal for the formation of the complex. The formation of the complex was (surprisingly) enhanced by 10mM DTT to a small extent, as analysed by substrate SDS-PAGE, but higher values (up to 50mM) failed to show any effect (results not shown).

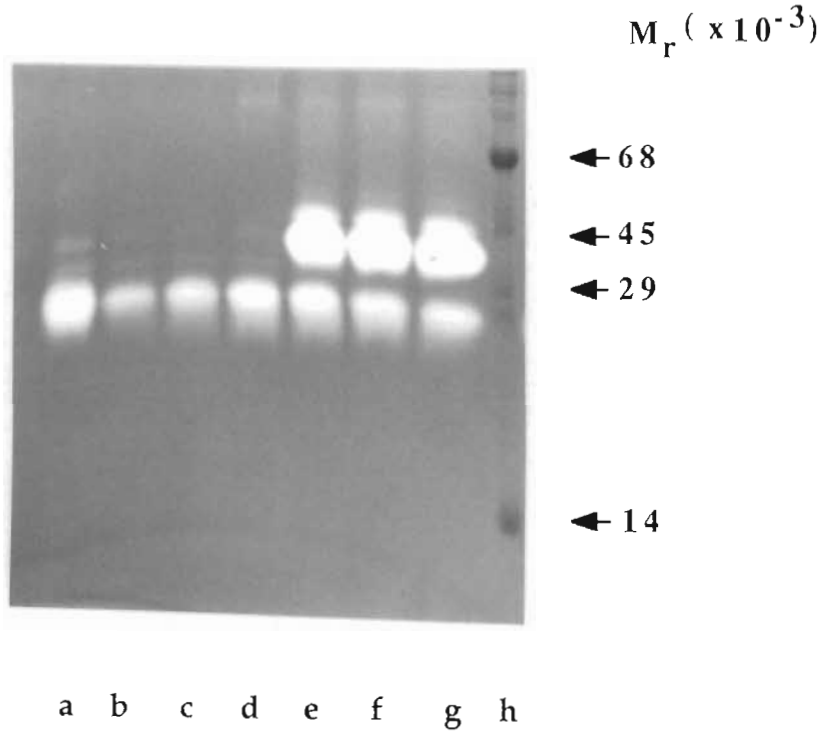


Figure 25: Substrate SDS-PAGE, showing the effect of pH on the *in vitro* formation of covalent complex between isolated sheep liver cystatin and cathepsin L fractions.
Sheep liver cystatin fraction (2,5 µg) and cathepsin L (2,5 µg) were incubated together for 1 h at 37 °C in 100mM buffers of the desired pH and 2,5 µg of protein was electrophoresed on a substrate gel as described in section 4.3.5. a) free cathepsin L; cystatin and cathepsin L incubated at b) pH 4,2; c) pH 4,5; d) pH 5,0; e) pH 5,5; f) pH 6,0; g) pH 6,5; h) MW standards (as before).

Normal SDS-PAGE analysis, however, showed that more covalent complex was apparently formed above pH 5,5 in the presence of 10mM DTT (Fig. 26). It should also be noticed from this figure, that the M_r 68 000 component was eliminated by DTT treatment, indicating that it was probably a loosely-bonded covalent agglomeration of cathepsin L and cystatin molecules, most probably in the ratio of two cathepsin L molecules to one cystatin molecule. The effect of pH seems to be dominant in the formation of the complex, however. The complex was found to be formed very rapidly at pH 6 (Fig. 27), since complete formation was attained at about 10 min and the complex could be detected after as little as 1 min of incubation.

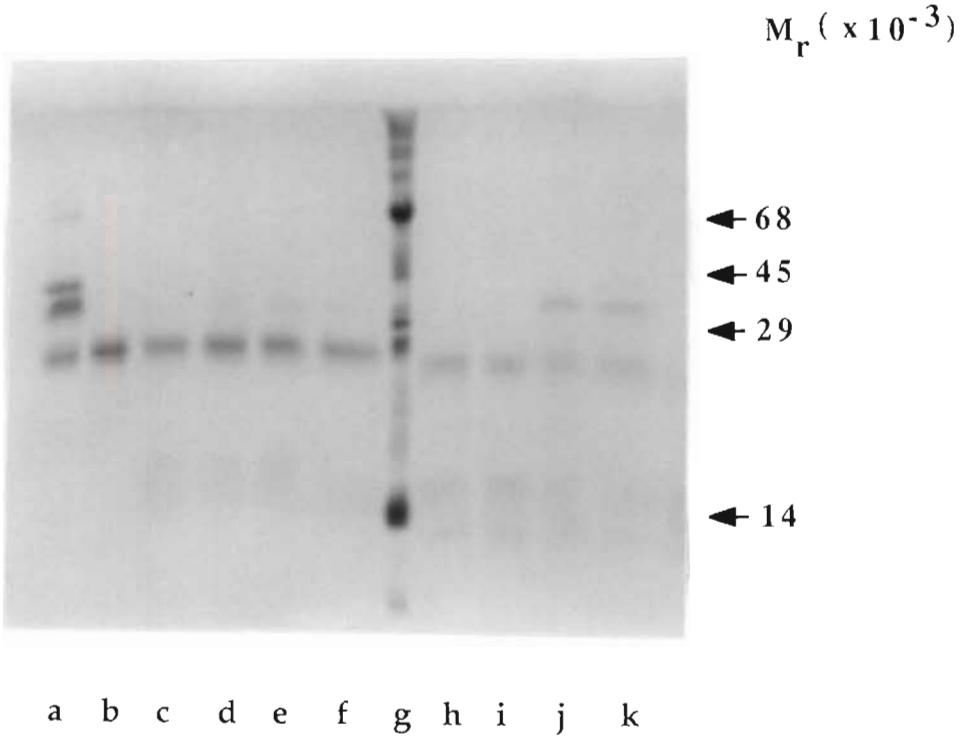


Figure 26: The effect of pH and DTT on the *in vitro* formation of the covalent complex between sheep liver cystatin and cathepsin L, as analysed by SDS-PAGE.
Samples were treated as in Fig. 25, with and without 10mM DTT. a) isolated complex; b) free cathepsin L; cystatin and cathepsin L incubated without DTT at c) pH 4,5; d) pH 5,0; e) pH 5,5; f) pH 6,0; g) MW standards (as before); cystatin and cathepsin L incubated with 10mM DTT at h) pH 4,5; i) pH 5,0; j) pH 5,5; k) pH 6,0.

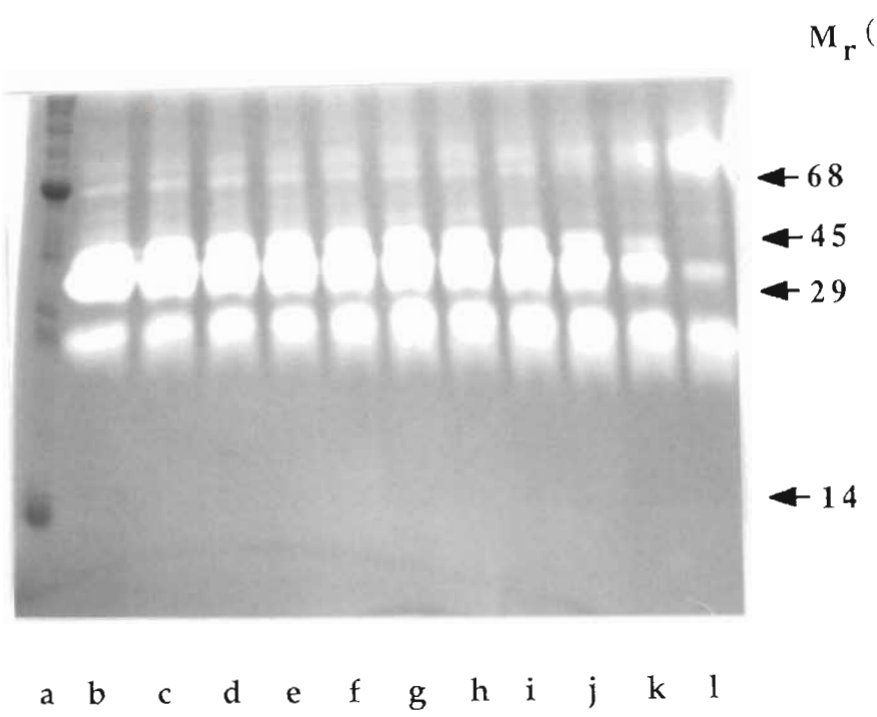


Figure 27: The *in vitro* formation of the covalent complex between sheep liver cystatin and cathepsin L over time.
Sheep liver cystatin fraction (2,5 µg) was incubated with cathepsin L (2,5 µg) at 37 °C in 100mM MES, pH 6,0 for:- b) 120 min; c) 90 min; d) 60 min; e) 45 min; f) 30 min g) 20 min; h) 15 min; i) 10 min; j) 5 min; k) 1 min; l) 0 min;. a) MW standards (as before).

Thus the rapid, pH-dependent formation of a proteolytically active, covalent complex between cystatin and cathepsin L could be demonstrated *in vitro*.

4.4.5 The purification of cathepsin L from human and sheep spleen. The purification procedure described in section 4.3.2 resulted in the purification to homogeneity of complexed cathepsin L from human spleen, the complex eluting late on the salt gradient in S-Sepharose chromatography (Fig. 28), and at a position corresponding to M_r 37 000 in MEC on Sephadex G-75 (Fig. 29). SDS-PAGE, with reduction (Fig. 30), showed that the active fraction was a complex of M_r 26 000 and 14 000 units. This is analogous to the situation observed with sheep liver, in which the M_r 26 000 component was identified as cathepsin L and the M_r 14 000 component was identified as a form of cystatin. Similarly, in the case of human spleen, the M_r 26 000 component was shown to be human cathepsin L by its strong reaction with anti-human cathepsin L peptide antibodies (see Fig. 50, ≥ 5). The M_r 14 000 component of the complex could not, however, be unequivocally identified as human cystatin, as the antibodies to sheep liver cystatin did not cross-react, but the circumstantial evidence is strong.

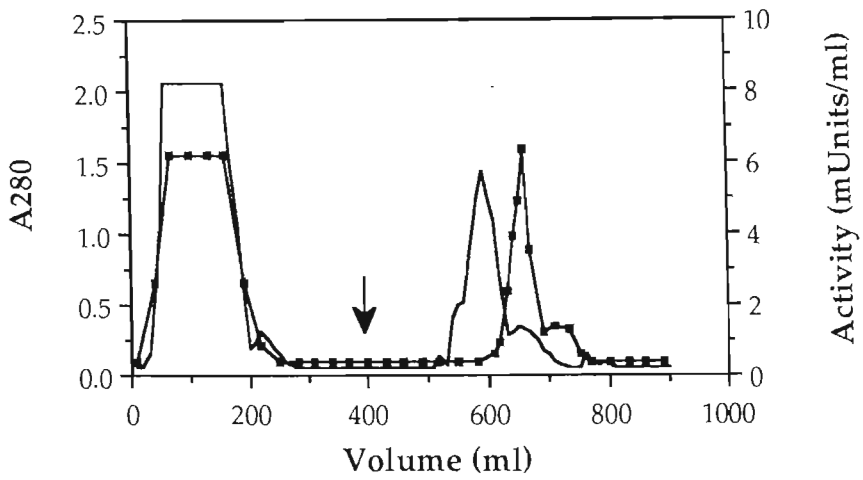


Figure 28: S-Sepharose chromatography of human spleen cathepsin L complex at pH 5,5. Experimental details were as for the S-Sepharose chromatography of sheep liver at pH 5,5 (Fig. 10). (-), A280; (◆), Activity against Z-Phe-Arg-NHMec.

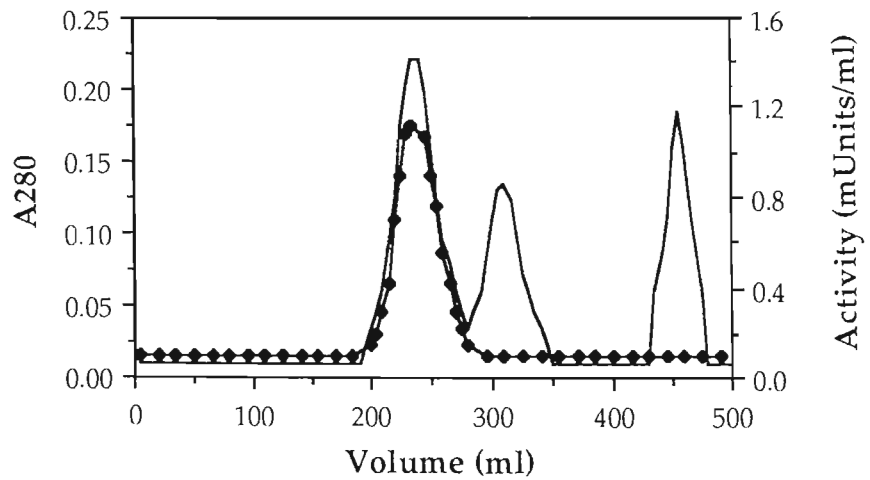


Figure 29: Molecular exclusion chromatography of complexed human spleen cathepsin L on Sephadex G-75.
Column, 2.5 x 87.5 cm (430 ml bed volume); Buffer, buffer A with 200mM NaCl; Flow rate, 25 ml/h (5 cm/h); Fractions, 5 ml (12 min). Void volume was 150 ml.
(-), A280; (◆), Activity against Z-Phe-Arg-NHMec.

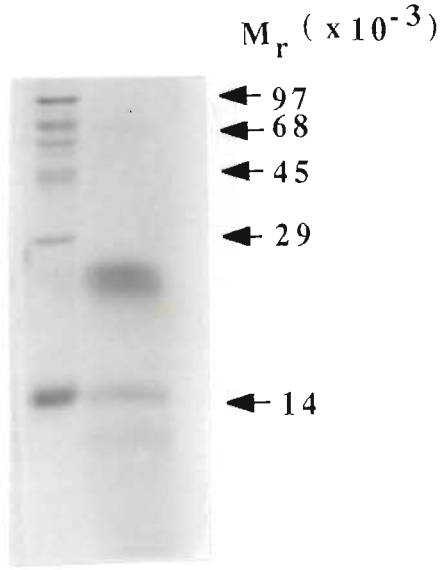


Figure 30: SDS–PAGE of human spleen cathepsin L.
Samples were electrophoresed on 12,5% SDS-PAGE. a) MW markers (Phosphorylase b, M_r 97 000; BSA, M_r 68 000; Ovalbumin, M_r 45 000; Carbonic anhydrase, M_r 29 000; Lysozyme, M_r 14 000); b) 5 µg of complexed human spleen cathepsin L .

The isolation procedure was also applied to sheep spleen, to ascertain the form(s) in which cathepsin L may be isolated from this organ. Similar results were obtained, especially with respect to the existence of the cathepsin L activity only in a M_r 37 000 form on MEC. This complex was isolated in very low yield, however, compared to that from the human spleen, and, in consequence, it was not extensively characterised. The spleen cathepsin L complexes from both species were only active against the synthetic substrate Z-Phe-Arg-NHMec, and not against azocasein, in contrast to the complex from sheep liver.

4.4.6 Inhibition characteristics of complexed and free cathepsin L from sheep liver. Both complexed and free forms of cathepsin L behaved similarly with a range of inhibitors and activators (Table 6). Both PMSF and pepstatin had no effect on either, indicating that they were not serine or aspartic proteinases. Their activation by DTT and inhibition by iodoacetate suggested that they were of the cysteine proteinase class. Leupeptin inhibited both to a large extent, indicating that neither was cathepsin H, which is insensitive to leupeptin. The high level of inhibition obtained with both Z-Phe-Phe-CHN_2 and Z-Phe-Tyr-(O-tBut)-CHN_2 after only 15 min, indicated that both forms were probably cathepsin L, as these inhibitors are fairly specific for cathepsin L, and only react slowly with cathepsin B. As mentioned previously, neither form of enzyme showed any activity against the cathepsin B substrate

Z-Arg-Arg-NHMec, or the cathepsin H substrate, Arg-NHMec. Due to the high level of activity of the enzyme against azocasein, it is unlikely to be cathepsin N (Maciewicz and Etherington, 1988), or cathepsin S, as it interacts too strongly with cation exchangers, is not from spleen (the major source of cathepsin S), and interacts strongly with Z-Phe-Phe-CHN_2, with which cathepsin S does not react (Kirschke *et al.*, 1989). Thus all the results confirm the identity of both forms of the enzyme isolated as cathepsin L.

Table 6. The effect of inhibitors and the absence of DTT on free and cystatin-complexed forms of sheep liver cathepsin L.

Inhibitor	Final concentration (mM)	% activity	
		Complexed	Free
Iodoacetate	1	20	10
PMSF	1	100	100
Pepstatin	1	100	100
DTT	0	10	10
Z-Phe-Phe-CHN_2	0,0001	7	4
Z-Phe-Tyr-(O-t-But)-CHN_2	0,0001	30	28
Leupeptin	0,0001	3	2

Active site titration of the complexed and free cathepsin L with E-64, (Barrett and Kirschke, 1981), showed some surprising results for the interaction of the complex with this inhibitor. As can be seen in Fig. 31, the free enzyme titrated almost to zero activity with E-64, and the amount of active enzyme was estimated to be 40% of that estimated by protein assay. This is a similar figure to that previously reported for the active site titration of cathepsin L (Mason *et al.*, 1985). Complex did not, however, completely titrate with E-64, with only 60% of its activity against Z-Phe-Arg-NHMec being available for titration. In order to find which proportion of the enzyme was proteolytically active, the proteolytic activity of the complex against azocasein was titrated with E-64. Fig. 32 shows that the proteolytic activity of the complex titrated completely with E-64, indicating that the 60% of activity against Z-Phe-Arg-NHMec, available to E-64, is also the proteolytically active proportion of the complex.

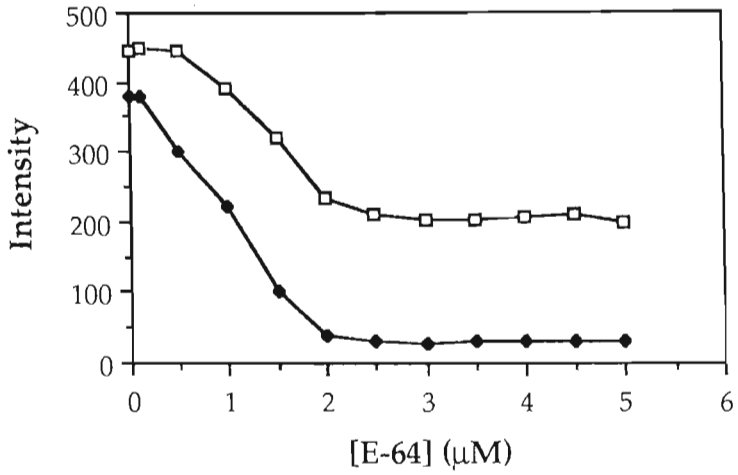


Figure 31: Active site-titration of cystatin-complexed and free forms of sheep liver cathepsin L, using E-64 in assays against Z-Phe-Arg-NHMec.
The titrations were carried out on 6,75 μM of complex and 7,6 μM of free cathepsin L, as described in section 4.3.3.1. (❑), complex and (◆), free cathepsin L.

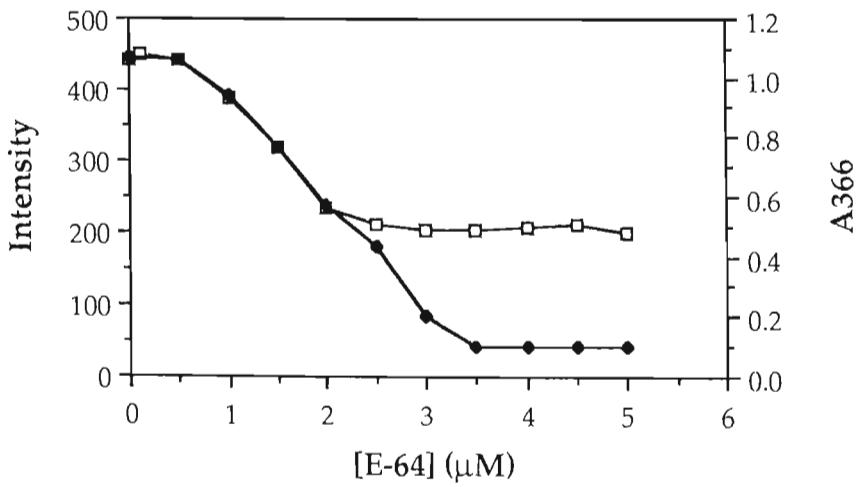


Figure 32: Comparison of the active site-titration of cystatin-complexed sheep liver cathepsin L, using E-64 in assays against azocasein and Z-Phe-Arg-NHMec.
Experimental details were as for Fig. 31 above. Complex titrated against Z-Phe-Arg-NHMec (❑) and azocasein (◆).

The proportion of the complex, active against Z-Phe-Arg-NHMec, but inaccessible to E-64, is therefore probably complexed with cystatin in a normal manner, and thus unavailable for E-64 binding. Cystatin has been found to bind to papain in spite of the presence E-64, but prior binding of cystatin to the papain has been found to preclude E-64 binding to the enzyme (Nicklin

and Barrett, 1984). Subsequent to the actual incubation of E-64 with the complex, the mixture is diluted 1000-fold, for the assay with the sensitive synthetic substrate, and thus the further 40% of non-titratable activity against this substrate is probably due to dissociation of the normal enzyme/inhibitor complex at this high dilution. The normal enzyme/inhibitor complex would not, however, have dissociated sufficiently at the higher concentrations at which E-64 was added, to allow binding of the inhibitor.

4.4.7 Characterisation of the pH-dependent behaviour of complexed and free forms of cathepsin L. Both the complexed and free cathepsin L have very similar pH profiles (Fig. 33), with pH optima of 6,5. Both have significant activity at pH 7 (80%), which is very different from published values for the action of human cathepsin L on Z-Phe-Arg-NHMec (Mason *et al.*, 1985), in which cathepsin L has an optimum of pH 5,5 and has very little activity at pH 7 and above. Results for the pH optimum of rabbit cathepsin L (Mason *et al.*, 1984) were more similar to those obtained with the above study, since this enzyme had 20% activity at pH 7 and an optimum of pH 6. The results of Brömme *et al.* (1989) for the optimum of rat cathepsin L on Z-Phe-Arg-NHMec, were very similar to the results for the sheep enzyme shown here. The results obtained here were tested over many replicates and with several different preparations of cathepsin L.

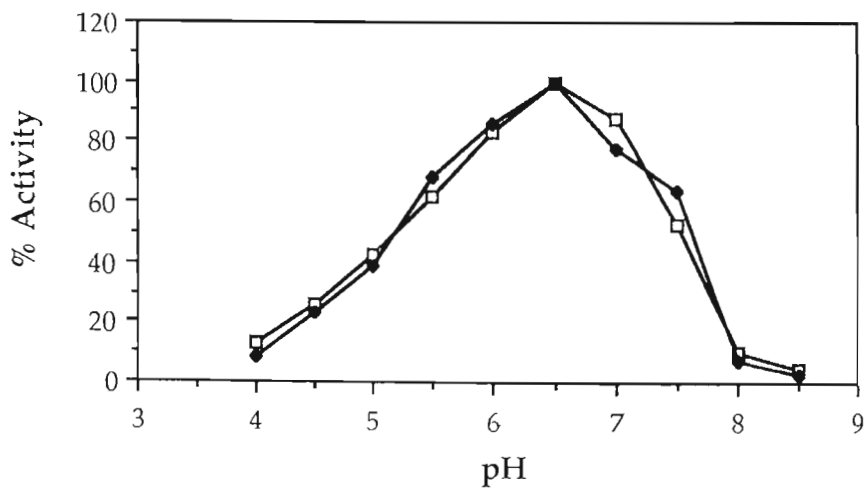


Figure. 33: The effect of pH on the hydrolysis of Z-Phe-Arg-NHMec by free and cystatin-complexed forms of sheep liver cathepsin L.

Z-Phe-Arg-NHMec hydrolysis was measured in a standard 10 min assay using the range of AMT buffers, described in section 4.2, containing 8 mM DTT. The co-efficient of variation for any one point over 5 replicates was never more than 2,9% and 6,3% for the complexed and free enzymes, respectively. The average standard deviation for the complexed and free enzymes were 1,5% and 3%, respectively. (◆), free cathepsin L; (❑) , cystatin-complexed cathepsin L.

The pH profile was also similar when constructed from the results of continuous monitoring at various pH values. The slope was fairly constant over time and the high activity recorded for values such as pH 7, was therefore not due to initial bursts of activity from the enzyme, followed by rapid denaturation. The pH optima for cystatin-complexed human spleen cathepsin L, and the commercially obtained human kidney cathepsin L (Fig. 34), were found to be very similar to that of sheep cathepsin L (Fig. 33).

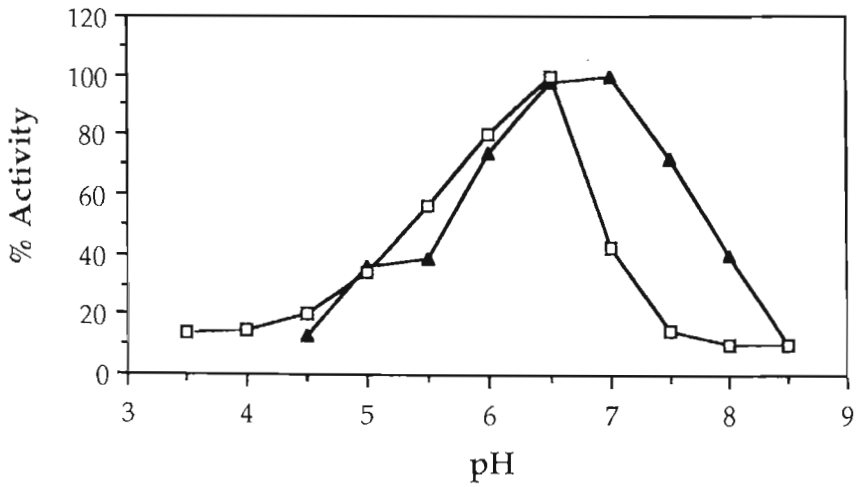


Figure 34: The effect of pH on the hydrolysis of Z-Phe-Arg-NHMec by complexed human spleen cathepsin L and human kidney cathepsin L.
Z-Phe-Arg-NHMec hydrolysis was measured in a standard 10 min assay using the range of AMT buffers, described in section 4.2, containing 8 mM DTT. (□), human kidney cathepsin L; (▲) , cystatin-complexed human spleen cathepsin L.

The pH stability of sheep liver cathepsin L has been published previously by Mason (1986) and, when measurements were made in the same way in this study, similar results were obtained (Fig. 35). The enzyme was maximally stable at pH 5,5, as was the case in Mason's study, and was not very stable at neutral pH. This method of determining the pH stability has merit for its purposes, since measurements of the residual activity at a single pH value, after incubation at various pH values, eliminates effects due to the variation of initial activity with pH, and is thus a fair measure of stability only. It does not, however, give an accurate reflection of situations which may occur *in vivo*, since the enzyme's activity and stability at a particular pH will determine its behaviour in various compartments, such as inside the lysosome or in the extracellular milieu. Thus in order to characterise the behaviour of an enzyme according to pH, in the context of a putative role in tumour invasion, it would be more useful to know its overall behaviour at certain pH values.

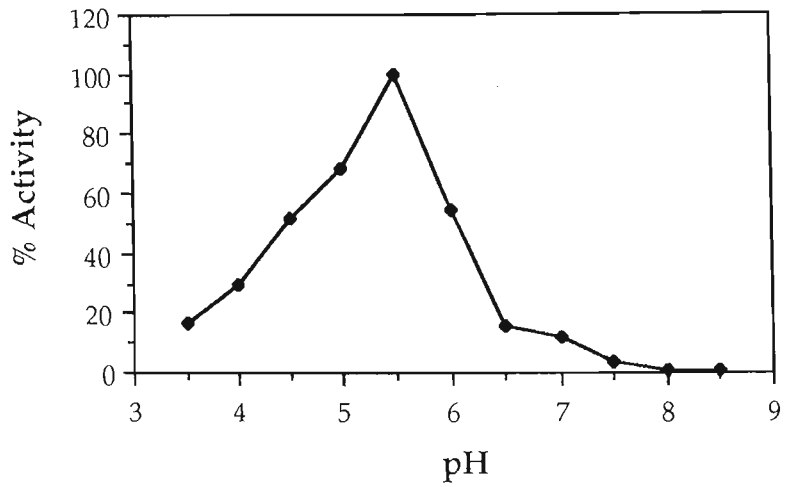


Figure. 35. pH stability of sheep liver cathepsin L determined according to Mason (1986). The enzymes were incubated in a range of AMT buffers for 1 h at 37°C, before being assayed at pH 5.5 in a 10 min stopped time assay as in section 2.4.2.2 .

The pH stability test of Kirschke *et al.* (1989), was therefore seen as being possibly of more relevance to this study, since it gave a measure of both the activity and stability of the enzyme to pH over time. In this test the enzyme is incubated in buffers of different pH, containing 2mM DTT, for 1 h at 37°C, substrate is added, and a 10 min assay is carried out. From the results in Fig. 36, it can be seen that the enzyme was maximally stable at pH 6, and the free and complexed forms were not very different in their stability in this test. These results were quite different from those of Kirschke *et al.* (1989) for rat cathepsin L, where their enzyme was maximally stable at pH 5,5 and did not display the same stability at pH 6 and above.

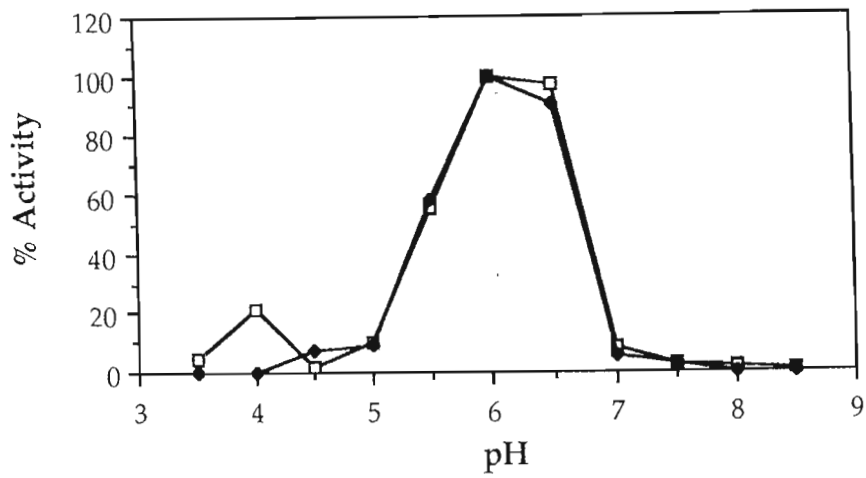


Figure 36: pH stability of cystatin-complexed and free cathepsin L determined according to Kirschke *et al.* (1989).

The enzymes were incubated in the range of AMT buffers, containing 2 mM DTT, for 1 h at 37°C, before being assayed against Z-Phe-Arg-NHMec for 10 min, in the same buffer in each case. The co-efficient of variation for any one point over 5 replicates was never more than 5,1% and 3,7% for the complexed and free enzymes, respectively. The average standard deviation for the complexed and free enzymes were 3% and 1,6%, respectively. (□), cystatin-complexed enzyme; (◆), free cathepsin L.

It was realised, however, that Kirschke's method was still unsatisfactory for our purposes, since it relied on measurement after incubation for an arbitary period (1 h), and did not reflect events which may be occurring before this time. Some way was therefore needed to measure the behaviour of the enzyme over time at a certain pH. In order to do this, a half-life measurement seemed to be most suitable, since it would give a prediction of the length of time over which an enzyme would be active at various pHs, and the level of activity expressed over time. At each pH, the enzyme was therefore incubated, in the presence of 2mM DTT, for increasing time intervals, at the end of which substrate was added, and a 10 min assay was carried out. The residual activity at each time interval was plotted semi-logarithmically for each pH, linear regression was carried out on the plots and the equations seen in Table 7 were generated, from which k_{obs} and $t_{1/2}$ values could be calculated.

Table 7: pH-dependent stability of cystatin-complexed and free sheep liver cathepsin L.

pH	Equation	R-value	k_{obs} (min^{-1})	$t_{½}$ (min)
Complex:				
4	y = 2,8361-0,0274x	0,96	0,063	11
5	y = 3,4974-0,0133x	1,00	0,031	23
5,5	y = 3,4162-0,0129x	1,00	0,030	24
6	y = 3,5157-0,0076x	0,96	0,0175	40
6,5	y = 3,7150-0,0073x	1,00	0,0168	41
7	y = 3,9044-0,0107x	0,99	0,025	28
7,5	y = 3,8116-0,0275x	0,99	0,063	11
8	not plotted[a]			
Free:				
4	y = 2,5915-0,0724x	1,00	0,166	4
5	y = 2,9738-0,0313x	1,00	0,072	10
5,5	y = 3,3148-0,0122x	0,96	0,028	25
6	y = 3,1890-0,0103x	0,98	0,024	29
6,5	y = 3,3401-0,0113x	0,98	0,026	27
7	y = 3,4060-0,0148x	0,93	0,034	20
7,5	y = 3,5507-0,0238x	1,00	0,055	13
8	y = 2,7500-0,0595x	0,90	0,137	5

[a]The complex seemed to denature very quickly at pH 8 and therefore activity fell to baseline values at the first measurement. It was therefore not possible to plot a line for the complex at this value.

As can be seen from the "R"-values (correlation coefficients), the lines were accurate reflections of the behaviour of the enzyme at a certain pH. The activities at pH 6-7 were fairly erratic, however, and therefore extra replicates were needed at these points, before acceptable results were obtained. The k_{obs} and $t_{½}$ values were calculated (see section 4.3.3.2) from the slope of the lines and the $t_{½}$ values were plotted relative to pH (Fig. 37). The complexed cathepsin L had maximal stability at pH 6,5, while the free enzyme was most stable at pH 6. The complex seemed to be more stable in the neutral region than the free enzyme, with a half-life of 41 min compared to 27 min at pH 6,5. Both forms of the enzyme had considerable stability at pH 7 (28 min and 20 min half-lives for the complexed and free forms respectively) and even at pH 7,5. Stability decreased above pH 7,5, however. Thus the above method seemed to be successful in obtaining information about the pH stability/activity of the enzyme over time.

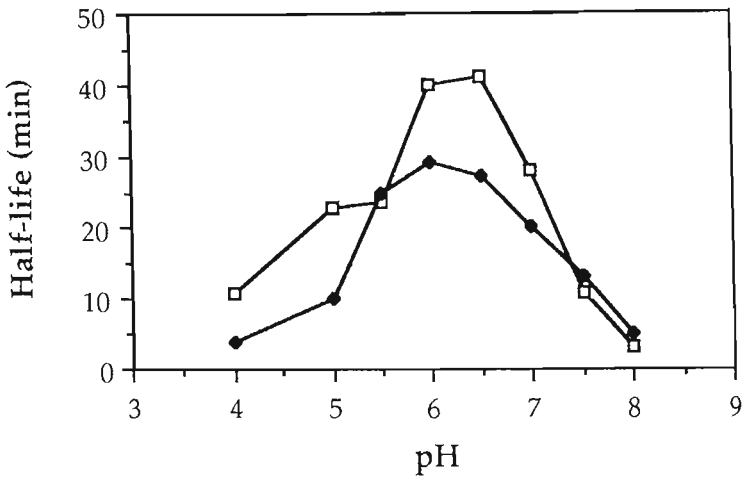


Figure 37: The half-lives of cystatin-complexed and free forms of sheep liver cathepsin L. Calculated half-life values (Table 7) for cystatin-complexed (❑) and free (◆) sheep liver cathepsin L

The enzyme's pH-dependent characteristics were also tested against azocasein (Fig. 38). This test takes place over 2 h and is, therefore, a test of both the activity and stability of the enzyme relative to pH. The results obtained using the synthetic substrate were confirmed to a large extent, in that the enzyme had more activity in the neutral pH range than has been reported previously (Kirschke *et al.*, 1977; Mason *et al.*, 1984). A common finding by these workers is that cathepsin L is more active against protein substrates in the acidic pH ranges, than it is against synthetic substrates. This finding was confirmed by the results here, since a broad activity against azocasein was found in the pH range from 5-7, whereas the enzyme was active from 5,5-7,5 against the synthetic substrate. The complexed and free forms of the enzyme did differ in their pH optima against azocasein, but overall were fairly similar in their activity profiles against this substrate.

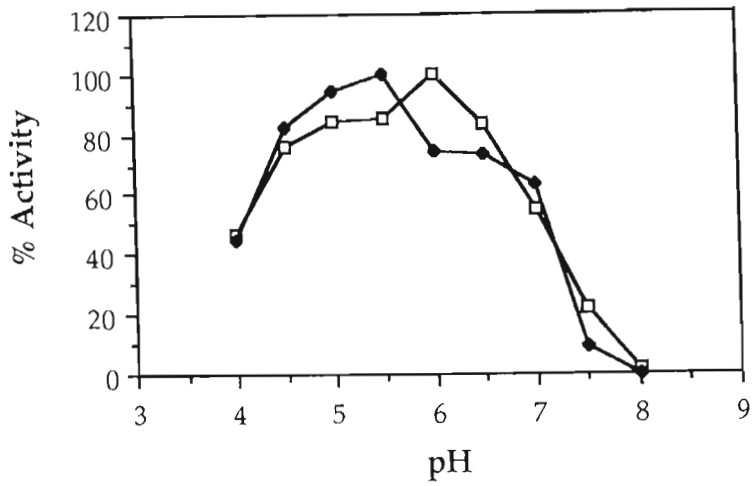


Figure 38: The effect of pH on the hydrolysis of azocasein by free and cystatin-complexed forms of sheep liver cathepsin L.
The enzymes were incubated together with 1% (w/v) azocasein for 2 h in the range of AMT buffers, containing 40 mM cysteine. The co-efficient of variation for any one point over 6 replicates was never more than 3% and 5% for the complexed and free enzymes, respectively. The average standard deviation for the complexed and free enzymes were 1,6% and 3,5%, respectively. (◆), free cathepsin L; (❑), cystatin-complexed cathepsin L.

4.5 Discussion

The purification procedure for cathepsin L, as devised above, resulted in the purification of cathepsin L in two forms: a single-chain free form and a proteolytically active complex with the cysteine proteinase inhibitor, cystatin. Both forms of the enzyme are different from that reported previously for cathepsin L from sheep liver (Mason, 1986).

Cystatins are tight-binding reversible inhibitors, with generally low K_i values, for papain and papain-like cysteine proteinases (Barrett, 1987). Homogenates of the sheep liver were found to yield only cystatin β-type inhibitors. The K_i for human cystatin B has been estimated as 0.23nM (Barrett *et al.*, 1986). The complex isolated from sheep liver, however, had an activity against azocasein higher than that of the free enzyme itself (Table 5). In order for a reversible, tight-binding inhibitor to be effective, Bieth (1980) estimated that the [I] must be > $10K_i$. In the situation of the active complex, the cystatin was present in concentrations 100-fold greater than K_i and therefore should have been effective as an inhibitor. The activity of the enzyme/inhibitor complex could not, therefore, be due to simple dissociation, suggesting

that the cystatin in the complex may be acting other than as a tightly bound, reversible, inhibitor.

The cystatin/cathepsin L complex was found to be abnormal in other respects, in that a proportion of the complex was found to be covalently bound by SDS-PAGE analysis. This is in contrast to normal complexes of this inhibitor to papain, which are non-covalently bound (Nicklin and Barrett, 1984). The covalently bound forms of the complex had M_r values of 68 000, 42 000 and 37 000. The M_r 37 000 form probably consists of cathepsin L bonded to one cystatin molecule in a covalent manner, while the M_r 42 000 component might be a conformational variant of this form. The M_r 68 000 form seems to be quite loosely covalently bound, since low concentrations of DTT were sufficient to break it down. This MW can be derived from two molecules of cathepsin L to one of cystatin, and thus it could conceivably consist of two cathepsin L molecules loosely bonded by a cystatin molecule in some way. The conversion of the M_r 37 000 form of the covalently bound complex to M_r 45 000 by treatment at pH 4,2, possibly reflects a conformational change which makes the complex more unstable, as is indicated by its inactivation on substrate SDS-PAGE after pH 4,2 treatment. The remainder of the complex appeared to be normal in its binding since it dissociated without reduction on SDS-PAGE. The covalently bound proportion of the complex was proteolytically active on substrate SDS-PAGE gels, while the dissociated proportion was not. The formation of the complex could not be prevented by omission of the autolysis step in the purification procedure, or by substitution of the TPP step with ammonium sulphate precipitation, thus eliminating these steps as the cause of the formation of the active, covalent complex.

These results were confirmed by E-64 titration of the active complex, in which it was found that 60% of the activity of the complex against the synthetic substrate, Z-Phe-Arg-NHMec, was titratable, and that this titratable proportion was the proteolytically active one. Normal binding of cystatin to cathepsin L would prevent E-64 binding at the dilutions at which the inhibitor is added to the complex (Nicklin and Barrett, 1984), and therefore from the E-64 results it may be seen that the 60% of activity which was titratable must be abnormally bound complex. The E-64 result therefore indicates that the inhibitor may be normally complexed to 40% of the active enzyme in the complex, and abnormally complexed to 60% of the active enzyme in the complex.

The formation of the complex was investigated using isolated cystatin β sub-types from sheep liver. Differences were noted in the formation of the complex by the different sub-types at pH 5,5, since sub-fractions II and III, and unfractionated cystatin reacted to form more complex than sub-fraction I and much more than sub-fraction IV. At pH 4,2 only sub-fraction I and, to a lesser extent, sub-fraction II formed active complex with cathepsin L. Sub-fraction I has a free

cysteine at position 3 and will react with cysteine proteinases in the absence of DTT, while the other sub-types will not. The formation of the complex at pH 4,2 by this inhibitor sub-type only, may indicate that the covalent binding between cathepsin L and the inhibitor is a spontaneous process that occurs more readily at higher pHs, except when the relevant cysteine group on cystatin β is always free to interact, as is the case with sub-fraction I. The lesser interaction of sub-fraction I with the enzyme at pH 5,5 may, however, indicate that its free N-terminal cysteine, which is thought to be less reactive than that of other sub-fractions, due to its lack of interaction with other substituents found on the other sub-types of the inhibitor, may simply react less with the enzyme at higher pHs where the covalent bonding of the inhibitor to the enzyme may occur more spontaneously. Thus at the higher pH, where the process of covalent bonding is more spontaneous, the cysteine in the more reactive sub-types may lose its substituent in preference for bonding to another cysteine group, in contrast to the less reactive sub-fraction I.

The effect of pH on the covalent interaction of the cystatin β with cathepsin L was investigated using the whole, unfractionated cystatin fraction from sheep liver. Results for this whole fraction will reflect the situation with sub-fractions II, III and IV to a much greater extent, than sub-fraction I, which is a very small proportion of the cystatin fraction (see Fig. 17),. The pH at which cathepsin L was incubated with the cystatin fraction was found to have a very pronounced effect on the formation of the complex, with active complex only formed at pH 5,5 and above. The effect of DTT was much less, and increased amounts of complex, seen on normal SDS-PAGE gels, were not visible on substrate gels. This suggests that the covalent bond between inhibitor and enzyme forms fairly spontaneously at higher pHs, which is fairly typical of disulphide bonds (Creighton, 1989), and that DTT has a very mild activating effect on this process. Formation of the complex was quite rapid as it was evident after only 1 min, and complex formation reached completion after 10 min. The profound effect that pH has on the formation of the complex indicates that the use of lower pHs throughout a purification procedure may prevent its formation, and allow the purification of higher yields of free cathepsin L.

The isolation of active, complexed cathepsin L from the purification procedure, despite the presence of a long incubation step at pH 4,2, which was found to inactivate the complex on substrate gels, indicates either that the complex is less stable to pH in the context of substrate gels, or that the complex may be forming in later steps of the chromatography procedure. The inactivation of the complex at pH 4,2 on substrate gels may indicate that the complex is destabilised by pH change, and that although it may be active in its native state, it is unable to withstand SDS treatment. This destabilisation may cause a conformational change which would explain the fact that the MW of the covalent complex changes from M_r 37 000 to M_r 45 000.

The presence of a free cysteine near the N-terminus of cystatin β-type cystatins, which were found to be essentially the only type of inhibitor in sheep liver, as in rat liver (Wakamatsu *et al.*, 1984), makes it highly likely that this group is involved in the covalent binding of the cystatin to cathepsin L. The binding of this group to cathepsin L must be occurring at a site other than the active site of the enzyme, however, and not to the active site cysteine, since the abnormally bound enzyme is very active. Papain has been found to contain a disulphide bond which is more labile to reduction than the other disulphide bridges in the molecule (Sluyterman and Wijdenes, 1980; Shapira and Arnon, 1969). This disulphide is between cysteine residues at positions 43 and 152 in the amino acid sequence of papain, a position which is removed from its active site. The partially reduced molecule is still active, although it is more susceptible to autolysis.

It is possible to extrapolate the results for papain to cathepsin L, since the molecules are similar in their structure, amino acid sequence, and their catalytic nature (Dufour, 1988). Thus the cystatin may be bound to cathepsin L through a free cysteine residue in the enzyme, released by a labile disulphide bond in the molecule, analogous to that in papain, which undergoes disulphide exchange with the free cysteine of the cystatin β-type inhibitor to which it then becomes covalently bound. A proportion of isolated papain has been found to be activatable by intramolecular disulphide exchange (Brocklehurst and Kierstan, 1973), demonstrating that in the cysteine proteinases, the disulphide bonds may be more fluid than in other molecules, allowing interactions such as the one found in this study. Possibly the cystatin becomes bound in this way during the association/dissociation cycle it undergoes being a reversible inhibitor. According to the binding mechanism established by Stubbs *et al.* (1990), the N-terminal "trunk" of cystatin, containing the reactive Cys 3 residue, would probably only be close to the disulphide bond between residues 22 and 63 in the papain structure, but would be further away from the supposedly more labile 43-152 disulphide bond. From this observation it is possible to deduce that either cathepsin L is slightly different in its three dimensional structure and/or its three-dimensional interaction with cystatin β and therefore the more labile disulphide is closer to the Cys 3 of the cystatin molecule, or the inhibitor becomes inappropriately bound as it moves towards docking with the active site of the enzyme in the normal manner.

The possibility that cystatin β may be bound to cathepsin L in a manner different from that previously described, raises the question as to why it is still found to be an inhibitor in this study and others. The binding mode seen here would not have been noticed in the studies of the three-dimensional complex between cystatin β and papain (Stubbs *et al.*, 1990), since in these studies recombinant cystatin β was used, which had the cysteine at position 3 substituted by a serine residue to prevent dimerisation of the inhibitor. Of the other studies on cystatin β and its

binding to proteinases, only that of Wakamatsu *et al.* (1984), who used cathepsin H, actually visualised the binding on PAGE. There are thus virtually no studies of the same nature as that conducted here. Parallel studies using related enzymes would be useful in this regard, but the papain obtained for this purpose did not seem to be stable on SDS-PAGE without reduction and thus similar studies on this enzyme were not possible. Cystatin β is able to inhibit the complex (results not shown), indicating that two inhibitor molecules may be required to fully inhibit the enzyme. Thus the inhibition of the enzyme may depend on the ratio of inhibitor to enzyme, with inhibition of the enzyme being seen as long as ratios in favour of the inhibitor are used.

The possible physiological roles of the enzyme/inhibitor complex found in this study are of interest, but are difficult to predict. Certainly, further investigations on whether the complex is found in tissue for instance, possibly through immunocytochemical methods, would be needed before the physiological relevance could be assessed. With regard to tumour invasion, if this mode of binding occurs between cathepsin L and the intracellular cystatins, released by tissue breakdown, the proteolytically active complex, which is slightly more stable at neutral pH, could have an active role in tumour invasion under conditions where it would not normally be expected to be active. These aspects will be further discussed in the general discussion.

With regard to the human spleen cathepsin L, if it is complexed to a cystatin, its activity may be due to simple dissociation of the reversible inhibitor from the enzyme at the high dilution required for the assay using a synthetic substrate. This would imply that the inhibition by cystatin was inefficient due to $[I]/ K_i < 10$ (Bieth, 1980). Assuming K_i values for human cystatins A and B (the inhibitors which might be expected in spleen) of 1.3nM and 0.23nM respectively (Barrett *et al.*, 1986), the $[I]/K_i$ ratios for cystatin A and B were calculated to be 9 and 48 respectively. The situation for cystatin A would therefore seem to be explained in terms of simple dissociation at the high dilution for the synthetic substrate assay, but for cystatin B, the amount of inhibitor should be sufficient to inhibit the enzyme effectively. Cystatin B, moreover, is the most abundant inhibitor in spleen (Kominami *et al.*, 1984), and is therefore most relevant.

Human and sheep spleens yield relatively little free cathepsin L in comparison to sheep liver, suggesting an organ difference in the ratios of cystatin(s) to cathepsin L. Radioactive probing of human tissues suggests that the quantity of cathepsin L in spleen is equivalent to that in the liver (Mason *et al.*, 1989), which agrees with immunological probing done on rat tissues, where equivalent amounts of the enzyme were shown in the two tissues (Bando *et al*, 1986). Cathepsin L has been successfully isolated from rabbit spleen (Maciewicz and Etherington, 1988) as part of a multi-enzyme purification procedure, but spleen has not been used as a source of the enzyme from other species. The reasons for the negligible yields of free enzyme from human and

sheep spleens are not known, but further studies, on the relative proportions of cathepsin L and cystatins in the different tissues, are required.

The isolation of free cathepsin L, in a single-chain form, from sheep liver is different from that reported for the previous isolation of this enzyme from sheep liver, where it was isolated in a two-chain form (Mason, 1986). There is some doubt as to whether cathepsin L exists *in vivo* as a two-chain form, since conflicting results have been obtained in various isolation procedures. Dufour *et al.* (1987) reported the isolation of a single-chain form of M_r 27 000, from chicken liver lysosomes, by a rapid procedure. This was in direct contrast to the result of Wada and Tanabe (1986), who isolated a two-chain form from chicken liver by a longer procedure. Similarly Bando *et al.* (1986) isolated a mixture of one-(M_r 30 000) and two-chain (M_r 25 000 and 5 000) forms from rat liver lysosomes, while Kirschke *et al.* (1977) isolated cathepsin L only in a two-chain form from the same source. Mason and co-workers have isolated the enzyme only in a two-chain form from various sources such as human (Mason *et al.*, 1985), sheep, bovine (Mason, 1986) and rabbit livers (Mason *et al.*, 1984).

The above evidence has led several authors (Mason, 1986, Bando *et al.*, 1986, Wiederanders and Kirschke, 1989) to postulate that the two-chain form of cathepsin L may be a consequence of limited proteolysis during the autolysis step used in several of the purification procedures. This contention is placed in doubt, however, by the observation that the procedure of Kirschke *et al.* (1977), in which cathepsin L was isolated directly from rat liver lysosomes, led to the isolation of a two-chain form of cathepsin L. The procedure of Wada and Tanabe (1986) did not involve an autolysis step, but did result in the isolation of a two-chain form, in contrast to the procedure of Dufour *et al.* (1987), which gave a single-chain form.

Intracellular processing studies in various cell types, using pulse-chase labelling and immunoprecipitation techniques, have also revealed conflicting results. Hara *et al.* (1988) found that in rat macrophages, conversion of cathepsin L from its pro-enzyme form to its single-chain form was dependent on a metalloproteinase, while the conversion of the single-chain form to the two-chain form was dependent on a cysteine proteinase, as evidenced by the effect of different inhibitors. In rat hepatocytes, the processing of the pro-enzyme form of cathepsin L to a M_r 30 000 single-chain form was demonstrated to be due to an aspartic proteinase (Nishimura *et al.* 1988a, 1988b, 1989). The rapid processing of the single-chain form to a two-chain form was also demonstrated, but the enzyme responsible for this was not identified. In rat fibroblasts, processing of the pro-enzyme to a single-chain form was also found to be accelerated by an aspartic proteinase (Wiederanders and Kirschke, 1989), but the conversion of single-chain form to two-chain form could not be demonstrated in this cell type. Incubation of the single-chain

form with cathepsins B, H and D *in vitro* also did not result in conversion to the two-chain form. Thus, depending on the cell type, different processing events may occur.

The occurence of the two-chain form may, therefore, correlate more with the length of an isolation procedure, or with the time taken before the enzyme is removed from other proteases in a purification procedure. In both the procedure developed in this study, and that of Dufour *et al.* (1987), chromatography steps which separate cathepsin L from other proteinases occur early in the procedure, while other procedures involve lengthy chromatography steps in the presence of other proteinases. Another possible explanation might be that certain purification procedures select for single-chain cathepsin L, by having a strong cation exchange step as their initial chromatography step, while other procedures generally employ weak cation exchangers in their initial steps.

The pH-dependent characteristics of the single-chain cathepsin L isolated in this study were studied by methods reported in the literature, and by a new method, which yields results which are more applicable to the elucidation of the possible roles of the enzyme in tumour invasion. The enzyme was found to have higher activities at neutral pH against both the synthetic substrate, Z-Phe-Arg-NHMec, and the protein substrate, azocasein, than previously reported. The results for the azocasein and half-life tests showed that there were relatively small differences between the complexed and free forms of the enzyme in their pH-dependent behaviour, with the complex slightly more stable in the neutral region than the free enzyme. The tests for pH optimum and stability according to Kirschke *et al.* (1989), revealed no differences between the two forms. The half-life test of pH stability yielded substantially more information on the enzyme's stability and the levels of activity it would be expressing at any one time, compared to previously described methods, and thus could prove a more useful test on which to base predictions about an enzyme's physiological role in future studies.

The results of the above tests suggest that the single-chain enzyme could have more activity at neutral pH and for a longer time than would be anticipated from results reported previously in the literature for cathepsin L. All the tests of the pH-dependent behaviour of cathepsin L carried out in the past have been on two-chain forms of the enzyme, and the tests carried out above suggest that there could be a difference between the single and two-chain forms in terms of their pH-dependent behaviour. It is unlikely that the results obtained could be ascribed to species difference since the results for human spleen and kidney cathepsin L were similar in the pH optimum tests. The pH-dependent characteristics of the single-chain enzymes isolated by other authors would be of great interest in this regard, to establish whether there are differences in the characteristics of the single and two-chain forms of cathepsin L.

CHAPTER 5

THE PRODUCTION OF IMMUNOINHIBITORY ANTI-SHEEP LIVER CATHEPSIN L POLYCLONAL ANTIBODIES AND ANTI-HUMAN CATHEPSIN L PEPTIDE ANTIBODIES.

5.1 Introduction

Following the purification of cathepsin L from sheep liver, it was possible to make polyclonal antibodies to the sheep enzyme. These antibodies would be anticipated to cross-react with human cathepsin L (Mason, 1986), and thus be useful in studies on the enzyme in human tissue. In an attempt to optimise the cross-reactivity of the antibodies so produced, antibodies were made in chickens in addition to the more conventional rabbit antibodies. The basis for this experiment was that the chicken is evolutionarily more distant from the sheep than the rabbit, and might thus make antibodies to different sites on the sheep enzyme compared to those to which the rabbit would respond. There is thus the possibility that these chicken antibodies, to the different sites on cathepsin L, could cross-react more strongly with human cathepsin L than rabbit antibodies, making them a more useful reagent for studies on this enzyme. Chicken antibodies found in the egg yolk (IgY), targeting different sites from the rabbit IgG antibodies, might also have novel immunoinhibitory properties compared to IgG antibodies. The potential importance of immunoinhibitory antibodies against proteinases has been discussed in the general introduction and by Dennison (1989).

The inhibitory effect of antibodies on several enzymes has been studied, such as bacterial β-lactamases (Richmond, 1977), cathepsin D (Dingle *et al.*, 1971; Weston and Poole, 1973) and, more recently, acetylcholinesterase (Olson *et al.*, 1990). In most of the cases reported, polyclonal or monoclonal antibodies to the whole enzyme were inhibitory, but cases where the anti-enzyme antibodies were not inhibitory, or even stimulatory, have been reported (Richmond, 1977). Inhibitory antibodies against cathepsin D were used to investigate the role of the enzyme in cartilage breakdown (Dingle *et al.*, 1971), demonstrating the potential these agents have in elucidating the role of enzymes in biological functions.

During the present study it was recognised that, while anti-sheep cathepsin L antibodies which cross-reacted with human cathepsin L could be generated, anti-human cathepsin L antibodies would obviously react to a much greater extent and to more sites on the human enzyme, which could be important in immunocytochemical studies, where antibody binding sites may be denatured by fixatives used in the procedure. They would thus be better tools for studies on human tumour invasion by immunocytochemistry, and for potential therapeutic applications. Human tissue was difficult to obtain, however [except for spleen, from which only

cystatin-complexed cathepsin L could be isolated, which was not satisfactory for the production of polyclonal antibodies (see Chapter 4)], and thus it was not possible to generate anti-human cathepsin L polyclonal antibodies in this study.

The technique of producing antibodies against a chosen peptide sequence in a protein was seen as a method of overcoming the foregoing problems. This technique involves the selection of an appropriate peptide sequence (predicted to be immunogenic), synthesis of the peptide, and immunisation of an animal with the peptide, conjugated to a carrier to make it more immunogenic, which elicits production of antibodies to the peptide. The antibodies so produced are tested for their reaction with the peptide and the parent protein. The advantage of this technique is that only small quantities of the original protein are required for characterising the antibodies, the antibodies are specific for the protein (by the nature of the peptide selection) and sequences which are not normally immunogenic may elicit antibodies by this technique (Van Regenmortel, 1988a). This technique was therefore thought to have the potential to produce specific immunoinhibitory antibodies, by virtue of the ability it confers on the researcher to choose the region of the molecule to be targeted, a level of predictability not available in the production of conventional polyclonal or monoclonal antibodies.

The challenge in this approach was to find a sequence in cathepsin L which fulfilled the above criteria, i.e. an immunogenic sequence which, when bound by antibodies, causes immunoinhibition of the enzyme. A secondary criterion was the targeting of cathepsin L across-species, in order to expand the usefulness of the antibody. Cathepsin L has been shown to be fairly homologous to papain (40-50%) in both its primary and secondary structures, particularly at or near the active site regions, presumably due to the influence of their common catalytic mechanism (Dufour, 1988). It was inferred, therefore, that there would be sufficient homology in the tertiary structure of these molecules to justify the use of a representation of papain's tertiary structure (Wolthers, 1970) (Fig. 39), as a guide to the selection of a peptide sequence from cathepsin L which was associated with the active site. The amino acid sequence for human cathepsin L is known (Gal and Gottesman, 1988, Ritonja *et al.*, 1988) as are the sequences for cathepsin L from rat (Ishidoh *et al.*, 1987), mouse (Portnoy *et al.*, 1986) and chicken (Dufour *et al.*, 1987, Wada *et al.*, 1987).

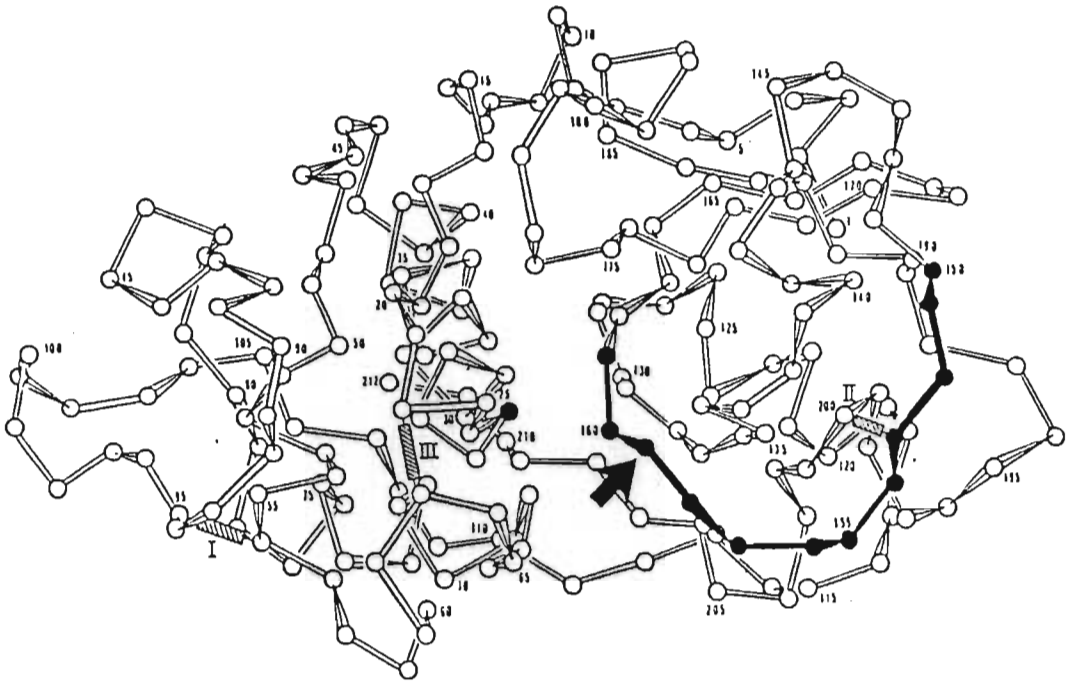


Figure 39: Representation of the tertiary structure of papain (from Wolthers, 1970). The selected peptide is indicated by the dark shaded region, as is the active site cysteine residue at position 25. The arrow indicates the active site histidine at position 159.

Three regions in the structure were thought to be potentially useful: a helical sequence around the active site cysteine residue at position 25, a more linear sequence around the active site histidine (residues 149-161 in papain) and a "hinge-like" region from residues 9-19. The helical region around the cysteine residue was thought to be too difficult to mimic using a linear peptide sequence, and was therefore not considered further. The "hinge-like" region was chosen for cathepsin B in a parallel study in this laboratory, but was unsuitable for cathepsins L or H, due to their large sequence homology in this region. The region around the active site-associated histidine was therefore of the most interest. The sequence here was found to be quite specific for cathepsin L when compared to cathepsins B and H (Fig. 40). In addition, the sequences of cathepsin L across species were very similar in this region (Fig. 41). The particular sequence chosen appeared to be accessible according to the tertiary structure of papain, becoming less accessible after the histidine residue. From these points of view the sequence appeared to be very suitable for the purpose of eliciting specific, immunoinhibitory antibodies to cathepsin L across species.

E-P-D-C-S-S-E-D-M-D-H-G-V	(human cathepsin L)
Q-H-V-T-G-E-M-M-G-G-H-A-I	(human cathepsin B)
S-C-H-K-T-P-D-K-V-N-H-A-V	(human cathepsin H)

Figure 40: The peptide sequence selected for human cathepsin L, in comparison to sequences from human cathepsins B and H. Residues which are the same or similar are underlined (sequences from Gal and Gottesman, 1988).

E-P-D-C-S-S-E-D-M-D-H-G-V	(human cathepsin L)
E-P-N-C-S-S-K-D-L-D-H-G-V	(rat cathepsin L)
E-P-N-C-S-S-K-N-L-D-H-G-V	(mouse cathepsin L)
E-P-D-C-S-S-E-D-L-D-H-G-V	(chicken cathepsin L)

Figure 41: The peptide sequence chosen for human cathepsin L, compared with other species for which the sequences are known. The references from which the sequences are taken are listed in the text above. Residues which are the same or similar are underlined.

In order to assess the potential immunogenicity of the sequence, the amino acid sequence of human cathepsin L was analysed in terms of the parameters known to be important in this regard i.e.:

1) The hydrophilicity of the peptide, which is thought to be a good means of predicting immunogenicity of protein regions, since it indicates whether the region is near the surface of the molecule and therefore accessible to antibody binding (Hopp and Woods, 1981, 1983).
2) Segmental mobility/flexibility of the peptide, since peptides have been found to be more immunogenic if they adopt a large number of conformations (Westhof *et al.*, 1984).
3) Immunogenicity of a peptide is enhanced if it is in the region of 10-20 amino acids in length (Van Regenmortel, 1988a).

The use of hydrophilicity and flexibility parameters for the prediction of the antigenicity of certain regions of a protein has recently been questioned by workers in the field (Van Regenmortel, 1988a), but they remain the best means of prediction available at present, apart from three-dimensional structures of the enzyme. The analysis of these two parameters was carried out by means of the computer programs of Hopp and Woods (1983), for hydrophilicity, and Westhof *et al.* (1984), for mobility. The sequence of interest is not on a peak of hydrophilicity or mobility but, as can be seen in Fig. 42, it is hydrophilic and mobile at its

N-terminus. This corresponds well with its position in the papain tertiary structure, as the hydrophilicity and mobility drops closer to the C-terminus of the peptide, where the sequence does in fact move towards the interior of the molecule. This correlation between the tertiary structure of papain, and the hydrophilicity/mobility plots, for human cathepsin L enhances the confidence in both methods as means of prediction of immunogenicity of regions in cathepsin L. The peptide chosen is 13 amino acids long and therefore conforms with the last criterion for immunogenicity.

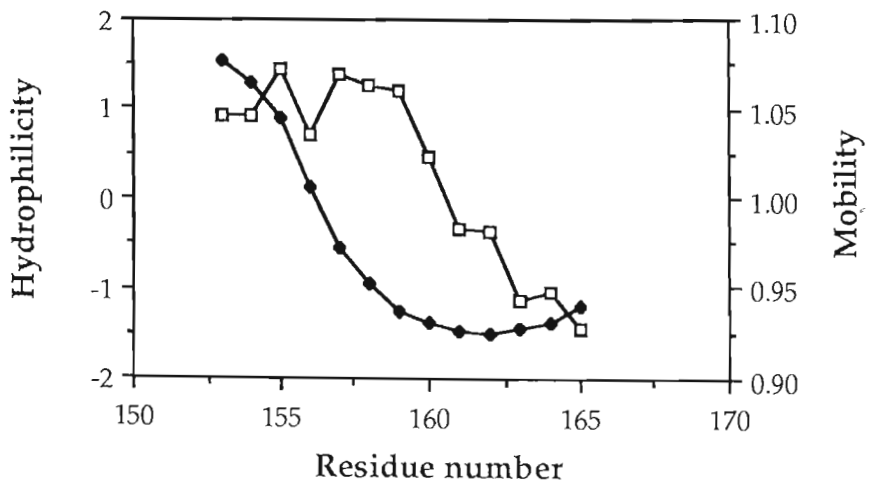


Figure 42: Hydrophilicity and segmental mobility profiles of the selected human cathepsin L sequence.
(□), hydrophilicity calculated according to Hopp and Woods (1981, 1983) and (◆), segmental mobility, calculated according to Westhof *et al.* (1984).

The peptide chosen therefore conforms to the criteria outlined i.e.:

(i) it is active site associated
(ii) it is specific for cathepsin L compared to related proteinases
(iii) it is similar across species
(iv) it is hydrophillic and mobile
(v) it is longer than 10 amino acid residues.

The greater hydrophilicity and mobility of the peptide towards its N-terminus, indicated that conjugation to a carrier should perhaps be through its C-terminus for maximum immunogenicity, but the necessity to expose the active site-associated histidine residue (near the C-terminus) overrode this consideration. The peptide was therefore conjugated through its N-terminus to the carrier, KLH, using glutaraldehyde. Glutaraldehyde conjugation (using 1% concentrations) was found to produce the most stable conjugates for the production of anti-peptide

antibodies (Briand *et al.*, 1985), and therefore was the method of choice for this study. The degree of conjugation achieved was analysed and the conjugate was used to immunise rabbits.

In this chapter will discuss the production of polyclonal antibodies to sheep cathepsin L, in both rabbits and chickens, and the differences between the antibodies from these two different species in their interaction with cathepsin L, will be discussed. The production of anti-peptide antibodies in rabbits, their characterisation and immunoinhibitory properties, and their potential therapeutic role will also be discussed.

5.2 Reagents

5.2.1 Synthesis of peptide. The peptide was custom synthesised by Multiple Peptide Systems, San Diego, California. The selected peptide was modified, before synthesis, by the substitution of the cysteine residue with α-amino butyric acid, which eliminates the formation of dimers between peptides via this highly reactive group, and prevents secondary reactions by cysteine in the glutaraldehyde conjugation procedure. An additional modification of the peptide was the conversion of the carboxy terminus to an amide, thus more effectively mimicking the peptide in the context of the protein, since the amide leaves the C-terminal group uncharged and bonded as it would be in the protein structure. This modification also ensures a higher yield from peptide synthesis (Multiple Peptide Systems technical bulletin).

5.2.2 Micro-biuret reagent. Na_3 citrate.$2H_2O$ (8,65 g) and Na_2CO_3 (5 g) were dissolved in 45 ml of dist.H_2O with gentle warming. The solution was cooled, $CuSO_4.5H_2O$ (0,865 g) was added and dissolved, and the volume was made up to 50 ml.

5.2.3 Human kidney cathepsin L. This enzyme (25 µg) was obtained from Novabiochem, U. K..

5.3 Procedures

5.3.1 Conjugation of peptide to KLH. Conjugation of the peptide to KLH was carried out via the bifunctional agent glutaraldehyde, using the method of Bulinski and Gundersen (1986).

KLH (14,8 mg, 0,074 µmoles, assuming a M_r of 200 000) was dissolved in 200mM sodium acetate buffer, pH 5,5 (10 ml). This solution was dialysed overnight against the acetate buffer and centrifuged (10 000 x g, 10 min, 4°C) to remove residual particulate matter. Peptide (10 mg, 7,4 µmoles, assuming a M_r of 1349) was dissolved in this solution, giving a molar ratio of 100:1 (peptide: KLH). A 50-fold molar excess of glutaraldehyde to peptide was added [0,138 ml

of a 25% glutaraldehyde solution (E.M. grade, Merck, d = 1,06)], dropwise, with stirring, over 5 min. The conjugation was allowed to proceed overnight at room temperature with continuous stirring. The reaction was stopped by the addition of 1 mg/ml of sodium borohydride, and the amount of conjugation was estimated by the method described in section 5.3.3.

5.3.2 Micro-biuret assay. Peptide solution (500 µl) was added to 3% (w/v) NaOH (4 ml) and micro-biuret reagent (200 µl), vortexed, and the A_{330} was read in quartz cuvettes after 20 min. A standard curve for the cathepsin L peptide was established by using a 1 mg/ml peptide solution, diluted to 4 levels from 0-500 µg. Linear regression analysis of the curve gave the equation:

$$y = 0{,}0127 + 0{,}0002176x \quad (R = 1{,}00).$$

Substituting the A_{330} as the "y"-value, allowed calculation of the "x"-value, which represents the peptide concentration (mg/ml) in a sample.

5.3.3 Estimation of the amount of peptide conjugated to the carrier. The conjugate mixture (10 ml) was applied to a Sephadex G-100 (superfine) column (2,5 cm x 40 cm = 200 ml), and the column was eluted at 25 ml/h (10 cm/h). The elution profile was determined using the micro-biuret reaction, and the free peptide peak was pooled, and quantitated by the same method. The amount of conjugation was estimated by subtracting the amount of free peptide from the total added, and expressing this as a percentage of the total. This figure was used to determine the amount of conjugate needed to immunise rabbits at the correct dose.

5.3.4 Production of antibodies in experimental animals. Antibodies to sheep liver cathepsin L were raised in rabbits by subcutaneous injections, at 4-5 sites on the back, with 50 µg of cathepsin L emulsified in Freund's complete adjuvant in a 1:1 ratio. Further inoculations (at the same dose) were administered in Freund's incomplete adjuvant in the same manner at two weeks, and thereafter at 4 week intervals. Rabbits were bled from the marginal ear veins at 8 weeks, and by cardiac puncture at 12 weeks.

Antibodies to the human cathepsin L peptide, conjugated to KLH, were raised in rabbits in a similar manner to that described for anti-sheep cathepsin L antibodies, except that the dose was 200 µg of conjugated peptide for each inoculation. To follow the progress of the rabbits' response to the antigen, the animals were bled from their marginal ear veins at 3 weeks and 8 weeks, and by cardiac puncture at 12 weeks.

Antibodies to sheep liver cathepsin L were also raised in chickens by intramuscular injections, at two sites in their breast muscles, with 50 µg of cathepsin L, emulsified in Freund's complete adjuvant in a 1:1 ratio. Two further inoculations (at the same dose) were administered

in Freund's incomplete adjuvant in the same manner at 1 week intervals, followed by two inoculations at 2 week intervals, and thereafter at 4 week intervals. Eggs were collected continuously throughout the immunisation period.

5.3.5 Removal of anti-KLH antibodies. KLH (12,5 mg) was dissolved in coupling buffer (5 ml) and dialysed overnight against this buffer (50 ml). The solution was centrifuged (10 000 x g, 10 min, 4°C) to remove any insoluble matter, and the KLH was added to Sepharose (5 g), activated by CNBr as described in section 2.10. The coupling was effected in an end-over-end mixer for 2 h at RT. The supernatant fluid was removed, and excess active groups were blocked by the addition of 1M ethanolamine, pH 8 (10 ml). Uncoupled ligand was washed from the adsorbent as described previously (section 2.10).

An immunoglobulin fraction (52 mg), purified from serum by PEG precipitation (Polson *et al.*, 1964), was applied to the column in PBS containing 500mM NaCl, and the column was washed with the same buffer. Bound antibodies were removed from the KLH-Sepharose by washing with two column volumes of 3,5M potassium isothiocyanate. Dot blots were carried out, on KLH adsorbed to nitrocellulose, to show that anti-KLH antibodies were removed from the immunoglobulin fractions.

5.3.6 Immunoinhibition of cathepsin L. Initially, immunoinhibition of cathepsin L was analysed by monitoring the cleavage of the substrate Z-Phe-Arg-NHMec on a continuous basis. Enzyme (25ng) was diluted in 0,1% (w/v) Brij 35 (250 µl), and to this was added the relevant IgG or IgY fraction at 1 mg/ml in 400mM sodium phosphate, 1mM EDTA and 0,1% (w/v) BSA, pH 6,0 (250 µl). This mixture was incubated at 30°C for 15 min, before being activated by the addition of the above buffer, containing 8mM DTT (250 µl), for 1 min. Substrate at 20µM (250 µl) was added, and the increase in fluorescence was monitored for 5 min on a Hitachi Model F-2000 fluorimeter. The slope of the linear increase in activity, in the presence of the different IgG or IgY preparations, was calculated, and the amount of inhibition was expressed in comparison to the slope in the presence of non-immune IgG or IgY at the same concentration, to account for non-specific competitive inhibition by the presence of the antibodies. Intrinsic proteolytic activity in the IgG fractions, probably due to serum kallikrein, was controlled both by the addition of SBTI (40 µg/ml), which had no effect on cathepsin L, and by measuring any residual activity in the IgG fractions.

The continuous monitoring method established whether a particular antibody preparation was immunoinhibitory, and allowed assessment of any unrelated effects in the system, for instance whether the slope of the line was linear at all times under the conditions employed, indicating that there was no substrate depletion.

For the measurement of the extent of immunoinhibition across a range of concentrations of antibody, the above method was less useful, however, since replication was difficult, and the method was too lengthy for a range of antibodies. Stopped time assays were therefore carried out over a range of antibody concentrations in a similar manner to that described above, except that the enzyme activity was stopped by the addition of the monochloroacetate reagent, as described in section 2.4.2. The inhibition of enzyme activity by the antibodies was expressed as a percentage of the activity in the presence of normal rabbit IgG or chicken IgY, with the subtraction of the intrinsic activity of IgG fractions as described above.

5.4 Results

5.4.1 Production and characterisation of polyclonal antibodies raised in rabbits against sheep liver cathepsin L. Antibodies were successfully raised in rabbits against pure cathepsin L using the immunisation procedure described by Mason (1986). The antibody titre was measured using an ELISA procedure in which cathepsin L was coated directly onto the ELISA plate, and the antibodies bound to the cathepsin L across a serial dilution were quantitated by HRPO-linked secondary antibodies. The results for only one rabbit is shown in Fig. 43, since the two rabbits immunised reacted similarly, both with titres in the range of 30 µg/ml of purified IgG (titres, in this study, are expressed as the concentration of antibody at which ELISA values are still noticeably higher than equivalent non-immune controls).

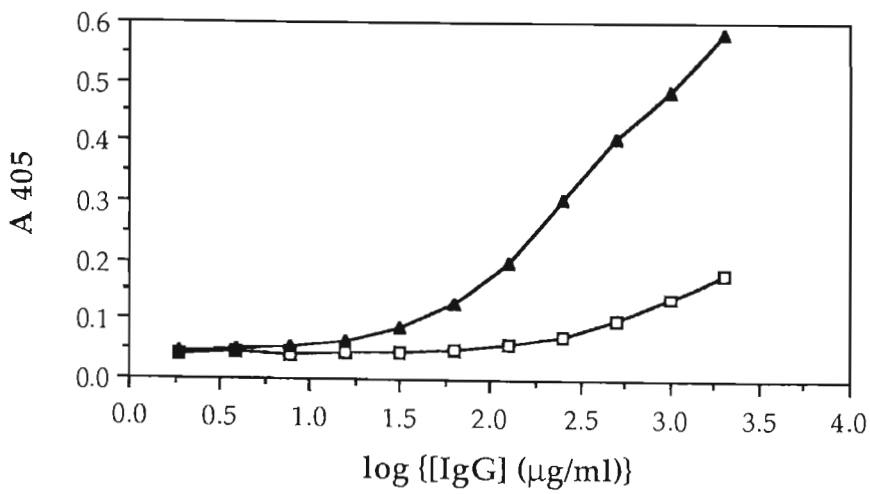


Figure 43: ELISA of the binding of rabbit anti-sheep cathepsin L IgG to sheep cathepsin L. Sheep cathepsin L was coated at 1 µg/ml and the ELISA was carried out as described in section 2.9. Binding by non-immune IgG (□) and immune IgG (▲).

The antibodies were also tested for their specificity by means of a western blot, using a dilution range derived from the above ELISA results, i.e. dilutions of IgG were chosen that corresponded to the steep part of the ELISA titration curve, just beyond the initial plateau region (30-200 µg/ml). The antibodies were tested against a crude ion-exchange sample, obtained by eluting the bound fraction from S-Sepharose with a step gradient of 600mM NaCl (shown in Fig. 44A). The western blot is shown in Fig. 44B, and shows that the antibodies are specific for cathepsin L in that they targeted only cathepsin L in the mixture of proteins provided by the crude ion-exchange sample. The rabbit anti-sheep cathepsin L antibodies were also found to cross-react with human cathepsin L in a western blot (Fig. 44C), thus confirming the results of the study of Mason (1986), in which a similar experiment was carried out.

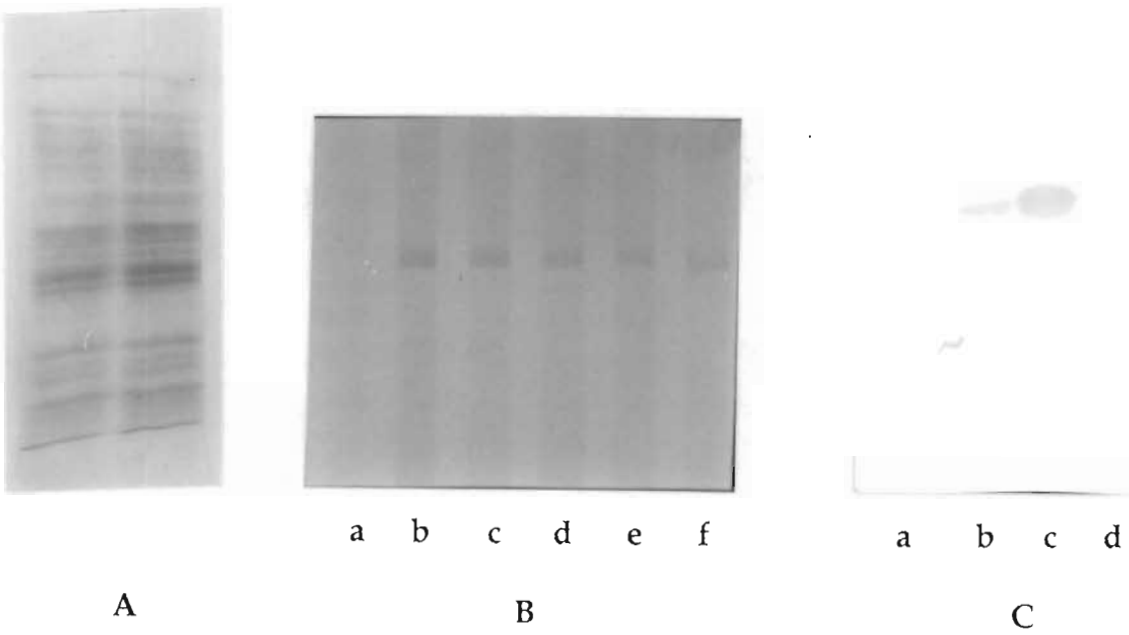


Figure 44: Targeting of sheep and human cathepsin L by rabbit anti-sheep cathepsin L antibodies on a western blot.

A) 12,5% SDS-PAGE of a crude S-Sepharose ion-exchange fraction from sheep liver.

B) targeting of sheep cathepsin L in the ion-exchange fraction, electroblotted onto nitrocellulose, by a) 0,2 mg/ml pre-immune IgG; b) 0,2 mg/ml; c) 0,125 mg/ml; d) 0,1 mg/ml ; e) 0,0625 mg/ml; and f) 0,05 mg/ml immune IgG, visualised using sheep anti-rabbit HRPO.

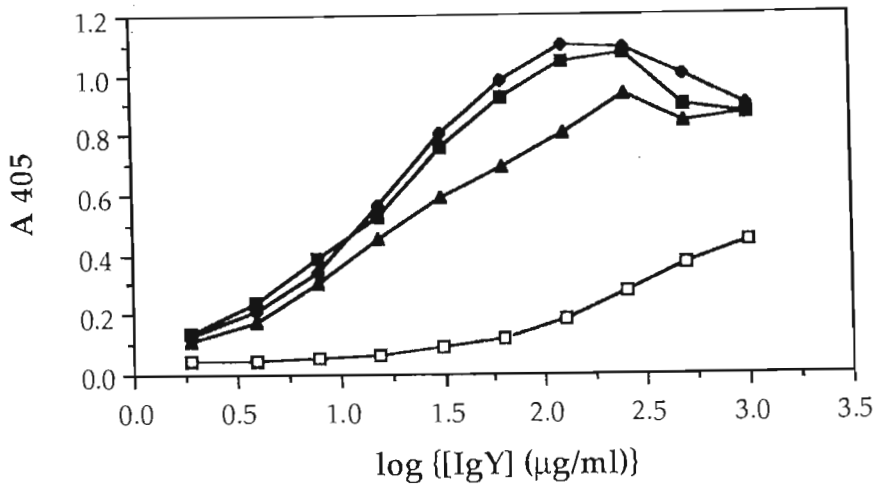
C) a) human cathepsin B (5 µg), b) human cathepsin L (5 µg), c) sheep cathepsin L (5 µg), d) MW markers, were electrophoresed on 12,5% SDS-PAGE, with reduction, electroblotted onto nitrocellulose, and incubated with 0,2 mg/ml rabbit anti-sheep cathepsin L, before targeting was visualised using protein A-gold, with silver amplification.

The antibodies were also tested for their immunoinhibition of sheep cathepsin L activity against the synthetic substrate Z-Phe-Arg-NHMec. No immunoinhibition of the

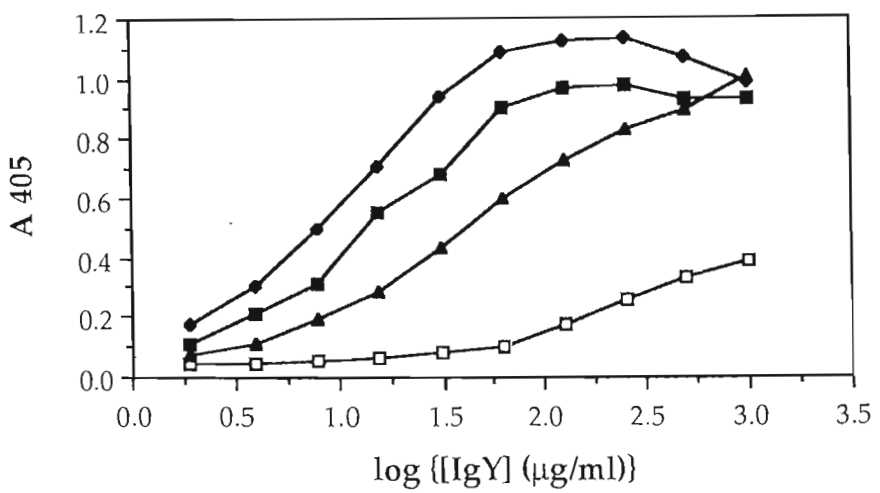
hydrolysis of this substrate was found with rabbit anti-cathepsin L IgG, as the slope of activity in the presence of the immune IgG was greater than that in the presence of the non-immune IgG (result not shown).

5.4.2 Production and characterisation of polyclonal antibodies raised in chickens against sheep liver cathepsin L. Cathepsin L was also used to immunise chickens by a more intensive procedure, devised by Polson *et al.* (1980). Antibodies produced by chickens are passed on to their offspring in the yolk of their eggs, thus making the egg yolk of immunised chickens a convenient source of the chicken antibody, IgY. This antibody is easily isolated from the egg yolk (Polson *et al.*, 1985), and thus large yields of antibodies are obtained relatively easily from chickens, in contrast to rabbits, where more difficult bleeding procedures are required.

An ELISA was carried out, for both chickens immunised, to follow the increase in titre of antibodies to cathepsin L (Fig. 45). A similar curve was not determined for rabbits as the timing of their response was known (Mason, 1986). The two chickens differed in the timing of their response, since, although both showed an increased response from 4 to 8 weeks, chicken 1 did not increase much from 8 to 12 weeks, while chicken 2 showed a large increase from 8 to 12 weeks. The titres of the two chickens were similar at 12 weeks, however, showing that chicken 1 simply responded faster than chicken 2. From the general results of the ELISAs, it was evident that the chicken antibodies against sheep liver cathepsin L, had approximately 10-fold higher titres (3 µg/ml) than the equivalent rabbit antibodies. While direct comparisons are not really possible between the rabbit and chicken anti-cathepsin L antibodies, due to the different secondary antibodies used as detection systems in the ELISAs, the chicken antibodies seemed to titrate much further against the enzyme in an ELISA, and thus seemed to be much stronger antibodies against cathepsin L than the rabbit antibodies.



(A)



(B)

Figure 45: ELISA of the binding of chicken anti-sheep cathepsin L IgY antibodies to sheep cathepsin L, over the period of the immunisation protocol.
Sheep cathepsin L was coated at 1 µg/ml and the ELISA was carried out as described in section 2.9. Curves for (A) chicken 1 and (B) chicken 2. Binding by non-immune IgY fraction (□), immune IgY after 4 weeks (▲), 8 weeks (■) and 12 weeks (◆).

The specificity of the chicken antibodies was confirmed in a western blot where only cathepsin L was targeted in a crude ion-exchange sample (Fig. 46). The light targeting of a band at about M_r 68,000 was attributed to keratins which contaminate protein samples to a certain degree, and non-specifically bind antibodies, especially in western blots (Ochs, 1983; Shapiro,

1987). This was confirmed by the reaction of the non-immune IgY with these bands, highlighting the non-specificity of the reaction.

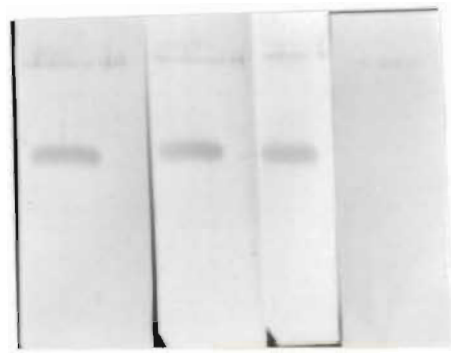


Figure 46: Targeting of sheep cathepsin L, in a crude ion-exchange fraction, by chicken anti-sheep cathepsin L antibodies on a western blot.
Crude ion-exchange sample, electroblotted onto nitrocellulose, was incubated with a) 30 µg/ml, b) 15 µg/ml, c) 7,5 µg/ml anti-sheep cathepsin L IgY and d) 30 µg/ml of non-immune IgY, and the reaction was visualised using rabbit anti-chicken IgG-HRPO.

The immunoinhibitory properties of the IgY fractions were tested and, contrary to rabbit IgG, were found to inhibit cathepsin L, since the slope of activity in the presence of the immune IgY was about half that of the non-immune IgY (result not shown). Immunoinhibition tests across a range of antibody concentrations (Fig. 47), revealed that the antibodies inhibited the enzyme weakly up to concentrations of 31,25 µg/ml, after which the inhibition increased up to 45% at 500 µg/ml, before dropping off again at 1 and 2 mg/ml. The reasons for the inhibition dropping off at higher concentrations of IgY are difficult to define, although it must be noted that activity against the substrate in the presence of 1 and 2 mg/ml non-immune IgY, decreased sharply compared to the 0,5 mg/ml values. The values in the presence of immune IgY continued to drop as well, but not to the same extent as the values with the non-immune IgY, to which the activity values in the presence of immune IgY are compared in order to obtain a controlled measure of the inhibition by these fractions. The drop in inhibition in these areas is therefore probably due to the large degree of non-specific inhibition of the enzyme by non-immune IgY, creating the impression of a drop in inhibition relative to the more specific effects of the immune fractions. Measurements of inhibition using these antibodies should therefore be avoided at values above 0,5 mg/ml.

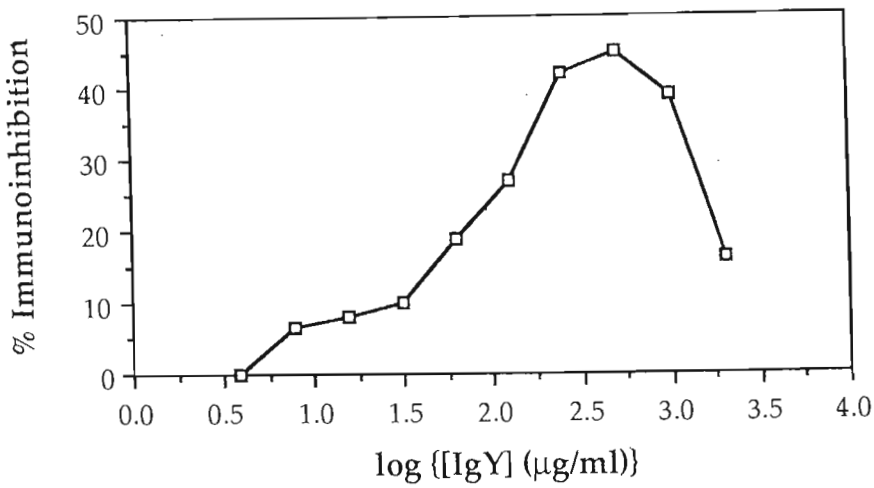


Figure 47: Immunoinhibition of sheep cathepsin L by chicken anti-sheep cathepsin L antibodies.
Stopped time assays were carried out using sheep cathepsin L as described in section 5.3.6, and the percentage inhibition was calculated relative to control assays with non-immune IgY.

5.4.3 Production and characterisation of antibodies raised in rabbits against the human cathepsin L peptide. In the estimation of the conjugation efficiency of peptide to KLH, free peptide was quantitated using the micro-biuret protein test. This test quantitates protein by reacting with peptide bonds, making it most suitable for peptides as well, especially the one used in this study, which contained none of the reactive groups considered necessary for other protein assay methods. The peptide did have absorbance maxima at A_{225} and A_{280}, but glutaraldehyde was found to interfere significantly with measurements at these wavelengths, and therefore quantitation by absorbance readings at 225 or 280 nm was not a useful method in this context.

Using this method, 47,5% of the cathepsin L peptide was found to have conjugated to the KLH, which is within the range obtained by Bulinski and Gundersen (1986). The limitations of the method used to make this estimation were appreciated, especially with reference to Briand *et al.* (1985), in which they point out that amino acid analysis of a conjugate to a protein whose amino acid composition is well known, such as ovalbumin or BSA, is possibly the only really valid method of estimating conjugation efficiency. Nevertheless, the method used here provided an estimation which allowed the implementation of the immunisation protocol.

An ELISA against the peptide, coated directly to the ELISA plate, was developed to assay for the presence of anti-peptide antibodies in the serum of immunised rabbits. Initially, ELISAs were carried out against peptide adsorbed via glutaraldehyde to BSA coated onto an ELISA plate, or peptide coated directly to a glutaraldehyde-activated plate. This was carried out due to the belief that the peptide stood a negligible chance of binding to the plate by itself (Bulinski *et al.*, 1983; Van Regenmortel, 1988b). Controls on these plates, where the above steps were left out and peptide was coated directly instead, indicated that this was sufficient, since efficient binding of the peptide was achieved, as evidenced by the stronger binding of the antibodies in this mode, compared to that with indirectly-bound mode. This mode of ELISA also meant that detection of carrier agent-modified residues (CAMOR) by the antibodies, possibly giving false positive results (Briand *et al.*, 1985), was avoided.

Using this ELISA, the progress of the rabbits' response to the immunisation protocol was monitored as shown in Fig. 48. The rabbits responded very similarly over time to the peptide, with maximal response after 8 weeks, with a slight decline at 12 weeks, and hence only the results for one rabbit are shown. Large amounts of blood were therefore collected from the rabbits by cardiac puncture in the 12th week, before the antibody levels dropped any further. IgG was purified from this serum and the anti-KLH antibodies were adsorbed from these fractions. Anti-KLH antibodies in the anti-peptide antibody fractions are known to cross-react with many proteins non-specifically (D. Buttle, Strangeways Research Laboratories, personal communication).

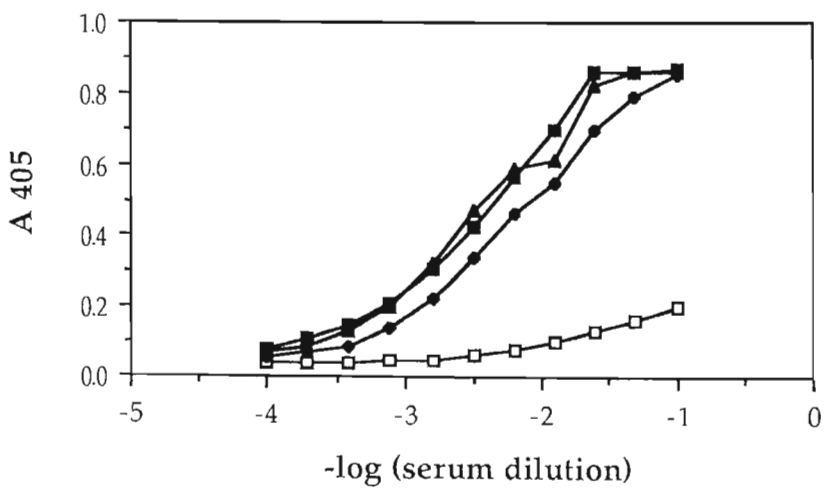


Figure 48: ELISA of the binding of rabbit anti-human cathepsin L peptide antibodies to human cathepsin L peptide over the time course of the immunisation protocol.
Human cathepsin L peptide was coated at 1 µg/ml and the procedure was carried out as described in section 2.9. Binding by non-immune IgG (□), immune IgG after 3 weeks (◆), 8 weeks (▲) and 12 weeks (■).

Following the verification that the rabbits responded to immunisation by the peptide, it was necessary to test the reactivity of the anti-peptide antibodies towards the whole protein. Pure sheep cathepsin L was available for this test, but the only human cathepsin L available in sufficient quantities, was that isolated from the human spleen. This cathepsin L was complexed to cystatin in a normal manner (relative to sheep liver cathepsin L, see Chapter 4), and thus the active-site directed anti-peptide antibody would be largely occluded from binding to the enzyme. Due to this factor the anti-peptide antibodies bound more strongly to sheep cathepsin L than to the human cathepsin L (Fig. 49). An interesting observation, was that anti-peptide antibodies had to be purified from serum before they showed any binding to cathepsin L, most probably indicating that the enzyme's active site was bound very strongly by cystatin present in the serum, precluding binding by the anti-peptide antibody.

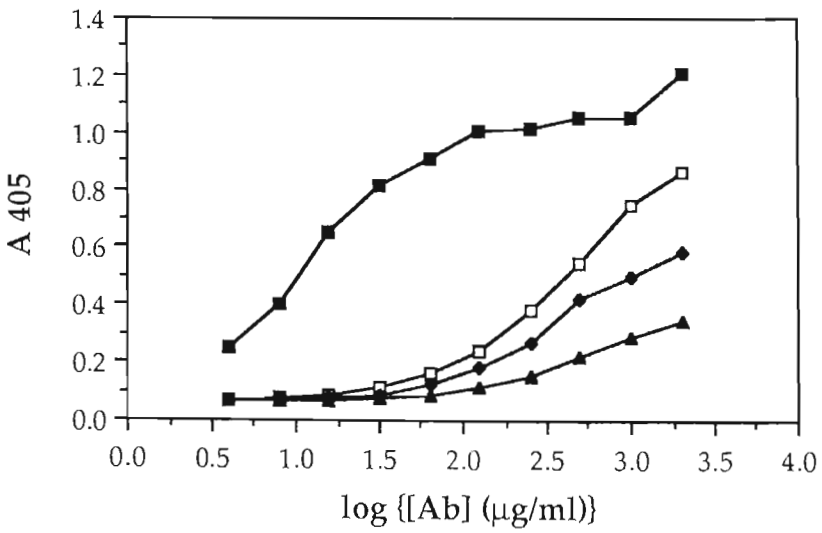
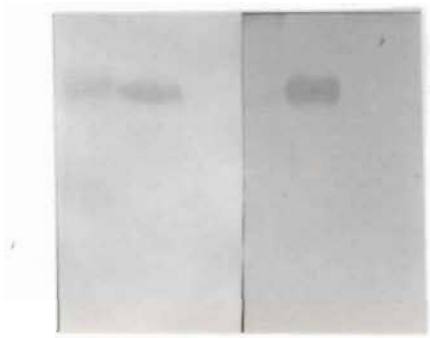


Figure 49: ELISA of the binding of anti-human cathepsin L peptide antibodies to whole, immobilised human and sheep cathepsin L.
The cathepsin L was coated at 1 μg/ml and peptide at 5 μg/ml, and the ELISA was carried out as described in section 2.9. Reaction with human (◆) and sheep (□) cathepsin L, peptide (■) and non-immune rabbit IgG (▲).

The ELISA demonstrated that the antibodies recognised the whole protein, and the level of interaction with the human enzyme was encouraging, despite the limitations of the test. The level of interaction with the whole enzyme dropped off quite sharply, giving quite a low titre of about 125 μg/ml, although once again due to the limitations of the test, this may be an underestimate. It was found that IgG fractions from which anti-KLH antibodies had not been purified, were equally suitable for ELISAs against cathepsin L, or the peptide itself, since values obtained with these fractions were not significantly different from those obtained with IgG fractions from which anti-KLH antibodies had been adsorbed. Adsorption of the anti-KLH

antibodies from the IgG fractions seemed, therefore, to be important mainly for western blots where significant non-specificity was obtained if the anti-KLH antibodies were not removed.

The anti-peptide antibodies were also tested against the protein in a western blot. In this test, the cystatin was removed from the human cathepsin L by the reducing SDS-PAGE conditions, and it was therefore a much better antigen than it was for ELISA purposes. As may be seen in Fig. 50, the antibodies targeted human cathepsin L very strongly (incidentally providing a positive identification of cathepsin L in this instance, see Chapter 4), but only targeted sheep cathepsin L quite weakly. The more sensitive Protein A-gold-silver amplification detection system was therefore employed to give a visible reaction with both the sheep and human enzymes. The antibodies were very specific since they didn't target human cathepsin B at all, and no interaction was visible with non-immune IgG.



a b c d e f

Figure 50: Targeting of human and sheep cathepsin L by rabbit anti-human cathepsin L peptide IgG, on a western blot.
a) sheep cathepsin L (5 µg), b) human cathepsin L (5 µg), and c) human cathepsin B (5 µg), were electrophoresed on 12,5% SDS-PAGE, with reduction, electroblotted onto nitrocellulose, and incubated with 0,5 mg/ml rabbit anti-human cathepsin L peptide, before targeting was visualised using protein A-gold, with silver amplification, as described in section 2.8.3. d), e) and f) were the enzymes in the same order, but detected with sheep anti-rabbit HRPO, as described in 2.8.2.

Following the success of the above tests, the immunoinhibitory properties of the anti-peptide antibodies were tested. Only a small amount of human kidney cathepsin L was purchased, and tests of immunoinhibition of proteolysis were therefore only possible against the very sensitive synthetic substrate. As shown in Fig. 51, the antibodies inhibited the human enzyme quite significantly (85%) at high levels of IgG, the level of inhibition dropping off with decreasing IgG concentration, as would be expected. The antibodies also successfully inhibited sheep cathepsin L (to 40%), although to a lesser degree than the human enzyme, showing that the antibodies target the native enzyme across species. The antibodies did not inhibit human cathepsin B at all.

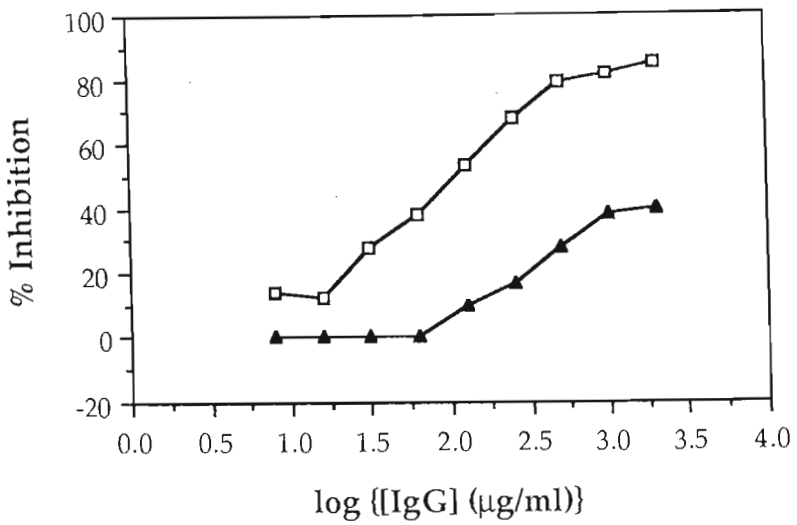


Figure 51: Immunoinhibition of human and sheep cathepsin L by anti-human cathepsin L peptide IgG.

Stopped time assays were carried out using human (□) and sheep (▲) cathepsin L as described in section 5.3.6, and the percentage inhibition was calculated relative to control assays with non-immune rabbit IgG.

Assays against the Z-Phe-Arg-NHMec substrate revealed that the IgG fractions had intrinsic activity against this substrate, which was probably attributable to contaminating plasma kallikrein which also cleaves this substrate (Barrett and Kirschke, 1981). Plasma kallikrein is often a contaminant of IgG purifications or *vice versa*, and IgG apparently stabilises this molecule (Colman and Bagdasarian, 1977). This activity was controlled by the addition of 40 μg/ml of SBTI, which inhibits kallikrein but not cathepsin L. An additional control was to subtract any residual activity in the antibody fractions from the measured cathepsin L activity. The controls outlined seem to be adequate enough to allow clear measurements of the immunoinhibition. In future, the use of plasma samples as sources for the isolation of IgG should be considered, since the clotting cascade is not activated in plasma preparations, in contrast to serum preparations (Lenney, 1983), thus preventing the contamination of the IgG samples by active kallikrein.

5.5 Discussion

This chapter was concerned with the production of antibodies which would prove useful in studies on the role of cathepsin L in tumour invasion. Two facets were explored in this regard: antibodies for use in immunocytochemical studies, and antibodies which specifically immunoinhibit cathepsin L, allowing studies on possible modes of therapy, should cathepsin L prove to be involved in tumour invasion.

For immunocytochemical studies, highly specific antibodies are required in order to prevent labelling not attributable to the protein of interest. The three approaches towards generating antibodies which recognised human cathepsin L were all successsful in generating antibodies which specifically recognised cathepsin L. The anti-peptide antibody appears to be the most specific and powerful of the three antibody-preparations, as might be expected, since the polyclonal antibodies against sheep cathepsin L rely on their cross-reactivity across species for the extent of their reaction with human cathepsin L. Anti-peptide antibodies might prove to be less useful for immunocytochemistry than polyclonal antibodies, however, since the single site targeted by these antibodies, might be labile upon treatment with the fixatives used for this technique, resulting in negligible labelling using this antibody. Future immuno-cytochemical work using the three antibodies should prove interesting in the comparison of the suitability of the different sources of antibody for this type of work.

Immunoinhibitory antibodies should prove to be useful in studies on the role of a specific enzyme in tumour invasion. Antibodies should be more specific than synthetic inhibitors in their action on an enzyme, since the inhibitors rely on the catalytic mode of action of the enzyme, and therefore, while specificity towards a class of enzyme may be simpler to obtain, discrimination within the enzyme class, relying on subtle differences such as those of substrate binding pockets, is more difficult to obtain. In the case of cathepsin L, for instance, a truly specific inhibitor or substrate has yet to be found. The inhibitors which come closest to meeting this criterion, Z-Phe-Tyr-(O-tBut)-CHN_2 and Z-Phe-Phe-CHN_2 (Kirschke *et al.*, 1988), also inhibit cathepsin B, although not as rapidly, nor to the same extent as cathepsin L. Up until this time, the use of synthetic inhibitors for studying the role of proteinases in tumour-associated functions has met with very limited success (Nelles and Schnebli, 1982). The use of antibodies, which are intrinsically quite specific for their target molecules, might thus meet with more success in this regard. Thus in the context of both the study of tumour invasion and potential therapeutic applications, the production of two types of novel, specific immunoinhibitory antibodies in this study holds good prospects for future studies in this area.

Chicken IgY polyclonal antibodies proved to be superior to rabbit IgG antibodies for the inhibition of cathepsin L. These antibodies are generally less commonly used than rabbit antibodies, but seem to produce more potent antibodies at higher yields, in a similar time span to rabbits. Initially, it was thought that the more intensive immunisation schedule used for the chickens might be the cause for this phenomenon, but subsequent studies, using the rabbit immunisation schedule on the chickens, revealed that chickens produced antibodies of similar, or even higher, titre with this protocol (Coetzer, Pike and Dennison, unpublished results). The difference between the strength of the antibodies produced by the rabbits and chickens is, therefore, most probably due to the greater evolutionary distance between mammals and the chicken. The target sites of the immunoinhibitory IgY antibodies is obviously of considerable interest in the context of optimising the production of immunoinhibitory anti-peptide antibodies to cathepsin L. Should the chicken prove to target immunoinhibitory sites different from that chosen for the anti-peptide antibody developed in this study, the use of peptides from these sites to make anti-peptide antibodies could provide better inhibitory antibodies than those developed thus far.

The anti-peptide antibody developed in this study has potential applications for the study of the specific role of cathepsin L in tumour invasion, and for tumour therapy. The antibody prepared was very versatile, in that it recognised native cathepsin L (as evidenced by imunoinhibition), partially denatured cathepsin L (according to Van Regenmortel, 1988a, enzymes are probably at least partially denatured on an ELISA) and fully denatured cathepsin L (on a western blot). It could thus be used for studies ranging from the quantitation of enzyme in a tumour homogenate, to inhibition of the enzyme in *in vitro* tumour invasion assays. For therapeutic applications, the antibodies in their present form are probably not acceptable, primarily due to the high levels of antibody required for a significant degree of inhibition.

To optimise the use of antibodies against the peptide for therapeutic applications, therefore, the most obvious route is monoclonal anti-peptide antibodies. A selection strategy in the production of a monoclonal antibody, which selects for antibodies which specifically inhibit cathepsin L to the highest degree, will allow production of an antibody population which consists entirely of antibodies inhibitory towards cathepsin L. This would mean that the antibodies could be used at a much lower dosage than that indicated by the present results, as there would not be a mixed population of antibodies recognising the enzyme in different ways, as is the case with the polyclonal anti-peptide antibodies made in this study. Also studies on the inhibitory sites targeted by chicken antibodies in cathepsin L, might generate peptide targets which allow an even higher degree of inhibition of cathepsin L.

CHAPTER 6

GENERAL DISCUSSION

Malignant cancer is particularly difficult to treat, due to its ability to metastasize and invade body parts at sites distant from the original tumour. Proteinases have been implicated in facilitating the processes of invasion necessary for metastasis to occur (Tryggvason *et al.*, 1987). The proteinase cathepsin L could be relevant in this regard, due to its ability to degrade many of the components of extracellular barriers such as BM (Baricos *et al.*, 1988), collagen (Kirschke *et al.*, 1982; Maciewicz *et al.*, 1990), elastin (Mason *et al.*, 1986a) and proteoglycan aggregates (Nguyen *et al.*, 1990). Pro-cathepsin L has been found to be secreted at high levels by transformed cells in the form of the MEP molecule (Mason *et al.*, 1987), and the mechanisms for this secretion have been shown to include large increases in the rates of synthesis of the enzyme, and lowered affinity for the receptor responsible for directing the proteinase to its intracellular targets (Dong *et al.*, 1989). This secretion is also enhanced by interaction with hormones such as PDGF (Prence *et al.*, 1990). The secretion of the MEP molecule has been linked to expression of the *ras* (Joseph *et al.*, 1987; Denhardt *et al.*, 1987) and the *fos* oncogenes (Taniguchi *et al.*, 1990), and has been correlated with the metastatic ability of the transformed cell lines in both these cases. The above evidence is supported by *in vitro* studies such as that of Yagel *et al.* (1989) where it was shown that cathepsin L inhibitors suppressed invasion by transformed cells. The presence of cathepsin L has been shown in metastatic murine and human melanoma cells (Rozhin *et al.*, 1989), human colorectal tumour cell lines (Maciewicz *et al.*, 1989) and in a metastatic human pancreatic carcinoma cell line (Yamaguchi *et al.*, 1990). Thus there is an ever growing body of evidence which links cathepsin L to the invasive and metastatic processes of tumour cells.

Most of the studies outlined above provide quite circumstantial evidence for the involvement of cathepsin L in tumour invasion, however, and do not clarify some of the areas of confusion which exist with regard to the putative role for this enzyme in these processes. For instance, it is not known how the enzyme could play a role in these processes at or near physiological pH values, since published values for its pH optimum and stability suggest that it should be highly unstable and hardly active at these pHs. Furthermore, the enzyme is bound very tightly by the ubiquitous inhibitors of cysteine proteinases, the cystatins, whenever outside the protective environment of the lysosome, and thus the mechanism by which it might overcome this inhibition, in order to play some role in tumour invasion, is obscure. Present attempts to study the specific role of the enzyme in tumour invasion are also hampered by the lack of specific inhibitors (Kirschke *et al.*, 1988). This study set out to clarify the above points

and to provide specific antibodies for immunocytochemical studies on the role of the enzyme in tumour invasion.

In order to study cathepsin L in the context described above, it was necessary to purify the enzyme from a convenient but suitable source. Sheep liver was chosen as the source since it was shown to provide enzyme similar to that from human liver at a high yield (Mason, 1986), and was easily accessible in the large quantities necessary for the development of a purification procedure. Generally, previous isolations of cathepsin L (Kirschke *et al.*, 1977; Mason, 1986; Bando *et al.*, 1986) have been multi-step procedures, often using HPLC techniques which were not available in this laboratory. After several attempts these procedures failed to yield an entirely pure enzyme, and a new procedure was consequently developed, of which a new crude fractionation procedure, TPP, was an essential component.

The TPP technique was extensively characterised in this study in order to find how the properties of a protein influence its behaviour in the technique. Generally it was found that proteins survive TPP best at or above their pI, and that an inverse relationship exists between the amount of salt needed to precipitate a protein and its MW. The effect of MW was found to be possibly the overriding consideration in the fractionation of a protein by this technique, although the pH and the protein concentration of the solution also have an effect on the protein's behaviour. Recognition of the above factors facilitated selection of the starting conditions for the fractionation of a protein, according to its properties.

Several of the test proteins were found to be irreversibly denatured during TPP. This was not a temperature dependent phenomenon, as might be expected in a technique involving an organic solvent, since the technique was found to be fairly insensitive to this parameter, within reasonable limits. The role of t-butanol was excluded in the process of denaturation, since CD-spectra indicated that it was not denaturing towards the protein in itself. The denaturing effect of TPP was therefore postulated to be due to the combined effects of the salt and the t-butanol in the procedure. The discovery that haemoglobin was extensively denatured by TPP, was used by Pol *et al.* (1990) in the purification of blood proteins, where this molecule is a particularly troublesome contaminant. The principles established in this study allowed TPP to be successfully applied to the purification of cathepsin D (Jacobs et al., 1989), collagenase (Coetzer and Dennison, unpublished results) and human IgG (Forsyth, Pike and Dennison, unpublished results). The technique was also applied to the crude purification of cathepsin L from rabbit, bovine and sheep livers, and found to be superior to ammonium sulphate precipitation, the technique used previously for this purpose (Mason, 1986).

The use of TPP for the crude fractionation of cathepsin L from sheep liver, seemed to leave the precipitated protein relatively free from salt, and thus obviated a desalting step before ion exchange chromatography in the purification procedure. The cation exchange chromatography step used as the initial chromatography step in the purification procedure developed, took advantage of the fact that cathepsin L binds very strongly to cation exchangers even at pHs very close to its pI (Kirschke *et al.*, 1977). The S-Sepharose ion exchanger used for this step was found to be even more exceptional in this regard, compared to CM-based cation exchangers, since it bound the enzyme very strongly even in the presence of salt, resulting in most contaminating proteins passing straight through the column. Cathepsin L then eluted very late on a salt gradient, away from most other proteins, resulting in a very large purification of the enzyme.

Initially a further step on this column at lower pH was thought to finally purify the enzyme, but the contamination of the final product by cystatin complexed to a proportion of the cathepsin L, meant that it was unlikely that this step would purify the enzyme to homogeneity, due to the similarity in the binding of the complex and free enzyme to the cation exchanger. The use of MEC instead was found to purify the free enzyme for use in later studies.

The free enzyme was found to be in a single-chain form, which was different from the cathepsin L previously isolated from sheep liver (Mason, 1986). Single-chain forms of the enzyme have been reported previously (Dufour *et al.*, 1987), but their isolation seems to require a faster procedure than the multi-step procedures often used for the purification of the enzyme. The purification procedure developed here seems to fulfill this criterion.

The isolated complex between cathepsin L and cystatin was unusual in that it was proteolytically active and covalently bound, which it should not have been if the binding of cystatin had been in the normal mode (Stubbs *et al.*, 1990). This complex was further characterised in an attempt to elucidate the manner in which it was bound to cathepsin L, and both the complexed and free forms of the single-chain enzyme were analysed in terms of their pH-dependent characteristics, to ascertain whether there were any differences between the two forms in this regard, and between the single-chain form and previously isolated forms of the enzyme.

A large proportion of the cystatin/cathepsin L complex was found to have novel binding characteristics. It was shown to be covalently bound in 60% of the fraction, and this covalently bound proportion was found to be proteolytically active by substrate gel SDS-PAGE and active site-titration. In order to study the formation of this covalent complex *in vitro*, cystatins were isolated from sheep liver and subfractionated by anion exchange chromatography. Analysis of the sub-fractionated fractions established that only cystatin β-type inhibitors were present in

this organ. Similar sub-types, differing on the basis of the interactions undergone by the free cysteine near the N-terminus of its structure, to those shown by Wakamatsu *et al.* (1984) in rat liver, were found, but an additional, slightly different form of the inhibitor was also found.

The demonstration that the complex could be formed very rapidly *in vitro* between the cystatin fraction and cathepsin L, at pH 5,5 and above, showed that the isolated complex was probably not just an artifact of the isolation procedure. This finding indicated that homogenisation of tissues at pHs below 5,5 might prevent the formation of the covalent complex, and therefore allow the purification of higher amounts of free enzyme, a strategy which is being employed with success in current purification studies (Coetzer, Pike and Dennison, unpublished results). Previous studies (Brocklehurst and Kierstan, 1973; Shapira and Arnon, 1969) have shown that papain has labile intra-molecular disulphide bonds, due to the interaction of the cysteine moieties, normally involved in these bonds, with the active site cysteine. The free, reactive cysteine residue near the N-terminus of cystatin β, was therefore postulated to be involved in forming the covalent bond to cathepsin L, after causing disulphide exchange within the enzyme in a similar manner to that in papain. The actual physiological role of this type of enzyme/inhibitor complex is obscure at present, but the fact that the inhibitor can interact in a manner different from that previously thought of as the normal mode of binding for cystatin β to cysteine proteinases (Stubbs *et al.*, 1990), is encouraging for further studies on this aspect.

The investigation into the presence of this type of complex *in vivo* would obviously require sophisticated antibodies, which would recognise the complex, but not cathepsin L or cystatin β. Anti-peptide antibodies specific for ubiquitin-histone conjugates, to the exclusion of either of the molecules participating in the complex, have previously been raised (Müller, personal communication), and antibodies of a similar nature would allow specific immunocytochemical studies of the complex *in vivo*, in order to elucidate its physiological role, if any.

The discovery of the alternative mode of binding of cathepsin L to cystatin β, obviously raises many questions about the role that this could play as a mechanism whereby cathepsin L could overcome inhibition by this inhibitor in tumour invasion. If cathepsin L was released by tumour cells in large quantities, there is every possibility that there would be insufficient cystatin in the tissues to cope with all of the cathepsin L being released. Over time the conversion of cathepsin L/cystatin β complexes to covalent, active molecules would mean that there would be a large deficiency in the control of the enzyme in this context, allowing it to play an active role in tumour invasion. The cystatin which is covalently bound to cathepsin L also seems to act as an activator for the enzyme, but obviously for the activity of this form of the enzyme to be relevant to tumour invasion it must be active and stable at the pH of the tumour

extracellular environment. Both the complexed and free, single-chain cathepsin L were therefore examined in terms of their pH-dependent behaviour in the context of tumour invasion.

Both forms were found to have considerably more activity and higher stability at neutral pH than would have been predicted from published values. Initially published enzyme assay methods (Mason *et al.*, 1985; Kirschke *et al.*, 1989) were used for the study of these characteristics, but they were all found wanting in the respect that they gave information from a particular slant, depending on what the authors were endeavouring to achieve in the assays. These methods did not, for instance, give results which would allow predictions about how long the enzyme would be active, at any particular pH. An assay of the half-life of the enzyme at a certain pH, was therefore developed in this study, and found to be very useful in yielding information on the overall behaviour of the enzyme at certain pH values. From these values a composite picture of how active and stable the enzyme would be under certain conditions could be obtained. In this way it was shown that the complexed enzyme was slightly more stable than the free enzyme at neutral pH. It was also shown, contrary to published information (Mason *et al.*, 1985; Mason, 1986; Kirschke *et al.*, 1989), that cathepsin L would be active enough for a sufficient length of time, at physiologically relevant pHs, to play a significant role in tumour invasion. These results, from assays against the synthetic substrate, were also corroborated by assays against the protein substrate, azocasein.

The purification procedure developed was used to purify cathepsin L from human spleen, a fairly readily available source of human tissue. The enzyme was only isolated as a complex with cystatin from this organ, however, and was not proteolytically active. A similar situation was observed for sheep spleen, suggesting that an organ difference was responsible for the differences between sheep liver and human spleen, rather than a species difference. The human enzyme obtained in this way, and that obtained from a commercial source, had similar pH optima compared to the sheep enzyme forms, and thus the differences in pH-dependent characteristics between these forms and those of published values, is probably not due to a species difference.

These findings indicate that cathepsin L could play a role in tumour invasion, since it may be sufficiently active at the pHs which may be found in tumour invasion. Also the cystatin/cathepsin L complex discovered in this study, provides an alternative mode of binding of the enzyme to cystatin β, enabling it to overcome inhibition by these molecules. Since the biochemical characterisation of the enzyme suggests this possibility, some means of studying the actual role of the enzyme in tumour invasion and metastasis, and potential ways of using this information for the treatment of cancer are needed. Immunocytochemistry, using specific antibodies to an enzyme, was regarded as a possible avenue for the further exploration of the

role of cathepsin L in tumour invasion. The development of specific polyclonal antibodies to the enzyme was therefore undertaken, and these were raised in rabbits and chickens. The antibodies were found to be highly specific and of a high titre, especially those from the chicken, and were therefore suitable for immunocytochemical studies now in progress.

A further way of investigating the enzyme's role in tumour invasion is through *in vitro* invasion assays and animal model systems, and the use of anti-enzyme agents to specifically suppress its action in these processes, allowing the effects of this suppression on the process to be examined. Thus the effect that specific anti-enzyme agents have on the metastasis of tumour cells could be examined in an experimental animal (Hart, 1982), possibly with concurrent immunocytochemical studies, as could the invasion of tumour cells through artificial barriers such as chorioallantoic membranes (Poste, 1982). Specific, inhibitory agents are obviously required for this work, but are not available at present, and seem unlikely to be developed using synthetic inhibitors (Kirschke *et al.*, 1988). The possibility of developing specific inhibitory antibodies for this purpose was therefore investigated in this study. The use of active site directed anti-peptide antibodies was examined in this regard, as were the polyclonal antibodies to cathepsin L raised in rabbits and chickens.

Careful examination of the tertiary structure of papain, which is considered to be analogous to cathepsin L (Dufour, 1988), allowed the selection of a peptide, from the active site of human cathepsin L, which was thought to have the potential to raise immunoinhibitory antibodies to the enzyme. The peptide was found to raise antibodies which recognised the enzyme in various tests, showing the presence of antibodies to the various forms of the enzyme, from completely denatured to native. This implies that these antibodies are very versatile and could therefore be used to examine tumours for the presence of cathepsin L using tests such as ELISAs, western blots or immunoinhibition assays. The antibodies were also shown to target cathepsin L across species. Most importantly, however, the anti-peptide antibodies were shown to inhibit cathepsin L quite specifically, especially in that they did not inhibit human cathepsin B, which has been the most similar to cathepsin L in its reactivity with synthetic inhibitors and substrates in the past. This antibody therefore has the potential to be of great use in future studies on the specific role of cathepsin L in tumour invasion. It is envisaged that the production of monoclonal anti-peptide antibodies to the same sequence of cathepsin L will further enhance the potential of these antibodies, since it would lower the dose required for their effective action, and therefore render them more useful in both the context of *in vitro* invasion assays and tumour therapy.

Polyclonal chicken IgY antibodies against cathepsin L were also found to be inhibitory towards the enzyme, in contrast to rabbit polyclonal antibodies which were not inhibitory. This

fact led to the hypothesis that they may be targeting a novel site on the enzyme, which could prove even better, for immunoinhibitory purposes, than that used for the anti-peptide antibodies. A further study which is envisaged, therefore, is to map the inhibitory binding sites of the IgY antibodies against cathepsin L, using peptide fragments of the enzyme to inhibit the antibodies' inhibition of the enzyme. The chicken antibodies were also generally of a higher titre than the rabbit antibodies, making them more useful in techniques such as immunocytochemistry, where high antibody concentrations may cause non-specific effects, rendering low titre antibodies less useful. Thus the ease of production of chicken antibodies and the novel binding characteristics that they possess, make them the reagents of choice over the more traditional rabbit antibodies.

The findings provided by this study will, hopefully, be significant in elucidating the role of the enzyme in tumour invasion. New light has been shed on the enzyme's pH-dependent characteristics with relevance to tumour invasion, which may strengthen the hypothesis that it has a potential role in tumour invasion. The discovery of a different mode of binding of the enzyme to one of its main endogenous inhibitors, cystatin β, is obviously contentious in many regards, not least in its physiological relevance and with respect to tumour invasion, but should this mode of binding be found *in vivo*, it would allow the enzyme to overcome cystatin binding in tumour situations and be proteolytically active. Two specific immunoinhibitory agents have been developed in this study which could allow further investigation of the specific role of cathepsin L in tumour invasion, and could have potential therapeutic applications.

The use of specific antibodies as tools in various techniques is fast becoming the cornerstone to a successful investigation of an enzyme's functions *in vivo*, and the use of a battery of antibodies to different enzymes can often give a very good idea of any one enzyme's contribution to a particular situation. It is hoped that the information gleaned about cathepsin L, with respect to its pH characteristics and interaction with its endogenous inhibitor, and the antibodies developed in this study, will contribute in a meaningful way to a fuller understanding of its role in tumour invasion and metastasis.

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PUBLICATIONS

rotein Fractionation by Three Phase artitioning (TPP) in Aqueous / -Butanol Mixtures

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Three-phase partitioning in aqueous/*t*-butanol mixtures TPP) is a method which may be used for the fractionation nd concentration of proteins.[1-4] Tertiary butanol is initely miscible with water, but can be induced to form a eparate phase by the addition of salt. If protein is present n the solution it may form a third phase, between the queous and *t*-butanol phases, consisting of concentrated nd dewatered protein. A claimed advantage of the method s that protein complexing agents, such as tannins and pheolics, and other enzyme inhibitors may be extracted into ne *t*-butanol phase, thus enhancing the purification of roteins from plants and micro-organisms. Lipids would e similarly extracted.

In studies to date the method has largely been applied mpirically and to the purification of enzymes. The present tudies were aimed at obtaining some insight into the facors which influence the TPP process so that it might subequently be used in a more systematic manner. The pproach adopted was to assemble a group of standard roteins, with known physico-chemical properties, and to nonitor their behavior in TPP under various conditions. Because enzymes *per se* have been the subject of previous tudies,[1-4] the standards were not chosen for enzymic ctivity, but rather to cover a range of pI and molecularveight values. The effect of the following factors upon the listribution of the standard proteins in the TPP system, nd upon their subsequent solubility, was studied: pH relative to the pI, protein molecular weight, protein concentraion, temperature, and the proportions of ammonium ulphate and *t*-butanol. To test the validity of the informaion gained from these studies, predictions were tested by ractionating a contrived mixture of standard proteins by TPP. The separation was monitored by SDS–PAGE.

MATERIALS AND METHODS

Standard Proteins

The standards used were: Bovine serum albumin molecular weight of 66,500 and pI of 4.8), cytochrome C molecular weight of 12.4×10^3 and pI of 10.0), γ-globulin (molecular weight of 1.6×10^5 and pI of ca. 6.0), hemoglobin (molecular weight of 6.8×10^4 and pI of 7.0), lysozyme (molecular weight of 14.3×10^3 and pI of 11.1), myoglobin (molecular weight of 1.7×10^4 and pI of 7) and ovalbumin (molecular weight of 4.5×10^4 and pI of 4.6) (Sigma Chemical Co. St. Louis, MO).

Three-Phase Partitioning

The protein sample was dissolved in buffer (0.01*M*), of the desired pH, at a concentration of 0.5 mg/mL (except where stated otherwise). The A_{280} of the solution was measured, and designated *A*. The *t*-butanol was added to constitute 30% (v/v) of the total mixture volume, and thoroughly mixed in. Ammonium sulphate crystals were added to the mixture to the desired concentration, expressed as a percentage (w/v) of the total mixture volume, and the solution was mixed vigorously to dissolve the salt. (The presence of *t*-butanol alters the physical properties of the solution so that frothing does not occur and there is no apparent denaturation of proteins, or loss of activity of the enzymes which we have tested before and after mixing.) During these operations the solution was maintained at 25°C, except where the effect of temperature was being investigated. The mixture was centrifuged ($10^4 g$, 10 min., room temperature), and the three phases were collected separately. The A_{280} value of the aqueous phase of the partitioned mixture was measured against a blank, treated identically, but with no protein present, and this value was designated *B*. The interphase (third phase) layer was dissolved (as far as possible) in a volume of 0.01*M* Phosphate buffer, pH 7.0, equal to that of the original buffer. Material which would not redissolve was removed by centrifugation and the A_{280} value of the resulting supernatant was measured, and designated C.

The percentage of the original protein which was extracted into the third phase, was calculated as follows:

$$\text{Percent protein} = \frac{A - [B(F/I)]}{A} \times 100$$

where *F* is the final volume, and *I* is the original volume of aqueous phase (It should be noted that no protein enters

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the *t*-butanol phase). The apparent percentage solubility of the protein after TPP was calculated as follows:

$$\text{Percent solubility of protein} = \frac{C}{(A - B)} \times 100$$

SDS–Polyacrylamide Gel Electrophoresis

Gradient SDS-PAGE (5–15% acrylamide) was carried out according to the method of Laemmli,[5] using a Hoeffer SE 600 slab gel apparatus. Electrophoresis was carried out at 30 mA/gel and the protein bands were stained with Coomassie brilliant blue R-250 stain.

Density Measurements

Solution densities at 25°C were measured in a Parr DMA 10 precision density meter.

Circular Dichroism Spectra

CD-spectra were measured using a Jasco JA-20 spectropolarimeter. The proteins were dissolved in 0.01*M* buffer, with pH = pI of the test protein, or in a mixture of buffer and *t*-butanol, up to 50% (v/v) *t*-butanol. The interpretation of CD spectra has been discussed by Tinoco and Cantor[6] and by Johnson[7].

Hydrophobicity Ranking

Test proteins were ranked for hydrophobicity by their order of elution from a phenyl-Sepharose column, in 0.01M Na-phosphate, pH 6.8. Elution was effected by a simultaneous gradient of decreasing $(NH_4)_2SO_4$ concentration and increasing concentration of ethylene glycol, as described in the Pharmacia handbook.

RESULTS

The Effect of Different Proportions of Ammonium Sulphate and t-Butanol

To determine the proportions of ammonium sulphate and *t*-butanol needed to form a two phase system, in the absence of protein, ammonium sulphate was added to a series of solutions containing increasing amounts [0–50% (v/v)] of *t*-butanol in distilled water, until two phases formed in each case. The resulting curve was used as a basis for partitioning of the proteins BSA and ovalbumin, to establish the amount of ammonium sulphate required to obtain a well-resolved third phase.

The results (Fig. 1), indicate a reciprocal relationship between the amounts of ammonium sulphate and *t*-butanol required to form a two phase system. However, it was observed that in the presence of protein more ammonium sulphate was required to get clearly resolved phases, than in the absence of protein. With both of the test proteins, it was possible to get a three phase system with a range of different proportions of ammonium sulphate and *t*-butanol. No particular proportion appeared to offer any advantage, although at the lowest levels of *t*-butanol less protein was extracted into the third phase. In an industrial context a least-cost mixture of ammonium sulphate and *t*-butanol might have an economic advantage.

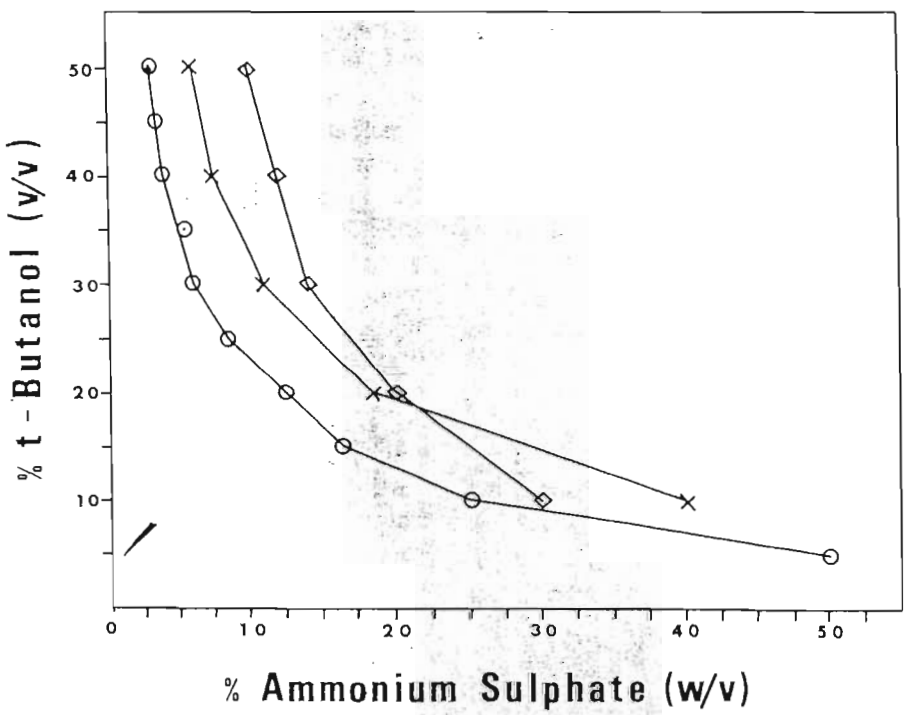

Figure 1. The proportions of ammonium sulphate and *t*-butanol required to form distinct phases: (O—O) two phase system in the absence of protein; (×—×) three phase system in the presence of ovalbumin at pH 4.6; and (◇—◇) three phase system in the presence of BSA at pH 4.8.

he Effects of Density of the Phases

Measurement of the solution densities revealed that, in ie TPP process (at a constant nominal *t*-butanol concen- ation of 30%), the densities of the phases are complex inctions of the nominal ammonium sulphate concentra- on, whereas with simple solutions of ammonium sulphate ie density increases linearly with the ammonium sulphate oncentration (Fig. 2). In the TPP process, proteins float n the aqueous phase whereas, in conventional salting out vith ammonium sulphate, they sink in ammonium sulphate olutions of the same density, e.g. BSA (2 mg/mL) is alted out, and sinks, in 30% ammonium sulphate in pH .8 buffer, but is precipitated out, and floats, on the queous (lower) phase of TPP conducted with a nominal oncentration of 25% ammonium sulphate (which has the ame density as 30% ammonium sulphate). Similarly, valbumin (2 mg/mL) is salted out, and sinks, in 60% am- nonium sulphate but is precipitated out, and floats, on the queous phase of TPP at a nominal 30% ammonium sul- hate. In the latter example, the TPP aqueous phase would ctually be *less* dense than the simple 60% ammonium sul- hate solution (Fig. 2).

The Effects of pH

TPP was carried out at a series of pH values for each of he standard proteins, except myoglobin which was totally lenatured by TPP. In each case the protein was initially lissolved in a 0.01*M* buffer (citrate, phosphate, or glyci- nate) of the required pH, and TPP was effected, using an ammonium sulphate concentration sufficient to extract at east 90% of the protein into the third phase at the opti- mum pH. Two indices of the efficacy of TPP were measured; firstly, the amount of protein extracted into the third phase (Fig. 3) and, secondly, the solubility of this extracted protein (Fig. 4). Both measures were expressed as a percentage.

From Figure 3 it may be seen that, with the exception of hemoglobin, the standard proteins are most readily extracted into the third phase at lower pH values, i.e. where the proteins have a net positive charge. However, the results presented in Figure 4 suggest that, in general, the solubility of the extracted protein is highest if the TPP process is carried out at the pI of the protein. This implies that the protein is less likely to be denatured if it has a neutral charge during the TPP process. Hemoglobin, the only one of the test proteins having a quaternary structure, was completely insoluble after TPP. Similarly, myoglobin is denatured by TPP and loses its noncovalently bound heme group (data not shown).

The Effect of Molecular Weight

The effect of molecular weight on TPP was measured in terms of the amount of $(NH_4)_2SO_4$ required to precipitate at least 90% of the test protein, at the pH which gives optimal precipitation of the protein concerned. The results (Fig. 5) reveal a reciprocal relationship between the protein molecular weight and the amount of $(NH_4)_2SO_4$ required to precipitate the test protein. The effect is apparently amplified in the lower molecular weight range, where proportionately more salt is required to precipitate the protein. However, the effect may not be solely due to molecular weight as cytochrome C, for example, is a relatively hydrophilic protein (see below) and this may in part explain the requirement for a greater salt concentration.

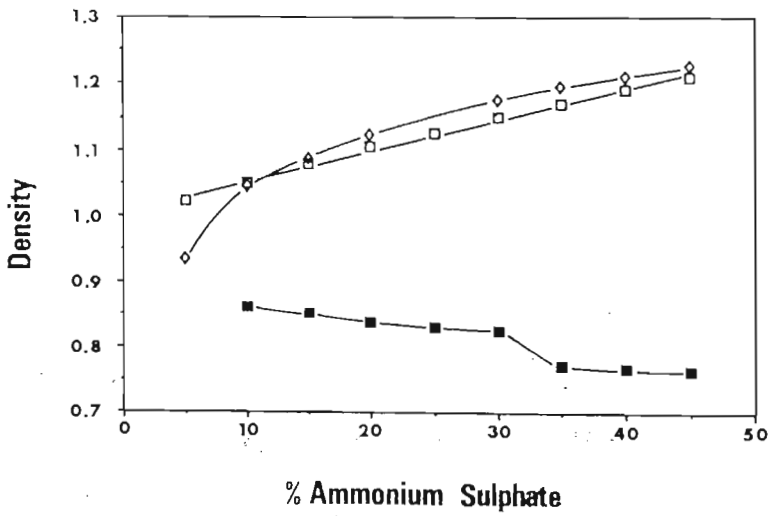

Figure 2. The effect of ammonium sulphate concentration on the density of the aqueous (lower) and *t*-butanol (upper) phases of two phase systems comprised of aqueous 0.01M citrate buffer (pH 4.8), *t*-butanol (nominally 30%) and ammonium sulphate, and the density of single phase aqueous ammonium sulphate solutions in the same buffer: (◇—◇) the aqueous phase of the TPP systems; (■—■) the *t*-butanol phase of the TPP systems; and (□—□) aqueous ammonium sulphate solutions.

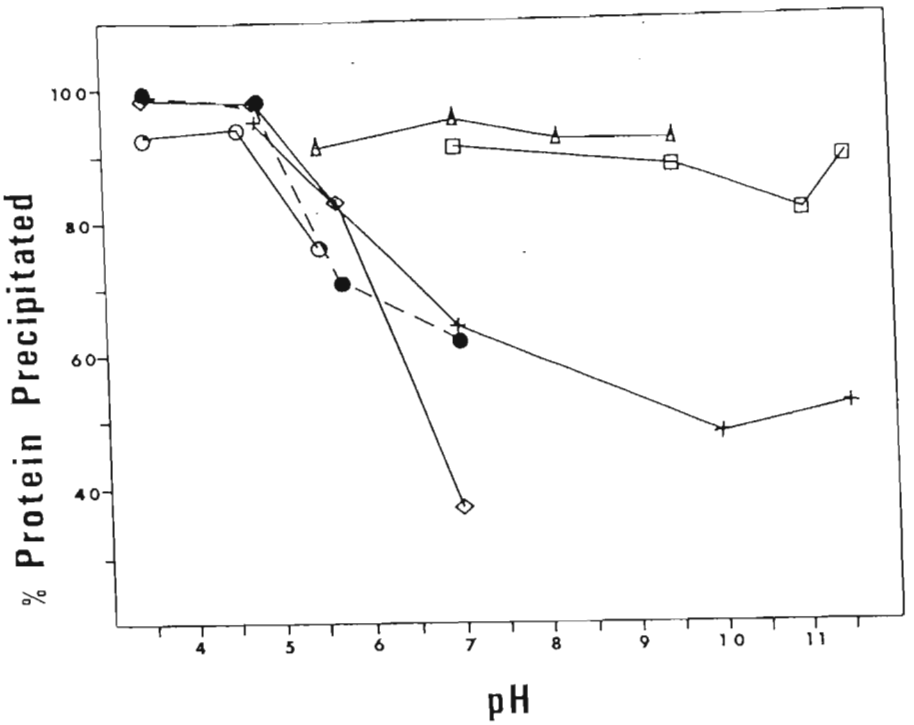

Figure 3. The effect of pH on the precipitation of proteins into the third phase in TPP. (◇—◇) BSA; (○—○) ovalbumin; (□—□) lysozyme; (×—×) cytochrome C; (●—●) γ-globulin; and (△—△) hemoglobin.

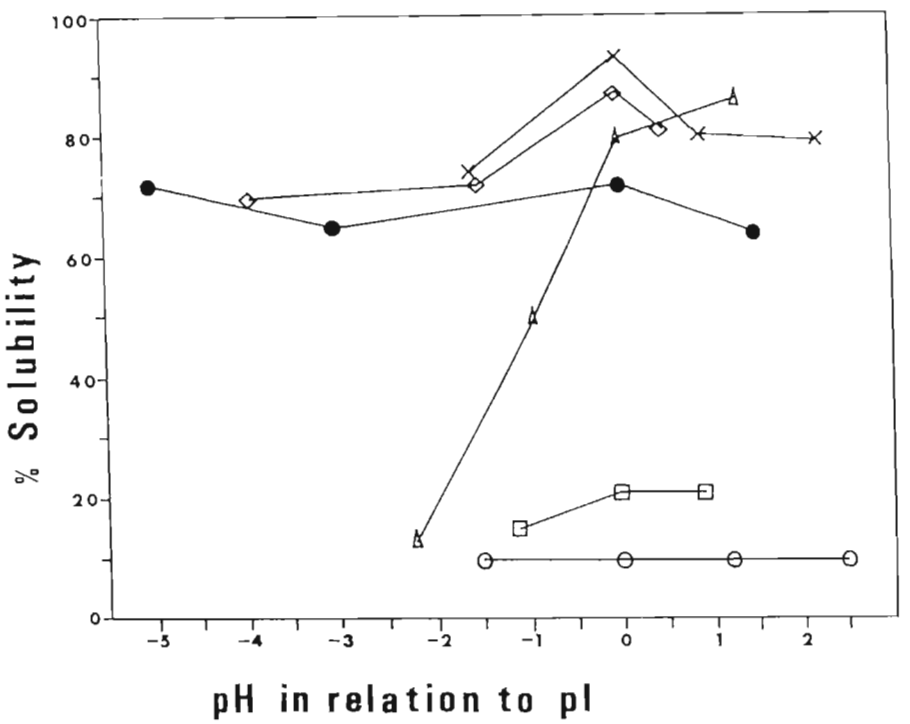

Figure 4. The effect of the pH at which TPP is conducted on the solubility of proteins after TPP: (×—×) BSA; (□—□) ovalbumin; (◇—◇) lysozyme; (●—●) cytochrome C; (△—△) γ-globulin; and (○—○) hemoglobin.

The Effect of Protein Concentration

The effect of protein concentration on TPP was determined using γ-globulin, BSA and ovalbumin as test proteins, at pH 5.7, 4.8, and 4.6 respectively, and at different initial concentrations. A fixed concentration of *t*-butanol [30%(v/v)] was used and the concentration of protein remaining in solution in the aqueous phase was measured, after TPP was effected by the addition of different amounts of ammonium sulphate, to replicate samples. The results,

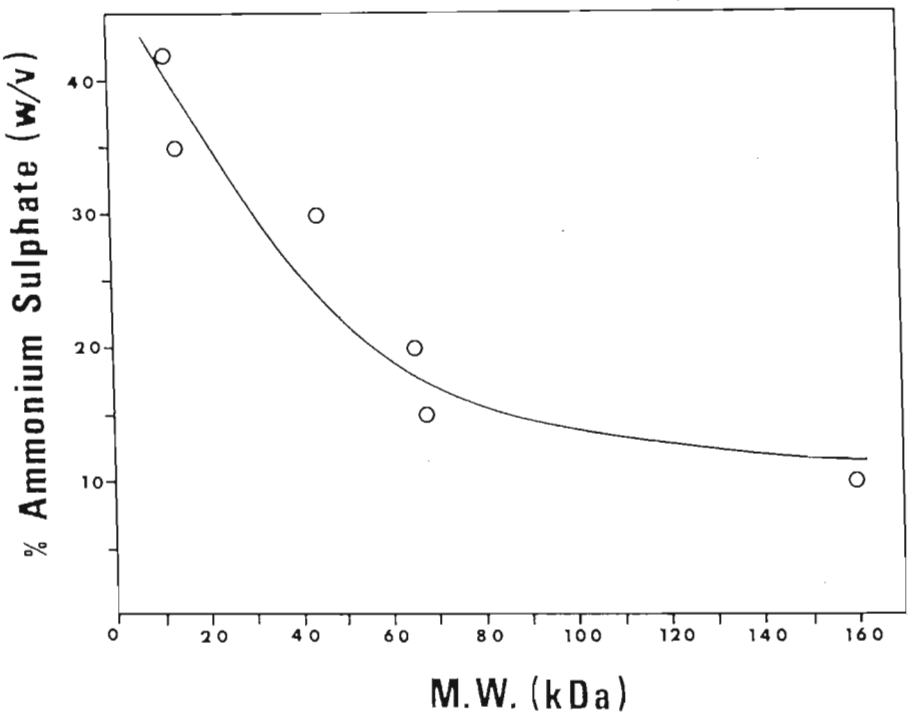

Figure 5. The effect of the molecular weight of the test protein upon the concentration of ammonium sulphate required to precipitate 90% of the protein into the third phase in TPP.

presented in Figure 6, suggest that protein concentration has an effect on the TPP process different from its effect on conventional salting out (ref. 8). In conventional salting out, a given protein has a single solubility curve, which is a function of the precipitating salt concentration, such that there exists a reciprocal relationship between the amount of protein in solution and the salt concentration required to begin to precipitate it (see Fig. 5 in ref. 8). In the case of TPP, however, this does not appear to be the case as a given protein gives rise to a family of precipitation curves, each being determined by the inital concentration of the protein.

At low ammonium sulphate concentrations, before the system splits into distinct phases, milky solutions are formed at higher protein concentrations. It was subsequently found that addition of 10% sucrose clarifies these solutions but does not result in phase separation.

The Effect of Temperature

This was measured at pH 4.8 and at an $(NH_4)_2SO_4$ concentration of 30% (w/v), using BSA as the test protein. Temperature has little effect on the TPP process, as neither the amount of protein precipitated into the third phase nor the subsequent solubility of this protein is markedly affected (Fig. 7). However, after TPP at 37°C, the protein redissolved less quickly than after TPP at lower temperatures.

The Effect of *t*-Butanol upon the Conformation of Proteins in Aqueous Solution

The addition of *t*-butanol — up to 50% (v/v) — to proteins in solution, at their pI and at 25°C, had little effect

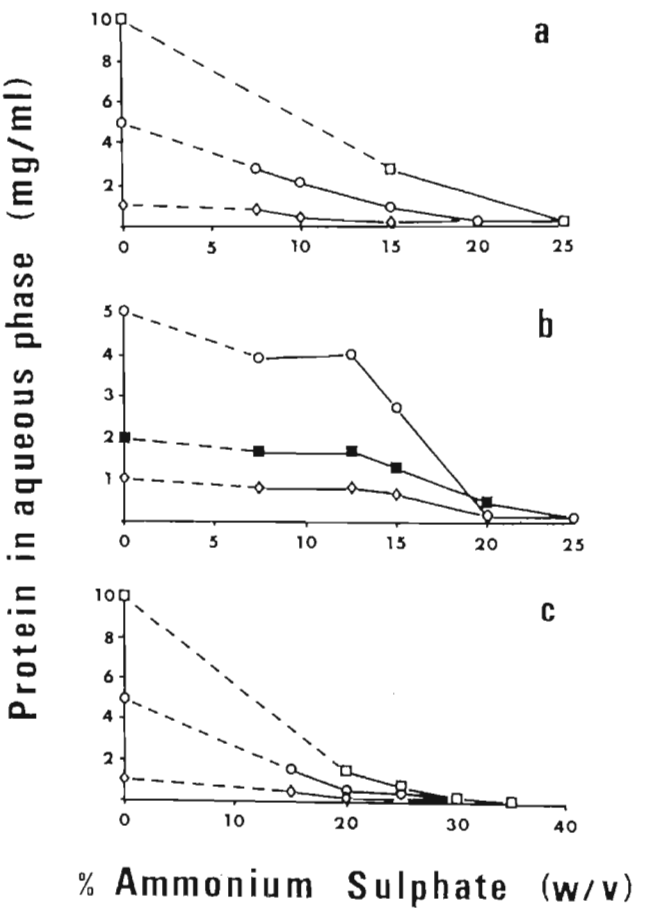

Figure 6. The effect of protein concentration on the TPP process: (a) bovine γ-globulin, (b) BSA, and (c) ovalbumin; the initial concentration in each case is indicated by the intercept on the *y*-axis. The dashed line joins successive points but the protein concentration could not be measured in this region as milky solutions were formed.

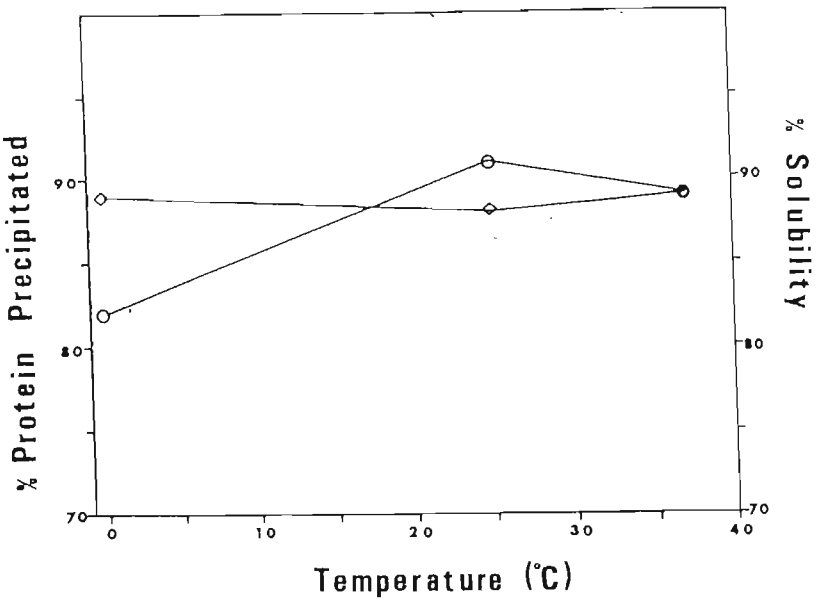

Figure 7. The effect of temperature on protein precipitation and solubility after TPP: (O—O) percent protein precipitated into the third phase and (◇—◇) percent solubility after TPP.

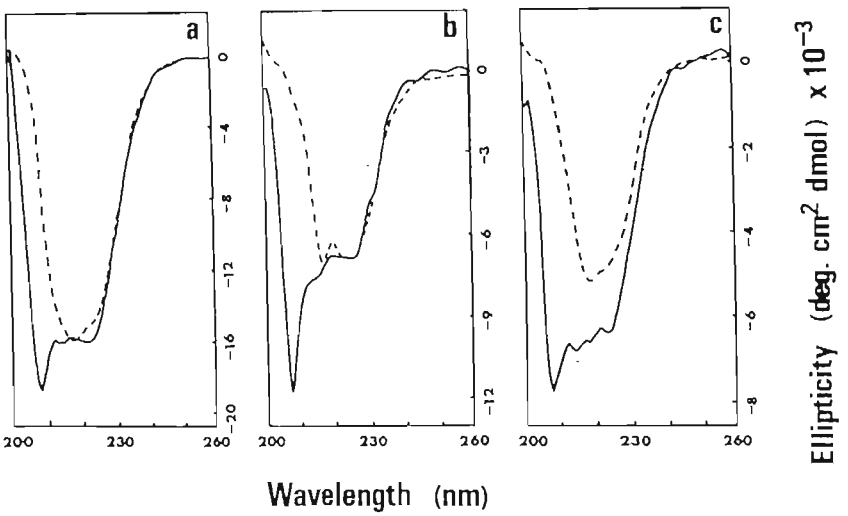

Figure 8. Circular dichroism spectra of (a) bovine serum albumin, (b) lysozyme, and (c) cytochrome C. The solid line represents the spectrum of sample in buffer containing 50% *t*-butanol while the dotten line represents the spectrum of sample in buffer alone.

upon the conformation of the proteins tested, i.e. BSA, lysozyme, cytochrome C, γ-globulin, and ovalbumin. In no case was there a shift to a random coil structure; the only obvious change was a slight increase in the α-helical content (The CD spectra of BSA, lysozyme and cytochrome C are shown in Fig. 8). This suggests that, at least for the proteins tested, *t*-butanol *per se* is not a denaturing agent.

Hydrophobicity Ranking

The test proteins eluted from phenyl-Sepharose in the order: myoglobin, cytochrome C, γ-globulin, ovalbumin, lysozyme, and bovine serum albumin. This may therefore be regarded as a tentative ranking order, from lesser to greater surface hydrophobic character. Hemoglobin appeared to precipitate on the column and therefore could not be ranked. However, the surface hydrophobic character, measured in this way, does not appear to be a significant factor in the separation by TPP as the ranking order did not correlate in any way with the precipitation of proteins into the third phase.

Fractionation of a Contrived Protein Mixture using TPP

On the basis of the evidence presented above, a number of predictions can be made regarding an optimal strategy

for separating a (contrived) mixture of the standard proteins. For example, from the results presented in Figure 5, a protein having the molecular weight of γ-globulin is predicted to precipitate at concentrations of ammonium sulphate of 10% and above (with 30% *t*-butanol). If the object were to isolate undamaged γ-globulin from the mixture, the procedure should be carried out at a pH of 7.0 (or above) as the results in Figure 4 indicate that γ-globulin is most soluble, after TPP, if the TPP process is carried out at a pH above its pI. In terms of yield, however, the results in Figure 3 suggest that a pH above the pI is not optimal, but this can be counteracted by an increase in the ammonium sulphate concentration to, say, 20%.

On the other hand, if we wished to isolate BSA, from the mixture, we should first remove the γ-globulin, as above, and then increase the ammonium sulphate concentration to 30% to precipitate the BSA. Again a pH of ca. 7 may be used to preserve the structure of the BSA (manifest in its solubility) (Fig. 4).

The next smallest protein, lysozyme, requires more than 35% ammonium sulphate to precipitate it under optimal conditions. Lysozyme has a very high pI of 11.1 and, at this pH, the protein precipitates minimally, but it is maximally soluble after TPP (Figs. 3 and 4). Significantly, however, at this high pH, cytochrome C is minimally precipitated. A TPP precipitation, on the sample remaining after removal of γ-globulin and BSA, using 35% ammonium sulphate and 30% *t*-butanol would therefore be predicted to precipitate lysozyme while leaving cytochrome C in solution.

An experiment was devised to test the above predictions. A series of mixtures was made up in solution, each containing 0.5 mg/mL of the standard proteins which had proved to be soluble after TPP, i.e. BSA, cytochrome C, lysozyme and γ-globulin. One mixture was fractionated with 20% (w/v) $(NH_4)_2SO_4$, at pH 7.0, conditions predicted to selectively precipitate γ-globulin into the third phase. A second mixture was fractionated with 30% (w/v) $(NH_4)_2SO_4$, at pH 7.0, conditions predicted to selectively precipitate γ-globulin and BSA. A third mixture was fractionated with 40% (w/v) $(NH_4)_2SO_4$, at pH 11.1, conditions predicted to precipitate all the proteins except cytochrome C.

The fractions arising from the three phase partitioning were analysed by SDS–PAGE (Fig. 9). As predicted, the 20% $(NH_4)_2SO_4$, at pH 7.0, removed most of the γ-globulin from solution into the third phase while leaving most of the remaining proteins in the aqueous phase. The separation was not absolute, which again is not unexpected. The 30% $(NH_4)_2SO_4$, at pH 7.0, as predicted, precipitated most of the BSA and γ-globulin into the third phase but, contrary to our expectations, a significant amount of lysozyme was coprecipitated. The 40% $(NH_4)_2SO_4$, at pH11.1, removed more of the lysozyme into the third phase. Cytochrome C, as indicated by the color of the precipitates and of the final solution, and confirmed by electrophoretic analysis, to a large extent remained in the aqueous phase.

It should be pointed out that in this experiment, the concentrations of the proteins were artificially predetermined. In the more real case of the separation of an unknown mixture, the concentration of the components would not be known, but their concentration would influence their precipitation as indicated in Figure 6.

DISCUSSION

The objective of the studies reported here was to determine how the physicochemical properties and concentration of a protein affect its behavior in the TPP process, as this

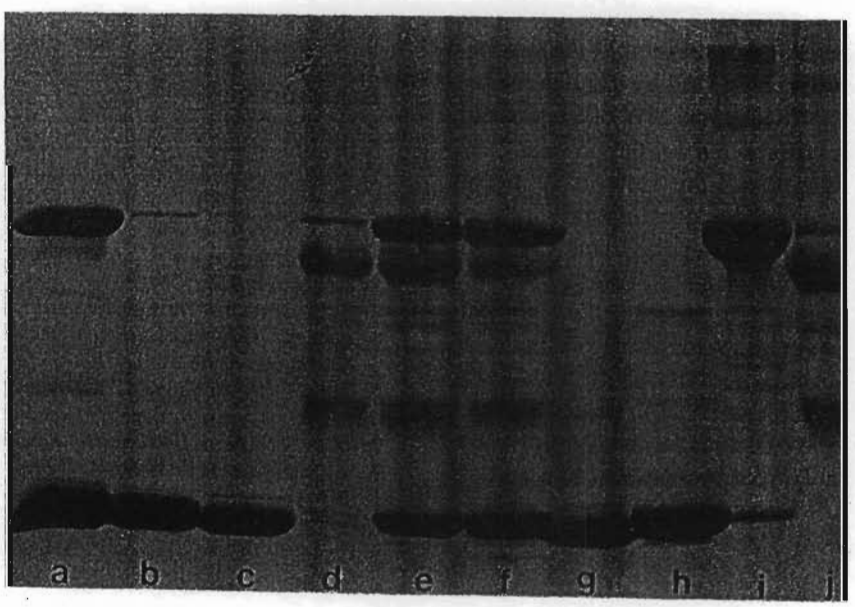

Figure 9. SDS–PAGE analysis of a fractionation of a mixture of standard proteins by TPP: (a) 20% aqueous phase; (b) 30% aqueous phase; (c) 40% aqueous phase; (d) 20% precipitate; (e) 30% precipitate; (f) 40% precipitate (in each case "%" refers to the % ammonium sulphate); (g) cytochrome C; (h) lysozyme; (i) BSA; and (j) γ-globulin.

knowledge would be useful in using the method to its best advantage. The results obtained indicate that proteins precipitate (into the third phase) most readily at their pI, but that proteins were most soluble after TPP when this was carried out above the pI of the protein. The amount of salt required to precipitate a protein was found to vary inversely with the protein molecular weight. Salt and *t*-butanol appear to have a similar effect in that more of one could compensate for less of the other. Protein concentration affects the precipitation of protein in TPP in a manner different from its effect in conventional salting out (ref. 8), in that each protein appears to yield a family of solubility curves, each curve being a function of the initial protein concentration.

The solubility of proteins after TPP provides some measure of the extent of their denaturation. Evidence provided by the measurement of CD-spectra suggests that simple exposure of the protein to *t*-butanol apparently does not denature single-chain proteins. However, the subsequent salting out with $(NH_4)_2SO_4$ — the step required to form the three phases — is apparently much more damaging to proteins in the presence of *t*-butanol than in its absence. Noncovalently bonded oligomeric proteins such as hemoglobin or myoglobin (in which the heme group is noncovalently attached), are completely denatured by the TPP process, as evidenced by their subsequent complete lack of solubility. Single chain proteins are denatured to a variable extent which appears to depend partly upon the pH at which the TPP process is carried out, relative to the pI of the protein. TPP therefore appears to be a method whereby stable single chain proteins might be separated from oligomers, by denaturation of the latter.

In general, proteins are denatured to the least extent at their pI. It is difficult to assign a physical significance to this, although the pI might be the pH at which the protein is hydrated to the least extent. Individual structural features of the proteins might also have some bearing on their susceptibility to denaturation, for example the extent to which the protein structure is stabilized by disulfide bridges. A factor can be derived by dividing the MW of the protein by the number of disulfide bridges and for the proteins examined in this study it appears that if this factor is 10^4 or less, then the protein will largely escape denaturation.

The results of the experiments reported here permit the construction of a tentative model to explain the TPP process. The observation that the protein phase floats upon the aqueous phase in TPP, in contrast to salting out in the absence of *t*-butanol when the precipitated protein usually sinks [in an ammonium sulphate solution of equal density (Fig. 2)], suggests that the *t*-butanol forms a complex with the protein. The *t*-butanol/protein complex (which floats) presumably has a density lower than that of the alternative water/protein complex (which sinks). The complexing of *t*-butanol with the protein might increase the apparent hydrophobic character of the protein, making this more easily salted out by ammonium sulphate, as in general less ammonium sulphate is required to effect "precipitation" in TPP than in conventional salting out. Of course, in the TPP process, the ammonium sulphate is largely concentrated into the aqueous phase when the solution separates into phases, but even allowing for this increase in concentration, it appears that in many cases less ammonium sulphate is required than in conventional salting out. Presumably pH affects the interaction of the *t*-butanol with the protein in some way. We cannot at this stage offer an explanation for the effect of molecular weight.

The TPP method may be used for protein concentration and has the advantage that relatively nonpolar contaminants may be removed in the *t*-butanol layer. The method can be optimized for the isolation of a particular single chain protein if its pI is known, although the protein concentration and MW have effects which make it difficult to predict the behavior of an unknown system. As a batch method it may be readily scaled up and could therefore find a use in industry. It would appear therefore that TPP constitutes a useful addition to the portfolio of methods available to the protein biochemist.

The authors wish to thank Professor Rex Lovrien of the Department of Biochemistry, University of Minnesota, for introducing them to the TPP process, Gary Osthoff of the National Chemical Research Laboratory, C.S.I.R. (Pretoria, Republic of South Africa) for assistance in running the CD-spectra, and Professor Leon Visser of the University of Pretoria, (Pretoria, Republic of South Africa) for assistance in the interpretation of the CD spectra. The data-reduction program used in the analysis of the CD-spectra was devised by Dr. L. S. Swart.

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ALYTICAL BIOCHEMISTRY 180, 169–171 (1989)

solation of Cathepsin D Using Three-Phase Partitioning n t-Butanol/Water/Ammonium Sulfate

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eceived November 28, 1988

A 6-h procedure for the isolation of bovine cathepsin is described. The procedure involves essentially only wo steps; three-phase partitioning in t-butanol/water/ mmonium sulfate followed by affinity chromatogra- hy on pepstatin–agarose. The major advantage of this ew method over previous methods is the greatly re- uced time required to obtain comparably pure cathep- in D.

Cathepsin D is a lysosomal aspartic protease which has been isolated from a number of sources, including porcine and bovine spleen (1,2), human liver (3), and porcine myometrium (4).

A number of approaches have been used for the isola- ion of cathepsin D. Takahashi and Tang (1) employed extended dialysis followed by DEAE and pepstatin affinity chromatography. Similarly, Nakao *et al.* (2) used mmonium sulfate precipitation, DEAE chromatogra- hy, and pepstatin affinity chromatography to obtain he pure enzyme. Babnik *et al.* (3) used ammonium sul- ate precipitation followed by four different chromatog- aphy steps, and Afting and Becker (4) used homogeni- ation and centrifugation steps prior to concanavalin A nd pepstatin affinity chromatography to obtain high ields of purified enzyme.

Three-phase partitioning (TPP)[1] in t-butanol/water/ mmonium sulfate has been used in the isolation of a umber of proteins (5–7), including cathepsin L (Pike nd Dennison, unpublished). Pike and Dennison (7) ave observed that TPP apparently causes the selective enaturation of oligomeric proteins, and, conversely, hat it might therefore be a method especially suited to he isolation of proteins not consisting of noncovalently ound subunits.

In the method described here, for the isolation of cathepsin D from bovine spleen, TPP proved effective in removing contaminants such as hemoglobin and reduced the time required for purification to 6 h.

MATERIALS AND METHODS

Chemicals. Aminohexyl agarose, pepstatin A, and hemoglobin were from Sigma. All other chemicals were of the highest chemical quality.

Spleen preparation. Spleen was processed essentially according to Takahashi and Tang (1). Thawed bovine spleen (200 g) was freed from its thick outer membrane, diced, and homogenized for 60 s in a Waring Blendor with cold distilled water (200 ml). The homogenate was centrifuged (10,000*g*, 30 min, 4°C), and the resultant supernatant was decanted and adjusted to pH 3.7 with HCl and again centrifuged as before.

Three-phase partitioning. TPP was effected on the pH 3.7 supernatant by the addition and mixing in of t-butanol to 30% (v/final volume) (7). This is conveniently performed with the protein solution at 4°C and the t-butanol at 30°C (above its crystallization temperature of about 25°C). Solid ammonium sulfate [$(NH_4)_2SO_4$] was added slowly to 20% (w/v) (based on the total volume of solution, including t-butanol, but before addition of the ammonium sulfate) and dissolved by magnetic stirring. The mixture was centrifuged (6000*g*, 10 min, 4°C) in a swing-out rotor. The interfacial precipitate (i.e., the solid "third phase"), which after centrifugation is a firm "button" of protein, was removed using a spatula and discarded. Further $(NH_4)_2SO_4$ was added to the mixture, to 35% (w/v) (again based on the initial total volume), and dissolved by magnetic stirring. Once the salt was completely dissolved, the mixture was centrifuged (6000*g*, 10 min, 4°C) and the interfacial precipitate was collected using a spatula. The precipitate was redissolved in column equilibration buffer (buffer A: 0.05 M sodium acetate, pH 3.5, containing 0.2 M NaCl) with gentle magnetic stirring for 1 h at 4°C. The volume of

[1] Abbreviations used: TPP, three-phase partitioning; SDS–PAGE, odium dodecyl sulfate–polyacrylamide gel electrophoresis.

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TABLE 1

Bovine Spleen Cathepsin D Purification

Fraction	Protein (mg)	Activity (units)[a]	Specific activity (units/mg)	Yield (%)	Purification (fold)
Supernatant	8750	108,750	12.43	100	1
Acid supernatant	1292	102,362	79.23	94.13	6.37
20–35% TPP	27.12	28,080	1035.40	25.82	83.30
Pepstatin column	2.18	8,346	3828.44	7.67	308

[a] One unit of enzyme activity = 1 μg of hemoglobin hydrolyzed/min at 37°C.

buffer used was equal to one-fifth of the original supernatant volume, before TPP. The solution was filtered (Whatman No. 4) to remove undissolved protein, and the filtrate was applied to the pepstatin affinity column.

Pepstatin affinity chromatography. The protein sample (20 ml) was loaded onto a column (1.0 cm i.d. × 3.0 cm) of pepstatin–aminohexyl–agarose, synthesized according to the method of Murakami and Inagami (8). Unbound material was eluted with buffer A, at a flow rate of 30 ml h^{-1}, until the A_{280} of the eluate reached a constant absorbance. Cathepsin D was then eluted using a 0.05 M Tris–HCl buffer, containing 0.2 M NaCl, pH 8.5 (buffer B).

Gel electrophoresis. Protein samples from each purification step were analyzed by SDS–PAGE on 12.5% gels, following the method of Laemmli (9).

Enzyme activity assay. Selected fractions were tested for protease activity by a modification (1) of the method of Anson (10), using hemoglobin (5%, w/v) at pH 3.5. Enzyme samples (40 μl) were incubated with hemoglobin solution (167 μl) for 30 min at 37°C. One unit of activity, defined by Schwartz and Barrett (11), effects the hydrolysis of 1 μg of hemoglobin per minute at 37°C.

Protein determination. Samples from each purification step were assayed using the Bradford dye-binding assay (12), as modified by Read and Northcote (13), using ovalbumin as the standard protein.

RESULTS AND DISCUSSION

When applied to the isolation of cathepsin D from bovine spleen, TPP was found to be effective in removing contaminating protein (Table 1) and thereby provides a useful step in the purification of the enzyme. Contaminating protein is largely precipitated in the first TPP cut [0–20% $(NH_4)_2SO_4$] while cathepsin D precipitates at a higher salt concentration (35%). TPP removes all the hemoglobin found in the spleen, probably by denaturing it as Pike and Dennison (7) have observed. In addition, pigments are removed in the t-butanol layer.

The second step in the purification was effected by affinity chromatography on pepstatin–aminohexyl–agarose. Pepstatin is a strong inhibitor of cathepsin D (14) which, when immobilized, adsorbs cathepsin D while contaminating proteins pass through. Elution of the column with buffer B yielded a sharp peak (Fig. 1), which contained most of the cathepsin D activity. Assay of

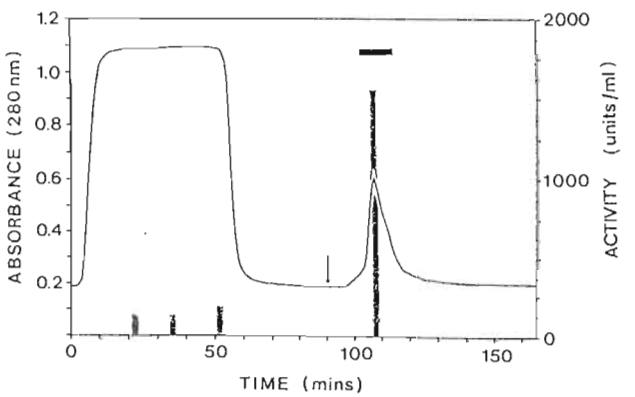

FIG. 1. Elution profile of the TPP-purified fraction on pepstatin–aminohexyl–agarose. Column, 1.0 × 3.0-cm pepstatin–aminohexyl–agarose; sample, 20 ml containing about 27 mg of protein; buffer, 0.05 M sodium acetate, pH 3.5, containing 0.2 M NaCl, followed by 0.05 M Tris–HCl, containing 0.2 M NaCl, pH 8.5, at point ↓; flow rate, 30 cm^3 h^{-1}; fractions, only the peak indicated (horizontal bar) was collected. The effluent was sampled at the positions indicated for the analysis of enzyme activity (vertical bars).

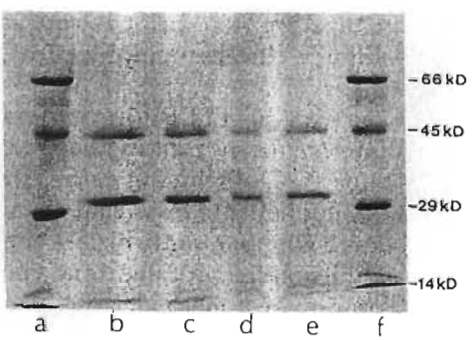

FIG. 2. SDS–PAGE of cathepsin D from pepstatin affinity chromatography. Lanes a and f contain reference proteins consisting of bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and lysozyme (14 kDa). Lanes b to e contain decreasing concentrations of purified enzyme.

fractions from this peak showed enzymatic activity to be 15-fold higher than activity in the breakthrough peak, which contained a low level of cathepsin D-like protease activity. Addition of pepstatin to these fractions resulted in a total loss of activity (data not shown). There was no significant cathepsin D-like activity in fractions outside of these two peaks.

Active cathepsin D occurs in two forms, a single polypeptide chain of 44 kDa and a noncovalent complex of two peptides of 30 and 14 kDa (15) which is the result of proteolytic processing of the 44-kDa single-chain form (16). The two-chain form is very similar in conformation to the single-chain enzyme (15), but the enzymatic activity of the complex is lower than that of the single-chain enzyme (approximately 80% of that of the single-chain enzyme) (17). The processing of the single-chain enzyme into the complex is a highly specific process and is apparently due to cysteine proteases (18). The site at which the cleavage occurs [at a unique insert of four residues between equivalent residues 91 and 92 of the pepsin sequence (19)] is very different from the processing site of pepsin, between residues 280 and 281, which are untouched in cathepsin D processing (20). Rat spleen cathepsin D has been found to be present only in the 44-kDa single-chain form (21), and cathepsin D from human liver is found only in the two-chain complex form (22).

SDS–PAGE of the purified enzyme under denaturing conditions shows three bands corresponding to the 44-, 30- and 14-kDa chains of the enzyme (single-chain and noncovalent complex, respectively) (Fig. 2), but no other significant bands, suggesting that the enzyme was pure, although polymorphic. Pike and Dennison (7) observed that TPP may destroy the quarternary structure of a protein. A suggestion that this might have occurred in the case of cathepsin D is provided by the strong staining of the bands at 44 and 30 kDa and the relatively weak staining of the band at 14 kDa. However, similar results have been obtained from previous purifications of cathepsin D from a variety of tissues (1,23,24), so the weak staining of the 14-kDa fragment may be an intrinsic property of this molecule.

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Three-phase partitioning in aqueous/*t*-butanol mixtures: a useful new protein fractionation method

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There are diverse reasons for isolating proteins, such as studying the protein's structure/function relationships, using the protein in a therapeutic or industrial context, and raising antibodies for therapy or immunocytochemistry. Similarly, there are diverse techniques for the isolation of proteins. Typically, proteins used in a research context are isolated in small amounts, often by means of expensive techniques, whereas those used industrially are isolated in relatively large amounts, using less expensive methods. The cost of scale-up is the main determinant of whether a technique, useful on a small scale, is suitable for industrial production, and not many techniques pass this test. As a result, there is a need for new protein isolation methods that may be readily scaled up. (Of course, protein biochemists are also always interested in new small-scale techniques that might provide easier routes to difficult isolations.)

A new technique that appears to hold promise for both small and large scale application is three-phase partitioning using *t*-butanol/water/salt mixtures.

Three-phase partitioning

Three-phase partitioning (TPP) was discovered serendipitously, in Rex Lovrien's laboratory at the University of Minnesota, in the course of studies on the effects of water-miscible organic solvents on the activity of enzymes.[1] The method was first documented by Odegaard *et al.*[2] when it was used in the purification of cellulases from *Trichoderma reesei*. They reported that *t*-butanol, normally completely miscible with water, separated from the aqueous phase on the addition of a salt such as ammonium sulphate. Any protein present in the initial mixture was precipitated into a third phase between the *t*-butanol and aqueous phases. An advantage of the technique was that it removed tannins, phenolics, lipids and many enzyme inhibitors from protein mixtures, thus improving the activity of enzymes, while simultaneously concentrating them, more or less specifically. Further advantages were that only low speed centrifugation was necessary to resolve the phases, and that the process could be carried out at room temperature, unlike many other protein fractionation procedures involving organic solvents.

These observations led Lovrien *et al.*[3] to make a further study of the utility of TPP in the fractionation of various crude enzyme preparations, mostly of plant and microbial origin. In all cases large increases in the total and specific activities of the enzymes were reported, emphasising the potential of the technique for protein purification in both industry and research. TPP was also used by Niehaus and Dilts[4] for the purification of mannitol dehydrogenase enzymes. A procedure similar to TPP, but using *n*-butanol in place of *t*-butanol, has also been used to isolate the enzyme lipase from the pancreas.[5]

Studies on standard proteins

TPP was introduced into our laboratory by Clive Dennison, following a sabbatical visit to the University of Minnesota. We were interested in TPP because it seemed to be a potentially useful technique for the purification of the proteolytic enzymes in which we have a special interest. Since it had been used only empirically, however, there were few systematic guidelines to its use. A study was therefore made of TPP so that it could subsequently be used in a more systematic, less empirical way. A group of standard proteins, of known physico-chemical properties, chosen for their range of molecular weights and isoelectric points (p*I*), rather than their enzymatic activity, was used for this study, as the effect of TPP on enzymatic activities had been studied by Lovrien's group.[3] The selected proteins were subjected to TPP under various conditions to establish the effect of the precipitation conditions on the proteins and their solubility after TPP. This was carried out on the proteins in isolation, and results were obtained by comparing protein concentrations in the aqueous phase before and after TPP, and in the solutions containing resolubilised, precipitated protein. The experiments have been reported in greater detail elsewhere.[6]

The results obtained on the effect of pH of the TPP aqueous phase indicate that proteins precipitate (into the third phase) most readily at or below their p*I*, but that proteins were most soluble after TPP when this was carried out above the p*I* of the protein. The effect of molecular weight was also monitored; the amount of salt required to precipitate a protein was found to vary inversely with the protein's molecular weight, at equal protein concentrations. Protein concentration affects the precipitation of protein in TPP differently from its effect in conventional salting out,[7] in that each protein appears to yield a family of solubility curves, each curve being a function of the initial protein

Practically, this implies that the initial concentration of a protein in solution will have a marked effect on the precipitation of the protein, and thus the protein concentration needs to be carefully controlled in order to obtain reproducible results with any given protein. Temperature, in the range of 0–30°C, was found to have very little effect on the TPP process. The effect of varying the ratio of *t*-butanol and ammonium sulphate was tested to determine the working range of the TPP system in both the presence and absence of protein. There was a reciprocal relationship between the amounts of ammonium sulphate and *t*-butanol needed to form a two-phase system, while more salt is required in the TPP system in the presence of protein in order to obtain a well resolved third phase.

During the experiments to find the effect on TPP of the various conditions mentioned above, several of the test proteins were found to be irreversibly denatured by the process. The denaturation appears to be related (inversely) to the number of disulphide bridges relative to the protein's molecular weight. With our range of test materials it appeared that the protein required a disulphide bridge for every 10 kilodaltons of its molecular weight to avoid denaturation. Haemoglobin, a non-covalently bound oligomer, was completely denatured, whereas myoglobin lost its non-covalently bound haem group.

Some recent applications of TPP

Selective denaturation of certain proteins by TPP during the purification of more robust compounds could, of course, be used to advantage. This proved to be the case in the purification of red blood cell proteins, for which haemoglobin is often a problematic contaminant due to its high concentration. At Pretoria University, Professor Harold Deutsch, on sabbatical from Wisconsin, Christine Pol and Professor Leon Visser were able to simplify the purification of several red blood cell proteins using the selective denaturation of haemoglobin by a variation of TPP, additionally using a small proportion of chloroform.

Similarly, we were able to exploit the haemoglobin denaturing ability of TPP in isolating cathepsin D from bovine spleen.[8] When TPP was used as a purification step before affinity chromatography, cathepsin D could be isolated in only six hours compared to the three days reported previously. We have also used TPP to advantage in the isolation of cathepsin L from sheep's liver. A twofold greater yield of cathepsin L was obtained, compared to the best previous method, and the subsequent chromatography steps required were also simplified.[9]

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A HIGH YIELD METHOD FOR THE ISOLATION OF SHEEP'S LIVER CATHEPSIN L

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ABSTRACT

A method, giving twice the yield of the previous method[1], for the isolation of sheep's liver cathepsin L is described. The method uses three phase partitioning (TPP) in t-butanol/water/ammonium sulphate mixtures, followed by two chromatographic steps, at different pH values, in a single column of S-Sepharose.

INTRODUCTION

Cathepsin L has been purified from various sources, including rat liver[2], rat kidney[3], rabbit liver[4], human liver[5] and sheep and bovine liver[1]. Mason[1] found that sheep's liver gave the highest yield of cathepsin L and that antibodies to the sheep enzyme cross-reacted with that from human liver, and from other species, indicating sheep's liver as the first choice for the isolation of cathepsin L for many purposes.

In the purification procedures of Kirschke et al.[2] and Bando et al.[3], lysosomes are first isolated, to partially purify cathepsin L, and to keep the enzyme separate from inhibitors in the cytosol. By contrast, in Mason's purification procedures[1,4,5], "autolysis" at pH 4.2 is used to remove inhibitors from the enzyme and salting-out is used for a preliminary crude fractionation. This obviates the need for the isolation of lysosomes and has the further advantage that stored, frozen, livers can be used. The new isolation procedure reported here is based firstly on the use of three phase partitioning (TPP) in t-butanol/water/ammonium sulphate[6], in place of ammonium sulphate precipitation, and secondly on improved cation exchange chromatography on S-Sepharose. Previous observations on the TPP process[7] suggested that TPP might be especially suited to the isolation of proteins, such as cathepsin L, which are not comprised of non-covalently associated sub-units. As reported here, applied to the fractionation of cathepsin L from sheep's liver, TPP gave an improved purification, compared to ammonium sulphate precipitation, and resulted in a simplification of the subsequent chromatography steps required for its isolation.

Cathepsin L is known to bind strongly to cation exchangers, even at pH values close to its pI[1]. Various cation exchangers have therefore been employed in previous purifications of cathepsin L, including CM-Sephadex[1-5] and the FPLC Mono S system[1,5]. In the procedure reported here, a low pressure equivalent of the Mono S system, S-Sepharose, was used to isolate cathepsin L.

The combination of TPP and S-Sepharose chromatography was found to constitute a rapid, simple and reproducible method for the isolation of cathepsin L in higher yields than have been reported previously.

MATERIALS AND METHODS

Chemicals. Azocasein, leupeptin, pepstatin, cysteine-HCl, S-Sepharose and E-64 were from Sigma. All other chemicals were of the highest chemical purity.

Enzyme Assays and Inhibition studies. Fractions were routinely tested for cathepsin L activity, using azocasein in 3M urea, at pH 5.0, as described by Barrett & Kirschke[8]. There is no substrate specific for cathepsin L, but the inclusion of urea, pepstatin, cysteine and EDTA make the azocasein assay relatively selective for cathepsin L activity[9]. The limitations of azocasein assays should, nevertheless, be appreciated[10]. One unit of activity, defined by Schwartz & Barrett[11], effects the hydrolysis of 1 μg of azocasein min^{-1} at 37°C. Activity against azocasein without urea was tested at pH 6.0 as described by Schwartz & Barrett[11]. Leupeptin (0.5 μM) was included in the activation buffer to test inhibition of the active fractions. Active site titration of the purified cathepsin L, using E-64, was carried out as described by Barrett & Kirschke[8].

Protein Determination. Column effluents were continuously monitored at 280 nm. Protein in pooled fractions was assayed using the Bradford dye-binding assay[12] as modified by Read & Northcote[13].

Purification Procedures.

Sheep's liver was frozen and, within two weeks, thawed, minced and mixed 1:2 with a solution of 2% n-Butanol/1% NaCl/0.1% EDTA and homogenized in a Waring blender for 2 minutes. The resulting homogenate was centrifuged (6,500 g, 30 min, 4°C); the supernatant was adjusted to pH 4.2 and stirred for 4 h at 37°C and left at 4°C overnight, to effect activation of cathepsin L, all as described by Mason et al.[5]. The mixture was centrifuged (6,500 g, 20 min, 4°C) and the supernatant was used for ammonium sulphate and TPP fractionation.

Ammonium sulphate precipitation was optimised in an experiment in which fractions, obtained by 10% increments in ammonium sulphate saturation, were assayed for protein content and cathepsin L activity. A fraction, obtained by a cut from 30-65% saturation, at pH 4.2 and 4°C, was found to be optimal. The TPP procedure was optimised in a similar manner, except that 5% increments in ammonium sulphate were used, and an

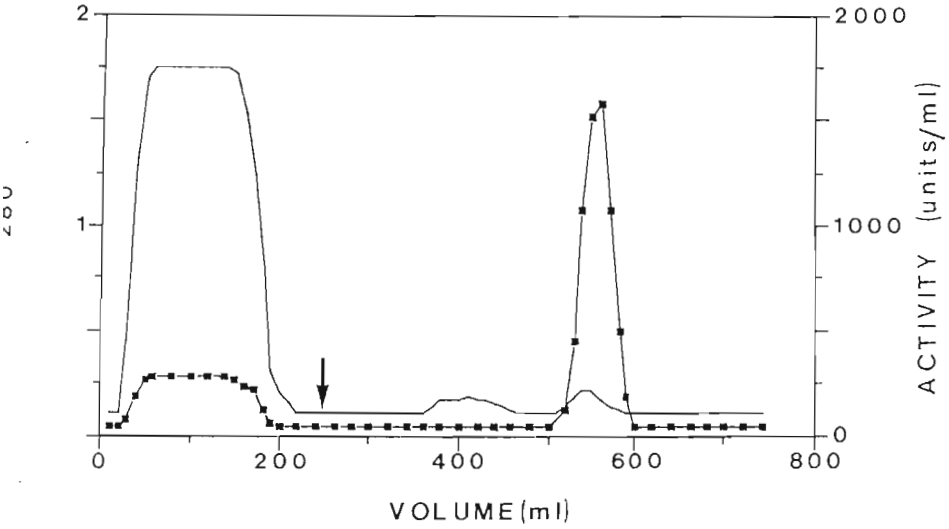

FIGURE 1

S-Sepharose chromatography, at pH 5.5, of TPP fraction containing sheep's liver cathepsin L.

Column; 2.5 x 14.5 (70 ml bed volume): Buffer; buffer A with 200mM NaCl, followed by 200-600mM NaCl gradient, in 5 x column volume, in buffer A, applied at point, followed by 2 x column volume of buffer A containing 600mM NaCl: Flow rate; 50 ml/h (10 cm/h): Fractions; 10 ml (12 min.).
——— = A_{280}; ■—■ = Activity

ptimal fraction was reproducibly obtained from the pH 4.2 upernatant by cutting between 20% and 30% $(NH_4)_2SO_4$, at pH 4.2, in the presence of 30% t-butanol. Percentages, i.e. %(w/v), were as defined by Pike and Dennison[7].

For the purification of cathepsin L from sheep's liver, 525 g was treated as outlined above. TPP was effected on the pH 4.2 supernatant by the addition and mixing in of 30% (v/v of final volume) of t-butanol (in this step, initially, the supernatant was at 4°C and the t-butanol was at 25°C: subsequent steps were done at 4°C). $(NH_4)_2SO_4$ (20% w/v)

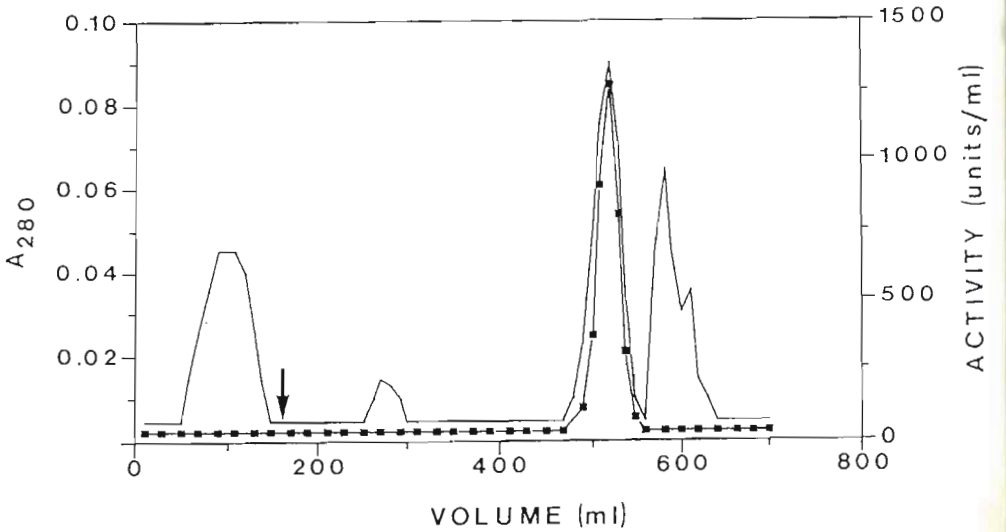

FIGURE 2

Rechromatography on S-Sepharose, at pH 4.5, of fraction containing cathepsin L.

As for Fig. 1, except that all buffers were at pH 4.5.
——— = A_{280}; ■—■ = Activity

(based on the volume of the original mixture of supernatant plus t-butanol) was added and dissolved by gentle stirring. The resulting mixture was centrifuged (6,000 g, 10 min, 4°C) in a swing-out rotor. The interfacial precipitate was removed, using a spatula, and discarded. A further amount of ammonium sulphate was added, to bring the solution to 30% (w/v) $(NH_4)_2SO_4$ and well stirred. Upon complete dissolution of the salt, the mixture was again centrifuged (6,000 g, 10 min, 4°C) and the interfacial precipitate was collected for further study.

The precipitate from TPP was re-dissolved, in one fifth of the pH 4.2 supernatant volume, in 0.02M Na-acetate buffer, pH 5.5, containing 1 mM EDTA and 0.02% sodium azide (buffer A,

pH 5.5). The pH was readjusted to 5.5 and the resuspended material was centrifuged (27,000 g, 10 min, 4°C) to remove any insoluble material.

The supernatant was loaded onto an S-Sepharose column, equilibrated with buffer A, containing 200 mM NaCl, and eluted with a 200-600 mM NaCl gradient in buffer A (Fig. 1). Fractions corresponding to the peak of activity, eluted at about 400 mM NaCl, were pooled and dialysed against 10 volumes of buffer A, pH 4.5, containing 200 mM NaCl, for 10 h. The dialysed solution was re-applied to the S-Sepharose column, equilibrated with buffer A, pH 4.5, containing 200 mM NaCl, and eluted with a 200-600 mM NaCl gradient (Fig. 2). The active fraction was pooled, concentrated by dialysis against 30% polyethylene glycol, and used for inhibition studies, active site titration and analysis by SDS-PAGE.

Gel Electrophoresis. Protein samples were analysed by SDS-PAGE on 12.5% gels as described by Laemmli[14], and either stained with Coomassie Blue R-250 or silver stained[15].

RESULTS AND DISCUSSION

The results obtained from a comparative study of the relative merits of TPP and ammonium sulphate precipitation, applied to the crude fractionation of cathepsin L from sheep's liver, are presented in Table I. A comparison of the specific activities obtained suggests that TPP is superior to ammonium sulphate precipitation, while yielding similar levels of cathepsin L. Similar results (not shown) were obtained for comparisons of the two methods for rabbit and bovine livers, showing that these results were not applicable only to sheep's liver.

TPP, however, must also be considered in relation to the chromatography steps subsequently needed to finally purify the enzyme. After TPP the sample has a much lower salt concentration, than after ammonium sulphate precipitation, and can therefore be applied directly to the S-Sepharose column, without prior desalting.

TABLE I

Comparison of ammonium sulphate precipitation and TPP for the crude fractionation of cathepsin L from sheep's liver.

Step	Specific Activity	Purification (fold)	Yield (%)
pH 4,2 Supernatant	5	1	100
$(NH_4)_2SO_4$ p'ptation	7	1.3	58
TPP	18	3.5	60

Equilibration of the column in relatively high salt concentrations, resulted in a larger unbound peak and a lesser amount of protein being bound to the column. Resolution of the cathepsin L peak from other bound proteins was thus simplified, and a large increase in specific activity was achieved in the first chromatography step (Table II). In spite of this, and the fact that cathepsin L activity eluted in a seemingly homogenous peak, the active fraction was not pure when analysed by SDS-PAGE (Fig. 3). Rechromatography at pH 4,5 removed the remaining contaminants, resulting in an apparently pure fraction, as judged by SDS-PAGE stained with Coomassie blue (Fig. 3), or by the more sensitive silver staining procedure (Fig. 4).

TABLE II
Purification of Sheep's Liver Cathepsin L

Step	Vol. (mℓ)	Total Protein (mg)	Total Activity (units)[a]	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	900	43600	48000	1.1	1	100
pH 4.2 Supernatant	650	4760	28000	5.9	5	58
TPP	140	1008	18667	18.5	17	39
S-Sepharose pH 5.5	57	7.1	7410	1044	949	15
S-Sepharose pH 4.5	35	2.45	3266	1333	1211	7

[a] 1 unit of activity = 1 µg of azocasein hydrolysed/min. at 37 °C

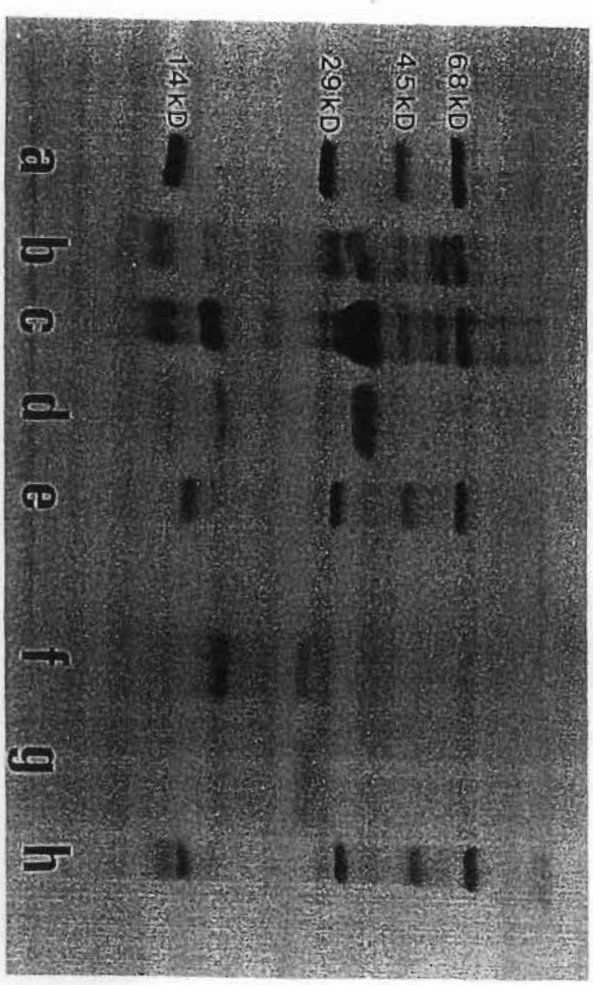

FIGURE 3

SDS-PAGE of fractions from sheep's liver.

a) Molecular weight standards, BSA (68kD), ovalbumin (45kD), carbonic anhydrase (29kD) and lysozyme (14kD); b) supernatant after pH 4.2 precipitation; c) TPP cut - 20-30% ammonium sulphate; d) unbound material from S-Sepharose, pH 5.5; e) molecular weight standards; f) S-Sepharose pH 5.5 fraction; g) S-Sepharose, pH 4.5 fraction; h) molecular weight standards.

Dialysis of the pH 5.5 fraction, to desalt and bring it to pH 4.5, must be accurately reproduced to ensure reproducible results in the second chromatographic step. Dialysis, rather than molecular exclusion chromatography, was used to effect buffer exchange at this point, due to the greater convenience of overnight dialysis. The resolution obtained between the active peak and the following two

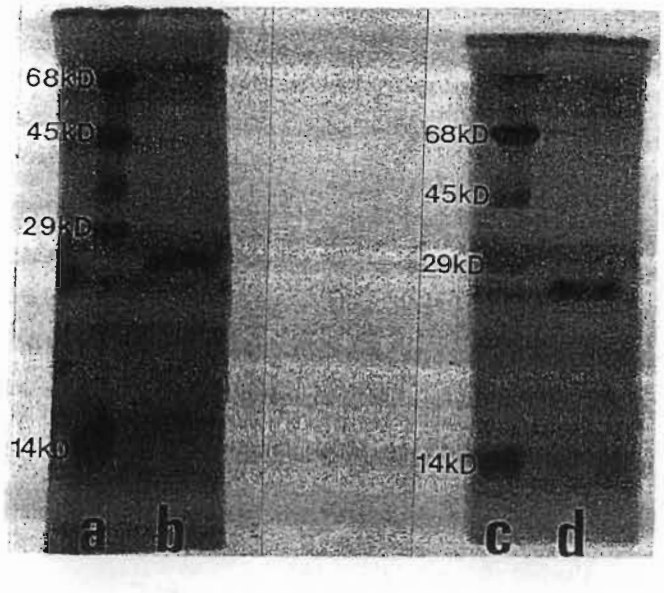

Figure 4

Silver stained SDS-PAGE of reduced and non-reduced pure sheep's liver cathepsin L

a) Reduced M.W. standards (as above); b) reduced cathepsin L; c) non-reduced M.W. standards (as above); d) non-reduced cathepsin L.

contaminants, in the pH 4.5 chromatography step, deteriorated if more than 500 g of sheep's liver was initially used, suggesting that the maximal ratio of liver to S-Sepharose is about 7 g/ml of packed column volume.

The isolation procedure reported here yields 2.4 mg of homogenous cathepsin L from 500 g of liver, which is approximately twice the yield reported for the previous method of isolating sheep's liver cathepsin L [1]. Active site titration, using E-64, revealed that the preparation apparently contained about 40% active enzyme which is

TABLE III

Inhibition and Activation Studies on Pooled Fractions

Sample	% activity[a]		
	- urea, pH 6.0	+ leupeptin	- cysteine HCl
Fraction from S-Sepharose, pH 4.5	70	10	50

[a] Values are expressed as a percentage of the activity against azocasein in 3M urea, using activation buffer (Barrett and Kirschke[8]).

comparable to the value obtained for the enzyme from human liver[5]. No such value has previously been reported for sheep's liver cathepsin L and thus direct comparisons are not possible. The percentage of active sites in the preparation may possibly be improved by protecting the enzyme reversibly with mercury ions, as reported by Kirschke et al.[16], but due to its toxicity, mercury is often an undesirable contaminant.

The enzyme isolated was identified as cathepsin L by the following criteria:

(i) It manifests a high activity against azocasein, which is enhanced by the presence of 3M urea (Table III),

(ii) It is highly sensitive to inhibition by leupeptin and its activity is markedly increased in the presence of a reducing agent, such as cysteine (Table III),

(iii) It has a MW of approx. 26kD (see below for a detailed discussion),

(iv) It is unstable at or above neutral pH,

(v) It binds strongly to cation exchangers at pH values close to the pI of cathepsin L.

In Fig. 4 the purified cathepsin L sample exhibits only one band, with and without reduction. This indicates that cathepsin L was isolated as a single-chain form by this procedure, since even the sensitive silver staining procedure was unable to show the presence of a lower M.W. subunit with reduction, and there was no change in the M.W. when no reduction took place. This is in contrast to the previous isolation procedure for sheep's liver cathepsin L by Mason[1] where a two-chain form was isolated.

There is some doubt as to whether cathepsin L exists *in vivo* as a two chain form, since Dufour *et al.*[17], reported the isolation of a single-chain form of 27kD, from chicken liver lysosomes, by a rapid procedure. This was in direct contrast to the result of Wada and Tanabe[18], who isolated a two-chain form from chicken liver by a longer procedure. Similarly, Bando *et al.*[3] isolated a mixture of one- (30kD) and two-chain forms (25kD and 5kD) from rat kidney lysosomes. The processing of cathepsin L in rat hepatocytes from a 37 kD higher M.W. form to 30 and 25 kD single-chain forms has been demonstrated by Nishimura *et al.*[18,19,20], and was found to be dependent on an aspartic protease. The conversion of single-chain cathepsin L to a two-chain form was found to be inhibited by a cysteine proteinase inhibitor by Hara *et al.*[21], which means that autocatalysis of the enzyme cannot be excluded. This indicates that although processing of cathepsin L to its lower M.W. forms is a demonstrable, known, event, further processing of the enzyme to a two-chain form may in fact be an artifact of more lengthy isolation procedures.

The above evidence has also led several authors[1,3,5] to postulate that the two-chain form of cathepsin L may be a consequence of limited proteolysis during the autolysis step (which is designed to remove inhibitors from the enzyme) in several of the purification procedures. This contention is placed in doubt, however, by the observation that the original procedure of Kirschke *et al.*[2], in which cathepsin L was isolated directly from rat liver lysosomes, led to the isolation of a two-chain form of cathepsin L. Also, the procedure of Bando *et al.*[3] for the isolation of cathepsin L from rat liver lysosomes led to a mixture of one- and two-chain forms. The procedure of Wada and Tanabe[18] did not involve a conventional autolysis step, but did result in the isolation of a two-chain form, in contrast to the procedure of Dufour *et al.*[17], which gave a single-chain form. The occurrence of the two-chain form, therefore, seems to correlate more with the length of the isolation procedure, rather than the use of an autolysis step, which may indicate that the isolation procedure reported here is rapid enough to ensure the survival of the single-chain form.

FOOTNOTES

Abbreviations used in this paper: E-64, L-3-carboxy-trans-2,3-epoxypropyl-leucylamido-(4-guanidino) butane. SDS-PAGE, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. FPLC, Fast Protein Liquid Chromatography (Pharmacia system).

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Anti-peptide antibodies to cathepsins B, L and D and type IV collagenase

Specific recognition and inhibition of the enzymes

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Anti-peptide antibodies were raised against synthetic peptides selected from the sequences of human cathepsins B and L, porcine cathepsin D and human type IV collagenase. Sequences were selected from the active site clefts of the cathepsins in the expectation that these would elicit immunoinhibitory antibodies. In the case of type IV collagenase a sequence unique to this metalloproteinase subclass and suitable for immunoaffinity purification, was chosen. Antibodies against the chosen cathepsin B sequence were able to recognize the peptide but were apparently unable to recognise the whole enzyme. Antibodies against the chosen cathepsin L sequence were found to recognise and inhibit the native enzyme and were also able to discriminate between denatured cathepsins L and B on Western blots. Antibodies against the chosen cathepsin D sequence recognised native cathepsin D in a competition ELISA, but did not inhibit the enzyme. Native type IV collagenase was purified from human leukocytes by immuno-affinity purification with the corresponding anti-peptide antibodies.

Key words: Anti-peptide antibody; Cathepsins B, L, D; Type IV collagenase; Immunoinhibition

Introduction

Cathepsins B, L and D and type IV collagenase have been implicated in tumour invasion and metastasis (Liotta et al., 1980; Sloane and Honn, 1984; Denhardt et al., 1987; Spyratos et al., 1989). The role of these enzymes in tumour invasion may be explored using specific antibodies and in this context anti-peptide antibodies (Briand et al., 1985) have many advantages. A sequence of ten or more amino acids has a very high probability of being unique to a particular protein and the corresponding anti-peptide antibody is, therefore, also likely to allow highly specific detection of the protein. Moreover, for immunocytochemistry, for example, with polyclonal anti-peptide antibodies against a linear peptide sequence, there is an intrinsically lower probability of the epitope(s) being destroyed during tissue processing, than in the case of a monoclonal antibody which may be targeted at a single, labile, discontinuous epitope.

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Abbreviations: ABTS, 2,2′-azino-di(3-ethyl)-benzthiozoline sulphonic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRPO, horseradish peroxidase; KLH, keyhole limpet haemocyanin; MBS, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester; M_r, relative molecular weight; NHMec, 7-(4-methyl)coumarylamide; PBS, phosphate-buffered saline; SBTI, soybean trypsin inhibitor; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; Z-, benzyloxycarbonyl.

The utility of anti-peptide antibodies may be increased if these are additionally able to inhibit enzymic activity. In the case of the cysteine cathepsins, B, H and L, for example, inhibiting anti-peptide antibodies might constitute tools with a unique ability to discriminate between these enzymes, and might thus aid in their identification. It has also been suggested (Dennison, 1989) that inhibiting anti-proteinase anti-peptide antibodies might be therapeutically useful.

To raise anti-peptide antibodies against the cathepsins, peptide sequences were selected from their primary sequences, mainly by consideration of their 3-dimensional structure, but also with reference to the mobility and hydrophilicity of the chosen peptide sequence. The cathepsins are involved in antigen processing (Takahashi et al., 1989; Van Noort and Van der Drift, 1989) and consequently may be regarded as integral parts of the immune system. The question thus arises as to whether there is any prejudice against production of anti-peptide antibodies to these proteinases, especially against their conserved sequences. As a basis for comparison, therefore, anti-peptide antibodies were also raised against a sequence in a non-lysosomal proteinase, type IV collagenase, similar to that previously shown to successfully elicit anti-peptide antibodies (Höyhtyä et al., 1988). We report here our observations on raising antibodies to the selected peptides and on the effectiveness of the resulting antibodies in binding to, and inhibiting, the target enzymes.

Materials and methods

Reagents

KLH and MBS were obtained from Sigma. Glutaraldehyde (E.M. grade) and cyanogen bromide were from Merck and ABTS was from Boehringer Mannheim. Human liver cathepsin B was a gift from Dr. D. Buttle, Strangeways Laboratory, Cambridge, U.K. Sheeps' liver cathepsin L was isolated by a modification of the method of Pike and Dennison (1989); chromatography on S-Sepharose, at pH 4.5, being substituted by chromatography on Sephadex G-75. Human spleen cathepsin L was similarly isolated, though in the form of a complex with cystatin, in a study to be reported elsewhere. Human kidney cathepsin L was purchased from Novabiochem, U.K. Cathepsin D was isolated from human, porcine and bovine spleens by the method of Jacobs et al. (1989). Type IV collagenase was purified from human leukocytes by immunoaffinity chromatography with the anti-peptide antibody immobilised on CNBr-activated Sepharose 4B. Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were obtained from Cambridge Research Biochemicals.

Selection of peptides

The peptide sequences from cathepsins B and L (Table I) were selected by considerations of 3-dimensional structure, based on a published structure of the analogous enzyme, papain (Wolthers et al., 1970). The 3-dimensional structures of cathepsins B, H and L have been deduced, from amino acid sequence information, to be comparable to that of papain (Kamphuis et al., 1985; Dufour, 1988).

The sequence selected for cathepsin B, corresponds to residues 13–22 in the structure of human liver cathepsin B (Turk et al., 1986). This sequence is in an accessible position, at one end of the substrate-binding cleft of the enzyme (Wolthers

TABLE I

THE PEPTIDE SEQUENCES SELECTED FOR THE GENERATION OF ANTI-PEPTIDE ANTIBODIES, FROM THE AMINO ACID SEQUENCES OF THE PROTEINASES INDICATED

Peptide	Sequence	Corresponding proteinase
B13–22	Q-C-P-T-I-K-E-I-R-D (+C) [a]	Human cathepsin B
L153–165	E-P-D-C-S-S-E-D-M-D-H-G-V	Human cathepsin L
D112–122	T-K-Q-P-G-L-T-F-I-A-A (+C)	Porcine cathepsin D
COL476–490	M-G-P-L-L-V-A-T-F-W-P-E-L-P-E	Human collagenase IV

[a] The selected peptides were modified for synthesis by the substitution of the cysteine residues in peptides B13–22 and L153–165 with α-amino butyric acid and by the addition of an extra cysteine residue to the C termini of B13–22 and D112–122 respectively, in addition to the acetylation of the N terminus of B13–22 and amidation of the C terminus of L153–165.

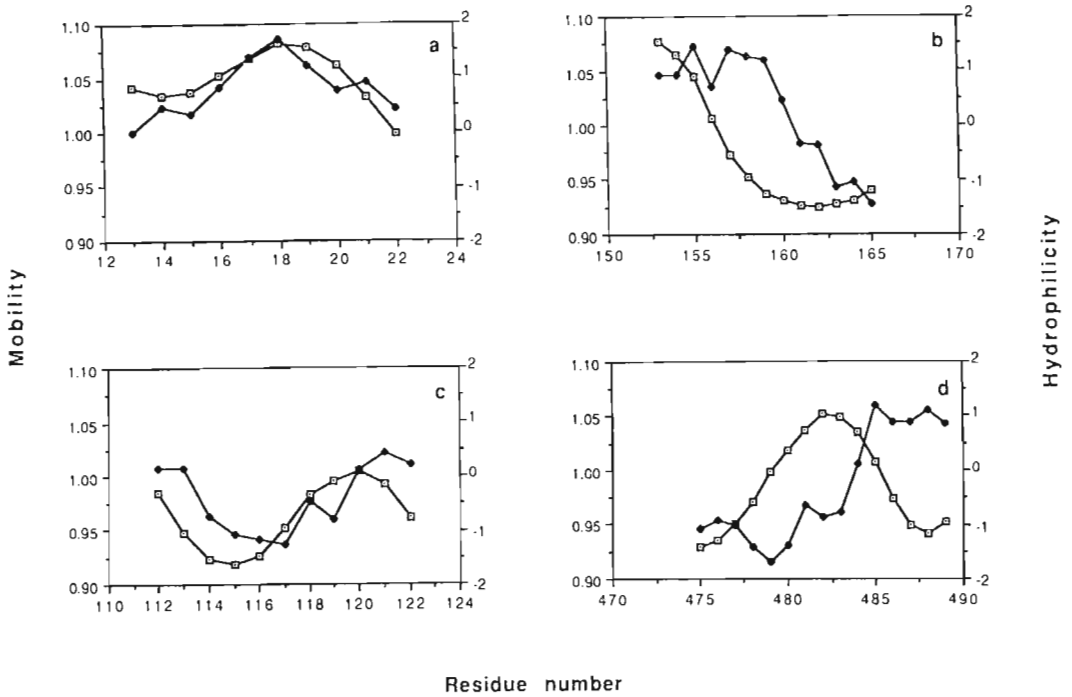

Fig. 1. Hydrophilicity and segmental mobility profiles of the selected peptides. ◆, hydrophilicity, calculated according to Hopp and Woods (1981, 1983); □, segmental mobility, calculated according to Westhof et al., (1986). Profiles indicated are for the peptides: (*a*) B13–22; (*b*) L153–165; (*c*) D112–122; and (*d*) COL476–490.

et al., 1970). It also corresponds to a peak of both hydrophilicity (Hopp and Woods, 1981, 1983) and segmental mobility (Westhof et al., 1984) (Fig. 1*a*).

A sequence different from that for cathepsin B was chosen for cathepsin L, to potentially maximise the information gained from the experiments. Also, the region chosen for cathepsin B is not a suitable choice for human cathepsin L since the human cathepsins L and H have analogous sequences in this region, with seven out of the 11 amino acids being similar or identical (Ritonja et al., 1988). There is thus an increased probability that an anti-peptide antibody to the sequence in cathepsin L may cross-react with cathepsin H.

By contrast, the loop of amino acids containing the active site histidine is also accessible (Wolthers et al., 1970), and there are marked differences in the sequences in this region between the different cysteine cathepsins. The presence of the active-site histidine in this sequence was also thought to increase the probability that antibodies targeting this region might be inhibitory. The chosen sequence corresponds to residues 153–165 in the amino acid sequence of human cathepsin L (Ritonja et al., 1988); in papain the comparable residues are 150–161. The sequence is largely hydrophilic but has a cluster of hydrophobic residues towards its C terminus (Fig. 1*b*). The L153–165 sequence is also relatively conserved between species and may be expressed as Glu-Pro-Asx-Cys-Ser-Ser-A-Asx-B-Asp-His-Gly-Val, where Asx is either Asp or Asn, A is Glu or Lys and B is Met or Leu (Dufour et al., 1987; Ishidoh et al.,1987; Ritonja et al., 1988). An additional criterion in its selection, therefore, was its potential to target cathepsin L across species.

The sequence chosen for cathepsin D (Table I) was based on the 3-dimensional structure of a related aspartic proteinase, penicillinopepsin (Hsu et al., 1977), since no 3-dimensional structure of cathepsin D has been published. The sequence corresponds to residues 112–122 in porcine cathepsin D (Faust et al., 1985), and corresponds to a loop on the rim of the substrate-binding groove of penicillinopepsin. It has low hydro-

philicity and mobility (Fig. 1*c*) and differs from human cathepsin D in a single, conservative, substitution of leucine for isoleucine at position 117 (Faust et al., 1985).

The sequence chosen for human type IV collagenase (Table I) is based on the sequence of a CNBr-generated fragment of this enzyme from human melanoma A2058 cells (CB4 peptide), reported by Höyhtyä et al. (1988) to elicit antibodies which bind only to type IV collagenase and not to related, secreted, extracellular matrix metalloproteinases, such as interstitial collagenase and stromelysin. The sequence corresponds to residues 476–490 in human type IV procollagenase (Collier et al., 1988) and is hydrophilic towards its C terminus and mobile in its centre (Fig. 1*d*). In the present study the C terminal Lys was omitted from the CB4 peptide to ensure that glutaraldehyde conjugation was effected exclusively through the N terminus, thereby exposing the hydrophilic part of the peptide.

Synthesis of peptides

The selected peptides were modified, before synthesis, by the substitution of the cysteine residues in peptides B13–22 and L153–165 with α-amino butyric acid and by the addition of an extra cysteine residue to the C-termini of B13–22 and D112–122 respectively. The resulting peptides were custom synthesised by Multiple Peptide Systems, San Diego, CA.

Conjugation

All four peptides were conjugated to KLH, using two different conjugation methods. Peptides B13–22 and D112–122 were conjugated, through their C termini to KLH, using MBS (Robertson and Liu, 1988). The maleimide content of KLH-MBS was determined by the addition of mercaptoethanol and subsequent assay for reduced thiol content (Kitagawa and Aikawa, 1976). Due to their solubility differences, it was necessary to treat B13–22 and D112–122 differently. B13–22 was dissolved in 200 mM sodium phosphate buffer, pH 8.0, and D112–122 was dissolved in the same buffer, but containing 8 M urea, before reduction and conjugation. The method of Sedlak and Lindsay (1968) was used to determine the peptide reduction. Peptides L153–165 and COL476–490 were conjugated to KLH, through their N termini, using 1% (v/v) glutaraldehyde, according to Briand et al. (1985). A carrier protein-to-peptide ratio of 1 : 40 was used.

TABLE II

INOCULATION PROTOCOL

Week	Freund's adjuvant	Site	Dose
0	Complete	s.c. [a]	200 μg conjugated peptide
2	Incomplete	s.c.	200 μg conjugated peptide
3			Bleed
6	Incomplete	s.c.	200 μg conjugated peptide
8			Bleed
10	Incomplete	s.c.	200 μg conjugated peptide or
	–	i.v. [b]	1 mg free peptide
12			Bleed
Monthly boosters as indicated for 10 weeks			

[a] s.c. = subcutaneous injection on the back at each of five sites.
[b] i.v. = intravenous in marginal ear vein.

Inoculation protocol

For each peptide two rabbits were inoculated with peptide conjugate according to the protocols summarized in Table II. For comparison the protocol of Richardson et al. (1985) was followed, in which conjugate was replaced by free peptide from week 10. B13–22 was only subjected to the latter protocol.

ELISA for anti-peptide antibodies

Wells of microtitre plates (Nunc Immunoplate) were coated overnight at room temperature with peptide solution in PBS, pH 7.2, at 5 μg/ml (B13–22 and L153–165), 0.5 μg/ml (D112–122) and 1 μg/ml (COL476–490). Wells were blocked with 0.5% BSA in PBS for 1 h at 37°C and washed 3 × with 0.1% Tween 20 in PBS (PBS-Tween). Dilutions of the primary antiserum in 0.5% BSA-PBS were then added, incubated at 37°C for 2 h, and excess antiserum was again washed out 3 × with PBS-Tween. A 1/200 dilution of sheep anti-rabbit IgG-horseradish peroxidase conjugate, in 0.5% BSA-PBS, was added and incubated for 30 min at 37°C. The ABTS substrate (0.05% in 150 mM citrate-phosphate buffer, pH 5.0, containing 0.0015% H_2O_2) was added and incubated for 15 min. The enzyme reaction was stopped by the addition of 0.1% NaN_3 in citrate-phosphate buffer

and the absorbance was read at 405 nm in a Bio-Tek EL307 ELISA plate reader.

ELISA for immobilized enzyme

The ability of anti-peptide antibodies to cross-react with the respective whole enzymes (not necessarily in their native form) was measured by coating the wells of microtitre plates with either cathepsin B or L (5 μg/ml and 1 μg/ml, respectively, in 50 mM carbonate buffer, pH 6.0, for 3 h at 37°C, followed by overnight at 4°C) or cathepsin D (2 μg/ml in PBS, pH 7.2, overnight at room temperature). The remainder of the procedure was as outlined above except that IgG was purified from serum, by the method of Polson et al. (1964), to remove serum inhibitors of the enzymes (e.g. cystatin). Species cross-reactivity of anti-peptide antibodies was measured using the same ELISA by coating with cathepsins purified from various sources.

Competition ELISA for native enzyme

The binding of the anti-peptide antibodies to the native cathepsins was tested in an ELISA in which free enzyme was permitted to compete with immobilized peptide for binding to the antibody and thus prevent a fraction of the antibody from being immobilized. Microtitre plates were coated with peptide as described above. Various amounts of antibody (between 10 and 450 μg/ml IgG) were pre-incubated at 37°C for 30 min with different levels of enzyme (molar ratios of peptide-to-enzyme from 1 : 24 to 1 : 0.5), before the incubation mixture was transferred to the peptide coated wells. After a further 1 h incubation at 37°C, the ELISA was developed as described above.

Removal of anti-KLH antibodies

KLH was coupled to cyanogen bromide activated Sepharose-4B according to Kohn and Wilchek (1982). Anti-KLH antibodies were removed from immunoglobulin fractions, purified from serum according to Polson et al. (1964), by passage through KLH-Sepharose.

Immunoblotting

The different enzymes were subjected to reducing SDS-PAGE (Laemmli, 1970), before transfer to nitrocellulose membranes (Schleicher and Schull, BA 85,0.45 μm) essentially as described by Towbin et al. (1979). Following electro-blotting for 16 h, the nitrocellulose membrane was air dried for 1.5 h and non-specific binding sites were blocked with low-fat dried milk powder (5% in TBS) for 1 h. After this, and at all subsequent steps, the membrane was washed (3 × 5 min) with TBS. Anti-peptide antibodies, from which anti-KLH antibodies had been removed, were diluted in 0.5% BSA-TBS and incubated with the membrane (2 h), followed by sheep anti-rabbit IgG-HRPO conjugate (1 h). All incubation steps were carried out at room temperature. The HRPO reaction was detected with 0.06% 4-chloro-1-naphthol in TBS, containing 0.0015% H_2O_2. The reaction was stopped by rinsing in TBS containing 0.1% NaN_3. Targeting of sheep and human cathepsin L by anti-L153–165 antibodies was also visualised by protein A-gold labelling with silver amplification (Moeremans et al., 1984).

Immunoinhibition assays

Assays for the immunoinhibition of cathepsins B and L were carried out using the substrates Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec, respectively, as described by Barrett and Kirschke (1981). Cathepsin B (250 ng) or cathepsin L (25 ng) were incubated at 30°C for 15 min with anti-peptide IgG, or normal rabbit IgG, at the appropriate concentration in 400 mM Na-phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.1% Tween 20. Assays against the Z-Phe-Arg-NHMec substrate revealed that the IgG fractions had intrinsic activity against this substrate, which is probably attributable to contaminating plasma kallikrein which cleaves this substrate (Barrett and Kirschke, 1981). This activity was controlled by the addition of 40 μg/ml of SBTI, and by subtracting the residual activity in the antibody fractions from the measured cathepsin L activity. SBTI inhibits kallikrein but not cathepsin L. Stopped time assays were carried out over the range of IgG concentrations, and the inhibition by anti-peptide antibodies was calculated in comparison to normal rabbit IgG. Immunoinhibition of cathepsin D was carried out using acid denatured hemoglobin as substrate, essentially as described by Dingle et al. (1971).

Results

Anti-peptide antibody production

All four peptide conjugates elicited antibodies, which reacted with the corresponding immobilized peptides in an ELISA (Fig. 2). In each case, it appears that the antibody titer peaked at about 8–12 weeks. No significant difference could be observed in titer obtained with the two inoculation protocols (using conjugate throughout or changing to free peptide after 10 weeks) when tested against immobilized peptide. Anti-B13–22 antibodies showed a decline after 12 weeks, but this could not be attributed to changing to inoculation with free peptide since anti-D112–122 antibodies, for instance, showed a similar decline in titer after 8 weeks with both inoculation protocols.

Recognition of enzymes coated to ELISA plates

The anti-B13–22 antibodies, although able to recognize the peptide B13–22, were unable to recognize the whole enzyme, coated to a multititer plate at pH 6.0, 7.2 or pH 9.6 (results not shown). By contrast, anti-L153–165 antibodies were able to recognize both human and sheep cathepsin L, immobilised on ELISA plates (Fig. 3*a*). They apparently reacted more strongly with the sheep than the human enzyme, from which the peptide sequence was selected, but this may merely be a concentration phenomenon. Human spleen cathepsin L, used in this test, was complexed to cystatin and the measured protein concentration was therefore not a true reflection of the amount of cathepsin L present per se. Anti-porcine cathepsin D, was able to recognize whole human, porcine and bovine cathepsin D enzymes, immobilised on an ELISA plate (Fig. 4). The peptide antibodies, raised against D112–122 (a sequence from porcine cathepsin D), apparently reacted better with human than with porcine or bovine cathepsins D. In the region corresponding to the chosen peptide, the human cathepsin D sequence shows a single substitution of leucine for isoleucine, at position 117, compared to the porcine

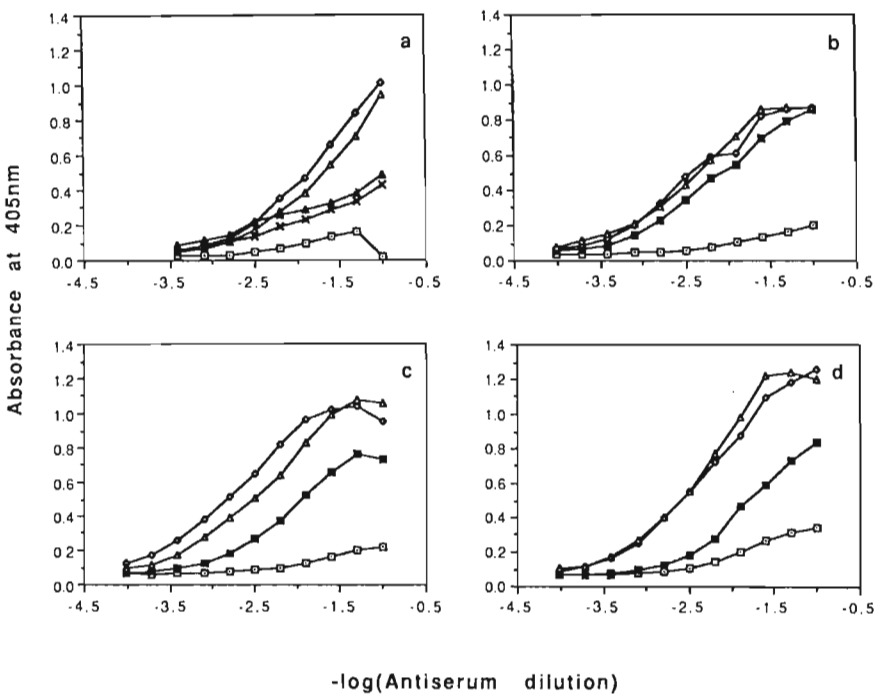

Fig. 2. Progress of immunisation with peptide conjugates as determined by ELISA. Peptides were coated to microtitre plates, (*a*) B13–22; (*b*) L153–165; (*c*) D112–122; and (*d*) COL467–490 and incubated with serial two-fold dilutions of antisera collected after 3 (■), 8 (◇), 12 (△), 30 (▲) and 32 weeks (×). Normal rabbit serum control (□). This was followed by incubation with HRPO- linked secondary antibody and ABTS as a chromogenic substrate, as described under materials and methods section. Each point is the mean absorbance at 405 nm of duplicate samples.

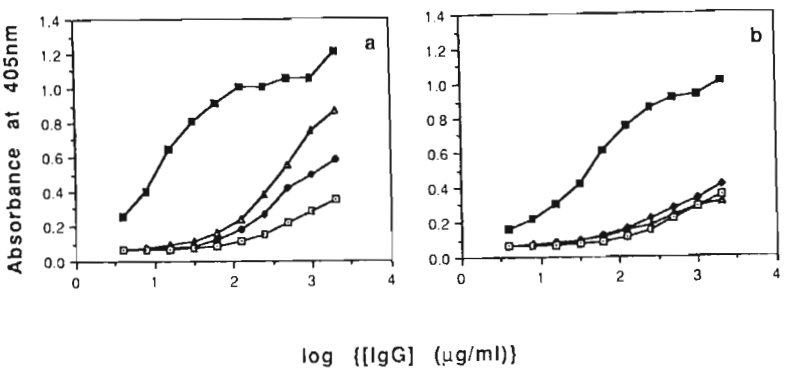

Fig. 3. ELISA of binding of anti-peptide antibodies to whole immobilised cathepsin L. Cross-reaction of anti-L153–165 antibodies with human (◆) and sheep (△) cathepsin L, and peptide L153–165 (■). Normal rabbit IgG (□). Experimental procedure as in Fig. 2 and in the materials and methods section. (*a*) anti-L153–165 antibodies elicited by use of conjugated peptide throughout. (*b*) anti-L153–165 antibodies elicited by use of conjugated peptide followed by free peptide in the inoculation procedure.

enzyme, and it may be inferred from the results that the bovine enzyme must also be very similar in this region. Due to the lack of sufficient enzyme, the anti-COL476–490 antibodies could not be tested against the collagenase IV enzyme, in an ELISA.

Although antibodies raised using the two different immunisation protocols apparently had the same titer against immobilised peptide, a clear difference was sometimes seen in their ability to target the immobilised whole enzyme. In the case of anti-L153–165 peptide antibodies, for example, where conjugate was used throughout, the resulting antibodies cross-reacted with the whole protein to a much higher degree (Fig. 3). This phenomenon was less marked in the case of cathepsin D (result not shown). Anti B13–22 antibodies, tested against whole cathepsin B, did not give a positive reaction at any stage, including at 8 weeks, before the switch to free peptide.

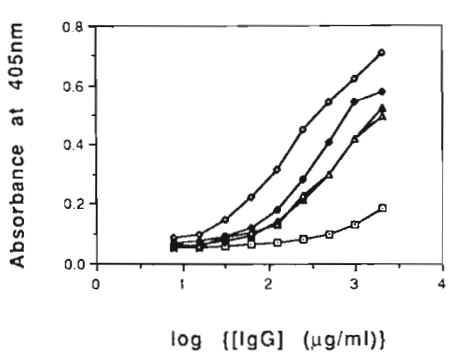

Fig. 4. ELISA of binding of anti-peptide antibodies to whole immobilised cathepsin D. Cross-reaction of anti-D112–122 antibodies with human (◆), porcine (△) and bovine (▲) cathepsin D, and peptide D112–122 (◇). Normal rabbit IgG (□). Experimental procedure as in Fig. 2 and in the materials and methods section.

Specificity of anti-peptide antibodies: Western blot analyses

In Western blot analyses it was found that a more specific reaction was obtained if anti-KLH antibodies were removed by passage through a column containing immobilised KLH. Anti-L153–165 antibodies targeted human cathepsin L to a much higher degree than the sheep enzyme and protein A-gold labelling with silver amplification was required to show the targeting of sheep cathepsin L (Fig 5*A*). The specificity of this targeting was evidenced by the fact that there was no cross-reactivity with human cathepsin B. Anti-B13–22 and D112–122 antibodies did not show any reaction with the corresponding enzymes on a Western blot (result not shown). The anti-COL476–490 antibodies detected a M_r 66,000 band of type IV collagenase purified from human leukocytes (Fig. 5*B*).

Recognition of native enzymes

Anti-B13–22 antibodies did not interact with the native form of cathepsin B when tested in a competition ELISA and immunoinhibition assays, all at pH 6.0 (results not shown). Cathepsin D inhibited the binding of anti-D112–122 antibodies (250 μg/ml) to the peptide coated to multititer plates, in a dose-dependent manner, up to 60% at

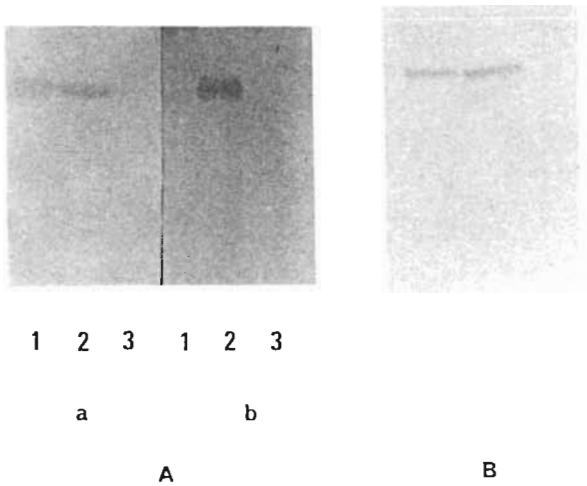

Fig. 5. Targeting of cathepsin L and type IV collagenase by anti-peptide antibodies on Western blots. *A*: samples ((1) sheep cathepsin L; (2) human cathepsin L; (3) human cathepsin B) were subjected to 12.5% reducing SDS-PAGE, electroblotted onto nitrocellulose and then incubated with anti-KLH-purified anti-L153–165 IgG, before developing with (a) protein A-gold with silver amplification or, (b) sheep anti-rabbit-HRPO conjugate as described in the materials and methods section. *B*: human type IV collagenase was electrophoresed on a 7.5% SDS-polyacrylamide gel with reduction, transferred to nitrocellulose and immunologically stained with anti-KLH-purified anti-COL476–490 IgG as described in the materials and methods section.

446 μg/ml (Fig. 6), suggesting that the antibody recognizes the native enzyme. Because of the relatively high concentrations of enzyme required for

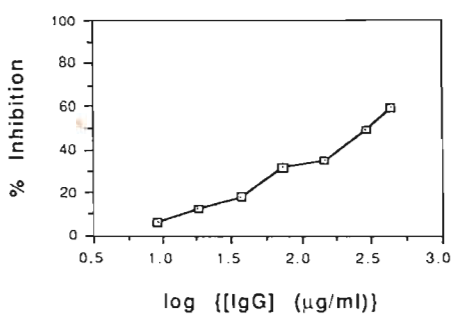

Fig. 6. Competition ELISA for native cathepsin D. The ability of cathepsin D to inhibit the binding of anti-D112–122 antibodies to immobilised D112–122 was measured by pre-incubating various amounts of IgG with different levels of enzyme before transfer of the incubation mixture to peptide coated plates. The ELISA was developed as in the materials and methods section. The percentage inhibition was calculated from control incubations containing either normal rabbit IgG or no competing cathepsin D.

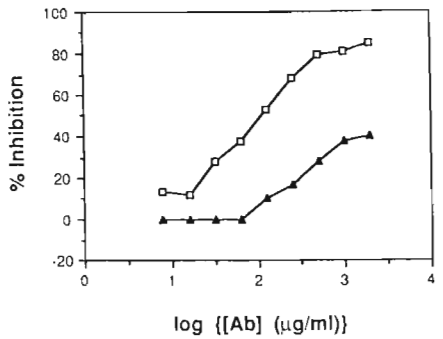

Fig. 7. Immunoinhibition of human and sheep cathepsin L by anti-L153–165 antibodies. Stopped time assays were carried out using human (□) and sheep (△) cathepsin L as described in the materials and methods section and the percentage inhibition calculated relative to control assays with normal rabbit IgG.

this assay, cathepsin L and type IV collagenase were not included in these tests. Cathepsin D activity was, however, not inhibited by anti-D112–122 antibodies in the enzyme immunoinhibition test.

An indication that anti-COL476–490 antibodies bind to native type IV collagenase is given by their effectiveness in immunoaffinity purification of the enzyme. Type IV collagenase thus purified from human leukocytes showed gelatinolytic activity on a gelatin zymogram (result not shown).

The L153–165 antibodies almost completely inhibited human cathepsin L at high antibody concentrations and inhibition decreased with decreasing antibody concentration until a plateau was reached at low antibody concentration (Fig. 7). Sheep liver cathepsin L was also inhibited, but to a lesser extent than the human enzyme. These results therefore show that the antibody was able to bind to and inhibit native human and sheep cathepsin L. Anti-L153–165 antibodies did not inhibit cathepsin B (results not shown), showing the specificity of this immunoinhibition for cathepsin L.

Discussion

The failure of the anti-B13–22 peptide antibodies to recognize whole human cathepsin B was not expected since the peptide corresponds to peaks of both hydrophilicity and mobility in the sequence

of cathepsin B (Fig. 1*a*) and, from a consideration of the 3-dimensional structure of papain, it would also appear to be on the surface of the molecule. It has been reported that, in general, segmental mobility is an important criterion for the recognition of the native protein by anti-peptide antibodies (Van Regenmortel, 1988a). This does not appear to hold for the peptide B13–22, and it may be speculated that the presence of a disulfide bridge might, perhaps, constrain the peptide in a particular way in the native protein. There is thus an apparent conflict between the high mobility value assigned to Cys-14, by Westhof et al. (1984) antigenicity prediction profile, and its participation in a constrained disulfide bridge. Comparison with the results obtained for the cathepsin L peptide, L153–165, may be instructive. The peptide L153–165 was conjugated through its N terminus, which is close to the Cys residue involved in a disulfide bridge, and in this case antibodies to the peptide were able to recognize the native protein. In both cases, the Cys residue was substituted by an α-amino butyric residue, but since the peptide L153–165 elicited competent antibodies, this substitution per se is probably not the reason why the peptide B13–22 failed to raise antibodies able to recognize the native enzyme. It may be interesting to examine the possible recognition of the native protein by antibodies raised against B13–22, but conjugated through its Cys residue, or its N terminus.

It must be noted that cathepsin B is generally a refractory enzyme with regard to antibody production and normal polyclonal antibodies, raised against whole cathepsin B, are only able to recognize denatured forms of the enzyme (Barrett, 1973). Monoclonal antibodies against native cathepsin B have been reported (Wardale et al., 1986). Monoclonal antibodies are produced in vitro, however, and it may be speculated that there is a prejudice against production of anti-cathepsin B antibodies in vivo, due to its involvement in antigen processing. By contrast, antibodies are easily raised against native cathepsin L, and it is interesting to note, in this regard, that Takahashi et al. (1989) have concluded that cathepsin B, and not cathepsin L, might be the major enzyme involved in antigen processing. Therefore, if human B13–22 shares sequence homology with its rabbit counterpart, any rabbit B cell clones producing anti-B13–22 antibodies capable of recognising native cathepsin B may be suppressed.

The cathepsin L peptide, L153–165, is much less hydrophilic and mobile at its exposed C terminus, than at its N terminus (Fig. 1*b*), but it was decided to conjugate it through its N terminus so as to expose the active site histidine. This stratagem appears to have been successful in eliciting anti-peptide antibodies able to inhibit native human and sheep cathepsin L (Fig. 7). Due to the specificity of this inhibition, anti-L153–165 peptide antibodies may be useful research tools, since the inhibitors currently in use are unable to discriminate qualitatively between cathepsins B and L (Kirschke et al., 1988). Anti-L153–165 antibodies also discriminate very specifically between cathepsins B and L on Western blots (Fig. 5*A*), which suggests that they may also be useful in immunocytochemistry. They may also be useful as therapeutic agents in pathologies arising from excessive cathepsin L activity.

A criterion in the selection of the peptide L153–165 was the potential of antibodies to this peptide to target cathepsin L across species. The cross-reactivity between anti-L153–165 antibodies and sheep cathepsin L, immobilised in ELISAs and on Western blots, and in the enzyme immunoinhibition assays (Figs. 3, 5*A* and 7), confirms this expectation.

Anti-D112–122 antibodies recognized the peptide as well as whole human, porcine and bovine cathepsin D enzymes, immobilised on multititer wells (Fig. 4). Nevertheless, the colour took a relatively long time (about 1 h) to develop in the ELISA assay against immobilised whole enzymes. There is evidence (Van Regenmortel, 1988b) that proteins become partially denatured or undergo conformational changes when adsorbed to solid phases, so the slow colour development may indicate that anti-D112–122 antibodies recognize the partially denatured enzymes only weakly or that only a small percentage of the enzyme adopts a conformation suitable for antibody binding. The antibody clearly recognizes the native form of human cathepsin D, as evidenced from the competition ELISA results (Fig. 6), but does not target the fully denatured enzyme on a Western blot. It may be inferred, from these results, that the epi-

tope in the native enzyme, recognized by anti-D112–122 antibodies, may be a continuous but conformationally specific epitope which is destroyed by reducing SDS-PAGE. Consideration of the 3-D structure of penicillinopepsin reveals a prominent spiral turn in the region corresponding to the D112–122 sequence and suggests that this may constitute such a conformational epitope.

Conjugation of the peptide D112–122, to KLH, was effected through its C terminus, since these residues appear to be less accessible in the native protein; a situation which may therefore be mimicked in the conjugate by the presence of the carrier protein. The more exposed N terminal residues proved to be antigenic and the resulting anti-peptide antibodies were able to bind to the native protein, but were not able to inhibit the enzyme. The paratope-epitope interaction is possibly too distant to occlude the substrate binding cleft.

From a methodological point of view it is of interest that although peptide D112–122 is largely hydrophobic, and was initially insoluble in all solvents tested except 8 M urea, it remained in solution after subsequent removal of the urea and could successfully elicit antibodies able to recognize the native enzyme. The significance of this is that many enzymes apparently have hydrophobic binding sites. From the results obtained using peptide D112–122, it would appear that generation of peptide antibodies against such hydrophobic sites is not impossible.

Omission of the CB4 C terminal Lys-residue (Höyhtyä et al., 1988), in COL476–490, used in the present study, ensured glutaraldehyde conjugation exclusively via the N terminus. This presentation, exposing the more hydrophilic C terminus, proved to be sufficiently immunogenic to elicit anti-peptide antibodies which are able to interact with native type IV collagenase from human leukocytes in immunoaffinity purification. This result confirms the finding of Höyhtyä et al. (1988) that the anti-CB4 antibody specifically immunoprecipitated native type IV collagenase from a mixture of metalloproteinases secreted by human melanoma cells and also recognized the denatured proteinase (M_r 68,000) on Western blots following SDS-PAGE. Targeting of a 67,000 band on a Western blot by anti-CB4 antibodies was also used by Spinucci et al. (1988) to positively identify the proteinase purified from c-Ha-*ras* oncogene transformed mouse NIH 3T3 fibroblasts. In the present study anti COL476–490 antibodies similarly recognized the denatured M_r 66,000 proteinase from human leukocytes (Fig. 5*B*). From these results it may be inferred that these anti-peptide antibodies recognize native and denatured type IV collagenase from both normal (leukocyte) and malignant (melanoma) human sources as well as oncogene transformed mouse NIH 3T3 fibroblasts.

From this study it is clear that there is as yet no reliable basis on which to predict which peptides will sucessfully elicit antibodies capable of recognising the native target protein. Consideration of the 3-D structure, when this is available, appears to be the most promising approach and was successful with cathepsin L, though not with cathepsin B. It will be interesting, in future, to further explore the structures of cathepsins B and D with a view to finding inhibitory peptide antibodies to these proteinases.

Acknowledgements

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