Properties of Cathepsin L in Relation to a Role in Invasive Cancer

by

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This thesis is dedicated to Heidi, for her unconditional support, love and encouragement.

PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg, from March 1992 to December 1997, under the supervision of Professor Clive Dennison.

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

Frieda Marie Dehrmann

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ABSTRACT

Cathepsin L, which has been implicated in many tissue degradative pathologies by virtue of its ability to degrade extracellular matrix components, was isolated by a novel, scaled-up protein purification method and purified to homogeneity in the single-chain form. In addition, the high molecular weight variant of cathepsin L covalently complexed with stefin B was isolated. Both cathepsin L and the complex were stable, in respect of their proteolytic activity, to the chaotropic agent urea, both showing enhanced activity in the presence of urea. Urea did not dissociate the complex.

The suitability of cathepsin L for a purported extracellular role was addressed by investigating its pH optimum and pH stability. Cathepsins L and B are affected by ionic strength and so buffers of constant ionic strength (rather than constant molarity, and therefore varying ionic strength) were used in determining their pH optima and stability. Cathepsins L and B had apparent pH optima of pH 6.5 and 7.5, respectively, (measured with synthetic substrates) and, contrary to the previous belief, were substantially stable at physiological pH. In Hanks' balanced salt solution, a model of the extracellular fluid, they were shown to be active and stable, cathepsin L having a half-life of 179 s at pH 7.2 and 657 s at pH 6.8 (the peritumour pH). It was also shown that prior reductive activation of these enzymes increased their stability to extracellular conditions, supporting the hypothesis that the active site thiolate-imidazolium ion pair contributes to their stability.

The nature of the bond between cathepsin L and stefin B in the covalent complex was examined, using CNBr cleavage, HPLC and amino acid sequencing. Stefin B was shown to be associated with residues 1-137 of cathepsin L via a reduction sensitive linkage which was deduced to be a thioester bond betwen Asp-71 of cathepsin L and Cys-3 of stefin B.

Polyclonal antibodies to cathepsin L and stefin B-complexed cathepsin L were raised in rabbits and chickens, and characterised with respect to their suitability for immunocytochemical localisation of these forms of cathepsin L.

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LIST OF ABBREVIATIONS

ABTS	2,2′-azino-di-(3-ethyl)-benzthiozoline sulfonic acid
AMT	acetate-MES-tris
AR	analytical reagent
B16-BL6	highly metastatic murine melanoma cell line
BDH	British Drug Houses
BM	basement membrane
BSA	bovine serum albumin
C3	third component of complement
CD	cation dependent
CD	circular dichroism
cDNA	complementary DNA
CI	cation independent
CNBr	cyanogen bromide
CP	"chemically pure" grade
CPs	cysteine proteinases
dist.H_2O	distilled water
DMEM	Dulbecco's minimal essential medium
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
GlcNAc	N-acetyl-glucosamine
GSH	reduced glutathione
GSSG	oxidised glutathione
GTP	guanosine triphosphate
Ha-*ras*	Harvey *ras* oncogene
HBSS	Hanks balanced salt solution
12(S)-HETE	12(*S*)-hydroxyeicosatetranoic acid
HPLC	high performance (price) liquid chromatography
HRPO	horse radish peroxidase
IgG	immunoglobulin G

IgY	immunoglobulin Y
K_{av}	availability constant
k_{cat}	enzyme turnover number
k_d	dissociation constant
K_i	inhibition constant
K_m	Michaelis constant
k_{obs}	observed equilibrium co-efficient
kDa	kilodaltons
KNIH 3T3	NIH3T3 cells transformed with Kirsten sarcoma virus
LAMPS	lysosome associated membrane proteins
LDL	low density lipid
LPR	lysosomal protein receptor
α_2-M	α_2-macroglobulin
MEM	minimal essential medium
MEP	major excreted protein (procathepsin L)
MES	2(N-morpholino)ethanesulfonic acid
MHC-	major histocompatibility
MMP	matrix metalloproteinase
M-6-P	mannose-6-phosphate
MPR	mannose-6-phosphate receptor
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix-metalloproteinase
NCD	National Co-operative Dairies
NEAA	non-essential amino acid solution
NHMec	7-amino-4-methyl coumarin
NIH 3T3	murine fibroblast cell line
p.s.i.	pounds per square inch
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
PAI-2	plasminogen activator inhibitor 2
PBS	phosphate buffered saline
PEG	polyethylene glycol
PDGF	platelet derived growth factor
PVDF	polyvinylidene difluoride
RT	room temperature
SDS	sodium dodecyl sulfate
T	acrylamide
TBS	Tris buffered saline
TCA	trichloracetic acid

TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TIMPS	tissue inhibitors of metalloproteinases
TPP	three phase partitioning
TRITC	tetramethylrhodamine isothiocyanate
uPA	urokinase-type plasminogen activator
VCAM1	vascular cell adhesion molecule 1
VLA-4	very late antigen-4 (a member of the integrin family)
Z	benzoylcarboxy

CHAPTER 1

INTRODUCTION

Diseases that are of major concern to humans, often arise from the body's inability to control normal cellular processes. In many instances, the regulation of the proteolytic enzymes becomes altered, resulting in pathologies such as arthritis, multiple sclerosis, emphysema, muscular dystrophy, bone resorption, Alzheimer's disease and tumour malignancy (Rich, 1986; Mason *et al.*, 1986; Ii *et al.*, 1993; Kakegawa *et al.*, 1993). Consequently, over the past decades, much research has centred on the biochemical properties and functions of proteolytic enzymes, in the hope that this information may point to the control of these aberrant processes, for both preventative and therapeutic treatments. The study reported here has centred on the biochemical characteristics of one such enzyme, cathepsin L, and its role in the development of malignancy and tumour invasion of the extracellular matrix (ECM).

1.1 The biology of cancer and metastasis

The formation of tumours is a multistage process, originating from the transformation of a single cell. This occurs at the genetic level, whereby stimuli (such as chemicals and/or radiation) cause, in sequence, a loss of heterozygosity of part of a chromosome, the activation of an oncogene, and the inactivation of tumour suppresser genes, resulting in the modification of DNA to the neoplastic state of the cell (Klein and Klein, 1986). The transformed cell exhibits altered metabolic characteristics, including uncontrolled cell division and growth to form a tumourigenic mass (Blood and Zetter, 1990). This neoplastic state may be benign or malignant. In the benign state, the tumour will remain localised and does not spread to other regions of the body. A malignant neoplasm, on the other hand, usually invades and destroys host tissue, often spreading to other parts of the body by a process of metastasis (Evans, 1991a).

The fate of the malignant tumour appears to depend on the vascularisation of the tumour spheroid (Blood and Zetter, 1990). Neovascularisation of tumours invariably arises in metastasis (Folkman and Shing, 1992). The process of formation of sprouting blood vessels in the direction of the tumour mass is referred to as angiogenesis. This enables the solid tumour to receive oxygen and nutrients, have

waste products cleared and become neovascularised (Blood and Zetter, 1990). The development of these blood vessels is stimulated by angiogenic factors released either by the tumour cell or by inflammatory cells which have been attracted to the tumour site (Blood and Zetter, 1990). The tumour cells encompass the new microvessels in a cylindrical fashion (Folkman and Shing, 1992). For metastasis to occur, cells must detach from the primary tumour and invade into the blood vessels enveloped by the tumour (McDonnell *et al.*, 1994), a process believed to occur more readily from surrounding neoplastic cells than normal cells (Nicolson, 1988).

The migratory tumour cells may be disseminated to other parts of the body via the bloodstream (via the tumour vasculature) or the lymphatic system, although the latter is less likely since neoplastic interstitium seldom possesses lymph vessels. In either event, tumour cells are required to traverse vascular epithelium. This process of intravasation occurs more readily through the tumour-induced vascular system, as these capillaries exhibit altered basement membrane structure and increased permeability (Nagy *et al.*, 1977).

A large number of tumour cells in the vasculature does not guarantee metastasis formation (Evans, 1991a). Several factors contribute to the short half-life of tumour cells in the bloodstream, e.g. shear forces in the vascular system, and the cells of the immune system which have anti-tumour activity (such as the cytotoxic T-lymphocytes, natural killer cells and mononuclear phagocytes). Furthermore, the nutrient requirements of tumour cells are thought not to be met by 100% serum (Blood and Zetter, 1990). Only tumour cells that survive this hostile environment have a chance of producing a metastatic tumour mass.

Circulating tumour cells which survive the vasculature are required to arrest at a site in the bloodstream. Although these cells may become passively trapped in the first vascular bed which they encounter, metastatic colonies are often found downstream from the first potential site of passive attachment. This has led some workers to investigate the selective adherence of certain tumour cells to endothelial cells of preferred secondary sites (Alby and Auerbach, 1984), allowing the discovery of tissue-specific glycoprotein molecules, which interact with adhesive receptor molecules on the tumour cell surface (Hynes, 1992). The vascular cell adhesion molecule (VCAM1) found on endothelial cells acts as a counter receptor for VLA-4

(very late antigen - a member of the integrin family), which has been identified on malignant melanoma cells. This suggests that VCAM-1 acts as a tumour cell adhesion receptor, allowing interaction of circulating melanoma cells with the endothelium prior to extravasation (Stetler-Stevenson *et al.*, 1993). Furthermore, tumour cells express integrins, which function as receptors for extracellular matrix proteins such as collagen, fibronectin, laminin and vitronectin (as well as mediating cell-cell adhesion). Gehlsen *et al.* (1988) showed that melanoma cell invasion *in vitro* and *in vivo* could be inhibited by disruption of integrin function upon addition of peptides containing sequences (RGD) common to the adhesive molecules (fibronectin, vitronectin and others). This suggests that the "sticky" factors are components of the basement membrane which create a haptotactic gradient towards the basement membrane (Kramer *et al.*, 1980). It is also possible that the factors responsible for adhesion to the endothelium are not proteinaceous in nature. Menter *et al*. (1987) showed that the presence of platelets enhanced the adhesion of tumour cells to endothelial cells, and Honn *et al.* (1989) showed that 12 (S)-hydroxy-eicosatetraenoic acid could induce reversible endothelial cell retraction, allowing tumour cells access to the sub-endothelial matrix. This molecule is, in fact, a platelet factor which can be transferred from activated platelets to endothelial cells during tumour cell - platelet - endothelial cell interactions.

Under normal circumstances, the endothelium does not contain pre-existing passageways for cells to access the basement membrane (Evans, 1991a), and therefore migratory cells should not have contact with sticky components of the basement membrane. During events such as wound healing, inflammation and tissue remodelling, the matrix of the basement membrane may be exposed to migratory tumour cells (if a tumour cell reaches it prior to platelets), which could allow adhesion between tumour cells and adhesion molecules of the subendothelium. Following adherence of the tumour cells to the endothelial cells of the vasculature, they may exit from the bloodstream via extravasation, which may be a destructive or non-destructive process (Evans, 1991a). The tumour cells may divide and increase in volume in the lumen, causing damage to the vessel, or they may exit following extravasating leukocytes. Alternatively, tumour cells may emigrate through pores or gaps between adjacent endothelial cells (Evans, 1991a). In all instances of extravasation of malignant cells, a variety of mechanisms may be employed to

produce retraction of the endothelium and exposure of the sub-endothelial basement membrane. Invasion of the basement membrane and interstitial connective tissue matrix follows to afford the tumour cells access to the tissue parenchyma, where the mass may form a secondary tumour, and subsequently repeat the metastatic event (Blood and Zetter, 1990).

Invasion of the extracellular matrix (basement membrane and interstitial stroma) involves the degradation of the components of these structures. The basement membrane is an acellular, sheet-like structure composed of three layers:

i. The *lamina lucida* (10-50 nm thick), immediately external to the basal plasma membrane of the overlying cells, and occasionally traversed by filaments joining the underlying *lamina densa* and the overlying cells.

ii. The *lamina densa* (20-300 nm thick), an amorphous matrix crossed by fine filaments.

iii. The *lamina fibroreticularis*, a discontinuous matrix with fibrils connecting collagen to the *lamina densa*, in a reticulating and anchoring manner, strengthening the overall structure and anchoring the overlying cells (Evans, 1991a).

It functions as a support for overlying epithelial and endothelial cells, aiding in the establishment of cellular polarity, and as a barrier to filtration and invasion (Evans, 1991a). Furthermore, it separates organ cells, epithelia and endothelia from the interstitial stroma. It is composed of a number of basement membrane-specific adhesive molecules such as laminin, fibronectin, proteoglycans (heparan- and chondroitin sulfate), enactin and nidogen, the amounts of which vary depending on the tissue source, embedded in a network of type IV collagen (Laurie *et al.*, 1982). The interstitial stroma is composed of a variety of cells, such as chondrocytes, osteoclasts, fibroblasts or macrophages (depending on the tissue type) embedded in a matrix of collagen fibres (types I, III and a minor type VI), proteoglycans and glycoproteins. Type V collagen fibres facilitate anchorage to the basement membrane (Tryggvason *et al.*, 1987). A schematic representation of the extracellular matrix is presented in Fig. 1.1.

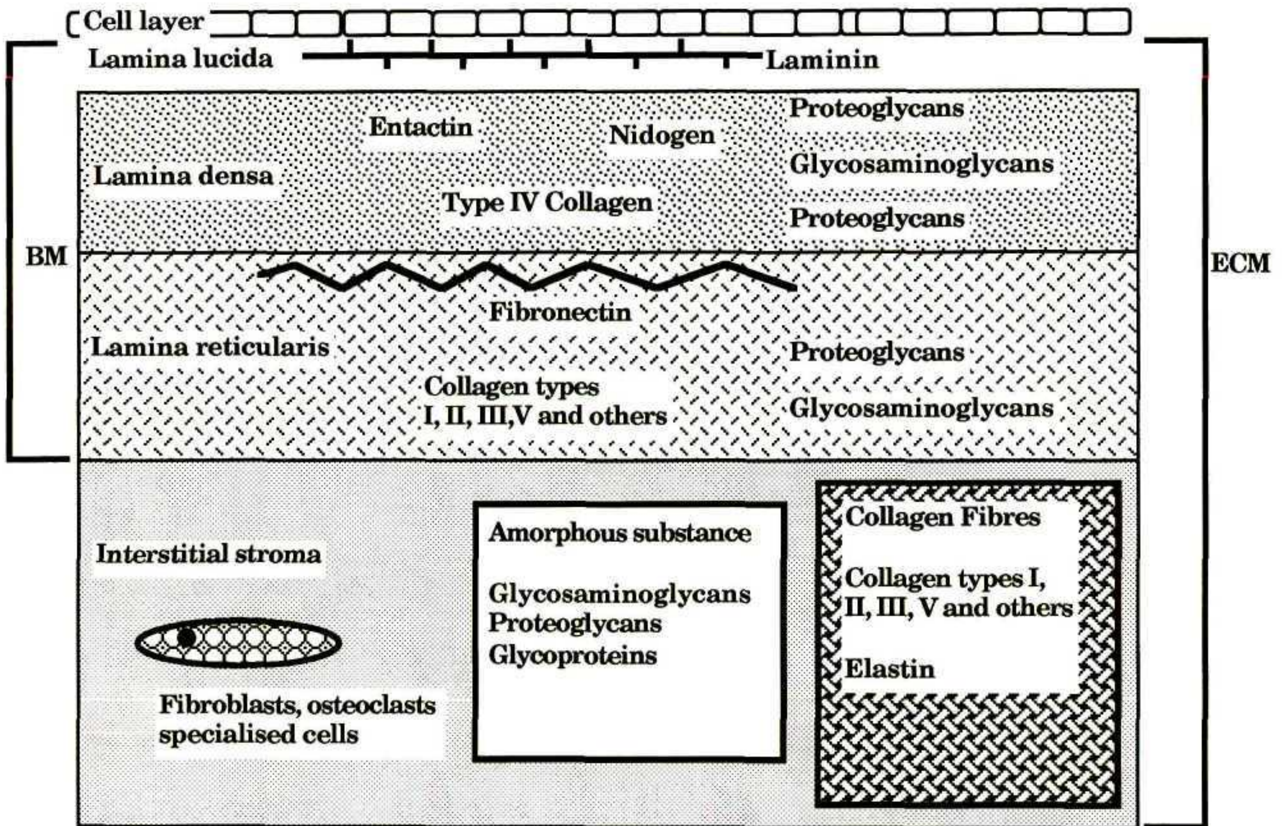


Figure 1.1. A schematic representation of the components of the extracellular matrix (not to scale) (adapted from Alexander and Werb, 1989).

1.2 The proteolytic enzymes

It is now acknowledged that tumour cells produce a plethora of enzymes capable of degrading the components of the extracellular matrix, and that this degradation is a hallmark of the metastatic process. Proteolysis must occur in a directed and controlled manner, otherwise degradative enzymes would destroy the matrix components necessary for traction (invasion involves a cyclic attachment to and release from matrix components in order for the metastatic cell to move forward). This behaviour is not unique to invading cancer cells, and at certain times, normal cells must invade normal tissues of the body. For example, circulating white blood cells invade out of the vasculature to reach sites of infection, and invasion also occurs during embryonic organ development. In such cases, the invasive mechanism employed by normal cells is likely to be the same as that employed by metastatic cells, but the fundamental difference is that normal cells arrest their behaviour when

the stimulus is removed. Metastatic cells migrate and invade relentlessly, at times and places that are inappropriate for normal cells (Liotta *et al.*, 1991).

Invasion of the ECM by tumour cells may be facilitated by matrix degrading enzymes secreted by the tumour cells (Tryggvason *et al.*, 1987), of which the proteolytic enzymes are of particular relevance. Table 1.1 shows the major classes of proteinases and their pH optima.

Table 1.1. The four classes of proteinases and their distinguishing characteristics (adapted from Barrett, 1980; Neurath, 1989; Evans, 1991a).

Type	Example	Characteristic active site residues	pH range
Aspartic	Cathepsin D, pepsin, renin	Asp-33, Asp-213	2-7
Serine	Chymotrypsin, plasmin, plasminogen activator, elastase, thrombin, cathepsin G, trypsin	Asp-102, Ser-195, His-57	7-9
Cysteine	Papain, cathepsins B, H, L & S	Cys-25, His-159, (Asp-158)	3-8
Metallo	Thermolysin, collagenases	Zn, Glu-270, Trp-248	7-9

Although a diverse selection of proteinases may contribute to the process of invasion, owing to the complexity of the ECM, those likely to be principally involved are the collagenases (of which there are specific types corresponding to the collagen types) (Liotta *et al.*, 1980), the cysteine proteinases such as cathepsin B (Sloane *et al.*, 1986) and the serine protease, plasminogen activator (Ossowski and Reich, 1983). However, in addition, a number of proteases and glycosidases, produced by tumour cells, are likely to selectively contribute to the destruction and invasion of the ECM.

1.2.1 The metalloproteases

In order to destroy the basement membranes and tissue stroma, the network of collagen must be degraded. This is accomplished by the family of zinc metalloproteases (matrix metalloproteases or MMPs). These are grouped into three broad categories depending on their substrate specificity [which consequently affords them a causal role in the destruction of the ECM (Matrisian, 1992)]: the type IV collagenases (gelatinases), interstitial collagenases (type I, II and III collagenases) and the stromelysins (Stetler-Stevenson *et al.*, 1993). The interstitial collagenases

degrade interstitial collagens, by a specific cleavage that denatures the helical structure of the collagens. The type IV collagenases degrade the basement membrane collagens and have strong activity against gelatin (denatured collagens). The third class of metalloproteases, the stromelysins, presently comprises four members. Their natural substrates appear to be glycoproteins and proteoglycans, although many degrade fibronectin, laminin, gelatins, the collagens and at least the globular form (non-collagenous) of type IV collagen (McDonnell *et al.*, 1994; Rucklidge *et al.*, 1994). The metalloproteases are enzymically active at pH values around neutrality, and are primarily secreted proteins. More recently, some have been shown to be membrane bound (Sato and Seiki, 1996). The MMPs are secreted as inactive zymogens and their activation is an important control step, postulated to occur by the "cysteine switch" mechanism (Sato and Seiki, 1996). This may occur by autoactivation, or by proteolysis by plasmin, cell surface associated serine proteases, or in some instances by membrane bound MMPs (Mignatti and Rifkin, 1993; Sato and Seiki, 1996). Cathepsins B and L are also thought to be able to activate the procollagenases (Eeckhout and Vaes, 1977; Yagel *et al.*, 1989; Sloane, 1990). There is an increasing body of evidence that shows a positive correlation between expression of MMPs (especially type IV collagenase) and metastatic potential of tumour cells (Liotta and Stetler-Stevenson, 1991).

It has been shown that the modulation of MMP activity, by monoclonal antibodies, reduces the invasive potential of human melanoma cells *in vitro* (Höyhtyä *et al.*, 1990). Tumour cell growth has also been shown to be reduced in murine melanoma cells (B16-F10) which overexpress the endogenous inhibitor of MMPs (TIMP-1) (Koop *et al.*, 1994), and addition of exogenous TIMP-1 to B16-BL6 (a murine melanoma variant) decreased the ability of the cells to invade through amnion membranes *in vitro* (Mignatti *et al.*, 1986). It would appear that the activity of MMPs is regulated by the presence of these tissue inhibitors, leading to speculation that the activity of the MMPs in tumour cells does not depend solely on their secretion and activation, but also on the balance between their activated forms and the endogenous TIMPS (Liotta and Stetler-Stevenson, 1991).

1.2.2 Urokinase type plasminogen activator

Although it has been suggested that the initial degradation of the ECM is facilitated by the MMPs (Liotta *et al.*, 1991), it is likely to be a complex cascade of events, in which the other classes of proteases play a vital role, owing to their ability to degrade several additional components of the ECM. A likely candidate is the serine protease, urokinase-plasminogen activator (uPA) as elevated levels of uPA have been determined in almost all cancer tissues (Chuchulowski *et al.*, 1992) and which correlates with malignancy (Schmitt *et al.*, 1992). uPA converts plasminogen to plasmin, which has broad trypsin-like specificity, hydrolysing proteins and peptides at arginine and lysine residues. It is also secreted as an inactive pro-form (pro-uPA) by normal and tumour cells, and exerts its proteolytic function after it is bound to a specific cell surface receptor (uPA-R). Pro-uPA can be converted to active uPA (HMW-uPA) by plasmin, plasma kallikrein, trypsin, thermolysin, cathepsin B and cathepsin L (Schmitt *et al.*, 1992) and can be inhibited by the plasminogen activator inhibitors (PAI-1 and PAI-2) (Cubellis *et al.*, 1989). Plasmin is of clinical significance as it can be active *in vivo* at neutral pH, contributing to the degradation of the components of the ECM, and the conversion of pro-collagenases to collagenases (Schmitt *et al.*, 1992). Research has indicated that in breast cancer, uPA may be an independent prognostic factor for early reoccurrence and shorter overall survival times (Chuchulowski *et al.*, 1992).

1.2.3 The cathepsins

There exists an association between levels and activities of lysosomal cathepsins and invasion.

Cathepsin D. Cathepsin D is an aspartic protease, active at acidic pHs, although this varies according to the structure of the substrate. It is overexpressed in most primary breast cancers, and is widely distributed in a number of lymphomas and carcinomas (Reid *et al.*, 1986). The measurement of cathepsin D levels in tissue has been used as a valuable prognostic marker for breast cancer and endometrial adenocarcinoma (Nazeer *et al.*, 1992), although conflicting reports exist concerning its accuracy (Duffy, 1996). The most compelling evidence for its involvement in invasion and metastasis comes from the (*in vitro*) work done by Westley and Rochefort (1980) who showed that the human oestrogen responsive metastatic breast

cell line (MCF-7), when treated with oestrogen, secreted a 52 kDa glycoprotein (procathepsin D) into the medium. Cathepsin D is synthesised as a preproenzyme which is cleaved cotranslationally to yield the pro-form (52 kDa), which in turn is activated by cleavage into single and two-chain forms (Huang *et al.*, 1979). The major role of cathepsin D appears to be in metastasis as overexpression is correlated with increased metastatic ability of tumour cells (Spyratos *et al.*, 1989; Rochefort, 1990). Recent work (Liaudet *et al.*, 1994) has shown, however, that it is the catalytic activity of cathepsin D which is required in the metastatic process, and that overexpression of the pro-form (52 kDa) of cathepsin D does not increase metastasis if it is not processed, and is retained in the ER.

Cathepsin D has been shown to be inactive under physiological conditions (C. Dennison and P. H. Fortgens, pers. comm.) which suggest that its role in tumour metastasis and progression may not be direct proteolysis of the ECM. In support of this, procathepsin D has been shown to act as an autocrine mitogen, stimulating cell proliferation (Vetvicka *et al.*, 1994). Antibodies to the propeptide of cathepsin D inhibited cell proliferation, and although the mechanism of this mitogenic activity is unknown, it has been suggested that procathepsin D activates latent forms of growth factors, by intra- or extracellular interaction.

Cathepsin B. Cathepsin B, a cysteine proteinase, has also been principally implicated in the ECM degradation associated with the malignant phenotype of tumours of the bladder, brain, breast, lung, pancreas, prostate, stomach and thyroid (Sloane *et al.*, 1986; Elliott and Sloane, 1996). It has been shown to degrade collagens and proteoglycans at acid pH (Morrison *et al.*, 1973). Recently, Buck *et al.* (1992) showed that human cathepsin B from normal and tumour tissue, degraded type IV collagen, fibronectin and laminin at acid and neutral pHs. This evidence supports the hypothesis of cathepsin B playing a role in the focal dissolution of ECM components. Cathepsin B is also thought to play a role in the processing of class II MHC-restricted antigens (Reyes *et al.*, 1991). Although it is active under acidic conditions (Barrett and Kirschke, 1981) a high M_r alkali-stable form has been reported (Buttle *et al.*, 1991) and identified as a non-covalent complex between cathepsin B and its pro-peptide (Mach *et al.*, 1994 a and b). Historically, cathepsin B is considered to be a lysosomal enzyme, although it has been shown to be secreted under normal physiological conditions [e.g. in sputum (Buttle *et al.*, 1991)] and pathologies such as breast

carcinomas (Gabrijelcic *et al.*, 1992). Surface-bound cathepsin B has been reported in H-*ras*-transformed human breast epithelial cells (Sloane *et al.*, 1994) and in murine melanoma (B16 variants) (Sloane *et al.*, 1986; Rozhin *et al.*, 1987), with the latter having a slightly higher pH optimum and resistance to inactivation by cysteine protease inhibitors.

The nucleotide sequence of cathepsin B indicates that the protein is synthesised in the prepro-form (37 kDa), and converted to the pro-form (35 kDa), which is activated by cleavage into the mature single chain form (28 kDa) or two-chain form (22 kDa and 5 kDa) (Sloane *et al.*, 1990). In addition, mRNA transcripts for cathepsin B suggest the existence of two truncated forms of the proenzyme, lacking the pre and part of the pro sequences. The truncated forms are believed to be cytoplasmic due to the absence of a signal peptide (Elliott and Sloane, 1996). Cathepsin B (as with all lysosomal proteins) is normally synthesised on membrane bound ribosomes and transferred co-translationally into the ER (where it is glycosylated), and transported to the Golgi (where it is further modified and phosphorylated). Trafficking from the Golgi to the lysosome occurs via the mannose-6-phosphate receptor (MPR) dependent pathway (Kornfeld and Mellman, 1989).

A large body of literature suggests that alterations in the expression, post-translational proteolytic processing, glycosylation and phosphorylation could contribute to the altered membrane association and secretion evident in malignant phenotypes (reviewed by Sloane *et al.*, 1994; Pagano *et al.*, 1989). In the case of recombinant cathepsin B, activation has been shown to be autocatalytic, especially at acidic pH (Rowan *et al.*, 1992). It therefore follows that any event which alters normal trafficking, processing or acidity could contribute to the formation of incompletely processed forms of the enzyme (Sloane *et al.*, 1994). Interestingly, Nishimura and co-workers (1988a) showed that pepstatin (an inhibitor of aspartic proteases) prevented the conversion of procathepsin B to the mature single chain form, while Hara *et al.* (1988) showed that inhibitors of metalloproteases inhibited this step. These findings suggest that precursor forms of cathepsin B released from tumours may be processed intracellularly by cathepsin D, or by metalloproteinases which are secreted extracellularly (Sloane *et al.*, 1990) presenting a manner in which the cysteine protease may be activated external to the cell. Taken together, these results suggest a multi-pronged proteolytic cascade that contributes to the activation,

secretion and expression of ECM degrading enzymes. Indeed, cathepsin B has been shown to activate both the soluble and receptor bound forms of pro-uPA (Kobayashi *et al.*, 1993) as well as the procollagenases (Eeckhout and Vaes, 1977) (see Fig. 1.2).

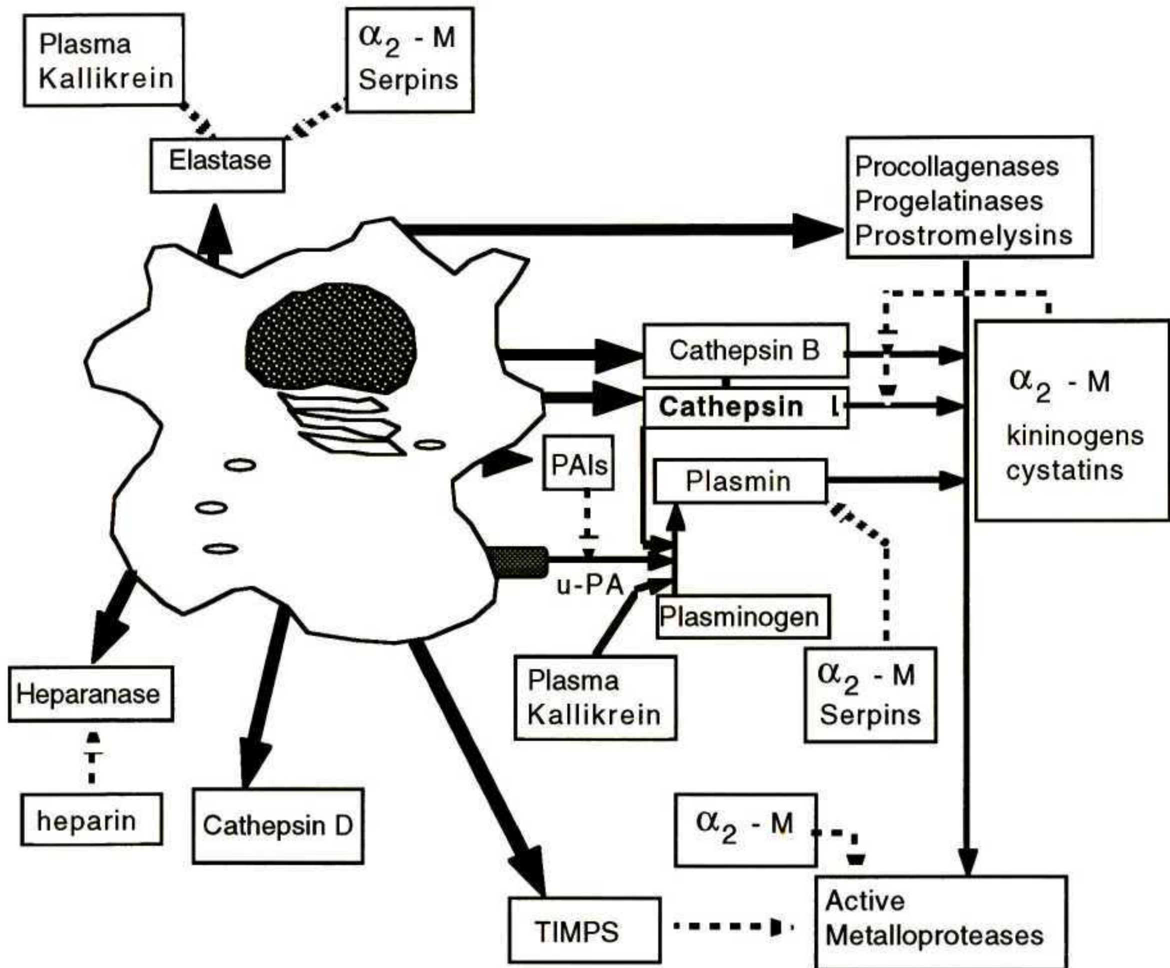


Figure 1.2. The possible roles of tumour-associated degradative enzymes, plasma proteinases and their inhibitors, in the proteolytic cascade.

Bold lines represent secretion, normal lines represent activation of pro-enzymes and broken lines represent inhibition (adapted from Nakajima *et al.*, 1987; Alexander and Werb, 1989).

Cathepsin L. The cysteine protease cathepsin L is the primary subject of the studies reported here. It was first isolated from rat liver lysosomes (Kirschke *et al.*, 1977), and subsequently from numerous sources, including human liver (Mason *et al.*, 1985), rat kidney (Bando *et al.*, 1986) rabbit liver (Mason *et al.*, 1984) and sheep liver

(Pike and Dennison, 1989b). Under normal cellular conditions, cathepsin L is now known to be synthesised as a 334 amino acid (39 kDa) precursor containing a 17 amino acid signal sequence, followed by the 96 amino acid propeptide (Erickson, 1989). The signal sequence allows entry into the endoplasmic reticulum, after which it is cleaved, and the protein undergoes N-linked glycosylation. A high mannose oligosaccharide is phosphorylated, affording a recognition signal for transport to the lysosomes. The pro-region appears to be necessary for the correct phosphorylation of the oligosaccharide, the specific determinant being a three-dimensional arrangement of lysine residues on the surface of the pro-sequence (Cuozzo and Sahagian, 1993). It has also been shown that the pro-region is required for proper folding of cathepsin L, as well as stability of the protein and exit from the endoplasmic reticulum (Tao *et al.*, 1994). The protein is further processed in the Golgi, by the removal of the terminal GlcNAc moieties, thereby allowing the mannose-6-phosphate receptors to bind the phosphorylated enzymes and transport them to the lysosomes. Once in acidic compartments the propeptide is cleaved, and the protein is activated (Cuozzo *et al.*, 1995).

Historically, the implication of cathepsin L in tumour invasion, comes from work conducted in the late 1970s, whereby the major excreted protein (MEP), found in large amounts of the medium of RNA virus transformed fibroblasts, was identified as a precursor of cathepsin L (Gottesman, 1978; Portnoy *et al.*, 1986; Mason *et al.*, 1987). This proform (39 kDa) was apparently not active (Gal and Gottesman, 1986a), but was shown to be autoconverted into lower molecular weight forms of 35 and 30 kDa, which had identical activity to cathepsin L. This autolysis occurred at pH 3 and not above pH 5, suggesting that the N-terminal peptide on procathepsin L is regulatory and affects the pH stability and activity of the enzyme (Mason *et al.*, 1986). In non-transformed cells, the synthesis and secretion of procathepsin L was shown to be increased by treatment of the cells with tumour promoters, growth promoters, platelet derived growth factors and fibroblast growth factors (Gottesman and Sobel, 1980; Nilsen-Hamilton *et al.*, 1981).

Although procathepsin L contains the mannose-6-phosphate carbohydrate moiety required for binding to the MPR (which trafficks such proteins to the lysosomes), most of the 39 kDa protein is secreted, while the 20 and 29 kDa forms remain in the lysosome (Gal and Gottesman, 1986b). This secretion of the proform provides a

vehicle for the expression of stable (and activatible) cathepsin L by tumour cells. It has been suggested that this aberrant trafficking of procathepsin L could be as a result of redistribution of the MPR to the cell surface (Achkar *et al.*, 1990), or as a result of saturated MPRs because of the high level of procathepsin L synthesis in transformed cells (Dong and Sahagian, 1990; Lazzarino and Gabel, 1990).

Cathepsin L has been identified in numerous human tumours; adrenal gland, bladder, breast, colon, kidney, lung, ovary, prostate, stomach, testis and thyroid (Chauhan *et al.*, 1991; Lah *et al.*, 1992a and b), the pancreatic cell line HPC-YC (Yamaguchi *et al.*, 1990) and pre-malignant and malignant colorectal cell lines (Maciewicz *et al.*, 1989) as well as metastatic murine melanoma (B16) cell lines (Qian *et al.*, 1989; Rozhin *et al.*, 1989) where it was localised to the cell surface. The invasion of an "*in vitro* basement membrane", the amnion membrane, by murine mammary carcinoma cells (C3-L1 and SP1neo5) is inhibited by the cysteine proteinase inhibitor Z-Phe-Phe-CHN_2 (specific for cathepsins B and L), albeit not to the same extent as by the MMP inhibitors (Yagel *et al.*, 1989). Hybridoma cells, formed by the fusion of murine myeloma cells (known to secrete an active precursor form of cathepsin L), with spleen cells secreting the antibody to cathepsin L showed a reduction in potential for forming solid tumours upon injection into mice (Weber *et al.*, 1994). These findings have led to the evaluation of the prognostic value of cathepsin L (and cathepsins B, D and H) in neck and head carcinomas (Budihna *et al.*, 1996) and breast cancer (Castiglioni *et al.*, 1994).

Cathepsin L is a likely candidate in metastasis owing to its ability to degrade components of the ECM, more effectively than the other cathepsins (Maciewicz *et al.*, 1987). It shows a strikingly higher specificity for physiological protein substrates than do other lysosomal proteases (Barrett and Kirschke, 1981). Kirschke and co-workers (1982) showed that under acidic conditions, rat liver cathepsin L hydrolysed the terminal non-helical peptides of type I collagen, destroying the crosslinks in the collagen molecules. This was confirmed by Maciewicz and Etherington (1988), who showed that rabbit cathepsin L cleaved type I collagen with a high specific activity. Subsequently (Maciewicz *et al.*, 1990), it was demonstrated that human cathepsin L could degrade types II, IX and XI collagens, at acidic and neutral pHs. Cathepsin L apparently cleaves these collagens in the non-helical domains of the teleopeptide regions, a mode of catalysis different from that of the metalloproteinases. The ability

of cathepsin L to degrade the glomerular basement membrane of kidney (composed primarily of laminin, fibronectin, proteoglycan and type IV collagen) at low pH values has also been shown (Baricos *et al.*, 1988). Cathepsin L was demonstrated to generate a matrix degrading proteolytic system from fibronectin at pH 6.5 (Blondeau *et al.*, 1993) and was shown to degrade type IV collagen, laminin and fibronectin at pH 6.8 (Guinec *et al.*, 1993). Clearly these results show that cathepsin L possesses the catalytic ability to degrade the components of the ECM, *in vitro*, under slightly acidic conditions.

For this catalytic ability to be implicated in pathological conditions, it would be necessary to show cathepsin L activity under physiological conditions as encountered in the extracellular milieu. Cathepsin L shows strong endopeptidase activity (Barrett and Kirschke, 1981) even at neutral pH, although its stability under these conditions is reportedly low (Mason *et al.*, 1985; 1986). However, it has been shown that the half-life (representing the stability) of cathepsin L is greater than previously expected (Dennison *et al.*, 1992). Extracellular conditions differ in pH and ionic strength to those found in the lysosomes, where a low pH environment appears to be necessary for the hydrolysis of internalised components of the ECM.

A number of mechanisms appear to exist whereby the cathepsins could retain their activity extracellularly. The first such mechanism was proposed following the observation of Silver *et al.* (1988) and Baron *et al.* (1985) that, during bone resorption, the region between the osteoclast and the bone matrix becomes acidified by the action of proton pumping ATPases. Lysosomal proteases are secreted into this "extracellular lysosome", where they actively participate in bone resorption. Furthermore, macrophages have been shown to secrete cathepsin L, which is active under the conditions of acidity produced by the macrophages (Reddy *et al.*, 1995), which rapidly acidify the cell-elastin interface using a vacuolar-type H^+-ATPase. It is known that within tumours, the rapidly mitosing cells acidify the medium (Boyer and Tannock, 1992), creating an acidic microenvironment where proteases may be active at the junction between the host and tumour cells. By this mechanism, the secreted lysosomal proteases may be responsible for ECM degradation external to the cell. What is required, then is the acidification of the microenvironment sufficient to autoactivate the procathepsins (Rozhin *et al.*, 1994). It has been shown that a decreased pericellular pH, or increased intracellular pH, is responsible for the

peripheral translocation of lysosomes in macrophages (Heuser, 1989) and in B16a and B16-F1 cells, the redistribution of these vesicles to the periphery was accompanied by the secretion of active (mature) cathepsin B (Rozhin *et al.*, 1994). Cathepsin L has been identified in the resorption lacunae, created at the ruffled border of osteoclasts during the degradation of bone matrix (Kakegawa *et al.*, 1993; Tagami *et al.*, 1994). In either instance, a plausible explanation exists for the activity of lysosomal proteases external to the cell.

The second mechanism whereby lysosomal proteases may be active extracellularly arises if the proteases are presented at the plasma membrane (as discussed previously with cathepsin B). Evidence supporting this was provided by Maciewicz *et al.* (1989), who immunolocalised cathepsin L to the plasma membrane in two epithelium-derived tumour cell lines. Rozhin *et al.* (1989) also showed the presence of cathepsin L in plasma membrane fractions isolated from B16a melanoma and tumour tissue, by sub-cellular fractionation of the tissue followed by enzyme assays. These results suggest that cathepsin L could play a role in the focal dissolution of the basement membrane during metastasis. The association of lysosomal enzymes with the plasma membrane may serve a number of purposes, such as,

i. the correct orientation of the enzyme, ensuring that they can be readily activated,
ii. concentration of the enzymes at the cell surface, increasing the local activity, and ensuring that activity is directed to the components immediately adjacent to the cell, and,
iii. presenting a way in which the enzymes may be protected from inhibitors (Moscatelli and Rifkin, 1988; Rozhin *et al.*, 1989).

It has been suggested that proteases may be associated with the membrane by one of two mechanisms:

i. They may be secreted as soluble proteins which associate with the membrane by a receptor (such as u-PA, as discussed previously). Procathepsin L and D were shown to bind acidic microsomal vesicles by a "protein-mediated" interaction which could be disrupted by incubation of microsomes with trypsin, suggesting the existence of a proteinaceous lysosomal proenzyme receptor (LPR) which could play a role in the sorting of these enzymes to the lysosomes and to the cell surface (McIntyre and Erickson, 1991). This putative lysosomal proenzyme

receptor binds the proenzymes (and note, not mature enzymes) under acidic conditions (pH 5.0 in the prelysosomal compartments) and it is postulated that the receptor-protein complex is transported to the endosomes where they are released from the receptors at slightly elevated pH's (pH 6). The physiological importance of this is not known as the precursor form of cathepsin L is not enzymatically active in endosomes of KNIH cells (Mason *et al.*, 1989). Although these workers admit that the microsomal pellets contain plasma membrane vesicles, they speculate that association of the proenzyme with the plasma membrane is unlikely as binding only arises under acidic conditions. However, the previously documented evidence for acidic microenvironments associated with the invasive front of tumour cells offers the ideal convenience for the association of the proenzyme form with the membrane (localised specifically to where it is required in high concentrations), which could be autocatalytically cleaved to release active enzyme. In bone metastatic tissue, a unique method of membrane association via receptors has been reported for mature cathepsin B, where secreted cathepsin B is entrapped by α_2-macroglobulin, and the resultant complex is bound to an endocytic receptor, the LDL-receptor related protein (LRP) on the plasma membrane (Arkona and Wiederanders, 1996).

ii. They may be integral membrane proteins that intercalate directly into the membrane (Kelly *et al.*, 1994), as suggested for cathepsin B which was shown to be localised to the membrane by mechanisms disruptable by solvents only (Rozhin *et al.*, 1987).

A third possibility for the activity of lysosomal enzymes in the extracellular matrix, is that the enzymes are, in fact, stable and sufficiently active under these conditions. This has been shown to be true for cathepsin B (Buck *et al.*, 1992), where cathepsin B degraded components of the extracellular matrix (including type IV collagen) at acidic and neutral pHs, and that autodegradation was reduced in the presence of additional extracellular proteins. Although Turk *et al.* (1994) showed cathepsin B to be irreversibly inactivated at high pH, they showed the half life of cathepsin B at pH 7 and 37°C to be 1281 s (*ca.* 20 min), a sufficiently long time to contribute to substantial proteolysis. More recently we have shown that under reducing conditions, cathepsins B and L are more stable at neutral pHs than previously believed, and cathepsin B is additionally stabilised by the presence of proteins

(Dehrmann *et al.*, 1996). Mature cathepsin L has similarly been shown to be active under extracellular conditions (Dehrmann *et al.*, 1995).

In any event, it is obvious that neoplastic cells possess the ability to redistribute a number of normally intracellular, matrix degrading enzymes to the exterior of the cell, and have devised mechanisms whereby the enzymes are or become active in a localised region to facilitate the destruction of barriers which impede the motility of metastatic cells. Under normal conditions (as with the MMPs and uPA), the cysteine proteases are subject to strict regulation by their intra- and extracellular inhibitors, the cystatins and α_2-macroglobulin (Mignatti and Rifkin, 1993; Barrett *et al.*, 1986), reinforcing the concept that invasion may be facilitated by an imbalance between proteases and their inhibitors (Liotta and Stetler-Stevenson, 1991).

1.2.4 The cystatins

The cystatins are a family of functionally related proteins, which are divided into three sub-classes:

i. The type I cystatins, which are approximately 100 amino acids in length, and lack intra-molecular disulfide bridges and carbohydrates. These include the human cystatins A and B (also known as stefins), and their mammalian counterparts.

ii. The type II cystatins, a group of single-chain polypeptides of 120 - 122 amino acids, with two intra-molecular disulfide bridges, lacking carbohydrates. Human cystatin C, S, SN, SA, D, and animal counterparts, including chicken egg-white cystatin are included in this group.

iii. The kininogens, which are a class of a high molecular weight plasma glycoproteins (60 - 120 kDa), showing inhibitory activity towards cysteine proteases, and having sequence homology to the type II cystatins (containing three copies of the type II cystatins) (Abrahamson, 1993).

The type I cystatins mainly occur intracellularly [although they have been isolated extracellularly (Mignatti and Rifkin, 1993)], while the type II molecules are found in secreted fluids. The kininogens are restricted to the plasma and synovial fluids. Some evidence implicates the cystatins in diseased tissue. Järvinen *et al.* (1987), showed that in malignant keratinocytes (and other carcinomas) staining for cystatin

A and B was visible in the differentiated cells, but was not visible in the outermost non-differentiated tumour cells. Keppler *et al.* (1994) report the expression of cystatin C (a type II cystatin and a powerful inhibitor of cathepsin B) in a number of cancerous cell lines, while Sloane *et al.* (1990) reported a reduction in the inhibitory activity of tumour cystatin (stefin) A towards cathepsin B. These and other findings allude to the possibility that altered cystatin expression, and/or affinity for cysteine proteases could result in increased protease activity, affording enhanced degradation of the ECM, especially as cystatins have been shown to arise where cysteine proteases are implicated.

The mechanism of inhibition of cystatins towards cysteine proteases is well documented, and will be described in detail in Chapter 4. Generally the cystatins display broad specificity and high affinity for the proteases, competing with the substrate for binding to the active site cleft in a reversible manner. In this way, the active site is blocked and the cystatin prevents proteolytic cleavage. Cystatins react with cysteine proteases in a 1:1 stoichiometry. In addition to the usual inhibitory association between these molecules, cathepsin L (from ovine and human liver) has been shown to form a covalent, non-inhibitory complex with a type I cystatin, stefin B (Pike *et al.*, 1992). This finding suggests a mechanism whereby cathepsin L released from tumour cells may be active in the presence of its normal inhibitor. The nature of the association between cathepsin L and stefin B appears to be a disulfide bond, as the complex is dissociated during reducing SDS-PAGE. The nature and occurrence of this complex is revisited in Chapters 3 and 4.

It would therefore appear that the balance between proteases and their inhibitors, rather than overexpression of proteases *per se*, is the key factor in tumour metastasis. This study has included an in-depth investigation into the activity of cathepsin L, under conditions expected to arise in the extracellular environment. The novel non-inhibitory interaction, between cathepsin L and its endogenous inhibitor, stefin B was investigated. Polyclonal antibodies were raised to cathepsin L and the complex formed between stefin B and cathepsin L. These were characterised and used to investigate the occurrence of cathepsin L and stefin B in a metastatic murine melanoma cell line, and a normal murine fibroblast cell line.

CHAPTER 2

MATERIALS AND METHODS

In this chapter methods are described that are considered fundamental in biochemical terms and were used routinely throughout this study. It also contains methods, especially immunological methods, and enzyme assays which could be considered relatively specialised but would hinder the intended structure of the relevant chapter. Methods and reagents that are considered more specialised will be described in the apposite chapters.

2.1 Materials

For convenience, the source of all specialised products used in this study is detailed here. Most common chemicals used were from Boehringer Mannheim, BDH or Merck, and were of the highest purity available (AR grade). Serva Blue G was from Serva; ovalbumin (Grade V), 4-chloro-1-napthol, azocasein, S-Sepharose, Sephadex G-25, 7-amino-4-methyl-coumarin and standard electrophoretic proteins were from Sigma Chemical Co., St. Louis, MO. Coomassie brilliant blue R-250 was from Merck; acrylamide and N,N'-methylenebisacrylamide were from BDH; N,N,N',N'-tetramethyl-ethylenediamine (TEMED) was from Bio-Rad; BSA (Fraction V) and 2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid (ABTS) were from Boehringer Mannheim, SA; Elite fat-free milk powder was from NCD; horseradish peroxidase (HRPO) was from Seravac, Nunc-Immuno Maxisorp F96 plates and Fluoronunc plates were supplied by Weil Organisation, S.A. Z-Phe-Arg-NHMec was from Cambridge Research Chemicals, UK. Freund's complete and incomplete adjuvants and gelatin were from Difco, MI, USA. Sheep anti-rabbit IgG was from NII, Pinetown, South Africa, rabbit anti-chicken IgY was from Dr T.H.T. Coetzer, Biochemistry Department, University of Natal, PMB, South Africa. In all instances, reagents were diluted with dist.H_2O except, where specified, Milli-Q Ultrapure water was used. Buffers were made according to the method of Dennison (1988).

2.2 Protein assays

The efficacy of protein purification procedures is determined by quantifying the isolated proteins. It follows that methods of protein quantification which are rapid, specific and yet sensitive are crucial in protein isolation. Spectrophotometry using

specific protein extinction coefficients at 280 nm is convenient and non-destructive. It is, however subject to interference by non-protein compounds which absorb at 280 nm. On the other hand not all proteins absorb (strongly) at 280 nm. An assay that to a large extent overcomes such problems, is the Bradford dye-binding assay (Bradford, 1976).

2.2.1 Bradford dye-binding assay

The Bradford assay is based on the observation that Coomassie brilliant blue G-250 dye exists in two colour forms, red and blue, the red form predominates at low pH but converts to the blue form upon binding of the dye to protein (Compton and Jones, 1985). The high extinction coefficient of the blue protein-dye complex at 595 nm results in sensitive protein measurement. Due to its rapidity, the assay is suitable for processing large sample numbers. Colour development is essentially complete within 2 min of the addition of the dye reagent to the protein sample and remains stable for up to 1 h. The important advantage of the assay is the lack of interference by most chemicals tested. Small effects due to Tris, acetic acid, 2-mercaptoethanol, sucrose, glycerol, EDTA, and trace amounts of Triton X-100 and sodium dodecyl sulfate can be eliminated by using the appropriate buffer blank. Detergents at concentrations of 1% or greater, however, produce abnormalities too great to overcome.

The greatest disadvantage of the dye-binding assay is the wide variation of colour-yield in response to different standard proteins (Read and Northcote, 1981). This would affect the validity of measurements extrapolated from the standard protein. When determining the concentration of purified proteins, this problem is best overcome by using the pure protein as its own standard, although this is seldom feasible due to the wastefulness of such an approach. To reduce response variation, Read and Northcote (1981) modified the method by substituting Coomassie brilliant blue G-250 with Serva blue G, and by increasing the dye concentration or decreasing the phosphoric acid concentration. In the present study it was found, however, that modification of dye and phosphoric acid concentrations caused the dye to precipitate. The reagent finally employed in this study was the same as that of Bradford (1976), except that Coomassie brilliant blue G-250 was replaced by Serva blue G. Ovalbumin was generally used as a calibration protein, since its response to

dye binding was comparable to the average of three other standard proteins (Pike, 1990).

The only disadvantage of the reagent employed compared to that of Read and Northcote (1981), is evidence of non-linearity at protein levels beyond 25 μg, presumably due to dye depletion. This can easily be overcome by diluting protein samples to within the 5-25 μg working range of the assay. For the determination of lower protein concentrations (1-5 μg), a micro-assay procedure (Read and Northcote, 1981) was used.

2.2.1.1 Reagents

Dye reagent. Serva blue G (50 mg) was dissolved in 89% phosphoric acid (50 ml). Absolute ethanol (23.5 ml) was added and stirred for 1 h. The solution was made up to 500 ml with dist.H_2O, stirred for a further 30 min and filtered through Whatman No. 1 filter paper. The dye reagent can be stored in a brown bottle at room temperature for several months. If precipitation had occurred upon storage, the reagent was filtered and re-calibrated before use.

Standard protein solution. Ovalbumin was dissolved at 1 mg/ml in dist.H_2O. This was diluted to 100 μg/ml for the micro-assay.

2.2.1.2 Procedure

Macro-assay. Standard protein solution (0-25 μl), or sample protein, was diluted with dist.H_2O or buffer to a final volume of 100 μl to give the desired concentration levels (0-25 μg). Dye reagent (5 ml) was added to standard solutions, samples and blanks (100 μl), vortexed and allowed to stand for 2 min. The absorbance was read at 595 nm against the buffer blank, for sample, or water for the ovalbumin standard solutions. Assays for a standard curve were carried out in triplicate at five concentrations of ovalbumin. Plastic cuvettes (3 ml) were used as the dye-reagent binds to quartz (Bradford, 1976) although it can be easily removed with dilute sodium hypochlorite.

Micro-assay. Standard protein solution (0-50 μl of the 100 μg/ml solution, i.e. 1-5 μg) or sample was diluted with dist.H_2O or buffer to a final volume of 50 μl. Dye reagent (950 μl) was added to standard solutions, samples and blanks, vortexed and allowed

to stand for 2 min. The absorbance was read at 595 nm against the buffer blank, for sample, or water for the ovalbumin standard solutions, in 1 ml plastic micro-cuvettes as above. A standard curve was generated for each batch of reagent made up, and subjected to linear regression analysis from which protein concentrations were calculated.

2.2.2 Spectrophotometry using protein extinction coefficients

Spectrophotometry using extinction coefficients is a highly specific method for determination of the concentration of specific pure proteins, but is inherently impractical for general protein quantification. Extinction coefficients at 280 nm of 1.43 and 1.25 ml/mg/cm respectively (Hudson and Hay, 1980; Coetzer, 1985), were used to determine the concentrations of pure immunoglobulin G and Y (IgG and IgY).

2.2.2.1 Procedure

The absorbance of the relevant sample at 280 nm was read in a quartz cuvette against the appropriate blank and the concentration calculated as follows:

$$A = E\,c\,l \qquad (2.1)$$

where A = absorbance at 280 nm

E = absorbance of a 0.1% solution (i.e. 1 mg/ml) solution in a 1 cm cuvette pathlength

c = protein concentration in mg/ml.

l = light path length (cm)

2.3 Methods of protein concentration

During some procedures it was necessary to concentrate proteins for protein assays, or for further analysis such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.1 Dialysis against sucrose or PEG

Where concentration of large volumes of dilute protein solutions was required, dialysis against a substance, which has high osmotic pressure when in solution, such as sucrose or polyethylene glycol (PEG, M_r 20 000) was utilised. Sample

concentration is based on the fact that a gradient of water concentration is established between the protein solution within the dialysis membrane and the substance at the exterior surface of the membrane. Water and buffer ions will thus move out of the membrane, while proteins larger than the membrane molecular weight cut-off limit, are retained. The overall volume in which the protein is dissolved is thus reduced, and the process can be halted when the required degree of concentration has been reached.

Sucrose, which is inexpensive, was most often used but is small enough to diffuse into the dialysis tubing. When concentrated sucrose-free protein solutions were required, as is the case of protein assays where a corresponding reagent blank is required, dialysis against PEG (20 kDa) was employed. This has a molecular weight greater than the size cut-off of the dialysis membrane and is thus excluded.

2.3.2 SDS/KCl precipitation

This method is based on the precipitation of SDS-protein complexes by KCl, and is a rapid technique, especially suitable for the concentration of small volumes, resulting in an overall 5-fold concentration.

2.3.2.1 Reagents

5% (m/v) SDS. SDS (0.5 g) was dissolved in dist.H_2O (10 ml).

3M KCl. KCl (2.24 g) was dissolved in dist.H_2O (10 ml).

2.3.2.2 Procedure

Sample (100 µl) was diluted with 5% SDS (10 µl) in a 1.5 ml polyethylene tube. The solution was mixed by inversion, and 3 M KCl (10 µl) was added. The mixture was vortexed and centrifuged (12 000 x g, 2 min, RT), the supernatant was discarded and the precipitate dissolved in buffer (usually stacking gel buffer, Section 2.4).

2.4 SDS-PAGE

SDS-PAGE, under reducing and non-reducing conditions was used to examine the purity of samples, and their molecular weights were determined under reducing conditions. This technique was also used with Western blotting to analyse samples

and to monitor antibody specificity. The inclusion of gelatin in the gels enabled SDS-PAGE to be used to monitor the proteolytic activity of the samples.

Electrophoresis describes the migration of charged particles in an electric field, with polyacrylamide gel electrophoresis being the most widely used electrophoretic technique for the analysis of proteins. The Ornstein-Davis PAGE system employs two different buffering systems to generate a sharp interface between the charged particles in accordance with Kohlrausch's regulatory function. This results in proteins being concentrated into very thin starting bands. These are separated according to size, charge and shape, and the homogeneity of a preparation may therefore be analysed.

The use of the anionic detergent, SDS, in PAGE enables the molecular weights of proteins to be estimated (Shapiro *et al.*, 1967). Molecules of SDS bind to the protein at a constant ratio of 1.4 g to 1 g of protein to produce rod-like complexes, the lengths of which vary according to the molecular weights of the protein constituents. The overriding negative charge of the SDS results in the complexes carrying an overall negative charge, and all protein/SDS complexes have the same charge/mass ratio. In a gel system, with the introduction of an electric field, the complexes will run according to their protein molecular weights. The migration distances may be compared with the migration distances of proteins with known molecular weights and that of the unknown may be determined.

The inclusion of a thiol reducing agent (such as 2-mercaptoethanol) to the sample will break disulfide bonds. If the sample is boiled in the presence of excess SDS and a reducing agent, the protein will be dissociated into individual polypeptide subunits. The molecular weights of the subunits may be determined and the presence of disulfide bridges in proteins may be established by this technique.

As SDS-PAGE affords the separation of proteins according to their molecular weights, it may be used on a preparative scale to purify proteins according to their molecular weights, although it is not often used due to the difficulty of removing the SDS and the denaturation of the proteins.

During this investigation SDS-PAGE was conducted using the method of Ornstein (1964) and Davis (1964) with the modifications of Laemmli (1970), who introduced

SDS. Gels were routinely stained with Coomassie brilliant blue G-250 or silver-stained (Blum *et al.*, 1987). For preparative PAGE, gels were stained using a rapid stain method of de Moreno (1985).

For gels requiring a broader resolving range, the Tris-Tricine gel system (Schägger and Von Jagow, 1987) was used. In this system, the mobility of the protein relative to the trailing ion is decreased, and lower molecular weight proteins may be separated from SDS micelles. In this study, a gel system with two porosities was used for the resolution of proteins of 5 - 100 kDa.

2.4.1 Tris-glycine SDS-PAGE

2.4.1.1 Reagents

Solution A (30% (m/v) acrylamide, 2.7% (m/v) N,N'-methylene-bisacrylamide. Acrylamide (58.4 g) and N,N'-methylene-bisacrylamide (1.6 g) were dissolved and made up to 200 ml with dist.H_2O. The solution was filtered and stored in an amber bottle at 4°C.

Solution B 4 x Running gel buffer (1.5 M Tris-HCl, pH 8.8). Tris (36.3 g) was dissolved in about 150 ml dist.H_2O, titrated to pH 8.8 with HCl and made up to 200 ml.

Solution C 4 x Stacking gel buffer (500 mM Tris-HCl, pH 6.8). Tris (3.0 g) was dissolved in about 40 ml of deionised dist.H_2O, titrated to pH 6.8 with HCl and made up to 50 ml.

Solution D 10% (m/v) Sodium dodecyl sulfate. SDS (50 g) was dissolved in dist.H_2O with gentle stirring and mild heating, cooled and made up to 500 ml.

Solution E 10% (m/v) Ammonium persulfate. Ammonium persulfate (0.5 g) was dissolved in dist.H_2O and made up to 5 ml. Fresh reagent was made up every two weeks.

Solution F Tank buffer [25 mM Tris, 192 mM glycine, 0.1% (m/v) SDS, pH 8.3]. Tris base (12.0 g), glycine (57.6 g) and 10% SDS were dissolved in dist.H_2O and made up to 4 litres. The pH was 8.3.

Crosslinker solution (TEMED). TEMED was from BDH.

Treatment buffer [125 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol]. Solution C (2.5 ml), solution D (4 ml) and glycerol (2 ml) were made up to 10.0 ml with dist.H_2O. This was used for non-reducing SDS-PAGE. For reducing SDS-PAGE, 2-mercaptoethanol (1 ml) was added to the solution prior to it being made up to 10 ml.

Stain stock [1% (m/v) Coomassie brilliant blue R-250]. Coomassie brilliant blue R-250 (2.0 g) was dissolved in dist.H_2O overnight with stirring. The solution was made up to 200 ml and filtered through Whatman No. 1 filter paper.

Stain [0.125% (m/v) Coomassie brilliant blue R-250, 50% methanol, 10% acetic acid]. Stain stock (62.5 ml), methanol (250 ml) and acetic acid (50 ml) were made up to 500 ml with dist.H_2O.

Destaining solution 1 (50% methanol, 10 % acetic acid). Methanol (500 ml) and glacial acetic acid (100 ml) were made up to 1 litre with dist.H_2O.

Destaining solution 2 (5% methanol, 7% acetic acid). Methanol (50 ml) and glacial acetic acid (70 ml) were made up to 1 litre with dist.H_2O.

Molecular weight markers (1 mg/ml standard protein). Bovine serum albumin (1 mg), ovalbumin (1 mg), carbonic anhydrase (1 mg) and cytochrome C (1 mg) were dissolved in treatment buffer (1 ml) and bromophenol blue tracking dye [0.1% (m/v) in stacking gel buffer] (15 μl) was added.

Both the Hoefer Scientific SE 250 and SE 600 were used during these investigations. The amounts of each reagent required to cast two gels (1.5 mm) for each apparatus is shown in Table 2.1.

Table 2.1. Reagent volumes required to cast running and stacking gels for the SE 250 and SE 600 apparatus.

	SE 250 12.5% T		SE 250 10% T		SE 600 10% T	
Reagent	Running gel(ml)	Stacking gel (ml)	Running gel (ml)	Stacking gel (ml)	Running gel (ml)	Stacking gel (ml)
A	6.25	0.94	5	0.94	20	2.66
B	3.75		3.75		15	
C		1.75		1.75		5
D	0.15	0.07	0.15	0.07	0.60	0.2
E	0.075	0.035	0.075	0.035	0.30	0.10
TEMED	0.0075	0.018	0.0075	0.018	0.020	0.010
dist.H_2O	4.75	4.2	6	4.2	24.1	12.2
Volume	15	7	15	7	60	20

The pore size of the running gel may be varied to achieve optimum resolution between bands of similar molecular weights. For the purpose of this investigation, running gel concentrations of 12.5% T were suitable for monitoring the molecular weight range 12-75 kDa, while a pore size of 10% T was suitable for monitoring a slightly lower range.

2.4.1.2 Procedure

Both types of apparatus were assembled according to the manufacturers' instructions. The aluminium and glass plates were washed thoroughly with detergent and water and rinsed with ethanol. The aluminium plates were placed on the gasket of the pod apparatus. Two 1.5 mm spacers were greased and placed vertically on the outside edges of the plate. On top of this, the glass plates were aligned so that the bottom edges of the aluminium and glass plates as well as the spacers were level with the bottom of the pod. The apparatus was secured with the clamps provided. A molten agarose solution (0.1%) was poured on a glass plate in two lines which corresponded with the bottom of the assembled pod. The pod was placed on the molten agarose in a manner which allowed the agarose to be drawn between the glass and aluminium plates by capillary action. Once the agarose had set, it formed a plug which sealed the bottom of the pod. The running gel (prepared

according to Table 2.1) was poured into the sandwich, using a large gauge needle, to a level about 3 cm from the top of the glass plate. Care was taken not to trap any air bubbles in the gel during this procedure. The gel was overlayered with dist.H_2O to exclude oxygen, which would prevent the acrylamide from polymerising. Polymerisation normally took 1 h, and was visualised by the formation of a visible gel-water interface. The water was poured off the gel, and the stacking gel buffer was added up to the top of the aluminium plate. The gel was sealed by the insertion of 15 or 10 well comb, and left to polymerise (20 - 30 min). Once the polymerisation had taken place, the combs were removed and the wells washed with dist.H_2O. The pod was placed in the container and cold tank buffer was poured into the wells and into the cathodic and anodic compartments.

Protein samples were treated with treatment buffer (1:1) and reduced samples were boiled for 90 s in a boiling water bath. The treated samples were underlayered into the wells using a fine-tipped Hamilton syringe. The lid was placed on the pod and the pod was connected to a circulating water bath set at 5°C. The lid was connected to a power supply and the gels were run at a constant 18 mA per gel. When the bromophenol blue tracking dye had reached the bottom of the gel, the power was switched off, the pod disassembled and the gels placed in a staining solution for 4 h. Gels were destained with destaining solution 1 overnight, and placed in destaining solution 2 until the background had faded sufficiently to view the bands. Gels were photographed and stored in zipseal bags at room temperature.

The Hoefer SE 600 was assembled according to the manufacturers instructions. Two glass plates were separated by 1.5 mm spacers and sealed with two long plastic clamps. The plates were placed in the stand and clamped to seal the bottom edge against the rubber of the stand. The running gel mixture, prepared according to Table 2.1, was poured into the space between the glass plates, to a level about 4 cm from the top This was overlayered with water and left to polymerise overnight. The stacking gel was added to a level of 2 cm from the top, and overlayered with water. No combs were used as this gel was used for preparative purposes. The stacking gel took 40-50 min to set.

When the gel was set, the cathode compartment was clamped securely onto the glass plates. The apparatus was placed in the tank and tank buffer was poured into the

lower chamber, so that no bubbles were present. Sample was applied to the top of the stacking gel. Tank buffer was added carefully to the reservoir to avoid disturbing the sample. The lid was placed on the apparatus and the gels were run at a constant voltage of 80 mV. Gels were removed from the pods when the electrophoretic run was completed (visualised by the passing of the bromophenol blue from the bottom of the gel).

2.4.2 Tris-tricine SDS-PAGE

2.4.2.1 Reagents

Monomer solution [49.5% (m/v) acrylamide, 3% (m/v) N,N'-methylene-bisacrylamide]. Acrylamide (120 g) and N,N'-methylene-bisacrylamide (3.8 g) were dissolved in dist.H_2O and made up to 250 ml. The reagent was stored at RT in an amber bottle.

Gel Buffer [3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris (90.38 g) was dissolved in 200 ml of dist.H_2O, adjusted to pH 8.45 with HCl, and made up to 250 ml.

Anode buffer [0.2 M Tris-HCl, pH 8.9]. Tris (24.22 g) was dissolved in 950 ml of dist.H_2O, adjusted to pH 8.9 with HCl and made up to 1 litre.

Cathode buffer [0.1 M Tris-HCl, 0.1 M Tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (12.11 g), Tricine (17.92 g) and 10% (m/v) SDS (10 ml) were made up to 1 litre with dist.H_2O.

Treatment buffer [125 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol]. Solution C (2.5 ml), solution D (4 ml) and glycerol (2 ml) were made up to 10.0 ml with dist.H_2O. This was used for non-reducing SDS-PAGE. For reducing SDS-PAGE, 2-mercaptoethanol (1 ml) was added to the solution prior to being made up to 10 ml.

2.4.2.2 Procedure

The composition of the gels was as defined in Table 2.2. The gels were run at 80 V until the dye had entered the separating gel, after which the voltage was increased to 120 V. When the marker dye reached the end of the gel, the current was stopped, and the gels were removed and stained by either Coomassie or silver staining.

Table 2.2. Preparation of the resolving and stacking gels for Tris-tricine SDS-PAGE

Reagent	Resolving gel 10% T, 3% C	Stacking gel 4% T, 3% C
Monomer	3 ml	1.5 ml
Gel buffer	5 ml	0.5 ml
Dist H_2O	14.95 ml	6.22 ml
Ammonium persulfate	50 μl	30 μl
TEMED	5 μl	3 μl

2.5 Serva blue G rapid stain

This is a modification of the Serva blue G silver stain method of de Moreno (1985) and was used to visualise bands from preparative SDS-PAGE rapidly, prior to excision from the gel.

2.5.1 Reagents

Serva blue G dye reagent [0.25% (m/v) Serva blue G, 50% (v/v) methanol, 12.5% (m/v) TCA. Serva blue G (1.25 g) was dissolved in methanol (250 ml) and dist.H_2O (125 ml) and 50% (m/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% (m/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.

2.5.2 Procedure

Following electrophoresis, strips were excised from the left, right and centre of the preparative gel, and soaked in 40% methanol, 10% acetic acid (100 ml, 15 min), and stained in Serva blue G dye reagent (100 ml, 5 min). The strips were destained in 5% TCA (100 ml, 2 x 10 min) and 40% methanol, 10% acetic acid (100 ml, 10 min). The

strips were aligned next to the original gel, areas of the gel corresponding to the stained bands of interest were excised, and macerated as described in Section 6.2.1.

2.6 Silver staining of electrophoretic gels

For gels where very small amounts of protein needed to be visualised, the silver stain technique of Blum *et al.* (1987) was used. This technique boasts greater sensitivity than other silver stain techniques, detecting as little as nanogram amounts of protein. In many silver staining procedures, the pH change causes the non-specific formation of silver salts on the gel, reducing the contrast. By treating the gel with thiosulfate, which complexes to and dissolves silver salts, Blum *et al.* (1987) found that the background could be reduced.

2.6.1 Reagents

Milli-Q distilled water was used for all reagents, and rinse steps were conducted in the same.

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.2% (v/v) formaldehyde]. Methanol (100 ml), glacial acetic acid (24 ml) and 37% formaldehyde (0.1 ml) were made up to 200 ml with dist.H_2O just before use.

Wash solution [50% (v/v) ethanol]. Absolute ethanol (100 ml) was made up to 200 ml with dist.H_2O.

Pre-treatment solution [0.02% (m/v) $Na_2S_2O_3.5H_2O$]. $Na_2S_2O_3.5H_2O$ (0.2 g) was made up to 1 litre with dist.H_2O.

Impregnation solution [0.2% (m/v) $AgNO_3$, 0.03% (m/v) formaldehyde]. $AgNO_3$ (0.4 g) and 37% formaldehyde (0.15 ml) were made up to 500 ml with dist.H_2O.

Development solution [6% (m/v) Na_2CO_3, 0.0004% (m/v) formaldehyde]. Na_2CO_3 (12 g), pre-treatment solution (4 ml) and 37% formaldehyde (0.1 ml) were made up to 200 ml with dist.H_2O.

Stop solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and glacial acetic acid (12 ml) were made up to 100 ml with dist.H_2O.

2.6.2 Procedure

Staining was conducted in clean glass containers, rinsed with chromic acid and ethanol. All steps were carried out on a rocker or orbital shaker. The gel was soaked in fixing solution (1 h), followed by wash solution (3 x 20 min) to neutralise the gel prior to treatment with the acid labile $Na_2S_2O_3$. The gel was treated with pre-treatment solution (1 min), rinsed with dist.H_2O (3 x 20 s) and soaked in impregnation solution (20 min). Gels were again rinsed thoroughly (3 x 20 s), to remove excess $AgNO_3$ from the gel surface and immersed in development solution until bands were evident against a lightly stained background (10 min). Occasionally the gels were allowed to develop for longer to visualise any additional lightly staining bands. Finally, the gel was rinsed in dist.H_2O (2 x 2 min), treated with stop solution (10 min) and stored in sealed plastic bags, in the dark, until photographed.

2.7 Substrate gel electrophoresis

SDS-PAGE can be conducted on polyacrylamide gels in which gelatin has been copolymerised. This allows the separation and visualisation of proteolytic enzymes on gels, in positions that correspond to their molecular weights. Following electrophoresis in the presence of SDS, the proteinases may be renatured by the removal of the SDS by incubation of the gel in Triton X-100 (Heussen and Dowdle, 1980). The gel is incubated in assay buffer containing the required activators and inhibitors to ensure specific protease activity. After staining of the gel, proteolytic bands are visualised as clear bands where the gelatin has been digested, at positions on the gel corresponding to specific molecular weights.

2.7.1 Reagents

1% (m/v) gelatin. Gelatin (0.1 g) was dissolved in 10 ml of dist.H_2O, with mild heating.

2.5% (v/v) Triton X-100. Triton X-100 (5 ml) was made up to 200 ml with dist.H_2O.

Assay buffer [100 mM sodium acetate, 40 mM cysteine, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (2.86 ml) and $Na_2EDTA.2H_2O$ (0.19 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 5.5 with NaOH, NaN_3 (0.1 g) was added, the pH checked and readjusted if necessary, and made up to 500 ml. Cysteine-HCl (0.70 g) was added to 100 ml buffer immediately before use.

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

Destaining solution [30% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (300 ml) and acetic acid (100 ml) were made up to 1 litre with dist.H_2O.

2.7.2 Procedure

The procedure for SDS-PAGE gels was modified to include 0.1% (m/v) gelatin in the gels prior to polymerisation. After electrophoresis, the gel was incubated in Triton X–100 (2 x 100 ml, 1 h, RT), followed by incubation in assay buffer, containing 40 mM cysteine (100 ml, 3 h, 37°C). The gel was stained in amido black stain solution (100 ml, 1 h) and destained overnight.

2.8 Western blotting

The procedure of Western blotting maximises the analytical potential of SDS-PAGE, by allowing the separated proteins to be transferred out of the polyacrylamide gel, onto a support matrix where they can be detected. The method used in this study was essentially devised by Towbin *et al.* (1979), with a few minor modifications.

The technique involves the electrophoretic transfer of proteins from a polyacrylamide gel to an adjacent nitrocellulose filter. This is achieved by placing the polyacrylamide gel-nitrocellulose stack between two electrodes, the gel closest to the cathode and the nitrocellulose closest to the anode. The electrodes and gel-nitrocellulose stack are submersed in a blotting buffer of relatively high pH and also containing SDS, the principles of transfer thus being essentially the same as those in SDS-PAGE. While the presence of SDS in the blotting buffer does give the proteins a high mobility, resulting in very efficient transfer out of the gel, some proteins do not adhere well to nitrocellulose membranes. This is probably due to the detergent action of SDS which disrupts hydrophobic interactions between protein and nitrocellulose. The absence of SDS, on the other hand, while maximising protein-nitrocellulose binding, compromises transfer. Methanol is often included in the blotting buffer as it improves adsorption of proteins onto nitrocellulose by virtue of its fixing properties.

The accessibility of a resolved protein mixture on the surface of a nitrocellulose membrane allows the use of a variety of detection methods, otherwise limited by the gel matrix structure. In this study it allowed for the targeting of antigens, both pure and in crude fractions, via an antibody specific for the antigen in question. The bound antibody is then recognised by a secondary antibody, conjugated to an enzyme whose coloured reaction product precipitates on the nitrocellulose and can easily be visualised against the white background of the membrane.

Such an immunoblot system is critical for characterising an antibody's specificity for purified antigen when tested alongside a crude preparation, and also for its ability to detect the antigen in the crude sample itself.

2.8.1 Reagents

Blotting buffer. Tris base (27.23 g) and glycine (64.8 g) were dissolved in about 3 litres of dist.H_2O. To this was added 10% (m/v) SDS (4.5 ml) and CP methanol (900 ml) and the volume made up to 4.5 litres with dist.H_2O. The pH is automatically 8.3.

Ponceau S reagent [0.1% Ponceau S in 1% acetic acid]. Glacial acetic acid (1 ml) was diluted to about 90 ml with dist.H_2O, Ponceau S (0.1 g) was added, dissolved, and the solution made up to 100 ml. This reagent was made fresh.

Tris buffered saline (TBS) (20 mM Tris-HCl, 200 mM NaCl, pH 7.4). Tris base (2.42 g) and NaCl (11.68 g) were dissolved in about 900 ml of dist.H_2O, titrated to pH 7.4 with HCl and made up to 1 litre.

Blocking agent [5% (m/v) fat-free milk powder in TBS]. Fat-free milk powder (5 g) was dissolved in about 90 ml of TBS and made up to 100 ml.

0.5% (m/v) Bovine serum albumin in TBS (BSA-TBS). Bovine serum albumin (0.4 g) was dissolved in about 50 ml of TBS and made up to 80 ml.

0.1% (v/v) Tween 20 in TBS (TBS-Tween). Tween 20 (0.5 ml) was diluted to 500 ml in TBS.

4-Chloro-1-napthol substrate solution [0.06% (m/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), and 2 ml of this solution diluted to 10 ml with TBS. Hydrogen peroxide (35%) (4 µl) was added.

0.1% (m/v) Sodium azide in TBS. NaN_3 (0.03 g) was dissolved in TBS (30 ml).

Alkaline phosphatase buffer (100 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 9.5). Tris (12.1 g) and $MgCl_2.H_2O$ (0.233 g) were dissolved in 800 ml dist.H_2O, adjusted to pH 9.5 with HCl and made up to 1 litre with dist.H_2O.

Nitroblue tetrazolium substrate solution [0.003% nitro-blue tetrazolium]. Nitro-blue tetrazolium (0.030 g) was dissolved in 70% (v/v) dimethyl formamide (1 ml).

Bromo-chloro-indolyl phosphate substrate solution (0.0015% bromo-chloro-indolyl phosphate). Bromo-chloro-indolyl phosphate (0.015 g) was dissolved in dimethyl formamide (1 ml). Immediately prior to use, the nitro-blue tetrazolium solution (1 ml) and bromo-chloro-indolyl phosphate solution (1 ml) were diluted to 100 ml with alkaline phosphatase substrate buffer (100 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 9.5).

2.8.2 Procedure

Two types of blotting apparatus were used. The first type, the Hoefer TE Transphor unit, is a liquid blotting system, while the second type, the Hoefer Semiphor unit, is a semi-dry blotting system. The later apparatus is composed of a platinum coated niobium anode and stainless steel cathode, situated between a body cover of inert plastic. The design allows for creation of a uniform electric field between the electrodes, so that proteins can be transferred by means of low current and voltage.

In the first instance, after the electrophoretic separation of proteins in the presence of SDS (Section 2.4), the polyacrylamide gel was removed, placed on 3 layers of filter paper and the pre-cut nitrocellulose membrane (Schleicher and Schuell, BA 85, 0.45 µm) laid squarely on the gel, care being taken to dislodge all air bubbles. Three further layers of wetted filter paper were placed over the nitrocellulose and placed in the blotting apparatus sandwich. The complete sandwich was transferred to the TE Transphor electroblotting unit, placed vertically between two electrodes and

submerged in blotting buffer, ensuring that the nitrocellulose is on the anodal side. Electroblotting was accomplished after 1.5 h at 200 mA constant current, the apparatus being cooled to 4°C by a circulating water-bath.

Using the semi-dry system, the transfer stack was assembled according to manufacturer's instructions. The mylar mask was placed on the anode. On top of this, 3 pieces of blotting paper (pre-soaked in buffer) were laid, the blotting paper being the same size as the gel or smaller. The pre-wet membrane was arranged on top of the blotting paper, and the gel carefully placed over this. Blotting paper (2-3 sheets) was placed on top of this. At each level of the stack, care was taken to remove air pockets, and components were stacked neatly with all edges parallel. The unit was assembled and a 1 kg weight was placed on the lid to ensure even contact between the electrode and the gel. The current was set to equal 0.8 mA/cm^2 of gel, with a maximum voltage of 50 V. Transfer was completed in 1 h.

Upon completion of blotting the apparatus was disassembled, the nitrocellulose marked with the positions of relevant wells and hung up to air-dry (1.5-2 h). Alternatively, the membrane was immediately immersed in 0.1% Ponceau S reagent (2 min) and rinsed in dist.H_2O until protein bands were visible. The positions of the molecular weight marker proteins were noted with needle marks and the nitrocellulose was rinsed until the pink colour faded completely. The membrane was cut with scissors into pieces corresponding to the sample wells, each piece being placed into a separate container and soaked with gentle rocking (1 h) in blocking agent to saturate additional protein binding sites. The membranes were washed with TBS (3 x 5 min) and antibody diluted in BSA-TBS added. Controls consisted of substituting a non-immune preparation for an immune antibody for each different antibody, antibody concentration or blotted protein sample tested. After incubation in the primary antibody (2 h or overnight), the nitrocellulose was washed with TBS-Tween (3 x 5 min). The presence of detergent decreases non-specific binding of antibody molecules to the nitrocellulose surface by acting as a further blocking agent, or by decreasing non-specific hydrophobic binding of the antibody. The nitrocellulose was incubated (1 h) in the appropriate secondary antibody-enzyme conjugate diluted in BSA-TBS, again washed in TBS-Tween (3 x 5 min) and incubated in the dark with freshly prepared substrate solution. Incubation in substrate was allowed to proceed until an optimal colour differential between specifically targeted

bands, and non-immune incubations was found. The reaction was stopped by briefly rinsing the membranes in 0.1% (m/v) NaN_3 in TBS (for HRPO-conjugated secondary antibodies only) and then allowing these to dry on a piece of filter paper. Blots were photographed, and stored in the dark to prevent yellowing.

2.9 Enzyme assays

Cathepsin L is a potent endoproteinase, which digests azocasein (Barrett and Kirschke, 1981), elastin (Mason *et al.*, 1986) and collagen (Kirschke *et al.*, 1982) notably faster than cathepsins B or H. The conventional assay for cathepsin L activity is the azocasein assay, in the presence of 3 M urea. The addition of urea enhances the specificity of the assay as cathepsin B is inactivated by 3 M urea at pH 5, and it sensitises the azocasein to proteolysis. Pepstatin is added to the buffer to inhibit any residual cathepsin D activity.

Cathepsin L does not have a specific synthetic substrate, although it has a requirement for a hydrophobic residue in position P_2 (Schechter and Berger, 1967). Consequently, Z-Phe-Arg-NHMec has been found to be a suitable and sensitive substrate for cathepsin L, although it is also cleaved by cathepsin B (Barrett and Kirschke, 1981).

In this study, the azocasein assay was used routinely during purifications, while those assays requiring higher sensitivity, employed the synthetic substrate assay.

2.9.1 Azocasein assay

The substrate azocasein is prepared by the derivitisation of the histidine and tyrosine residues of casein with diazotised sulfanilic acid or sulfanilimide under alkaline conditions (Barrett and Kirschke, 1981). Upon proteolysis, the cleaved peptide fragments are soluble in TCA, while the larger uncleaved portions are precipitated by TCA. The coupled azo-groups give a yellow colour to the fragments in the supernatant, which can be quantified spectrophotometrically by reading the absorbance of the supernatant at 366 nm. Although the absorption maximum of the chromogenic groups is 334 nm (Langner *et al.*, 1973), absorbance readings at 366 nm are acceptable as this corresponds to 75% of the extinction at 334 nm. In this study the azocasein assay was conducted as described by Barrett and Kirschke (1981).

2.9.1.1 Reagents

Assay buffer [100 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, 1 µg/ml pepstatin, 40 mM cysteine, pH 5.0]. Glacial acetic acid (2.86 ml), $Na_2EDTA.{_2}H_2O$ (0.185 g) and NaN_3 (0.1 g) were dissolved in 450 ml dist.H_2O. The pH was adjusted to 5.0 with NaOH, pepstatin (500 µg) was added and the solution was made up to 500 ml with dist.H_2O. Prior to use, cysteine.HCl (0.04 g) was added to 5 ml of assay buffer.

6% (m/v) Azocasein. Azocasein (3 g) was weighed into a glass beaker, and dissolved in 50 ml dist.H_2O with stirring, at room temperature, for approx. 1 h.

Azocasein/3 M urea solution. Urea (54 g) was dissolved in 6% azocasein solution (50 ml) by magnetic stirring with gentle heat (30°C). Upon dissolution, the volume was made up to 150 ml with assay buffer (excluding cysteine).

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

2.9.1.2 Procedure

Sample (200 µl) was diluted with assay buffer (containing cysteine) (200 µl), and activated for 5 min at 37°C. The azocasein/urea solution (400 µl) was added, and a sample (200 µl) was removed immediately, mixed with 5% TCA (1 ml) in a polyethylene microfuge tube. This served as the blank for the reaction. After a specified time (usually 30 min, or 2 h for dilute samples), a further 200 µl was withdrawn and mixed with TCA (1 ml). The samples were centrifuged (12 000 x g, 5 min, RT). The resultant supernatants were transferred to blacked-out glass micro-cuvettes, and the A_{366} was determined. Cuvettes with blacked-out sides were used to prevent transmission of light around the sample, which was found to cause a non-linear response to the assay.

A standard curve was prepared in a similar fashion, by treating the azocasein with cathepsin L for several days to ensure complete digestion of the substrate. The resultant digest was precipitated with 5% TCA in the same proportions as described above, and was treated as an 100% hydrolysate (i.e. the azocasein had been cleaved at all possible cleavage sites). Dilutions of this solution gave points on the curve corresponding to % hydrolysis vs A_{366}. Linear regression of this curve gave an equation used to calculate % hydrolysis from any A_{366} value:

$$\% \text{ Hydrolysis} = \frac{A_{366} - 0.0233}{0.0436} \quad \text{.......} 2.2$$

From this, the units of activity can be calculated, using the definition that 1 unit = 1 μg of azocasein digested in 1 min (Schwartz and Barrett, 1980):

$$\text{Units} = \frac{(\%H \times 10 - 2) \times 2000 \text{ mg azocasein}}{\text{time}} \quad \text{.......} 2.3$$

Hence: Units/ml = 5 x units.

2.9.2 Synthetic substrate assay

The synthetic substrate Z-Phe-Arg-NHMec is the most commonly used substrate for cathepsin L. Upon cleavage, the intensely fluorescent leaving group, 7-amino-4-methyl coumarin is liberated (Barrett and Kirschke, 1981). This substrate is also subject to cleavage by cathepsin B, tissue and plasma kallikreins, so where necessary, the activity of cathepsin L can be determined by the use of specific inhibitors. Cathepsin B hydrolyses the synthetic substrate Z-Arg-Arg-NHMec (while cathepsin L does not) and so the activity of cathepsin B can be accounted for in this manner. The synthetic assays carried out in this study were essentially as described by Barrett and Kirschke (1981). However, at a later stage, the importance of buffer ionic strength, and buffer ions became apparent, and the assays were conducted in the AMT (acetate-MES-Tris) buffer system. This buffer system affords a constant ionic strength across a pH range (Ellis and Morrison, 1982). This is discussed in detail in Chapter 5.

2.9.2.1 Reagents

Z-Phe-Arg-NHMec assay buffer [340 mM Na-acetate, 60 mM acetic acid, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 8 mM 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, adjusted to pH 5.5 with NaOH and made up to 500 ml. DTT (0.0062 g) was added to 5 ml of the buffer just before the assay.

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

20 μM substrate solution. Substrate stock solution (0.1 ml) was diluted to 5 ml with dist.H_2O.

0.1% (m/v) Brij 35 diluent. Brij 35 (0.1 g) was dissolved in dist.H_2O (100 ml).

Stop reagent (100 mM monochloroacetate, 30 mM Na-acetate, 70 mM acetic acid, pH 4.3). Monochloroacetate (sodium salt) (9.45 g), Na-acetate.$3H_2O$ (4.08 g) and glacial acetic acid (4 ml) were dissolved in 950 ml dist.H_2O, titrated to pH 4.3 with NaOH, and made up to 1 litre. Caution was exercised with this highly corrosive reagent.

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

AMT buffer (100 mM acetate, 100 mM MES and 200 mM Tris, 4 mM Na_2EDTA). Glacial acetic acid (1.43 ml), MES (4.88 g) Tris (6.06 g) and $Na_2EDTA.2H_2O$ (0.375 g) were dissolved in 200 ml of dist.H_2O. This was aliquotted into 20 ml volumes, each of which was titrated to the required pH with HCl or NaOH (pH 4-8.5), and diluted to 25 ml with dist.H_2O.

2.9.2.2 Procedure

Cathepsin L (usually 1-5 ng) was diluted to 500 μl with 0.1% Brij 35, followed by the addition of assay buffer (250 μl). This solution was activated by incubation for 1 min, 30°C, and substrate (250 μl) was added and incubated for 10 min at 30°C. Stop reagent was added, and the tubes vortexed. Blanks were prepared by adding stop reagent prior to substrate solution. The fluorescence was recorded on a Hitachi F-2000 fluorimeter, with excitation at 370 nm and emission at 460 nm.

Continuous assays were conducted in the same manner, with the assay solution contained in the cuvette, resting in a temperature controlled cell. In this way, the linearity of the reaction could be observed, and the addition of stop reagent was not necessary.

From the standard 7-aminomethyl coumarin solution, units of activity could be assigned to fluorescence. According to Barrett and Kirschke (1981), the fluorescence obtained from a 0.5 μM standard is equal to 1000 arbitrary enzyme units, which is

equal to 0.1 mU of enzyme activity. The fluorescent reading of the 0.5 μM standard was 10 400 and activity could be calculated as follows:

$$\text{mUnits activity} = \frac{\text{Fluorescent intensity} \times 0.1 \text{ mU}}{10\,400} \quad \text{.......... 2.4}$$

Activity could be calculated as total activity or activity per ml.

2.10 Fractionation of IgG and IgY

A simple and convenient method of purification of IgG and IgY was found in the protein precipitating properties of PEG, a water-soluble linear polymer. Polson *et al.* (1964) found that relatively low concentrations of high molecular weight polymers were able to precipitate proteins, but high concentrations of low molecular weight species are required to effect the same degree of precipitation. The conclusion drawn was that precipitation by PEG was not due to dehydrating effects of the polymer on the protein molecules as is the case with ammonium sulfate precipitation. It was apparent, however, that the concentration of the polymer required to precipitate a protein is a function of the net charge on the protein as determined by the pH of the medium in which it is dissolved.

The protocol used to purify IgG from rabbit serum was that of Polson *et al.* (1964) while the method used for IgY purification from chicken egg yolks was as a result of more recent developments in the investigation of antibodies from chicken eggs (Polson *et al.*, 1980; Rowland *et al.*, 1986).

2.10.1 Reagents

10 mM sodium borate buffer, pH 8.6. Boric acid (2.16 g), NaOH (0.2 g), 37% (v/v) HCl (0.62 ml) and NaCl (2.19 g) were added to dist.H_2O and made up to 1 litre. The pH should automatically be 8.6.

100 mM phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. NaH_2PO_4 (13.8 g) and NaN_3 (0.2 g) were dissolved in about 800 ml of dist.H_2O, titrated to pH 7.6 with NaOH and made up to 1 litre.

2.10.2 Isolation of IgG from rabbit serum

Rabbits were bled from the marginal ear vein and the blood allowed to clot overnight at 4°C. Supernatant serum was carefully drawn off the clot, and remaining serum recovered by centrifugation (3 000 x g, 10 min, RT) of the clot. The serum was preserved with NaN_3, added to 0.02% (m/v). Rabbit serum (1 volume) was diluted with borate buffer (2 volumes). 15% (m/v) 6 kDa polyethylene glycol was dissolved in the protein solution with stirring and the resulting IgG precipitate sedimented (12 000 x g, 10 min, RT). The pellet was redissolved in phosphate buffer (3 volumes) and the precipitation procedure repeated to remove remaining contaminants. The final pellet was redissolved in half the initial serum volume with phosphate buffer. In determination of IgG concentration, a 1/40 dilution of IgG in phosphate buffer was made and the absorbance read at 280 nm in a quartz cuvette against a buffer blank. To calculate the protein concentration an extinction coefficient of 1.43 ml/mg/cm (Section 2.2.2) was used.

2.10.3 Isolation of IgY from chicken egg yolk

Individual yolks were freed of adhering albumin (egg white) by careful washing in a stream of water. The yolk sac was punctured, the yolk volume measured and phosphate buffer, equivalent to 2 volumes of yolk, was added and thoroughly mixed. Solid PEG (6 kDa) was added to a final concentration of 3.5% (m/v of diluted yolk). The PEG was dissolved with stirring and the mixture was centrifuged (4 420 x g, 30 min, RT) to separate three phases, a casein-like vitellin fraction, a clear fluid, and a lipid layer on the surface. The supernatant fluid contaminated with some of the lipid layer was filtered through a loose plug of cotton wool in the neck of a funnel. The volume of clear filtrate was measured and the PEG concentration increased to 12% (m/v). The precipitated IgY fraction was centrifuged (12 000 x g, 10 min, RT), the pellet redissolved in phosphate buffer to the volume after filtration and the IgY again precipitated with 12% (m/v) PEG and centrifuged. The final IgY pellet was dissolved overnight in a volume of phosphate buffer equal to one sixth of the original yolk volume. Immunoglobulin Y concentration was determined as in Section 2.10.2 using an extinction coefficient of 1.25 ml/mg/cm (Section 2.2.2).

2.11 Enzyme-linked immunosorbent assay

Immunoassays use the specific interaction of an antibody with antigen to provide information about antibody (or antigen) concentration in unknown samples. In principle, the labelling by chemical conjugation of an enzyme to either antibody or antigen allows the detection of immune complexes formed on a solid phase. This forms the basis of the ELISA, first introduced by Engvall and Perlmann (1971). One of the simplest and most commonly used ELISAs for the detection of antibodies, is a three layer system. Briefly, antigen is coated to the plastic surface of the wells of polystyrene microtitre plates, and the primary antibodies to be quantified allowed to form a complex with the immobilised antigen. After excess antibody has been washed away, the degree or amount of reactivity is quantified with an appropriate detection system. In an ELISA this would take the form of an enzyme conjugated to a secondary antibody which will recognise the primary antibody bound to the immobilised antigen. The enzyme reacts with a substrate which yields a coloured product which can be measured spectrophotometrically. This quantitative system complements western blotting (Section 2.8), which gives qualitative information about antibody specificity.

During the course of this work, an ELISA was most commonly used as a means to monitor the progress of immunisation of rabbits and chickens, and in enzyme cross-species reactivity studies. It also aided in the determination of suitable antibody dilutions for use in Western blot analysis (Section 2.8).

2.11.1 Reagents

Phosphate buffered saline (PBS). NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in about 800 ml of dist.H_2O, adjusted to pH 7.4 with HCl and made up to 1 litre.

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

0.1% (v/v) Tween 20 in PBS (PBS-Tween). Tween 20 (1 ml) was diluted to 1 litre in PBS.

Substrate buffer (150 mM citrate-phosphate, pH 5.0). Na_2HPO_4 (2.84 g) and citric acid (2.29 g) were each dissolved in dist.H_2O and made up to 100 ml. The citric acid solution was titrated against the Na_2HPO_4 (50 ml) solution to pH 5.0.

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

0.1% (m/v) Sodium azide in 150 mM citrate-phosphate buffer, pH 5.0. For each ELISA plate NaN_3 (15 mg) was dissolved in citrate-phosphate buffer (15 ml).

100 mM Na-borate buffer, pH 7.4. Na-borate (6.18 g) was dissolved in 950 ml dist.H_2O, adjusted to pH 7.4 with NaOH and made up to 1 litre.

1 mM Na-acetate buffer, pH 4.4. Glacial acetic acid (1 ml) was diluted to 950 ml with dist.H_2O, adjusted to pH 4.4 with NaOH, and made up to 1 litre.

200 mM Na_2CO_3 buffer, pH 9.5. Approximately 18.6 ml $NaHCO_3$ (1.68 g/ 100 ml) was titrated to pH 9.5 with approximately 6.4 ml Na_2CO_3 (2.12 g/100 ml).

100 mM sodium periodate solution. Sodium periodate (0.2139 g) was dissolved in 10 ml dist.H_2O. This was prepared freshly before use.

Sodium borohydride solution (4 mg/ml). Sodium borohydride (8 mg) was dissolved in 2 ml dist.H_2O, just before use.

HRPO linked secondary antibodies. The coupling 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 freshly prepared 100 mM sodium periodate (200 µl) was added. The mixture was stirred (20 min, RT), and turned a greenish brown. This was dialysed against Na-acetate buffer, pH 4.4, overnight at 4°C. The pH of the solution was raised by the addition of 200 mM Na_2CO_3 buffer, pH 9.5 (20 µl) and 1 ml of IgG (8 mg/ml) was added. This was left for 2 h at RT, followed by the addition of fresh sodium borohydride (100 µl) to reduce any free enzyme. This was left at 4°C, for 2 h, and dialysed against 100 mM Na-borate buffer overnight at 4°C. An equal volume of 60% glycerol in Na-borate buffer was added and stored at 4°C. The dilution of conjugate to be used was determined by checkerboard ELISA. The dilution which

gave a steep titration curve, above the background, over a serially diluted primary antibody range (usually 1:400 - 1:600 dilution), was used.

2.11.2 Procedure

Wells of microtitre plates (Nunc Immunoplate) were coated with antigen (150 µl) at predetermined dilutions (1.0 µg/ml for most antigens, as determined by a checkerboard ELISA) in PBS overnight at room temperature. Wells were blocked with BSA-PBS (200 µl) for 1 h at 37°C and washed 3 times with PBS-Tween. Serial two-fold dilutions of the primary rabbit antiserum, IgG or IgY solution in BSA-PBS (starting at 1 mg/ml) were added (100 µl), incubated for 1 h at 37°C and excess antibody washed out 3 times with PBS-Tween. A suitable dilution of sheep anti-rabbit IgG-HRPO conjugate or rabbit anti-chicken IgY-HRPO conjugate in BSA-PBS, was added (120 µl) and incubated (30 min) at 37°C. The ABTS substrate (150 µl) was added and incubated in the dark for optimal colour development (usually 10-20 min). The enzyme reaction was stopped by the addition of 50 µl of 0.1% (m/v) NaN_3 in citrate-phosphate buffer and absorbances read at 405 nm in a Bio-Tek EL307 ELISA plate reader. Titration curves were constructed from the spectrophotometric values.

CHAPTER 3

ISOLATION OF FREE AND STEFIN B-COMPLEXED-SHEEP CATHEPSIN L

3.1 Introduction

In developing a rapid method for the isolation of sheep liver cathepsin L, Pike (1990) found that three phase partitioning (TPP) was a useful method which enabled cathepsin L to be isolated from a liver homogenate in a three-step process (Pike and Dennison, 1989a; 1989b). In the course of those studies Pike (1990) first observed the occurrence of proteolytically **active** complexes of stefin B and cathepsin L. These complexes were further studied by Coetzer (1992), who showed that the complexes were not limited to sheep tissue, but also occurred in homogenates of human and baboon liver (Coetzer *et al.*, 1995). Pike *et al.* (1992) also showed that the complexes were not artefacts generated by the novel TPP method, but could also be isolated via conventional ammonium sulfate precipitation.

Pike *et al.* (1992) showed that the active complex fraction eluting from Sephadex G-75 at 37 kDa probably consisted of a mixture of a covalent, proteolytically active, complex and the known, inactive, tightly bound non-covalent complex. Kirk (1992) explored a number of possible ways of separating the two forms of complex, but without success. Among the methods that she tried were electroelution of the two forms of complex using a Schleicher and Schuell Biotrap BT1000® and an H-tube (Kirk, 1992).

In the present study, the method of isolating free and complexed forms of cathepsin L, developed by Pike *et al.* (1992), was adopted but was scaled up several-fold. Attempts were made to separate active and inactive complexes on the basis of their differential activity, using affinity chromatography with either cystatin or bradykinin as the ligand, but without success. The effect of urea on the cathepsin L forms was also explored with the prospect of separating active (covalent) forms of stefin B-cathepsin L complexes from inactive (non-covalent) forms, by incorporating urea in the buffer during molecular exclusion chromatography. It was thought that 8 M urea might dissociate the non-covalent complex, while leaving the covalent complex intact. Separation of the two forms of complex would facilitate subsequent structural analysis of the active complex, aimed at determining the nature of the

linkage between the stefin B and cathepsin L molecules. For such structural studies it would be of little moment if the cathepsin L were denatured by urea, though it had been reported that cathepsin L is relatively stable to urea (Kirschke *et al.*, 1984).

Kirschke *et al.* (1984) reported that cathepsin L has substantial azocaseinolytic activity in 3 M urea, its activity being increased 2.5-fold, compared to that in the absence of urea. This characteristic has been used to discriminate between the activities of cathepsins L and B, the latter being depressed by urea (Kirschke *et al.*, 1980). An early observation in the present study was that the azocaseinolytic activity of both free and stefin B-complexed cathepsin L was increased by urea, up to at least 6 M, activity of the complex being increased proportionately more than that of the free enzyme. It was decided to explore this phenomenon, since it held the prospect of throwing some light on the nature of cathepsin L and the stefin B-complexed cathepsin L.

A detailed analysis of the effect of urea on cathepsin **B** has been reported (Agarwal and Khan, 1988). These authors followed the urea-induced denaturation of cathepsin B by monitoring changes in fluorescent emission, at the emission λ_{max} of 372 nm, which is thought to be due to changes in the interaction of tryptophan and tyrosine residues, brought about by conformational changes (Agarwal and Khan, 1988; Franks, 1988). The present study of the effect of urea on cathepsin L was modelled on that of Agarwal and Khan (1988) on cathepsin B. However, cathepsin L, unlike cathepsin B, is active in urea and so the approach of Agarwal and Khan (1988) could be extended to include studies of the **activity** of cathepsin L in urea as well as urea-induced changes in conformation. These studies have revealed a fundamental difference in the behaviour of cathepsin L towards protein substrates, compared to small synthetic substrates.

3.2 Methods and Materials

3.2.1 Isolation of cathepsin L and stefin B-complexed-cathepsin L

3.2.1.1 Reagents

Sheep livers were from Abakor, Cato Ridge. Cysteine, pepstatin, Z-Phe-Arg-NHMec and S-Sepharose were from Sigma Chemical Co., St Louis, MO, USA. Ultra-pure

urea and TCA were from BDH. All other chemicals were of the highest analytical grade.

Homogenisation solution [1% (m/v) NaCl, 0.1% (m/v) Na_2EDTA, 2% (v/v) n-butanol]. NaCl (12 g) and Na_2EDTA (1.2 g) were dissolved in 1100 ml cold dist.H_2O and n-butanol (22 ml) was added. The solution was thoroughly mixed and made up to 1.2 litres.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (2.29 ml), Na_2EDTA (0,74 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres dist.H_2O, titrated to pH 5.5 with NaOH, and made up to 2 litres.

S-Sepharose fast flow. S-Sepharose was prepared by diluting 600 ml of the supplied hydrated gel in 400 ml of buffer A, and packing the resulting slurry into a column (5 x 30 cm) under gravity. The column was regenerated by passing two column volumes of 1 M NaCl in buffer A through it, followed by five column volumes of 200 mM NaCl in buffer A, before use. The column was washed with 1 M NaCl in buffer A and 200 mM NaCl in buffer A between purifications, just before use.

Sheep Liver. Fresh sheep livers were collected from the Cato Ridge abattoir, cut into strips and frozen (-70°C) for at least 3 days before use. The livers were generally not stored for longer than 1 month.

3.2.1.2 Procedure

Cathepsin L was isolated on two scales, the "standard" scale, using 400-600 g of liver per run and a scaled-up method using 1200-1500 g of liver per run.

Standard scale isolation. This was done as described by Pike and Dennison (1989b), with the modifications described by Pike *et al.* (1992). Frozen livers were cut into small pieces and mixed 1:2 with homogenisation buffer, and homogenised 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 1 M HCl and stirred. The mixture was centrifuged (6 000 x g, 20 min, 4°C) and the supernatant harvested. Three-phase partitioning was conducted by adding and mixing t-butanol to a final concentration of 30% (v/v). In this step the supernatant was at 4°C and the t-butanol at room temperature. All subsequent steps were conducted at 4°C. Ammonium

sulfate [20% (m/v) based on the total volume of supernatant plus t-butanol] was added and dissolved by gentle stirring. The resulting mixture was centrifuged (6 000 x g, 15 min, 4°C) in a swing out rotor. The supernatant and subnatant were collected and the precipitate was discarded. A further amount of $(NH_4)_2SO_4$ was added to bring the final concentration to 30% (m/v) $(NH_4)_2SO_4$ (based on the total volume of supernatant plus t-butanol) and stirred well. The mixture was centrifuged (6 000 x g, 15 min, 4°C) and the interfacial pellet was collected and dissolved in buffer A, to a final volume of one fifth of the pH 4.2 supernatant volume. The pH was adjusted to pH 5.5 with NaOH, and the resuspended material was centrifuged (27 000 x g, 10 min, 4°C) to remove any insoluble material. The supernatant was loaded directly onto an S-Sepharose column (70 ml) equilibrated with 200 mM NaCl in buffer A. The loaded material was washed with 10 column volumes of buffer A containing 200 mM NaCl and eluted with a salt gradient (200-600 mM NaCl in buffer A in five column volumes). Fractions corresponding to the peaks of activity eluted at about 400 mM NaCl, and were pooled and concentrated by dialysis against sucrose. The concentrated fractions were loaded onto a Sephadex G-75 column (450 ml) equilibrated with buffer A containing 200 mM NaCl. Peaks with activity against azocasein or Z-Phe-Arg-NHMec were collected for further study.

Scaled-up procedure. In the scaled up procedure, liver (about 1200 g) was processed as described above, with the resulting TPP fraction (100-120 ml) being loaded onto columns of S-Sepharose (5 x 30 cm = 590 ml) and Sephadex G-75 (5 x 134 cm = 2630 ml). Protein peaks showing activity against azocasein and Z-Phe-Arg-NHMec were collected and concentrated by dialysis against sucrose for further analysis using SDS-PAGE.

In an attempt to separate the complex forms, molecular exclusion of the 37 kDa forms of stefin B complexed-cathepsin L was repeated, on an analytical scale, in the presence of 8 M urea. The column (2.5 x 90 cm) was pre-equilibrated with 200 mM sodium acetate buffer, pH 5.5, containing 8 M urea and urea was added to the sample to 8 M. The effluent was monitored by absorbance at 280 nm and fractions were analysed for activity against azocasein as described previously (Section 2.9).

3.2.2 Activity assays in the presence of urea

These experiments were conducted to monitor the effect of urea on the activity of free cathepsin L and stefin B complexed cathepsin L against azocasein and the small synthetic substrate, Z-Phe-Arg-NHMec. The assays were conducted essentially as described by Barrett and Kirschke (1981), except that urea was added to all solutions to the required final concentration. All reagents requiring urea were made fresh, to reduce the formation of cyanates which arise on standing and heating, especially of alkaline urea solutions (Stark *et al.*, 1960; Stark, 1964). The reagents and procedures that are different from those outlined in Section 2.9 are given below.

3.2.2.1 Reagents

Assay buffer [100 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3 and 1 µg/ml pepstatin] pH 5.0], containing 40 mM cysteine and varying amounts of urea. Glacial acetic acid (2.9 ml), $Na_2EDTA.2H_2O$ (0.19 g) and NaN_3 (0.1 g) were dissolved in 440 ml of dist.H_2O. The pH was adjusted to 5.0 with NaOH, pepstatin (500 µg) was added and the volume made up to 450 ml. The solution was aliquotted into 45 ml samples and urea was added to each to the desired concentration. The volumes were made up to 50 ml and the pH was checked. Immediately before use, cysteine.HCl (0.04 g) was added to the buffer (5 ml).

2% Azocasein solutions. These were made up as described previously (Section 2.9) except that urea was added to give final concentrations of 0.375, 0.75, 1.5, 3 and 6 M, respectively.

5% Trichloroacetic acid (TCA). TCA (25 g) was dissolved in 500 ml of dist.H_2O.

Acetate-MES-Tris (AMT) assay buffers, pH 5.5, 6.0, 6.5 and 7.0, containing 50/150 mM cysteine and urea. These were made up as described previously (Section 2.9.2), except that urea was added to give final concentrations of 1, 2, 3, 4, 5, 6, 8 and 12 M urea, when made to volume. The 12 M urea solution required gentle heating, and did not precipitate out as the experiment was conducted at 30°C. Upon dilution, the urea concentrations were halved to give final assay concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 4 and 6 M urea. Cysteine (0.24 g or 0.73 g) was added, and the pH titrated to 5.5, 6.0, 6.5 or 7.0 with either HCl or NaOH, and the buffers made up to volume (10 ml).

0.1% (m/v) Brij. Brij 35 (0.1 g) was dissolved in dist.H_2O (100 ml).

1 mM substrate stock solution. This was prepared as described in Section 2.9. Z-Phe-Arg-NHMec (2 mg) was dissolved in DMSO (3 ml) and stored at 4°C.

20 µM substrate solutions containing urea. Substrate stock solution (0.2 ml) was diluted to 5 ml with dist.H_2O, and varying amounts of urea were added to give final concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 4 and 6 M urea in 10 ml. The volumes were made up to 10 ml with dist.H_2O.

Stopping reagent (100 mM monochloroacetate, 30 mM Na-acetate, 70 mM acetic acid, pH 4.3). This was made up as described previously (Section 2.9). Monochloroacetate (sodium salt) (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 and made up to 1 litre.

3.2.2.2 Procedure for azocasein assays

Enzyme sample (200 µl) was mixed with sodium acetate assay buffer (200 µl), containing urea, and activated at 37°C for 5 min. The 2% azocasein solution, containing urea of the required concentration (400 µl), was added and incubated for 2 h at 37°C. A blank was prepared as described previously (Section 2.9) by withdrawing an aliquot (200 µl) immediately after addition of the azocasein solution and mixing it with 5% TCA (1 ml) in a microfuge tube. Further aliquots (200 µl) were withdrawn after the required time and mixed with 5% TCA solution (1 ml) and centrifuged (12 000 x g, 5 min, RT). The absorbance of the supernatants was read at 366 nm in micro-cuvettes with blacked-out sides. Assays were conducted in triplicate and units of activity were calculated from a standard curve as described previously (Section 2.9).

3.2.2.3 Procedure for synthetic substrate enzyme assay

Enzyme (5 ng) was diluted to 375 µl with Brij 35 (0.1%), assay buffer containing urea (375 µl) was added, and incubated at 30°C for 1 min. Z-Phe-Arg-NHMec substrate (250 µl), containing urea at the required concentration, was added and the reaction allowed to proceed for 10 min. Stopping reagent (1 ml) was added and tubes were vortexed. The fluorescence emission at 460 nm was read with excitation at 370 nm.

A blank was prepared by adding stopping reagent prior to addition of substrate. The activity in the absence of urea was designated 100% activity.

3.2.3 Denaturation experiments

The use of intrinsic fluorescence of a protein to monitor conformational changes relies on the absorption of energy by the protein, with a concomitant change from the ground state to one of several excited states. This is followed by a rapid internal conversion to the lowest energy state and finally to the ground state with the emission of photons of light (Brand and Witholt, 1986). The emission in proteins is due to the presence of phenyalanine, tyrosine and tryptophan residues and consequently it is highly sensitive to conformational changes of proteins. The addition of chaotropic agents, such as urea and guanidine hydrochloride, causes alterations in the native conformation of proteins, which can be monitored by changes in the intrinsic fluorescence of the molecules (Pace, 1986). Generally, the greater the change in the physical property of the protein in the unfolded (denatured) state, the greater the change in fluorescence. Practically, a wavelength should be chosen that reflects the greatest change in fluorescent intensity between the native and unfolded state, irrespective of whether this is the wavelength maximum of the two forms (Pace, 1986).

Denaturation experiments were conducted to monitor the effects of urea on single-chain sheep cathepsin L. Difference fluorescence spectra were determined, in order to determine the wavelength at which urea-induced conformational changes could best be measured.

3.2.3.1 Reagents

AMT buffer, pH 5.5. This was made as described in Section 2.9.

3.2.3.2 Procedure

Difference fluorescence spectra. Cathepsin L (10 ng) was diluted in AMT buffer, pH 5.5 (2 ml) containing increasing amounts of urea, at 30°C. Emission spectra of these solutions, and solutions in the absence of protein, were recorded from 300-480 nm, with excitation at 280 nm. Difference spectra were obtained by subtraction of the emission spectra of the buffer and urea solutions from that of the enzyme solutions.

Unfolding spectra. Cathepsin L (10 ng) was diluted with AMT buffer, pH 5.5, containing increasing amounts of urea (1 ml). The solutions were mixed thoroughly and allowed to equilibrate (10-12 h, 30°C). Fluorescence emission was read at 390 nm, with excitation at 280 nm.

3.2.4 Renaturation experiments

3.2.4.1 Reagents

AMT buffer, pH 5.5. This was made as described in Section 2.9.

3.2.4.2 Procedure

To monitor the refolding of cathepsin L, enzyme (10 ng) was equilibrated for 10 h with AMT buffer, pH 5.5 (100 µl) containing 1.5 M or 6 M urea at 30°C. These solutions were diluted to decreasing concentrations of urea (in 1 ml) and the fluorescence emission recorded at 390 nm, with excitation at 280 nm.

To monitor the residual enzyme activity following unfolding with urea, cathepsin L was treated as for the refolding experiments (above), except that following dilution to decreased urea concentrations, enzyme (0.5 ng) was removed and assayed for residual activity against Z-Phe-Arg-NHMec at the corresponding urea concentrations, as described in Section 3.2.2.5. Enzyme untreated with urea, but similarly diluted, was taken as 100% active. All assays were conducted in triplicate.

3.2.5 Stability experiments

3.2.5.1 Reagents

AMT buffer, pH 5.5. This was made as described in Section 2.9.

6% Azocasein solution. Azocasein (1.5 g) was dissolved in pH 5.5 AMT buffer (25 ml) with gentle stirring.

2% Azocasein/6 M Urea. The 6% azocasein solution (25 ml) was mixed with urea (27 g) and diluted to 75 ml with pH 5.5 AMT buffer. The solution was stirred, with gentle heating, to dissolve the urea.

3.2.5.2 Procedure

To monitor the stabilising effect of azocasein on cathepsin L, in the presence of 3 M and 6 M urea, cathepsin L (10 ng) was diluted to 500 µl, in AMT buffer, pH 5.5. Azocasein/urea solution was added (500 µl) to a final concentration of 1% azocasein, 3 M urea. To obtain a final concentration of 6 M urea, urea was added to the dilution buffer to a final concentration of 6 M, so that on addition of azocasein/urea solution, the urea concentration remained 6 M. The fluorescence emission was immediately recorded from 300-480 nm, with excitation at 280 nm.

To monitor the stabilising effect of stefin B on cathepsin L, in the presence and absence of urea, cathepsin L (10 ng) was reacted with stefin B (30 ng) and diluted to 1 ml with AMT buffer, pH 5.5, in the absence and presence of 6 M urea. The fluorescence emission was immediately recorded from 300-480 nm, with excitation at 280 nm.

3.3 Results

3.3.1 Isolation of cathepsin L and stefin B-complexed-cathepsin L

The purification procedure facilitated the isolation of free cathepsin L and stefin-B complexed cathepsin L. S-Sepharose ion exchange chromatography resulted in the elution of a bound fraction, showing activity against azocasein, that was well resolved from the unbound fraction (Fig. 3.1; conventional isolation procedure). Molecular exclusion chromatography on Sephadex G-75, resulted in the separation of the S-Sepharose peak into two proteolytically active peaks, with M_r 37 000 and 26 000. Both peaks were active against azocasein and Z-Phe-Arg-NHMec (Fig. 3.2; conventional isolation procedure). The activity was characterised as being that of cathepsin L, as rationalised previously (Coetzer, 1992). The two peaks were divided into six fractions, to obtain optimal purity of the components, and designated MEC I - MEC VI.

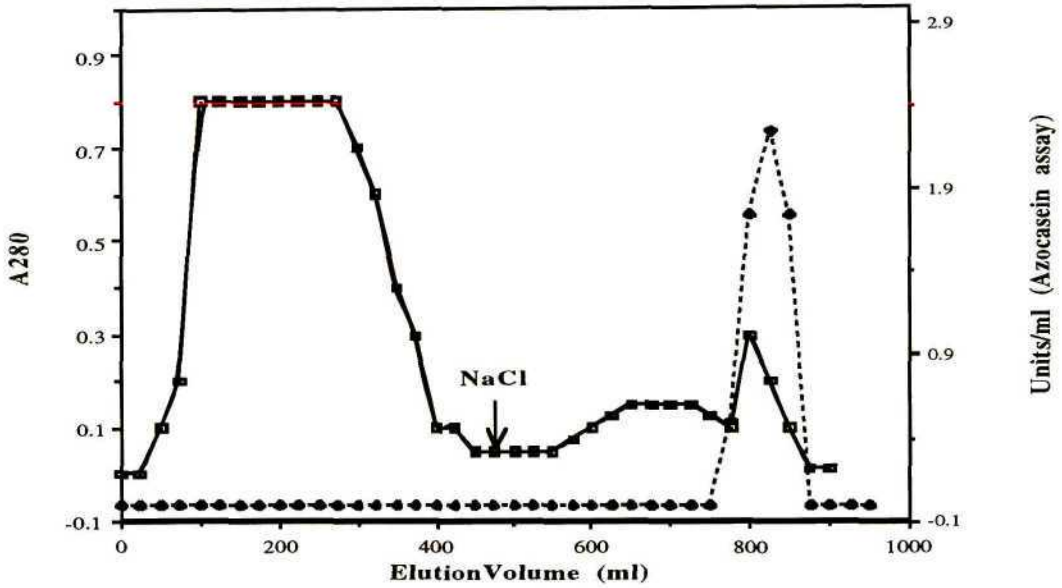


Figure 3.1. Chromatography of the TPP fraction from sheep liver on S-Sepharose.

Column (5 x 30 cm), Buffer A with 200 mM NaCl, followed by 200-600 mM NaCl gradient, in five column volumes, in buffer A, applied at ↓, followed by two column volumes of 600 mM NaCl in buffer A. Flow rate, 10 cm/h (196 ml/h). Fractions 16.3 ml (5 min/tube). ( ), A_{280} and (♦), enzyme activity in units/ml from the azocasein assay.

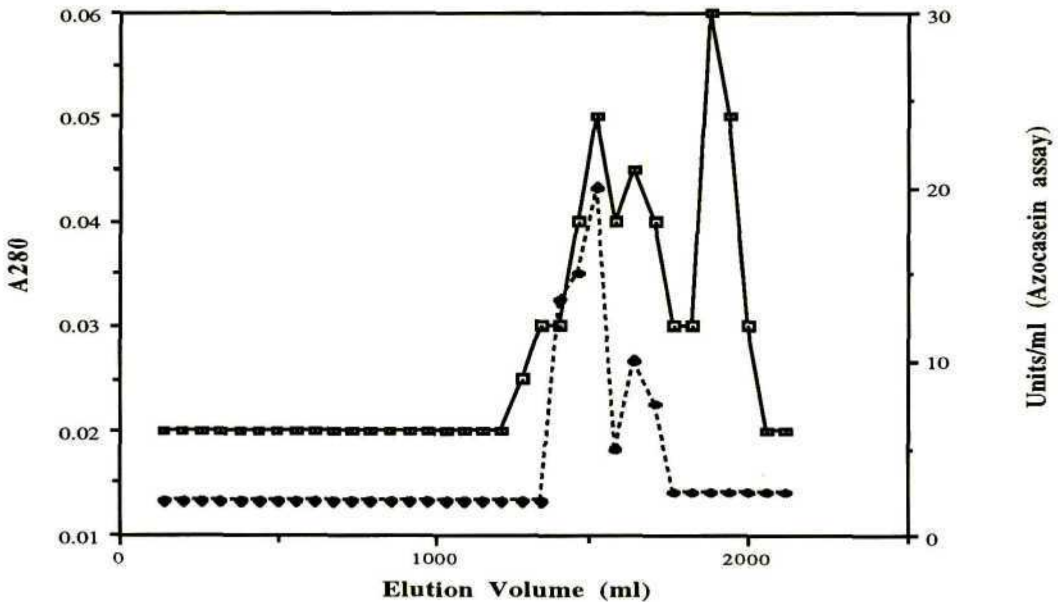


Figure 3.2. Chromatography of the S-Sepharose peak on Sephadex G-75.

Column (5 x 134 cm), Buffer A with 200 mM NaCl. Flow rate, 5 cm/h (98 ml/h). Fractions, 19.6 ml (12 min). ( ), A_{280} and (♦), enzyme activity in units/ml from the azocasein assay.

Both fractions from Sephadex G-75 showed proteolytic activity on substrate SDS-PAGE (Fig. 3.3). Analysis of these two peaks on non-reducing SDS-PAGE showed that the higher molecular weight peak contained a number of components, with M_r 42 000, 37 000 and 26 000, and an additional faint band at M_r 68 000 (Fig. 3.4). In some preparations, the M_r 26 000 band was more prominent than the 37 000 band, and an additional band at M_r 14 000 was clearly visible. On reducing SDS-PAGE, two bands of Mr 26 000 and 14 000 were obtained (Fig. 3.5). These results indicate that a proportion of the M_r 37 000, 42 000 and 68 000 forms, which do not dissociate without reduction, are covalently bonded to the M_r 14 000 moiety. The remainder of the complex appears to be non-covalently associated as it does not require reduction to yield the M_r 26 000 and 14 000 bands. The lower molecular weight peak (M_r 26 000) from Sephadex G-75, gave a single band at M_r 26 000 on SDS-PAGE gels, with and without reduction (Fig. 3.4). These results suggest that this cathepsin L is in fact a single-chain form, which differs from the cathepsin L isolated by Mason (1986) from sheep liver. This has been confirmed by amino acid sequencing of cathepsin L (Coetzer, 1992).

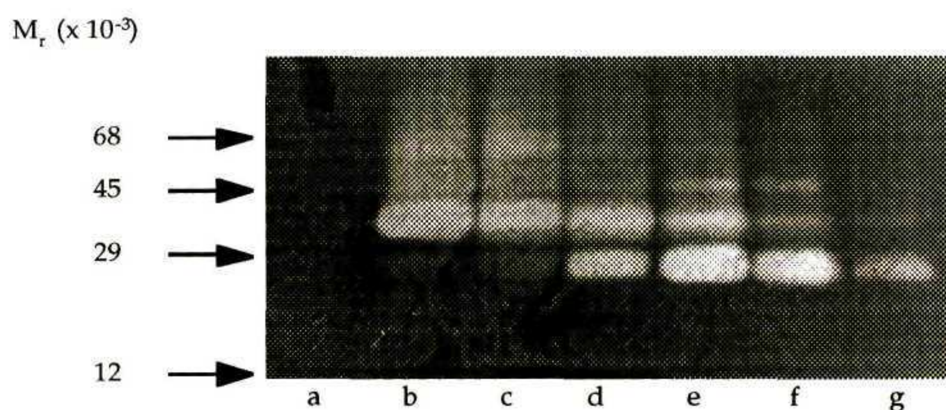


Figure 3.3. Substrate gel electrophoresis of Sephadex G-75 fractions.
(a) Molecular weight markers (BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa and cytochrome C, 12 kDa), (b) - (g) Fractions (5 μg) from molecular exclusion chromatography showing activity against azocasein, designated MEC I - MEC VI, performed on a 12.5% acrylamide gel, containing 0.1% gelatin, and stained as outlined in Section 2.7.2

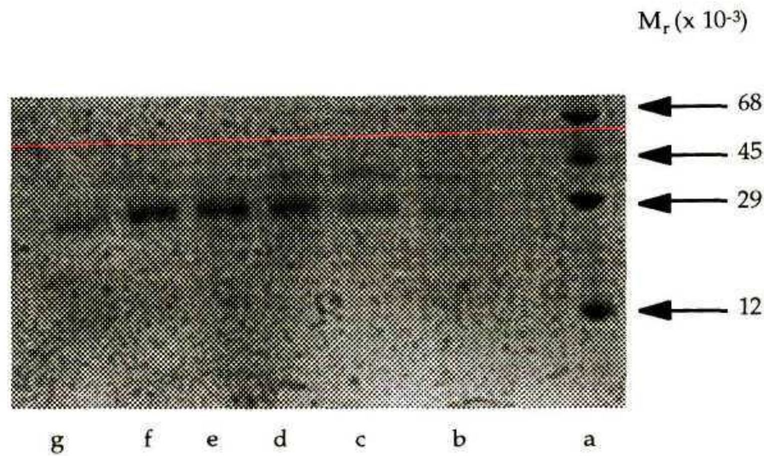


Figure 3.4. Non-reducing SDS-PAGE of Sephadex G-75 fractions.

(a) Molecular weight markers (BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa and cytochrome C, 12 kDa), (b) - (g) Fractions (10 μg) from molecular exclusion chromatography showing activity against azocasein, designated MEC I - MEC VI, performed on a 12.5% acrylamide gel, stained with Coomassie as outlined in Section 2.4.1.2.

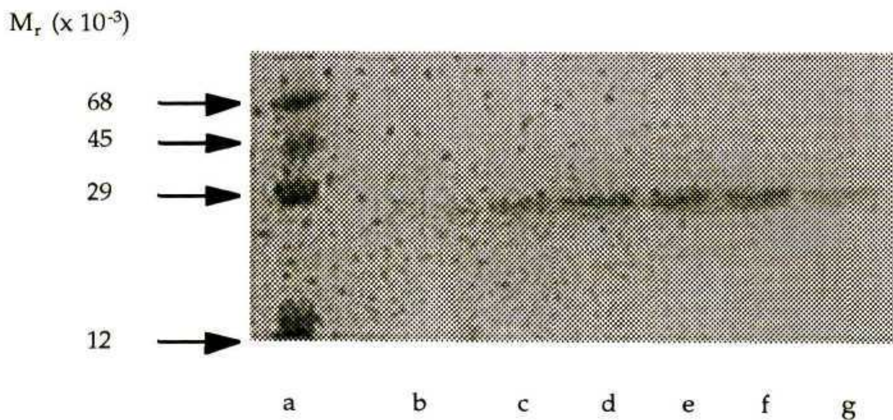


Figure 3.5. Reducing SDS-PAGE of Sephadex G-75 fractions.

(a) Molecular weight markers (BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa and cytochrome C, 12 kDa), (b) - (g) Fractions (10 μg) from molecular exclusion chromatography showing activity against azocasein, designated MEC I - MEC VI, treated with reducing treatment buffer, performed on a 12.5% acrylamide gel, stained with Coomassie as outlined in Section 2.4.1.2.

Table 3.1 summarises the purification of cathepsin L from sheep liver. The conventional isolation results may be compared with those of the scaled-up procedure.

Table 3.1. Purification table of sheep liver cathepsin L, for the conventional and scaled up procedure.

Scaled-up Procedure	Vol (ml)	Total Protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Purification (fold)	Yield (%)
Liver Homogenate	2870	60754	110208	1.814	1	100
pH 4.2 supernatant	1835	28940	44498	1.5	0.82	40
TPP	320	4395.7	37973	9	4.9	34
S-Sepharose	60	17.6	17160	975	537.4	15
MEC (complex)	23	4.93	6127	1243	592.6	5.6
MEC (free)	18.5	5.14	3699	720	351.7	3.4
Conventional Procedure	**Vol (ml)**	**Total Protein (mg)**	**Total activity (mU)**	**Specific activity (mU/mg)**	**Purification (fold)**	**Yield %**
Liver Homogenate	870	17924	57357	3.2	1	100
pH 4.2 supernatant	650	2392	26312	11	3.4	46
TPP	140	1189	22591	19	6	39
S-Sepharose	35	4.27	4176	1000	313	7
MEC (complex)	8	0.45	645	1435	448	1.1
MEC (free)	6	0.55	562	1023	320	1

3.3.2 Molecular exclusion chromatography in the presence of urea

Rechromatography in the presence of 8 M urea, of the 37 kDa peak from Sephadex G-75 (Fig. 3.6) resulted in a single peak of 37 kDa, which exhibited enhanced activity against azocasein.

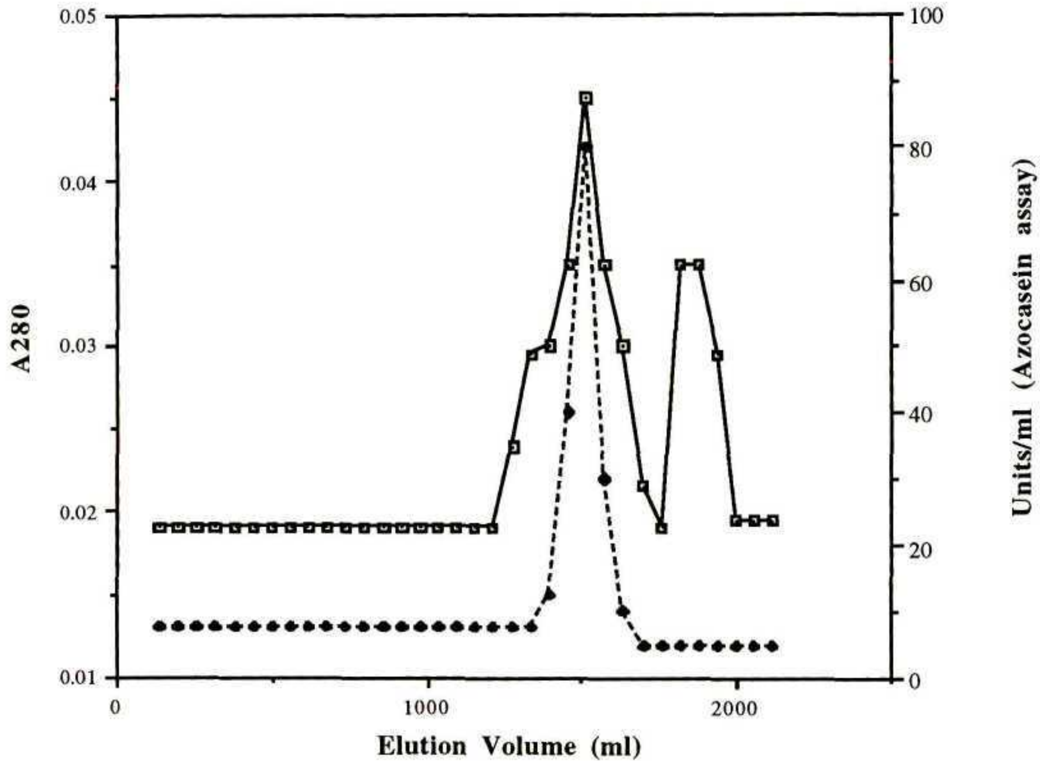


Figure 3.6. Chromatography of complexed cathepsin L on Sephadex G-75 in 8 M urea.

Column (2.5 x 90 cm), Buffer A containing 8 M urea. Flow rate, 5 cm/h (25 ml/h). Fractions 5 ml (12 min/tube). ( ), A_{280} and (♦), enzyme activity in units/ml from the azocasein assay.

3.3.3 The effect of urea on activity against azocasein and Z-Phe-Arg-NHMec

The effects of increasing urea concentration on the activities of cathepsin L and stefin B-complexed-cathepsin L against azocasein are shown in Fig. 3.7. In 6 M urea the activity of free cathepsin L is nearly doubled while that of stefin B complexed-cathepsin L is increased almost three-fold.

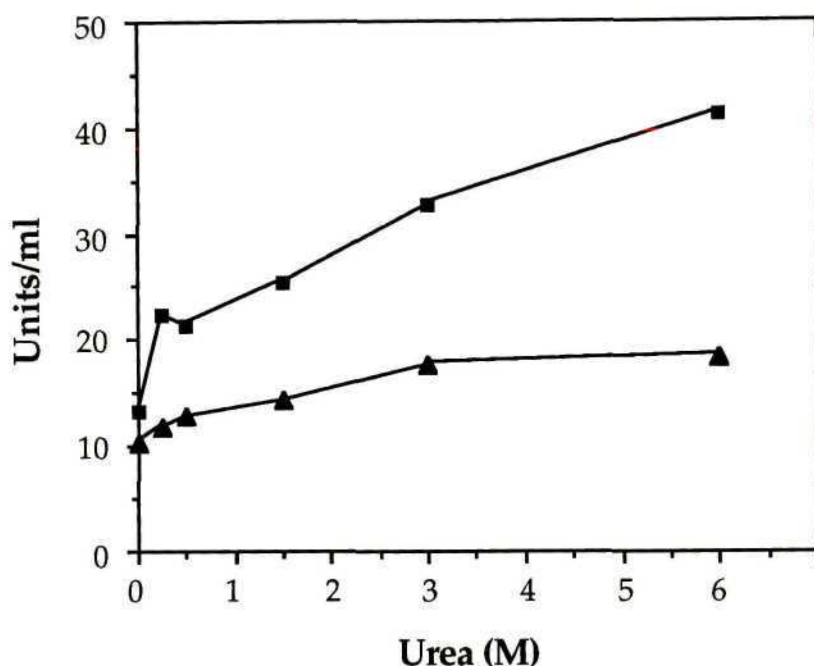


Figure 3.7. The effect of urea on the azocaseinolytic activity of cathepsin L and stefin B-complexed cathepsin L.
▲ - cathepsin L, ■ - stefin B complexed cathepsin L. Buffer, 100 mM Na-acetate assay buffer, pH 5.5, containing 40 mM cysteine.

In contrast to the effects of urea on the azocaseinolytic activity of the different cathepsin L forms, increasing amounts of urea, above 0.5 M, resulted in a **decrease** in their activity against Z-Phe-Arg-NHMec (Fig. 3.8).

At pH 5.5, and 150 mM cysteine, urea concentrations of 0.5 M caused an increase in enzyme activity for both the free and complexed forms of cathepsin L, but this declined with increasing urea concentration. At 6 M urea, the complex had retained 60% of its activity and free cathepsin L only 20%. At 50 mM cysteine, less than 10% of the activity of both the free and complexed cathepsin L was retained in 6 M urea (Fig. 3.8 A).

At pH 6.0, 6.5 and 7.0 (Fig. 3.8 B, C and D respectively) the increase in enzyme activity at 0.5 M urea was less marked. At pH 7.0, at most urea levels, higher activities were observed with 150 mM cysteine, than with 50 mM cysteine (Fig. 3.8 D); this accords with the observation that reductive activation increases the stability of cathepsin L at extracellular pH (see Chapter 5) (Dehrmann *et al.*, 1995).

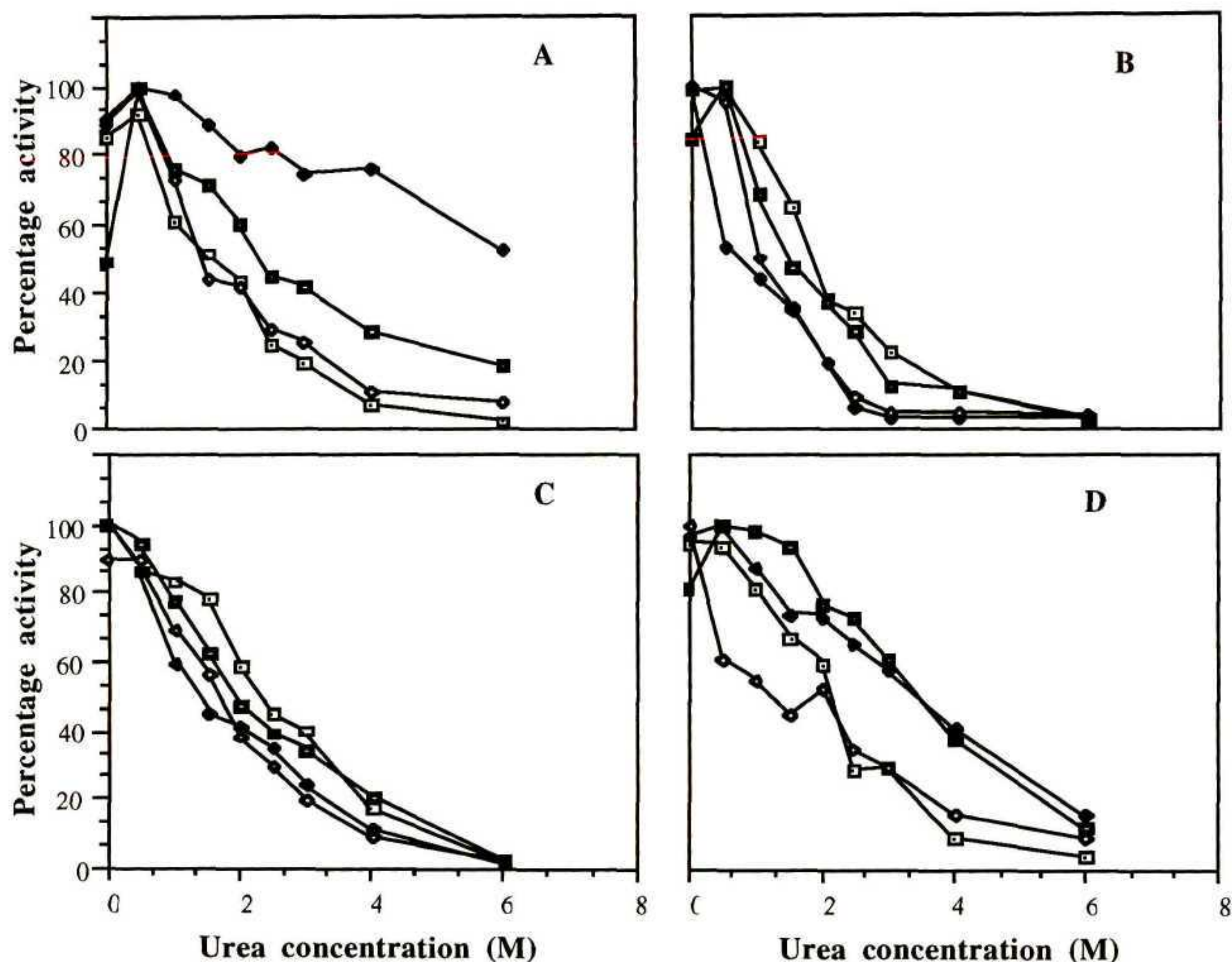


Figure 3.8. The effect of urea on the activity of cathepsin L and stefin B-complexed cathepsin L on Z-Phe-Arg-NHMec.

A, pH 5.5; B, pH 6.0; C, pH 6.5; D, pH 7.0.

❑	- cathepsin L,	50	mM cysteine;
■	- cathepsin L,	150	mM cysteine;
◇	- complex,	50	mM cysteine;
◆	- complex,	150	mM cysteine.

3.3.4 Difference fluorescence spectra

Free cathepsin L showed an emission maximum at 330 nm with a shoulder at 305 nm (Fig. 3.9). This is indicative of tryptophan and tyrosine fluorescence respectively, with possible transfer of excitation energy from tyrosine to tryptophan (Franks, 1988). An increase in urea concentration resulted in a red shift in the 330-338 nm region (typical of solvent exposed tryptophan [Knappskog and Haavik, 1995]) and the emergence of an emission peak at 390 nm, notably at urea concentrations above 1.5 M. This wavelength (390 nm) was consequently used to monitor emission

intensity in subsequent denaturation experiments, as the changes in spectral properties were greatest at this wavelength.

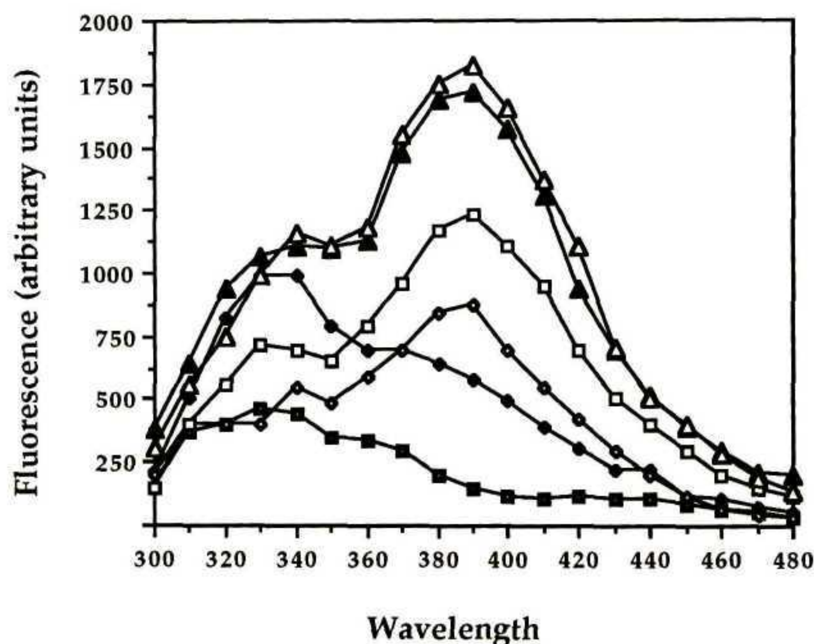


Figure 3.9. The effect of urea on the fluorescent emission of single-chain cathepsin L.

■ - 0 M urea; ◇ - 1 M urea; ❑ - 1.5 M urea; ◆ - 3 M urea,
Δ - 4 M urea and ▲ - 6 M urea. Excitation λ, 280 nm.

3.3.5 Denaturation and renaturation experiments

The urea-induced conformational changes in sheep liver cathepsin L, monitored by fluorescent emission at 390 nm, proceeded through two discernible transition states, denoted U1 and U2, between the native (N) and denatured (D) forms. Forms U1, U2 and D are obtained at urea concentrations of *ca.* 1.8 M, 3.8 M and 5.6 M respectively (Fig. 3.10). Renaturation experiments showed that the transition was apparently not exactly reversible below 3.5 M urea (Fig. 3.10). Similarly, treatment of a sample with 1.5 M urea, showed that the conformational transition was not reversible on dilution of the urea (Fig. 3.11).

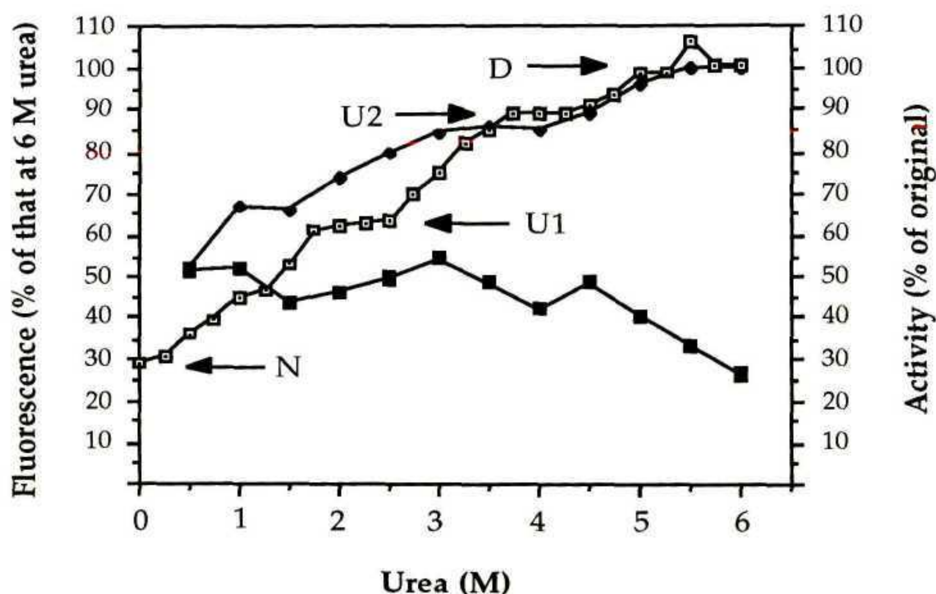


Figure 3.10. Denaturation of single chain cathepsin L with urea, up to 6 M.

Cathepsin L was treated for 10 h with urea of different concentrations, up to 6 M, and the fluorescence emission was read at 390 nm (excitation λ, 280 nm). Cathepsin L in 6 M urea was then diluted to the indicated urea concentration and the fluorescence, and activity against Z-Phe-Arg-NHMec, measured immediately.

❑ - denaturation curve (N, native conformation; U1 and U2, transitional conformations; D, denatured conformation); ◆ - renaturation curve upon dilution of urea from 6 M; ■ - activity after renaturation in the indicated concentration of urea. Activity is expressed as a percentage of the original activity before exposure to the urea.

Following treatment with 6 M urea for 10 h, 30% of the activity of cathepsin L, against Z-Phe-Arg-NHMec, remained (Fig. 3.10). Upon subsequent dilution of the urea, 50% of the original activity, before exposure to urea, was recovered (Fig. 3.10). Treatment under the same conditions with 1.5 M urea reduced activity to 80% of the original. Dilution of the urea completely restored the activity (Fig. 3.11), even though the original conformation was not regained.

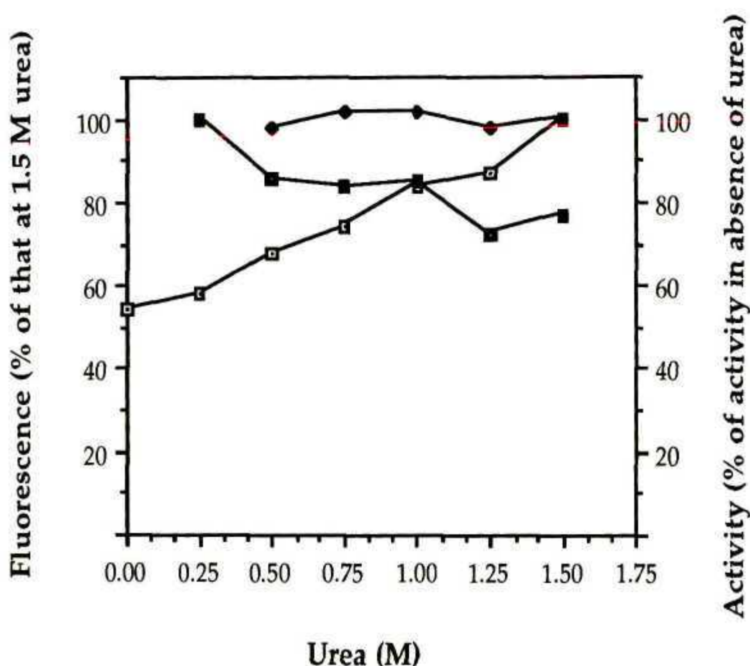


Figure 3.11. Denaturation of cathepsin L with urea, up to 1.5 M, and the reversibility thereof.

Cathepsin L was treated for 10 h with urea of different concentrations, up to 1.5 M. Cathepsin L in 1.5 M urea was then diluted to the indicated concentration and the fluorescence emission at 390 nm, and activity against Z-Phe-Arg-NHMec, was measured.
❑ - denaturation curve; ◆- renaturation curve upon dilution of urea from 1.5 M; ■ - activity after renaturation in the indicated concentration of urea.

3.3.6 Stability experiments

The stabilising effect of stefin B could be clearly observed (Fig. 3.12). The denaturation curve of cathepsin L in 6 M urea, in the presence of stefin B, was significantly reduced, compared to that in the absence of stefin B. The stabilising effect of azocasein in the presence of urea, could not be demonstrated, owing to the quenching effect of the azocasein (Fig. 3.12). The effect of casein could not be demonstrated, as the casein could not be solubilised (although it had been treated with alkali/acid to facilitate dissolution, followed by dialysis to remove the salt).

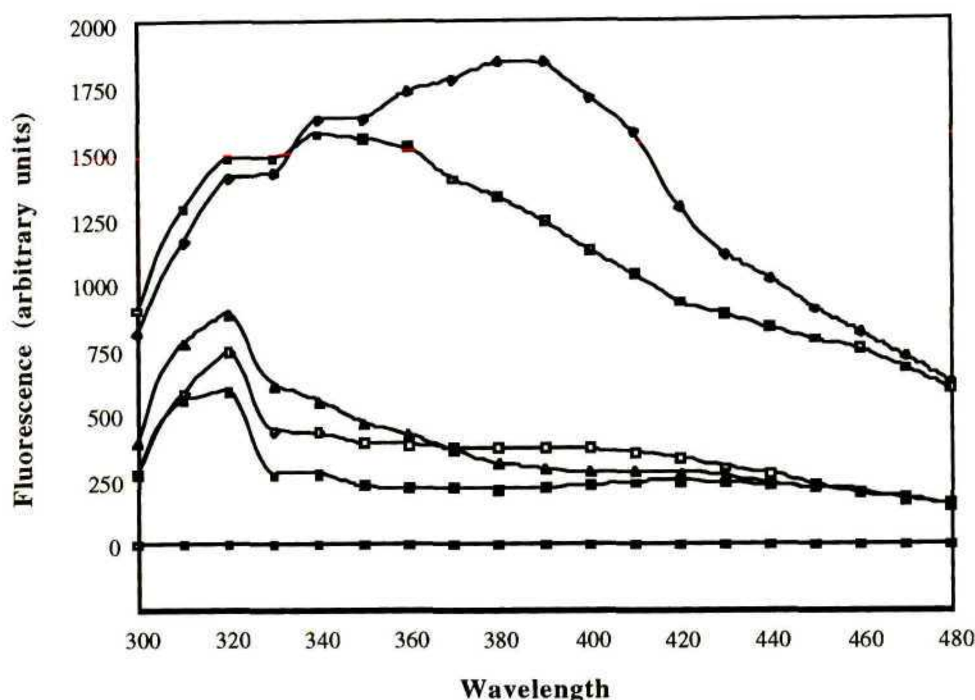


Figure 3.12. The effect of proteins on the denaturation of cathepsin L by urea.

Cathepsin L (10 ng) and stefin B (30 ng) were reacted together (2 min, RT) and diluted with AMT buffer, pH 5.5 (1 ml), in the presence (6 M) or absence of urea. The fluorescence was recorded at 300 - 480 nm (excitation $\lambda = 280$ nm).

□ - azocasein; ■ - cathepsin L; □ - stefin B; ▲ - cathepsin L-stefin B complex; ◇ - cathepsin L, 6 M urea; ❑ - cathepsin L stefin B complex, 6 M urea.

3.4 Discussion

This chapter detailed studies on the large-scale isolation of free and stefin B complexed cathepsin L for the complete enzymatic characterisation of cathepsin L, and investigation into the nature of the association between the covalent cathepsin L/stefin B complexes (Chapter 4). Cathepsin L (single-chain) and the stefin B-complexed cathepsin L forms (active and inactive) were isolated from sheep liver essentially as described previously (Pike, 1990; Kirk, 1992; Coetzer, 1992; Pike and Dennison, 1989b; Pike *et al.*, 1992) but in a scaled-up procedure.

The conventional isolation procedure compared favourably with that reported by Pike (1990) when the autolysis step was omitted following acidification to pH 4.2. However, yields (%) were considerably less when compared to isolations employing autolysis (pH 4.2, 25°C, 16 h), where yields were routinely between 7 and 15% (Kirk, 1992; Pike and Dennison, 1989a). The autolysis step had been found to be necessary

to activate cathepsin L, presumably by destroying endogenous cytosolic inhibitors such as cystatins A and B, although this mechanism is not thought to be proteolytic (Mason *et al.*, 1985). It should be noted that cathepsin L activity is responsible for less than 25% of the Z-Phe-Arg-NHMec hydrolysing activity (Mason *et al.*, 1985). Autolysis has been employed for the isolation of chicken liver cathepsin L (Dufour *et al.*, 1987), human kidney cathepsin L (Baricos *et al.*, 1988) and cathepsin L from human, rabbit, sheep and ox livers (Mason *et al.*, 1985). The autolysis step was thought initially to be instrumental in the production of proteolytically active (covalent) and normal inhibitory (non-covalent) complex, but it was shown by Coetzer (1992) that the autolysis step was not necessary for the formation of the complexes, nor did it dissociate either type of complex (both forms are still purified). Coetzer (1992) showed that omission of the autolysis step caused a two-fold increase in the formation of proteolytically active stefin B-complexed cathepsin L. Considering that this study was concerned with the isolation of both of these forms of cathepsin L, the autolysis step was omitted to allow for large scale purification of both. Treatment of the isolated complex to a pH 4.2, and subsequent SDS-PAGE analysis, showed that the 37 kDa band was converted to a 42 kDa band, an apparently contradictory effect. It was therefore speculated that the active, covalent complexes may be formed at a later stage of the purification (Coetzer, 1992).

The use of TPP in the isolation procedure, [shown to be superior to ammonium sulfate precipitation (Pike and Dennison, 1989a)] allowed the sample to be loaded immediately onto the cation exchanger, without prior desalting, as the salt concentration is low following TPP. TPP depends on the water-miscible nature of t-butanol, which, in the presence of a salt, separates into a solvent layer. If proteins are present they are precipitated into a third phase between the t-butanol and the aqueous layer. The protein is more or less specifically concentrated, and removed from other contaminating substances, such as pigments, phenolics, lipids and enzyme inhibitors (Pike and Dennison, 1989b). The efficiency of this procedure can be seen in the purification table (Table 3.1) which shows a marked increase in the purification following TPP, particularly in the scaled-up procedure. The employment of cation exchange, following TPP, exploits the ability of cathepsin L to bind strongly to cation exchangers at pH values close to its pI (Mason, 1986). Cathepsin L has been shown to have a pI of 5.5 - 6 (Kirschke *et al.*, 1980) and

therefore during the purification, will be neutral or slightly positively charged. The S-Sepharose step shows a noteworthy increase in purification when compared to the results of Mason *et al.* (1985), in the purification of human cathepsin L, and is a cost-effective low pressure substitute for the FPLC Mono-S system.

Scaling-up of the cathepsin L method of Pike *et al.* (1992) affords a rapid and effective method of cathepsin L isolation. The scaled-up procedure resulted in increased yields of both free cathepsin L and stefin B-complexed cathepsin L (Table 3.1) when compared to the conventional isolation procedure. Significantly higher amounts of protein (4.93 and 5.14 mg of complexed and free cathepsin L) were isolated from the scaled up procedure when compared to the normal protocol (0.45 and 0.55 mg respectively). Note that the scaled-up procedure uses approximately three times the amount of starting material, resulting in approximately a ten times increase in yield compared to the normal protocol. From the purification table (Table 3.1), it is apparent that the improved yields are attributable to improved molecular exclusion chromatography on Sephadex G-75. The scaled-up procedure employed a longer, wider column (5 x 134 cm), which allows a greater sample volume to be loaded, resulting in an improved difference in the elution volumes (V_e), sufficient to allow complete resolution (and separation) of the complexed and free cathepsin L. From these results, it would appear that the critical step in the purification is efficient resolution during MEC.

This purification method yields largely single-chain cathepsin L, which differs from the two-chain cathepsin L isolated from sheep (Mason, 1986) and human liver (Mason *et al.*, 1985). Dufour *et al.* (1987) isolated a single-chain form (27 kDa) from chicken liver lysosomes by a rapid procedure, in contrast to Wada and Tanabe (1986) who isolated a two-chain form by a longer procedure, from the same source. Interestingly, Bando *et al.* (1986) isolated a mixture of single-chain (30 kDa) and two-chain (25 kDa and 5 kDa) cathepsin L from rat kidney lysosomes, although only the single-chain form could be detected by Western blots of the homogenates. Dalet-Fumeron *et al.* (1991) isolated the single and two-chain form of cathepsin L from human liver, using a rapid three-step HPLC isolation procedure. These results have led to speculation about the *in vivo* existence of the two-chain form, with many authors postulating that the two-chain form of cathepsin L may be a consequence of limited proteolysis during the autolysis step (Kirschke *et al.*, 1977; Mason, 1986;

Bando *et al.*, 1986). However, the procedure employed by Kirschke *et al.* (1977), isolated two-chain cathepsin L directly from rat liver lysosomes, while that employed by Bando and co-workers (1986) isolated a mixture of single and two-chain forms from the same source. The occurrence of the two-chain form therefore, seems to correlate more with the length of the isolation procedure rather than the presence of an autolysis step.

The conversion of high molecular weight forms of cathepsin L to lower molecular weight forms has been demonstrated (Nishimura *et al.*, 1988a; 1989) where rat liver procathepsin L (37 kDa) was converted to a single-chain form of 30 kDa by an aspartic protease, while the rapid conversion to the two-chain form was processed by an unidentifiable protease (Nishimura *et al.*, 1988b). Hara *et al.* (1988) showed that a similar conversion from the pro-form to the mature form was metalloprotease dependent, while conversion to the two-chain form could be inhibited by a cysteine protease inhibitor. Wiederanders and Kirschke (1989) showed that in rat fibroblasts, the conversion of pro-form to the mature form was accelerated by cathepsin D, although no further processing to the two-chain form was evident. In NIH 3T3 cells (murine fibroblasts), the conversion of the pro-enzyme to the mature enzyme was shown to be cathepsin D dependent, while subsequent processing to the two-chain form was thought to be cathepsin L dependent (Salminen and Gottesman, 1990). Procathepsin L secreted from mouse fibroblasts was also shown to be converted to the mature form by contact with negatively charged particles such as dextran sulfate at the physiological lysosomal pH 5.5 (Mason and Massey, 1992). Procathepsin L from guinea pig sperm was also shown to be autoactivated at pH 3 (McDonald and Kadkhodayan, 1988). Smith and Gottesman (1989) showed that procathepsin L, expressed in *E. coli*, was autoprocessed to the single-chain form (29 kDa) after gel filtration at acidic pH (pH 6.5). Work conducted on recombinant human procathepsin L, expressed in mouse myeloma cells, showed that the autocatalytic conversion to the active form was intramolecular (Nomura and Fujisawa, 1997). McDonald and Emerick (1995) showed that guinea pig procathepsin L, from spermatozoa, readily self-processed to the mature single chain form by an intramolecular event (which they deduce from a non-sigmoidal linear progress curve). This was accelerated in the presence of dextran sulfate approximately 3.6 fold. In more recent work (Ménard *et* al., 1998), the autocatalytic processing of

recombinant human procathepsin L was shown to be intermolecular, initiated by decreasing the pH from pH 8.0 to 5.3, to form the single-chain enzyme. Ritonja *et al.* (1996) showed that the isolation of cathepsin L from sheep liver at low pH (pH 4.5 as opposed to pH 5.0) selectively increased the amount of two-chain cathepsin L, suggesting that the conversion of single chain to the two-chain form may be a result of autocatalysis.

From the literature, it is evident that the processing of procathepsin L to the mature form (single chain) relies on a cascade of proteolysis, which appears to be cell type and pH/charge dependent. The conversion of the single chain to the two-chain form, however, may be an artefact of a lengthy purification procedure, although this process also appears to be cell type and pH dependent. The recently elucidated crystal structure of procathepsin L indicates that the major cleavage sites for processing of the pro-form are situated near the N-terminus of the mature enzyme (the C-terminus of the pro-fragment) and correspond to recognised cleavage residues for cathepsin L (Lys-Val-Phe, Val-Phe-Gln, Leu-Phe-Tyr and Phe-Tyr-Glu). On examination of the ribbon structure (Ménard *et al.*, 1998) these sites are accessible to cleavage by an intermolecular event, as they appear to be situated far away from the active site triad (for intramolecular processing). Nonetheless, the isolation protocol employed here ensures the rapid separation of cathepsin L from other proteinases allowing the isolation of single-chain cathepsin L.

From the molecular exclusion chromatography results, it is evident that stefin B complexed cathepsin L is not notably dissociated in 8 M urea. Thus, contrary to expectations, chromatography in 8 M urea proved not to be a practicable method for the separation of the covalent and non-covalent complexes. Nevertheless, the increase in azocaseinolytic activity occasioned by urea was intriguing and the effect of urea was consequently studied further, in the hope of gaining insight into the phenomenon and what it may reveal about cathepsin L.

The stability of the non-covalent complex to 8 M urea implies that the association of the two molecules is significantly robust to withstand MEC in the presence of the chaotrope. The 37 kDa complex, when subjected to non-reducing SDS-PAGE, manifests bands at 37 kDa, and 26 kDa and 14 kDa. This suggests that the 37 kDa complex is composed of dissociable and non-dissociable species which may be

distinguished by non-reducing SDS-PAGE. This SDS-PAGE "dissociable" non-covalent complex is apparently destroyed by SDS-PAGE, suggesting that the presence of SDS, or an electromotive force, breaks the association between cathepsin L and stefin B. The "non-SDS-PAGE dissociable" complex may, however, be dissociated by **reducing** SDS-PAGE, which suggests that the bond between the stefin B and the cathepsin L may be a disulfide bridge (Pike *et al.*, 1992; Coetzer *et al.*, 1995). [Preparative non-reducing SDS-PAGE was subsequently used as a means of isolating the covalent complex from the non-covalent complex (Chapter 6)]. In both forms of complex (SDS-PAGE dissociable and non-SDS-PAGE dissociable), however, the association between the two molecules can withstand the chaotropic effect of urea. Proteins in 8 M urea normally approach a randomly coiled conformation (provided their disulfide bonds are broken) which is reversible (Pace, 1986). Although, in this instance the disulfide bonds are not broken, this suggests that the associations are well secured or deeply anchored within the conformation of the molecule to avoid the destabilising effects of the urea.

Noteworthy is the significant increase in azocaseinolytic activity, especially of the complex fraction, following molecular exclusion in 8 M urea. This is supported by the results shown in Fig. 3.7, that increasing concentrations of urea increased the activity of free and complexed cathepsin L. The activity of the free cathepsin L was increased 0.5-fold and that of the complex 3-fold. It has been suggested that urea may sensitise azocasein to hydrolysis (Kirschke *et al.*, 1984) or that it may increase the solubility of cleaved azocasein peptides (Mason *et al.*, 1984). Either of these may explain the apparent enhanced activity of cathepsin L in the presence of 8 M urea but neither explains the asymmetric increase in activity between free cathepsin L and complexed-cathepsin L. The differential effect of urea upon the activity of free cathepsin L against azocasein (Fig. 3.7) and against Z-Phe-Arg-NHMec (Fig. 3.8), also requires explanation.

It is noted that 8 M urea is apparently unable to dissociate the non-covalent complex of stefin B and cathepsin L. This suggests that multiple-site binding of stefin B might stabilise cathepsin L against the chaotropic effect of urea. In a similar manner it may be envisaged that multiple-site binding of a polymeric substrate, such as azocasein, may draw the cathepsin L into an active conformation, in a manner which a small substrate such as Z-Phe-Arg-NHMec is unable to do. Clearly, in the absence of

substrate, urea induces a conformational change in cathepsin L, perhaps making it more flexible. Co-operative multi-site binding of a macromolecular substrate might thus be able to readily induce a fit to the substrate, with little change in energy state, and this may be reflected in the enhanced activity in urea. Pre-binding of stefin B in the active complex may define part of the structure, while leaving other parts flexible. This may make attainment of the active conformation more likely, as reflected in the greater enhancement of activity in the case of the complex. This suggests that the binding of stefin B to cathepsin L (in the active complex), not only facilitates increased activity, but also conformational stability, as verified by the results presented in Fig. 3.12.

The increased activity of the complex (though not in urea) has been previously published (Dennison *et al.*, 1992) suggesting that the binding of stefin B acts as an activator, similar to the effect of increased cysteine (or reducing agent). These results, along with the observation that reducing SDS-PAGE destroys the active 37 kDa complex, suggest that the linkage between cathepsin L and stefin B is a covalent linkage. The nature of this bond will be discussed in further detail in Chapter 4.

The above explanation is plausible with respect to the macromolecular substrate azocasein. The results obtained using the synthetic substrate assays should be considered separately. Owing to the limited size of the substrate, it is unlikely that urea enhances solubilisation of the molecules resulting in increased exposure before cleavage, or increased solubility of the fluorescent leaving group. At pH 5.5, the optimal pH for activity and stability of cathepsin L, 0.5 M urea results in increased activity. At this concentration, sufficient unravelling or extending of the enzyme may have occurred to increase the exposure of the active site to increased turnover of substrate, without denaturation of the active tertiary conformation. Elevated concentrations of activating reagents (150 mM cysteine) effects additional stabilisation of the complex (where 60% of the activity is retained in 6 M urea) and free cathepsin L (20% residual activity in 6 M urea). A more "presented" active site might require higher amounts of cysteine for continued reduction and activation. Reduction is necessary for stability (Dehrmann *et al.*, 1996 and Chapter 5) because in the reduced state, the active site conformation is retained. At pHs 6.0 and 6.5, increasing urea concentrations caused a decrease in activity, suggesting that the tertiary conformation of the active site could not be maintained under these

conditions. At pH 7.0 this decrease in activity was also seen although some residual activity remained in all preparations except for free cathepsin L (50 mM cysteine). This suggests that there is a slight difference in the stability of the enzymes between pH 6.5 and pH 7.0. This may be due to deprotonation of the active site imidazole moiety. Turk *et al.*, (1994) have proposed a mechanism for the irreversible denaturation of cathepsin B which is due to this deprotonation mechanism, followed by the subsequent "unzipping" of the active site conformation. This could explain the reduced stability and activity at the higher pH values.

The denaturation curve of the fluorescent emission of single chain cathepsin L at increasing concentrations of urea, showed a biphasic step increment, suggesting that the denaturation of cathepsin L proceeds through two transitional conformational states before reaching full denaturation. Renaturation (upon dilution of urea) showed that cathepsin L does not return to the native state, but remains in transitional state U1, even when the urea has been diluted to 0.5 M. In this conformation, cathepsin L retains 50% of its activity. The denatured state (D) of cathepsin L retained 20% of its activity, while U2 retained 30 - 40% activity (Fig. 3.10). When denatured with 1.5 M urea, cathepsin L conformation could not be restored to the native state, suggesting that an irreversible transition had occurred. However, activity could be fully restored upon dilution of the urea. This suggests that the native conformation of the enzyme is not necessary for full catalytic activity. It may be speculated that the active site groove of cathepsin L is maintained despite the unfolding to the first transitional state. Although the 3-D crystal structure of mature cathepsin L is not known, by analogy with papain, the active site groove of cathepsin L would appear to be lined by aromatic amino acids, creating a hydrophobic micro-environment (Drenth *et al.*, 1971). It appears that the active site is sufficiently protected from the denaturing effects of 1.5 M urea, but as this concentration is increased, so the hydrophobic interactions are destroyed and the active site conformation is altered, resulting in the U2 and D forms. The results of the renaturation studies with cathepsin L suggest the following simple model:

$$N \rightarrow U1 \leftrightarrow U2 \leftrightarrow D$$

where U1 represents an intermediate conformation distinct from the native state, which retains full kinetic activity. This suggests a separate transitional state in the

case of cathepsin L, as with cathepsin D, where a 40% increase in activity is observed in 0.5 M guanidinium chloride (Lah *et al.*, 1984) which was not reversed on removal of the denaturant (assayed against the proteinaceous substrate haemoglobin). This is in contrast to goat spleen cathepsin B, which shows no stimulatory effect with urea, even at low concentrations of 0.5 M (assayed against Bz-Arg-NH-Nap, at 37°C, 20 mM Na-phosphate buffer, pH 6.5), and shows completely reversible denaturation with 1.5 M urea, but not with 6.4 M. It must be noted that urea and guanidine hydrochloride apparently have differential effects, owing to the ionic nature of the latter (Ward *et al.*, 1995), a consideration in light of the effect of buffer ionic strength on the stability and activity of cathepsins L and B (Chapter 5).

The results presented in this chapter, showing the increased stability of free and complexed cathepsin L, to the chaotropic effect of urea, suggest that the catalytic site of cathepsin L is stabilised and effectively "presented" by the pre-binding of macromolecules (such as large proteinaceous substrates). This phenomenon could explain the apparent increased activity of the active stefin B-cathepsin L complex compared to free cathepsin L at equimolar concentrations (Dennison *et al.*, 1992).

The ability of stefin B to complex with cathepsin L forming an active complex raises the question of the importance of the single chain nature of cathepsin L, compared to the two chain form conventionally isolated. To date, no other research has identified this complex, which suggests that the single chain form of cathepsin L could be the only form of cathepsin L capable of forming this complex. The mode of association between cathepsin L and stefin B will be revisited and discussed in Chapter 4.

The results presented in this chapter suggest that cathepsin L is more stable, both to urea and to physiological pH values, than previously thought and has a higher pH optimum for activity than previously believed. In the light of these findings, it would appear that its properties do not preclude cathepsin L from playing a role, extracellularly, in normal conditions such as tissue remodelling and in pathologies such as invasive cancer.

CHAPTER 4

THE COVALENT BOND BETWEEN CATHEPSIN L AND STEFIN B

4.1 Introduction

The existence of an apparently novel, proteolytically active, complex of cathepsin L and stefin B described in Chapter 3, raised the question of the nature of the interaction between the two molecules in these complexes.

Stefin B (a type I cystatin) and the chicken cystatins (type II) have been shown to form inhibitory, tight-binding, non-covalent, reversible complexes with papain (Stubbs *et al.*, 1990; Björk and Ylinjärvi, 1989). By comparison with the X-ray crystal structure of chicken egg white cystatin (Bode *et al.*, 1988), the stefin family has been shown to contain a contiguous "wedge", consisting of the amino terminal segment Gly-9→Ala-10, a β-hairpin loop containing the conserved QVVAG region, and a second hairpin loop composed of the doublet Pro-103→Trp-104. This "wedge" was shown to be complementary to the active site groove of papain as illustrated in Fig.4.1.

In an elegant work, Stubbs *et al.* (1990) showed that recombinant human stefin B (mutant C8→S8) binds to papain with a 1:1 stoichiometry, in a manner which is fundamentally different to that observed for serine proteinase inhibitors. The two hairpin loops of the stefin B form extensive hydrophobic interactions along the "primed" sites of the papain, and the amino-terminus interacts with the "unprimed" region of the surface of the enzyme, acting like a docking trunk. The amino terminal segment interacts with the active site of papain, with Leu-7, Leu-8 and Gly-9 occupying the S_3, S_2 and S_1 subsites respectively, with a tight turn between Leu-7 and Ala-10 necessitating the conservation of Gly-9. In this manner, the Gly-9→Ala-10 doublet is sufficiently removed (and in an inappropriate conformation) to preclude catalytic attack from the active site Cys-25. This model explains why complex formation can still occur when the active site of papain is blocked by small thiol binding reagents (Björk and Ylinjärvi, 1989); the inhibitor forms a "curtain" across the active site of the papain molecule, preventing substrates from binding in the active site for catalytic processing. In this model, the inhibitor complex should have

no enzyme activity as the inhibitor occupies the substrate binding sites, through side chain interactions (Abrahamson, 1993) at the hydrophobic interface.

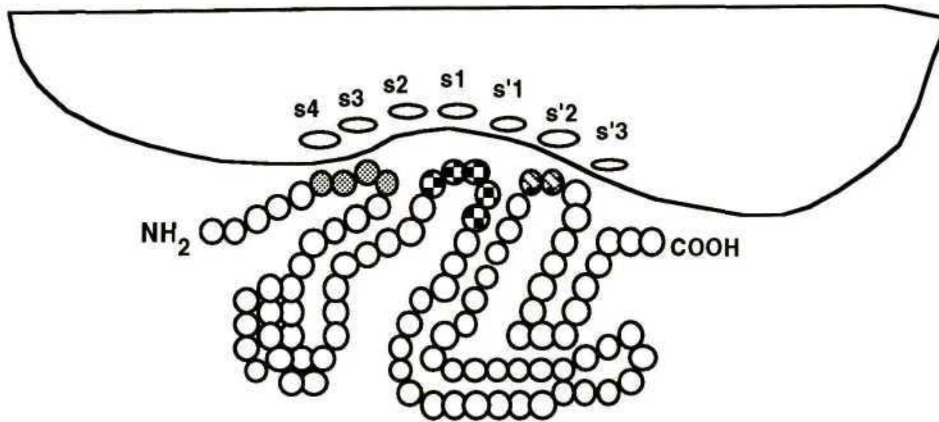


Figure 4.1. A schematic representation of the interaction between type 2 cystatin and a thiol protease (Abrahamson, 1993).
The active site of the enzyme is shown above, while the shaded regions of the cystatin represent residues 6-9, the conserved QVVAG, and residues 105-106. These regions form a tripartite "wedge" with good complementarity to the active site groove.

Some evidence suggests that the central conserved QVVAG region is not essential for this inhibition, as stefin B with mutations in the QVVAG region still inhibited thiol proteases (Jerala *et al.*, 1990). Furthermore, in sheep and bovine stefin B, the conserved region is QVLAG, and this appears to have little effect on the K_i of the normal inhibitory interactions (Coetzer, 1992). Truncation at the N-terminal region of oryzacystatin appears to have little effect on the inhibition of papain (Abe *et al.*, 1991), although chicken cystatin truncated in the N-terminal region manifests a loss of inhibition (Abrahamson *et al.*, 1987). Yamamoto *et al.* (1992) conducted experiments on the interaction between succinyl-Gln-Val-Val-Ala-Ala-p-nitroanilide (a potent, reversible, non-competitive inhibitor of papain) and papain. They suggested that the conserved sequence, QVVAG, itself is not necessarily inhibitory, but rather the tertiary structure which it forms (the β-hairpin loop), and the spatial orientation of the N-terminal region are responsible for inhibition. This interaction is therefore possible with both the type 1 and type 2 cystatins

The formation of the inhibitor complex between papain and chicken egg-white cystatin has been shown to be pH-dependent, occurring optimally at pH 6-8 (Björk

et al., 1989). The reaction of recombinant human cystatin C and the cysteine proteases, papain and actinidin, have been shown to be similar to that of papain and chicken egg white cystatin (Lindahl *et al.*, 1992), forming at pH 7.4 and causing inactivation of the enzyme in a manner highly similar to that of stefin B with papain. Turk *et al.*, (1993) have shown that, under acidic and neutral conditions, inhibitory complexes form between cathepsin L and stefin B which may be dissociated by dilution of the complex and addition of substrate. They demonstrated that catalytically active cathepsin L may be released from a complex with stefin B, under pH conditions where the cathepsin L would usually be inactivated.

These findings have led numerous workers to speculate on the purpose of this complex formation. These complexes may form a "reservoir" system of control (Bieth, 1980), whereby, on cellular destruction and release of the lysosomal proteases into the neutral extracellular milieu, extracellular cystatins complex with the proteases, inhibiting their activity. The proteases may subsequently be released (by some signal mechanism) under conditions where dissociation is favoured, and where they can perform a biological function (Turk *et al.*, 1993).

The novel, proteolytically active, complex apparently forms during isolation. This complex is active against Z-Phe-Arg-NHMec and azocasein, and against gelatin in a substrate-containing PAGE gel (under non-reducing conditions). E-64 titration typically shows the isolated complex to be about 60% active. The complex shows significant activity and stability at pH 7, especially in the presence of elevated (150 mM) cysteine levels (Dennison *et al.*, 1992). Complex, which shows proteolytic activity when analysed by substrate PAGE, may also be formed *in vitro*, by the addition of cathepsin L (or papain) to stefin B (or chicken cystatin, stefin A and kininogen domain 2) in a 1:2 stoichiometry, (Pike, 1990; Dennehy, 1994). Formation of the complex *in vitro* is pH-dependent, forming only above pH 5.5, and its formation is apparently enhanced by DTT (Pike, 1990).

The fact that this novel, active complex could be dissociated by treatment with mercaptoethanol, prior to SDS-PAGE, hinted at the association perhaps being a disulfide linkage between the cathepsin L and the stefin B (Pike, 1990; Coetzer, 1992; Dennison *et al.*, 1992; Pike *et al.*, 1992). This would require the presence of a cysteine residue in the stefin B molecule. In sheep, human and bovine stefin B, there is a

single Cys residue, at position 3 (Ritonja *et al.*, 1996), which could conceivably be involved in this interaction. Dennehy (1994) attempted to demonstrate the formation of active complex between cathepsin L and stefin A [which lacks an exposed cysteine residue (Machleidt *et al.*, 1983)] and chicken cystatins (which vary in amino terminal truncation and contain no exposed cysteinyl residue [Bode *et al.*, 1988]). He showed the occurrence of low levels of proteolytically active complexes at molar ratios of cathepsin L to stefin/cystatin of 1:10. In direct contrast to this, however, Coetzer (1992) found that coincubation of cathepsin L with chicken cystatin (1:2) did not result in the formation of active complex. Because the active complex had only been reported from this laboratory, it was postulated that the complex may form only with single chain cathepsin L. Papain, a single chain cysteine protease, was consequently reacted with sheep stefin B, and chicken cystatin I and II, and appeared to form active complexes when analysed by substrate PAGE (Dennehy, 1994).

The aim of the study reported in this chapter was to determine the position and the nature of the link between cathepsin L and stefin B in the isolated covalent complex. The amino acid sequences of sheep cathepsin L and stefin B are known (Ritonja *et al.*, 1996). Sheep cathepsin L contains 7 cysteine residues, with the active site cysteine (Cys-25), being free. By analogy with papain, the remaining cysteine residues may be linked by intramolecular disulfide bridges, i.e. C22–C63, C54–C95, and C154–C200. By mapping the CNBr-cleavages at Met residues, in the absence of reduction (and, at the outset, using the null hypothesis that the two molecules may be associated by a disulfide bridge) putative cyanogen bromide cleaved fragments of the complex were determined (Figure 4.2). The null hypothesis suggests a link involving the Cys-3 residue of the stefin B. Fortuitously, the only two methionine residues in sheep stefin B occur at the N-terminus, prior to the single cysteine residue envisaged to be involved in the disulfide link. This means that cleavage of the stefin B should occur before the putative link. Therefore the stefin B should remain attached to a fragment of cathepsin L, and serve as a good indicator.

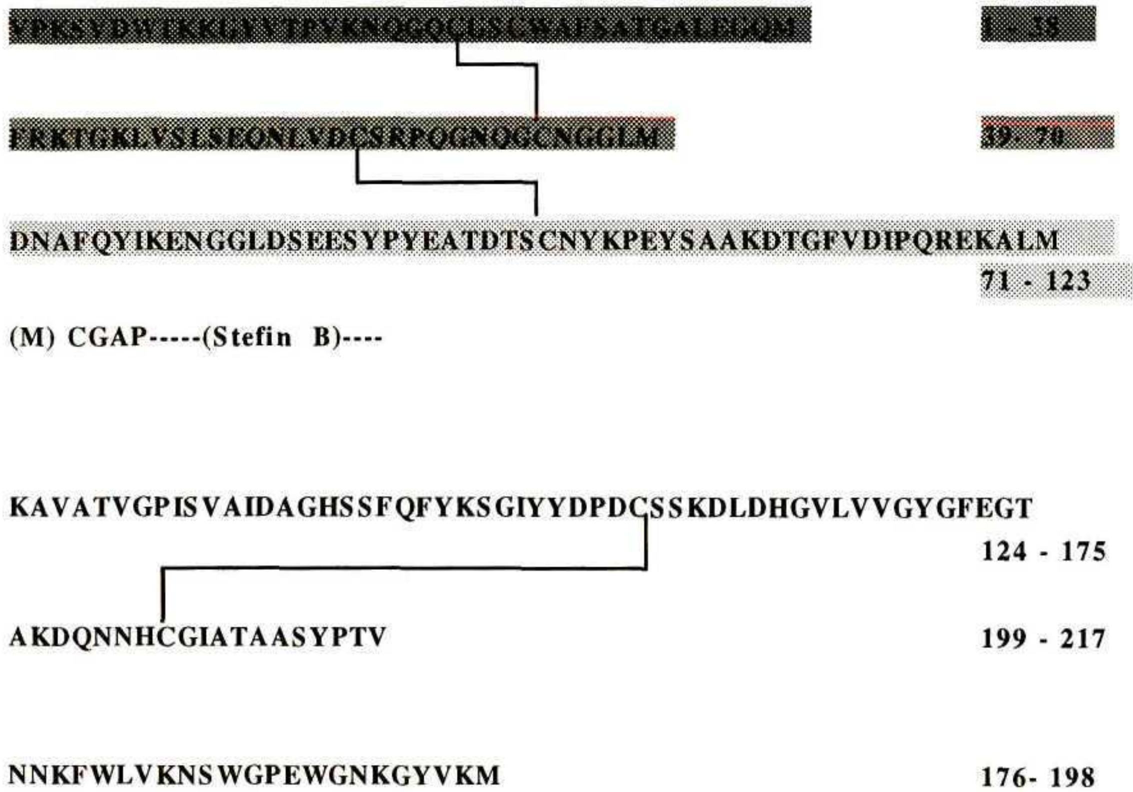


Figure 4.2. Putative fragments of cathepsin L complexed stefin B obtained by cyanogen bromide cleavage.

The molecular masses of these fragments were calculated to enable their identification following SDS-PAGE.

Table 4.1. Molecular masses of peptide fragments generated by cyanogen bromide cleavage of cathepsin L.

Fragment	Molecular mass (kDa)
1 - 38	4056
39 - 70	3434
71 - 123	5995
124 - 175	5563
176 - 198	2795
199 - 217	1959
(1 - 38)+	
(39 - 70)+	
(71 - 123)	13485
(124 - 175)+(199 - 217)	7522
Stefin B	11150

4.2 Materials and Methods

4.2.1 Cyanogen bromide cleavage

4.2.1.1 Reagents

Cyanogen bromide (analytical grade) was from Merck (Darmstadt, Germany). Formic acid, acetone and trifluoracetic acid (Spectrosol grade) were from BDH Laboratories (Poole, England). Cathepsin L-stefin B complex was isolated as described in Section 3.2.1. In all instances, Ultrapure Milli-Q water was used.

100% (m/v) Trichloracetic acid. Trichloracetic acid (100 g) was dissolved in ultrapure Milli-Q water, made up to 100 ml, and stored in an amber bottle.

70% (v/v) Formic acid. Redistilled formic acid (700 µl) was added to 300 µl of ultrapure Milli-Q water. This reagent was made fresh, before each cleavage.

20 µg/ml Cyanogen bromide solution. Cyanogen bromide (0.02 mg) (weighed in a fume hood) was dissolved in 70% formic acid (1 ml). This reagent was made fresh, before each cleavage, and excess was disposed of in special waste containers.

6 M Guanidinium chloride, 0.05% trichloracetic acid. Guanidinium chloride (5.73 g) was dissolved in Milli-Q water (6 ml), 100% (m/v) trichloracetic acid (5 µl) was added, and made up to 10 ml.

4.2.1.2 Procedure

Active (determined by activity against azocasein and Z-Phe-Arg-NHMec) stefin B complexed cathepsin L (33-50 µg, in 1.5-2 ml) and cathepsin L (20-30 µg) were precipitated by the addition of 100% TCA solution to a final concentration of 10%, in eppendorf tubes. The mixtures were incubated at 0°C (30 min) and centrifuged (12 000 x g, 5 min, 4°C). The resulting transparent pellet was washed with cold acetone (100 µl) and centrifuged (12 000 x g, 5 min, 4°C), this procedure being repeated. The white, rubbery pellet was redissolved in 70% formic acid (100 µl). The cyanogen bromide solution was added to this to give a 100 x mass excess of cyanogen bromide over protein. The tubes were capped with nitrogen (to replace oxygen), sealed and reacted for 16 h in the dark. The reaction mixtures were

evaporated with nitrogen gas (3 h) to remove residual formic acid and cyanogen bromide, and diluted with Milli-Q water (1 ml), frozen in liquid nitrogen, and freeze-dried overnight. The lyophilisate was dissolved in 6 M guanidinium chloride, 0.05 % trifluoracetic acid solution (100 µl), prior to HPLC separation and SDS-PAGE analysis and Western blotting.

4.2.2 HPLC separation of cyanogen bromide cleaved fragments

4.2.2.1 Reagents

Acetonitrile 190 (far UV) was from Romil, distributed by Microsep Johannesburg. The chromatography column was a Phenomenex Selectosil 5 µ, 300 Å, C8 reverse phase column (3.2 x 250 mm), fitted with a guard column (3.2 x 30 mm).

Solvent A (0.1% trifluoracetic acid). Trifluoracetic acid (500 µl) was added to Milli-Q water (499.5 ml). The solution was degassed with sonication under vacuum (5 min).

Solvent B (80% acetonitrile, 0.1% trifluoracetic acid). Acetonitrile (400 ml) was diluted to 499.5 ml with Milli-Q water and trifluoracetic acid (500 µl) was added. The solution was degassed with sonication under vacuum (5 min).

4.2.2.2 Procedure

HPLC was performed on a Millipore Waters system. (Waters 501 pumps, WISP 710B and Waters 994 photodiode array detector), using Millennium software. The column was pre-equilibrated with 90% solvent A, 10% solvent B. The chromatography conditions were as follows, all at a flow rate of 0.5 ml/min:-

0-10 min:	90% solvent A,
10-55 min:	10 - 70% solvent B in 45 min,
55-65 min:	70% solvent B,
65-75 min:	70 - 10% solvent B.

Detection was at 214 nm. The column was conditioned by one gradient prior to application of the sample. Cyanogen bromide cleaved complex, redissolved in guanidinium hydrochloride (Section 4.2.1.1) (100 µl) was loaded onto the column. Fractions were collected manually in eppendorf tubes, frozen in liquid nitrogen and

freeze dried. The peaks were analysed by non-reducing SDS-PAGE and Western blotting (as described in Section 2.8). Rabbit anti-stefin B antibody was a gift from Dr T. Coetzer, Biochemistry, UNP, and rabbit anti-cathepsin L antibodies were raised as outlined in Chapter 6. Secondary antibody enzyme conjugate (Goat anti-rabbit IgG-alkaline phosphatase) was from Sigma (A-0418) and used at the recommended working dilution of 1:5000.

4.2.3 N-terminal amino acid sequencing of cyanogen bromide fragments

4.2.3.1 Reagents

Problott PVDF membranes, from Applied Biosystems (product 400944), were a gift from Dr Roy Geddes, University of Auckland, New Zealand. All sequencer grade reagents were from Applied Biosystems.

4.2.3.2 Procedure

Following SDS-PAGE analysis of the peaks, the gels were blotted onto PVDF membranes, previously moistened with methanol, (as described in Section 2.5.1) and stained with amido black (Section 2.7.1). Bands of interest were excised and N-terminally sequenced (5 cycles) on a Perkin Elmer Procise Automated Protein Sequencer, using the Pulsed Liquid Cycle.

4.3 Results

4.3.1 Cleavage of cathepsin L with cyanogen bromide

Cathepsin L cleaved with cyanogen bromide gave two peaks when separated by HPLC (Fig. 4.3). The second peak contained a shoulder, which could not be resolved from the main peak by changes in the flow rate or gradient (data not shown). The peaks were collected in fractions I - V, with peak 1 corresponding to fraction I, fraction II was the trough between the 2 peaks, and peak 2 divided into fractions III - V. Analysis of the fractions I - V on non-reducing SDS-PAGE showed that peak 1 contained a fragment of approximately 14 kDa, while peak 2 contained two low molecular weight fragments (7 and 9 kDa) and one larger component (30 kDa) (Fig. 4.4).

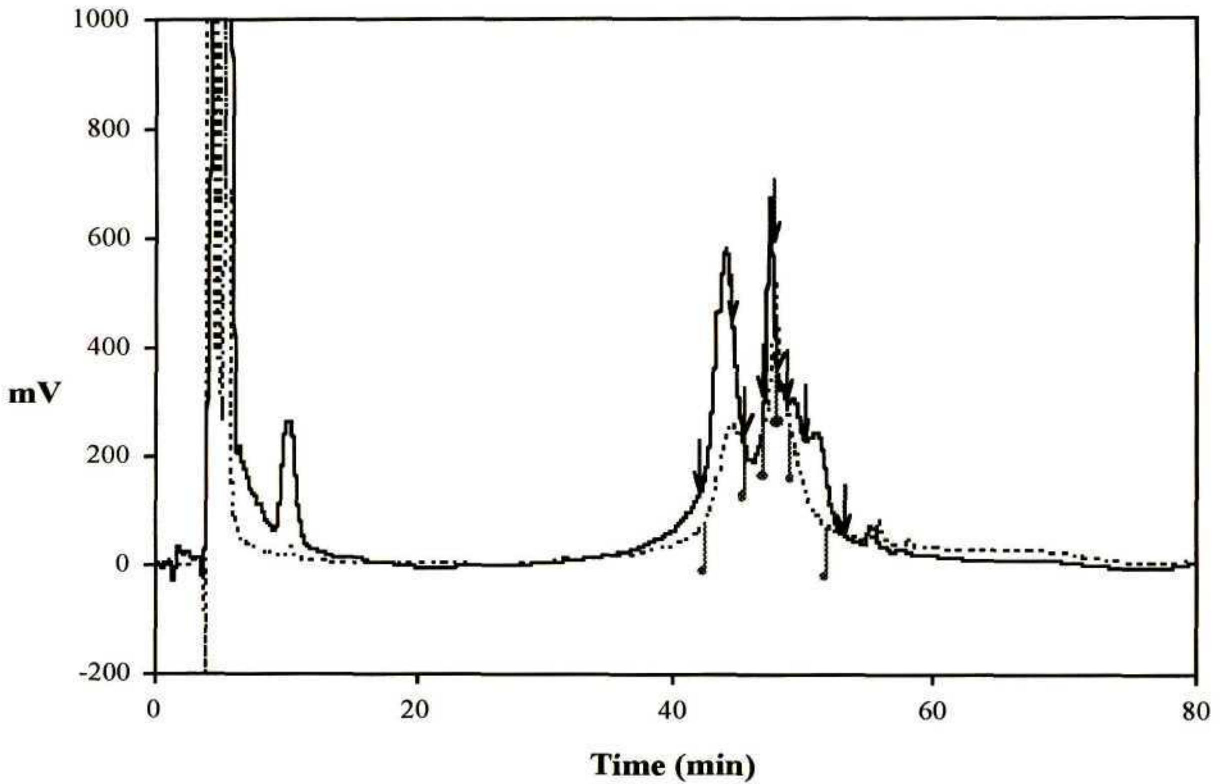


Figure 4.3. Chromatographic separation of cathepsin L and complex fragments generated by cleavage with cyanogen bromide.
(....), cathepsin L; (—), stefin B complexed cathepsin L. Anchors delineate the fractions which were collected manually.

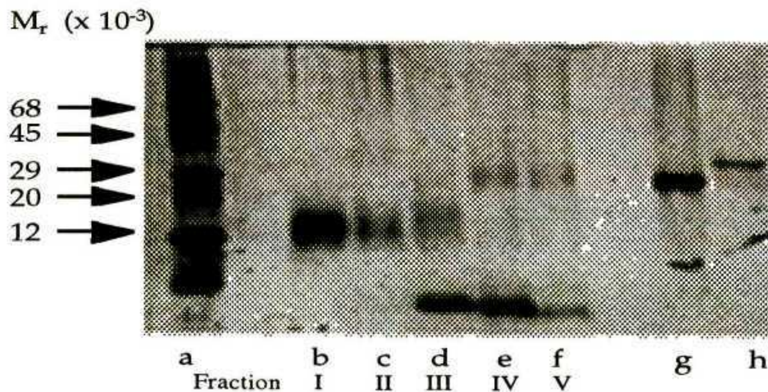


Figure 4.4. Non-reducing SDS-PAGE analysis of CNBr-fragments of cathepsin L separated by HPLC separation and complex fractions.
(a) Molecular weight markers (BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa, soybean trypsin inhibitor, 20 kDa and cytochrome C, 12 kDa), (b) - (f) - Fractions collected manually from HPLC (10 µl), designated I - V, (g) cathepsin L (200 ng), (h) cathepsin L-stefin B complex (200 ng), performed on a 10 % Tris-tricine gel, and silver stained as outlined in Section 2.6.2.

Blotting of these fragments onto nitrocellulose, followed by detection with rabbit anti-cathepsin L antibody, showed no recognition of any fragments (results not shown). N-terminal analysis of peak 1 identified three N-termini, which by comparison to the known sequence of sheep cathepsin L were identified as VPKS, FRKT and DNAF. These correspond to the N-termini of fragments 1-38, 39-70 and 71-123 respectively (Fig. 4.2). From peak 2, both the 7 and 9 kDa fragments contained two N-termini, KAVA and AKDQ, i.e. fragments 124-175 and 199-217. The larger band from peak 2 appeared to contain more than 3 N-termini which could not be positively identified.

4.3.2 Cleavage of stefin B complexed cathepsin L with cyanogen bromide

Stefin B complexed cathepsin L cleaved with cyanogen bromide resulted in the generation of two major peaks separated by HPLC, the second of which showed three poorly resolved shoulders. The effluent was manually collected into 8 fractions, as shown in Fig. 4.3. Analysis of the fractions on non-reducing SDS-PAGE showed that peak 1 contained a single fragment (14 kDa). Fraction 2 (trough between two peaks) showed no protein (or a small amount of carry over from peak 1). Fraction 3 and 4 contained two major components (ca. 25 kDa and 7 kDa), fraction 5 contained two components giving distinct bands at 30 kDa and 11 kDa, which carried over into fractions 6 and 7. Fraction 8 showed no distinct bands, while fraction 9 showed a band at 39 kDa (Fig. 4.5). The results of N-terminal sequencing of the various fractions is shown in Table 4.2.

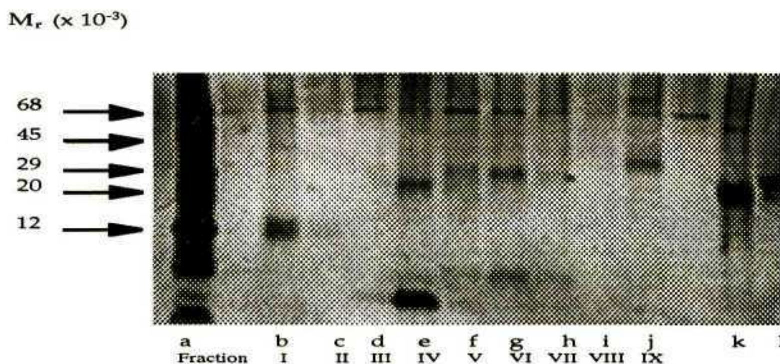


Figure 4.5. Non-reducing SDS-PAGE analysis of CNBr-fragments of stefin B-complexed cathepsin L separated by HPLC.
(a) Molecular weight markers (BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa, soybean trypsin inhibitor, 20 kDa and cytochrome C, 12 kDa), (b) - (j) - Fractions collected manually from HPLC (10 µl), designated I - IX (k) cathepsin L (200 ng), (l) cathepsin L-stefin B complex (200 ng), performed on a 10 % Tris-tricine gel, and silver stained as outlined in Section 2.6.2.

Immunoblotting of these fractions and detection with anti-cathepsin L antibody, and anti-stefin B antibody showed no recognition for cathepsin L, but recognition for stefin B, in fraction 4, at ca. 25 kDa, and fraction 5, at ca. 25 - 30 kDa, 39 kDa and 65 kDa (Fig. 4.6).

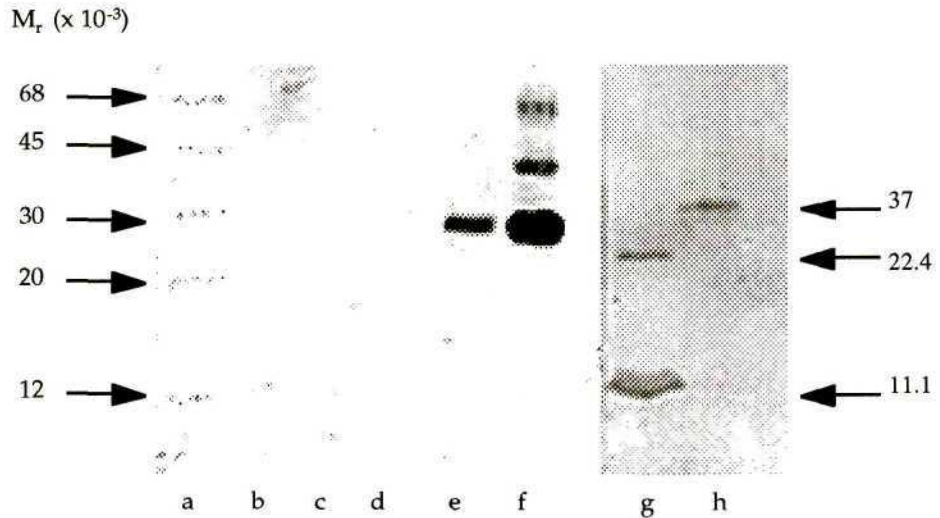


Figure 4.6. Western blot analysis of complex fractions I - V with anti-stefin B antibody.

(a) Molecular weight markers (BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa, soybean trypsin inhibitor, 20 kDa and cytochrome C, 12 kDa), (b) - (f) - Fractions collected manually from HPLC (50µl), designated I - V, (g) stefin B (10 ug), (h) cathepsin L-stefin B complex (10 µg), performed on a 10 % Tris-tricine gel, blotted, and detected with rabbit anti-stefin B (50 µg/ml), goat anti-rabbit IgG alkaline phosphatase as secondary antibody, and developed as outlined in Section 2.8.2. Lanes g and H were performed on a different blot, the blots were aligned so that molecular weights corresponded. Lane g contains stefin B, monomer (11.1 kDa) and dimer (22.4 kDa), while lane h contains stefin B cathepsin L complex (37 kDa).

4.3.3 Sequencing data of cathepsin L and stefin B complexed cathepsin L

The results of the N-terminal sequence analysis are presented in Table 4.2.

Table 4.2. The N-terminal sequences of the cyanogen bromide fragments separated by HPLC.

Fragment	N-terminal Sequence
Cathepsin L peak 1 (fraction I)	VPKS FRKT DNAF
Cathepsin L peak 2 (fractions III - V, 2 fragments, 7 and 9 kDa)	KAVA AKDQ
Cathepsin L, peak 2 (fractions IV - V, 1 fragment, 30 kDa)	More than 3 N-termini, results indefinite
Complex, peak 1 (fraction I, 1 fragment, 14 kDa)	VPKS FRKT DNAF
Complex peak 2 (fractions III - VI, 2 fragments, 7 and 11 kDa)	KAVA AKDQ
Complex peak 2 (fraction IV, only the 25 kDa fragment was sequenced)	VPKS FRKT DNAF

4.4 Discussion

The fragments generated by cyanogen bromide cleavage of cathepsin L itself correlated exactly with the postulated fragments, thereby confirming the cleavage system, as well as acting as a control for the hypothesis. The first peak contained the fragment 1-123, cleaved at M38 and M70, and crosslinked by the postulated C22–C63, and C57–C95 disulfide bridges. The appearance of two fragments (*ca.* 7 and 10 kDa) in peak two, with the same N-termini, but differing in molecular weights, may be explained by the existence of single and two-chain forms of cathepsin L. In the case of the single chain form, fragment 176-198 would remain associated with fragment 124-175, adding an additional 2 795 Da, to give a total of 10 317 Da. The two-chain form would be missing this fragment, consequently having a molecular weight of 7 522 Da.

The results obtained from cleavage of stefin B-complexed cathepsin L suggest that stefin B is associated with the 1-123 fragment of cathepsin L, as shown by the fact

that the complex fraction 4 (Table 4.2) is recognised by anti-stefin B antibodies, at ca. 25 - 30 kDa. N-terminal sequence analysis of this fraction showed that it contained three N-termini, VPKS, FRKT and DNAF, confirming that it was fragment 1-123 of cathepsin L. A trace of the stefin B N-terminal sequence was also found in this fraction, although Ritonja *et al.* (1996) found it to be N-terminally blocked. The 1-123 fragment has a calculated molecular weight of *ca.* 13 kDa. The *ca.* 11 kDa of stefin B added to this gives a total of *ca.* 24-25 kDa which agrees with the MW estimated from SDS-PAGE analysis and Western blotting. The higher molecular weight bands in Fraction 5 (39 and 65 kDa) showed the same three N-terminal sequences as fraction 4, and were assumed to be uncleaved complex (39 kDa) and a possible dimer (65 kDa).

It must be remembered that it has thus far proven to be not possible to physically separate the covalent complex from the non-covalent complex of cathepsin L and stefin B, thought to be concurrently present (on the basis of non-reducing SDS-PAGE analysis). It is thought that, being comprised of the same two molecules, the two complexes behave very similarly on most forms of chromatography tried (Chapter 3). Because of this the "proteolytically active complex fraction" might actually consists of a mixture of covalent and non-covalent complexes.

Upon fragmentation with CNBr, the non-covalent complex would be expected to give a fragment, consisting of residues 1-123, with the N-termini VPKS, FRKT and DNAF and with a molecular weight of *ca.* 13 kDa. These criteria agree very well with the observed properties of complex peak 1 (Table 4.2). The non-covalent complex would be expected to liberate stefin B upon CNBr fragmentation, perhaps with the Met-1 and Met-2 residues cleaved off but otherwise intact. However, no free stefin B could be detected by Western blotting after CNBr fragmentation.

The association of stefin B with the 1-123 fragment of cathepsin L may be most easily rationalised by assuming a disulfide bridge between stefin B and the active site Cys residue (Cys-25) of cathepsin L, as this should be the only free Cys in the 1-123 fragment. However, if the stefin B had reacted with the active site Cys-25, the complex should not be proteolytically active. Treatment of the active complex with radiolabelled iodoacetic acid, before CNBr fragmentation and chromatography, showed that the residues 1-123 fraction became radioactively labelled, suggesting the presence of at least one free thiol group, which is most likely the active site thiol,

since the complex is active. This is consistent with work done by Coetzer (1992) who showed that *in vitro* covalent complexes could still form between cathepsin L and stefin B after treatment of cathepsin L with E-64 or Z-Phe-Phe-CHN_2, inhibitors which alkylate the active site Cys residues of cysteine proteases, indicating that the active site residue may not be involved in complex formation.

In order to interpret the results from the stefin B complexed cathepsin L samples, it is necessary to examine the possible bonds which could exist between the two molecules. Since the bond is reduction sensitive, there are two most-likely possibilities, a disulfide or a thioester. Assuming that the link is a disulfide bridge, there are a number of possibilities for this. In the absence of a 3-D structure for cathepsin L itself, papain, which is believed to approximate the 3-D structure of cathepsin L (Dufour *et al.*, 1988), may be used as a model. Examination of the 3-D structure of stefin B docking into papain (Figure 4.7), shows that the free Cys-3 residue of stefin B is in close proximity to Cys-22, Cys-25 and Cys-63, and could possibly bond with any of these residues.

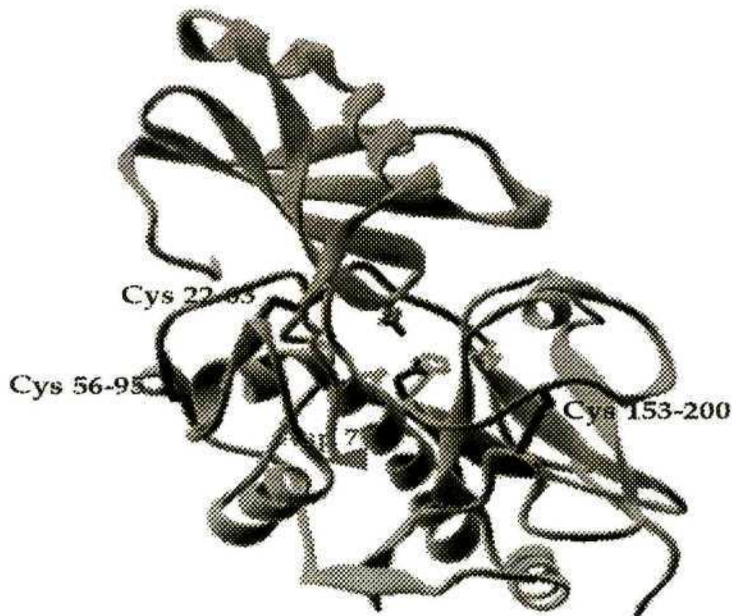


Figure 4.7. The ribbon presentation of the complex formed between stefin B (beige) and papain (magenta) (after Stubbs *et al.*, 1990, from Swiss Prot Data Bank, S3D0059/60/61/62). The active site triad is shown in turquoise. The disulfide links are shown in blue, the interactions between the QVVAG region on the hairpin loop are shown in navy, and Asp 71 which forms the putative thioester (see Discussion) is shown in green.

The Pro-6 residue, present in sheep (and human) stefin B introduces a bend in the N-terminal trunk, containing the Cys residue, and may facilitate covalent complex formation. Stefin B could conceivably form a disulfide bridge by a thiol-disulfide interchange mechanism, as proposed by Brocklehurst and Kierstan (1973) for the activation of papain. In their hypothesis, the inactive form of the enzyme is envisaged as having the active site Cys-25 disulfide linked to Cys-63. Upon reductive activation, the active site Cys-25 may undergo a thiol-disulfide exchange, resulting in Cys-22 bonding with Cys-63 and freeing Cys-25 to form the active site Cys-25/His-159 thiolate-imidazolium ion pair (Fig. 4.8).

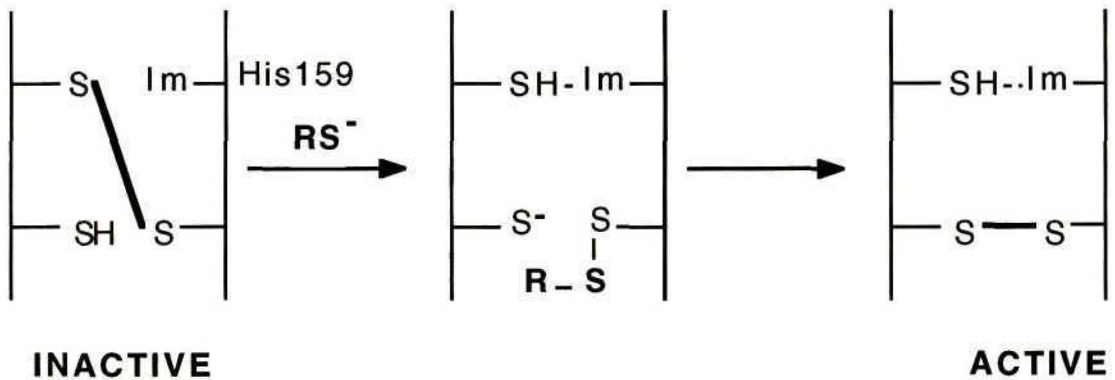


Figure 4.8. Reductive activation of papain (after Brocklehurst and Kierstan, 1973).
An active site Cys-25 thiolate-His-159 imidazolium ion pair (SH-Im) is formed by the production of a disulfide bond between Cys-22 and Cys-63. Either of these Cys residues may be disulfide linked to Cys-25, prior to activation by a reducing agent, RS^-.

It has been shown, by alkylation of papain, that the C22–C63 disulfide bond is not necessary for activity (Brocklehurst and Kierstan, 1973). If stefin B bound to either of these residues in a manner such that the active site was not occluded, it may be possible for cathepsin L to be active, and possibly stabilised by this interaction (see Chapter 5).

Stefin B could also conceivably form a disulfide link with either Cys-56 or Cys-95, as the C56–C95 disulfide bond is also not essential for activity (Shapira and Arnon, 1969). Although the three-dimensional structure of papain suggests that this disulfide is situated too far from the docking site of the Cys-3 residue, Dufour (1988) demonstrated that the residues 71-117 region shows the lowest sequence homology with papain. This, coupled to the fact that there may be an additional α-helix in

position 85-108 [at least in avian cathepsin L (Dufour, 1988)], suggests that there may be differences in the overall tertiary structure which, in cathepsin L, may bring the C56–C95 disulfide bond into closer proximity to Cys-3 of stefin B, thereby facilitating thiol-disulfide exchange.

The final candidate for thiol-disulfide interchange with Cys-3 of stefin B is the C153–C200 disulfide bridge. This bond is situated on the opposite lobe of papain (when compared to the other disulfide bonds), and is some distance from the active site. It has also been shown (by deletion analyses) to be essential for enzyme activity (Smith and Gottesman, 1989). Cys-153 is positioned close to the active site His-159, and it may be required for stabilisation of the active site. It is therefore unlikely that stefin B undergoes thiol-disulfide interchange with either of Cys-153 or Cys-200, to form active covalent complexes.

An enigmatic problem, is the fact that any thiol-disulfide interchange between stefin B and any of the Cys residues of the 1-123 CNBr fragment of cathepsin L would cause the release of one of the three chains comprising the 1-123 fragment, either as a free chain or linked to stefin B. In turn, this would reduce the MW to less than 24 kDa and would also cause the loss of one of the three N-termini of the 1-123 fragment. Neither of these is observed, which casts doubt on the linkage being a disulfide bridge.

An alternative may be a thioester bond (as these are also reduction sensitive), formed by the linking of a cysteine thiol to a carboxyl group, contributed by either glutamic or aspartic acid. If a thioester is indeed the link between cathepsin L and stefin B, the thiol contributor must be the stefin B, since the cysteine residues in the 1-123 fragment of cathepsin L are involved in disulfide bridges. An exception is the active site cysteine which must, however, be free for the complex to be active. By elimination, therefore, it may be deduced that the thiol contributor must be Cys-3 of stefin B.

But to which acid residue might Cys-3 be attached? Re-examination of the 3-D structure of stefin B docking into papain (Figure 4.7), suggests that the only acid groups within ready reach of Cys-3 of stefin B is Asp-71 of cathepsin L. Moreover, Asp-71 is almost ideally positioned. Upstream, the next acidic residue is Asp-55 and downstream is Glu-79. However, neither of these is very close to Cys-3 of stefin B in

the docking complex. It is consequently hypothesised that the covalent link between the cathepsin L and stefin B molecules is a thioester formed between Cys-3 of the stefin B with Asp-71 of cathepsin L, since only such a linkage is consistent with all of the experimental data currently available.

Thioester bonds are high-energy bonds, and are obligatory intermediates in several key processes in which ATP is either used or regenerated. They are involved in the synthesis of all esters, including those found in complex lipids, fatty acids, sterols, terpenes, porphyrins and peptides. Thioesters are encountered in C3 and C4 complement proteins, and α_2-macroglobulin, where they play a fundamental role in the functions of these proteins. When these proteins are cleaved by proteases, the thioester becomes exposed to the surroundings and is subject to nucleophilic attack from amino and hydroxyl groups. This results in covalent attachment of the thioester-containing protein to the surface of the activatory particle (Iijima *et al.*, 1984; Sottrup-Jensen, 1989).

It has been shown that liver homogenate possesses factors which can catalyse the formation of thioesters in C3 (Iijima *et al.*, 1984). In addition, thioester formation between Cys and Gln residues on different proteins have been shown to be catalysed by calcium-dependent transglutaminases (Folk, 1980). While thioesters are identified as high energy bonds, some evidence suggests that thioester formation may occur spontaneously *in vivo*, without the participation of ATP or similar high energy sources (Pangburn, 1992).

It may be speculated that during homogenisation of the liver, lysosomal cathepsin L, and cytosolic stefin B, along with enzymes and cofactors, may be brought into close proximity to each other. Thioester formation may be favoured as the proteins associate, and may be promoted by the presence of other factors, not necessarily requiring high energy metabolites. For example, Pangburn (1992) suggest that a proximal His group (such as His-159 of the active site?) might aid in polarising the participatory Cys group, thereby facilitating thioester formation.

Under certain cellular conditions, type 1 cystatins may exist as disulfide bridged dimers, or in a mixed disulfide with glutathione (as discovered for rat stefin B) (Wakamatsu *et al.*, 1984). Depending on the glutathione to oxidised glutathione ratio, disulfide bond formation may be regulated, and indirectly favour thioester

formation. In the ER, the GSH:GSSG ratio favours protein disulfide bond formation (Lodish *et al.*, 1995). Depending on the ratio of oxidised to reduced glutathione during homogenisation, the Cys-3 of stefin B may be more available for nucleophilic attack on acidic residues in cathepsin L. In this model, the formation of complexes is dependent on the redox potential which exists in the milieu. This could explain the variation in the proportion of active complex formed, as the homogenisation environment, and length of procedure would ultimately affect the reducing conditions of the homogenate.

The active complex has been identified in sheep, human and baboon liver, as well as sheep kidney, but only inactive complexes were isolated from sheep and human spleen (Pike, 1990). Although cathepsin L is known to be present in limited amounts in spleen (Mason *et al.*, 1989) these results suggest the presence of tissue/organ specific factors and/or conditions which allow the formation of complex.

CHAPTER 5

THE pH-DEPENDENT ACTIVITY AND STABILITY OF CATHEPSIN L

5.1 Introduction

The endopeptidase activity of cathepsin L could contribute to the ability of a tumour cell to degrade extracellular matrix. It may function intracellulary, mediating protein catabolism in the conditions encountered in the endosomal system, or it could be involved in extracellular protein degradation. In the latter instance, it would be necessary to prove that cathepsin L, conventionally assigned an acidic pH optimum, is significantly active and stable in the conditions of the extracellular milieu. The purpose of the studies reported in this chapter was to reinvestigate the pH optimum and stability of cathepsin L (and cathepsin B), paying particular attention to the question of its activity in the extracellular environment.

By analogy with members of the cysteine protease family of known 3-D structure, cathepsin L is most likely a bilobular globular protein, in which the active site groove contains subsites into which the substrate can bind (Figure 5.1) (Schechter and Berger, 1967).

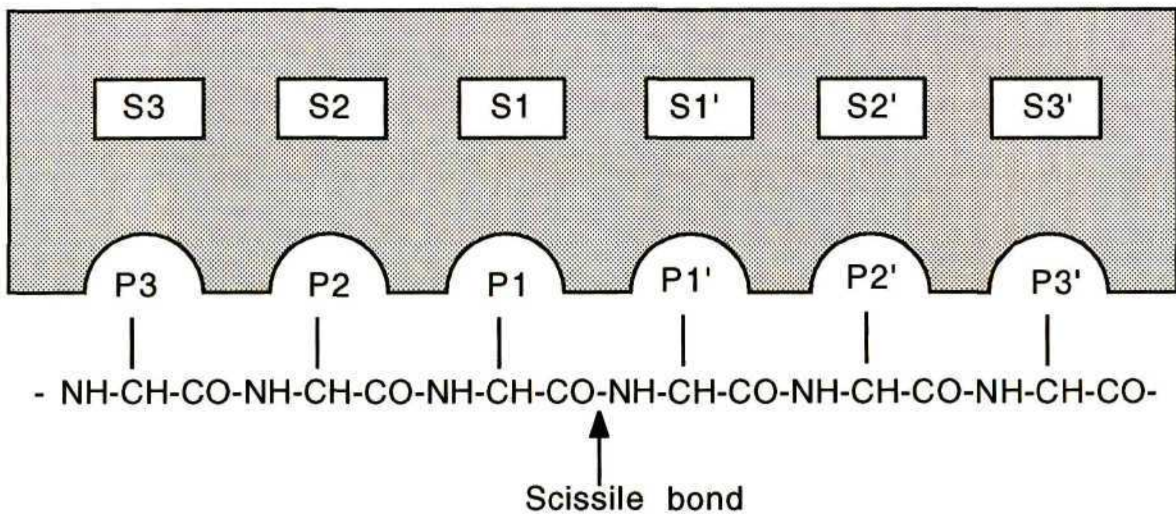


Figure 5.1. The Schechter and Berger nomenclature for binding of a peptide substrate to a peptidase.
The protease is represented by the shaded area.

These subsites are denoted S_3, S_2, S_1, S_1', S_2' and S_3' while the corresponding substrate amino acid residues are termed P_3, P_2....... P_3'. The numbering is inverted on either

side of the scissile bond. Cathepsin L has a specificity for hydrophobic residues in positions P_2 and P_3, (Katanuma *et al.*, 1983), and will tolerate large hydrophobic side chains in position P_1, in contrast to cathepsin B (Kirschke *et al.*, 1988). With proteinaceous substrates the specificity is largely determined by the conformation of the substrate molecules, and for this reason, the effect of pH on the conformation of substrate proteins should also be considered (Barrett *et al.*, 1988). Cathepsins B and L are most commonly assayed using azocasein as a proteinaceous substrate, and fluorogenic dipeptidyl derivatives, such as Z-Phe-Arg-NHMec, as synthetic substrates (Barrett and Kirschke, 1981). Z-Arg-Arg-NHMec is specific for cathepsin B, allowing cathepsin L activity to be measured in the presence of cathepsin B by differential hydrolysis of Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec. Inubushi *et al.* (1994) designed a more specific method for assaying cathepsins B and L activity employing a range of inhibitors.

Cathepsins B and L have been reported to show maximal activity at acidic pH, suggesting that the catalytic sites are different to that of papain (Barrett *et al.*, 1988) which demonstrates a broad pH profile. Consequently, most assays are conducted under acidic conditions, and a number of authors have reported a pH optimum of ca. 5.5-6 for cathepsin L (Table 5.1) with the enzyme reportedly being largely inactive at neutral pH. This is in contrast to previous work conducted in this laboratory, which showed cathepsin L to have a pH optimum of 6.5 (Dennison *et al.*, 1992). It has been well established, however, that cathepsin B is active for limited periods at extracellular pH (Mort *et al.*, 1984; Machleidt *et al.*, 1986).

Table 5.1. Some pH optima reported for cathepsin L

Reference	Source of cathepsin L	Substrate	pH optimum
Mason *et al.*, 1984	rabbit liver	azocasein	5.5
		Z-Phe-Arg-NHMec	6.0
		collagen	3.5
Mason *et al.*, 1985	human liver	Z-Phe-Arg-NHMec	5.5
Mason *et al.*, 1986	human liver	elastin	5.0
Dufour *et al.*, 1988	chicken liver	Z-Phe-Arg-NHMec	5.5
Kirschke *et al.*, 1989	rat liver	Z-Phe-Arg-NHMec	5.5
Brömme *et al.*, 1989	rat liver	Z-Phe-Arg-NHMec	6.0
Dennison *et al.*, 1992	sheep liver	Z-Phe-Arg-NHMec	6.5

A difference, however, was that Dennison *et al.* (1992) used acetate-MES-Tris (AMT) buffers (Ellis and Morrison, 1982) of constant ionic strength whereas most others have used buffers of constant molarity, typically 0.1 M phosphate. An advantage of AMT buffer, besides its constant ionic strength, is that it effectively buffers over a wide pH range (Ellis and Morrison, 1982) whereas phosphate, for example, will effectively buffer only in the limited range of pKa ± 1 (i.e. 6.1-8.1). Despite this, phosphate has been used as low as pH 4.6 and as high as pH 8.5.

It has been reported that, besides pH, the ionic strength also affects the activity and stability of these cathepsins (Khan and Ahmad, 1987; Turk *et al.*, 1994; Dehrmann *et al.*, 1995), but this does not appear to have been widely appreciated, or taken into account, by previous workers in this field. It has been previously reported that the activity of cathepsin L is decreased by an increase in ionic strength (Dennison *et al.*, 1992). With anionic buffers of constant molarity, therefore, the increase in ionic strength with increasing pH (Fig. 5.2) may skew the pH profile, depressing the activity at higher pH values and resulting in a lower apparent pH optimum. These observations, and the consequent realisation that the true pH optimum of cathepsin L (and its behaviour in the specific ionic milieu of the extracellular medium) has not previously been established, motivated the present study of the effects of ionic strength and pH on the activity of cathepsin L.

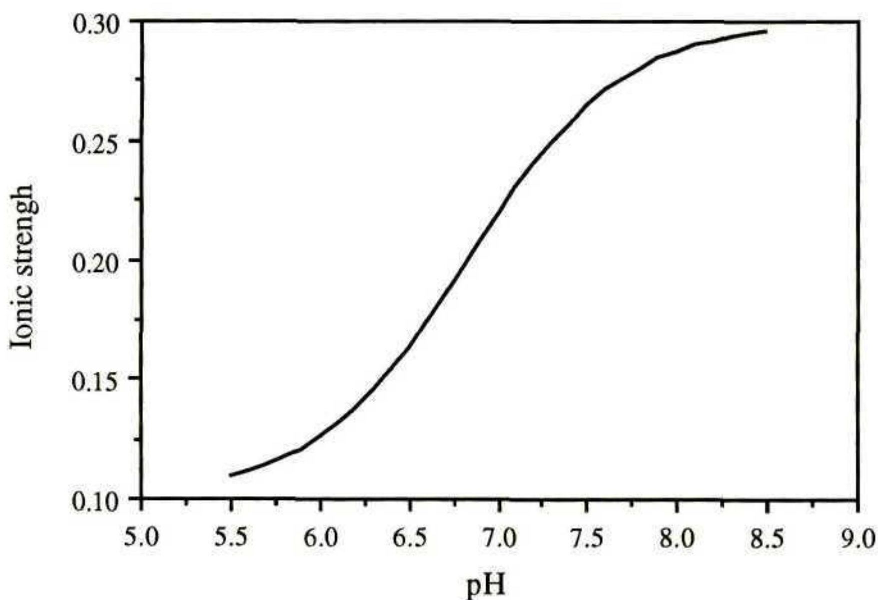


Figure 5.2. The effect of pH on the ionic strength of a 0.1 M phosphate buffer.

Ionic strength values were calculated by an adaptation of the programme of Ellis and Morrison (1982).

In measuring pH-activity profiles, most authors have reported "relative activity" which, as discussed below, does not relate linearly to the second order rate constant, k_{cat}/K_m, when [S] is of the same order as K_m, which is the case with cathepsin L acting upon Z-Phe-Arg-NHMec. In these circumstances it is difficult to assign other than a superficial meaning to relative activity values, and this has motivated the present study of the effects of changes in pH and ionic strength on the kinetic constants k_{cat} and K_m, and hence on k_{cat}/K_m.

In the context of the possible extracellular activity of cathepsin L, not only the activity but also the stability of the enzyme is of interest. The pH-dependent stability of human kidney cathepsin L has previously been studied by Turk *et al.* (1993), who used phosphate buffer of constant molarity (100 mM) (or Tris, at pH 8.0). In the light of the results presented in this chapter, it is evident that their findings cannot be extrapolated to physiological conditions. Moreover, since the ionic strength of the buffers used by these workers would have varied with the pH, it is difficult to distinguish the effects of pH and of ionic strength. Consequently, in the present study, the stability of cathepsin L in AMT and phosphate buffers was measured, varying the pH and ionic strength independently. The stability was also measured in Hanks' balanced salt solution, a buffer modelling the extracellular fluid.

5.2 Expression of pH-activity profiles

The velocity of a reaction such as that catalysed by cathepsin L is described by the equation (Knight, 1977):-

$$V_o = \frac{k_{cat}}{K_m} \cdot [E].[S] \qquad \text{5.1}$$

from which k_{cat}/K_m can be readily identified as a second order rate constant. The relationship between the concentration of the free enzyme, [E] (which is usually unknown), and that of the total enzyme, $[E]_o$ (which is known), is given by the equation:-

$$[E] = \frac{[E]_o}{(\frac{[S]}{K_m} + 1)} \qquad \text{5.2}$$

Modelling of equation 5.2 reveals that [E] approximates $[E]_o$, only when $[S] \leq \frac{K_m}{40}$. The fluorogenic substrate commonly used to assay cathepsin L is Z-Phe-Arg-NHMec, for which cathepsin L has a K_m roughly comparable to the concentration at which this substrate is used and a concentration of $[S] \leq \frac{K_m}{40}$ is impractical, as cathepsin L activity is not measurable at this substrate concentration. An experimental approach different from that used with cathepsin B, where k_{cat}/K_m can be calculated from V_o, assuming $[E] = [E]_o$ (e.g. Khouri *et al.*, 1991), is thus necessary with cathepsin L.

Changes in pH and ionic strength, as explored here, affect V_o by their influence on either or both of the constants comprising the second order rate constant, k_{cat}/K_m (equation 5.1), and it is therefore most appropriate to express their influences in terms of their effects on k_{cat}/K_m. When [S] is not substantially smaller than K_m, however, $[E] \neq [E]_o$ and so equation 5.1 cannot be used to determine k_{cat}/K_m. Instead, it is necessary to measure V_o at more than one substrate concentration, which enables values for k_{cat} and K_m to be calculated from the Michaelis-Menten equation:-

$$V_o = \frac{k_{cat}}{K_m + [S]} . [E]_o . [S] \qquad \text{5.3}$$

It may be noted that, under conditions where the Michaelis-Menten equation applies, if the factor being investigated, e.g. pH or ionic strength, affects only k_{cat}, this should have a linear affect on V_o and the shape of a profile of "relative activity" will be similar to that of k_{cat}/K_m vs pH or ionic strength. If on the other hand K_m is affected, the effect on V_o will be non-linear and will be a function of [S]. In these circumstances it may be difficult to assign other than a superficial meaning to the term "relative activity".

In this study, the effects of pH- and ionic strength on k_{cat}, K_m, k_{cat}/K_m and relative activity were explored, the latter for comparison with previous reports.

5.2 Buffers

The ionic strengths of buffers of defined molarity were calculated using the BASIC programme of Ellis and Morrison (1982) and, conversely, the molarities of buffers of defined ionic strengths were calculated by a simple adaptation of this programme,

using an iterative loop to home in on the molarity value. Acetate-MES-Tris buffers of constant ionic strength were made up according to Ellis and Morrison (1992) and other buffers were made up following the procedure previously described (Dennison, 1988).

5.2.1 AMT buffers

AMT buffers of ionic strength $I = 0.533$ were prepared at pH 5.5, 6.0, 6.5, 7.0 and 7.5. Molarities were calculated to be 0.266 M for acetic acid and MES, and 0.533 M for Tris.

AMT buffer pH 5.5-7.5. Acetic acid (1.68 ml), MES (5.20 g) and Tris (6.45 g) were diluted to 80 ml with dist.H_2O, titrated to the appropriate pH with NaOH or HCl, and made up to 100 ml with dist.H_2O. Upon 3→4 dilution in the modified assay described below, the final ionic strength of each of these buffers becomes $I = 0.4$

Serial 2-fold dilutions of these buffers gave additional buffers that, after dilution in the assay, yielded ionic strengths of $I = 0.2$ and 0.1 respectively. The pH of the buffers was checked after dilution; deviations from the nominal pH were insignificantly small. The solutions were stored in stoppered vials and kept at 4°C.

5.2.2 K-phosphate buffers

K-phosphate buffers were prepared at pH 6.0, 6.5, 7.0 and 7.5 (pH 5.5 being outside of phosphate's buffering range); the required molarities being calculated to be 0.4258, 0.3277, 0.2444, and 0.2019, respectively.

K-phosphate buffers. Samples of KH_2PO_4 (5.79 g, 4.46 g, 3.33 g and 2.75 g) were separately diluted to 80 ml with dist.H_2O, titrated to the required pH with KOH, and made up to 100 ml with dist.H_2O. Upon 3→4 dilution in the modified assay described below, the final ionic strength of each of these buffers becomes $I = 0.4$.

As with the AMT buffers, serial 2-fold dilutions of these buffers gave additional buffers that, after dilution in the assay, yielded ionic strengths of $I = 0.2$ and 0.1 respectively. The pH of the buffers was checked after dilution; deviations from the nominal pH were insignificant.

5.2.3 Hanks' balanced salt solution (HBSS)

Hanks' balanced salt solution was made up according to Freshney (1983), but without glucose or phenol red, and at 4/3 the nominal concentration to allow for dilution in the assay.

4/3 HBSS (1.68 mM $CaCl_2$, 7.15 mM KCl, 0.59 mM KH_2PO_4, 0.65 mM $MgCl_2$, 0.55 mM $MgSO_4$, 0.183 M NaCl and 0.45 mM NaH_2PO_4, pH 7.4). $CaCl_2$ (0.187 g), KCl (0.53 g), KH_2PO_4 (0.08 g), $MgCl_2.6H_2O$ (0.133 g), $MgSO_4.7H_2O$ (0.133 g), NaCl (10.67 g) and $NaH_2PO_4.7H_2O$ (0.12 g) were diluted to 900 ml, titrated to pH 7.4 with NaOH and made up to 1 litre. Immediately before use, the solution was titrated to pH 7.2 with CO_2 gas and, for the pH 6.8 buffer, further titrated to pH 6.8 with lactic acid.

5.3 Enzyme assays

5.3.1 pH-activity profiles

pH-activity profiles of cathepsin L and cathepsin B were determined using Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec as substrates, respectively.

5.3.1.1 Reagents

Sheep liver cathepsin L was isolated as described in Section 3.2.1. Human liver cathepsin B, isolated by the method of Rich *et al.* (1986), was from Athens Research and Technology (Athens, Georgia, USA) and according to the suppliers is free of cathepsin L.

Buffers were prepared as described in Section 5.2 and substrate and Brij solutions were prepared as described in Section 2.9.

5.3.1.2 Procedure

To determine pH-activity profiles, and ionic strength-activity profiles, expressed in terms of relative activity, enzymes were assayed either by the method of Barrett and Kirschke (1981) (Section 2.9) or in an assay modified to minimise the dilution of the buffer. In the latter case, the assay solution consisted of 750 µl of buffer, either 32 µl or 64 µl of DTT solution (154.2 mg DTT per ml water) and a complementary volume of 0.1% Brij, the total summing to 865 µl. All solutions were equilibrated to 37°C, except the diluted enzyme solution which was kept on ice. Appropriately diluted

enzyme (10 μl) was added, allowed to activate for 2 min at 37°C, and 40 μM Z-Phe-Arg-NHMec (or Z-Arg-Arg-NHMec) solution (125 μl) was mixed in. The change in fluorescence was measured continuously at 37°C in a temperature-controlled recording spectrofluorimeter, with excitation at 370 nm and emission at 460 nm. Data was sampled at 10-second intervals over a 5 min period and activity was determined from the initial slope of the plot of fluorescence intensity versus time.

5.3.2 Stability assays

Stability was determined as the half-life of the enzyme activity, as previously described (Dennison *et al.*, 1992), using an assay of appropriate duration (20 min). Each assay was conducted in triplicate, and semilogarithmic plots of the mean fluorescence intensity values against time were plotted, and analysed by linear regression. The slopes of the regression lines were used to calculate the observed (pseudo first-order) inactivation constant k_{obs} using the equation (Segel, 1976)

$$k_{obs} = \text{slope} \times -2.3 \quad \text{.......... 5.4}$$

The first-order decay constant, k_{obs}, is related to the half-life by the equation,

$$t_{1/2} = \frac{0.693}{k_{obs}} \quad \text{.......... 5.5}$$

5.3.2.1 Reagents

Reagents were prepared as described in Section 5.3.1.

5.3.2.2 Procedure

To determine half-lives, enzymes were assayed by the modified procedure described in Section 5.3.1.2. Data was sampled at 10-second intervals over a 20 min period. The log of the slope of the fluorescent intensity vs time was plotted against time, and the slope of the regression lines were used to calculate k_{obs} and hence $t_{1/2}$.

5.3.3 K_m and k_{cat} determinations

To determine K_m and k_{cat} values for cathepsin L, a semi-continuous assay in a fluorescence microplate reader (Cambridge Technology, Model 7620) was used. For each determination, buffer (75 μl) and DTT plus Brij (11.5 μl) was added to each of 14 wells of a white Fluoronunc maxisorp microtitre plate. The DTT plus Brij mixture

consisted of 42.9 mg DTT/500 µl Brij. Enzyme (1 µl), of an appropriate dilution, was added (using a microsyringe) to each well, incubated for 2 min, and serial two-fold dilutions of Z-Phe-Arg-NHMec (12.5 µl) were added to duplicate wells in a row of seven, giving final substrate concentrations ranging from 50 µM to 0.78125 µM. After 2, 4 and 6 min, the wells were read, with excitation at 360 nm and emission at 460 nm. Reading at three different times provides a check on the linearity of the progress curve. It was empirically established that before 2 min the reaction rate increased with time, but the reaction was linear between 2 and 6 min. K_m and V_{max} values were determined using the direct linear plot of Eisenthal and Cornish-Bowden (1974), implemented using the programme HYPER obtained from Dr J. S. Easterby, University of Liverpool, UK. $[E]_o$ values were established by E-64 titration, as described by Barrett and Kirschke (1981) (Section 5.4), and the relationship:

$$k_{cat} = V_{max}/[E]_o \quad \text{.........} 5.6$$

was used to calculate k_{cat} from V_{max} values.

For cathepsin B, k_{cat}/K_m values were determined from V_o determinations as described in Section 5.3.1.

5.4 Active site titration with E-64

The irreversible inhibitor E-64 was used routinely to determine the amount of active enzyme present in the sample, according to the method of Barrett and Kirschke (1981). This is a stoichiometric titration where the absolute molarity of the enzyme solution may be determined.

5.4.1 Reagents

<u>1 mM E-64 stock solution</u>. Dry E-64 (3.6 mg) was dissolved in dimethyl sulfoxide (100 µl) and diluted to 10 ml with dist.H_2O (this solution was stored at 4°C for up to one week). As required, working solutions of 1, 2, 3 to 10 µM were prepared.

<u>Enzyme solution</u>. Cathepsin L, or stefin B complexed cathepsin L (Section 3.3) or cathepsin B of approximately 10 µM (as estimated from molecular weights) was diluted in 0.1% Brij 35 to the appropriate volume.

Assay buffer pH 5.5. As described in Section 3.2, containing 40 mM cysteine, or 8 mM DTT.

5.4.2 Procedure

Enzyme (25 µl) was diluted with buffer (50 µl), and inhibitor (25 µl) of varying molarity (1-10 µM) was added. This was incubated at 30°C (30 min), after which the solutions were diluted to 5 ml with 0.1% Brij 35. Aliquots were assayed for residual activity against Z-Phe-Arg-NHMec as described previously (Section 3.) The plot of residual activity vs E-64 molarity bisects the X-axis (zero activity) at the molarity of the enzyme solution (active). The slope of the graph shows the specific activity of the enzyme in the assay.

5.5 The effect of pH and the presence of proteins on stability

The stability ($t_{1/2}$) of cathepsins B and L was assayed as described in Section 5.3, at pH 7.2 and 6.8, in HBSS, in the presence of 0.1 and 0.2% BSA and gelatin. BSA and gelatin (0.1 and 0.2 g/100 ml) were dissolved with gentle stirring (and mild warming in the case of gelatin) in HBSS.

5.6 The effect of activation and dilution on stability

The residual activity of cathepsins B, L and stefin B-complexed cathepsin L was assayed after dilution to monitor the effect of reductive activation.

5.6.1 Reagents

All reagents were made up as described in Section 5.3.1.

5.6.2 Procedure

For assay with simultaneous dilution and activation (the normal approach), reagents were added in the following order:- 750 µl of buffer, 115 µl DTT/Brij, and 10 µl of diluted enzyme. The enzyme was activated for 1 min at 37°C, and 40 µM substrate (125 µl) was mixed in. Note that "dilution" also entails a pH change, from pH 5.5 to the stated value.

For assay with enzyme dilution before activation, the order of addition was: buffer, Brij, enzyme and DTT. The DTT was added either immediately or after 10 min, followed by substrate 1 min later.

As a third option (denoted "enzyme added last"), buffer, Brij, DTT and substrate were mixed together and enzyme was added at zero time.

In each case the change in fluorescence was measured continuously at 37°C and activity was determined from the initial slope of the curve of fluorescence vs time.

5.7 Results

5.7.1 pH-activity profiles of cathepsin L

The pH-activity profiles shown in Fig. 5.3. illustrate that in a buffer of constant ionic strength, cathepsin L has an apparent pH optimum higher than in a phosphate buffer of constant molarity (and which therefore has an increasing ionic strength, with increasing pH).

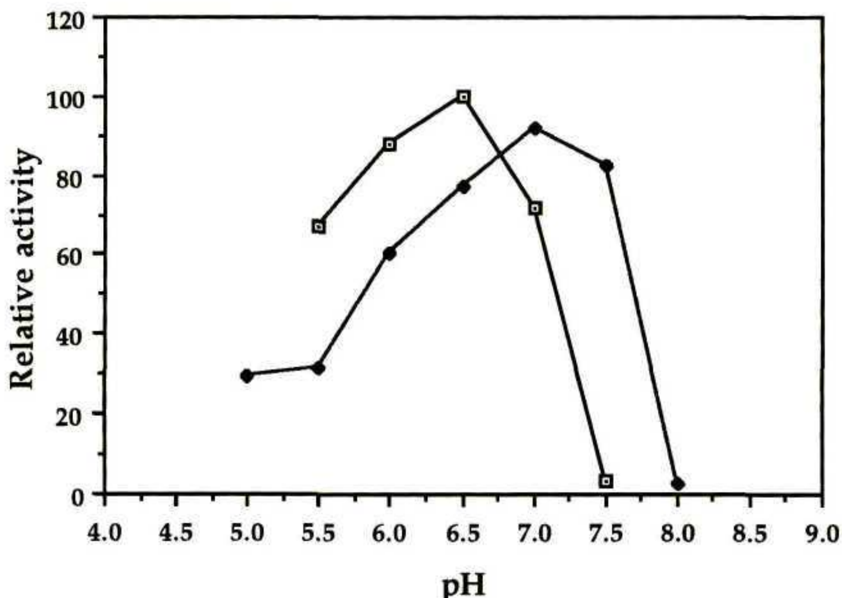


Figure 5.3. Effect of constant ionic strength buffer versus constant molarity buffer upon the apparent pH-activity profile of sheep liver cathepsin L.

Cathepsin L was assayed by the method of Barrett and Kirschke (1981) using 100 mM K-phosphate buffers (❑), or AMT buffers (◆) of $I = 0.1$ (These were diluted 4-fold in the assay, giving a final concentration of 25 mM and $I = 0.025$, respectively).

5.7.2 k_{cat} and K_m determinations

Measurement of k_{cat} and K_m in AMT and phosphate buffers of different pH and ionic strengths revealed a general trend for k_{cat} to peak (at *ca.* 20→30 s^{-1}) at about pH 6.5 in both buffers and essentially independently of ionic strength (Tables 5.2 and 5.3). K_m values, of *ca.* 5 μM, manifested a general slight rising trend with increasing ionic strength, with a sharp increase to 20-25 μM, specifically at pH 6.5 and I = 0.4, in both buffers. It has been previously noted that the reported K_m values of single-chain forms of cathepsin L appear to be higher than those of two-chain forms (Dennison *et al.*, 1992). This may be a consequence of a greater flexibility of the two-chain forms, enabling them to bind substrate with higher affinity. The second order rate constant, k_{cat}/K_m, generally declined with increasing ionic strength, in both buffers, and declined above pH 6.5, in phosphate buffer, but only above pH 7, in AMT buffer. At I = 0.4, however, there was a general decline in k_{cat}/K_m, with increasing pH in both buffers.

Table 5.2. The effect of pH and ionic strength of AMT buffer upon the K_m, k_{cat} and k_{cat}/K_m of sheep liver cathepsin L.

Ionic strength		pH				
		5.5	6.0	6.5	7.0	7.5
	K_m	7.17	4.21	8.97	5.96	8.79
0.1	k_{cat}	11.26	9.87	22.13	14.88	9.10
	k_{cat}/K_m	1.6	2.3	2.5	2.5	1.0
	K_m	7.38	7.92	9.37	5.96	n.m.
0.2	k_{cat}	11.43	16.22	17.51	20.25	n.m.
	k_{cat}/K_m	1.5	2.0	1.9	3.4	-
	K_m	7.52	17.38	25.43	16.73	n.m.
0.4	k_{cat}	13.54	22.95	24.48	15.09	n.m.
	k_{cat}/K_m	1.8	1.3	1.0	0.9	-

K_m = μM, k_{cat} = s^{-1}, k_{cat}/K_m = $M^{-1}s^{-1} \times 10^{-6}$

n.m. = not measurable

Table 5.3. The effect of pH and ionic strength of K-phosphate buffer upon the K_m, k_{cat} and k_{cat}/K_m of sheep liver cathepsin L.

Ionic strength		pH			
		6.0	6.5	7.0	7.5
	K_m	6.81	3.94	5.79	n.m.
0.1	k_{cat}	19.74	24.81	8.41	n.m.
	k_{cat}/K_m	2.9	6.3	1.5	-
	K_m	6.68	8.94	7.26	n.m.
0.2	k_{cat}	17.71	23.85	9.15	n.m.
	k_{cat}/K_m	2.7	2.7	1.3	-
	K_m	9.93	22.09	13.46	n.m.
0.4	k_{cat}	24.20	29.81	9.36	n.m.
	k_{cat}/K_m	2.4	1.3	0.7	-

K_m = μM, $k_{cat} = s^{-1}$, $k_{cat}/K_m = M^{-1}s^{-1} \times 10^{-6}$

n.m. = not measurable

In Hanks' balanced salt solution (HBSS), a model of the extracellular fluid, measured values for cathepsin L were:

k_{cat}, 17.4 s^{-1}; K_m, 11.7 μM, and k_{cat}/K_m, 1.5×10^{-6} $M^{-1}s^{-1}$

Cathepsin B showed a second order rate constant (k_{cat}/K_m) which declined with increasing ionic strength in both buffers, in phosphate buffer above pH 7.0, while in AMT buffer above pH 7.5 (Fig. 5.4)

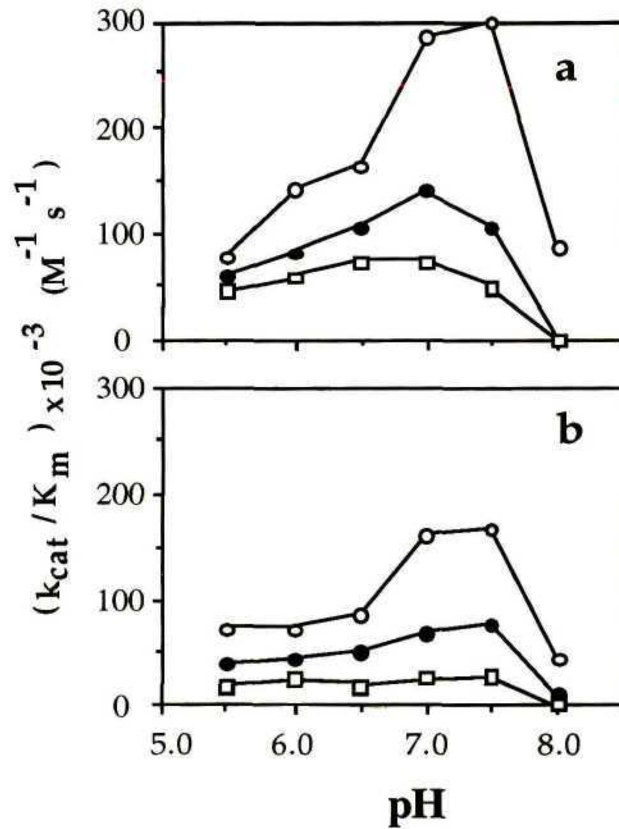


Figure 5.4. The effect of pH and ionic strength on the Z-Arg-Arg-NHMec hydrolysing activity of human liver cathepsin B.

a) K-phosphate buffers; b) AMT buffers. Ionic strength, ○ = 0.1; ● = 0.2; □ = 0.4

In HBSS, k_{cat}/K_m for cathepsin B acting on Z-Arg-Arg-NHMec was 220 x 10^{-3} $M^{-1}s^{-1}$, at pH 7.2. Gullino *et al.* (1965) have reported that the peritumour pH is 6.8. In HBSS titrated to pH 6.8 with lactic acid, k_{cat}/K_m for cathepsin B acting on Z-Arg-Arg-NHMec was 168 x 10^{-3} $M^{-1}s^{-1}$.

5.7.3 Stability assays

The stability of cathepsin L, in the physiological pH range was generally higher in AMT buffer than in phosphate (Table 5.4). In both buffers there was a trend to shorter half-lives with increasing pH and with increasing ionic strength.

Table 5.4. The effect of buffer ionic composition, ionic strength and pH on the stability of sheep liver cathepsin L

Ionic strength	$T_{1/2}$ (s)			
	Phosphate buffer		AMT buffer	
	pH 7.0	pH 7.5	pH 7.0	pH 7.5
0.05	733	186	∞	374
0.1	531	83	∞	493
0.2	360	64	∞	213
0.4	327	77	530	105

∞ = no decrease in rate of reaction could be measured over the period of the assay

In phosphate and AMT buffers, a general correlation between stability and activity is evident, i.e. stability declines in parallel with activity in either buffer. Since initial velocities were measured, there would appear to be no *a priori* reason why stability and activity should be affected in concert, and the results suggest that the two phenomena may be mechanistically linked.

In HBSS the half-life of cathepsin L was 179 s at pH 7.2 and 657 s at pH 6.8 (Table 5.5). The half life was affected differentially by the additional presence of BSA or gelatin (Table 5.5), being decreased by BSA at both pH values, and gelatin at pH 6.8, but increased by gelatin at pH 7.2. Consistent with the report of Buck *et al.* (1992), the half-life of cathepsin B was increased by the presence of 0.1 to 0.2% BSA, most markedly at pH 6.8, where it was increased from *ca.* 14 min to 24-32 min.

Table 5.5. The effect of pH and the presence of protein on the half-life of sheep liver cathepsin L and human liver cathepsin B in Hanks' balanced salt solution.

	$T_{1/2}$ (s)[a]			
	pH 7.2		pH 6.8	
	Cathepsin L	Cathepsin B	Cathepsin L	Cathepsin B
HBSS	179 ± 5.2	245 ± 11.3	657 ± 6.1	857 ± 50.1
+ 0.1% BSA	178 ± 11.2	343 ± 62.2	277 ± 31.1	1954 ± 379.0
+ 0.2% BSA	178 ± 3.7	360 ± 36.3	341 ± 20.4	1461 ± 138.9
+ 0.1% gelatin	231 ± 6.7	240 ± 12.0	484 ± 11.4	762 ± 216.0
+0.2% gelatin	187 ± 24.5	335 ± 16.3	575 ± 78.1	709 ± 154.9

[a]each figure is the mean of three determinations.

5.7.4 The effect of reductive activation

With dilution (to the stated pH) before activation, stability was markedly reduced and large proportions of the activities of both cathepsins B and L were lost before they could be measured (Table 5.6). With stefin B complexed cathepsin L significantly higher proportions of activity were retained with activation immediately after dilution. When the enzyme was added last, generally lower activities were obtained than with simultaneous dilution and activation (Table 5.6).

Table 5.6. Effect of activation before or after pH change and dilution upon the stability of cathepsins B and L and stefin B complexed cathepsin L in Hanks' balanced salt solution.

Protocol	Residual activity (%)[a]					
	Cathepsin B		Cathepsin L		Stefin B complexed cathepsin L	
	pH 7.2	pH 6.8	pH 7.2	pH 6.8	pH 7.2	pH 6.8
Simultaneous dilution and activation	(100)	(100)	(100)	(100)	(100)	(100)
Activation immediately after dilution	38 ± 13.9	81 ± 6.5	14 ± 2.8	59 ± 4.9	64 ± 2.9	80 ± 6.0
Activation 10 min after dilution	3 ± 0.2	14 ± 1.8	1.5 ± 0.4	3 ± 1.4	10 ± 4.9	27 ± 2.6
Enzyme added last	81 ± 3.3	94 ± 4.2	95 ± 10.7	64 ± 2.6	77 ± 6.3	84 ± 2.5

[a] each figure is the mean and s.d. of the results from three replicate experiments.

5.8 Discussion

The pH-activity profile of cathepsin L has been studied by several authors (Table 5.1), but a common error has been a failure to take into account the effects of ionic strength on the activity of this and other cathepsins. In a buffer of constant molarity, the ionic strength is a function of the buffer pH, as is evident from the Henderson-Hasselbalch equation. For example, in the case of constant-molarity phosphate, the ionic strength increases sigmoidally, about threefold from pH 5.5→8.5, with the greatest increment occurring at the pKa (Fig. 5.2) (Ellis and Morrison, 1982). Failure to take this into account has led to an exaggeration of the effect of increasing pH since, as we have shown here, increasing pH and increasing ionic strength have a co-operative effect in decreasing the activity and stability of cathepsin L. The net effect has been a relative depression of the activity and stability of both cathepsins L and B at higher pH and a consequent general underestimate of the pH optimum and the stability at physiological pH, of both enzymes.

The 3-D structure of mature cathepsin L is presently not known but deductions have been made by analogy with the known structures of papain and actinidin, which have primary structures homologous to that of cathepsin L (Dufour *et al.*, 1988). The conclusion reached by Dufour and co-workers is that cathepsin L is probably similar to papain in its 3-D structure but may have an extra α-helix on the outer aspect of the domain containing the central helix. The 3-D structure of cathepsin B has been published (Musil *et al.*, 1991)) and its catalytic mechanism studied (Khouri *et al.*, 1991; Hasnain *et al.*, 1992; Turk *et al.*, 1994) and, by extrapolation, this may provide insights into the mechanism of the pH and ionic strength dependence of the activity of cathepsin L.

Turk *et al.* (1994) have proposed a mechanism explaining the pH and ionic strength dependence of the irreversible denaturation of cathepsin B, which invokes the deprotonation of the imidazole moiety of the active site $-S^-/^+HIm-$ ion pair, with subsequent "unzipping" of the structure along the active site groove. As these authors have suggested, this is a mechanism which might apply to more than just cathepsin B and it is noted that it does explain the correlation we have observed between activity (measured as initial velocity) and stability of cathepsin L: in other words, that conditions which promote/reduce activity, similarly promote/reduce stability and vice versa. In this model, inactivation, caused by the deprotonation of the imidazole moiety of the active site $-S^-/^+HIm-$ ion pair, is mechanistically linked to the subsequent inactivation by "unzipping" and both steps would be sensitive to ionic strength as well as pH. This model is also consistent with the observations of Dufour *et al.* (1988) who noted a marked decrease in α-helix content of chicken cathepsin L at higher pH, suggesting that the enzyme is denatured under these conditions.

A comparison of the primary structures of cathepsin L (Ritonja *et al.*, 1988) with that of cathepsin B (Musil *et al.*, 1991) indicates, however, that the charged amino acids proposed by Turk *et al.* (1994) to be involved in the zipper mechanism in cathepsin B, have few exact counterparts in cathepsin L. The existence of fewer, or different, links in the zipper might underlie the differences in the pH optima of cathepsin B and cathepsin L. In the absence of a 3-D structure for cathepsin L, however, it is not possible to comment further on the extent to which the proposed model may be applicable to cathepsin L.

One difference between cathepsins B and L is that both hydrolyse Z-Phe-Arg-NHMec, whereas only cathepsin B hydrolyses Z-Arg-Arg-NHMec. This can be explained by the fact that in cathepsin B, a negatively charged Glu-245 residue occupies the S_2 site and this presumably interacts with the positively charged Arg residue in the P_2 position. The equivalent residue in cathepsin L is an uncharged Ala-209 and deletion of the charge at this position apparently removes the possibility of interaction with Z-Arg-Arg-NHMec. It can be imagined that binding of Z-Phe-Arg-NHMec might require a negatively charged group at the S_1 position and a hydrophobic pocket at S_2 [nomenclature of Schechter and Berger (1967)]. The fact that the K_m increases with increasing ionic strength, at least between pH 6.0 and pH 7.0, suggests, however, that in this range ionic interactions predominate, since an increase in ionic strength should weaken ionic interactions and strengthen hydrophobic interactions.

The measured stability of cathepsin L was greatest in AMT buffer and less in phosphate buffer and HBSS. Also, activity declined at a higher pH in AMT buffer than in phosphate buffer. A commonality between phosphate buffer and HBSS is the size of the cations, K^+ and Na^+ respectively, which are smaller than the $Tris^+$ and MES^+ cations in AMT buffer. Moreover, with Na-phosphate buffer the concentration of Na^+ ions increases with increasing pH. These results are thus not inconsistent with a special role for small cations in the inactivation and destabilisation of cathepsin L.

The fact that the activity and stability of cathepsin L at physiological pH is different in AMT and phosphate buffers suggests the existence of specific ion effects, over and above the effects of ionic strength *per se* and pH. In turn, this implies that if physiological relevance is to be explored, it is necessary (as performed in this study) to use a buffer modelling the compartment of interest in its specific ionic composition, as well as pH and ionic strength. When this is done, we find that mature single-chain cathepsin L has an apparent extracellular half-life in the region of 3 to 8 minutes. These values are consistent with a previous report by Machleidt *et al.* (1986), though the buffer used was not specified in that study. Reports of much shorter half-lives (Turk *et al.*, 1993), while valid for the specific buffers used, probably cannot be extrapolated to physiological conditions.

With regard to the possible extracellular activity of cathepsin L, if cells secrete cathepsin L at a constant rate (say, D moles s^{-1}) then, theoretically, an equilibrium should be set up between secretion and subsequent first-order decay of the enzyme. The amount of active enzyme (E) present in the extracellular space is described by the equation:-

$$E = \frac{D}{k_{obs}}(1 - e^{-k_{obs}t}) \qquad 5.7$$

which indicates that E is asymptotic to $\frac{D}{k_{obs}}$, i.e. the amount of enzyme in the extracellular space at equilibrium is:-

$$\frac{D}{k_{obs}} \text{ moles.}$$

In the case of tumour cells, Gullino *et al.* (1965) have reported that the pericellular space surrounding tumour cells is slightly acidified, to *ca.* pH 6.8. In Hanks' balanced salt solution, lowering the pH from pH 7.2 to 6.8, with lactate, increased the half-life of cathepsin L from 185 s (3.1 min) to 506 s (8.4 min), and that of cathepsin B from 245 s (4.1 min) to 857 s (14.3 min). This would have the effect of increasing the amount of active enzyme present in the extracellular space, at equilibrium, by a factor of 506/185, i.e. almost 3-fold in the case of cathepsin L, and 857/245, i.e. 3.5-fold in the case of cathepsin B. The additional presence of specific extracellular proteins would have a modifying effect, however (Table 5.4). In the digestion of extracellular proteins cathepsin L would most probably encounter an excess of substrate in the form of scissile bonds. Under these circumstances, V_o would be largely influenced by k_{cat}, which peaks at pH 6.5. At pH 6.8, the pericellular pH of tumour cells, therefore, cathepsins B and L would be almost optimally active and markedly stable. While the involvement of the activity of mature cathepsin L, extracellularly, in tumour invasion and metastasis is as yet unproved, the present study suggests that the properties of the mature enzyme do not preclude such a role.

The effect of reductive activation in markedly increasing the stability of both cathepsins B and L can be explained by the "unzipping" model proposed by Turk *et al.* (1994) for the pH-dependent inactivation of cathepsin B. Initiating the unzipping is disruption of the active site thiolate-imidazolium ($-S^-/^+HIm-$) ion pair,

either by deprotonation of the imidazole moiety at high pH, and/or by ionic competition. It follows from this model that in the absence of the -S^-/$^+$HIm- ion pair, which in isolated cathepsins B and L is presumably generated by activation with the reducing agent, the enzyme would be less stable. The results of the present study support this model.

It may be envisaged that during isolation, due to the oxidative environment obtaining *in vitro*, intermolecular or intramolecular (Brocklehurst and Kierstan, 1973) mixed disulfides may be formed, leading to inactivation of the enzyme. Subsequent reductive activation would be effected by removal of the group blocking the active site thiol. Consequent reconstitution of the -S^-/$^+$HIm- ion pair would lead to enhanced enzyme stability. In this model, inactive-but-activatable cysteine cathepsins are perceived as isolation artefacts. Evidence that at least a proportion of endogenous cathepsins B and L are active has been provided by *in vivo* labelling with radio-iodonated inhibitors (Mason *et al.*, 1989) and by the use of fluorogenic substrates (Assfalg-Machleidt *et al.*, 1992). Endogenous and thus secreted cathepsins may, therefore, be in an active form which would be maximally stable. Their subsequent inactivation and denaturation would nevertheless depend, among others, upon the redox potential of the environment into which they were secreted.

In previous studies (Mason, 1986; Kirschke *et al.*, 1989), the stability of cathepsin L was determined by pre-incubation of the unactivated enzyme for 1 h at various pH values, before measuring residual activity. Here we have shown that activation of the enzyme markedly increases its stability which suggests that pre-incubation of the unactivated enzyme is not a valid approach to determining its physiologically relevant stability.

The relative instability of isolated, unactivated, cathepsin B may underlie the difficulties which have been reported in obtaining polyclonal antibodies which react with the native enzyme (Mort and Recklies, 1986). In eliciting antibody production, the normal practice is to inoculate with unactivated enzyme. In the light of the results reported here, it would be expected that the unactivated enzyme would survive in its native conformation for only a very short time under extracellular conditions.

The extracellular activity of cathepsins is subject to modulation by inhibitors such as the cystatins (Barrett, 1987) and, in the blood, α_2-macroglobulin (Mason, 1989). Since ionic strength and buffer composition influence the interaction of cathepsin L with substrate, it is not implausible that the interaction with inhibitors might be similarly affected. To obtain physiologically relevant data on inhibition, therefore, it would seem prudent to also measure this in a buffer modelling the extracellular fluid, though to our knowledge this does not seem to be common practice. Nevertheless, it can be envisioned that the cystatins' role *in vivo* may depend upon the rate at which these can diffuse to the pericellular area versus the rate at which cathepsins are secreted.

In conclusion, we have shown here that the activity and stability of cathepsins L and B are affected by specific buffer ions as well as by pH, ionic strength, and the presence of protein substrates. Overall it may be concluded that their properties are such that these enzymes may well function extracellularly. Also, the stability of these enzymes is affected by their activation state, an observation which partially explains the markedly different results which have been previously reported concerning the stability of these enzymes. In their activated state the enzymes are also more stable than they were previously thought to be.

CHAPTER 6

PRODUCTION OF POLYCLONAL ANTIBODIES.

6.1 Introduction

Since the introduction of immunological assay methods in the 1950's, the manner in which analytes are detected has been greatly enhanced. Immunoassays are able to detect and quantify compounds with high specificity, which has revolutionised clinical and therapeutic biochemistry (Hamilton and Adkinson, 1988). In the field of invasive cancer, immunoassays have been used in diagnosis to identify potential metastatic markers in serum and tissue biopsies (Lah *et al.*, 1992a and b), while the production of specific antibodies to active enzymes offers a possible therapeutic approach (Dennison, 1989). This chapter details the production of antibodies to sheep liver cathepsin L, and stefin B complexed cathepsin L, and the immunolocalization of these molecules in the murine B16-BL6 melanoma and NIH 3T3 fibroblast cell lines.

6.1.1 Antibody production

For immunoassays to be functional, a specific antibody, produced against a pure antigen is required. Protein antigens will elicit a polyclonal immune response, derived from the stimulation of a number of B-cells, each responding to a particular immunogenic epitope. The affinity of the antibody preparation for the antigen will therefore be the collective efect of the numerous affinities and specificities of the individual antibodies produced. Polyclonal antisera have the advantage of increased chance of antigen recognition, as a number of epitopes of the antigen may be recognised. Concurrent with this, however, is the increased chance of cross reactivity with a closely related protein, as epitopes may be sufficiently similar to be co-recognised. Monoclonal antibodies minimise this possibility, as they are derived from a single B-cell clone, and the antibodies produced all target a single, specific epitope (Köhler and Milstein, 1975). In the 1980's, the introduction of anti-peptide antibodies, where a predetermined peptide (10-20 amino acids) is used to elicit a polyclonal response (avoiding the technical expertise required and the expense associated with monoclonal antibody production), has resulted in the production of antibodies which recognise a pre-determined epitope of a native protein, thereby

increasing the antibody specificity (Lerner, 1984). Prudent selection of the peptide sequence enhances the successful production of protein-reactive antibodies. This may be effected by examining the tertiary conformation of the enzyme (Pike, 1990), or by using computer based prediction algorithms which correlate possible epitope regions with physical parameters such as mobility and hydrophilicity (Westhof *et al.*, 1984; Hopp and Woods, 1981, 1983).

The additional advantage of antibodies with pre-determined specificities is that the epitope region may be selected to correspond with a specific region of the protein, such as the active site, thereby modulating, for example, enzyme activity. This may provide immunoinhibitory antibodies, which may improve therapeutic potential (Dennison, 1989). They may also aid in mapping of the active site of enzymes: for example, an immunoinhibitory antibody to cathepsin L, raised in chickens, recognised a peptide associated with the active site of cathepsin L (Coetzer *et al.*, 1992). Furthermore, the role of a particular enzyme in a metabolic process may be discovered. For example, the use of immunoinhibitory anti-cathepsin D antibodies shows the role of cathepsin D in the breakdown of cartilage (Dingle *et al.*, 1971). Heidtmann and co-workers (1993), used an anti-peptide antibody to the propiece of pro-cathepsin L to show that pro-cathepsin L is secreted from lung carcinoma cell lines. This antibody did not cross-react with precursors of the lysosomal cathepsins B, H or D.

Despite the advantages of monoclonal and anti-peptide antibodies, polyclonal antibodies continue to be raised to native proteins, and they continue to successfully distinguish closely related proteins. Bando *et al.*, (1986) immunised rabbits with rat kidney cathepsin L. The resulting IgG fraction did not cross-react with cathepsins B or H, but did recognise cathepsin L isolated from the liver, spleen and lung of rat. Mason (1986) raised antibodies to human cathepsin L in rabbits, which did not recognise cathepsins B and H (showing specificity for a particular enzyme), but which recognised species variants of cathepsin L. The general ability of polyclonal antibodies to recognise species variants has been exploited beneficially in the diagnostic field. Maciewicz *et al.* (1989) employed a polyclonal goat anti-rabbit cathepsin L to detect the levels of cathepsin L in human malignant and pre-malignant colorectal tumour cell lines.

Polyclonal anti-peptide antibodies have been raised to the peptide fragment of cathepsin L corresponding to amino acids 153-165. This peptide was selected after consideration of the 3-dimensional structure of the analogous cysteine proteinase, papain. Antibodies raised in rabbits, to this peptide, recognised the partially denatured enzyme in an ELISA, and the denatured enzyme in a Western blot, and immunoinhibited human and sheep cathepsin L activity (Coetzer *et al.*, 1991). These results were obtained with a peptide representing a continuous epitope. Most epitopes are, however, discontinuous (Van Regenmortel, 1986), and the authors suggested that the linear continuous peptide may form part of a larger, discontinuous, epitope (Coetzer *et al.*, 1992). In contrast to the effect of a targeted peptide antibody, polyclonal rabbit anti-sheep cathepsin L, raised to the whole, native enzyme was immunoactivatory rather than inhibitory (Coetzer *et al.*, 1992). These results emphasise the utility of anti-peptide antibodies as they clearly target a specific and different epitope to the polyclonal antisera against the whole enzyme.

At this point, attention should be drawn to the difference between immunoinhibitory antibodies and immunoprecipitatory antibodies. The former interact with the antigen enzyme in a manner that occludes or alters the active site. The latter type of antibody may apparently inhibit the enzyme, but it may not involve the active site at all. In the correct proportions, immunoprecipitatory antibodies react with antigen to form a network lattice which precipitates the enzyme and thus may render the active site inaccessible. Immunoprecipitation requires at least two epitopes on the antigen, which react with two different paratopes on two different antibody molecules (Clark, 1986), in order to form the immunoprecipitin lattice. By definition, therefore, monoclonal antibodies and anti-peptide antibodies are not immuno-precipitatory.

The difference in reactivity between antibodies produced in rabbits and chickens, suggests that their immune systems may respond to different epitope regions. The evolutionary distance between mammals and birds may enhance the immunogenicity of a mammalian antigen in birds, producing antibodies which respond to a different epitope. This may offer recognition of the antigen when the mammalian counterpart does not. For example, in immunocytochemistry, the fixation employed may alter the epitope presentation, and antibodies which recognise additional epitopes may increase the probability of successful targeting.

The present study raised the question: may certain antibodies recognise a region of association between two proteins only, and not the individual molecules comprising the complex? More specifically, in the case of stefin B-complexed cathepsin L, could antibodies be produced that would only recognise the complex and not cathepsin L or stefin B individually? Antibodies such as these would be extremely useful in the immunolocalisation of the complex in tumour biopsies and transformed cell lines. One approach to the formation of such antibodies would be to raise peptide antibodies to a peptide representing the link region between the two molecules. However, the present studies were done concurrently with the structural studies presented in Chapter 4 and as the region of association between the two molecules had not yet been elucidated, it was not possible to synthesise a peptide representing this region. It was consequently decided that an initial approach should be the production of polyclonal antibodies to the complex fraction which would be further purified by preparative SDS-PAGE.

Consequently, the purpose of the study reported here was to produce polyclonal antibodies to cathepsin L and stefin B-complexed cathepsin L, raised in both rabbits and chickens, and to assess their suitability for the immunocytochemical detection of cathepsin L and stefin B-complexed cathepsin L in various cell lines. This chapter reports on the production, isolation, characterisation (titre and specificity) and subsequent utilisation of the antibodies.

6.1.2 Cell culture

Given the ethical constraints concerning experimentation on humans and animals it is not surprising that a number of models have been developed to aid research into the mechanisms underlying tumour cell spread. For this reason and in an attempt to provide a degree of consistency concerning experimental approaches, many workers have employed tumour cell lines which can be propagated *in vitro*. The advantages of cell lines include their global availability, and their provision of sufficient quantities of tissue for biochemical and molecular studies (Evans, 1991b).

Rozhin *et al.* (1987; 1989) localised cathepsins B and L to the surface of the murine B16-BL6 melanoma, and this prompted the use of this cell line to immunolocalise cathepsin L using immunocytochemistry. This cell line is derived from a murine melanoma, which arose spontaneously in a C57BI/6J mouse. In the original work,

these cells were adapted to grow in tissue culture, detached from confluent layers using trypsin, and injected into the tail veins of recipient mice. The colonies which arose in the lungs were removed and grown *in vitro*, the pigmented colonies which arose from this were passaged and allowed to grow to confluence. Cells from these cultures were injected into recipient mice, and originally this procedure was repeated 5 times (Hart, 1979; Poste *et al.*, 1980). Subsequently, two variants have become established, the B16-F1 and B16-F10 lines, with 1 and 10 passages through the lungs respectively (Evans, 1991b). Hart (1979) selected an invasive variant by injecting B16-F10 cells into the bladder of male C57Bl6 mice via the *vas deferens*, following which the bladder was ligated, excised and cultured on semi-solid agar. Tumour cells which migrated through the bladder wall were collected, grown *in vitro*, and recycled through the same process a total of 6 times to yield the B16-BL6 variant. Compared to the parent B16-F10 cell line, this was less motile, more resistant to trypsinisation and produced less plasminogen activator (Hart, 1979). The B16-BL6 variant was shown to produce more tumours than the parent line when injected intravenously or intramuscularly into host mice (Weiss *et al.*, 1982) providing evidence that the B16-BL6 cells have increased metastatic capacity.

As a comparison to the metastatic murine cells, the murine fibroblast cell line NIH 3T3 was employed. This is a normal cell line derived from mouse embryo cells which has undergone several passages (Evans, 1991b). This cell line has also been transformed by transfection with viral oncogenes, to produce derivative cell lines, such as KNIH 3T3 which was transfected with the Kirsten virus murine sarcoma oncogene and was used to identify MEP (procathepsin L) as the major protein subsequently secreted (Dong *et al.*, 1989).

6.2 Materials and Methods

Those specific experimental protocols employed in this chapter are outlined below. Methods employed generally are detailed in Chapter 2. For the immunisation of experimental animals, cathepsin L was isolated as previously described (Section 3.2.1). Stefin B complexed cathepsin L required additional purification in order to separate the covalent and non-covalently bound complex and this was effected by preparative SDS-PAGE.

6.2.1 The isolation of covalently complexed stefin B-cathepsin L

Stefin B complexed cathepsin L, from MEC (peak 1, Fig. 3.7) was subject to preparative SDS-PAGE (Section 2.4.1) under non-reducing conditions. Typically, a sample volume (2.5 ml) was treated with treatment buffer (2.5 ml) and loaded into the trough. Following electrophoresis, the gel was disassembled, and strips of gel (0.5 cm) were excised from the left and right side of the gel. These strips were stained using the rapid Serva blue G staining procedure (Section 2.5) and the intact gel was stored at 4°C for the duration of the staining procedure to limit diffusion. The stained strips were placed alongside the unstained gel on either side, and the region of gel corresponding to the stained 37 kDa band on the strips was excised. The gel was passed through a 2.5 ml syringe (without the needle), mixed with buffer A (Section 3.2) (2 ml) and dialysed against buffer A (16 h). The dialysate was concentrated against sucrose to a typical volume of 5-6 ml. Aliquots were removed to determine enzyme activity against Z-Phe-Arg-NHMec (Section 2.9.2), protein concentration (Section 2.2.1) and to determine purity by SDS-PAGE (Section 2.4.1) and the sample was stored at -75°C.

6.2.2 Production of antibodies in experimental animals

Antibodies to cathepsin L and the isolated complex were raised in both rabbits and chickens. Two animals were immunised with each antigen, except for the isolated complex, where one animal was immunised because The amount of sample available was limited. Cathepsin L and isolated complex (50 µg) were emulsified with Freund's complete adjuvant (1:1 ratio) and injected subcutaneously at 3-4 sites on the back of the rabbits. Further inoculations (at the same dose) were administered in Freund's incomplete adjuvant, in the same manner, at two weeks and thereafter at four week intervals. Rabbits were bled from the marginal ear vein at eight weeks and by non-lethal cardiac puncture at 12 weeks. Serum was separated from the blood clots and the IgG was isolated (Section 2.10.2), and stored at -20°C.

Chickens were inoculated in the same manner, by intramuscular injections at a single site in each of the breast muscles with cathepsin L and complex (50 µg) and Freund's complete adjuvant (1:1). Further inoculations were administered in Freund's incomplete adjuvant at two weeks and thereafter at monthly intervals (at the same concentration). Eggs were collected daily and stored at 4°C.

The progress of the animals' immune response to the immunogens was followed by ELISA analysis of egg yolk extracts and serum samples, prior to isolation of IgY or IgG (Section 2.10). The use of yolk extracts offered a satisfactory method for determining which eggs should be selected for IgY isolation. Yolk extracts were prepared by mixing the washed egg yolk with two volumes of 100 mM phosphate buffer, pH 7.6.

The IgY and IgG fractions were subjected to analysis using ELISA to determine titres (Section 2.11), and Western blots to monitor specificity (Section 2.8). Following characterisation, these antibodies were employed in the immunocytochemical investigations of cathepsin L and complex distribution in various cell lines.

6.2.3 Cell culture

B16-BL6 cells were cultured as recommended by Dr Carl Albrecht, in McCoy's 5A medium, with 10% foetal calf serum. NIH 3T3 cells were grown as recommended by Dr Elke Bey, Highveld Biological, in MEM, non-essential amino acids and 15% foetal calf serum.

6.2.3.1 Reagents

The B16-BL6 cell line was obtained from Dr Carl Albrecht, Tygerberg Hospital, Tygerberg, South Africa, and NIH 3T3 cells were purchased from Highveld Biological, Johannesburg, South Africa. Powdered culture medium, McCoy's 5A, minimal essential medium (MEM), Hanks' balanced salt solution, sterile 200 mM L-glutamine solution and trypsin-EDTA (x 1) solution were purchased from Sigma Chemical Co., St. Louis, MO. Sterile non-essential amino acid solution (NEAA) (x 100) was from Highveld Biological, Johannesburg, South Africa. Foetal calf serum was from Gibco, Paisley, U.K. and sodium hydrogen carbonate (AR) was from Sigma. Where applicable, solutions were filtered through a 0.22 μm filter (Millipore, Microsep, Pinetown, South Africa). Bibby Sterilin sterile plastic cultureware was from Weil Organisation, Pinetown, South Africa. All water used was ultra-pure grade, supplied by a Millipore Milli-Q Plus system. Glassware was washed in phosphate-free detergent, 7X-PF (ICN Biomedicals, Ltd, Irvine, Scotland), rinsed well in tap water and in double distilled water prior to autoclaving.

B16-BL6 culture medium (McCoy's 5A, 10% FCS, pH 7.3). Powdered medium and sodium hydrogen carbonate (2.2 g) were added consecutively to 900 ml ultra-pure water and adjusted to pH 7.2 with 1 M NaOH. This solution was filter sterilised (0.22 µm filter) and aseptically dispensed into sterile containers containing a volume of FCS to give a final concentration of 10% FCS. Complete medium was stored at 4°C.

NIH 3T3 culture medium (MEM, 10% NEAA, 15% FCS, pH 7.0). Powdered medium was added to 900 ml of ultra-pure water and stirred until dissolved. Additional water was added to the final volume minus the volume of glutamate and sodium bicarbonate. The solution was autoclaved (121°C, 15 p.s.i., 15 min) and allowed to cool to 15-20°C. A volume of filter sterilised 7.5% (m/v) sodium bicarbonate (29.3 ml) and 200 mM sterile L-glutamine solution (10 ml) was added to each litre. An aliquot of medium (5 ml) was removed to check pH. If necessary, the pH was adjusted to pH 7.0 with sterile NaOH, using sterile technique. Medium was aseptically dispensed into storage bottles, containing volumes of NEAA (x 100) and FCS to give final concentrations of 10 and 15% respectively. Complete medium was stored at 4°C.

HBSS (Hanks' balanced salt solution, pH 7.2). Powdered medium was dissolved with gentle stirring in 900 ml of ultra-pure water. Sodium bicarbonate (0.35 g) was added to this and the pH was adjusted to pH 7.1 with 1 M NaOH. Water was added to the final volume and the solution was sterile filtered (0.22 µm filter), aseptically dispensed into sterile containers and stored at 4°C.

6.2.3.2 Procedure

Cells were maintained in 25 cm^3 flasks in complete medium in a humidified atmosphere containing 5% CO_2 at 37°C. Upon reaching confluence cells were passaged by washing with pre-warmed HBSS (x 3) and rinsing with a small volume of (pre-warmed) trypsin-EDTA to leave a thin film covering the monolayer. The flasks were incubated at 37°C and observed microscopically to monitor cell detachment. Cells were diluted in complete medium and split in a ratio of 1:3. The B16-BL6 cells grew faster than the NIH 3T3 cells, requiring splitting twice a week, while the fibroblasts were passaged approximately once a week.

6.2.4 Immunofluorescence microscopy

This technique allows the observation of the distribution of an antigen in a cell or tissue, by exploiting the high specificity of immunodetection in combination with low magnification light microscopy. It was used to monitor the distribution of some proteolytic enzymes in the B16-BL6 and NIH 3T3 cell lines.

6.2.4.1 Reagents

Nunc multiwell plates were from Weil Organisation, Pinetown, South Africa, round glass coverslips (15 mm) were from Chance Propper (supplied by Lasec, Durban, South Africa). Slow-Fade™ was from Molecular Probes, Eugene, Oregon. Paraformaldehyde was from BDH, Poole, England, and saponin was from Sigma Chemical Co., St Louis, MO.

Phosphate buffered saline (PBS) (8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 136.89 mM NaCl, 2.68 mM KCl, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.3). Na_2HPO_4 (1.145 g), KH_2PO_4 (0.2 g), NaCl (7.99 g), KCl (0.199 g), $CaCl_2$ (0.147 g) and $MgCl_2$ (0.1015 g) were dissolved in about 800 ml of dist.H_2O, adjusted to pH 7.3 if necessary and made up to 1 litre.

16% (m/v) Paraformaldehyde stock solution. Paraformaldehyde (16 g) was dissolved in dist.H_2O (100 ml), warmed to 60°C and cleared with a minimum amount of 1 M NaOH. The solution was stored at -10°C until required.

3.7% (v/v) Paraformaldehyde in PBS. Stock solution (3.47 ml) was added to PBS (11.53 ml) and adjusted to pH 7.3 with 1 M HCl.

2% (m/v) Bovine serum albumin in PBS (BSA-PBS). Bovine serum albumin (0.8 g) was dissolved in PBS and made up to 40 ml.

0.1% (m/v) Saponin in PBS. Saponin (0.1 g) was dissolved in PBS and made up to 100 ml.

5% Foetal calf serum. Foetal calf serum (0.5 ml) was mixed with PBS and made up to 10 ml.

Antibodies. Chicken anti-porcine cathepsin D IgY (a gift from Philip Fortgens, Biochemistry, UNP, Fortgens, 1997) was used at 50 μg/ml, rabbit anti-human

cathepsin H peptide (a gift from Dr Theresa Coetzer, Biochemistry, UNP) at 300 μg/ml, chicken anti-human cathepsin S peptide IgY (Liesl Morrison, Biochemistry, UN) at 300 μg/ml, rabbit anti-human cathepsin B IgG (a gift from Dr Edith Elliott, Biochemistry, UNP) at 40 μg/ml, chicken anti-sheep cathepsin L-stefin B complex (raised in this study) at 150, 300 and 600 μg/ml, rabbit and chicken anti-sheep cathepsin L (raised in this study) at 150, 300 and 600 μg/ml, rabbit anti-chicken IgG-FITC (Sigma, F-888) at 1/320 dilution, and goat anti-rabbit IgG-TRITC (Sigma, T-5268) at 1/250 dilution. Non-immune sera were used as controls at the lowest dilutions, i.e. 600 μg/ml.

6.2.4.2 Procedure

Cells were seeded at a low density into duplicate 12-well Nunc Multiwell plates, each well containing a 15 mm sterile glass coverslip. Before confluence was reached, cells were washed three times with warmed (37°C) PBS, fixed (10 min, RT) with 3.7% formaldehyde in PBS and washed again in PBS (x 3). Non-specific binding sites were blocked by incubating cells in BSA-PBS (45 min, RT). Coverslips were incubated in the primary antibody (1 h, RT) diluted in saponin in PBS. After six rapid washes with saponin in PBS, 5% foetal calf serum block solution was applied (30 min, RT). Cells were incubated in the appropriate secondary antibody (1 h, RT) washed six times in saponin in PBS, re-fixed in 3.7% formaldehyde (10 min, RT), washed three times in PBS followed by dist.H_2O and the coverslips allowed to dry. The coverslips were mounted face-down in a minimum amount of Slow-Fade™, sealed with clear nail varnish, and viewed in an Olympus BH2 microscope equipped with a BH2-RFC epifluorescence attachment, with a 460 nm interference filter and a 520 nm barrier filter. Representative cells were photographed with Agfacolor XRG 200 or Ilford XP2 film. A non-immune antibody preparation was substituted for immune antibody at a concentration equal to the lowest dilution used for the immune antibody. Non-immune incubations were assessed for acceptably low background fluorescence; since this was generally obtained, non-immune controls are not shown in the results.

6.3 Results

6.3.1 SDS-PAGE of covalently bound stefin B-cathepsin L complex

Preparative SDS-PAGE effected the isolation of the covalently bound complex. Protein determination by the modified micro-Bradford assay revealed that typically a 40% yield was obtained (Table 6.1). Allowance for the fact that the starting fraction contained a heterogeneous mixture of covalent and non-covalent complex, and that the protocol was subject to a certain percentage loss, this yield was considered to be acceptable. This method was chosen above others such as electroelution, owing to its ease of use and greater yield, which far outweighed the limited yields obtained by these other techniques (Kirk, 1992). Enzyme activity against Z-Phe-Arg-NHMec showed that the isolated complex had lost most of its proteolytic ability (Table 6.1).

Table 6.1. The yield and activity of the covalent cathepsin L - stefin B complex, isolated by preparative SDS-PAGE.

Sample	Protein concentration (mg/ml)	Volume (ml)	Total Protein (µg)	Protein yield (%)	Activity yield (%)
MEC peak (complex)	0.225	2.5	562.5	(100)	(100)
Complex isolated by SDS-PAGE	0.380	6.0	228.0	40	2

SDS-PAGE analysis under non-reducing conditions, however, showed the presence of 37 kDa, 26 kDa and 14 kDa bands (Fig. 6.1). These proteins correspond to free cathepsin L and stefin B respectively, and are believed to be as a result of the effect of electrophoresis on the covalently bound complex. Substrate gel electrophoresis showed activity in the region of 37 kDa (Fig. 6.2).

Figure 6.1. Non-reducing SDS-PAGE analysis of the isolation of covalent cathepsin L-stefin B complex.

(a) Molecular weight markers (BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa, and cytochrome C, 12 kDa), (b) - (d) cathepsin L isolated by preparative SDS-PAGE, 250, 500 and 750 ng respectively, (e) - f) stefin B complexed cathepsin L isolated by preparative SDS-PAGE, 250, 500 and 750 ng respectively.

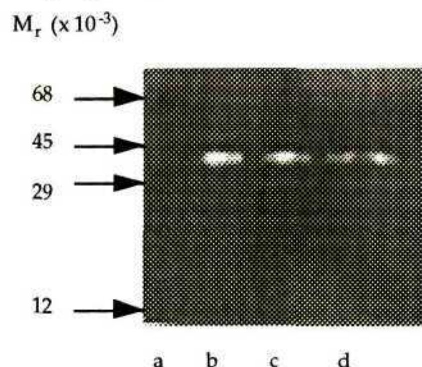


Figure 6.2. SDS-PAGE zymogram of the isolated cathepsin L-stefin B complex.

(a) Molecular weight markers (BSA, 68 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 29 kDa, and cytochrome C, 12 kDa), (b) - (d) cathepsin L complexed stefin B isolated by preparative SDS-PAGE, 750, 500 and 250 ng respectively, performed on a Laemmli gel containing 0.1% gelatin, and treated as outlined in Section 2.7.

6.3.2 Production and characterisation of anti-cathepsin L antibodies

Polyclonal antibodies to sheep liver cathepsin L were produced using both the conventional mammalian (rabbit) and the avian (chicken) immune systems. The rabbit IgG fractions from the two different animals showed a good but differing response (Fig. 6.3), with significantly different titres. The antibody titre, which is expressed as the concentration of antibody at which the A_{405}-values are notably higher than the equivalent absorbance of the non-immune controls, is a good indication of the antibody concentration (although this is not necessarily an indication of antibody affinity - see discussion) (Devey and Steward, 1988), and may be used to evaluate the anticipated performance of the antiserum. Rabbit 1 exhibited a limited antibody response at 8 weeks, but an improved response at 12 weeks. In both instances, however, the titre was 18 µg/ml. Rabbit 2 showed an improved response at 8 weeks which did not vary notably at 12 weeks, although even at

0.4 μg/ml the antibody had not titrated to completion. These results suggest that rabbit 2 elicited a superior immune response compared to rabbit 1.

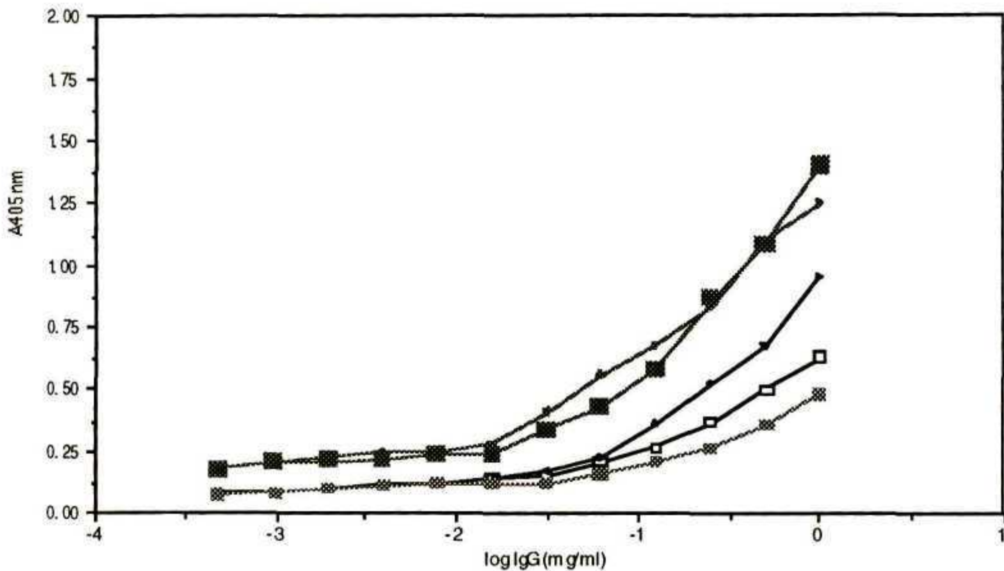


Figure 6.3. The progress of immunisation and titres of rabbit antibodies raised against sheep liver cathepsin L.

Sheep liver cathepsin L was coated onto the ELISA plates at a concentration of 1 μg/ml, and incubated with serial two-fold dilutions of rabbit (1 and 2) anti-sheep liver cathepsin L at 8 and 12 weeks.
❑ - rabbit 1 anti-sheep liver cathepsin L (8 weeks),
◆ - rabbit 1 anti-cathepsin L (12 weeks),
■ - rabbit 2 anti-sheep liver cathepsin L (8 weeks),
◇ - rabbit 2 anti-sheep liver cathepsin L (12 weeks),
▩ - pre-immune serum. Each point is the mean of duplicate A_{405} values.

The corresponding progress of the avian immune response showed that both birds had responded similarly to the immunogen, eliciting a strong response at 8 weeks which remained so at 12 weeks (Fig. 6.4). The titre of all preparations was 4 μg/ml, which compares favourably to results obtained previously (Coetzer, 1992). However, previous immunisation of chickens with cathepsin L showed that the response increased notably between, 4, 6, 8 and 12 weeks (Coetzer, 1992), but this was not the case in this instance. There may have been an increase in immune response at 5 weeks, but both birds ceased egg production between 4 and 6 weeks, and therefore no IgY was isolated for this period.

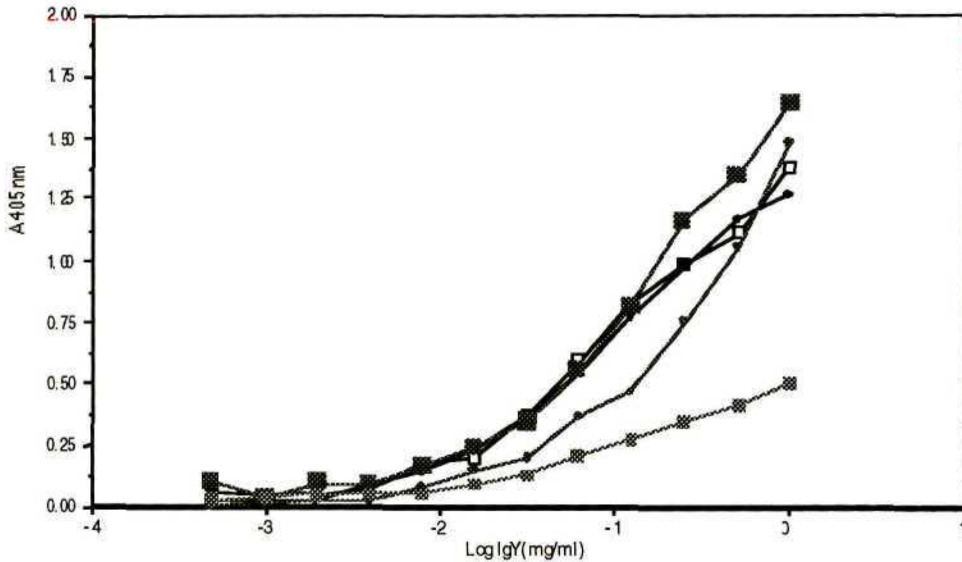


Figure 6.4. The progress of immunisation and titres of chicken antibodies raised against sheep liver cathepsin L.

Sheep liver cathepsin L was coated onto the plates (1 μg/ml) and titrated with serial two-fold dilutions of IgY anti-sheep liver cathepsin L (chicken 1 and 2) at 8 and 12 weeks.
❑ - chicken (1) anti-sheep liver cathepsin L (8 weeks),
◆ -chicken (1) anti-sheep liver cathepsin L (12 weeks),
■ - chicken (2) anti-sheep liver cathepsin L (8 weeks),
◇ - chicken (2) anti-sheep liver cathepsin L (12 weeks),
▩ - pre-immune IgY. Each point is the mean of duplicate A_{405} values.

The specificities of the rabbit and chicken antibodies were verified using Western blot analysis against a crude ion-exchange fraction of sheep liver, obtained by a modification of the purification procedure in which the entire fraction of proteins bound to the S-Sepharose was eluted with NaCl (1 M), and against cathepsin L, complex and *in vitro* formed complex (Fig. 6.5).

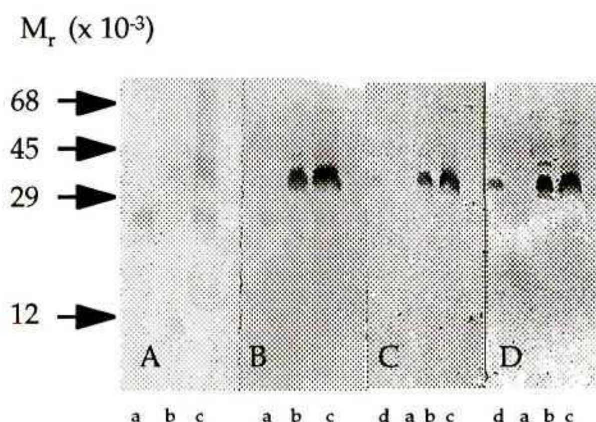


Figure 6.5. Western blot analysis of antibodies against sheep liver cathepsin L and stefin B complexed cathepsin L.

(a) cathepsin L (5 µg), (b) complex (10 µg), (c) crude S-Sepharose fraction (50 µg), (d) *in vitro* formed complex (ca. 30 µg), on a 12.5% gel, blotted and probed with antibodies as described in Section 2.8.

A: Rabbit anti-sheep cathepsin L, 50 µg/ml.
B: Chicken anti-sheep cathepsin L, 50 µg/ml.
C: Rabbit anti-sheep complex, 50 µg/ml.
D: Chicken anti-sheep complex, 50 µg/ml.

The antibody preparations targeted the cathepsin L at 26 kDa and higher bands and complex fractions successfully. Three bands were recognised in the crude ion exchange fraction, at 37, 42/45 and 68 kDa, which were presumed to be higher molecular weight variants of cathepsin L, as discussed below.

6.3.3 Production and characterisation of anti-complex antibodies

Antibodies to the cathepsin L - stefin B complex isolated by preparative SDS-PAGE were successfully raised in both rabbits and chickens. ELISA results showing the progress of immunisation, employing the isolated complex as antigen, exhibited a strong response at 8 weeks, which had subsided at 12 weeks (Fig. 6.6). The titres were 6 µg/ml and 4 µg/ml for 12 and 8 week IgG fractions respectively. Using cathepsin L as the antigen coated onto the plate, showed a weaker immune response, with titres of about 30 µg/ml. Employing stefin B and *in vitro* formed complex as the antigen in the ELISA, resulted in strong recognition by the rabbit IgG fraction in both cases (Fig. 6.7).

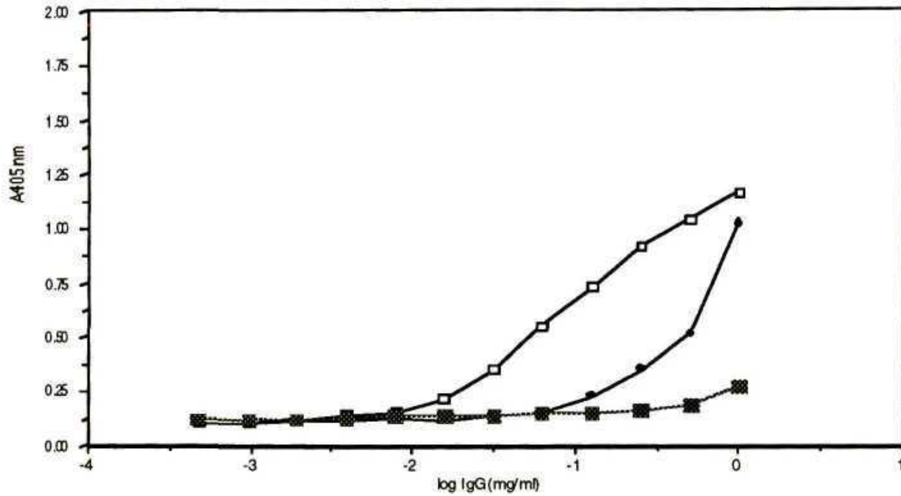


Figure 6.6. The progress of immunisation and titres of rabbit antibodies raised against the isolated sheep liver cathepsin L-stefin B complex .

Complex was coated onto ELISA plates at a concentration of 1μg/ml and incubated with serial two-fold dilutions of rabbit anti-complex antibody (8 and 12 weeks).

❑ - rabbit anti-complex (8 weeks),

◆ - rabbit anti-complex (12 weeks),

■ - pre-immune serum. Each point is the mean of duplicate A_{405} values.

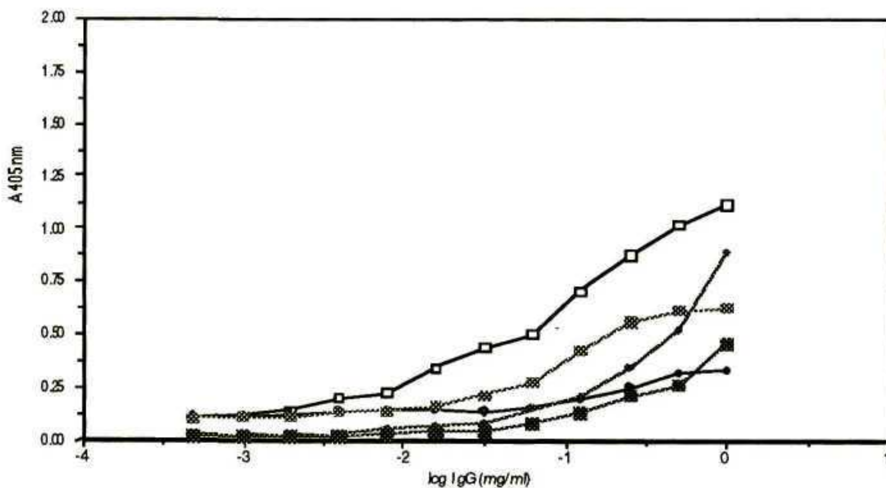


Figure 6.7. The recognition of cathepsin L, stefin B and *in vitro* formed complex by rabbit anti-complex IgG.

Each antigen was coated at 1 μg/ml and the relevant pre-immunes are plotted.

❑ - rabbit anti-complex (12 weeks, stefin B as the coated protein),

◆ - pre-immune serum (stefin B as the coated protein),

■ - rabbit anti-complex (12 weeks, *in vitro* formed complex as the coated protein),

◇ - pre-immune serum (*in vitro* formed complex as the coated protein),

▩ - rabbit anti-complex (12 weeks, cathepsin L as the coated protein). Each point is the mean of duplicate A_{405} values.

Antibodies raised in chickens to the isolated complex targeted the isolated complex strongly in an ELISA (Fig. 6.8). The antibodies recognise cathepsin L strongly as an antigen, but do not recognise in vitro formed complex or stefin B very well (Fig. 6.9).

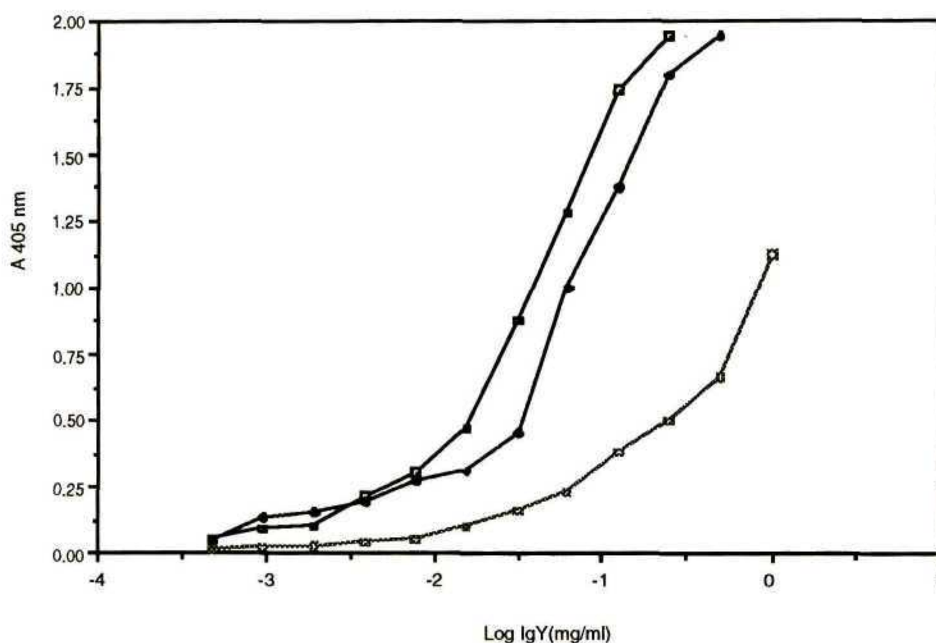


Figure 6.8. The progress of immunisation and titres of chicken antibodies raised against the isolated complex.

Complex was coated onto ELISA plates at a concentration of 1μg/ml and incubated with serial two-fold dilutions of chicken anti-complex antibody (8 and 12 weeks).
❑ - chicken anti-complex (8 weeks),
◆ - chicken anti-complex (12 weeks),
■ - pre-immune serum. Each point is the mean of duplicate A_{405} values.

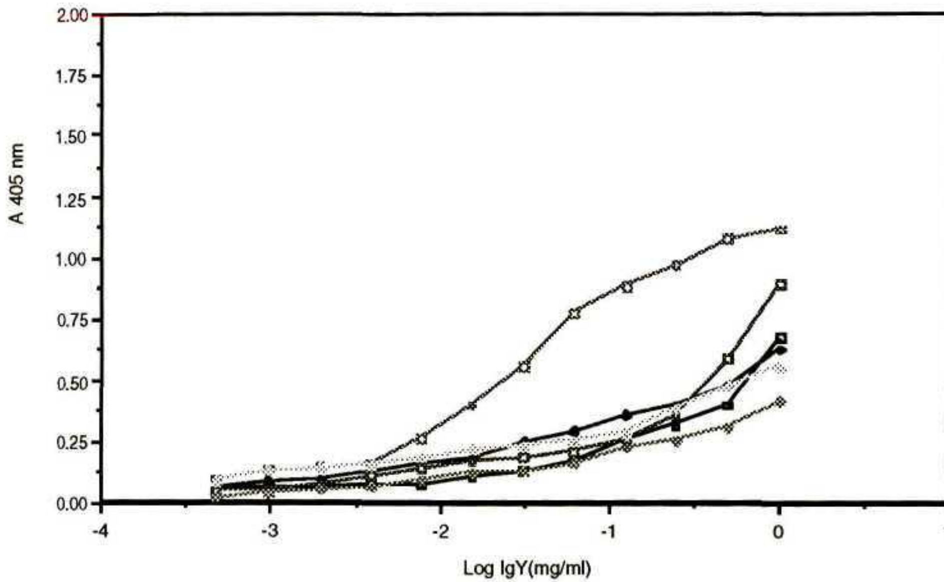


Figure 6.9. The recognition between anti-isolated complex IgY and cathepsin L, stefin B and *in vitro* formed complex.

Each antigen was coated at 1 µg/ml and the relevant pre-immunes are plotted.

❑ - chicken anti-complex (12 weeks, stefin B as the coated protein),

◆ - pre-immune serum (stefin B as the coated protein),

■ - chicken anti-complex (12 weeks, *in vitro* formed complex as the coated protein),

◇ - pre-immune serum (*in vitro* formed complex as the coated protein),

❑ - chicken anti-complex (12 weeks, cathepsin L as the coated protein),

▲ - pre-immune serum (cathepsin L as the coated protein). Each point is the mean of duplicate A_{405} values.

The chicken anti-isolated complex recognised cathepsin L very weakly but recognised the complex and high molecular weight variants quite strongly when tested in a Western blot. The crude ion exchange fraction showed strong recognition at 37 kDa, and weak targeting at 26, 45 and 68 kDa bands. The complex fraction showed strong recognition at 37 kDa, weaker recognition at 45 kDa, and no recognition at 26 kDa, while the cathepsin L fraction showed weak recognition at 26 kDa only. The antibody recognised a 37 kDa band in the isolated complex fraction. No recognition was observed at 14 kDa (stefin B) (Fig. 6.5 D). Remarkably, the rabbit anti-isolated complex recognised the 37 kDa band of the *in vitro* complex in Western blot analysis. In the crude ion exchange fraction, it recognised 37, 45 and 68 kDa bands only, in the *in vivo* and *in vitro* formed complex fraction, it targeted the 37 and

bands only, in the *in vivo* and *in vitro* formed complex fraction, it targeted the 37 and 45 kDa bands, while the cathepsin L and stefin B bands were not detected in any of the fractions (Fig 6.5 C).

In order to facilitate interpretation of the immunofluorescence studies report in Section 6.3.4, the reactivities of the rabbit and chicken anti-cathepsin L and anti-complex antibodies are summarised in Tables 6.2 and 6.3 respectively.

Table 6.2. Summary of reactivity of rabbit and chicken anti-cathepsin L antibodies.

Protein (sheep)	MW (kDa)	Western Blot	
		Anti-sheep cathepsin L	
		Rabbit	Chicken
stefin B	14	–	–
cathepsin L	26	++	++
in vitro complex	37	+	+
isolated complex	37	+++	++++
crude component	45	+	++
crude component	68	+	++

Table 6.3. Summary of reactivity of rabbit and chicken anti-isolated cathepsin L-stefin B complex antibodies

Protein (sheep)	MW (kDa)	ELISA		Western Blot	
		Anti-isolated sheep cathepsin L-stefin B complex			
		Rabbit	Chicken	Rabbit	Chicken
stefin B	14	++	–	–	–
cathepsin L	26	±	++	–	±
in vitro complex	37	–	+	-	++
isolated complex	37	++	+++	+++	+++
crude component	45	NT	NT	+	++
crude component	68	NT	NT	+	++

Points to be noted from Table 6.3 are:-

- The differential reactivity between the cathepsin L-stefin B complex formed *in vitro* (which would be largely the known, tight-binding, non-covalent, complex) and the isolated, covalent, complex confirm that there are structural differences between these complexes, with a cathepsin L epitope being obscured by stefin B in the complex formed *in vitro.*
- Rabbit anti-isolated complex appears to recognise only isolated complex and higher molecular weight forms of cathepsin L, in Western blots, but does not recognise the individual cathepsin L and stefin B components of the isolated complex.

6.3.4 Immunofluorescence microscopy

In this study cathepsins L, B, D, S and H were immunolocalised in the B16-BL6 and NIH 3T3 murine melanoma and fibroblast cell lines.

B16-BL6 mouse melanoma cells.

- Labelling for cathepsin L. From Table 6.2 it can be seen that, in Western blots, both rabbit and chicken anti-sheep cathepsin L antibodies seemed to target cathepsin L in all molecular forms. However, in the B16-BL6 cells generally, the antibodies showed poor recognition. With the rabbit antisera, labelling was achieved only at low dilutions [i.e. high concentrations (600 μg/ml)] and no labelling was observed at concentrations of 150 and 300 μg/ml. Rabbit anti-cathepsin L showed a medium intense fluorescence distributed over the whole cell, and thus possibly being at the surface, and being slightly more intense at the nuclear region (Fig. 6.10A). The chicken anti-cathepsin L IgY (600 μg/ml) showed a similar diffuse labelling (Fig. 6.10B), though with greater intensity at the peri-nuclear regions, suggesting a surface distribution and possible labelling of the endoplasmic reticulum and Golgi network. As cathepsin L is a lysosomal enzyme, more distinct labelling of vesicles was expected, but this was not observed and the specificity of the labelling, at the high antibody concentrations used, may be questionable.

- Labelling for cathepsin L-stefin B complex. Rabbit anti-complex antibody (150 μg/ml) (Fig. 6.10C, D) showed diffuse general labelling as well as punctate labelling of vesicular bodies within the cytoplasm. Chicken IgY anti-complex antibody showed intense fluorescent labelling of the perinuclear region, possibly the endoplasmic reticulum and Golgi network, and vesicular labelling, suggesting possible trafficking to endosomes (Fig. 6.11A, B, C and D). Anti-complex antibodies may or may not target cathepsin L itself, but they do appear to target higher molecular mass forms, including the covalent cathepsin L-stefin B complex (Table 6.3). In normal circumstances, stefin B should not encounter cathepsin L, as it is a cytoplasmic protein whereas cathepsin L occurs in vesicles of the endosome/lysosome system. The higher molecular mass entities targeted are thus most likely to be precursor forms of cathepsin L, and labelling of the ER and Golgi, through to secretory vesicles or the late endosome, is thus not unexpected.

However, when cells are homogenised, in order to do a Western blot, the cathepsin L and stefin B would be able to interact. The most likely outcome of this is the formation of the normal non-covalent complex (since the purported enzyme which catalyses synthesis of the covalent complex does not occur in all tissues), and a consequent decline in immunoreactivity (Table 6.3). This may partly explain why it has proved difficult to demonstrate the presence of cathepsin L in Western blots of B16-BL6 homogenates (blots not shown). However, exactly why antibodies raised against the complex should target procathepsin L is at present only a matter for conjecture. It may be speculated that the propiece of procathepsin L may hold the enzyme moiety in a similar conformation to that in which it is held by stefin B in the covalent complex, leading to a common structure which can be recognised in both molecules (the complex and the proenzyme).

- Labelling for cathepsin D. Cathepsin D is a classic marker of "lysosomes". However, anti-mature cathepsin D antibodies would also label precursor forms, which may occur in secretory vesicles as well as vesicles in transit to lysosomes. Chicken anti-porcine cathepsin D IgY (50 μg/ml) showed clear punctate labelling distributed throughout the cytoplasm (Fig. 6.12 A, B), to the ends of the cellular extensions. This may indicate a lysosomal distribution, but previous work in our laboratory using Western blotting, suggests that the cathepsin D present in B16-BL6 cells is larger than the 30 kDa mature form (results not shown) which may indicate that it is on a secretory pathway, which would explain the peripheral distribution.

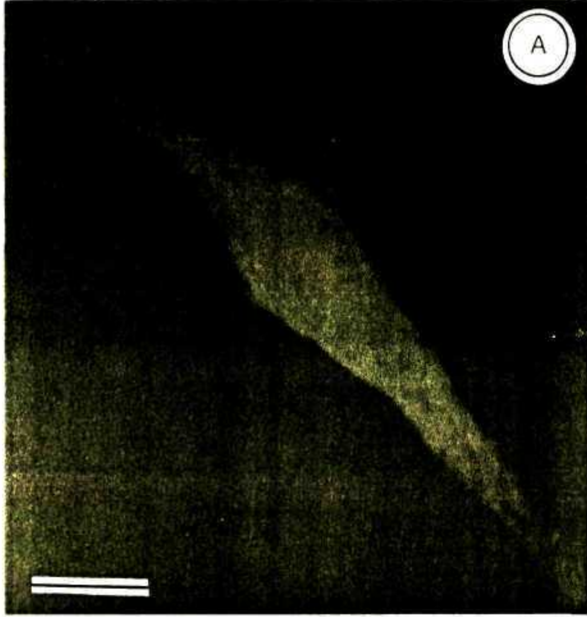
- Labelling for cathepsin H. Rabbit anti-cathepsin H showed defined labelling in small and large compartments, with the larger vesicles localising closer to the nucleus, and the smaller vesicles being more peripherally distributed (Fig. 6.12 C, D). Claus *et al.* (1998) have reported finding that cathepsin H occurs largely in early endosomes and the labelling observed here is consistent with this.

- Labelling for cathepsin B and S. No labelling for cathepsin B or S was found in this cell line.

Figure 6.10. Immunofluorescent labelling of B16-BL6 cells for cathepsin L and stefin B-complexed cathepsin L.

B16-BL6 cells were grown overnight on coverslips, and labelled (Section 6.2.4.2) with (A) rabbit anti-sheep cathepsin L (600 μg/ml), (B) chicken anti-sheep cathepsin L (600 μg/ml), (C) and (D) rabbit anti-sheep cathepsin L-stefin B complex (150 μg/ml).

Bar scales: (A) - (D) 10 μm.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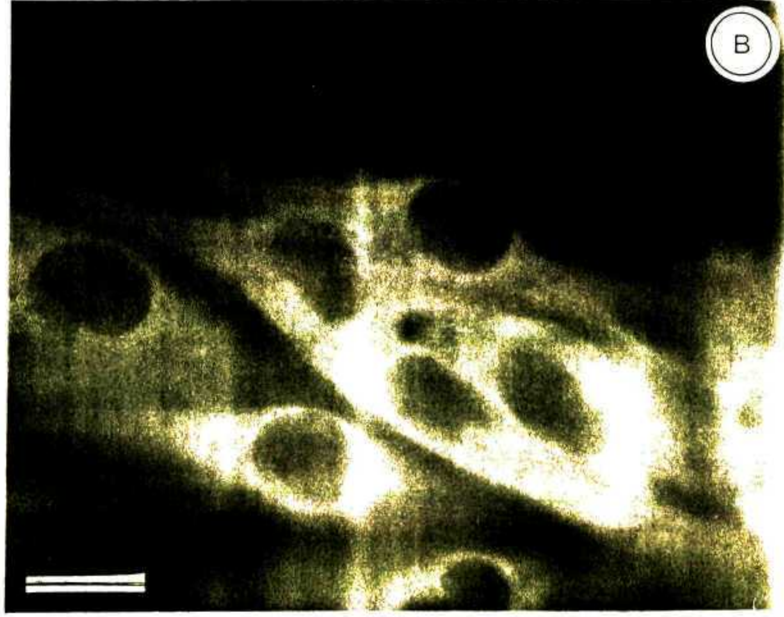
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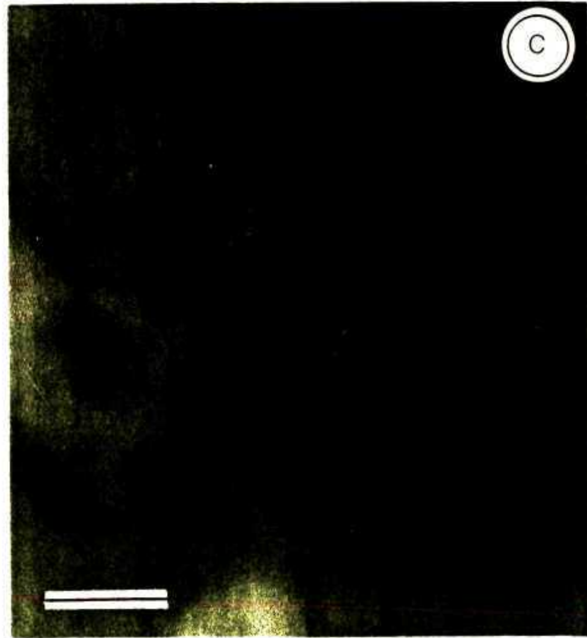
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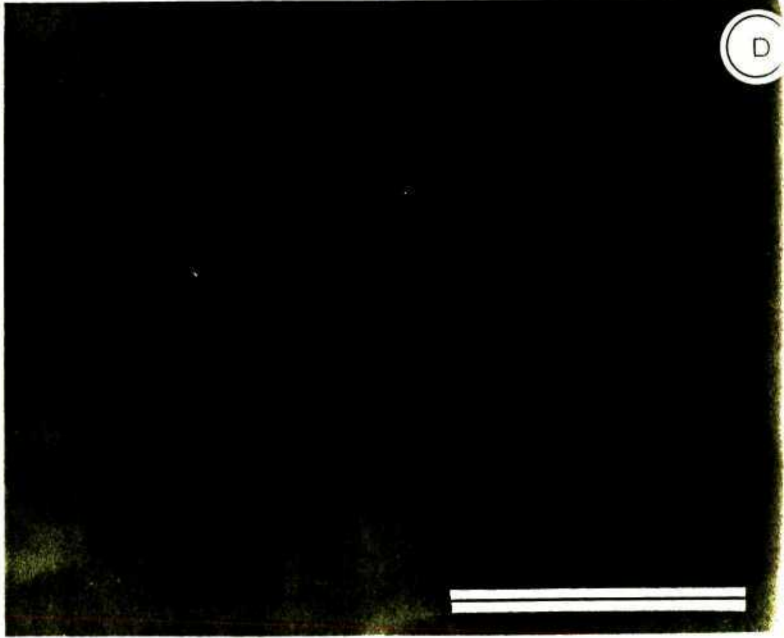
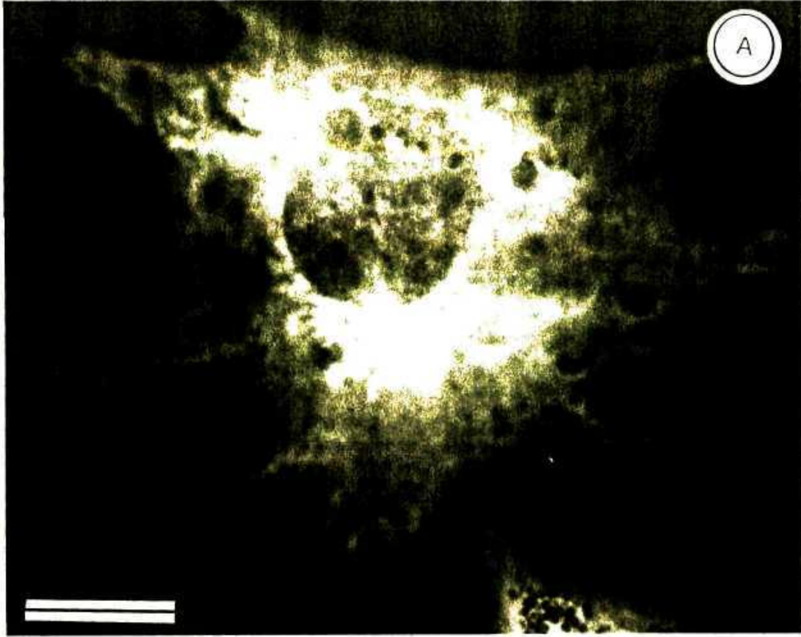

D

Figure 6.11. Immunofluorescent labelling of B16-BL6 cells for cathepsin L and stefin B-complexed cathepsin L.

B16-BL6 cells were grown overnight on coverslips, and labelled (Section 6.2.4.2) with (A) - (D) chicken anti-sheep stefin B complexed cathepsin L (600 μg/ml).

Bar scales: (A) - (D) 5 μm.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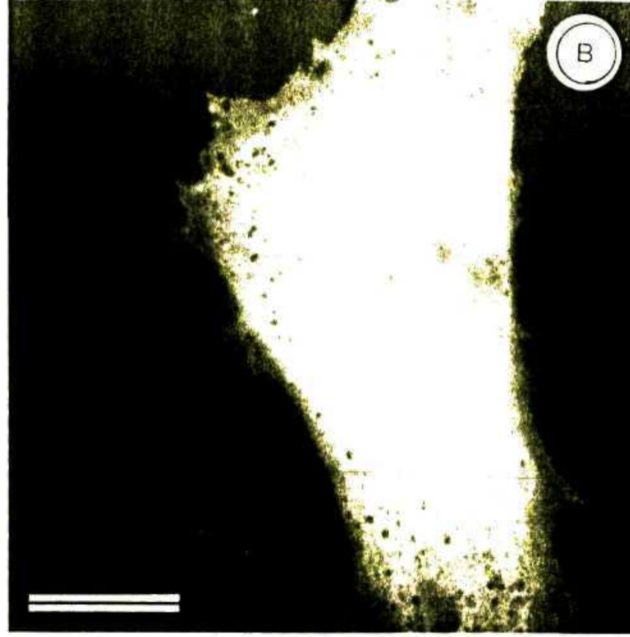
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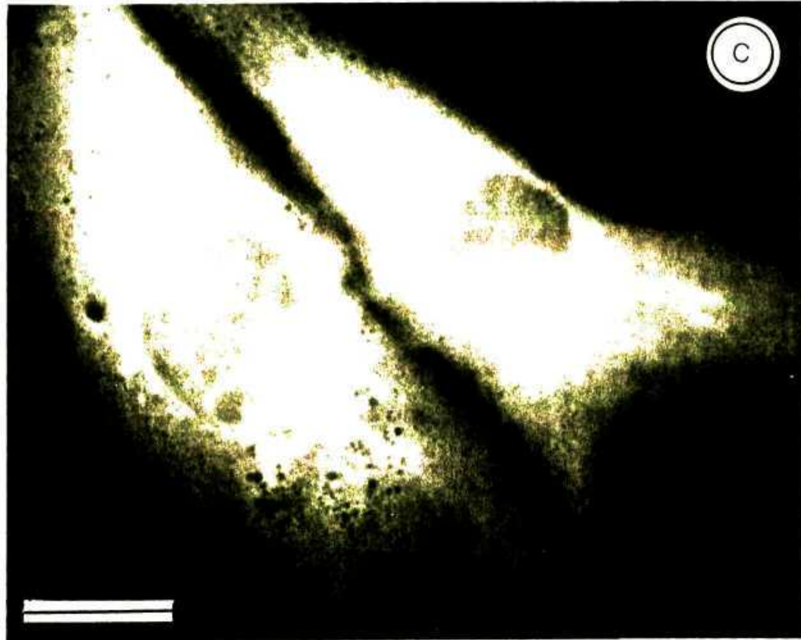
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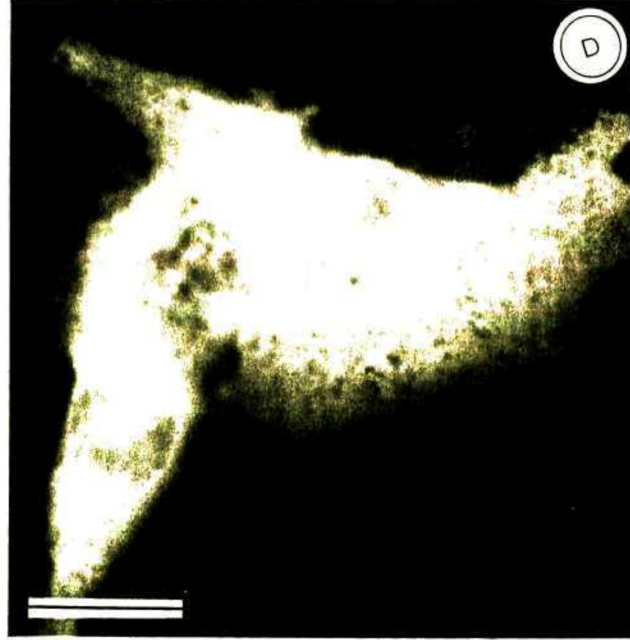
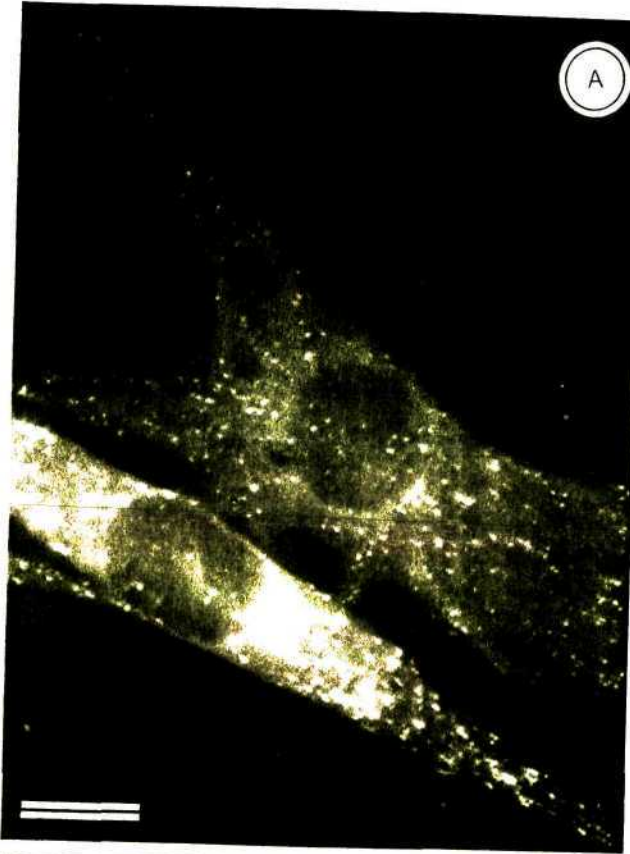

D

Figure 6.12. Immunofluorescent labelling of B16-BL6 cells for cathepsins D and H.

B16-BL6 cells were grown overnight on coverslips, and labelled (Section 6.2.4.2) with (A) - (B) chicken anti-porcine cathepsin D (50 μg/ml), and (C) - (D) rabbit anti-human cathepsin H peptide (300 μg/ml).

Bar scales: (A) - (D) 10 μm.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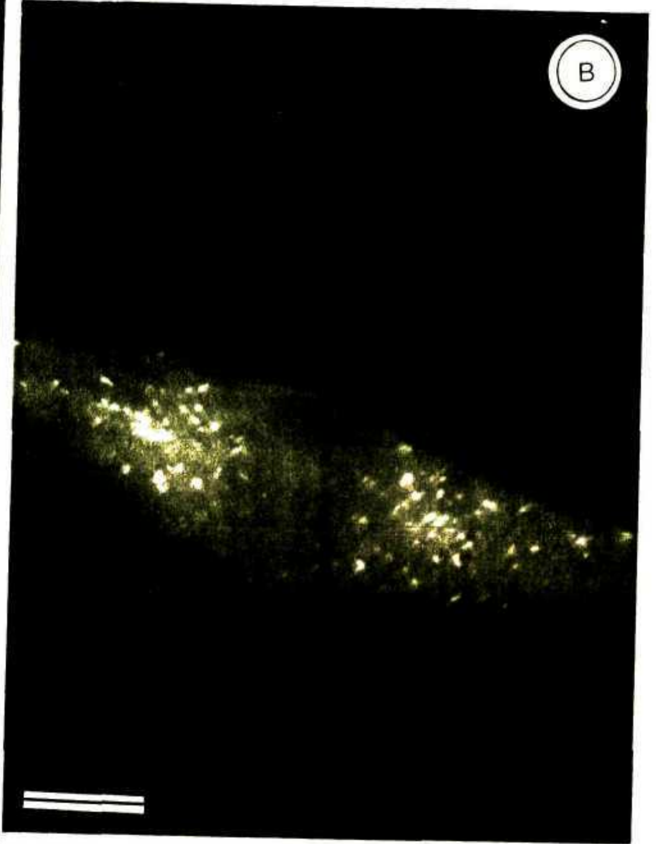
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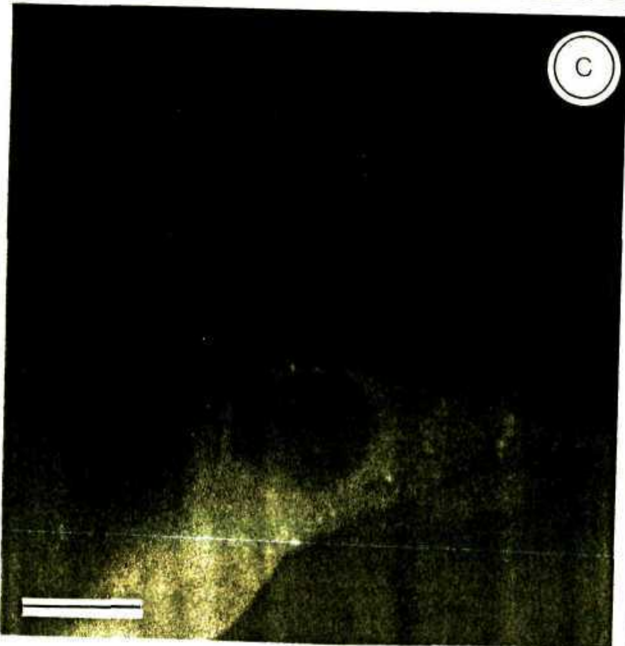

C

D

The NIH 3T3 cell line.

- Labelling for cathepsin L. Generally poor results were obtained with the NIH 3T3 cell line using rabbit antisera, so these are not shown. With IgY preparations, the fibroblasts showed a different labelling pattern, especially for cathepsin L and complex, compared to the B16-BL6 melanoma cells. Localisation of cathepsin L was "smudgy", with punctate labelling being observed apparently within the cytoplasm and extracellularly (Fig. 6.13 A, B). This labelling resembled either antibody or FITC aggregates although both preparations were centrifuged to remove particulate components, and fluorescence in the "extracellular" environment is negligible.

- Labelling for cathepsin L-stefin B complex. A similar distribution was observed with IgY anti-complex, although more vesicles appeared to have labelled (Fig. 6.13 C, D). Some labelling suggests that vesicles may be exocytosing.

- Labelling for cathepsin D. Cathepsin D was detected in small punctate vesicles evenly distributed throughout the cytoplasm (Fig. 6.14 C).

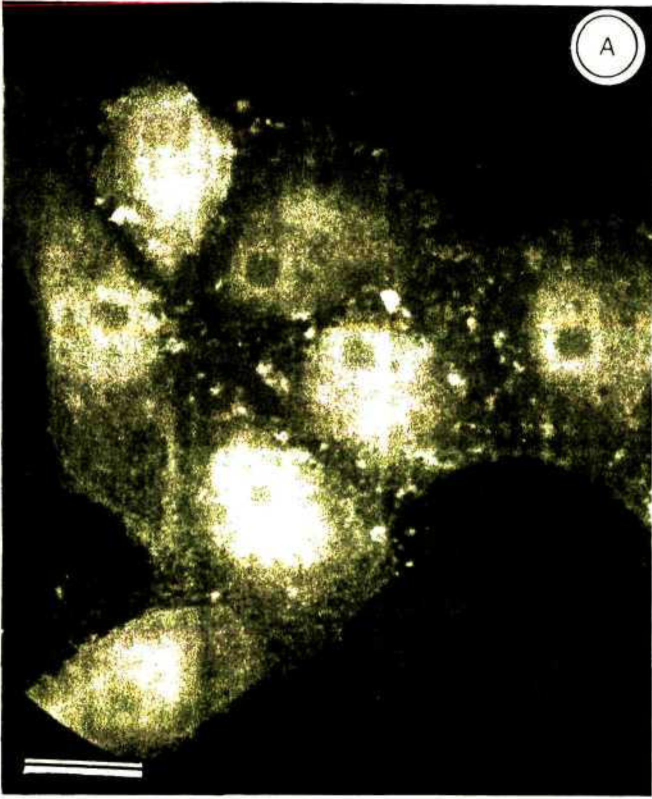
- Labelling for cathepsin H. Cathepsin H was detected in small and large vesicles throughout the cytoplasm, though in fewer vesicles than cathepsin D (Fig. 6.14 D). Compared to the B16-BL6 melanoma cell line (Fig. 6.12 C, D), there were more and smaller vesicles labelling for cathepsin H.

- Labelling for cathepsins B and S. Cathepsin B could not be detected in this cell line by immunolabelling, which is curious since cathepsin B is considered to be a ubiquitous lysosomal enzyme. On the other hand, anti-cathepsin S antibodies gave some indistinct labelling of the perinuclear region (Fig. 6.14 A, B). Cathepsin S is not expected in this fibroblast cell line, since it is an enzyme associated with cells of mononuclear-phagocytic origin (Patanceska *et al.*, 1996), and so this may represent non-specific labelling.

Figure 6.13. Immunofluorescent labelling of NIH 3T3 cells for cathepsin L and stefin B-complexed cathepsin L.

NIH 3T3 cells were grown overnight on coverslips, and labelled (Section 6.2.4.2) with (A) - (B) chicken anti-sheep cathepsin L (300 µg/ml), and (C) - (D) chicken anti-sheep stefin B complexed cathepsin L (300 µg/ml).

Bar scales: (A) - (D) 10 µm.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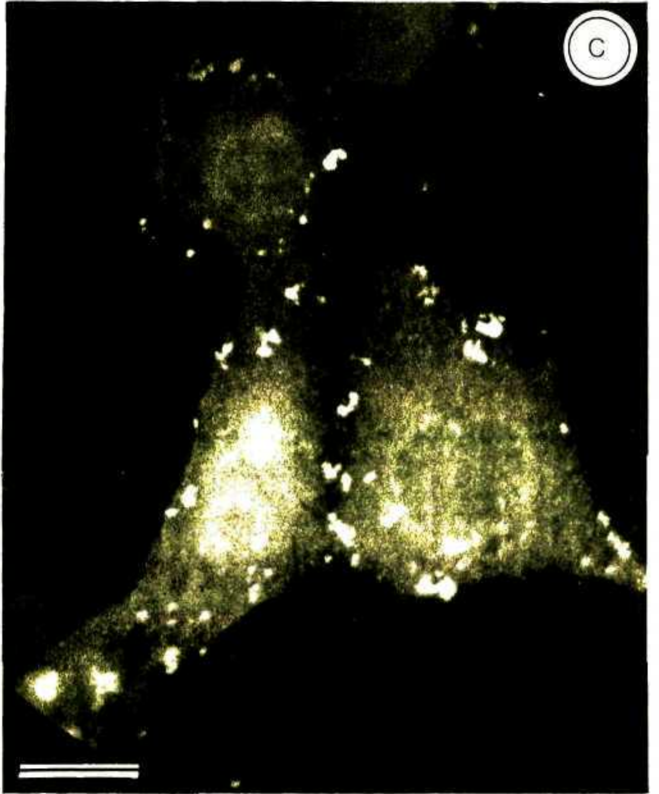
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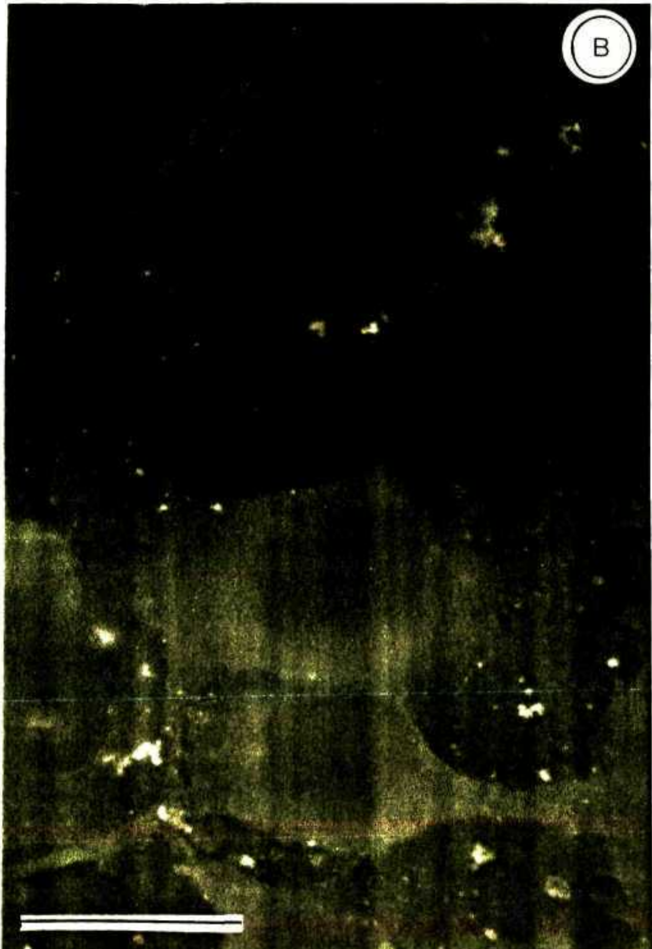
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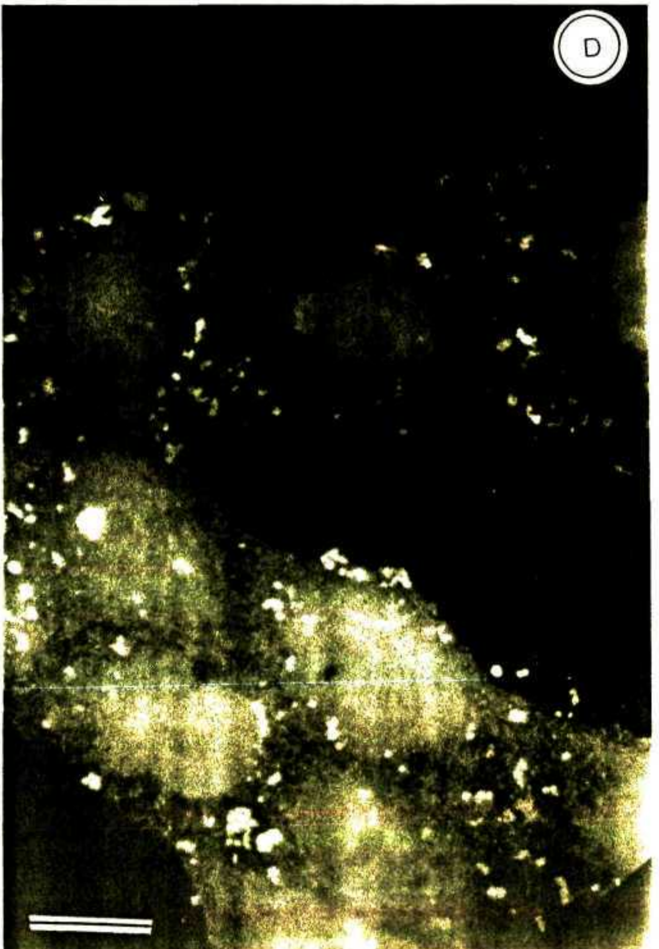
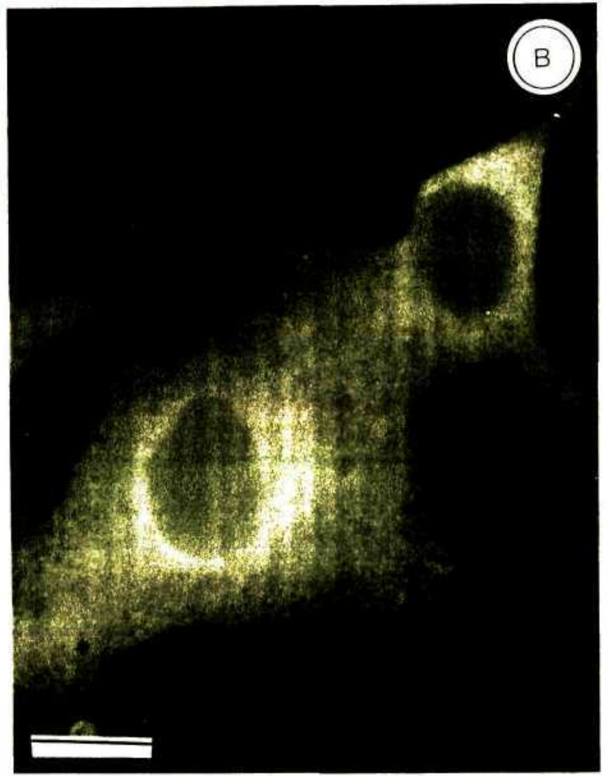

D

Figure 6.14. Immunofluorescent labelling of NIH 3T3 cells for cathepsins D, H and S.

NIH 3T3 cells were grown overnight on coverslips, and labelled (Section 6.2.4.2) with (A) - (B) chicken anti-human cathepsin S (300 μg/ml), (C) chicken anti-porcine cathepsin D (50 μg/ml) and (D) rabbit anti-human cathepsin H peptide (300 μg/ml)

Bar scales: (A) - (D) 10 μm.

A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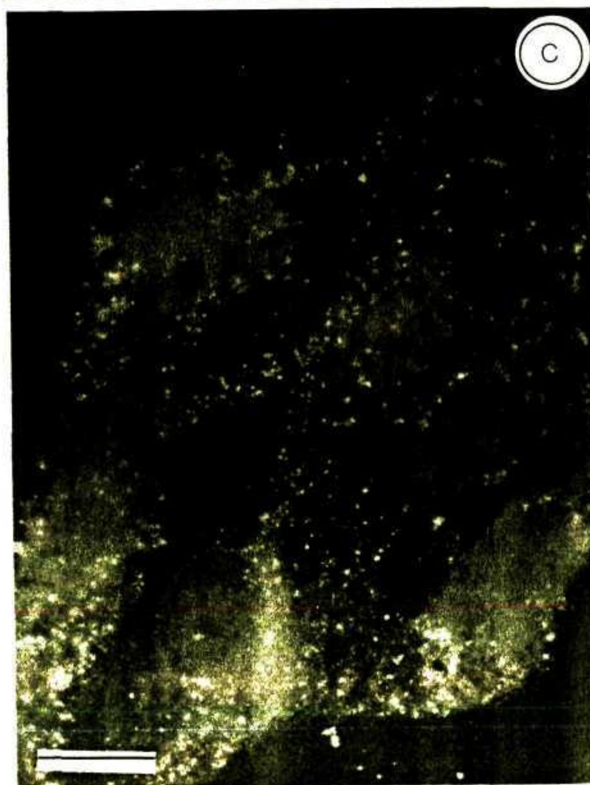
C

D

6.4 Discussion

6.4.1 Antibody production

In this chapter the production and characterisation of polyclonal antibodies to cathepsin L and covalent stefin B-cathepsin L complex is described. It was hoped that these antibodies would prove valuable in the investigation of these proteins in tumour invasion, in studies being conducted in parallel with the present study by other workers in this laboratory. The present study, therefore, had the limited objective of production and characterisation of the antibodies, and a preliminary assessment of their suitability for immunolabelling, while more extensive application of the antibodies, it was envisioned, would be done by colleagues in this and other laboratories. For the preliminary assessment of their suitability for immunolabelling, two mouse cell lines which were to hand were used.

The theory that sheep cathepsin L (and stefin B-complexed cathepsin L) is more immunogenic in chickens than in rabbits (Pike, 1990), owing to the greater evolutionary distance between chicken and sheep, was supported in the present study by the greater immune response elicited in chickens than in rabbits. The immunogenicity of proteins is enhanced by their ability to elicit fully adaptive immune responses by engaging T-cell lymphocytes required for immune memory. As part of an adaptive immune response, proteins must be taken up by professional antigen presenting cells and be proteolytically cleaved to form peptide fragments which become bound to MHC II (major histocompatibility) molecules expressed on cell surfaces for presentation to T-cells. T-cells recognise this complex and activate B cells to proliferate and differentiate into antibody producing plasma cells (Janeway and Travers, 1994). Antigen presenting cells preferentially take up aggregated or particulate antigens, and the larger and more complex a protein, and the more distant its relationship to a self protein, the more likely it is to be immunogenic.

The use of class-specific inhibitors has suggested that the lysosomal proteinases cathepsins B, L and D are involved in the proteolytic processing of antigens (Buus and Werdelin, 1986; Takahashi *et al.*, 1989; Diment, 1993). Diment (1993) showed cathepsin B to be responsible for the processing of myoglobin and Matsunaga *et al.* (1993) showed cathepsin B to be pivotal in the production of vaccines to hepatitis B and rabies by processing the antigens for presentation to the MHC class II molecules.

Cathepsin L has been isolated in a complex with the invariant chain of MHC II molecules from human kidney (Ogrinc *et al.*, 1993), which showed enhanced stability at neutral and slightly alkaline pH, and reduced enzyme activity. These lysosomal enzymes participate in the as-yet-poorly-understood proteolytic events of antigen presentation, which involve the processing of the protein antigen into peptides of 13-25 amino acids, and the proteolytic degradation of the MHC class II invariant chain. The physiological role for the complex of cathepsin L and the invariant chain of the MHC II molecule may be explained in light of its involvement in the latter event. Reyes *et al.* (1991) have also shown the involvement of cathepsin B in the cleavage of the invariant chain from MHC class II molecules. The involvement of these enzymes in these immunological roles could prejudice the immune response against these enzymes, and may offer an explanation as to why the evolutionary distance between chickens and sheep produces an enhanced immune response to the sheep antigen in chickens.

That antigen presenting cells prefer more complex antigens may explain why the titre of the anti-cathepsin L-stefin B complex antibody preparations was superior to that of anti-free cathepsin L preparations. Polson *et al.* (1980) reported that smaller molecules ($M_r \leq 70$ kDa) are apparently poorly immunogenic in chickens. However, in the present study, cathepsin L and the complex (26 and 37 kDa) both proved to be immunogenic. Coetzer (1992) noted that the observations of Polson *et al.* (1980) were based on demonstration of immunoprecipitating antibodies, and not necessarily antibody titre *per se*. Immunoprecipitation requires at least two epitopes and the smaller the protein, the more likely it is to have only one epitope.

The observation that the stefin B molecule of the complex affords stability to cathepsin L (Chapters 3 and 5) could further explain the greater immune response to complex compared to cathepsin L. The envisaged three dimensional structure of the complex could be one of greater restraint, and might therefore better reflect the native state of free cathepsin L. An immune response mounted by either the humoral (B cell path) or T cell mediated adaptive response would be elicited against epitopes of the stable cathepsin L-stefin B complex, and the antibodies produced are therefore more likely to recognise the native immunogen. This was the rationale presented by Dehrmann *et al.*, (1996) suggesting that the formation of antibodies to

cathepsin B would be enhanced by immunisation of reductively stabilised cathepsin B into the bloodstream of the host animal.

Coetzer (1992) observed that rabbit polyclonal antibodies targeted different epitopes to the chicken polyclonal antibodies, when tested for immunoinhibition of cathepsin L. In the present study, chicken antibodies produced against the complex recognised all forms of cathepsin L and complex, but not stefin B alone, suggesting that the epitope(s) recognised is/are situated on cathepsin L, or at the site of cathepsin L-stefin B association (Table 6.3). This contrasts with the rabbit antibodies, which recognised only complex forms in Western blots, but in ELISAs also recognised stefin B and free cathepsin L. This may be due to the difference in presentation of the antigen by the two assay procedures. In Western blots the enzyme is likely to be more fully denatured (after exposure to SDS-PAGE and binding to nitrocellulose) while binding to an ELISA plate may only partially denature the protein.

Polyclonal antibodies offer distinct advantages in diagnostic and immuno-therapeutic approaches to diseases such as invasive cancer, emphysema, and arthritis. This is owing to their ability to distinguish individual enzymes such as the cathepsins (Pike, 1990). Synthetic proteinase inhibitors, which have been employed in diagnostic studies, are usually only class specific and are unable to distinguish between the individual enzymes in a class. The ability of the antibodies raised against forms of cathepsin L, as reported in this chapter, to recognise the different cathepsins was exploited in determining their distribution in two murine cell lines, one normal and one transformed.

6.4.2 Immunofluorescence microscopy

Immunocytochemistry requires the antibody to be highly specific, and preferably have a high affinity for the antigen, in order to recognise the immunogen, while minimising non-specific labelling, i.e. labelling not attributable to the antigen. This was largely achieved by the antibodies produced against cathepsin L and stefin B-complexed cathepsin L by chickens and to a lesser extent by those produced by rabbits. Nevertheless, the results presented here require careful interpretation.

In the first instance, the lower affinity of the rabbit antisera for especially the NIH 3T3 cell line suggests that the chicken and rabbit antibodies target different epitope regions. Additionally, chicken IgY fractions raised against the sheep cathepsin L antigens show strong cross species recognition in the murine cells, while the rabbit antisera showed limited recognition. This may be due to poor cross species recognition, or it may be due to changes which arise during fixation of cells, destroying the native antigen so that it is not recognised by the antibodies. To test this hypothesis, Western blot analysis of cell supernatants and homogenates could be conducted. Recognition in Western blots would suggest that chemical fixation alters the three dimensional structure of the epitope regions during immunofluorescence. This is an area requiring further investigation.

Tumour cells exhibit many altered features, both phenotypically and genotypically. Notably altered is the production and secretion of enzymes and hormones. Many malignant tumour cells show raised levels of lysosomal enzymes, which may assist in the digestion of connective tissue during invasion (Evans, 1991a), and this has led many workers to correlate the elevated expression and activity of proteases with a malignant phenotype (Qian *et al.*, 1991; Kane and Gottesman, 1990). Results presented here corroborate these observations.

Pro-cathepsin L has been shown to be the major protein synthesised and released from *ras* transformed murine fibroblasts (Gal and Gottesman, 1986a; 1986b; Denhardt *et al.*, 1987), although the upregulation of cathepsin L expression in "normal" NIH 3T3 cells did not induce the transformed phenotype when tested by serum and anchorage independent growth and injection into nude mice. Association of cathepsins B and L with the cell surface (i.e. in plasma membrane fractions) in the B16 amelanotic melanoma was demonstrated biochemically by Rozhin *et al.* (1987, 1989). Results obtained in the present study, that cathepsin L may be associated with the cell surface in the B16-BL6 cells, are consistent with these findings. In the present study, neither cathepsin B nor pro-cathepsin B was detected, although these were demonstrated in the B16a cell line (Rozhin *et al.*, 1987).

Work on the *ras* transfected MCF-10AneoT human breast cell line has shown that constitutive cathepsin B expression is increased 2.5 fold (Rozhin *et al.*, 1994), compared to the control cell line which was not transfected, and the mature two-

chain form of cathepsin B is membrane associated (Sloane *et al.*, 1994). In the transfected cell line, cathepsin L was found to be more peripherally associated (Moin *et al.*, 1992). Cathepsins D and H were found to be vesicular, in both the normal and transfected cell lines, suggesting that their main role may be in the intracellular processing of other proteases, and/or in the proteolysis of endocytosed proteins. Similar results were obtained for cathepsins D and H in the present study on murine cell lines.

These models of malignancy are not the only cells that suggest a more peripheral or cell surface association of cathepsins B and L. Maciewicz *et al.* (1989) showed by immunofluorescence that cathepsin L is membrane associated in cultured human colon carcinoma cells, activated macrophages secrete lysosomal enzymes (Reilly *et al.*, 1989), while osteoclasts transport lysosomes to the apical surface so that they are in close proximity to the resorption lacunae formed between the osteoclast and bone (Vaes *et al.*, 1992). The peripheral mobilisation of lysosomes may be instrumental in the delivery of lysosomal enzymes to the cell surface, or in the secretion of these enzymes, where it has been shown that they would be able to actively degrade the proteinaceous barriers surrounding the cells.

The intense labelling obtained with the IgY anti-complex antibody should be interpreted with caution. This antibody targeted free cathepsin L in Western blots, although not as strongly as the higher molecular weight bands, which may include procathepsin L. It is not unlikely that the labelling seen, which appears to be associated strongly with the rough endoplasmic reticulum, is indeed labelling for procathepsin L. As transformed cells often show increased expression of cathepsin L, procathepsin L must also be hyperexpressed as a precursor of the mature enzyme. The specificity of this antibody appears to be that it recognises stabilised forms of cathepsin L (the antigen being the stefin B complexed cathepsin L. although stefin B alone is not targeted by Western blots), the pro-form probably being held in a more restricted native conformation, thereby affording enhanced recognition. It seems unlikely that complexes of cathepsin L and stefin B could exist in the Golgi apparatus. Cathepsin L itself does passage through the Golgi, probably as the pro-enzyme. However, stefin B is a cytoplasmic protein, with a typical acetylated N-terminus, so under normal circumstances cathepsin L should not encounter stefin B.

The labelling of the NIH 3T3 cells showed a pattern consistent with secretion of cathepsin L, while cathepsins D and H show punctate vesicular labelling throughout the cytoplasm, being more profusely distributed in the case of cathepsin D. Non-transformed NIH 3T3 cells have been shown to secrete cathepsin L and uPA activity (Zhang and Schultz, 1992) which is significantly increased by the transformation with various *ras* mutated oncogenes. They showed that the elevated expression was a result of increased transcription rates, and proposed that the dissimilar patterns of protease expression suggest two discrete biochemical signal transduction pathways arising from *ras* transformation. Transformation of murine BALB/3T3 fibroblasts with Moloney murine sarcoma virus resulted in elevated secretion of pro-cathepsins B and L (Achkar *et al.*, 1990), although it was shown that the non-transformed cells secreted the pro-forms as well as β-glucuronidase when grown in serum free medium. Human skin fibroblasts have been shown to contain cathepsins B, L, D, S and H in a vesicular distribution throughout the cytoplasm, suggesting a lysosomal distribution (Drs Edith Elliott and Lukas Mach, pers. comm.).

Fibroblasts in cell culture are characterised by their property of secreting proteins necessary for the formation and maintenance of connective tissue, and therefore the importance of secreted proteins/proteases can be imagined in the role of remodelling of interstitial stroma. However, although these cells are considered to be "normal", it has been noted that under certain growth conditions, they may show a relatively high frequency of spontaneous transformation, and thus the physiological importance of the above observations must be interpreted with caution.

In conclusion, the antibodies produced as described in this chapter, to specifically target cathepsin L and stefin B-complexed cathepsin L, have proved useful in this pilot study in showing the altered distribution of cathepsin L in the metastatic murine melanoma cell line B16-BL6. The surface associated labelling, and intense peri-nuclear immunofluorescence (suggested to be procathepsin L) is consistent with literature reports of the elevated expression, and peripheral mobilisation of this protease, providing circumstantial evidence for its role in invasion and metastasis. The anti-cathepsin L antibodies generally were less-than-satisfactory in the sense that they had to be used at a high concentration, where non-specific labelling became a consideration. Similar problems have been found with anti-cathepsin L antibodies from other sources and this may be a characteristic of anti-cathepsin L antibodies in

general (Dr Edith Elliott, pers. comm.). Nevertheless, the anti-cathepsin L antibodies gave significantly higher labelling than the controls. The anti-complex antibodies were better, in the sense that they could be used at much lower concentrations, but exactly what they are targeting remains a question. The usefulness of these antibodies is being further explored in on-going studies being undertaken by colleagues in this laboratory.

CHAPTER 7

GENERAL DISCUSSION

7.1 Benign vs malignant tumours

Tumours may be characterised into two general types, benign and malignant. The former do not invade tissues, but may displace them as they increase in size. In general, benign tumours may be successfully dealt with surgically. Malignant tumours, on the other hand, invade and destroy local tissue, often spreading through the blood or in the lymphatic system to distant, secondary sites. It is this process that makes malignant tumours so difficult to treat, as they have often spread to unknown sites, before they come to attention. It follows, therefore, that an understanding of the process of metastasis might afford potential avenues for treatment aimed at preventing this often fatal process.

As will be outlined below, cysteine proteases and their inhibitors show a number of alterations in tumours, which are often correlated with the progression of a benign to a malignant tumour (Berquin and Sloane, 1994). The metastatic cascade is a multi-step process, with several of the stages requiring the activity of proteases. Berquin and Sloane (1994) have categorised the metastatic cascade into three distinct stages which require proteolytic activity:

i. Tumour cells exhibit increased growth, which may be stimulated directly or indirectly by proteases.

ii. Tumours must induce the formation of new blood vessels surrounding the tumour cells in order to grow beyond a diameter of 2 mm. This process of angiogenesis requires the movement of endothelial cells towards the tumour in a process similar to invasion.

iii. Tumour invasion, a process which requires the degradation of the extracellular matrix, the traversal of the basement membrane of the endothelia to permit intravasation into the vascular system, transport and arrest in the vasculature, followed by extravasation at the metastatic site, requiring degradation of normal proteinaceous barriers such as the basement membrane and interstitial

connective tissue. Once the tumour cells have invaded the target tissues and formed a secondary tumour, the process can be repeated (Fig. 7.1).

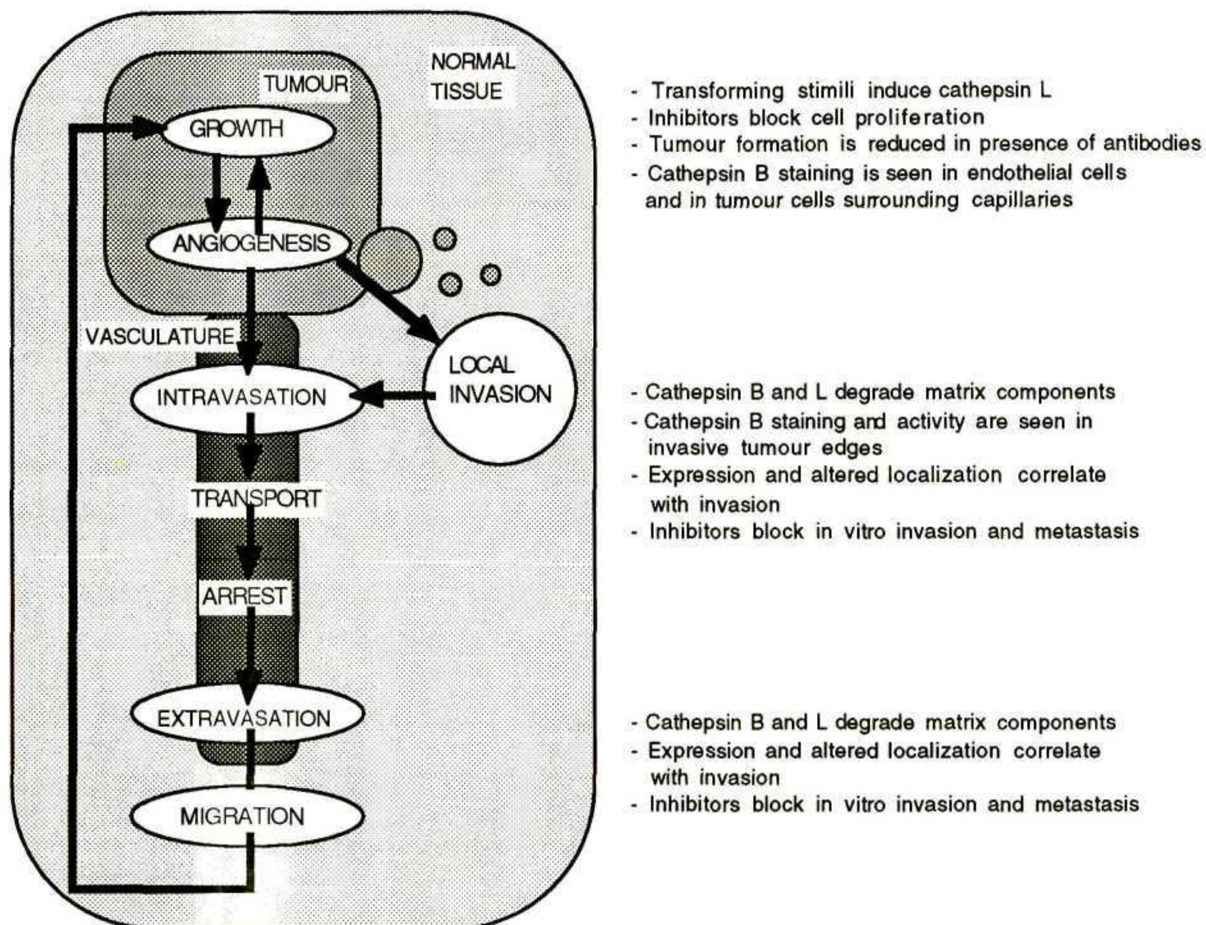

Figure 7.1. Evidence for the involvement of cysteine proteases in tumour metastasis.
The metastatic cascade is represented in the diagram, with the normal tissue, tumour and blood vessel compartments boxed. Steps where the proteases are thought to be involved are circled, and evidence for the role of cysteine proteases in those steps is listed on the right. (After Berquin and Sloane, 1994).

7.2 Cathepsin L (and B) and tumour metastasis

It is now widely accepted that tumour cells release and recruit an array of enzymes necessary for the development of metastatic disease. Significant evidence points to the involvement of the cysteine proteases (CPs), including the following:

- CPs are involved in normal tissue remodelling and repair,

- levels of CPs correlate with metastatic potential in model systems,
- transfection of cells with cDNA encoding for CPs increases the metastatic potential of cells,
- levels of CP inhibitors, and antibodies to various CPs decreases invasion in model systems, and,
- transfection of cells with cDNA encoding for CP inhibitors decreases the metastatic potential of these cells.

Indeed, cathepsin L has been identified in all of the above cases. It is ubiquitously present in all cells, where it plays a role in normal protein turnover (Uchiyama *et al.*, 1994). The role of (pro-) cathepsin L in more specialised cells is also documented. For example, in bone resorption, it is secreted by osteoclasts (Delaisse *et* al., 1991; Kakegawa *et al.*, 1993; Page *et al.*, 1993), and by macrophages (Reddy *et al.*, 1995) and more recently, experimental evidence suggests that cathepsin L mediates thyroid hormone release in thyroid epithelial cells (Brix *et al.*, 1996). Furthermore, intra and extracellular proteolysis mediated by (pro-) cathepsin L is implicated in the differentiation and maturation of spermatozoa in the mammalian genital tract (Peloille *et al.*, 1997), as well as being the major secreted product of the Sertoli cells (Erickson-Lawrence *et al.*, 1991). The ubiquity of cathepsin L endopeptidase activity is demonstrated by the presence of cathepsin L-like cysteine proteases in the parasites *Trypanosoma brucei* and *T. congolense* (Lonsdale-Eccles *et al.*, 1995), and of cathepsin L in *Schistosoma mansoni* (Smith *et al.*, 1994b) and *Fasciola hepatica* (Smith *et al.* 1994a).

Cathepsin L activity has been found to be associated with numerous transformed cells (Mason, *et al.*, 1987; Heidtmann *et al.*, 1993; Yamaguchi *et al.*, 1990), and the level of mRNA expression of cathepsin L has been correlated with the metastatic potential of H-*ras* transformed murine fibroblast cells (Denhardt *et al.*, 1987). In addition, murine myeloma cells (which secrete procathepsin L), when fused with spleen cells of mice immunised with cathepsin L, result in hybridoma cells which have lost their potential for producing solid tumours after implantation into mice (Weber *et al.*, 1994). Yagel *et al.* (1989) showed that cysteine protease inhibitors suppressed invasion of human amnion membranes by murine cancer cells, suggesting that the

cysteine proteases (cathepsins L and B) contribute significantly to the invasive capacity of cells, perhaps by facilitating the action of the metalloproteases.

In order to play a role in invasion, these enzymes must be active in the conditions encountered in the extracellular milieu, in close proximity to the transformed cells. In addition, these enzymes would need to circumvent the inhibitory action of endogenous protease inhibitors. The potential role of these inhibitors remains to be identified, since somewhat contradictory results are reported in the literature. However, a protease/protease inhibitor imbalance could just as easily result in decreased inhibition as would increased enzyme activity. Accordingly, invasive cells probably employ a number of mechanisms for degrading the ECM, ranging from increased expression of proteases, to cytokine-mediated stimulation of protease expression from surrounding cells. Although the identity of the key-players appears to be tumour specific, it is probable that optimal matrix degradation is achieved by a variety of proteases. Control of tumour cell proteolysis may therefore only be achieved by targeting more than one class of protease.

In normal cells, the cysteine proteases are strictly regulated at nearly every step of their biosynthesis, from transcription, translation, post-translational modification and trafficking to the lysosomes. They are also subject to regulation by their interactions with the endogenous inhibitors. In tumour cells however, alterations frequently occur at one or more levels of cathepsin expression, including:

- increased expression of mRNA, of protein or of activity,
- altered processing and/or trafficking, and,
- abnormal activity of endogenous inhibitors.

7.2.1 Expression and activity

Cathepsin B has been extensively studied in a variety of animal and human tumours, and tumour cell lines. Those human tumours found to overexpress cathepsin B include glioma and carcinoma of the colon and rectum, bladder, breast, cervix, oesophagus, liver, lung, ovary, pancreas, stomach and thyroid (Berquin and Sloane, 1994). Cathepsin L, in contrast to cathepsin B, has not been extensively studied in human tumours, although Chauhan *et al.* (1991) showed by quantitative slot blot

analysis that human tumours express higher levels of cathepsin L than normal. They showed that cathepsin L expression is highest in tumours of the kidney and testis, and progressively lower in non-small cell lung, ovary, colon, adrenal, bladder, prostate and thyroid cancers. Berquin and Sloane (1994) have reviewed some of the recent findings of cathepsin L in human tumours, including human lung tumours, murine lung tumour cell lines, human thyroid tumours, human breast cancers, murine D2 mammary tumour cells and human colorectal carcinomas.

7.2.2 Altered trafficking

In addition to the increased expression of cysteine proteases, many tumour cell lines exhibit altered trafficking of cathepsin L. Alterations in trafficking of the proteases may occur at any stage of the biosynthetic pathway, resulting in the proteases being redistributed to different vesicular compartments, secreted, or redirected to the plasma membrane.

As discussed in Chapter 1, cathepsin L is synthesised in the preproform, which is translocated into the ER, where it undergoes N-linked glycosylation of the Asn-204 (prosequence numbering included) (Kane, 1993). Some evidence suggests that the propeptide plays an important role in the proper trafficking and folding of the molecule. Tao and co-workers (1994) expressed two deletion/chimera cathepsin L proteins, one of which lacked the propeptide, while the other contained the prosequence from a related cysteine protease (aleurain). Both of these recombinant proteins were retained in the ER and degraded over a period of 2-6 h, suggesting that the propeptide is important to correct trafficking.

Normal trafficking of lysosomal enzymes from the ER to the lysosomes depends on the proteins containing a mannose-6-phosphate moiety on their asparagine linked oligosaccharides side chains. This is the critical first step in the sorting of the lysosomal enzymes to the lysosomes via the cation-independent (CI) or cation-dependent (CD) mannose-6-phosphate (MPR) pathway. A phosphotransferase enzyme is responsible for the transferral of GlcNAc-1-phosphate from UDP-GlcNAc to the C-6 hydroxyl groups of mannose on the side-chains of the newly glycosylated enzymes (Cuozzo and Sahagian, 1993). This occurs in the ER (Tao *et al.*, 1994). In the Golgi, an N-acetylglucosaminidase removes the GlcNAc group to expose the mannose-6-phosphate groups. Subsequent binding to the CI-MPR in the *trans*-Golgi

network results in the transport of the lysosomal enzymes to endosomal compartments which in turn, fuse with the mature lysosomes. The CD-MPR appears to play a role in the transport of these enzymes to the lysosomes, but is apparently less efficient, and may be involved in lysosomal enzyme secretion (Cuozzo and Sahagian, 1993). Both pathways depend on the recognition and phosphorylation of the lysosomal enzymes by phosphotransferase. This recognition of the oligosaccharide moiety was shown to be dependent on a specific three-dimensional arrangement of lysine residues (Lys-54 and Lys-99) in the prosequence of cathepsin L (Cuozzo *et al.*, 1995). Mature cathepsin L, lacking the prosequence was not phosphorylated but when the propiece was added phosphorylation was restored. These results suggest that phosphotransferase recognition resides in the interaction of the enzyme with a structural determinant in the propeptide, and infer that correct trafficking relies on correct glycosylation and subsequent phosphorylation. On the other hand, McIntyre and Erickson (1991) have demonstrated a proenzyme receptor responsible for the trafficking of cathepsins L and D, in a mannose-6-phosphate independent manner. This receptor has been identified as a 43 kDa integral membrane protein, which specifically binds the N-terminus of procathepsin L at pH 5.0 (McIntyre and Erickson, 1991). Mannose-6-phosphate independent pathways for the transport of pro-cathepsin D to acidic vesicles have also been reported (Rijnboutt *et al.*, 1991).

Tao *et al.* (1994) further conclude that not only the trafficking but the correct folding of pro- and mature cathepsin L also relies on the presence of the propeptide. They suggest that the propeptide forms an integral part of the protein, and is not just a structural domain whose function is to block off substrate access to the active site. They suggest a general function for lysosomal protein propeptides, as the folding of these proteins occurs at neutral pH in the ER, but must produce a protein which is capable of functioning at acidic pH. For many proteins, the formation of the "low pH conformation" may be energetically or kinetically unfavourable at neutral pH. They suggest that the propeptides direct folding by providing interactions which favour formation of the required structure. They note that the propeptides of cathepsins L, B, D and H are especially rich in basic amino acids, which may play a role in neutralising the anionic forms of acidic amino acids which predominate at

neutral pH (i.e. in the ER). The propeptide stabilises cathepsin L against the denaturing effects of neutral to alkaline pH (Nomura and Fujisawa, 1997).

The propeptide of cathepsin L has also been shown to be a potent and selective, pH-dependent, inhibitor of cathepsin L (Carmona *et al.*, 1996). Deletion peptides were assayed for their inhibitory activity and it was shown that propeptide sequences containing two conserved motifs, located between residues 29-46 (ERFNIN motif) and residues 59-65 (GNFD motif), exhibited potent and selective inhibition of cathepsin L. The association between the propeptide and the mature enzyme relies more on the N-terminal region of the propeptide than the C-terminus and near-UV CD spectra suggest that hydrophobic interactions between Trp residues and the surface of the mature enzyme are responsible for the binding of the propeptide to the enzyme. On cleavage from the full protein, this structure can associate with the mature enzyme and result in inhibition. This is not uncommon for various protease zymogens, for example the propeptide of rat cathepsin B is also a potent inhibitor of the mature enzyme (K_i = 0.4 mM) (Fox *et al.*, 1992), and of papain. The occurrence of noncovalent complexes between cathepsin B and its propeptide has been documented in culture media of human and murine tumour explants (Recklies *et al.*, 1982) and in the sputum of patients with purulent bronchiectasis (Buttle *et al.*, 1991), as well as in the culture media of yeasts transfected with the cDNA for human procathepsin B (Mach *et al.*, 1994a; 1994b). Combined, these results suggest a mechanism whereby the processed protease may be stabilised as a noncovalent complex with the propiece, until cellular or extracellular conditions initiate dissociation and consequent activation of the enzyme.

Besides their action in stabilising the enzymes, the propeptides of cathepsin L (Kasai *et al.*, 1993) and of cathepsin D (Fusek and Vetvicka, 1994) act as autocrine growth factors or mitogens. This may provide a mechanistic link between mis-trafficking of the enzymes, and tumour growth. For example, growth factor stimulated, or malignantly transformed, murine fibroblasts have enhanced synthesis and secretion pro-cathepsin L (Kane and Gottesman, 1990) - in turn the propeptide of the pro-cathepsin L may act as an autocrine growth factor, in essence closing a vicious circle.

7.3 Cysteine protease inhibitors in cancer

Evidence on the role of the endogenous cysteine protease inhibitors remains somewhat contradictory, as inhibitor activity is sometimes elevated and sometimes decreased in tumours. The effect not only depends on the balance between cysteine proteinases and their endogenous inhibitors, but also on the kinetics of the interactions between the proteases and their inhibitors. Lah *et al.* (1989) have shown that stefin A, purified from human sarcoma, has reduced inhibitory activity against papain and cathepsin B, which seems to be as a result of higher inhibition constants reflecting a slower rate of association and a faster rate of dissociation between the enzyme and inhibitors. Cysteine proteases inhibitors are frequently secreted from tumours, and it is suggested that this may be a physiological attempt to regulate the balance between the proteases and inhibitors. Alternatively, the inhibitors may function as a "reservoir" for the secreted proteases, protecting them from autocatalysis, and releasing them in an activable form (Turk *et al.*, 1994; Berquin and Sloane, 1994).

The therapeutic use of protease inhibitors to prevent tumour dissemination has been investigated. Endogenous protease inhibitors occur naturally, and appear always to be proteinaceous, while non-physiological inhibitors are either chemically synthesised or produced by micro-organisms. The application of protease inhibitors appears to have limited application, however, owing to the fact that prolonged administration of these compounds may lead to toxicity. Protease inhibitors are also often relatively non-specific and many of the target proteases are involved in normal, non-pathological tissue remodelling and repair, reproduction and menstruation (matrix metalloproteinases) (Marbaix *et al.*, 1996), and blood coagulation (serine proteases). To overcome this, one approach is to utilise antibodies, directed to tumour specific antigens, and to couple these antibodies to a cytotoxic compound, such as a radioisotope (Larson, 1990). A similar approach has been employed, using prodrugs, which require activation by proteases, utilising the phenotypic characteristic of malignant cells of expressing proteases on their cell surface. In one such case, cathepsin B is used to trigger the action of α-haemolysin, a pore-forming toxin (Panchal *et al.*, 1996). α-Haemolysin induces pore formation resulting in permeabilisation and cell death. Another approach has been to inhibit the secretion of the proteases (rather than inhibit their activity). Wang and Stearns (1988) used

estramustine to prevent the secretion of type IV collagenase from mouse melanoma and human prostate carcinoma, thereby preventing the invasion of ECM barriers. This drug prevents the secretion of the protease by interfering with the microtubular system responsible for the intracellular vesicular traffic.

Despite the toxicity associated with the use of protease inhibitors in chemotherapy, a number of protease inhibitors have undergone limited clinical trials as antitumour drugs. Examples are, Batimastat, a metalloproteinase inhibitor (Brown, 1994), α_1-antitrypsin inhibitor, hirudin (a thrombin inhibitor) (DeClerck and Imren, 1994) and the Bowman-Birk soybean proteinase inhibitor (Kennedy, 1994). The Bowman-Birk soybean proteinase inhibitor has been shown to prevent carcinogenesis by a variety of carcinogens in different species and tissues, involving different tumours. The exact mechanism of inhibition is unknown, but it is thought to be related to the ability of this molecule to inhibit the expression of the c-*myc* and c-*fos* proto-oncogenes (Kennedy, 1994). Unfortunately, the therapeutic application of proteinase inhibitors is likely to only ever be limited, owing to the fact that most cancer sufferers have developed metastases at the time of detection of the primary tumour. As such, the use of proteinase inhibitors to prevent metastasis would be ineffective.

7.4 Some achievements of this study and resultant questions

7.4.1 The isolation procedure

At the outset, the aim of this study was first to isolate sheep liver cathepsin L [a good model of human liver cathepsin L, owing to the high sequence homology (Ritonja *et al.*, 1996)] and cathepsin L-stefin B complexes, and to separate the two forms (active and inhibited) of these complexes. As discussed above, the interaction of proteinases and their inhibitors is central to invasive cancer and, with the discovery of an unusual, proteolytically active, complex of cathepsin L and stefin B it was decided that this warranted further study as it may cast light on a mechanism whereby cysteine proteinases may escape inhibition by their endogenous inhibitors.

It was also felt that the evidence implicating cathepsin L in tumour progression and metastasis warranted a re-examination of the kinetic activity and stability of cathepsin L itself, especially concerning the activity and stability of the enzyme under extracellular conditions. At that time a paradox existed. On the one hand,

much indirect evidence had suggested that cathepsin L plays an extracellular role in many normal and pathological processes and yet, on the other hand, analyses of the pH dependence of the activity and stability of cathepsin L had suggested that this enzyme would be completely inactive and very unstable at extracellular pH (i.e. pH 7-7.2). Consequently it had been proposed that mechanisms must exist for acidifying the extracellular milieu, under certain circumstances, to permit the action of cathepsin L

To these ends, based on the method of Pike (1990), a scaled-up method for the isolation of cathepsin L and complexed cathepsin L was developed. The cathepsin L was isolated predominantly in the single-chain form, in contrast to the two-chain form most often isolated by other workers (see Chapter 3). Previous workers in this laboratory have found that the proportion of two-chain cathepsin L obtained depends upon the isolation method used. For example, Ritonja *et al.* (1996) found their sample to be approx. 60% single-chain form, and when the purification was performed under more acidic conditions, the relative proportion of two-chain cathepsin L increased (which, by providing more of the light-chain fragment, was useful for their purposes of sequencing the molecule). The fact that single-chain cathepsin L was isolated by the procedure used in the present study may be significant, in light of the further investigations conducted in this study, as it may be uniquely the single-chain form that has the properties described. In any event, the isolation protocol employed in this investigation demonstrated a proportionately superior yield and it was shown that the improved exclusion chromatography step was critical to this higher yield. This approach may be useful in the scaling-up of the isolation of other proteases.

It has been speculated that the two-chain form arises as a result of lengthy purification procedures, where cathepsin L is in contact with other proteinases for long periods prior to separation (Coetzer, 1992) and this brings into question the relevance of the two-chain form *in vivo*. In re-visiting this question, the intracellular processing of procathepsin L was reviewed in Chapter 3, in the hope that an understanding of the processing may illuminate the possible occurrence of single *vs* two-chain forms *in vivo*. Although it appears that the major proteolytic processing occurs in the lysosomes, the literature is not conclusive as to how the processing occurs. Clearly, however, the two-chain form is downstream from the single-chain

form and while the two-chain form could be an isolation artefact, this cannot be true of the single-chain form. It may be speculated that *in vivo* the single-chain form may be processed to a two-chain form as the first step in the degradation of the enzyme - what is needed to answer the questions is information on the biological half-lives of the single and two-chain forms but, unfortunately, this is not available.

7.4.2 The covalent complex

Determination of the structure of the novel, proteolytically active, complex of cathepsin L and stefin B proved to be challenging as described in Chapter 4. The results of these studies suggest that cathepsin L and stefin B are associated by a thioester bond, between Cys-3 of stefin B, and Asp-71 of cathepsin L. The question then arises as to whether these complexes are of physiological relevance or whether they are merely isolation artefacts?

The latter possibility arises because, under normal circumstances, cathepsin L should occur exclusively in the lysosome/endosome system and stefin B exclusively in the cytoplasm, and "the twain should never meet". Upon homogenisation of tissue, however, compartmentalisation is completely broken down and these molecules may well then react. Moreover, in the presence of air, the redox conditions obtaining in the homogenate may become relatively oxidising, thereby favouring the formation of disulfide and thioester bonds. Evidence supporting this view is the fact that isolated cysteine cathepsins are inactive and require reduction before they regain their activity, i.e. their required free thiol does not seem to survive in the homogenate. If the appropriate enzymes were also present in the homogenate, these could perhaps catalyse the formation of disulfides and/or thioester bonds. Thioester bonds are ubiquitously formed in the synthesis of fatty acids and the presence of thioester synthesising enzymes in the liver may thus be inferred. With the general tissue disruption obtaining in a homogenate, it is perhaps not unexpected that these enzymes might synthesise novel combinations such as the cathepsin L-stefin B complex. However, formation of the complex is nevertheless not an entirely random occurrence and must depend in the first instance upon the specific interaction of cathepsin L with stefin B

Tissue homogenisation is, however, an extreme example of tissue destruction and one wonders if there is any physiological equivalent - qualitatively, if not

quantitatively? Could necrotic regions of tumours represent something similar? If so, is it possible that complexes may be formed during such tissue destruction, and could this be a mechanism whereby cathepsin L may escape protease inhibitors such as α_2-M?

α_2-M is a major protease inhibitor in blood, which binds and clears proteases, thereby being a general defence against inappropriate proteolytic activity. The native α_2-M is a tetramer, composed of four identical 185 kDa subunits, where pairs of subunits are disulfide bonded. Proteases form complexes with α_2-M by attacking internal Cys-Glu thioester bonds of α_2-M, resulting in covalent attachment of the protease to α_2-M. This is accompanied by proteolysis, resulting in large conformational changes of α_2-M, which "traps" the protease, in a manner which allows the protease to remain active against small substrates (Chen *et al.*, 1992). This interaction is well documented for the cysteine proteases, cathepsins B, L and H (Mason, 1989). The possibility exists that active stefin B-complexed cathepsin L, might not be "baited" by α_2-M, as the presence of stefin B may mask the interaction with cathepsin L. Therefore the complex may have physiological relevance in tissue degradative pathologies, and the interaction between the active complex and α_2-M is an avenue for further research.

On a more philosophical note, if it proves to have no physiological relevance, the complex may nevertheless serve as a caveat that artefacts should not be unexpected after the massive tissue destruction involved in forming a homogenate.

7.4.3 The effect of urea on cathepsin L

The study of the effect of urea on single chain cathepsin L activity is noteworthy in that it shows that cathepsin L is stable to up to 8 M urea. It has previously been recognised that lower concentrations of urea enhance cathepsin L activity by either sensitising azocasein to hydrolysis (Kirschke *et al.*, 1984) or by increasing the solubility of cleaved azocasein fragments (Mason *et al.*, 1984). This characteristic of cathepsin L has been used to discriminate between the activities of cathepsin L and cathepsins B and H (Barrett and Kirschke, 1981; McDonald and Kadkhodayan, 1988). The activities of cathepsins B and H are diminished in the presence of urea and cathepsin B shows completely reversible denaturation with up to 1.5 M urea, but not

with 6.4 M. This contrasts with cathepsin L, which shows a 50% increase in activity (up to and including 8 M urea) when assayed against azocasein, and although this was not the case when assayed against a synthetic substrate, at 0.5 M urea (pH 5.5 and 6.0) there was a distinct increase in activity. The presence of low amounts of denaturant appear to have a similar effect as the association with stefin B, suggesting that the pre-binding of the stefin B prevents the full denaturing effects of the urea. By association with stefin B, and by treatment with low concentrations of chaotrope, the active site may be more "presented" and therefore more active. Although the stability of cathepsin L to 8 M urea has not been directly reported previously, recombinant human procathepsin L, expressed in *E. coli*, required treatment with 8 M urea at alkaline pH, and this was the only solubilising agent from which active cathepsin L could be recovered (Smith and Gottesman, 1989). This supports the finding in the present study that the enzyme survives exposure to 8 M urea. The present study suggests, however, that the enzyme exists in more than one active conformation and that the conformational change induced by urea is not completely reversible.

7.4.4 The effect of pH on cathepsins L and B

Cathepsin L (and B) have been shown to be active against extracellular matrix components at physiological pHs, suggesting a role in the invasive steps of metastasis. In the present study the activity of cathepsin L, especially on the alkaline side of the pH profile, was reinvestigated in light of the observation that cathepsin L is affected by buffer ionic strength (Dennison *et al.*, 1992). Using buffers of constant ionic strength, rather than of constant molarity, the pH optimum for cathepsin L was determined as pH 6.5, and that for cathepsin B as *ca.* pH 7.5. This study showed that the previous use of buffers of constant molarity (and therefore with differing ionic strength) with differing pH has led to the erroneous conclusion that cathepsins B and L are not active at extracellular pH.

A plausible, but incorrect, hypothesis can become solidly entrenched and may thus be very enduring and damaging. For example, in the case of the cathepsins it was originally observed that their measured pH optima agreed with the measured pH of lysosomes. Since it was believed that these enzymes worked in lysosomes, the coincidence of their pH optima with the pH of the lysosome lumen was interpreted

as an example of evolutionary "fitness" for their task. More seriously, perhaps, it also entrenched the incorrect pH optima values in the minds of most researchers. Only recently has it been appreciated that the lysosome/endosome system is complex and dynamic with "lysosomes" (by the old definition of "dense bodies containing lysosomal enzymes"), in fact being mere storage organelles, where lysosomal enzymes would be required to **not** function (Griffiths, 1996). The main site of proteolysis is the late endosome (Tjelle *et al.*, 1996), which has a lumenal pH significantly higher than that of the dense lysosomes - and which agrees with our revised pH optima for cathepsins L and B. The books can therefore be rewritten: lysosomal proteases **are** fitted to their task, but this task is proteolysis in the late endosome, not the lysosome. In fact, research in this laboratory - a spin off of the present study - suggests that the density of lysosomes may be a consequence of their function as storage organelles, and may be brought about by complexing of lysosomal enzymes with LAMPS at low pH, with consequent electro-constriction. This also provides an answer to the enigmatic observation of glycogen being stored in lysosomes (Geddes *et al.*, 1992).

The present study has also shown that the stability of cathepsins L and B is related to their activity, especially under alkaline pH conditions, and that their stability is markedly increased under reducing conditions. We have speculated that the active site $-S^-/^+HIm-$ ionic bond plays a key role in stabilising the enzyme - a hypothesis first proposed by Turk *et al.* (1993) for cathepsin B. The enzyme becomes labile when ionic competition, at high ionic strength, or deprotonation of the imidazole, at high pH, breaks the $-S^-/^+HIm-$ bond, or when oxidation of the thiol prevents it being formed. Activation with a reducing agent such as cysteine or DTT would reconstitute the $-S^-/^+HIm-$ ionic bond and the enzyme would concomitantly become more stable. Again, previously reported studies on the stability of cysteine proteinases have been misleading because not all experiments were performed on activated enzymes. We have hypothesised that the requirement for reductive activation is possibly an isolation artefact, as *in vivo*, the enzymes are most probably reduced and therefore stable.

In order for these findings to be of physiological relevance, it was necessary to examine the extracellular stability of these enzymes, in an activated state and in a buffer modelling the extracellular environment in its pH as well as its ionic

composition. The discovery that cathepsin B has a half-life of 4 min at pH 7.2, and 14 min at 6.8, while that of cathepsin L is 3 min at pH 7.2 and 11 min at pH 6.8 in Hanks' balanced salt solution (a model of the extracellular fluid) shows that both of these enzymes are markedly stable under these conditions, especially at the peritumoural pH (pH 6.8). In addition, the stability of cathepsin B was increased in the presence of substrate proteins (half-life *ca.* 30 min) and, although not demonstrated in this study, a similar observation has been noted for cathepsin L (Dr B. F. Sloane, pers. comm.). Therefore, this work has shown that secreted cathepsins L and B would be substantially active and sufficiently stable to contribute to extracellular proteolysis, and thus have the potential to play a role in tumour invasion.

7.4.5 Anti-cathepsin L antibodies

The production of polyclonal antibodies to cathepsin L proved useful in the immunolocalization of this protease in two murine cell lines. The present work showed a distinct difference in the distribution of cathepsin L in the "normal" fibroblastic NIH 3T3 cells, (where diffuse surface labelling was obtained) compared to that of the transformed B16-BL6 murine melanoma cells where cathepsin L labelled in distinct vesicles (possibly lysosomes). This suggests that these antibodies may be generally useful in exploring the occurrence and distribution of cathepsin L in tumour tissues. However, although these anti-cathepsin L antibodies appeared to be specific they suffered from the problem apparently common to all anti-cathepsin L antibodies produced so far, i.e. that they have a low avidity and so must be used at a high concentration, where non-specific protein-protein interactions are more likely to occur.

The observation that the IgY anti-complex antibodies appear to recognise "stabilised" forms of cathepsin L, i.e. the pro- and the complexed forms, suggests that this antibody is not suitable for the specific detection of cathepsin L-stefin B complexes. However, the apparent cross-reactivity raised the question of a comparison of the pro-sequence and stefin B. Could the pro-sequence and stefin B have similar 3-D structures, or does their association with cathepsin L result in the same stabilised conformation? Comparison of the amino acid sequences revealed primary sequences of similar length (95 and 98 amino acids for the pro-sequence and stefin B, respectively), but sequence alignment showed no significant amino acid

homology. It may be speculated, however, that association with either of these molecules may result in cathepsin L of a particular conformation which allows enhanced detection with antibodies. If true, this would be an important conclusion for the production of antibodies, as it suggests that immunogens should be maximally stable and presented to the immune system in as native a conformation as possible, in order to elicit effective antibody production. The failure to do this may explain the general ineffectiveness of previously-produced anti-cathepsin L antibodies, as discussed in Chapter 6.

Previous work in this laboratory has shown that chicken anti-sheep liver cathepsin L antibodies were immunoinhibitory, while rabbit anti-sheep liver cathepsin L antibodies were not, suggesting that the avian and mammalian immune systems recognise different epitopes on the sheep immunogen. Coetzer *et al.* (1992) located the epitope recognised by the chicken antibodies to a peptide region at the active site. This work has highlighted a problem with relatively small molecules such as cathepsin L, i.e. that they may have few natural epitopes. Consequently, in a further study - another spin off of the present study - the effect of conjugation of cathepsins L and B to a carrier molecule is being investigated, with the aim of increasing the number of T-cell epitopes. Normally, antibodies to cathepsin B only recognise the denatured enzyme but, in a study which has been largely overlooked, Pierart-Gallois *et al.* (1977) reported that conjugation of cathepsin B to BSA resulted in the production of polyclonal antibodies which recognised, and immunoinhibited, the native enzyme. In this way it is hoped that more effective anti-cathepsin L and B antibodies may be produced and that these will open the way to more definitive immunocytochemical studies and, down the road, perhaps to novel immunotherapy approaches.

7.5 The way forward

Initially, this study intended to address the specific issue of the possible involvement of cathepsin L in tumour invasion, especially in light of its purported instability in the environment of the extracellular milieu. It also intended to address the intriguing existence of the "active" stefin B-cathepsin L complex, and its localisation in tumour cells. As is often the case, this work appears to have generated more questions than answers. These may include:

- The physiological relevance of the single *vs* two-chain form of cathepsin L? Although the literature suggests that the formation and conversion to the mature and two-chain form is pH and cell-type dependent, is there a further effector, such as redox potential, which may control maturation and activity? Is the single chain form more (or less) active and/or stable than the two-chain form?
- What is the role of the propeptide in the folding, processing and transport of mature cathepsin L. Do aberrations in trafficking lie in incorrectly cleaved prosequences, which subsequently alter delivery of cathepsin L to incorrect destinations? Does the cleaved propeptide offer a mechanism of inhibition/stabilisation of the mature enzyme during transport and delivery?
- Does the active stefin B-cathepsin L complex ever occur *in vivo*, and what, if any, is its role? Does it stabilise cathepsin L as the proform might, until the conditions are correct for activation? Is its association with mature cathepsin L similar to that described for the cleaved prosequence? Under what conditions does it form? Is it an artefact of homogenisation?
- What role does the association between cathepsin L and stefin B play in the metastatic potential of tumour cells. Is only the single chain cathepsin L involved? Is tumour stefin B aberrant or altered in any manner?
- Based on the literature implicating cathepsin L in tumour invasion and other protein degradative diseases, could this and other proteinases be targeted with antibodies which would inhibit their proteolytic activity, thereby providing an immunotherapeutic approach to cancer intervention?

There are many unanswered questions, and research such as this, merely provides another piece of the greater puzzle. However, each piece that is put in place contributes to the greater clarity of the overall picture and increasing insight is perhaps the only sure result of persistent effort.

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PUBLICATIONS

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Mature Cathepsin L Is Substantially Active in the Ionic Milieu of the Extracellular Medium

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The activity of cathepsin L is affected by ionic strength, resulting in the measured pH optimum being higher in acetate-4-morpholineethane sulfonic acid (MES)–Tris buffers of constant ionic strength than in phosphate buffers of constant molarity (and hence varying ionic strength). In acetate–MES–Tris and phosphate buffers of constant ionic strength across the pH range, the catalytic constant, k_{cat}, generally peaked at ca. pH 6.5 and essentially independently of ionic strength. K_m values, of ca. 5 μM, manifested a slight rising trend with increasing ionic strength, with a sharp increase to 20–25 μM, specifically at pH 6.5 and I = 0.4. At physiological ionic strengths, the specific buffer ions present affected the activity of mature cathepsin L, k_{cat}/K_m declining above pH 6.5 in phosphate buffer, but only above pH 7 in acetate–MES–Tris buffer. In Hanks' balanced salt solution, a model of the extracellular fluid, measured values at pH 7.2 were k_{cat}, 18.9 s^{-1}; K_m, 13.5 μM; and k_{cat}/K_m, 1.4 × 10^6 M^{-1} s^{-1}. The stability of cathepsin L in the physiological pH range was also differentially affected by the specific buffer ions, generally in parallel with the enzyme activity. In Hanks' balanced salt solution, mature cathepsin L was substantially active and stable, having a half-life of 179 s at pH 7.2 and 657 s at pH 6.8 (the peritumor pH). © 1995 Academic Press, Inc.

Cathepsin L is a lysosomal cysteine proteinase that is thought to play an extracellular role in normal tissue remodeling (1) and in pathologies, such as liver fluke infection (2). It has also been implicated in tumor invasion and metastasis (3–6), which may also involve extracellular activity, and so the question of the pH and ionic strength dependence of the activity and stability of cathepsin L is of interest in the context of such purported extracellular activities.

In a previous determination of the pH optimum of sheep liver cathepsin L (7) we found that this enzyme had an apparent optimum of pH 6.5 and was substantially active at ca. physiological pH. This finding was atypical, however, since most authors have reported a pH optimum of ca. 5.5–6.0 (Table I), with the enzyme reported to be largely inactive at physiological pH. A difference, however, was that we used acetate–MES[2]–Tris (AMT) buffers (8) of constant ionic strength, whereas most others have used buffers of constant molarity, typically 0.1 M phosphate. An advantage of AMT buffer, besides its constant ionic strength, is that it effectively buffers over a wide pH range (8), whereas phosphate, for example, will effectively buffer only in the limited range of $pK_a \pm 1$. Despite this, phosphate has been used as low as pH 4.6 and as high as pH 8.5.

It does not appear to be widely appreciated by previous workers in this field that cathepsin L is influenced by ionic strength. We have previously reported that the activity of cathepsin L is apparently decreased by an increase in ionic strength (7). With anionic buffers of constant molarity, therefore, the increase in ionic strength with increasing pH may skew the pH profile, depressing the activity at higher pH values and resulting in a lower apparent pH optimum. These observations, and the consequent realization that the true pH optimum of cathepsin L (and its behavior in the specific ionic milieu of the extracellular medium) has not previously been established, motivated the present study of the effects of ionic strength and pH on the activity of cathepsin L.

In measuring pH–activity profiles, most authors have reported "relative activity" which, as discussed below, does not relate linearly to the second-order rate constant k_{cat}/K_m, when $[S]$ is of the same order as K_m,

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[2] Abbreviations used: MES, 4-morpholineethane sulfonic acid; AMT, acetate–MES–Tris; DTT, dithiothreitol; HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin.

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TABLE I

Some pH Optima Reported for Cathepsin L

Reference	Source of cathepsin L	Substrate	pH optimum
Mason *et al.* (20)	Rabbit liver	Azocasein	5.5
		Z-Phe-Arg-NHMec	6.0
		Collagen	3.5
Mason *et al.* (21)	Human liver	Z-Phe-Arg-NHMec	5.5
Mason *et al.* (22)	Human liver	Elastin	5.0
Dufour *et al.* (23)	Chicken liver	Z-Phe-Arg-NHMec	5.5
Kirschke *et al.* (24)	Rat liver	Z-Phe-Arg-NHMec	5.5
Brömme *et al.* (25)	Rat liver	Z-Phe-Arg-NHMec	6.0
Dennison *et al.* (7)	Sheep liver	Z-Phe-Arg-NHMec	6.5

which is the case with cathepsin L acting upon Z-Phe-Arg-NHMec. In these circumstances it is difficult to assign other than a superficial meaning to relative activity values, and this has motivated us to explore the effects of changes in pH and ionic strength on the kinetic constants k_{cat} and K_m and hence on k_{cat}/K_m.

In the context of the possible extracellular activity of cathepsin L, not only the activity but also the stability of the enzyme is of interest. The pH-dependent stability of human kidney cathepsin L has previously been studied by Turk *et al.* (9), who used phosphate buffer of constant molarity (100 mM) (or Tris, at pH 8.0). In light of the results presented in the present paper, it is evident that their findings cannot be extrapolated to physiological conditions. Moreover, since the ionic strength of the buffers used by these workers would have varied with the pH, it is difficult to distinguish the effects of pH and of ionic strength. Consequently, we have measured the stability of cathepsin L in AMT and phosphate buffers, in which the pH and ionic strength were varied independently, and also in Hanks' balanced salt solution, a buffer modeling the extracellular fluid.

EXPERIMENTAL

Materials. Z-Phe-Arg-NHMec was from Sigma (St. Louis, MO), and sheep liver cathepsin L was purified as previously described (10). The purification procedure involves homogenization of frozen liver, pH 4.2 precipitation, three-phase partitioning of the pH 4.2 supernatant at pH 4.2, and chromatography on S-Sepharose and Sephadex G-75. Cathepsin L is well separated from related cysteine proteinases, such as cathepsins B, H, and S, by chromatography on S-Sepharose. Chromatography on Sephadex G-75 effects separation of free cathepsin L from complexes of cathepsin L and stefin B (10).

Expression of pH–activity profiles. The velocity of a reaction such as that catalyzed by cathepsin L is described by the equation (11)

$$V_0 = \frac{k_{cat}}{K_m}\cdot[E]\cdot[S] \qquad [1]$$

and the relationship between the concentration of the free enzyme $[E]$ (which is usually unknown) and that of the total enzyme $[E]_0$ (which is known) is given by the equation

$$[E] = \frac{[E]_0}{\left(\frac{[S]}{K_m}+1\right)}. \qquad [2]$$

Modeling of Eq. [2] reveals that $[E]$ approximates $[E]_0$, only when $[S] \leq K_m/40$. The fluorogenic substrate commonly used to assay cathepsin L is Z-Phe-Arg-NHMec, for which cathepsin L has a K_m roughly comparable to the concentration at which this substrate is used and a concentration of $[S] \leq K_m/40$ is impractical, as cathepsin L activity is not measurable at this substrate concentration. An experimental approach different from that used with cathepsin B (e.g., 12) is thus necessary with cathepsin L.

Changes in pH and ionic strength, as explored here, affect V_0 by their influence on either or both of the constants comprising the second-order rate constant, k_{cat}/K_m (see Eq. [1]) and it is therefore most appropriate to express their influences in terms of their effects on k_{cat}/K_m. When $[S]$ is not substantially smaller than K_m, however, $[E] \neq [E]_0$ and so Eq. [1] cannot be used to determine k_{cat}/K_m. Instead, it is necessary to measure V_0 at more than one substrate concentration, which enables values for k_{cat} and K_m to be calculated from the Michaelis–Menten equation

$$V_0 = \frac{k_{cat}}{K_m + [S]}\cdot[E]_0\cdot[S].$$

It may be noted that, under conditions where the Michaelis–Menten equation applies, if the factor being investigated, e.g., pH or ionic strength, affects only k_{cat}, this will have a linear affect on V_0 and the shape of a profile of relative activity will be similar to that of k_{cat}/K_m vs pH or ionic strength. If on the other hand K_m is affected, the effect on V_0 will be nonlinear and will be a function of $[S]$. In these circumstances it may be difficult to assign other than a superficial meaning to the term relative activity.

In this study, the effects of pH and ionic strength on k_{cat}, K_m, k_{cat}/K_m, and relative activity were explored, the latter for comparison with previous reports.

Buffers. The ionic strengths of buffers of defined molarity were calculated using the BASIC program of Ellis and Morrison (8) and, conversely, the molarities of buffers of defined ionic strengths were calculated by a simple adaptation of this program, using an iterative loop to home in on the molarity value. AMT buffers of constant ionic strength were made up according to Ellis and Morrison (8) and other buffers were made up following the procedure previously described (13).

AMT buffers of ionic strength $I = 0.533$ were prepared at pH 5.5, 6.0, 6.5, 7.0, and 7.5. K-phosphate buffers were prepared at pH 6.0, 6.5, 7.0, and 7.5 (pH 5.5 being outside of phosphate's buffering range); the required molarities being calculated to be 0.4258, 0.3277, 0.2444, and 0.2019, respectively. Upon 3–4 dilution in the modified assay

described below, the final ionic strength of each of these buffers becomes $I = 0.4$. Serial twofold dilutions of these buffers gave additional buffers that, after dilution in the assay, yielded ionic strengths of $I = 0.2$ and 0.1, respectively. The pH of the buffers was checked after dilution; deviations from the nominal pH were insignificantly small.

Hanks' balanced salt solution was made up according to Freshney (14), but without glucose or phenol red, and at 4/3 the nominal concentration to allow for dilution in the assay. Immediately before use, the solution was titrated to pH 7.2 with CO_2 gas and, for the pH 6.8 buffer, further titrated to pH 6.8 with lactic acid.

Enzyme assays. To determine pH–activity profiles, and ionic strength–activity profiles, expressed in terms of relative activity, enzymes were assayed either by the method of Barrett and Kirschke (15) or in an assay modified to minimise the dilution of the buffer. In the latter case, the assay solution consisted of 750 μl of buffer, either 32 μl or 64 μl of DTT solution (154.2 mg DTT/ml water), and a complementary volume of 0.1% Brij, the total summing to 865 μl. All solutions were equilibrated to 37°C, except the diluted enzyme solution which was kept on ice. Appropriately diluted enzyme (10 μl) was added, allowed to activate for 2 min at 37°C, and 40 μM Z-Phe-Arg-NHMec solution (125 μl) was mixed in. The change in fluorescence was measured continuously at 37°C in a temperature-controlled recording spectrofluorometer, with excitation at 370 nm and emission at 460 nm. Data were sampled at 10-s intervals over a 5-min period and activity was determined from the slope of the plot of fluorescence intensity versus time. Stability was determined as the half-life of the enzyme activity, as previously described (7), using an assay of appropriate duration. The first-order decay constant k_{obs} is related to the half-life by the equation

$$t_{1/2} = \frac{0.693}{k_{obs}}.$$

To determine K_m and k_{cat} values, a semicontinuous assay in a fluorescence microplate reader (Cambridge Technology, Model 7620) was used. For each determination, buffer (75 μl) and DTT plus Brij (11.5 μl) was added to each of 14 wells of a white Fluoronunc maxisorp microtiter plate. The DTT plus Brij mixture consisted of 42.9 mg DTT/500 μl Brij. Enzyme (1 μl), of an appropriate dilution, was added (using a microsyringe) to each well, incubated for 2 min, and then serial twofold dilutions of Z-Phe-Arg-NHMec (12.5 μl) were added to duplicate wells in a row of seven, giving final substrate concentrations ranging from 50 to 0.78125 μM. After 2, 4, and 6 min, the wells were read, with excitation at 360 nm and emission at 460 nm. Reading at three different times provides a check on the linearity of the progress curve. It was empirically established that before 2 min the reaction rate increased with time, but the reaction was linear between 2 and 6 min. K_m and V_{max} values were determined using the direct linear plot of Eisenthal and Cornish-Bowden (16), implemented using the program HYPER obtained from Dr. J. S. Easterby (University of Liverpool, UK). $[E]_0$ values were established by E-64 titration, as described by Barrett and Kirschke (15), and the relationship

$$k_{cat} = V_{max}/[E]_0$$

was used to calculate k_{cat} from V_{max} values.

RESULTS

Cathepsin L was isolated from sheep liver in a single-chain form which, as previously reported, gives a single band on SDS–PAGE, corresponding to a molecular weight of 26 kDa (Ref. 10, Fig. 3A(b), and Ref. 17, Fig. 2e). As previously reported (10), the isolated enzyme

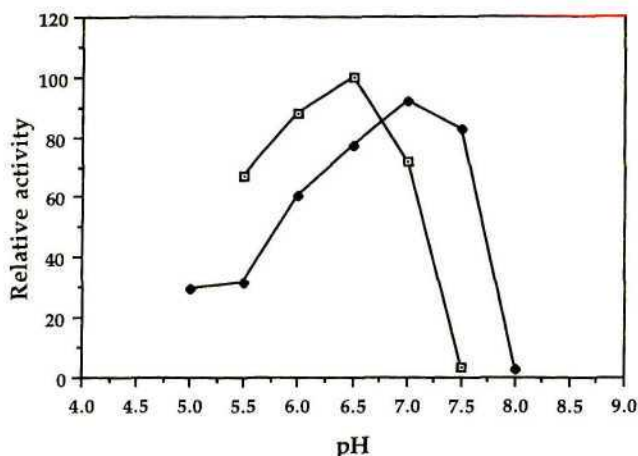

FIG. 1. Effect of constant ionic strength buffer versus constant molarity buffer upon the apparent pH–activity profile of sheep liver cathepsin L. Cathepsin L was assayed by the method of Barrett and Kirschke (15) using 100 mM K-phosphate buffers (□) or AMT buffers (♦) (8) of $I = 0.1$. (These were diluted fourfold in the assay, giving a final concentration of 25 mM and $I = 0.025$, respectively.)

was not active against Z-Arg-Arg-NHMec, which indicates that it is not contaminated by cathepsin B, and was rapidly inhibited by 1 μM Z-Phe-Phe-CHN_2 (18), which distinguishes it from cathepsin S. Cathepsins B and S elute from S-Sepharose much earlier than cathepsin L; moreover, cathepsin S has not been detected in liver (19).

The pH–activity profiles shown in Fig. 1 illustrate that in a buffer of constant ionic strength, cathepsin L has an apparent pH optimum higher than in a phosphate buffer of constant molarity (and which therefore has an increasing ionic strength, with increasing pH).

Measurement of k_{cat} and K_m in AMT and phosphate buffers of different pH and ionic strengths revealed a general trend for k_{cat} to peak (at ca. 20–30 s^{-1}) at about pH 6.5 in both buffers and essentially independently of ionic strength (Tables II and III). K_m values, of ca. 5 μM, manifested a general slight rising trend with increasing ionic strength, with a sharp increase to 20–25 μM, specifically at pH 6.5 and $I = 0.4$, in both buffers. We have previously noted that the reported K_m values of single-chain forms of cathepsin L appear to be higher than those of two-chain forms (Ref. 17, Table 3). This may be a consequence of a greater flexibility of the two-chain forms, enabling them to bind substrate with higher affinity. We have also argued that two-chain forms may be isolation artefacts (17). The second-order rate constant, k_{cat}/K_m, generally declined with increasing ionic strength, in both buffers, and declined above pH 6.5, in phosphate buffer, but only above pH 7, in AMT buffer. At $I = 0.4$, however, there was a general decline in k_{cat}/K_m, with increasing pH in both buffers. In Hanks' balanced salt solution (HBSS), a model of the extracellular fluid, measured values were k_{cat}, 17.4 s^{-1}; K_m, 11.7 μM; and k_{cat}/K_m, $1.5 \times 10^6\ M^{-1}\ s^{-1}$.

TABLE II

The Effect of pH and Ionic Strength of AMT Buffer upon the K_m, k_{cat}, and k_{cat}/K_m of Sheep Liver Cathepsin L

Ionic strength	pH				
	5.5	6.0	6.5	7.0	7.5
0.1					
K_m	7.17	4.21	8.97	5.96	8.79
k_{cat}	11.26	9.87	22.13	14.88	9.10
k_{cat}/K_m	1.6	2.3	2.5	2.5	1.0
0.2					
K_m	7.38	7.92	9.37	5.96	n.m.
k_{cat}	11.43	16.22	17.51	20.25	n.m.
k_{cat}/K_m	1.5	2.0	1.9	3.4	—
0.4					
K_m	7.52	17.38	25.43	16.73	n.m.
k_{cat}	13.54	22.95	24.48	15.09	n.m.
k_{cat}/K_m	1.8	1.3	1.0	0.9	—

Note. K_m, μM; k_{cat}, s^{-1}; k_{cat}/K_m, $M^{-1}\ s^{-1} \times 10^{-6}$. n.m., not measureable.

TABLE IV

The Effect of Buffer Ionic Composition, Ionic Strength, and pH on the Stability of Sheep Liver Cathepsin L

Ionic strength	$t_{1/2}$ (s)			
	Phosphate buffer		AMT buffer	
	pH 7.0	pH 7.5	pH 7.0	pH 7.5
0.05	733	186	∞	374
0.1	531	83	∞	493
0.2	360	64	∞	213
0.4	327	77	530	105

Note. ∞, no decrease in rate of reaction could be measured over the period of the assay.

The stability of cathepsin L, in the physiological pH range was generally higher in AMT buffer than in phosphate (Table IV). In both buffers there was a trend to shorter half-lives with increasing pH and with increasing ionic strength. In HBSS the half-life of cathepsin L was 179 s at pH 7.2 and 657 s at pH 6.8 (Table V). The half-life was affected differentially by the additional presence of BSA or gelatin (Table V), being decreased by BSA at both pH values, and gelatin at pH 6.8, but increased by gelatin at pH 7.2.

In phosphate and AMT buffers, a general correlation between stability and activity is evident; i.e., stability declines in parallel with activity in either buffer. Since initial velocities were measured, there would appear to be no *a priori* reason why stability and activity should be affected in concert, and the results suggest that the two phenomena may be mechanistically linked.

TABLE III

The Effect of pH and Ionic Strength of K-Phosphate Buffer upon the K_m, and k_{cat}/K_m of Sheep Liver Cathepsin L

Ionic strength	pH			
	6.0	6.5	7.0	7.5
0.1				
K_m	6.81	3.94	5.79	n.m.
k_{cat}	19.74	24.81	8.41	n.m.
k_{cat}/K_m	2.9	6.3	1.5	—
0.2				
K_m	6.68	8.94	7.26	n.m.
k_{cat}	17.71	23.85	9.15	n.m.
k_{cat}/K_m	2.7	2.7	1.3	—
0.4				
K_m	9.93	29.81	13.46	n.m.
k_{cat}	24.20	22.09	9.36	n.m.
k_{cat}/K_m	2.4	1.3	0.7	—

Note. K_m, μM; k_{cat}/K_m, $M^{-1}\ s^{-1} \times 10^{-6}$. n.m., not measureable.

DISCUSSION

The pH–activity profile of cathepsin L has been studied by several authors (e.g., Table I), but a common error has been a failure to take into account the effects of ionic strength on the activity of this and other cathepsins. In a buffer of constant molarity, the ionic strength is a function of the buffer pH, as is evident from the Henderson–Hasselbalch equation. For example, in the case of constant molarity phosphate, the ionic strength increases sigmoidally, about threefold from pH 5.5 to 8.5, with the greatest increment occurring at the pK_a (8). Failure to take this into account has led to an exaggeration of the effect of increasing pH since, as we have shown here, increasing pH and increasing ionic strength have a cooperative effect in

TABLE V

The Effect of pH and the Presence of Protein on the Half-Life of Sheep Liver Cathepsin L in Hanks' Balanced Salt Solution

HBSS	$t_{1/2}$ (s)[a]
pH 7.2	179 ± 5.2
+0.1% BSA	178 ± 11.2
+0.2% BSA	178 ± 3.7
+0.1% gelatin	231 ± 6.7
+0.2% gelatin	187 ± 24.5
pH 6.8	657 ± 6.1
+0.1% BSA	277 ± 31.1
+0.2% BSA	341 ± 20.4
+0.1% gelatin	484 ± 11.4
+0.2% gelatin	575 ± 78.1

[a] Each figure is the mean of three determinations.

decreasing the activity and stability of cathepsin L. The net effect has been a relative depression of the activity and stability of cathepsin L at higher pH and a consequent general underestimate of both its pH optimum and its stability at physiological pH.

The 3-D structure of cathepsin L is presently not known but deductions have been made by analogy with the known structures of papain and actinidin, which have primary structures homologous to that of cathepsin L (23). The conclusion reached by Dufour *et al.* (23) is that cathepsin L is probably similar to papain in its 3-D structure but may have an extra α-helix on the outer aspect of the domain containing the central helix. The 3-D structure of cathepsin B has been published (26) and its catalytic mechanism studied (12, 27, 28) and, by extrapolation, this may provide insights into the mechanism of the pH and ionic strength dependence of the activity of cathepsin L.

Turk *et al.* (28) have proposed a mechanism explaining the pH and ionic strength dependence of the irreversible denaturation of cathepsin B, which invokes the deprotonation of the imidazole moiety of the active site -S^-/$^+$HIm- ion pair, with subsequent "unzipping" of the structure along the active site groove. As these authors have suggested, this is a mechanism which might apply to more than just cathepsin B and we note that it does explain the correlation we have observed between activity (measured as initial velocity) and stability of cathepsin L: in other words, that conditions which promote/reduce activity, similarly promote/reduce stability and vice versa. In this model, inactivation, caused by the deprotonation of the imidazole moiety of the active site -S^-/$^+$HIm- ion pair, is mechanistically linked to the subsequent inactivation by unzipping and both steps would be sensitive to ionic strength as well as pH. This model is also consistent with the observations of Dufour *et al.* (23) who noted a marked decrease in α-helix content of chicken cathepsin L at higher pH, suggesting that the enzyme is denatured under these conditions.

A comparison of the primary structures of cathepsin L (29) with that of cathepsin B (26) indicates, however, that the charged amino acids proposed by Turk *et al.* (28) to be involved in the zipper mechanism in cathepsin B, have few exact counterparts in cathepsin L. The existence of fewer, or different, links in the zipper might underlie the differences in the pH optima of cathepsin B and cathepsin L. In the absence of a 3-D structure for cathepsin L, however, it is not possible to comment further on the extent to which the proposed model may be applicable to cathepsin L.

One difference between cathepsins B and L is that both hydrolyze Z-Phe-Arg-NHMec, whereas only cathepsin B hydrolyzes Z-Arg-Arg-NHMec. This can be explained by the fact that in cathepsin B, a negatively charged Glu-245 residue, occupies the S2 site and this presumably interacts with the positively charged Arg residue in the P2 position. The equivalent residue in cathepsin L is an uncharged Ala-209 and deletion of the charge at this position apparently removes the possibility of interaction with Z-Arg-Arg-NHMec. It can be imagined that binding of Z-Phe-Arg-NHMec might require a negatively charged group at the S1 position and a hydrophobic pocket at S2 (nomenclature of Schechter and Berger [30]). The fact that the K_m increases with increasing ionic strength, at least between pH 6.0 and pH 7.0, suggests, however, that in this range ionic interactions predominate, since an increase in ionic strength should weaken ionic interactions and strengthen hydrophobic interactions.

The measured stability of cathepsin L was greatest in AMT buffer and less in phosphate buffer and HBSS. Also, activity declined at a higher pH in AMT buffer than in phosphate buffer. A commonality between phosphate buffer and HBSS is the size of the cations, K^+ and Na^+, respectively, which are smaller than the $Tris^+$ and MES^+ cations in AMT buffer. Moreover, with Na-phosphate buffer the concentration of Na^+ ions increases with increasing pH. These results are thus not inconsistent with a special role for small cations in the inactivation and destabilisation of cathepsin L.

The fact that the activity and stability of cathepsin L at physiological pH is different in AMT and phosphate buffers suggests the existence of specific ion effects, over and above the effects of ionic strength per se and pH. In turn, this implies that if physiological relevance is to be explored, it is necessary (as we have done in this study) to use a buffer modeling the compartment of interest in its specific ionic composition, as well as pH and ionic strength. When this is done, we find that mature single-chain cathepsin L has an apparent extracellular half-life in the region of 3 to 8 min. These values are consistent with a previous report by Machleidt *et al.* (31), though the buffer used was not specified in that study. Reports of much shorter half-lives (9), while valid for the specific buffers used, probably cannot be extrapolated to physiological conditions.

With regard to the possible extracellular activity of cathepsin L, if cells secrete cathepsin L at a constant rate (say, D mol s^{-1}) then, theoretically, an equilibrium should be set up between secretion and subsequent first-order decay of the enzyme. The amount of active enzyme (E) present in the extracellular space is described by the equation

$$E = \frac{D}{k_{\mathrm{obs}}}(1 - e^{-k_{\mathrm{obs}}t})$$

which indicates that E is asymptotic to D/k_{obs}, i.e., the amount of enzyme in the extracellular space at equilibrium is D/k_{obs} mol.

In the case of tumor cells, Gullino *et al.* (32) have

reported that the pericellular space surrounding tumor cells is slightly acidified, to ca. pH 6.8. In Hanks' balanced salt solution, lowering the pH from pH 7.2 to 6.8, with lactate, increased the half-life of cathepsin L from 179 to 657 s. This would have the effect of increasing the amount of active enzyme present in the extracellular space, at equilibrium, by a factor of 657/179 (i.e., almost fourfold). The additional presence of specific extracellular proteins would have a modifying effect, however (Table IV). In the digestion of extracellular proteins cathepsin L would most probably encounter an excess of substrate in the form of scissile bonds. Under these circumstances, V_0 would be largely influenced by k_{cat}, which peaks at pH 6.5. At pH 6.8, the pericellular pH of tumor cells (32), therefore, cathepsin L would be almost optimally active and markedly stable. While the involvement of the activity of mature cathepsin L, extracellularly, in tumor invasion and metastasis is as yet unproven, the present study suggests that the properties of the mature enzyme do not preclude such a role.

The extracellular activity of cathepsins is subject to modulation by inhibitors such as the cystatins (33) and, in the blood, α_2-macroglobulin (34). Since ionic strength and buffer composition influence the interaction of cathepsin L with substrate, it is not implausible that the interaction with inhibitors might be similarly affected. To obtain physiologically relevant data on inhibition, therefore, it would seem prudent to also measure this in a buffer modeling the extracellular fluid, though to our knowledge this does not seem to be common practice. Nevertheless, it can be envisioned that the cystatins' role *in vivo* may depend upon the rate at which these can diffuse to the pericellular area versus the rate at which cathepsins are secreted.

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Short Communication

Reductive Activation Markedly Increases the Stability of Cathepsins B and L to Extracellular Ionic Conditions

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Cathepsins B and L are thought to function extracellularly in pathological conditions. pH-Activity profiles of cathepsin B, measured in phosphate and acetate-Mes-Tris buffers of constant ionic strength, indicated that cathepsin B is sensitive to specific buffer ions, as previously reported for cathepsin L. In assessing the activity of these enzymes *in vitro* the influence of the buffer must therefore be taken into account. In Hank's balanced salt solution, a buffer modeling the extracellular fluid, the half-life of activated human liver cathepsin B at 37 °C is 245 ± 11.3 s, at pH 7.2, and 857 ± 50.1 s, at pH 6.8 (the peritumor pH), indicating that cathepsin B is markedly stable under these conditions. The stability was increased by the additional presence of proteins. Without immediate activation, however, the stabilities of both cathepsins B and L were markedly decreased, a large proportion of their activity being lost before it could be measured. Enzymes injected into the extracellular space in the unactivated state would therefore survive for only a very short time in their native conformation. It is proposed that the active site thiolate-imidazolium ion pair contributes substantially to the stability of cathepsins B and L to extracellular ionic conditions.

Key words: Activation / Cathepsins / Extracellular / Reduction / Stability.

Cathepsins B and L are lysosomal cysteine proteinases that play a role in endogenous protein turnover and in the degradation of endocytosed proteins (Barrett and Kirschke, 1981). They have also been implicated in various pathologies in which they are thought to function extracellularly, so the question of their stability under extracellular conditions is important.

It has been reported that cathepsins B and L are unstable at the extracellular pH (Turk *et al.*, 1993; Turk *et al.*, 1994), though it is well established that cathepsin B is active for limited periods at this pH (Mort *et al.*, 1984; Machleidt *et al.*, 1986). However, besides pH, the ionic strength also affects the activity and stability of these cathepsins (Turk *et al.*, 1994; Khan and Ahmad, 1987; Dehrmann *et al.*, 1995). Dehrmann *et al.* (1995) additionally showed that not only ionic strength *per se*, but also the specific buffer ions present can influence the activity and stability of cathepsin L. Similarly, the specific buffer ionic composition also affects cathepsin B, since at equal pH and ionic strengths, phosphate and acetate-MES-Tris (AMT) buffers have different effects upon cathepsin B (Figure 1).

In general, the activity of cathepsin B is higher in phosphate buffer (Figure 1A), compared to AMT buffers at the same ionic strength (Figure 1B). For physiological relevance, therefore, in relation to its purported extracellular activity, the behavior of cathepsin B was measured in Hank's balanced salt solution (HBSS) (Freshney, 1983), a buffer modelling the extracellular milieu in ionic composition, ionic strength and pH. In HBSS, k_{cat}/K_m for cathepsin

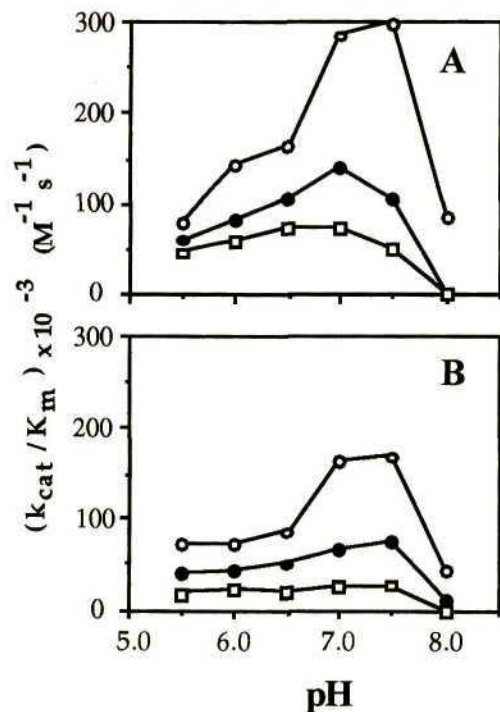

Fig. 1 The Effect of Different Buffers on the Z-Arg-Arg-NHMec-Hydrolysing Activity of Human Liver Cathepsin B.
(A) K-phosphate buffers; (B) AMT buffers. Ionic strength (after 3 – 4 dilution in the assay), ○ = 0.1; ● = 0.2; □ = 0.4
Buffers were prepared, and the assay conducted, as described in Dehrmann *et al.*, 1995.

B acting on Z-Arg-Arg-NHMec was 220 x $10^3 M^{-1}s^{-1}$, at pH 7.2. Gullino *et al.* (1965) have reported that the peritumor pH is 6.8. In HBSS titrated to pH 6.8 with lactic acid, k_{cat}/K_m for cathepsin B acting on Z-Arg-Arg-NHMec was 168 x $10^3 M^{-1}s^{-1}$.

The pH optimum obtained for cathepsin B in the present study (pH 7 → 8) (Figure 1) is consistent with previous reports (Willenbrock and Brocklehurst, 1985; Khouri *et al.*, 1991; Hasnain *et al.*, 1992; Moin *et al.*, 1992). An optimum corresponding to the pH of the extracellular fluid is clearly significant for any purported extracellular activity of cathepsin B, but of equal importance is the stability of the enzyme to extracellular conditions.

The stability of cysteine cathepsins has been measured in different ways. Mason (1986) and Kirschke *et al.* (1989) incubated the enzyme for 1 h at various pH values, before measuring residual activity. In this approach the enzyme is not in the activated state during the incubation. We and others have measured the half-life of cathepsin L and/or cathepsin B using continuous monitoring (Mort *et al.*, 1984; Machleidt *et al.*, 1986; Baici *et al.*, 1988; Dennison *et al.*, 1992; Turk *et al., 1993* Turk *et al.*, 1994; Dehrmann *et al.*, 1995). For such continuous monitoring of activity it is necessary that the isolated enzyme be pre-activated with a reducing agent such as DTT, cysteine or 2-mercaptoethanol. Here we show that this activation itself markedly increases the stability of cathepsins B and L.

Using the normal assay procedure involving simultaneous dilution, pH change, and activation of the enzyme, the half-life of cathepsin B in HBSS at 37 °C was measured to be about 4 min at pH 7.2 and increased to about 14 min at pH 6.8 (Table 1). Consistent with the report of Buck *et al.* (1992), the half-life of cathepsin B was increased by the presence of 0.1 to 0.2% BSA, most markedly at pH 6.8, where it was increased from 14 min to 24 → 32 min. [Studies that have concluded that cathepsin B is much less stable at extracellular pH can be traced back to observations using buffers of constant molarity at different pH (and therefore of differing ionic strength) – see Dehrmann *et al.* (1995) for a discussion]. It has been reported that in HBSS the half-life of cathepsin L is 3 min at pH 7.2 and 11 min at pH 6.8 and that cathepsin L is apparently not stabilized by the presence of BSA or gelatin (Dehrmann *et al.*, 1995).

With dilution (to the stated pH) before activation, stability was markedly reduced and large proportions of the activities of both cathepsins B and L were lost before they could be measured (Table 2). When the enzyme was added last, generally lower activities were obtained that with simultaneous dilution and activation (Table 2). Here the substrate is already present when the enzyme is added, so it appears that under these circumstances substrate does not markedly stabilize the enzymes.

The effect of reductive activation in markedly increasing the stability of both cathepsins B and L can be explained by the model proposed by Turk *et al.* (1994) for the pH-dependent inactivation of cathepsin B, but which also probably applies to cathepsin L (Dehrmann *et al.*, 1995). In

Table 1 The Effect of pH and the Presence of Protein on the Half-Life of Human Liver Cathepsin B in Hank's Balanced Salt Solution, at 37 °C.

HBSS	T 1/2 (s)[a]
pH 7.2	245 ± 11.3
+ 0.1% BSA	343 ± 62.2
+ 0.2% BSA	360 ± 36.3
+ 0.1% gelatin	240 ± 12.0
+ 0.2% gelatin	335 ± 16.3
pH 6.8	857 ± 50.1
+ 0.1% BSA	1954 ± 379.0
+ 0.2% BSA	1461 ± 138.9
+ 0.1% gelatin	762 ± 216.0
+ 0.2% gelatin	709 ± 154.9

[a] Each figure is the mean and standard deviation of results from three replicate experiments.
Human liver cathepsin B, isolated by the method of Rich *et al.* (1986), was from Athens Research and Technology (Athens, Georgia, USA). According to the suppliers, the product is free of cathepsin L.
Buffers were prepared as previously described (Dehrmann *et al.*, 1995) and the half-life of the enzyme activity was determined according to Dennison *et al.*, 1992.

Table 2 Effect of Activation before or after pH Change and Dilution upon the Stability of Cathepsins B and L in Hank's Balanced Salt Solution.

Protocol	Residual activity (%)[a]			
	Cathepsin B		Cathepsin L	
	pH 7.2	pH6.8	pH 7.2	pH 6.8
Simultaneous dilution and activation	(100)	(100)	(100)	(100)
Activation immediately after dilution	38 ± 13.9	81 ± 6.5	14 ± 2.8	59 ± 4.9
Activation 10 min after dilution	3 ± 0.2	14 ± 1.8	1.5 ± 0.4	3 ± 1.4
Enzyme added last	81 ± 3.3	94 ± 4.2	95 ± 10.7	64 ± 2.6

[a] Each figure is the mean and s.d. of the results from three replicate experiments.
Sheep liver cathepsin L was isolated as previously described (Pike *et al.*, 1992). Substrates were Z-Arg-Arg-NHMec for cathepsin B and Z-Phe-Arg-NHMec for cathepsin L.
Assays were as previously described (Dehrmann *et al.*, 1995). For assay with simultaneous dilution and activation, (the normal approach), reagents were added in the following oder: – 750 μl of buffer, 115 μl DTT/Brij, and 10 μl of diluted enzyme. The enzyme was activated for 1 min at 37 °C, and 40 μM substrate (125 μl) was mixed in and the change in fluorescence was measured continuously at 37 °C. Note that 'dilution' also entails a pH change, from pH 5.5 to the stated value.
For assay with enzyme dilution before activation, the order of addition was: buffer, Brij, enzyme and DTT. The DTT was added either immediately or after 10 min, followed by substrate 1 min later.
As a third option (denoted 'enzyme added last'), buffer, Brij, DTT and substrate were mixed together and enzyme was added at zero time.

this model, inactivation of cathepsin B is envisaged to be a consequence of 'unzipping' of the two domains, along the active site groove, the zipper being constituted by a series of ionic bonds. Initiating the unzipping is disruption of the active site thiolate-imidazolium (-S^-/$^+$HIm-) ion pair, either by deprotonation of the imidazole moiety at high pH, and/or by ionic competition. It follows from this model that in the absence of the -S^-/$^+$HIm- ion pair, which in isolated cathepsins B and L is presumably generated by activation with the reducing agent, the enzyme would be less stable. The results of the present study support this model.

It may be envisaged that during isolation, due to the oxidative environment obtaining *in vitro*, intermolecular or intramolecular (Brocklehurst and Kierstan, 1973) mixed disulfides may be formed leading to inactivation of the enzyme. Subsequent reductive activation would be effected by removal of the group blocking the active site thiol. Consequent reconstitution of the -S^-/$^+$HIm- ion pair would lead to enhanced enzyme stability. In this model, inactive-but-activatable cysteine cathepsins are perceived as isolation artefacts. Evidence that at least a proportion of endogenous cathepsins B and L are active has been provided by *in vivo* labeling with radioiodonated inhibitors (Mason *et al.*, 1989) and by the use of fluorogenic substrates (Assfalg-Machleidt *et al.*, 1992). Endogenous and thus secreted cathepsins may, therefore, be in an active form which would be maximally stable. Their subsequent inactivation and denaturation would nevertheless depend, among others, upon the redox potential of the environment into which they were secreted.

The relative instability of isolated, unactivated, cathepsin B may underlie the difficulties which have been reported in obtaining polyclonal antibodies which react with the native enzyme (Mort and Recklies, 1986). In eliciting antibody production, the normal practice is to inoculate with unactivated enzyme. In the light of the results reported here, it would be expected that the unactivated enzyme would survive in its native conformation for only a very short time under extracellular conditions. [Thus an alternative route for eliciting anti-cathepsin B antibodies may be to inoculate activated cathepsin B directly into the bloodstream. Not only is the activated enzyme more stable but it would be immediately inhibited and stabilized by cystatins (Turk *et al.*, 1993) and either presented in this form or perhaps passed on to α_2-macroglobulin (Chu *et al.*, 1994), for immune presentation.]

In conclusion, we have shown here that, like cathepsin L, the activity and stability of cathepsin B is affected by specific buffer ions as well as by pH, ionic strength, and the presence of protein substrates. Also, that the stability of these enzymes is affected by their activation state, an observation which partially explains the markedly different results which have been previously reported concerning the stability of these enzymes.

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