TYPE IV COLLAGENASE AND CATHEPSINS L AND H: PROTEINASES INVOLVED IN TUMOUR INVASION

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This thesis is dedicated to my parents, Elizabeth and Gochie Coetzer.

PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg and the Department of Biochemistry, University of Georgia, Athens, Ga., USA from December 1988 to December 1992, under the supervision of Prof. Clive Dennison.

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

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ABSTRACT

The collagenolytic proteinases, type IV collagenase and cathepsins L and H, have been implicated in tumour invasion and metastasis, by virtue of their degradative action on the extracellular matrix barriers traversed by migrating tumour cells. Type IV collagenase was isolated from human leucocytes using anti-peptide antibody immunoaffinity chromatography. The highly specific targeting of both native and denatured forms of human type IV collagenase by these anti-peptide antibodies holds much promise for immunolocalisation studies in human tumour tissue. Cathepsin L was purified in both a free, single-chain form from sheep liver, and as complexes with the endogenous cysteine proteinase inhibitor, stefin B. These complexes comprised mixtures of the usual tight-binding non-covalent, inhibitory complexes, and novel, proteolytically active, covalent cathepsin L/stefin B complexes. The latter form spontaneously in a pH-dependent manner *in vitro* from purified, active constituents. The primary structures of these complexing moieties from sheep liver are reported here for the first time, and showed a high degree of sequence homology with their human counterparts. Single-chain cathepsin L, both in the free, and novel, covalently complexed forms, manifested stability and increased activity at neutral pH, thus suggesting a role in extracellular tissue destruction. This potential involvement in tumour invasion was strengthened by demonstrating that the single-chain form of the enzyme, and similar covalent complexes, active under physiological conditions, could be isolated from liver tissue homogenates of higher primates, baboon (*Papio ursinus*) and man.

A battery of versatile polyclonal anti-sheep cathepsin L and anti-human cathepsins L and H peptide antibodies were raised in chickens and rabbits. The chicken egg yolk antibodies were often of a higher titre than the corresponding rabbit serum antibodies, and additionally manifested unique immunoinhibitory properties. In the case of the polyclonal chicken anti-sheep cathepsin L antibodies, this was derived from their ability to target a peptide located in the active site of cathepsin L. The chicken anti-human cathepsins L and H peptide antibodies constitute the immunological probes of choice for immunolocalisation and *in vitro* tumour invasion studies to elucidate the relative contributions of these collagenolytic cathepsins to tumour invasion, and could ultimately find application in tumour immunotherapy.

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ABBREVIATIONS

ABTS	2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (6)]
AMT	acetate-MES-Tris buffer
APMA	4-Aminophenylmercuric acetate
BSA	bovine serum albumin
CAMOR	carrier agent-modified residues
CDR	complementarity determining region
CM	carboxymethyl
CPI	cysteine proteinase inhibitor
dpm	disintegrations per minute
kDa	kilodaltons
DEAE	diethyl aminoethyl
dist.H_2O	distilled water
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
E-64	L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane
Fab	fragment, antigen binding
Fc	fragment, crystallisable
Fv	fragment, variable region
FGF	fibroblast growth factor
HIV	human immunodeficiency virus
HRPO	horse radish peroxidase
IgG	immunoglobulin G
IgY	immunoglobulin Y
K_{av}	availability constant
k_d	dissociation constant
K_i	inhibition constant
k_{obs}	observed equilibrium co-efficient
KLH	keyhole limpet haemocyanin
α_2M	α_2-macroglobulin
MBS	*m*-maleimidobenzoyl-N-hydroxysuccinimide ester
MEC	molecular exclusion chromatography
MES	2(N-morpholino)ethanesulfonic acid

MCP	mouse cysteine proteinase
MEP	major excreted protein
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MPR	mannose-6-phosphate receptor
mRNA	messenger ribonucleic acid
M_r	relative molecular weight
MW	molecular weight
NHMec	7-amino-4-methyl coumarin
PAGE	polyacrylamide gel electrophoresis
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PBS	phosphate-buffered saline
pI	isoelectric point
PMA	see TPA
PMNL	polymorphonuclear leucocyte
PMSF	phenylmethylsulfonyl fluoride
p21	protein of M_r 21 000
RT	room temperature
R-value	correlation coefficient
SBTI	soya bean trypsin inhibitor
scFvs	single chain variable fragment regions
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TCA	trichloracetic acid
TEA	triethylamine
TEMED	N,N,N',N', tetramethyl ethylene diamine
TGF-β1	transforming growth factor β1
TIMP	tissue inhibitor of metalloproteinases
TLCK	tosyl lysyl chloromethyl ketone
TPCK	tosyl phenylalanine chloromethyl ketone
TPA	12-O-tetradecanoylphorbol-13-acetate, also called PMA
TPP	three-phase partitioning
V_H	varible region of immunoglobulin heavy chain
V_o	void volume
Z	benzoylcarboxy

CHAPTER 1

INTRODUCTION

Tumour invasion of the extracellular matrix (ECM) and the subsequent formation of distant metastases are the hallmarks of malignant neoplasia, and constitute the principal reasons for the high rate of cancer mortality. Cancer treatment involving surgery, chemo- or radiotherapy has only a 50% success rate in patients developing malignant tumours, because of the heterogenous cell composition and dispersed anatomical location of tumour metastases (Liotta *et al.*, 1986). Major challenges are, therefore, the development of improved methods of predicting the metastatic potential of tumours, prevention of local tumour invasion and identification and treatment of clinically silent micrometastases. The present study focused on the biochemical events associated with tumour invasion, with a view to developing methods for arresting this process.

Malignant transformation, which may be triggered by molecular and cellular events such as oncogene activation or chemical carcinogenesis, could lead to the formation of a primary tumour (Klein and Klein, 1986). Following acquisition of the tumourigenic phenotype, cells no longer respond to normal regulatory mechanisms that control cellular migration and proliferation. Progressive growth of primary tumours, leading to tumour expansion, depends on sustained oxygen and nutrient supply (Fidler *et al.*, 1978). A new blood supply is generated by angiogenesis, a process by which capillaries grow from pre-existing microvessels toward a growing tumour. Neovascularisation takes place in response to angiogenic molecules, released by both tumour cells and host inflammatory cells attracted to the tumour site (Blood and Zetter, 1990; Folkman and Shing, 1992).

In order to metastasise, tumour cells become detached from the primary tumour and start migrating into adjacent tissue, probably under the influence of chemotactic factors, where they could penetrate host blood and lymphatic vessels and become disseminated (Goldfarb, 1982). Intravasation may also readily occur into the newly formed capillary blood vessels embedded in the tumour, which have been found to be more permeable than normal blood vessels, primarily because of their discontinuous basement membrane structure (Netland and Zetter, 1989).

During circulation in the blood and/or lymphatic systems, tumour cells or small tumour emboli must be able to survive attack by tumouricidal immune cells and destruction by mechanical shear forces in the bloodstream, to form metastases at distant sites. Although tumour cells become mechanically arrested in the first capillary bed they encounter, their

subsequent metastatic distribution often does not correlate with the initial arrest pattern and instead they show a high degree of organ selectivity (Hart, 1982; Nicolson, 1988). Circulating tumour cells, which escaped destruction at the site of initial arrest, may recirculate to a specific organ where adhesion to microvessel endothelial cells take place (Nicolson, 1988). Adhesion is facilitated by the expression of organ-specific cell surface glycoproteins on microvessel endothelial cells (Auerbach *et al.*, 1987; Nicolson *et al.*, 1989). Following adhesion, tumour cells stimulate retraction of the endothelial cells, thus leading to exposure of the subendothelial basement membrane, which contains a variety of tumour cell adhesion molecules (Kramer and Nicolson, 1979). These adhesion molecules are regular basement membrane components and provide a much better adhesive substrate for tumour cells than the endothelial cell surface.

In order to gain access to the organ parenchyma, metastasising cells extravasate through the vascular wall, and invade the underlying interstitial stroma, where secondary tumours could be formed. Directional tumour cell motility is induced by substratum bound gradients of ECM molecules which act as haptotaxins, or soluble gradients of ECM derived fragments acting as chemotaxins. Following angiogenesis and expansion of the secondary tumour, a repeat of the metastatic cascade could lead to the formation of further metastases.

It, therefore, becomes apparent that at various stages during tumour invasion, neoplastic cells would encounter different ECM barriers which do not normally contain pre-existing passageways for cells. Earlier hypotheses suggested that mechanical pressure, exerted by the expanding tumour mass (Eaves, 1973), or a decrease in adhesion between transformed cells, which could lead to an increase in cellular locomotion (Coman and Anderson, 1955; Vesely and Weiss, 1973), provide the major means of penetrating the ECM during tumour invasion. It has, however, become well established that invasive tumour cells secrete a variety of matrix-degrading proteinases, or induce their secretion by the surrounding host cells, to cause focal lysis of the basement membrane at points of contact with tumour cells, to allow penetration of tumour cell pseudopodia through the basement membrane (Liotta *et al.*, 1980; 1982; Sloane and Honn, 1984; Dano *et al.*, 1985; Spyratos *et al.*, 1989). Tumour invasion does involve active locomotion of tumour cells, but only once the surrounding ECM has been altered by the action of proteolytic enzymes. Furthermore, the chemotactic factors, such as collagen and fibrinogen, facilitating subsequent colonisation of neighbouring tissue, are the products of proteolytic degradation of the ECM (Nicholson, 1984).

Invasion, following proteolytic modification of the ECM, is not a process restricted to tumour cells, but also occurs in normal tissue during wound healing and tissue remodelling

(Werb and Burleigh, 1974). Proteolysis is also required for normal trophoblast invasion of the uterine epithelial basement membrane, interstitial stroma and blood vessels during implantation of the placenta (Lala and Graham, 1990). Leucocytes, such as PMNLs, display invasive behaviour when they extravasate from the bloodstream and migrate towards inflammatory foci (Murphy *et al.*, 1989a). Angiogenesis by normal endothelial cells also requires proteolysis, in fact tumour invasion and angiogenesis have many common features (Blood and Zetter, 1990; Liotta *et al.*, 1991; Folkman and Shing, 1992). Pathologies such as rheumatoid arthritis, osteoarthritis and emphysema are also characterised by proteolytic degradation of the ECM (Etherington *et al.*, 1988; Dean *et al.*, 1989; Johnson *et al.*, 1986).

The ECM, to which tumour cells bind, and which is subsequently degraded during tumour invasion, consists of a dense meshwork of protein, mainly collagen and elastin, embedded in a ground substance comprising glycoproteins and proteoglycans (Yamada and Olden, 1978; 1979; Stone *et al.*, 1982; Pauli *et al.*, 1983). Two types of ECM, interstitial connective tissue stroma and basement membranes are distinguished, which differ in composition (notably in the type of constituent collagen), function and location. Interstitial connective tissue is a complex matrix composed, depending on the tissue type, of cells such as chondrocytes, fibroblasts, macrophages and osteoblasts, located in a matrix of collagen fibres, glycoproteins and proteoglycans (Tryggvason *et al.*, 1987). This tissue has major mechanical and structural functions and is present in bone, tendons, cartilage, ligaments and stroma. Types I and III collagen and an additional minor type VI collagen are usually present in all interstitial connective tissues, while type II collagen and the minor collagen types IX, X and XI are specifically found in cartilage (Kühn, 1986). Type V collagen forms fibres which anchor interstitial collagen fibres to the basement membrane (Tryggvason *et al.*, 1987).

Basement membranes are acellular sheet-like structures found at the base of epithelial and endothelial tissues, separating the cells of these tissues from the underlying interstitial connective tissue stroma (Martin *et al.*, 1988). The basement membrane is a highly cross-linked matrix consisting of the basement membrane specific type IV collagen, which forms the structural skeleton to which the glycoproteins laminin and fibronectin, heparan sulfate proteoglycans and nidogen (enactin) are bound (Timpl *et al.*, 1981; Laurie *et al.*, 1982; Martin *et al.*, 1988). Basement membranes provide a physical support for orderly growing cells, play an important role in cell adhesion, are responsible for maintaining tissue architecture and form selective permeability membranes for proteins, as in capillaries and kidney glomeruli.

An appraisal of the complexity of the ECM emphasises that a diverse group of proteolytic enzymes may be required to effect penetration of these barriers during tumour invasion. Proteinases from all four classes of endopeptidases, distinguished on the grounds of their catalytic mechanisms, pH optima and susceptibility to specific inhibitors, have thus been implicated in tumour invasion (Table 1). These include the serine proteinases, urokinase-type plasminogen activator (Dano *et al.*, 1985) and elastase (Zeydel *et al.*, 1986), cysteine proteinases, cathepsin B (Sloane and Honn, 1984) and cathepsin L (Gal and Gottesman, 1986b), an aspartic proteinase, cathepsin D (Spyratos *et al.*, 1989), and metalloproteinases, interstitial collagenase (Harris *et al.*, 1972) and type IV collagenase (Liotta *et al.*, 1980). Additionally proteoglycanases, such as hyaluronidase (Biaci, 1980) and the heparan sulfate-specific endoglycosidase, heparanase (Nakajima *et al.*, 1983), have been shown to aid in the removal of the proteoglycan ground substance from interstitial and basement membrane ECM respectively, which results in exposure of collagen to proteinases.

Table 1. Distinguishing characteristics of the four classes of proteinases (adapted from Barrett, 1980; Neurath, 1989).

Class	Representative proteinase	Characteristic active site residues[a]	pH range for activity	Typical inhibitors
Serine	**Chymotrypsin** Trypsin, elastase, cathepsin G, plasminogen activators	Asp-102, Ser-195, His-57	7-9	Di-isopropyl fluoride
Cysteine	**Papain** Cathepsins B, H, L,S	Cys-25, His-159, Asp-158	3-8	Iodoacetate, cystatins
Aspartic	**Penicillopepsin** Cathepsin D, renin	Asp-33, Asp-213	2-7	Pepstatin
Metallo	**Thermolysin** Collagenases	Zn, Glu-270, Trp-248	7-9	EDTA, 1, 10-phenan-throline

[a] Residue numbers correspond to the amino acid sequences of the enzymes listed in bold in the adjacent column

These correlations between the action of proteolytic enzymes and tumour invasion have been based on observations of increased proteinase activity in malignant cells compared to normal cells, an increase in proteinase secretion by cultured transformed cells, and in some cases a direct relationship has been demonstrated between the production of certain proteinases and the metastatic potential of tumour cells (Liotta *et al.*, 1980; Sloane and Honn, 1984). From these studies, and the fact that a single class of enzyme is not able to digest all components of the ECM, it became evident that different tumours may not employ their own unique proteolytic enzyme(s) to effect invasion, but that a cascade of enzymes, involving members of all classes of proteinases may operate in tumour invasion, each filling a specific functional and temporal niche in the degradation of ECM components.

Collagen is an important substrate for the proteolytic enzymes involved in tumour invasion since, as stated above, it constitutes the structural scaffolding upon which both interstitial stroma and basement membrane components of the ECM are assembled. Proteolytic enzymes capable of degrading collagen could, therefore, play a major role in the initiation of tumour invasion, by either loosening of the ECM, which may be sufficient for migration of tumour cells into neighbouring tissue (Sträuli, 1980), or rendering collagen more susceptible to degradation by other proteinases. Specific collagen-degrading matrix metalloproteinases (MMPs), as well as collagenolytic cathepsins have been identified in normal conditions, where they play a role in normal connective tissue remodelling (Werb and Burleigh, 1974; Etherington *et al.*, 1986), and inflammatory diseases such as rheumatoid arthritis (Etherington *et al.*, 1988) and also in tumour invasion (Liotta *et al.*, 1982; Sloane and Honn, 1984).

The matrix metalloproteinase family has been divided into three broad groups depending on their ECM substrate specificity, namely, interstitial collagenases, type IV collagenases (gelatinases) and stromelysins (Table 2). The nomenclature of the MMPs has been confusing and different names have been given to the same enzyme by different workers. Amino acid sequencing of the different members of the MMP family, especially the type IV collagenases, has facilitated their classification (Collier *et al.*, 1988; Wilhelm *et al.*, 1989). Matrix metalloproteinases show considerable sequence homology and have a number of common structural features, including an active site Zn^{2+}-binding domain (HEXGHL) and, with the exception of matrilysin, a C-terminal domain with sequence similarity to hemopexin, a haem-binding protein. The partial overlap in substrate specificity may be related to the well conserved Zn^{2+}-binding domain, which presumably constitutes a part of the active site (Woessner, 1991; Matrisian, 1992). The MMPs are all secreted as

Table 2. Classification and natural substrates of the matrix metalloproteinases (adapted from Woessner, 1991; Matrisian, 1992).

Groups	Enzyme names	M_r (kDa)	ECM substrates
I	Interstitial collagenase	52 Deduced	Collagen types I, II, III,
	MMP-1	52 Latent	VII, X
	Fibroblast collagenase	42 Active	
	Neutrophil collagenase	53 Deduced	Collagen types I, II, III
	MMP-8	85 Latent	
		65 Active	
II	72-kDa type IV collagenase	72 Deduced	Gelatin type I
	MMP-2	72 Latent	Collagen types IV, V, VII, X
	Type IV collagenase	66 Active	Fibronectin, elastin
	72-kDa gelatinase		
	92-kDa type IV collagenase	80 Deduced	Gelatins type I, V
	MMP-9	92 Latent	Collagen types IV, V
	Type V collagenase	84 Active	
	92-kDa gelatinase		
III	Stromelysin	52 Deduced	Proteoglycan, link protein,
	MMP-3	57 Secreted	Fibronectin, laminin,
	Transin	48 Active	Gelatins I, III, IV,V
	Proteoglycanase		Collagens III, IV, V, IX
	Stromelysin-2	53 Deduced	Gelatins I, III, IV,V
	MMP-10	53 Secreted	Weak on collagens III, IV, V
	Transin-2	47 Active	
	Matrilysin	28 Deduced	Gelatins I, III, IV, V
	MMP-7	28 Secreted	Proteoglycan
	Pump-1	20 Active	Fibronectin
	Uterine metalloproteinase		

latent proenzymes and organomercurial activation results in autocatalytic removal of an N-terminal 80-residue propeptide which contains a conserved amino acid sequence immediately adjacent to the cleavage site.

Interstitial and neutrophil collagenases cleave the native triple-helical fibrillar collagen types I, II and III molecules at a single distinctive site, and the resulting fragments are labile at physiological temperatures, and upon unfolding become susceptible to general proteolysis (Birkedal-Hansen, 1987). The two enzymes differ in their cellular origin and their specificity for fibrillar collagens in that interstitial collagenase, which is produced by fibroblasts, preferentially degrades type I collagen and neutrophil collagenase, expressed by cells of the neutrophil lineage, has a preference for type III collagen. Although high interstitial collagenolytic activity has been demonstrated in tumour tissues and transformed cells in culture (Robertson and Williams, 1969; Yamanishi *et al.*, 1973; Woolley, 1982; Tarin *et al.*, 1982), a direct relationship between secretion of collagenases and the metastatic potential of tumour cells has not been established (Tryggvason *et al.*, 1987). There has in fact been conflicting evidence as to whether tumour or host cells produce interstitial collagenases (O'Grady *et al.*, 1981; Huang *et al.*, 1986). The main function of interstitial collagenases in tumour invasion may be the initial degradation of collagen fibrils for further hydrolysis by other proteinases such as the cathepsins.

Since basement membrane separates epithelial and endothelial cells from their underlying interstitial stroma, it represents a critical barrier which needs to be crossed during the transition from *in situ* to invasive tumour, and during tumour cell migration in and out of blood vessels. The basement membrane type IV collagen is, however, not susceptible to interstitial collagenases, but separate basement membrane degrading metalloproteinases, called type IV collagenases, have been identified in cell cultures derived from highly metastatic murine tumours and mononuclear phagocytes and also in human polymorphonuclear leukocytes (PMNLs) (Liotta *et al.*, 1979; 1980; Uitto *et al.*, 1980; Garbisa *et al.*, 1986).

Further evidence for a direct relationship between basement membrane type IV collagenolytic activity and the metastatic propensity of tumour cells was obtained from studies on rat embryo fibroblasts transfected with activated *ras*-oncogenes (Garbisa *et al.*, 1987). This transfection, which has been shown to induce the metastatic phenotype in suitable recipient cells (Thorgeirsson *et al.*, 1985), resulted in the secretion of high levels of an M_r 72 000 type IV collagenase by all transfectants, whereas tumourigenic, nonmetastatic cells did not produce significant amounts of this enzyme (Garbisa *et al.*, 1987). The human analogue of this rodent tumour type IV collagenase (Liotta *et al.*, 1979; 1980; Garbisa *et al.*,

1987) was shown to be the 72 kDa type IV collagenase, the only MMP secreted by *ras*-transformed human bronchial epithelial cells, which in turn was found to be identical to the enzyme found in normal human skin fibroblasts (Collier *et al.*, 1988).

A different (92 kDa) molecular form of type IV collagenase, which shares gelatin and type IV collagen substrate specificity with the 72 kDa enzyme form, but which is the product of a distinct gene, was similarly expressed in *ras*- transfected murine NIH3T3 cells (Ballin *et al.*, 1988), as well as SV-40 transformed human lung fibroblasts (Wilhelm *et al.*, 1989). This 92 kDa type IV collagenase, which was also isolated from rat mammary carcinoma cells (Lyons *et al.*, 1991), was shown to be similar to the enzyme secreted by cytotrophoblasts, normal human macrophages and PMNLs (Wilhelm *et al.*, 1989; Murphy *et al.*, 1989a; Librach *et al.*, 1991). Similarly to invading tumour cells, cytotrophoblasts invade the uterine epithelium, while PMNLs and monocytes traverse the vascular endothelial basement membrane when they exit the circulation into tissue during an inflammatory response, and to mature into macrophages, respectively.

In addition to *ras*-oncogenes, tumour promoters and growth factors have also been shown to play a significant role in regulation of the expression of type IV collagenases (Thorgeirsson *et al.*, 1985). Expression of the 92 kDa type IV collagenase is induced by epidermal growth factor, interleukin-1 and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Wilhelm *et al.*, 1989), whereas transforming growth factor β1 (TGF-β1) effected an increase in the mRNA levels and secretion of the 72 kDa proteinase in invasive tumour cells (Brown *et al.*, 1990).

Type IV collagenases, therefore, seem to be closely associated with tumour invasion by virtue of their strong proteolytic activity against key components of basement membranes, traversed by metastasising tumour cells. Moreover, their synthesis and secretion is regulated by oncogenes and growth promoters, which also induce the phenotype for invasion and metastasis. Futher studies are, however, required to determine the relative contributions of the 72 and 92 kDa forms of type IV collagenase to invasion by different human tumours, and to elucidate the cell source and the *in vivo* mode of activation of these ECM degrading enzymes.

Degradation of the ECM, especially the collagen components, by MMPs during tumour invasion and other matrix degradative pathological conditions, is often complemented by the action of collagenolytic cathepsins (Tryggvason *et al.*, 1987). These enzymes are normally lysosomal cysteine proteinases, reported to be optimally active at acidic pH, and unstable and inactive at neutral or alkaline pH (Table 3; Mason *et al.*, 1985).

Extralysosomal cysteine proteinase activity is controlled by endogenous cysteine proteinase inhibitors (CPIs), the cystatins. Within the cystatin superfamily, the stefin family (stefins A and B) controls intracellular proteolysis (Barrett, 1987). Cathepsin L has been shown to be a particularly powerful proteolytic enzyme on ECM components, usually at acidic pH-values, and has a higher affinity for many of these components than the other collagenolytic cysteine cathepsins B, H, N and S (Maciewicz *et al.*, 1987; Guinec *et al.*, 1990).

Table 3. General characteristics of the collagenolytic lysosomal cysteine proteinases (adapted from Kirschke *et al.*, 1980).

Proteinase	M_r	pI	ECM substrate
Cathepsin B	24-27 000	5.0-6.5	Collagen types I[a,c,d,j], IV[e], gelatin[d], laminin[h], fibronectin[h], proteoglycan[g], link protein[g]
Cathepsin H	28 000	6.2-7.1	Collagen type IV[h], laminin[h], fibronectin[h]
Cathepsin L	21-29 000	5.8-6.1	Collagen types I[a,c,d], II[i], IV[e], IX[i], XI[i], gelatin[d,j], elastin[b], fibronectin[h], laminin[h], proteoglycan[g], link protein[g]
Cathepsin N	20-35 000	5.1-6.5	Collagen types I[c,d]
Cathepsin S	24 000	6.3-6.9	Collagen type I[c,d,f], elastin[k,l]

[a] Kirschke *et al.*, 1982; [b] Mason *et al.*, 1986a; [c] Maciewicz *et al.*, 1987 ; [d] Maciewicz and Etherington, 1988; [e] Maciewicz *et al.*, 1989; [f] Kirschke *et al.*, 1989; [g] Nguyen *et al.*, 1990; [h] Guinec *et al.*, 1990; [i] Maciewicz *et al.*, 1990; [j] Pike *et al.*, 1992; [k] Shi *et al.*, 1992; [l] Xin *et al.*, 1992.

Substrates for cathepsin L include the N-terminal non-helical telopeptides of type I collagen, resulting in the depolymerisation of collagen fibres (Kirschke *et al.*, 1982), as well as bone collagen during bone resorption at acidic pH (Delaisse *et al.*, 1991). Baricos *et al.* (1988) found that cathepsin L degrades the kidney glomerular basement membrane during its

normal turnover, and type IV collagen, laminin and fibronectin of basement membranes were also found to be degraded *in vitro* (Maciewicz *et al.*, 1989; Rozhin *et al.*, 1989; Guinec *et al.*, 1990). Cathepsin L is specific for the non-helical domains of the cartilage collagen types II, IX, and X, even at pH-values near neutrality. These cathepsin L cleavage sites differ from those recognised by the interstitial collagenases, and the removal of type IX collagen, which coats the type II collagen fibres, is also a prerequisite for collagenolytic degradation by MMPs, illustrating the cooperative degradation by these two classes of proteinases to effect cartilage remodelling or destruction, as in arthritis. Cathepsin L also destabilises cartilage proteoglycan aggregates by hydrolysing the link proteins (Nguyen *et al.*, 1990).

Although it has been shown that cathepsin L is able to degrade cartilage collagens *in vitro* at physiological pH (Maciewicz *et al.*, 1990), an acidic milieu has been suggested to be usually required for the degradation of collagen and other ECM components by cysteine proteinases (Maciewicz *et al.*, 1987; Etherington *et al.*, 1986). In contrast to the low pH inside the lysosomes where the final hydrolysis by cysteine cathepsins of internalised ECM fragments take place, neutral pH conditions exist extracellularly where ECM degradation is initiated during pathological conditions. Mechanisms are, therefore, required whereby cysteine proteinases, which have been reported to have low stability and activity at neutral pH (Mason *et al.*, 1985; 1986a), could retain activity extracellularly against ECM components.

It was shown that the extracellular compartment, between the attached osteoclast and the bone matrix, where bone resorption takes place, becomes actively acidified to pH 3.0 by ATP-dependent proton pumps present in the ruffled border membrane of the osteoclast (Baron *et al.*, 1985; Silver *et al.*, 1988). Acidification of the subosteoclastic microenvironment results in the polarised secretion of lysosomal enzymes by the osteoclast into this "extracellular lysosome", allowing the degradation of the organic phase of bone matrix. Similarly, macrophages, which were shown to secrete active cathepsin L (Reilly *et al.*, 1989; Bird *et al.*, 1989), were found to produce a localised acidic environment when attached to collagen films, thus controlling their microenvironment to facilitate extracellular hydrolysis of ECM components by cathepsins (Silver *et al.*, 1988). Since low-pH zones have been observed in tumours, and metastatic cells appear to acidify their media more rapidly than non-metastatic cells, invasive tumour cells may employ a similar mechanism to create an acidic microenvironment at the tumour-host junction for local dissolution of the ECM (Sutherland, 1988; Maciewicz *et al.*, 1989).

Although this mechanism provides for extracellular ECM degradation by cysteine proteinases, these "extracellular lysosomes" have not been observed *in vivo* in tumour tissue. It has, therefore, been suggested that enzyme activity and stability could instead be maintained by the localisation of proteolytic enzymes at the cell surface (Moscatelli and Rifkin, 1988). This could additionally result in optimal orientation of the proteinases for ECM degradation. Cathepsin L has been found in association with the plasma membrane of malignant colorectal tumour and metastatic melanoma cell lines (Maciewicz *et al.*, 1989; Rohzin *et al.*, 1989). It has been proposed that the advantages of the binding of degradative enzymes to the cell surface are, firstly, that they are more readily activated, secondly, it provides a means of concentrating the enzymes participating in the ECM degrading cascade, thereby increasing the rate of reaction, thirdly, restriction of the activity of the enzymes to ensure the focal degradation of ECM components in close proximity to the invading cells, and fourthly, bound to the cell surface, enzymes may be protected from inhibitors (Zucker *et al.*, 1987; Moscatelli and Rifkin, 1988; Rohzin *et al.*, 1989).

In the present study two observations were made which may be relevant to the question of the extracellular activity of cathepsin L. Firstly, it was found that when isolated by a rapid procedure single-chain cathepsin L is obtained. Moreover, unlike the two-chain forms previously reported, the single-chain form is stable and active at physiological pH. Secondly, it was found that cathepsin L can form proteolytically active complexes with its endogenous inhibitor, stefin B, which have enhanced activity and are stabile under physiological conditions. These studies are reported in Chapter 4 of this thesis.

More direct evidence for the involvement of cathepsin L in tumour invasion, than its ability to degrade a wide variety of components of the ECM, was provided by the characterisation of the major excreted protein (MEP), found in large amounts in the medium of RNA virus transformed fibroblasts, as a precursor of cathepsin L (Gottesman, 1978; Portnoy *et al.*, 1986; Mason *et al.*, 1986b, 1987). Synthesis and secretion of MEP was also significantly enhanced by treatment of non-transformed cells with either the tumour promoter, TPA, or growth promoters, platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) (Gottesman and Sobel, 1980; Nilsen-Hamilton *et al.*, 1981; Frick *et al.*, 1985). Increased synthesis of MEP in transformed and treated cells was shown to be due to increased translatable MEP mRNA levels (Gottesman and Sobel, 1980; Scher *et al.*, 1983).

Despite containing the mannose-6-phosphate lysosomal recognition marker, most of the M_r 39 000 MEP form is secreted, while a further proportion was found to be processed to M_r 29 000 and 20 000 forms, which remain in the lysosomes (Gal and Gottesman,

1986a). Inappropriate trafficking of MEP could reflect the situation *in vivo* resulting in the secretion of (pro)cathepsin L by tumour cells. Augmented MEP secretion could either be the result of saturation of the mannose-6-phosphate receptors (MPRs) because of the high level of MEP synthesis in transformed cells, or of low intrinsic affinity of MEP for MPRs (Dong *et al.*, 1989; Dong and Sahagian, 1990; Lazzarino and Gabel, 1990). Alternatively, enhanced secretion could be the result of either PDGF-induced redistribution of MPRs to the cell surface, or a transformation-induced decrease in surface receptor binding activity (Prence *et al.*, 1990; Achkar *et al.*, 1990). Further work is required to elucidate the mechanism of MEP secretion, but it is evident that there are a number of steps which could be independently regulated and which would affect the trafficking of procathepsin L (MEP) and its mature forms under normal and tumourigenic conditions.

In a manner analogous to type IV collagenase, expression and secretion of cathepsin L correlates with the metastatic potential of *ras*-transformed murine fibroblasts (Denhardt *et al.*, 1987; Joseph *et al.*, 1987). This augmented expression of *ras* correlated with the increased expression of cathepsin L mRNA, which suggests that the cathepsin L gene is also under control of the *ras* gene (Denhardt *et al.*, 1987). This control may be effected indirectly by the *ras* gene product p21 (Hiwasa *et al.* 1988). The mechanism for p21 regulation is uncertain and inhibition of cathepsin L, or cathepsin L-mediated degradation of epidermal growth factor (EGF)-receptors by p21, leading to uncontrolled growth, have been suggested (Hiwasa *et al.* 1988; 1991).

It was similarly found that the levels of cathepsin L and c-Ha-*ras* mRNA rise and fall in parallel during the development of the murine placenta, a normal, rapidly growing, highly invasive tissue. The coincident waves of cathepsin L and *ras* expression, which reached peak levels during trophoblast invasion and immediately before parturition, are indicative of a connection between the two, as has also been found in synovial cells from patients with rheumatoid arthritis (Trabandt *et al.*, 1990). The association between *ras* and cathepsin L expression is thus probably a normal mechanism controlling the secretion of large amounts of cathepsin L for the purpose of tissue invasion. The presence of both in normal tissue, such as the placenta, indicate that they are usually under strict control, but provide a potential mechanism by which tumour invasion could be effected. Overexpression of the secreted forms of cathepsin L *per se* was shown not to be responsible for introducing the metastatic phenotype in transformed cells (Kane *et al.*, 1988).

Increased secretion of pro-cathepsin L was also shown by a highly metastatic and invasive v-*fos* transfected cell line (Taniguchi *et al.*, 1990). Since TPA- and PDGF-treated cells showed a similar response, it suggests that the expression of cathepsin L is also

regulated through the *fos*-gene. Elucidation of the gene structure of cathepsin L is shedding light on the regulation of gene expression *in vivo* by polypeptide growth factors and oncogenes. The positive regulation by TPA is consistent with the presence of TPA enhancer elements in the 5'-upstream region of the cathepsin L gene (Ishidoh *et al.*, 1991). This is indicative of the participation of cathepsin L not only in intracellular proteolysis, but also cell growth and tumourigenesis.

Evidence for the participation of cathepsin L in tumour progression has also been obtained from studies on a variety of tumour cells. Whereas the majority of earlier studies demonstrated an association between malignancy and increased levels of only cathepsin B in tumours (Sloane *et al.*, 1981; Recklies *et al.*, 1982), mature basement membrane-degrading forms of both cathepsins B and L were found to be secreted by malignant human colorectal tumour cell lines (Maciewicz *et al.*, 1989). Activation of these enzymes might have occurred in an acidic microenvironment at the tumour-host interface in a similar fashion as was shown to occur for MEP in acidic lysosomes (Gal and Gottesman, 1986a). Studies using specific cysteine and metalloproteinase inhibitors, showed that cathepsin L, rather than cathepsin B, was involved in human amnion basement membrane penetration by murine melanoma and mammary carcinoma cells *in vitro* (Yagel *et al.*, 1989). Complete blocking of invasion was only accomplished with a metalloproteinase inhibitor, however, suggesting that although cathepsin L contributes significantly to invasion, it possibly does so by facilitating the action of MMPs. Cathepsin L could possibly induce their activation or the inactivation of their inhibitors, or exert its effect by enhancing basement membrane accessibility.

Cathepsin L was suggested to be a possible prognostic marker for breast carcinoma, since although the specific activity of the enzyme was lower than that of cathepsin B, significantly elevated levels were observed in tumour homogenates (Lah *et al.*, 1992). The presence of increased amounts of cathepsins B and L in breast carcinoma homogenates and patients' sera, compared to controls, was confirmed using specific antibodies, but in this instance cathepsin B, rather than cathepsin L, was directly correlated with the stage of tumour dedifferentiation (Gabrijelcic *et al.*, 1992).

The same study showed, however, that the level of cathepsin H, measured by enzyme-linked immunosorbent assay (ELISA), was higher than those of cathepsins B and L in breast tumour tissue (Gabrijelcic *et al.*, 1992). Cathepsin D has previously been shown to be the most prominent prognostic marker for breast carcinoma (Spyratos *et al.*, 1989), but was not included in this study. Elevated levels of cathepsin H activity were also found to parallel levels of cathepsins B and L in breast cancer tissue (Vashista *et al.*, 1988). From the limited number of studies on the presence of cathepsin H in tumour tissue, it appears that this

enzyme may play a significant role in breast tumours. Very little is known about the ECM hydrolysing ability of cathepsin H, which has both amino- and endopeptidase activity (Kirschke *et al.*, 1977b; Mason, 1989). Although interstitial collagen has been shown to be poorly digested by cathepsin H (Kirschke *et al.*, 1982), of greater importance in the context of tumour invasion, is the *in vitro* degradation of basement membrane constituents, type IV collagen, laminin and fibronectin in the pH 5.5-7.0 range reported by Guinec *et al.* (1990). Studies on the role of cathepsin H in the invasion by different tumours is clearly an area which requires attention.

One of the most important regulatory mechanisms of invasive processes is the delicate balance between proteolytic enzymes and their natural endogenous inhibitors, which are often produced by the same cells which secrete the enzymes (Green *et al.*, 1984; Goldberg *et al.*, 1989). Tumour invasion may be the result of an imbalance which favours proteolysis. A marked association was found, for example, between decreased expression of the tissue inhibitor of metalloproteinases (TIMP) and the invasive potential of fibrosarcoma cell lines. Reduced TIMP expression observed following transfection with a plasmid carrying anti-sense TIMP RNA, was shown to result in the conversion of fibroblasts to a tumourigenic and metastatic phenotype (Hicks *et al.*, 1984; Khokha *et al.*, 1989). An immunohistochemical study of the distribution of collagenase and TIMP in colorectal tumours showed increased amounts of both enzyme and inhibitor in the stroma of malignant tissue, but that there was more collagenase than TIMP at the invasive edge of the carcinoma (Hewitt *et al.*, 1991). It has been suggested that TIMP acts as a tumour suppressor protein since it inhibits ECM proteolysis and blocks tumour cell invasion of the human amnion *in vitro* and prevent metastasis in animal models (Stetler-Stevenson, 1990).

Recent studies on the distribution of different cysteine proteinases in breast carcinoma tissue, showed a correlation between increased cathepsin B and L activity and a decrease in the levels of CPI. The lower CPI activity was ascribed to the lower transcriptional rate of stefin A which binds more tightly to cathepsin B than L (Lah *et al.*, 1992). Stefin B, which shows a preference for cathepsin L, was, however, not included in this study. Kolár *et al.* (1989) found that stefins A and B were present both inside breast tumour cells and in the surrounding connective tissue. In contrast, in an earlier study on colorectal carcinoma, there did not seem to be any difference in CPI activity between the carcinoma and normal colon mucosa (Sheahan *et al.*, 1989). More studies, especially involving stefin B, are required to determine whether the CPIs also act as tumour suppressor proteins or in the novel activating way found in the present study.

Although the ECM degrading proteinases, type IV collagenases and cathepsin L, have been shown to be major effector gene products associated with the oncogene induced metastatic phenotype *in vitro*, a number of areas concerning their involvement in tumour invasion *in vivo*, still need to be clarified. These include, for instance, the relative contribution of each proteinase to the multistep process of tumour invasion and metastasis, which may differ between different tumours, and also their *in vivo* activation mechanisms. The majority of the studies on these proteinases in the context of tumour invasion have employed *in vitro* transformed cells, usually of non-human origin and maintained in tissue culture. This approach gives only limited information on the role of the proteinases in tumour invasion *in vivo*, which is a significantly more complex situation.

The ultimate goals of the present study were to address the question of the role of the collagenolytic proteinases, type IV collagenase and cathepsins L and H, in tumour invasion, particularly in human malignancies, and to devise a strategy to inhibit the invasion process and consequently suppress metastasis. Specific proteinase reactive antibodies were considered to constitute appropriate tools with which to pursue both avenues. By virtue of the specificity of antibodies, immunoassays and immunolocalisation studies could distinguish between the different molecular forms of type IV collagenase and different cysteine cathepsins, a level of discrimination not afforded by the synthetic substrates and inhibitors presently available (Kirschke *et al.*, 1988). The antibody approach would also allow identification of the latent proenzyme forms or enzyme-inhibitor complexes often present in the extracellular milieu. Antibodies targeting discrete active-site regions may additionally inhibit enzyme activity, and could find application in tumour invasion model studies *in vitro* using reconstituted basement membranes, and ultimately, perhaps, in tumour immunotherapy.

In an endeavour to meet these objectives, firstly type IV collagenase was purified from a suitable human source for the production of specific antibodies. As outlined in Chapter 3, human PMNLs constituted such a source and a simplified immunoaffinity isolation procedure was developed employing protein-reactive anti-peptide antibodies, prepared against a type IV collagenase specific peptide sequence.

In order to identify candidate immunoinhibitory epitopes in the active-site of cathepsin L for the production of immunoinhibitory anti-peptide antibodies, both a suitable source of cathepsin L and the primary structure of the enzyme were required. Although the aim was to develop immunoinhibitory agents which would target the human enzyme, these studies were impeded by the unavailability of sufficient quantities of human tissue for purification purposes, or alternatively a gene expression system. However, sheep liver was

previously identified as a good source of cathepsin L, which shares immunological and catalytic identity with the human enzyme (Mason, 1986), and by employing the rapid isolation method developed by Pike and Dennison (1989b) in this laboratory, large amounts of single-chain cathepsin L could be purified. Since the primary structure of sheep cathepsin L was not available, purification was initially aimed at obtaining sufficient amounts of enzyme for sequencing purposes (Chapter 4).

During the course of the development of a purification method for cathepsin L, a proportion of cathepsin L was found to be complexed to a protein moiety, tentatively identified as stefin B and, surprisingly, found to be proteolytically active (Pike, 1990). In an attempt to improve the yield of free cathepsin L, it was discovered that a substantial fraction of the complex comprised cathepsin L bound in an unusual manner to stefin B. Both single-chain cathepsin L and these novel cathepsin L/stefin B complexes revealed properties which could implicate them in tumour invasion. Consequently it was decided to characterise these complexes further in an endeavour to elucidate their potential role in tumour invasion. This also prompted investigations into whether similar complexes, as well as single-chain cathepsin L *per se* might occur in higher primates.

Polyclonal antibodies produced against cathepsin L in chickens and rabbits, revealed differential targeting specificities. While both antibody preparations recognised the enzyme in ELISAs and Western blots, only the chicken antibodies were immunoinhibitory. These polyclonal chicken antibodies were subsequently used to localise an immunoinhibitory epitope in cathepsin L (Chapter 5). Additionally, protein-reactive anti-peptide antibodies were raised in chickens against a human cathepsin L active-site sequence. These antibodies were not only immunoinhibitory, but also targeted cathepsin L across species in ELISAs and Western blots. The avian immune system also succeeded in eliciting antibodies against the equivalent active-site sequence in cathepsin H. These anti-peptide antibodies manifested superior immunoinhibitory and immune-targeting properties than the corresponding rabbit antibodies.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

The experimental methods used in this study mainly involved the production and characterisation of antibodies directed against synthetic peptides and purified proteins, obtained through various chromatographic procedures. Both antibodies and purified proteinases were further studied using enzyme assays and different electrophoretic techniques. A number of these fundamental biochemical techniques were common to the different areas covered in this study and will be described in this chapter. In cases where individual methods pertained to a specific area, they will be described in the relevant chapters.

2.2 Materials

For convenience, the source of specialised products used in this study will be described here. Most of the common chemicals used in the study were from BDH, Merck or Boehringer Mannheim, and were of the highest purity available. 4-Aminophenylmercuric acetate (APMA), azocasein, *Clostridium histolyticum* collagenase, 7-amino-4-methyl coumarin, cysteine.HCl, DEAE-Trisacryl, dithiothreitol (DTT), L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), keyhole limpet haemocyanin (KLH), leupeptin, all standard proteins, papain (2 x crystallised), pepsin, pepstatin, soybean trypsin inhibitor (SBTI), Sephadex G-25, Sephadex G-75, Sephadex G-100, Sepharose-4B, CM-Sepharose, S-Sepharose, Q-Sepharose, Tosyl lysyl chloromethyl ketone (TLCK) and Tosyl phenylalanine chloromethyl ketone (TPCK) were from Sigma Chemical Co., St. Louis, Mo. 2,2 Azino-di-[3-ethylbenzthiazoline sulphonate (6)] (ABTS) and phenyl methyl sulfonyl fluoride (PMSF) were from Boehringer Mannheim, SA. Horse radish peroxidase (HRPO) was from Seravac. May-Grünwald and Giemsa stains were from BDH. Arg-NHMec, Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were from Cambridge Research Chemicals, UK. Z-Phe-Phe-CHN_2 was from Enzyme System Products, Livermore, Ca., USA. Human kidney cathepsin L was from Novabiochem, UK and human cathepsin H from Athens Research Technology, Ga., USA. Recombinant human cystatin C was a kind gift from Dr Jan Potempa, Jagiellonian University, Cracow, Poland. Serva blue G dye was from Serva. The ELISA plates used in this study were Nunc-Immuno Maxisorp F96 plates

supplied by the Weil Organisation, S.A.. Freund's complete and incomplete adjuvants and gelatin were from Difco, Mi., USA.

2.3 Protein Assay

The Bradford dye-binding assay (Bradford, 1976) was used routinely for protein determination, since it provides a very sensitive (microgram level) and rapid quantitative method. A further advantage is that it is essentially devoid of interference by common laboratory reagents, with the exception of relatively high concentrations (1%) of detergents, flavenoids and basic buffers (Bradford, 1976). The assay is based on the binding of Coomassie brilliant blue G-250 dye to basic amino acid side chains, mainly arginine, and to a lesser extent to histidine, lysine, tyrosine, tryptophan and phenylalanine amino acid side chains. This binding causes a shift in the absorbance maximum of the dye from the cationic red form at 465 nm to the anionic blue form at 595 nm (Compton and Jones, 1985). Read and Northcote (1981) modified the Bradford method to decrease the level of protein-to-protein variability in dye binding and to increase the sensitivity of the assay, by replacing the Coomassie brilliant blue G-250 with Serva blue G dye, and by increasing the amount of dye and decreasing the phosphoric acid component in the reagent.

Since the concentrations of the dye and acid/alcohol components, suggested by Read and Northcote (1981), led to precipitation of the dye upon storage, the assay used in this study employed the original Bradford method, but using Serva blue G dye. The dye reagent described here was found to effectively eliminate the variation in dye-binding to different standard proteins (Pike, 1990). Ovalbumin was generally used as calibration protein, since its response to the dye reagent was found to be comparable to the average of three other commonly used standard proteins: lysozyme, BSA and γ-globulin (Pike, 1990).

A macro-assay was used to measure protein in the 10-25 μg range, which is less sensitive than the method of Read and Northcote (1981), probably because of the smaller amounts of dye used, but which was adequate for the levels of protein measured in this study. A micro-protein assay was also employed to quantify protein in the 1-5 μg range, using the quantities of dye reagent suggested by Read and Northcote (1981). This assay was found to be very useful where only small amounts of purified protein were available from proteinase purification procedures.

2.3.1 Reagents

Dye Reagent. Serva blue G dye (50 mg) was dissolved in 88% phosphoric acid (50 ml) and 99,5 % ethanol (23,5 ml). The solution was made up to 500 ml with dist.H_2O and stirred for 30 min on a magnetic stirrer. The resulting solution was filtered through Whatman No. 1 filter paper and stored in a brown bottle. The solution could be stored for up to 6 months, although visual checks for precipitation were made before use. If precipitation was visible, the reagent was filtered and re-calibrated before use.

Standard protein solution. A 1 mg/ml ovalbumin solution was made up in dist.H_2O. This was diluted to 100 µg/ml for the micro-assay.

2.3.2 Procedure

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

Micro-assay. Protein standard (0-50 µl of the 100 µg/ml solution, i.e. 1-5 µg) or sample was diluted to 50 µl with dist.H_2O, or buffer, respectively, in 1.5 ml polyethylene microfuge tubes. Dye reagent (950 µl) was added, and the mixture was mixed by inversion of the tube. The colour was allowed to develop for 2 min after mixing, and the A_{595} was read, in 1 ml plastic micro-cuvettes, as above. Assays for a standard curve were usually carried out in quintuplicate at 5 concentrations of ovalbumin.

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

2.4 Concentration of Samples

It was usually necessary to concentrate dilute protein samples, before subjecting them to further purification, or for SDS-PAGE analyses or innoculation procedures. A simple, low cost concentrating method, employing dialysis against commercial sucrose or polyethylene glycol (PEG) (M_r 20 000), was found to be sufficiently mild to retain enzyme activity and was the method of choice for the concentration of cathepsin L samples, since it was found that rabbit liver cathepsin L was denatured by ultrafiltration (Mason *et al.*, 1984). Dialysis against the more expensive, high M_r PEG, which will be excluded from the M_r 12 000 cut-off dialysis bags, was only used when the presence of small amounts of sucrose, which diffuse into the dialysis bags, was not desirable. Concentration by these dry, highly soluble polymers is based on the principle of movement of water along a concentration gradient out of the dialysis bag to dissolve the dry polymer. In cases where additional concentration of samples was required, for reducing SDS-PAGE analyses, a quick, small-scale method, based on the precipitation of SDS-protein complexes by KCl, was used and resulted in an overall 50-fold concentration. The SDS-KCl precipitation method was found to be unsuitable for concentrating samples for non-reducing SDS-PAGE analyses, possibly because it resulted in an unacceptably high salt concentration in the samples.

2.4.1 Dialysis against sucrose or PEG

The protein sample was placed in a dialysis bag (M_r 12 000 cut-off), which was surrounded by a thick layer of commercial white sugar or PEG (M_r 20 000) in a polystyrene tray at 4°C. Once the sample had been sufficiently concentrated (usually 5 to 10-fold in 2-4 h), the bag was briefly rinsed in dist.H_2O and the sample was squeezed out. During the preparation of samples for SDS-PAGE, and throughout the entire electrophoresis and Western blotting procedures, rubber gloves were worn to prevent contamination of the samples by skin keratins. These proteins are troublesome contaminants which cross-react with most antibodies non-specifically and appear as contaminants at a M_r of 68 000 on reducing SDS-PAGE (Ochs, 1983; Shapiro, 1987).

2.4.2 SDS/KCl Precipitation

2.4.2.1 Reagents

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

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

2.4.2.2 Procedure

5% SDS (10 µl) was added to the sample (100 µl) in a 1.5 ml polyethylene microfuge tube. The solution was mixed by inverting the tube and 3 M KCl (10 µl) was added. The mixture was again inverted and centrifuged (12 000 x g, 2 min, RT), the supernatant was discarded and the precipitate was dissolved in stacking gel buffer (10 µl) and reducing treatment buffer (10 µl) (solution G, Section 2.5.1).

2.5 SDS-PAGE

SDS-PAGE, under either reducing or non-reducing conditions, was used to evaluate the homogeneity of purified protein samples. The molecular weights of proteins were determined using reducing SDS-PAGE, while covalent enzyme-inhibitor complexes were studied under non-reducing conditions. These techniques were also used in conjunction with Western blotting (Section 2.8) to identify proteins using specific antibodies or to assess the specificity of antibodies raised against the purified proteins. Enzymatic activity of proteinases was illustrated by non-reducing SDS-PAGE in gelatin-containing gels (Section 2.6).

The anionic detergent, SDS, binds tightly to most proteins, thereby converting them from globular native amphoteric proteins into highly negatively charged rodlike complexes, the length of which varies with the MW of the protein moiety of the detergent-protein complex (Reynolds and Tanford, 1970). Concomitant treatment with a disulfide reducing agent, such as 2-mercaptoethanol, will break the proteins down into their constituent subunits, which will also bind SDS and will more effectively open up the protein structure, giving a truer estimate of M_r. These negatively charged detergent-polypeptide complexes will have similar charge-to-mass ratios, resulting in size dependent anodal migration in an electrical field. Since an inverse relationship exists between the logarithm of the M_r of a

protein and the distance migrated in a gel, a standard curve can be generated for M_r estimation by running standard proteins, of known M_r, alongside the polypeptides to be characterised. SDS-PAGE was carried out using the discontinuous buffer and gel system described by Laemmli (1970) and the Hoefer SE 250 Mighty Small II vertical slab electrophoresis unit.

2.5.1 Reagents

Solution A: Monomer Solution [30% (m/v) acrylamide, 2.7% (m/v) Bis-acrylamide]. Acrylamide (73 g) and Bis-acrylamide (2 g) were dissolved and made up to 250 ml with dist.H_2O and stored in an amber coloured bottle at 4°C.

Solution B: 4 x Running Gel Buffer (1.5 M Tris-HCl, pH 8.8). Tris (45.37 g) was dissolved in approximately 200 ml of dist.H_2O, adjusted to pH 8.8 with HCl and made up to 250 ml.

Solution C: 4 x Stacking Gel Buffer (500 mM Tris-HCl, pH 6.8). Tris (3 g) was dissolved in 40 ml dist.H_2O, adjusted with HCl to pH 6.8 and made up to 50 ml. This buffer was made up weekly, because, as a result of its poor buffering capacity at 2.1 pH units below its pKa at 4°C (Pharmacea products catalogue), pH drift led to anomalous running patterns in non-reducing SDS-PAGE.

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

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

Solution E: Initiator [10% (m/v) ammonium persulfate]. Ammonium persulfate (0.2 g) was made up to 2 ml just before use.

Solution F: Tank Buffer [250 mM Tris-HCl, 192 mM glycine, 0.1% (m/v) SDS, pH 8.3]. Tris (15 g) and glycine (72 g) were dissolved and made up to 5 litres with dist.H_2O. Prior to use, 2.5 ml of SDS stock (solution E) was added to 250 ml for use in the Mighty Small II apparatus.

Solution G: Reducing Treatment Buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution D), glycerol (2 ml) and 2–mercaptoethanol (1 ml) were made up to 10 ml with dist.H_2O.

Solution H: Non-reducingTreatment Buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution D) and glycerol (2 ml) were made up to 10 ml with dist.H_2O.

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

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

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

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

Table 4. Preparation of running and stacking gels of different acrylamide concentrations.

Reagent	Volume (ml)					
	Running gel (%)				Stacking gel (%)	
	15.0[a]	12.5[b]	7.5[c]	5.0[d]	4.0[e]	3.0[f]
A	7.5	6.25	3.75	2.5	0.94	0.71
B	3.75	3.75	3.75	3.75	0	0
C	0	0	0	0	1.75	1.75
D	0.15	0.15	0.15	0.15	0.07	0.07
E	0.75	0.75	0.75	0.75	0.35	0.35
dist.H_2O	3.5	4.75	7.25	8.5	4.3	4.53
TEMED	0.075	0.075	0.075	0.075	0.015	0.015

Running gels used in: [a] Section 4.4 (stefin B); [b] Sections 4.2, 4.3 and 4.5 (Cathepsin L); [c] Section 3.11.3 (type IV collagenase) and [d] Section 3.2 (type IV collagen). Stacking gels used in combination with running gels of: [e] 15.0, 12.5, and 7.5%, and [f] 5%.

2.5.2 Procedure

For SDS-PAGE, the Hoefer SE 250 Mighty Small II vertical slab electrophoresis unit was assembled as described in the manufacturer's manual. This involved cleaning one notched aluminium plate and one glass plate with ethanol for each of the two sides of the apparatus, and clamping these with two 1.5 mm polyethylene spacers separating them at the edges. The bottom space was filled with molten 1% agarose and this was allowed to solidify. The running gel solution was run into the space between the plates, to a depth 3 cm from the top of the glass plate, and overlayered with dist.H_2O to allow for even polymerisation. Once the gel had set (evidenced by the appearance of the interface between gel solution and water, usually about 1 h), the water was removed with a syringe. Stacking gel solution was poured in, up to the notch of the aluminium plate, and a 10- or 15-well comb was inserted to form the sample application wells. Once this gel had set (about 30 min) the comb was removed and the wells were rinsed with dist.H_2O.

Tank buffer, containing SDS, was poured into the upper and lower electrode compartments. For reducing SDS-PAGE, samples were combined with an equal volume of reducing treatment buffer (solution G) and incubated in a boiling waterbath for 90 s, before they were placed on ice until loaded onto the gel. Samples for non-reducing SDS-PAGE were combined with half their volume of non-reducing treatment buffer (solution H) before loading. A marker dye, bromophenol blue (5 µl), which migrates with the buffer front, was added to each sample before loading onto the gels. Suitable amounts of samples (at least 1 µg of protein per band for the Coomassie blue R-250 staining procedure) were applied into the wells using a Hamilton microsyringe. The gel unit was connected to a power pack and run at 18 mA per gel until the bromophenol blue tracker dye was about 0.5 cm from the bottom of the running gel. At this point, the apparatus was disconnected from the power supply, the plates were removed, and levered apart using a plastic spacer. The gel was removed using gloves and placed into Coomassie blue R-250 staining solution for 4 h. The staining solution was removed at the end of this time and, following rinsing with dist.H_2O, the gel was placed into destain I, overnight, and then into destain II to effect complete destaining. Gels were stored in polythene zip-seal bags and kept well hydrated until photographed. They were stable in this form for long periods.

2.5.3 Combined Serva blue G/ silver stain procedure

In cases where very small amounts of protein, separated on SDS-PAGE, needed to be visualised, a significantly higher degree of sensitivity than that obtained with Coomassie brilliant blue R-250 staining, was achieved with the combined Serva blue G/silver stain procedure (De Moreno *et al.*, 1985). A modification of the Serva blue G part of the staining procedure provides rapid visualisation of protein bands where immediate results are required.

2.5.3.1 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), following which 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.

3.4 mM $K_2Cr_2O_7$ - 3.2 mM HNO_3. $K_2Cr_2O_7$ (0.25 g) and 55% HNO_3 (0.068 ml) were dissolved in 250 ml of dist.H_2O.

12 mM $AgNO_3$. $AgNO_3$ (0.51 g) was dissolved in 250 ml of dist.H_2O.

280 mM Na_2CO_3. Na_2CO_3 (14.84 g) was dissolved in 500 ml of dist.H_2O and 35% formaldehyde (0.5 ml) was added to every litre.

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

2.5.3.2 Procedure

All steps were carried out on an orbital shaker (50 rpm; RT) and in scrupulously clean glass containers to minimise background staining. The electrophoresis gel was soaked in 40% methanol-10% acetic acid (100 ml, 3 x 20 min), and stained in Serva blue G dye reagent (50 ml, 30 min.). Destaining was effected in 5% TCA (100 ml, 3 x 10 min), 40% methanol-10% acetic acid (100 ml, 2 x 10 min) and 10% ethanol-5% acetic acid (100 ml, 2 x 10 min). Following destaining, the gel was incubated in 3.4 mM K_2CrO_7 - 3.2 mM HNO_3 (50 ml, 10 min), washed in dist.H_2O (100 ml, 6 x 10 min), soaked in 12 mM silver nitrate (50 ml, 30 min) and washed in dist.H_2O (100 ml, 2 min). Silver stained bands were developed by three changes of the 0.28M Na_2CO_3 buffer (50 ml), the solution being removed whenever too much precipitate formed in the solution. Development was stopped by immersing the gel in 5% acetic acid (50 ml, 5 min), following which it was placed in dist.H_2O and stored in polythene zip-seal bags.

For rapid visualisation of protein bands the electrophoresis gel was soaked in 40% methanol-10% acetic acid (100 ml, 15 min), and stained in Serva blue G dye reagent (50 ml, 30 min.). Destaining was effected in 5% TCA (100 ml, 2 x 10 min), 40% methanol-10% acetic acid (100 ml, 10 min) and stored in 10% ethanol-5% acetic acid

(100 ml, overnight), before the gel was transferred to destaining solution II (Section 2.5.1) to prevent fungal growth in the gel.

2.6 Gelatin-containing substrate SDS-PAGE

Electrophoretic analyses of proteinases separated in non-reducing SDS-polyacrylamide gels containing copolymerised gelatin, give estimates of the MWs of the proteolytic enzymes. Following SDS-PAGE, the proteinases are renatured upon removal of SDS by incubation of the gel in Triton X-100 (Heussen and Dowdle, 1980). The gel is then incubated in the relevant assay buffer, containing the required activators and inhibitors to ensure specific enzyme activity, and protein activity is visualised, following staining of the gel, by clear bands of gelatin digestion.

2.6.1 Reagents

1% (m/v) gelatin in running gel buffer. Gelatin (0.1 g) was dissolved in running gel buffer (10 ml, solution B, Section 2.5.1) with gentle heating.

Overlay solution for substrate gels [0.05% (m/v) gelatin]. Gelatin (0.005 g) was dissolved in running gel buffer (2 ml, solution B, Section 2.5.1) with gentle heating and diluted to 10 ml with dist.H_2O.

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

Assay buffer. See sections 3.5.1 and 4.6.1.1

0.1% (m/v) amido black. Amido black (0.1 g) was dissolved in methanol:acetic acid:dist.H_2O in the proportions 30:10:60 (100 ml), and filtered through Whatman No. 1 filter paper before use.

2.6.2 Procedure

The procedure for SDS-PAGE was modified from that described in Section 2.5.2, in that 0.1% (m/v) gelatin was incorporated into the gel, to allow the detection of proteinases (Heussen and Dowdle, 1980). This was carried out by adding 1% (m/v) gelatin

in running gel buffer (1.5 ml), to running gel buffer (2.25 ml) and the rest of the solution for casting a 12.5% gel (section 2.5.1), all at 37°C, and pouring the gel as quickly as possible. The gel was overlaid with the overlay solution for substrate gels, and allowed to set. The SDS-PAGE was carried out as normal.

After electrophoresis, the running gel was soaked in two changes of 2.5% (v/v) Triton X-100 (50 ml) over 1 h at RT. Following this, the gel was incubated in the pH 5.0 assay buffer, containing 40 mM cysteine (50 ml), described for azocasein assays (section 4.6.1.1), for 3 h at 37°C. It was stained in 0.1% (m/v) amido black solution for 1 h, and destained in several changes of methanol:acetic acid:dist.H_2O (30:10:60). The presence of proteolytically active components in the gel was indicated by clear bands in the gel after staining, due to the digestion of the gelatin.

2.7 Isolation of Antibodies

Antibodies were isolated using PEG precipitation methods which proved to be very simple and efficient in the purification of both IgG and IgY to near homogeneity. Polson *et al.* (1964) introduced the use of this neutral, water-soluble, high-M_r polymer for the fractional precipitation of proteins. PEG is a mild precipitating agent which operates on a steric exclusion mechanism, whereby proteins are concentrated in the extrapolymer space, until they exceed their solubility limit. IgY was isolated from egg yolks using the method of Polson *et al.* (1985), while IgG from serum was isolated using a method derived from findings about the precipitation of serum components using PEG (Polson *et al.*, 1964).

2.7.1 Reagents

Borate buffered saline, pH 8.6. A sodium borate solution was prepared by dissolving boric acid (2.16 g), NaCl (2.19 g), NaOH (0.7 g) and 37% HCl (0.62 ml) in 950 ml of dist.H_2O, checking and adjusting the pH if necessary with HCl or NaOH, and making the solution up to 1 litre.

100 mM Na-phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. $NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, titrated to pH 7.6 using NaOH, and made up to 1 litre.

2.7.2 Procedure for the isolation of IgG from rabbit serum

One volume of rabbit serum was mixed with two volumes of borate buffered saline. Solid polyethylene glycol (M_r 6 000) was added to the diluted serum to 14% (m/v), dissolved with constant gentle stirring, and the mixture was centrifuged (12 000 x g, 10 min, RT). The pellet was re-dissolved in the original serum volume, using 100 mM Na–phosphate buffer, pH 7.6. PEG was again added to 14% (m/v), dissolved with stirring, and the solution was centrifuged (12 000 x g, 10 min, RT). The pellet was redissolved in half the original serum volume, using 100 mM Na–phosphate buffer, pH 7.6, containing 60% (v/v) glycerol, and stored at -20°C.

2.7.3 Procedure for the isolation of IgY from chicken egg yolks

Egg yolks were separated from the egg white and carefully washed under running water to remove all traces of albumin. The yolk sac was punctured and the yolk volume determined in a measuring cylinder. Two volumes of 100 mM Na-phosphate buffer, pH 7.6, were added and mixed in thoroughly. Solid PEG (M_r 6 000) was added to 3.5% (m/v) and dissolved by gentle stirring. The precipitated vitellin fraction was removed by centrifugation (4 420 x g, 30 min, RT), and the supernatant fluid was filtered through absorbent cotton wool to remove the lipid fraction. The PEG concentration was increased to 12% [i.e. 8.5% (m/v) was added], the solution was mixed thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant was discarded and the pellet was dissolved in 100 mM Na-phosphate buffer, pH 7.6, in a volume equal to the volume obtained after filtration. The final concentration of PEG was brought to 12% (m/v), the solution was stirred thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant fluid was discarded and the final antibody pellet was dissolved in 1/6 of the original egg yolk volume, using 100 mM Na-phosphate buffer, pH 7.6, and stored at 4°C.

2.7.4 Determination of [IgY] and [IgG]

The A_{280} of a 1 in 40 dilution of IgY and IgG solutions in 100 mM phosphate buffer was determined and the concentration of IgY and IgG in the undiluted solution was calculated [extinction coefficient of IgY, $E_{280}^{1\ mg/ml} = 1.25$ (Coetzer, 1985) and that of IgG, $E_{280}^{1\ mg/ml} = 1.43$ (Hudson and Hay, 1980c)].

2.8 Western blotting

In this technique specific antibodies are used for the identification and characterisation of proteins separated by SDS-PAGE and electrophoretically transferred to a matrix with a high protein binding capacity, such as nitrocellulose. Western blotting is also used for the qualitative evaluation of antibodies raised against a purified protein. The method used in this study was essentially that of Towbin *et al.* (1979), with a few minor modifications. Methanol is included in the transfer buffer to enhance binding of protein-SDS complexes to the nitrocellulose membrane. Following blocking of all the unoccupied binding sites on the membrane with non-fat milk, the antigens are allowed to react sequentially with the primary antibody and a secondary detection system. The detection system comprises a secondary antibody directed against the primary antibody, labelled with an enzyme such as HRPO, which catalyses a reaction leading to the formation of a precipitating coloured product.

2.8.1 Reagents

Blotting buffer. Tris (27,23 g) and glycine (64,8 g) were dissolved in 3.5 litres of dist.H_2O, and methanol (900 ml) was added. The volume was made up to 4.5 litres in a large beaker, the exact volume not being critical. Prior to use, 10% (m/v) SDS was added (4.5 ml, solution D, Section 2.5.1).

Tris buffered saline (20 mM Tris, 200 mM NaCl, pH 7.4). Tris (2.42 g) and NaCl (11,69 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 7.4 with HCl, and made up to 1 litre.

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

HRPO linked secondary antibodies. The conjugation of HRPO to immunoglobulin was carried out according to Hudson and Hay (1980b). HRPO (4 mg) was dissolved in 1 ml of dist.H_2O and a freshly prepared 100 mM sodium periodate solution (200 µl) was added. The mixture was stirred for 20 min at RT (the mixture usually turned a greenish-brown colour at this stage). This mixture was dialysed against a 1 mM Na-acetate buffer, pH 4.4, overnight at 4°C. The pH was raised to 9-9.5 by the addition of 200 mM Na_2CO_3 buffer, pH 9.5 (20 µl), and 1 ml of an 8 mg/ml IgG fraction was immediately added. This solution was left at RT for 2 h. Freshly prepared 4 mg/ml Na-

borohydride solution (100 µl) was added and the solution was left at 4°C for 2 h to reduce any free enzyme. This mixture was dialysed against 100 mM Na-borate buffer, pH 7.4, overnight at 4°C, an equal volume of 60% glycerol in 100 mM Na-borate buffer, pH 7.4, was added, and the conjugate was stored at 4°C. The dilution of conjugate to be used was established in a checkerboard ELISA. The conjugate dilution used was that which gave a steep titration curve, sufficiently high above background values, over a serially diluted primary antibody range.

4-chloro-1-naphthol substrate solution [0.06% (m/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2]. 4-chloro-1-napthol (0.03 g) was dissolved in methanol (10 ml). Two ml of this solution was diluted to 10 ml with TBS, with the addition of 30% hydrogen peroxide (4 µl).

2.8.2 Procedure

Following SDS-PAGE, usually on duplicate gels, one gel was stained to show the total protein pattern, while the other was used for blotting. Nitrocellulose was cut to a suitable size and, to avoid entrapment of air, carefully floated onto blotting buffer, before being totally immersed. The immersed nitrocellulose was sandwiched, with the gel lying squarely on top of it, between three pieces of Whatman No. 4 filter paper and two pieces of Scotchbrite foam, also totally immersed in blotting buffer. The sandwich was placed into the Western blotting apparatus and filled with blotting buffer. The whole apparatus was immersed in a tank of cold water, kept at 8°C by a refrigerated circulator. The apparatus was connected to a power supply so that the nitrocellulose was on the anodal side of the gel, and blotting was effected for 16 h at 200 mA. The buffer was stirred by a magnetic stirrer throughout the process to ensure even distribution of cooling. After 16 h, the sandwich was removed and the filter paper was peeled off the gel. The gel was carefully removed, and stained to assess the efficiency of the blotting, which was usually very good.

The nitrocellulose sheet was removed from the filter paper and air dried for about 1.5 h. The nitrocellulose strip was blocked for 1 h with 5% low fat milk powder in TBS, washed in TBS (3 x 5 min) and incubated for 2 h with primary antibody in 0.5% BSA-TBS. Following washing in TBS (2 x 5 min), it was incubated in HRPO-linked secondary antibody in 0.5% BSA-TBS for 1 hour, and again washed in TBS (3 x 5 min). It was immersed in substrate solution and reacted in the dark until bands were clearly evident against a lightly-stained background. Finally, the strip was removed from the substrate

solution, and washed in dist.H_2O and dried between filter paper. This last step ensured good preservation of the bands before photography.

2.9 Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA technique was used to evaluate the progress of polyclonal and anti-peptide antibody production during immunisation procedures and to test cross-reactivity between immobilised peptides and corresponding whole proteinases. The peptides used in this study were found to adsorb directly to the wells of microtitre plates, in agreement with the general notion that 15-residue long peptides become adsorbed to ELISA plates following overnight incubation (Van Regenmortel, 1988b). This technique complements Western blotting, which gives qualitative information about antibody specificity, by measuring the quantitative properties of antibodies. While Western blotting gives an indication of antibody targeting of fully denatured proteins, partial denaturation of proteins usually accompanies adsorption to ELISA plates (Van Regenmortel, 1988a,b).

2.9.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 1 litre of dist.H_2O.

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

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

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

Substrate solution [0.05% (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.

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

Carbonate coating buffer. $NaHCO_3$ (0.21 g) was dissolved in 45 ml of dist.H_2O, titrated to pH 6.0 with HCl, and made up to 50 ml.

2.9.2 Procedure

The peptides COL476-490, L153-165 and H157-168 were coated at a concentration of 1 µg/ml in PBS (150 µl, 16 h), while cathepsins L and H were coated at a concentration of 1 µg/ml in carbonate coating buffer (150 µl, 3 h at 37°C, followed by 16 h at 4°C for cathepsin L or, 16 h at 4°C for cathepsin L and H when testing cross-reactivity between anti-peptide antibodies and corresponding whole proteinases). All coating concentrations were determined using a checkerboard ELISA, the coating concentration chosen being that which gave the most even titration over a range of serially diluted antibody. Non–specific binding of antibody was prevented by blocking the wells with 0.5% BSA-PBS (200 µl, 1 h at 37°C), and the plates were washed three times with PBS-Tween.

Serial two-fold dilutions, starting from 1/10 serum dilution (or 1mg/ml IgG or IgY) was prepared on the plate in 0.5% BSA-PBS and incubated (100 µl, 2 h at 37°C), the plates were washed three times with PBS-Tween, the HRPO-linked secondary antibody, at a suitable dilution in 0.5% BSA-PBS, was added to each well and incubated (120 µl, 1 h at 37°C), and the plates were washed three times with PBS–Tween. Substrate solution (150 µl) was added to each well and colour was allowed to develop in the dark against the background of the controls (usually 10-15 min). The enzyme reaction was stopped by addition of citrate–phosphate–NaN_3 buffer (50 µl) and the A_{405} of each well was measured on a Titertek ELISA plate reader.

2.10 Preparation of cyanogen bromide-activated Sepharose-4B

This activated matrix was used for immobilising KLH (Section 3.9), anti-COL476-490 peptide antibodies (Section 3.10) and carboxymethyl(CM)-papain (Section 4.4) for the isolation of anti-KLH antibodies, type IV collagenase and cystatins, respectively, and was prepared by activating Sepharose-4B with cyanogen bromide (CNBr) by the method of Kohn and Wilchek (1982). This method is based on the formation of active cyanate esters on the resin, by enhancing the electrophilicity of CNBr using a cyano-transfer reagent, TEA, rather than enhancing the nucleophilicity by a strong base. This allows the activation to be carried out at neutral pH, which decreases the hydrolysis of CNBr

and thereby the amount of CNBr required, while increasing the reaction yield. Moderate activation of the gel is achieved by using 10 mg CNBr/g gel and was preferable for immobilising large molecules such as KLH and antibodies, where too much ligand on the activated gel may cause steric hindrance and therefore decrease the binding efficiency and selectivity of the adsorbent. The smaller ligand, CM-papain, was adsorbed to a slightly more activated gel, prepared with 15 mg CNBr/g gel.

2.10.1 Reagents

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

1.5 M triethylamine (TEA) in 60% acetone. TEA (2.09 ml) was dissolved in 60% acetone (10 ml).

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

Coupling buffer [100 mM Na_2CO_3, 100 mM $NaHCO_3$, 500 mM NaCl, 0.02% (m/v) NaN_3, pH 8.3]. Na_2CO_3 (2.65 g) and $NaHCO_3$ (2.1 g) were dissolved separately, each with NaCl (7.3 g) and NaN_3 (0.1 g) in 250 ml dist.H_2O. Approximately 4 parts of the $NaHCO_3$-containing solution was titrated to pH 8.3 using one part of the Na_2CO_3-containing solution.

2.10.2 Procedure

Wet Sepharose-4B (20 g) was weighed out and washed on a Buchner funnel with ice-cold 30% acetone, followed by 60% acetone, and resuspended in 60% acetone. This suspension was cooled to -20°C in the freezer. 1 M CNBr (1.89 ml for moderate activation; 2.8 ml for higher activation) was added to the Sepharose in an ice-bath, with stirring, to bring the Sepharose to 10 (15) mg CNBr/g. Following this, 1.5 M TEA (1.98 ml or 2.8 ml) was added dropwise over 2-3 min, with stirring. The Sepharose was poured into 125 ml of ice cold washing medium, and washed with 60% acetone, 30% acetone, dist.H_2O and coupling buffer (all ice cold).

CHAPTER 3

PURIFICATION OF HUMAN LEUCOCYTE TYPE IV COLLAGENASE BY ANTI-PEPTIDE ANTIBODY IMMUNOAFFINITY CHROMATOGRAPHY

3.1 Introduction

Evidence in support of a positive correlation between type IV collagenase activity and tumour cell invasion has mainly come from tissue culture studies, employing highly metastatic murine and rat tumour cell lines (Liotta *et al.*, 1979, 1980; Turpeenniemi-Hujanen *et al.*, 1985; Nakajima *et al.*, 1987; Yamagata *et al.*, 1988; Lyons *et al.*, 1991), or *ras*-transfected NIH3T3 cells (Ballin *et al.*, 1988). Similar studies were also done on human tumourigenic cell lines (Ballin *et al.*, 1988; Mackay *et al.*, 1990; Brown *et al.*, 1990) or *ras*-transformed human bronchial epithelial cells (Collier *et al.*, 1988) and similarly transformed fibroblasts (Spinnuci *et al.*, 1988). Compared to their normal counterparts, increased type IV collagenase production was demonstrated in conditioned media from the tumourigenic cell lines, using radiolabelled type IV collagen assays (Liotta *et al.*, 1981a) and zymograms with either gelatin or type IV collagen co-polymerised with SDS-polyacrylamide gels. These *in vitro* studies, however, only give an indication of the potential which both tumour and normal cells have for producing type IV collagenase. More direct evidence for a functional role *in vivo* , and identification of the cells secreting the enzyme, could come from immunolocalisation studies, especially at the electron microscope level.

In contrast to interstitial collagenases, only a small number of immunolocalisation studies have been reported for type IV collagenase. Cytoplasmic immunoreactivity was demonstrated in invasive human breast carcinoma cells (Barsky, *et al.*, 1983), but not in normal breast, benign lesions or *in situ* carcinomas. Monteagudo *et al.* (1990) showed that, with the progression of the severity of breast lesions, from a typical hyperplasia, through lobular carcinoma *in situ* to invasive ductal and lobular carcinomas, there is an increase in the immunohistochemical staining for type IV collagenase specifically associated with breast epithelial cells and in lymph node metastases. Immunostaining for type IV collagenase was also found within large mononuclear cells in the inflamed synovium of rabbits with antigen-induced arthritis (Murphy *et al.*, 1989b). In the only study at the electron microscope level, Hibbs and Bainton (1989) immunolocalised type IV collagenase to the specific granules of polymorphonuclear leucocytes. There is, therefore, a clear need for more studies on the distribution of type IV collagenase in different tumours, especially human tumours.

Immunolocalisation studies at the electron microscope level may ascertain whether the type IV collagenase originates from normal host or transformed tumour cells. The aim of this study was therefore to purify type IV collagenase from a suitable human source for the production of specific antibodies for use in subsequent immunolocalisation studies at the electron microscope level.

The two genetically distinct 72 kDa and 92 kDa forms of type IV collagenase have been isolated from different sources, and in some instances both forms could be obtained simultaneously from the same source. The 72 kDa type IV collagenase has been isolated from metastatic murine tumour cells (Liotta *et al.*, 1981a; Salo *et al.*, 1983; Fessler *et al.*, 1984), human tumourigenic cell lines of varying invasive potential (Brown *et al.*, 1990), *ras*-transformed human bronchial epithelial cells (Collier *et al.*, 1988) and similarly transformed fibroblasts (Spinucci *et al.*, 1988). This form was also isolated from rabbit bone culture medium (Murphy *et al.*, 1985), human mononuclear phagocytes (Garbisa *et al.*, 1986) and normal human plasma (Vartio and Baumann, 1989). The reported molecular masses for these enzymes varied between 68 000 and 72 000 for the latent, and between 62 000 and 66 000 for the active enzyme in the different studies. Amino acid sequence determination (Collier *et al.*, 1988), established that type IV procollagenase is secreted as a single, unmodified polypeptide chain with a predicted M_r of 72 000. Activation by the organomercurial, APMA, led to a loss in M_r of 6 000. The 68 and 72 kDa latent forms of the proenzyme, with their respective activated 62 and 66 kDa forms, are now considered to be one and the same enzyme (Collier *et al.*, 1988; Stetler-Stevenson *et al.*, 1989, 1990).

Glycosylated 92 kDa type IV collagenase was originally purified from human PMNLs (Murphy *et al.*, 1980; 1982, Hibbs *et al.*, 1985). A latent 97 kDa gelatinase, purified from porcine PMNLs, could be activated by APMA or autolysis to an 88 kDa form (Murphy *et al.*, 1989a), while similar latent and active forms were purified from culture media of rat mammary carcinoma cells (Lyons *et al.*, 1991). The enzyme was also isolated from SV-40 transformed human lung fibroblasts and shown to be identical to the enzyme secreted by normal alveolar macrophages, polymorphonuclear leucocytes and phorbol ester-differentiated monocytic leukemia U937 cells (Wilhelm *et al.*, 1989). Both the 72 and 92 kDa forms could be purified from human plasma (Vartio and Baumann *et al.*, 1989), porcine and human PMNLs (Murphy *et al.*, 1989a,b) and various human tumour cell lines (Mackay *et al.*, 1990).

For the purposes of the present study, and since preparative cell culture facilities were not available, a suitable source for the purification of human type IV collagenase seemed to be polymorphonuclear leucocytes, which can be isolated from the buffy coat layers from the blood of regular donors.

Purification of type IV collagenase usually involves an initial salting out step with ammonium sulfate, followed by one or more affinity chromatography steps, either preceded or followed by molecular exclusion and/or anion exchange chromatography on DEAE-Sepharose (Murphy *et al.*, 1982, 1985; Ward *et al.*, 1991). In the present study three-phase partitioning (TPP) in t-butanol/water/ammonium sulfate (Lovrien *et al.*, 1987; Pike and Dennison, 1989a) was used instead of ammonium sulfate precipitation for the crude fractionation of type IV collagenase from human leucocytes. The TPP process has been shown to be especially suited to the purification of proteins which are not comprised of non-covalently associated subunits (Pike and Dennison, 1989a) and was successfully applied to the purification of cathepsins L (Pike and Dennison, 1989b) and D (Jacobs *et al.*, 1989). Due to its denaturing effect on oligomeric proteins, TPP is especially effective in removing haemoglobin, a common contaminant of leucocyte samples.

Both forms of type IV collagenase can be partially purified by gelatin-Sepharose (Hibbs *et al.*, 1985; Collier *et al.*, 1988; Murphy *et al.*, 1989a,b) or Engelbreth-Holm-Swarm (EHS) sarcoma type IV collagen-agarose affinity chromatography (Salo *et al.*, 1983; Höyhtyä *et al.*, 1988). Zinc chelate-Sepharose chromatography is often included for adsorption of interstitial collagenase and other contaminating proteins (Murphy *et al.*, 1982, 1985, 1989b; Collier *et al.*, 1988). The 72 kDa and 92 kDa forms can be separated by exploiting their differential binding to either concanavalin A-Sepharose or heparin-Sepharose, since the glycosylated 92 kDa form adsorbs to concanavalin A (Murphy *et al.*, 1989a), whereas the 72 kDa form adsorbs to heparin (Murphy *et al.*, 1982, 1985, 1989b; Collier *et al.*, 1988). Another possible way to separate the two forms is to adsorb the 72 kDa form to reactive green-agarose (Collier *et al.*, 1988) and the 92 kDa form to reactive red-agarose (Wilhelm *et al.*, 1989).

While the above procedures are effective, they are lengthy and immunoaffinity purification could potentially provide a more rapid method. Höyhtyä *et al.* (1988) used monoclonal antibodies raised against whole type IV collagenase, purified from culture media of human melanoma cells, for subsequent immunoaffinity purification of the enzyme. Since type IV collagenase, recognised by the monoclonal antibody, shares sequence homology with interstitial collagenase and stromelysin (Höyhtyä *et al.*, 1988), it could lead to non-

specific interaction in an immunoaffinity procedure. This could be the case when using starting material such as rheumatoid synovial fibroblasts, which contain type IV collagenase (Murphy *et al.*, 1989b), stromelysin (Chin *et al.*, 1985) and fibroblast interstitial collagenase (Stricklin *et al.*, 1977; Goldberg *et al.*, 1986), or polymorphonuclear leucocytes which contain type IV (Murphy *et al.*, 1982, 1989a) and neutrophil interstitial collagenase (Hasty *et al.*, 1986, 1990). If a peptide sequence unique to type IV collagenase could be identified, anti-peptide antibodies could, potentially, provide discriminatory ligands for immunoaffinity purification of the enzyme. A cyanogen bromide digest of the human melanoma-derived type IV collagenase yielded such a peptide (Höyhtyä *et al.*, 1988). The peptide, CB4, corresponds to residues 476-491 in the native enzyme and is very similar to one of the prominent tryptic digest peaks, P18, prepared by Collier *et al.* (1988), which stretches from residues 473-491. Anti-CB4 peptide antibodies were used in Western blotting to identify the enzyme purified from human melanoma (Höyhtyä *et al.*, 1988) and from H-*ras* transformed murine NIH3T3 cells (Spinucci *et al.*, 1988).

Thus at the outset antibodies prepared against this peptide were considered to be potentially suitable ligands for anti-peptide antibody immunoaffinity chromatography, and practical points with regard to the use of this peptide as an immunogen were considered. For immunisation purposes Höyhtyä *et al.* (1988) conjugated the CB4-peptide, MGPLLVATFWPELPEK, to a carrier protein, using glutaraldehyde. This method links the peptide via either its N-terminus or C-terminal Lys-ε-NH_2-group to the carrier protein (Avrameas and Ternynck, 1969), to give a mixture of exposed N- and C-termini. A study of the hydrophilicity (Hopp and Woods, 1981, 1983) and segmental mobility (Westhof *et al.*, 1984) parameters, which aid in predicting the location of continuous epitopes in proteins (Van Regenmortel, 1988a), showed that the C-terminus is more hydrophilic and the peptide is mobile in the middle of the sequence (Fig. 1). Immunogenicity of this sequence could, therefore, be enhanced if the C-terminus were exposed. To effect conjugation with glutaraldehyde through the N-terminus exclusively, the CB4-peptide C-terminal Lys-residue was omitted in the present study and the resulting peptide, designated COL476-490 was conjugated to KLH and used for raising antibodies in rabbits (Coetzer *et al.*, 1991).

In this chapter the production of anti-COL476-490 peptide antibodies and the subsequent purification of human leucocyte type IV collagenase, using TPP and anti-peptide antibody immunoaffinity chromatography, will be discussed. In order to assess the efficiency of the purification procedure, it was necessary to first optimise an assay procedure, using radioactively labelled type IV collagen, purified from human placental material. Finally the characterisation of the purified enzyme will be presented.

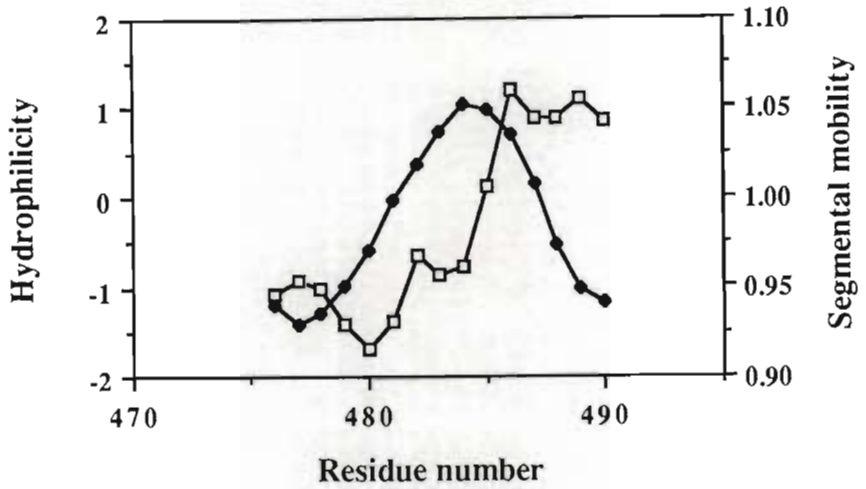

Figure 1. Hydrophilicity and segmental mobility profile of the peptide COL476-490, selected from human type IV collagenase.
(❑), hydrophilicity, calculated according to Hopp and Woods (1981; 1983); and (◆), segmental mobility, calculated according to Westhof *et al.* (1984).

3.2 Isolation of type IV collagen from placental tissue

Type IV collagen has been isolated from organ cultures of EHS tumours maintained in mice (Tryggvasson *et al.*, 1980), or by limited proteolysis, using pepsin, of basement membranes, such as in kidney glomerulus (Daniels and Chu, 1975) or lens capsules (Uitto *et al.*, 1980). A convenient source of type IV collagen, which has been reported to give better yields, is placental tissue (Glanville *et al.*, 1979), which was the tissue of choice in this study. Collagens were initially solubilised with pepsin and a collagen IV-rich fraction was selectively precipitated from neutral salt solutions. This fraction was reduced and carboxymethylated under non-reducing conditions and subjected to a second pepsin digestion. Acidic non-collagenous contaminants were removed using anion exchange chromatography on DEAE-Trisacryl and traces of type III collagen were removed by cation exchange chromatography on CM-Sepharose.

3.2.1 Reagents

Pepsin (EC 3.4.23.1). Pepsin powder from porcine stomach mucosa, containing 2250 units/mg, was obtained from Sigma Chemical Co., Mo., USA.

400 mM Sodium acetate. $CH_3COONa.3H_2O$ (272.16 g) was dissolved in 5 litres of dist.H_2O.

500 mM Formic acid. Formic acid (112.8 ml of an 85% solution, d = 1.2) was diluted to 5 litres with dist.H_2O.

100 mM Acetic acid. Glacial acetic acid (11.43 ml) was diluted to 2 litres with dist.H_2O.

Buffer A [300 mM Tris-HCl, 200 mM NaCl, 0.02% (m/v) NaN_3, pH 7.6]. Tris (363.3 g), NaCl (116.88 g), and NaN_3 (2.0 g) were dissolved in 9.9 litres of dist.H_2O, adjusted to pH 7.6 with HCl, and made up to 10 litres.

Buffer B [500 mM Tris-HCl, 0.02% (m/v) NaN_3, pH 8]. Tris (121.1 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 8 with HCl, and made up to 2 litres.

Buffer C [300 mM Tris-HCl, 200 mM NaCl, 2 M Urea, 0.02% (m/v) NaN_3, pH 8.6]. Tris (72.66 g), NaCl (23.37 g), urea (240 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 8.6 with HCl, and made up to 2 litres.

Buffer D [40 mM Na-acetate, 2 M Urea, 0.02% (m/v) NaN_3, pH 4.8]. Glacial acetic acid (4.57 ml), urea (240 g), and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 4.8 with NaOH, and made up to 2 litres.

DEAE-Trisacryl. DEAE-Trisacryl was prepared by diluting 120 ml of the supplied hydrated gel in an equal volume of buffer C, and packing the resulting slurry into a glass column (5 x 6 cm) under gravity. The column bed was initially regenerated with one column volume of buffer C, containing 1 M NaCl. The gel was equilibrated with five column volumes of buffer C, before use.

CM-Sepharose. CM-Sepharose (250 ml) was prepared as described for DEAE-Trisacryl, except that all preparative steps were carried out in buffer D and the gel was packed into a 2.5 x 50 cm column.

Human placentas. Human placental tissue from normal births, from HIV and hepatitis B negative mothers, was obtained from Grey's Hospital, Pietermaritzburg, and frozen at -20°C immediately after parturition.

3.2.2 Procedure

Placental tissue was thawed at 4°C for 16 h, the umbilical cord and chorionic/amnionic membrane was removed, and the chorionic plate with its attached villi, was cut into smaller sections and minced. The minced placental material (1 kg) was stirred into 2.5 litres of 400 mM acetate and centrifuged (6 370 x g, 20 min, 4°C). The resulting pellet was resuspended in the same volume of 400 mM Na-acetate and the washing procedure repeated until all visible blood was removed from the tissue residue. The pellet was finally washed in 500 mM formic acid, before the 1.5 kg residue was resuspended in 4.5 litres of 500 mM formic acid at 25°C. Pepsin (150 mg) was added and, after the mixture was incubated for 18 h at 25°C with magnetic stirring, the digest was centrifuged (6 370 x g, 20 min, 4°C) and the pellet discarded. Sodium chloride was added slowly to the supernatant to a final concentration of 6% (w/v), the precipitate collected by centrifugation (11 300 x g, 20 min, 4°C), and resuspended in one litre of buffer A. The pH of the mixture was adjusted to 7.6 with NaOH and the mixture was dialysed against buffer A for 16 h at 4°C. The precipitate, formed during dialysis, was removed by centrifugation (8 670 x g, 20 min, 4°C).

Types III and IV collagen were separated from types I and V by adjusting the NaCl concentration to 1.8 M, which caused the former to precipitate. Following centrifugation (11 300 x g, 20 min, 4°C), the pellet was resuspended in buffer A and precipitated once more using 1.8 M NaCl. The pellet obtained by centrifugation (11 300 x g, 60 min, 4°C), was resuspended in buffer B, 2-mercaptoethanol was added to a final concentration of 20 mM and the mixture was incubated for 17 h at 25°C. Following this, free sulfhydryl groups were carboxymethylated with 18 mM iodoacetate for 1 h at 25°C. The preparation was dialysed for 17 h at 25°C against 500 mM formic acid (four changes), before a second pepsin digest was carried out with 1 mg of pepsin/g wet mass of original 1.8 M NaCl precipitate, for 20h at 25°C, with magnetic stirring. Collagen was precipitated by adjusting the NaCl concentration to 6% (m/v) and pelleted by centrifugation (8 670 x g, 20 min, 4°C).

The collagen pellet was dissolved in buffer C and dialysed against the same buffer for 48 h to inactivate pepsin. The sample was loaded onto a DEAE-Trisacryl column (5.0 x 6.0 cm = 118 ml), equilibrated with buffer C. The unbound fraction was dialysed against buffer D and applied to a column (2.5 x 50 cm = 245 ml) of CM-Sepharose, equilibrated with buffer D, and eluted with a 0-200 mM NaCl gradient in five column volumes of buffer D. Fractions corresponding to the protein peak, eluted at about 100 mM

NaCl, were pooled, dialysed for 48 h against several changes of 100 mM acetic acid and lyophilised.

3.3 Protein assays for the quantification of type IV collagen

In order to radiolabel the correct amount of type IV collagen with the 250 mCi [1-^{14}C] acetic anhydride, which has to be used at one single time, the lyophilised protein had to be quantitated. A similar amount of [1-^{14}C] acetic anhydride was used by Cawston and Barrett (1979) to label 250 mg collagen. Since different protein assays give different responses depending on the amino acid composition of the test protein, the Bradford dye-binding assay (Bradford, 1976) as modified by Read and Northcote (1981) [see Section 2.3], the micro-biuret (Itzhaki and Gill, 1964) and Lowry (Lowry *et al.*, 1951) protein assays were compared using both ovalbumin and gelatin as standard proteins.

3.3.1 Micro-biuret protein assay

In the biuret assay, copper (II) ions bind to the peptide nitrogen of peptide bonds under alkaline conditions to give a purple colour which absorbs maximally at *ca.* 540 nm. In order to increase the sensitivity of the assay, the absorbance is, however, read at 310 nm, a wavelength where the ratio of absorption of copper blank to copper-protein complex is sufficiently low (Itzhaki and Gill, 1964).

3.3.1.1 Reagents

Micro-biuret reagent [600 mM Na-citrate, 940 mM Na_2CO_3, 69 mM $CuSO_4$]. Tri-sodium citrate.$2H_2O$ (8.65 g), Na_2CO_3 (5 g), and $CuSO_4.5H_2O$ (0.865 g) were dissolved in 40 ml dist.H_2O with careful heating and made up to 50 ml after cooling.

3% (m/v) NaOH. NaOH (15 g) was dissolved in 500 ml dist.H_2O.

Standard protein solutions. Ovalbumin and gelatin solutions were made up at 2 mg/ml in dist.H_2O.

Type IV collagen samples. Type IV collagen samples were dissolved in 3% (m/v) NaOH.

3.3.1.2 Procedure

Standard ovalbumin or gelatin solutions (0-500 µl), or sample protein, were diluted to a final volume of 500 µl with dist. H_2O to give the desired protein content (0-1 000 µg) and 3% (m/v) NaOH (4 ml) was added. Micro-biuret reagent (200 µl) was added and the mixture was vortexed. The colour was allowed to develop for 15 min and the A_{310} was read in quartz cuvettes against a blank of dist.H_2O for the standard proteins, or 3 % (m/v) NaOH for type IV collagen samples, treated in the same way as the protein samples. Assays were carried out in quintuplicate at five concentrations of standard protein. Linear regression analysis of the calibration curve gave the equation:

$$A_{310} = -0.0011 + 0.00024[\text{protein concentration (mg/ml)}] \quad (R = 1.00)$$

3.3.2 Lowry protein assay

In the Lowry assay (Lowry *et al.*, 1951), the copper-catalysed oxidation of aromatic amino acids, which causes the Folin-Ciocalteu phosphomolybdicphosphotungstic acid to be reduced to heteromolybdenum blue, is exploited.

3.3.2.1 Reagents

Lowry reagent A [2% (m/v) Na_2CO_3 in 100 mM NaOH]. $Na_2CO_3.H_2O$ (11.7 g) was dissolved in 500 ml of 100 mM NaOH [NaOH (2 g) dissolved in 500 ml dist.H_2O] and stored at 4°C in a polyethylene bottle.

Lowry reagent B [1% (m/v) $CuSO_4$]. $CuSO_4.5H_2O$ (0.1 g) was dissolved in 10 ml dist.H_2O and stored at 4°C.

Lowry reagent C [2% (m/v) Potassium-tartrate]. Potassium-tartrate (0.2 g) was dissolved in 10 ml dist.H_2O and stored at 4°C.

Lowry reagent D. This reagent was freshly prepared by mixing the components in the correct sequence. Lowry reagent C (1.25 ml) was placed in a beaker and Lowry reagent B (1.25 ml) was added, followed by Lowry reagent A (125 ml), with magnetic stirring .

1 M Folin-Ciocalteu reagent. Folin-Ciocalteu reagent (5 ml) was diluted to 10 ml with dist.H_2O and stored in a dark brown bottle at 4°C. Great care was taken with this extremely poisonous reagent.

Standard protein solutions. Ovalbumin and gelatin solutions were made up at 2 mg/ml in dist.H_2O.

Type IV collagen samples. Type IV collagen samples were dissolved in 3% (m/v) NaOH.

3.3.2.2 Procedure

Standard ovalbumin or gelatin solutions (0-75 µl), or sample protein, were diluted to a final volume of 500 µl with dist.H_2O to give the desired protein content (0-150 µg). Lowry reagent D (2.5 ml) was added and the mixture was incubated for 15 min at 25°C, before Folin-Ciocalteu reagent (250 µl) was added and immediately vortexed. The colour was allowed to develop for 30 min at 25°C and the A_{540} was read in plastic cuvettes against a blank of dist.H_2O for the standard proteins or 3 % (m/v) NaOH for type IV collagen samples, treated in the same way as the protein samples. Assays were carried out in quintuplicate at five concentrations of standard protein. Linear regression analysis of the calibration curve gave the equation:

$$A_{540} = 0.0146 + 0.0033[\text{protein concentration (mg/ml)}] \quad (R = 1.00)$$

3.4 Radio-labelling of type IV collagen

Type IV collagen was radiolabelled with [1-^{14}C] acetic anhydride, according to Cawston and Barrett (1979). Acetic anhydride reacts predominantly with the ε-amino groups of lysine residues in proteins. The highest degree of incorporation of radiolabel is achieved at high protein concentration and pH. In order to prevent precipitation of collagen at these high concentrations, $CaCl_2$ is added to the preparation.

3.4.1 Reagents

0.2 M Acetic acid. Glacial acetic acid (1.14 ml) was diluted to 100 ml with dist.H_2O.

Acetylation buffer [10 mM Na-borate, 200 mM $CaCl_2$, 0.02% (m/v) NaN_3, pH 9.0]. $Na_2B_4O_7.10H_2O$ (15.25 g) and $CaCl_2$ (117.6 g) were each dissolved separately in 4 litres of dist.H_2O, in which NaN_3 (0.8 g) was previously dissolved, before the two solutions were combined and titrated to pH 9.0 with NaOH.

Dialysis buffer [50 mM Tris, 200 mM NaCl, 5mM Ca-acetate, 0.03% (v/v) toluene, 0.02% (m/v) NaN_3, pH 7.6]. Tris (12.11 g), NaCl (23.37 g), $(CH_3COO)_2Ca.H_2O$ (17.62 g), toluene (6 ml) and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, titrated to pH 7.6 with HCl and made up to 2 litres.

[1-^{14}C] Acetic anhydride. [1-^{14}C] Acetic anhydride, 257 μCi/mg (9.5 MBq/mg), was from Amersham, United Kingdom.

Type IV collagen. Type IV collagen was purified from human placentas as described in Section 3.2.2.

3.4.2 Procedure

Type IV collagen (288 mg) was dissolved in 200 mM acetic acid (50 ml) and dialysed against acetylation buffer (2 litres) at 4°C for 20 h with two buffer changes. The collagen was placed in a conical flask containing a large magnetic stirring bar and suspended in a waterbath at 10°C and stirred slowly. The vial containing the [1-^{14}C] acetic anhydride was placed in a slurry of dry ice and acetone to condense the [1-^{14}C] acetic anhydride to one end of the vial. The glass hook in the vial was broken with a magnetic hammer, according to the manufacturers instructions, and anhydrous dioxane (2 ml) was pipetted into the hook containing area, which was then covered with a B12 socket. The seal to the ampoule inside the vial was broken with the magnetic hammer to bring the dioxane into contact with the [1-^{14}C] acetic anhydride. The vial was rotated carefully to ensure proper mixing and the break-seal of the ampoule was broken to allow the contents to flow into the conical flask containing the collagen. The ampoule was rinsed with a further 2 ml of anhydrous dioxane, which was added to the conical flask, and the mixture was stirred for 1 h at 10°C. Radiolabelled collagen was dialysed at 4°C against dialysis buffer for several days. The radioactivity in the dialysis buffer was checked daily and the buffer changed until the radioactivity of the diffusate had fallen to background levels. Radiolabelled collagen was diluted with 200 mM acetic acid to 1 mg/ml and aliquoted into 1.2 ml volumes, snap-frozen in liquid nitrogen and stored at -75°C.

3.5 ^{14}C-type IV collagen assay

Assays for type IV collagen are based on the degradation of radiolabelled type IV collagen at a temperature below the melting temperature of the substrate. The larger cleavage fragments and intact molecules are then separated from small, TCA-tannic acid soluble peptides released due to type IV collagenase activity, and the radioactivity solubilised is measured in a scintillation counter. An assay procedure based on the methods of Liotta *et al.*, (1981a) and Murphy *et al.*, (1985) was used in this study. A sensitive coumarin-labelled synthetic peptide substrate for the matrix metalloproteinases was developed only subsequent to this study (Knight *et al.*, 1992).

3.5.1 Reagents

Assay buffer [50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, 0.02% (m/v) NaN_3, 2 mM 4-aminophenylmercuric acetate (APMA), pH 7.5]. Tris (6.05 g), NaCl (11.685 g), $CaCl_2$, (1.47 g) and NaN_3 (0.2g) were dissolved in 950 ml of dist.H_2O, titrated to pH 7.5 with HCl and made up to 1 litre. Prior to the assay, APMA (7 mg) was dissolved in 200 mM NaOH (3 ml), added to the assay buffer (3 ml) and was first titrated with dilute HCl to pH 10.5, and then further titrated to pH 7.5 with 5 x concentrated assay buffer to a final volume of 10 ml.

Precipitating solution [15% (m/v) TCA, 0.75% (m/v) tannic acid]. TCA (1.5 g) and tannic acid (0.075 g) were dissolved in 10 ml dist.H_2O.

Scintillation cocktail. Beckman Ready-Solv EP scintillation cocktail for aqueous solutions, was used.

^{14}C-type IV collagen substrate. ^{14}C-acetylated type IV collagen, prepared as described in Section 3.4.2, was dialysed against dialysis buffer (Section 3.4.1) for 16h at 4°C before use.

Clostridium histolyticum collagenase (EC 3.4.24.3). A lyophilysed preparation containing 550 units/mg was obtained from Sigma Chemical Co.

3.5.2 Procedure

Sample (185 µl) was mixed with assay buffer containing APMA (375 µl) and activated for 4 h at 37°C. The temperature of the waterbath was decreased to 34°C and

^{14}C-type IV-collagen (90 µl) was added and incubated for 15 h. The reaction was stopped by the addition of precipitating solution (100 µl) to a final concentration of 2% (m/v) TCA and 0.1% (m/v) tannic acid, incubation on ice for 30 min and centrifugation (4 800 x g, 15 min, 25°C). Supernatant fluid (500 µl) was combined with scintillation cocktail (5 ml) and assayed for radioactivity, expressed as disintegrations per minute (dpm), in a Packard Tri-Carb Liquid Scintillation counter.

A standard curve, to calculate units of activity, was prepared by incubating ^{14}C-type IV collagen for 50 h at 34°C with *C. histolyticum* collagenase (50 µg), to allow complete digestion of the substrate. The resulting mixture was precipitated with a final concentration of 2% (m/v) TCA and 0.1% (m/v) tannic acid at the end of the 50 h period, in the same proportions as in the procedure described above. The supernatant was treated as the 100% hydrolysate, i.e. the radioactivity measured was treated as the value obtained when all the ^{14}C-type IV collagen was proteolytically cleaved at all possible sites.

Increasing dilutions of this supernatant in assay buffer, gave further points on the standard curve of % hydrolysis of ^{14}C-type IV collagen versus dpm. Linear regression analysis of this curve gave an equation, which was used to calculate % hydrolysis from any dpm-value:

$$\% \text{ Hydrolysis } (\%H) = \frac{\text{dpm} - 10.7951}{17.766} \quad [R = 1.00]$$

One unit of enzyme activity has been defined by Harris and Vater (1982) as the degradation of 1 µg collagen per hour at 35°C, and therefore to calculate the number of units given by a %H-value, the following equation was used:

$$\text{Units} = \frac{(\%H \times 10^{-2}) \times 90 \text{ µg type IV collagen}}{\text{time}}$$

Hence Units/ml = 5.4 x units

3.6 Conjugation of peptide COL476-490 to KLH

3.6.1 Reagents

Synthetic peptide COL476-490. The peptide, MGPLLVATFWPELPE, corresponding to residues 476-490 in human type IV collagenase (Collier *et al.*, 1988) was custom synthesised by Multiple Peptide Systems, San Diego, Ca. The selected peptide was

modified, before synthesis, by the conversion of the carboxy terminal glutamate residue to the corresponding amide, thus more effectively mimicking the peptide conformation in the native protein, since amidation renders the C-terminal residue uncharged and bonded as it would be in a peptide bond in the native protein. This modification also ensures a higher yield from peptide synthesis (Multiple Peptide Systems technical bulletin).

Conjugation buffer [100 mM Na-phosphate buffer, 0.02% (m/v) NaN_3, pH 7.0]. $NaH_2PO_4.H_2O$ (13.8 g) and NaN_3 (0.2 g) were dissolved in 950 ml dist.H_2O, titrated to pH 7.0 with NaOH and made up to 1 litre.

3.6.2 Procedure

Peptide COL476-490 was conjugated to KLH via the bifunctional agent, glutaraldehyde, according to the method of Briand *et al.* (1985). A peptide:carrier molar ratio of 1:40 and 1% glutaraldehyde was used.

KLH (14.7 mg, 0.0735 µmoles, assuming an M_r of 200 000) was dissolved in conjugation buffer (1.1 ml) and dialysed against the same buffer for 16 h at 4°C, before the solution was centrifuged (10 000 x g, 10 min, 4°C), to remove undissolved particulate material. Peptide (5 mg, 2.94 µmoles, M_r = 1 700) was dissolved in the KLH solution. Glutaraldehyde [568 µl of a 25% solution (Merck, E.M Grade, d = 1.06)] was added dropwise, with stirring, to the KLH-peptide mixture at 4°C over a period of 5 min. The reaction was allowed to proceed for 1 h, and stopped by incubation for 1 h at 4°C after the addition of $NaBH_4$ (10 mg/ml). Following this, conjugated peptide was separated from free peptide and the amount of peptide conjugated to carrier protein was estimated as described in Section 3.7.

3.7 Estimation of amount of peptide conjugated to KLH

The peptide-KLH conjugate mixture (2 ml) was applied to a Sephadex G-75 column (1.8 x 20 cm = 56 ml) and eluted at a flow rate of 12.7 ml/h (5 cm/h). The A_{280} was monitored and fractions (1 ml) were collected. The free peptide peak was pooled and quantitated by means of a calibration curve, constructed using the A_{280}-values of 6 dilutions (0-1mg) of the standard peptide solution (peptide COL476-490 made up in PBS to a concentration of 1 mg/ml). Linear regression analysis of the calibration curve gave the equation:

$$A_{280} = 0.0172 + 1.689[\text{peptide conc. (mg/ml)}] \quad (R = 1.00)$$

The percentage conjugation was estimated by subtracting the amount of free peptide from the original amount used for conjugation, and expressing this as a percentage of the total amount. This figure was used to determine the amount of conjugate needed to immunise rabbits at the correct dose.

3.8 Production of anti-COL476-490 peptide antibodies in rabbits

3.8.1 Procedure

Antibodies against the COL476-490-KLH conjugate, were raised in rabbits by immunising rabbits subcutaneously at 5-6 sites on the back, with 200 μg of the conjugated peptide emulsified in a 1:1 ratio with Freund's complete adjuvant. Further inoculations were administered, in the same manner, in Freund's incomplete adjuvant, using the same dose, after two weeks and then at 4 week intervals. The progress of the rabbits' response to the immunogen was followed by ELISA analysis of blood collected from the marginal ear vein at 3 and 8 weeks, and by non-lethal cardiac puncture at 12 weeks (Section 2.9).

3.9 Removal of anti-KLH antibodies

3.9.1 Reagents

CNBr-activated Sepharose 4B. This was prepared as described in Section 2.10.

Coupling buffer [100 mM Na_2CO_3, 100 mM $NaHCO_3$, 500 mM NaCl, 0.02% (m/v) NaN_3, pH 8.3]. Na_2CO_3 (2.65 g) and $NaHCO_3$ (2.1 g) were dissolved separately, each with NaCl (7.3 g) and NaN_3 (0.1 g) in 250 ml dist.H_2O. Approximately 4 parts of the $NaHCO_3$-containing solution was titrated to pH 8.3 using one part of the Na_2CO_3-containing solution.

1 M ethanolamine, pH 8.0. Ethanolamine (420 μl) was diluted in coupling buffer, adjusted to pH 8.0 with HCl, and made up to 10 ml with coupling buffer.

3.5 M Na-thiocyanate, pH 8.3. Na-thiocyanate (7.1 g) was dissolved in coupling buffer (25 ml).

3.9.2 Procedure

KLH (12.5 mg) was dissolved in coupling buffer (5 ml) and dialysed for 16 h at 4°C against the same buffer. The KLH solution was centrifuged (10 000 x g, 10 min, 4°C) to remove any insoluble material and the clarified solution was added to CNBr-activated Sepharose 4B (5 ml). Coupling was facilitated by end-over-end mixing for 2 h at 25°C. The adsorbent was allowed to settle and the supernatant fluid was removed by aspiration. Excess active groups were blocked by end-over-end mixing with 1 M ethanolamine (10 ml) for 2 h at 25°C. Uncoupled ligand and residual ethanolamine were washed from the adsorbent as described previously (Section 2.10). The KLH-Sepharose was packed in a column (1 x 6 cm = 4.7 ml) and weakly adsorbed ligand was eluted with one column volume of 3.5 M Na-thiocyanate in coupling buffer. The column was equilibrated with 10 column volumes of coupling buffer at a flow rate of 7.8 ml/h (10 cm/h).

Rabbit anti-COL476-490 IgG (50 mg), purified by PEG precipitation as described in Section 2.7, was dialysed against coupling buffer (16 h, 4°C) and applied to the KLH-Sepharose column. The column was washed with coupling buffer and bound anti-KLH antibodies were eluted with two column volumes of 3.5 M Na-thiocyanate in coupling buffer. Dot blots were carried out with KLH adsorbed to nitrocellulose, to demonstrate the absence of anti-KLH antibodies in the unbound rabbit anti-COL476-490 IgG fraction.

3.10 Preparation of anti-COL476-490 peptide antibody immunoaffinity column

3.10.1 Reagents

CNBr-activated Sepharose 4B. Prepared as described in Section 2.10.

Loading buffer [50 mM Tris, 1 M NaCl, 10 mM $CaCl_2$, 0.5% (m/v) Brij-35, 0.02% (m/v) NaN_3, pH 7.4]. Tris (6.06 g), NaCl (58.44 g), $CaCl_2$ (1.47 g), Brij-35 (0.5 g), and NaN_3 (0.2 g) were dissolved in 950 ml dist.H_2O, titrated to pH 7.4 with HCl and made up to 1 litre.

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

3.10.2 Procedure

The rabbit anti-COL476-490 IgG [from which anti-KLH antibodies had been removed (100 mg from two rounds of anti-KLH antibody removal, Section 3.9)] was coupled to CNBr-activated Sepharose 4B (10 ml) as described for KLH in Section 3.9. The immunosorbent was finally washed with loading buffer and packed in a column (1.8 x 3.6 cm = 9. 2 ml). Weakly bound ligand was eluted with one column volume of elution buffer, before the column was equilibrated with 10 column volumes of loading buffer, at a linear flow rate of 10 cm/h.

3.11 Purification of type IV collagenase from human leucocytes

3.11.1 Separation of leucocytes from buffy coat layers

A polymorphonuclear leucocyte enriched fraction was obtained from buffy coat layers, essentially as described by Bretz and Baggiolini (1974).

3.11.1.1 Reagents

Isotonic PBS [150 mM Na-phosphate, 150 mM NaCl, 0.02% (m/v) NaN_3, pH 7.2]. $NaH_2PO_4.H_2O$ (20.69 g), NaCl (8.77 g) and NaN_3 (0.2 g) were dissolved in 950 ml dist.H_2O, titrated to pH 7.2 with NaOH and made up to 1 litre.

6% (m/v) dextrose in isotonic PBS. Dextrose (60 g) was dissolved in isotonic PBS (1 litre).

Buffy coat layers. Buffy coat layers from the blood of regular blood donors, collected in the presence of acid citrate dextrose anticoagulant, were obtained from the Natal Blood Transfusion Service, Pietermaritzburg.

3.11.1.2 Procedure

Pooled buffy coat layers were stored at 4°C for 4-6 h after collection, to allow most of the erythrocytes to settle to the bottom of the storage bottle. The upper layer consisting of plasma, leucocytes and residual contaminating erythrocytes was carefully removed by aspiration and placed in a measuring cylinder. The erythrocyte containing layer was centrifuged (185 x g, 20 min, 4°C) in a swing-out rotor and the

supernatant, containing leucocytes and plasma, was combined with the first fraction in the measuring cylinder. This leucocyte containing fraction was diluted with one fifth of its volume of 6% (m/v) dextrose in isotonic PBS and incubated at 25°C for 30-60 min, to allow sedimentation of the aggregated erythrocytes. The leucocytes and plasma in the upper layer were centrifuged (4 400 x g, 20 min, 4°C), the plasma discarded and the leucocyte pellet washed three times in isotonic PBS with centrifugation (4 400 x g, 20 min 4°C) between washes. The morphology of the leucocytes was assessed after each step by staining a cell sample with May-Grünwald/Giemsa stain (Hudson and Hay, 1980a) and viewing the stained cells by light microscopy. The leucocyte pellet was resuspended in isotonic PBS, containing 2 mM PMSF and stored at -75°C to effect lysis of the cells. Thawed cells were homogenised in two volumes of extraction buffer (see Section 3.11.2) by 10 downward strokes, at 5 000 rpm, in a Braun Potter S homogeniser. Cell debris was collected by centrifugation (27 200 x g, 60 min, 4°C) and the supernatant was further fractionated as described in Section 3.11.2.

3.11.2 Optimisation of three-phase partitioning of human leucocyte supernatant and comparison with ammonium sulfate precipitation.

Ammonium sulfate precipitation and three-phase partitioning (Pike and Dennison, 1989a) were tested for their relative effectiveness in the purification of type IV collagenase from human polymorphonuclear leucocytes.

3.11.2.1 Reagents

Extraction buffer A [50 mM Tris, 10 mM $CaCl_2$, 0.05% (m/v) Triton X-100, 2 mM PMSF, 0.02% (m/v) NaN_3, pH 7.5]. Tris (1.51 g), $CaCl_2$ (0.367 g), Triton X-100 (125 ml), PMSF (0.087 g dissolved in anhydrous methanol), and NaN_3 (0.05 g) were dissolved in 220 ml dist.H_2O, titrated to pH 7.5 with HCl and made up to 250 ml.

Extraction buffer B [50 mM Tris, 10 mM $CaCl_2$, 2 mM PMSF, 0.02% (m/v) NaN_3, pH 7.5]. Tris (1.51 g), $CaCl_2$ (0.367 g), PMSF (0.087 g dissolved in anhydrous methanol) and NaN_3 (0.05 g) were dissolved in 220 ml dist.H_2O, titrated to pH 7.5 with HCl and made up to 250 ml.

Extraction buffer C [50 mM Na-cacodylate, 10 mM $CaCl_2$, 2 mM PMSF, 0.02% (m/v) NaN_3, pH 6.5]. Na-cacodylate (0.267 g), $CaCl_2$ (0.147 g), PMSF (0.087 g dissolved in anhydrous methanol) and NaN_3 (0.02 g) were dissolved in 80 ml dist.H_2O, titrated to pH 6.5 with NaOH and made up to 100 ml.

Extraction buffer D [50 mM Na-acetate, 10 mM $CaCl_2$, 2 mM PMSF, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (0.715 ml), $CaCl_2$ (0.367 g), PMSF (0.035 g dissolved in anhydrous methanol) and NaN_3 (0.05 g) were dissolved in 220 ml dist.H_2O, titrated to pH 5.5 with NaOH and made up to 250 ml.

Extraction buffer E [50 mM Na-acetate, 10 mM $CaCl_2$, 2 mM PMSF, 0.02% (m/v) NaN_3, pH 4.2]. Glacial acetic acid (0.715 ml), $CaCl_2$ (0.367 g), PMSF (0.087 g dissolved in anhydrous methanol) and NaN_3 (0.05 g) were dissolved in 220 ml dist.H_2O, titrated to pH 4.2 with NaOH and made up to 250 ml.

3.11.2.2 Procedure

Ammonium sulfate fractionation was carried out on four leucocyte sub-fractions, prepared in extraction buffers A-D, with 10% increments in $(NH_4)_2SO_4$ saturation. The redissolved $(NH_4)_2SO_4$-fractionated samples were dialysed against several changes of assay buffer, for 16 h at 4°C, before being assayed, to prevent increasing amounts of $(NH_4)_2SO_4$ interfering with the soluble ^{14}C-type IV collagen assay (Section 3.5). Ammonium sulfate is not miscible with the scintillation cocktail. An ammonium sulfate cut between 25-50% saturation was found to be optimal in all the buffer systems tested.

Three-phase partitioning was optimised on leucocyte extracts prepared in extraction buffers A-E. Tertiary butanol (2.2 ml) was added to leucocyte extract (5 ml) to constitute 30% (v/v) of the total volume and mixed in, before solid $(NH_4)_2SO_4$ was added to the desired concentration (expressed as w/v of the total solution), including t-butanol, and mixed thoroughly at 25°C to dissolve the salt. The mixture was centrifuged (3 000 x g, 10 min, 25°C) and protein was precipitated into the third phase between the t-butanol and aqueous phases. The protein pellet was redissolved in 5 x concentrated assay buffer (1 ml, Section 3.5.1) and assayed for activity (Section 3.5.2) and protein content (Section 2.3). A cut from 15-30% (m/v) of $(NH_4)_2SO_4$ was found to give the highest specific activity in the different buffer systems tested (in the absence of Triton X-100).

3.11.3 Immunoaffinity purification of type IV collagenase

A type IV collagenase fraction was obtained from TPP of the clarified leucocyte extract, prepared in extraction buffer D (see Section 3.11.2.1). Tertiary butanol was added to a concentration of 30% (v/v) and mixed in, before $(NH_4)_2SO_4$ [15% (w/v), based on the original volume of leucocyte extract plus t-butanol] was added and dissolved completely by gentle stirring. The resulting mixture was centrifuged (6 000 x g, 15 min, 4°C) in a swing-out rotor. The TPP super- and subnatant fractions were poured from the precipitate, which was discarded. The $(NH_4)_2SO_4$ concentration was increased to 30% (m/v) by the addition of solid $(NH_4)_2SO_4$, which was completely dissolved before the mixture was centrifuged (6 000 x g, 15 min, 4°C). The super- and subnatant were discarded and the precipitate was redissolved in one fifth of the leucocyte extract volume, in loading buffer (see Section 3.10.1). Any undissolved material was removed by centrifugation (27 200 x g, 15 min, 4°C) and the clarified sample was loaded onto the anti-COL476-490 peptide antibody immunoaffinity column (see Section 3.10). Following elution of the unbound proteins, the column was eluted with a further ten column volumes of loading buffer, before the bound protein fraction was eluted with two column volumes of elution buffer (see Section 3.10.1). The fractions were dialysed against the assay buffer (see Section 3.5.1) for 16 h at 4°C.

3.12 Molecular exclusion chromatography

Crude fractions from TPP and $(NH_4)_2SO_4$ fractionation, as well as the immunoaffinity purified fraction, were separated by molecular exclusion chromatography as an additional purification step and to confirm the M_r obtained by reducing SDS-PAGE. Fractions were concentrated against sucrose (see Section 2.4) to 2% (v/v) of the gel volume before application to the Sephadex G-100 column (2.5 x 90 cm = 442 ml) and elution with the loading buffer (see Section 3.10.1).

The column was calibrated with the standard proteins, myoglobin (M_r 17 000), ovalbumin (M_r 45 000) and BSA (M_r 68 000)[15 mg each in loading buffer (3 ml), containing 2 mg/ml blue dextran]. Linear regression analysis of the calibration curve constructed from K_{av} vs log M_r gave the equation:

$$\log M_r = - (K_{av} - 3.61730/0.1765),$$

from which the M_r could be calculated.

3.13 Results

3.13.1 Isolation of type IV collagen from placental material

The isolation method of Glanville *et al.* (1979) (Section 3.2) for the separation of types I, III, IV and V collagen from human placental material, proved to be reproducible. A very small peak of acidic contaminants was removed by adsorption to the DEAE-Trisacryl anion exchange resin (Fig. 2), and the unbound type III/IV collagen fraction was subsequently fractionated on CM-Sepharose (Fig. 3). A very small peak of unbound material was eluted, while a large type IV collagen peak could be resolved from a shallow spread-out peak, containing type III collagen, consistent with the elution profile reported by Glanville *et al.* (1979).

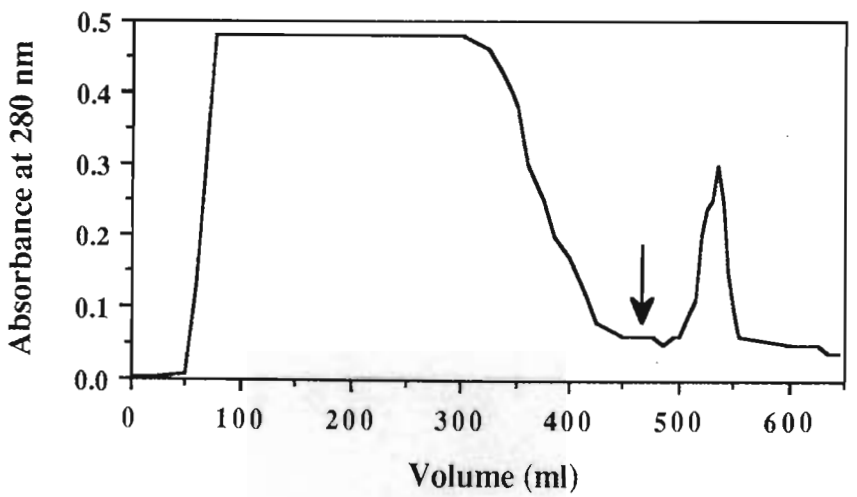

Figure 2. DEAE-Trisacryl anion exchange chromatography of pepsin digest of human placenta, containing type IV collagen.
Column, 5.0 x 6.0 cm (118 ml bed volume); buffer, 300 mM Tris-HCl, 200 mM NaCl, 2 M urea, pH 8.6, followed at ↓ by two volumes of the same buffer containing 1 M NaCl. Flow rate, 70 ml/h (3.5 cm/h); fractions, 9 ml (8 min). (—) A_{280}.

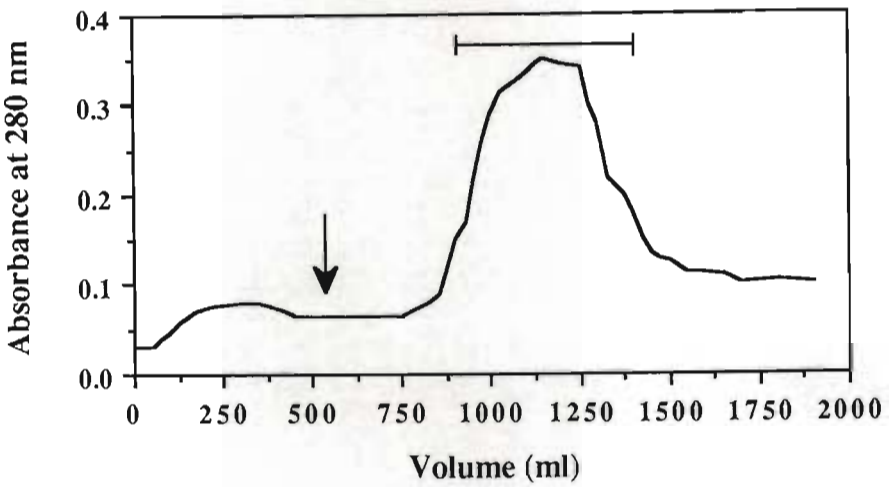

Figure 3. CM-Sepharose cation exchange chromatography of unbound type IV collagen containing fraction obtained from anion exchange chromatography.
Column, 2.5 x 50 cm (245-ml bed volume); buffer, 40 mM Na-acetate, 2 M urea, pH 4.8, followed at ↓ by a 0-200 mM NaCl gradient in five column volumes of the same buffer. Flow rate, 35 ml/h (7 cm/h); fractions, 10 ml (17 min). (—) A_{280}; (⟷) type IV collagen fraction pooled for reducing SDS-PAGE.

SDS-PAGE analysis of the purified type IV collagen (Fig. 4) showed major bands at M_r 150 000, 100 000 and 70 000. Similar banding has been reported for placental type IV collagen (Glanville *et al.*, 1979; Bailey *et al.*, 1979; Sage *et al.*, 1979). Additional minor collagenous bands, smaller than M_r 50 000, present in the type collagen IV sample, visible in higher % gels (7.5%), may be the result of pepsin digestion during the isolation procedure. Similar degradation products have been observed during the preparation of placental (Glanville *et al.*, 1979), lens capsule (Uitto *et al.*, 1980) and kidney glomerular (Daniels and Chu, 1975) type IV collagen, consistent with the presence of non-helical sequences, which are susceptible to pepsin (Glanville *et al.*, 1979), within the triple helical domains. Type IV collagenase, however, specifically cleaves type IV collagen in a pepsin-resistant triple helical domain, to generate the characteristic N-terminal and C-terminal fragments (Liotta *et al.*, 1981a; Fessler *et al.*, 1984), and the substrate isolated by pepsin digestion should therefore be suitable and specific for type IV collagenase assays.

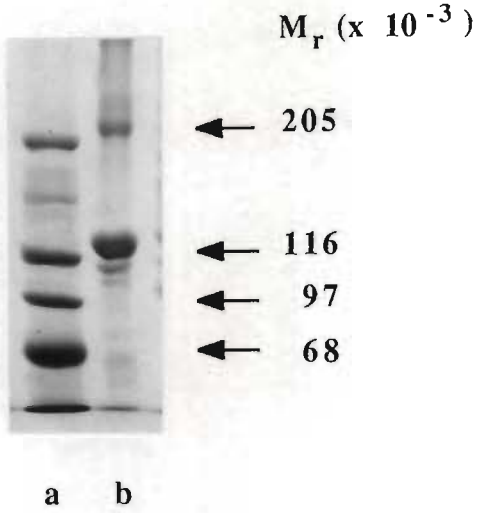

Figure 4. Reducing SDS-PAGE of type IV collagen isolated from human placental tissue. The sample (10 μg) was reduced and loaded onto a 3% stacking-5% running gel. (a) MW markers (myosin, M_r 205 000, β-galactosidase, M_r 116 000; phosphorylase b, M_r 97 400 and BSA, M_r 68 000); (b) type IV collagen.

Initially the Bradford dye-binding assay (Bradford, 1976), as modified by Read and Northcote (1981) (see Section 2.3), was used for the quantification of type IV collagen, using ovalbumin as standard protein, but a poor correlation was found between the amount of type IV collagen weighed out and protein content estimated by the assay (Table 4). Type IV collagen was extensively dialysed against 100 mM acetic acid prior to lyophilisation, and the discrepancy could, therefore, not be ascribed to a high salt content. The variation between different standard proteins in the Bradford assay has been found to be effectively eliminated by using Serva blue dye and the concentration of dye and acid/alcohol components originally specified by Bradford (1976) (Pike, 1990). The low level of dye binding found in this study with type IV collagen, may be ascribed to a relatively small number of arginine residues present in the protein (Miller and Gay, 1982), which are required for dye binding (Compton and Jones, 1985). This was recently confirmed by Stoscheck (1990) who reported that bovine skin gelatin gave a very poor response in the standard Bradford assay.

Since the quantification of type IV collagen proved to be problematic, different dye-binding protein assays, using ovalbumin and later gelatin as standard proteins, were compared to find the optimal system for the quantification of type IV collagen (Table 5). The micro-biuret and Lowry assays gave 43% and 54% of the weighed out amounts respectively, when ovalbumin was used as standard protein. When gelatin was employed as standard protein, a very good correlation was found between the protein content determined by the respective assays, and the amount of type IV collagen weighed out, i.e. 91% in the Lowry assay and 82% in the micro-biuret assay. The amount of type IV

collagen weighed out for acetylation (Section 3.4) was, therefore, adjusted by a factor of 1.16, based on the average protein content, estimated by these two assay methods. Both micro-biuret and Lowry assays are based on the coordination of a copper atom with four peptide nitrogen atoms, with the concomitant loss of a proton from each of the four substituted amide groups (Rising and Yang, cited by Itzhaki and Gill, 1964). The copper-gelatin complex has a relatively low extinction coefficient (Itzhaki and Gill, 1964) and this can be ascribed to the high proline and hydroxyproline residue content of the protein. These residues form imine linkages in the polypeptide chain and, therefore, have no ionisable amide hydrogens to form ultraviolet absorbing complexes with copper. By using gelatin as the standard protein for the quantitation of type IV collagen in this study, this protein-to-protein variation was effectively minimised.

Table 5. Comparison of protein assays for placental type IV collagen.

Lyophilised type IV collagen[a] (μg)	Protein assay method	Standard protein	Type IV collagen content[b] (μg)	% of weighed out amount
2000	Bradford [c]	Ovalbumin	19	10
250	Micro-biuret [d]	Ovalbumin	110	42
500			205	43
100	Lowry [e]	Ovalbumin	56	56
150			80	53
1000	A_{280}	Ovalbumin	138	14
250	Micro-biuret	Gelatin	207	83
500			410	83
100	Lowry	Gelatin	90	90
200			183	91

[a] Total amount of lyophilised type IV collagen weighed out; [b] Total amount of type IV collagen measured in protein assay; [c] Bradford (1976); [d] Itzhaki and Gill (1964); [e] Lowry *et al.* (1951)

3.13.2 Optimisation of soluble ^{14}C-type IV collagen assay

After dilution of ^{14}C-acetylated type IV collagen to 1 mg/ml with unlabelled collagen, the type IV collagen contained 35 000 dpm/mg, compared to the 100 000 dpm/mg obtained by Cawston and Barrett (1979) for rat skin type I collagen. Since the radiolabel is incorporated into Lys-residues, a comparison of the number of Lys-residues available in types I and IV collagen, could be indicative of the theoretical degree of radiolabel incorporation. Although the percentage of hydroxylysine residues is significantly higher in type IV collagen than in type I collagen (Table 6), most of these residues are glycosylated in the α(IV) chains. Type IV collagen contains 30% of the number of Lys residues compared to type I collagen [1.3% of the total number of residues in the α(IV) chains, as opposed to 4.4% of the total number of residues in the α(I) chains]. This difference in Lys content corresponds to the amount of radiolabel incorporated into type IV collagen, i.e. 35% of the amount reported to be incorporated into type I collagen.

Table 6. Lysine residue content (residues/1 000 total residues) of types I and IV collagen chains (adapted from Miller and Gay, 1982).

Residue	Collagen chain type					
	α1 (I)	α2 (I)	Σ[α (I)]	α1 (IV)	α2 (IV)	Σ[α (IV)]
Lysine	26	18	44	6	7	13
Hydroxylysine	9	12	21	50	36	86
Gal-hydroxy-lysine	1	1	2	2	2	4
Glc-Gal-hydroxylysine	1	2	3	44	29	73

The soluble ^{14}C-type IV collagen assay was initially carried out at 37°C (Liotta *et al.*, 1981a), but degradation of the substrate was observed in control samples incubated in the absence of enzyme in 15 hour assays. The incubation temperature was reduced to 34°C, which is below the *in vitro* melting temperature of 37°C reported for type IV collagen (Uitto *et al.*, 1980) and specific enzyme cleavage was observed over this assay period. Bovine serum albumin, used by Liotta *et al.* (1979, 1981a), was also omitted, since complete precipitation of undegraded substrate was obtained with the TCA-tannic acid levels used. Furthermore, the presence of BSA complicated the interpretation of

SDS-PAGE analysis of type IV collagen degradation by type IV collagenase. The BSA M_r 66 000 band, and additional bands often present in commercial BSA samples, masked the M_r 70 000 type IV collagen band and other degradation bands.

3.13.3 Production of anti-COL476-490 peptide antibodies

Free, unconjugated peptide was separated from high molecular weight conjugated peptide and unconjugated KLH by molecular exclusion chromatography, in order to estimate the percentage peptide conjugated to KLH. Since peptide COL476-490 absorbs strongly at 280 nm, by virtue of the presence of Phe and Trp residues, glutaraldehyde did not interfere significantly with absorbance measurements at this wavelength, and free peptide could be quantitated using a calibration curve constructed with standard peptide COL476-490 solutions (Section 3.7). By subtracting the amount of free peptide from the original amount used for conjugation, the percentage of peptide conjugated to KLH could be estimated, and was found to be 78%. This is in good agreement with a coupling yield of 70-80% reported by Briand *et al.* (1985). Amino acid analyses of a conjugate and a carrier protein of known amino acid sequence, such as ovalbumin or BSA, have been reported to provide an accurate method for determining conjugation efficiency (Briand *et al.*, 1985). Because of the size of KLH and the presence of impurities in commercially available samples, this method is not suitable for KLH-conjugates, however, and the method used in this study seemed to be a satisfactory alternative for estimating the amount of immunogen to administer to rabbits in the inoculation protocol.

An ELISA was developed to detect anti-peptide antibodies in the sera of immunised rabbits. Since it has been reported that peptides adsorb poorly to the wells of multititre ELISA plates (Bulinski *et al.*, 1983), the wells were initially coated with BSA, and peptide COL476-490 was linked to the immobilised BSA via glutaraldehyde or carbodiimide. Alternatively, the peptide was coated directly to glutaraldehyde- or carbodiimide-activated plates. Specific recognition of peptide COL476-490 by the corresponding antisera was manifested, using non-immune serum as controls. Controls, where the above coating steps were omitted, and the peptide was coated directly on to the ELISA plate, indicated that efficient adsorption of the peptide itself was obtained, as evidenced by increased antiserum binding in this mode, compared to the indirectly adsorbed mode. During the polyclonal response to a peptide-carrier protein conjugate, antibodies are not only raised against the peptide, but also against the carrier protein and residues in the carrier protein, modified by the conjugation reaction, i.e. carrier agent modified residues (CAMOR) (Briand *et al.*, 1985). Detection of these antibodies may therefore give false positive results. In order to allow for this, anti-peptide antibodies are usually also tested in

an ELISA against the peptide conjugated to a different carrier, by means of another coupling agent. Therefore, the specificity of the anti-peptide antibody ELISA would be enhanced by the direct adsorption of the peptide to the ELISA plate, since none of these interfering constituents will be present. Since the peptides used in this study were found to adsorb readily to the wells of ELISA plates, the direct adsorption mode was the method of choice for anti-peptide antibody ELISAs.

Once the optimal peptide concentration for coating the ELISA plates was established, this ELISA format was used to follow the anti-peptide antibody production over the course of the immunisation protocol. Both rabbits responded similarly over time to the peptide and a typical result is shown in Fig. 5. The titre peaked at 8-12 weeks and larger volumes of blood were therefore collected from the rabbits by cardiac puncture in the twelfth week, before the antibody levels could start to decrease. The IgG fraction was purified from the serum, and anti-KLH antibodies were adsorbed from the purified IgG on a KLH-immunoaffinity column, before this was used for the preparation of an anti-COL476-490 peptide immunoaffinity column. Removal of anti-KLH antibodies was necessary as these antibodies, present in the anti-peptide antibody preparations, are known to cross-react non-specifically with many proteins (Dr David Buttle, Strangeways Research Laboratories, personal communication).

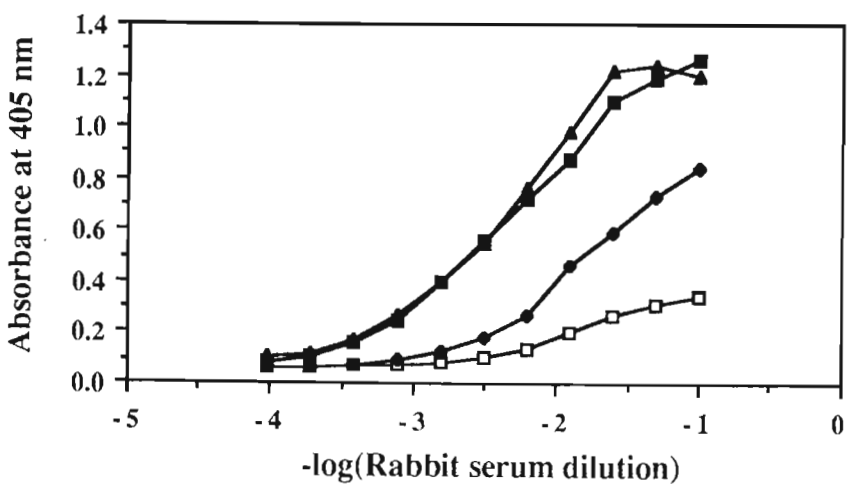

Figure 5. Progress of immunisation with peptide COL476-490-KLH-conjugate as determined by ELISA. Peptide COL476-490 was coated at 1 µg/ml to the microtitre plate and incubated with serial two-fold dilutions of antisera collected after 3 (◆), 8 (■) and 12 weeks (▲) and non-immune serum (❑). Binding was visualised by incubation with sheep anti-rabbit-HRPO conjugate and ABTS as described in Section 2.9. Each point is the mean absorbance at 405 nm of duplicate samples.

3.13.4 Separation of leucocytes from buffy coat layers

Initially, pooled buffy coat layers were obtained, within a week of separation, from blood packs of regular blood donors, but very few PMNLs were observed after staining with May-Grünwald/Giemsa stain. Collection of buffy coat layers on a daily basis resulted in a marked increase in the number of PMNLs. Large losses in the numbers and morphological integrity of PMNLs occurred during the subsequent separation from contaminating erythrocytes and plasma. Most methods for the removal of erythrocytes from buffy coat layers involve hypotonic lysis of the erythrocytes (Engelbrecht *et al.*, 1982), followed by the addition of NaCl to restore the isotonicity. This approach was not entirely successful for the lysis of erythrocytes, and had the added disadvantage that the leucocytes were damaged, as evidenced by enlarged and fragile PMNLs. Removal of the bulk of erythrocytes by low speed centrifugation, and the remainder by dextran sedimentation in isotonic PBS, followed by centrifugation, proved to be more successful in maintaining leucocyte morphology. The yield of leucocytes was further improved by separating them from the buffy coat layers within 4-6 h after collection.

3.13.5. Optimisation and comparison of ammonium sulfate precipitation and three-phase partitioning for the fractionation of type IV collagenase from the leucocyte extract

The results obtained from a comparative study of ammonium sulfate precipitation and TPP for the crude fractionation of type IV collagenase from human leucocytes, prepared in different extraction buffer systems, are summarised in Table 7.

Initially ammonium sulfate precipitation, with Triton X-100 present in the pH 7.5 extraction buffer, as used by most workers in the field (Murphy *et al.*, 1980, 1982; Callaway *et al.*, 1986), appeared to be superior to TPP for the crude fractionation of type IV collagenase, resulting in a higher specific activity and superior yield of the enzyme. When Triton X-100 was omitted from the extraction buffer, there was only a small decrease in specific activity of the leucocyte homogenate, indicating that most of the enzyme was released from the leucocytes by the freezing and thawing process. A comparison of the specific activity after ammonium sulfate precipitation or TPP on these preparations, in the different buffer systems and in the absence of Triton X-100, indicated that under these conditions TPP was superior to ammonium sulfate precipitation. Performing TPP at pH 5.5 gave the highest specific activity and yield of enzyme, significantly higher than those obtained by ammonium sulfate precipitation in the Triton X-100 containing buffer at pH 7.5.

Table 7. A comparison of ammonium sulfate precipitation and TPP for the crude fractionation of type IV collagenase from the leucocyte extract at different pH values.

Buffer	Step	Specific activity (units/mg)	Purification (fold)	Yield (%)
pH 7.5 Triton X-100	Homogenate	4	1	100
	$(NH_4)_2SO_4$ precipitate	12	3	63
	TPP	5.3	1.3	25
pH 7.5	Homogenate	3.2	1	100
	$(NH_4)_2SO_4$ precipitate	8.2	2.5	50
	TPP	12	3.6	53
pH 6.5	Homogenate	3.4	1	100
	$(NH_4)_2SO_4$ precipitate	6.2	1.9	35
	TPP	10	3.1	47
pH 5.5	Homogenate	2.8	1	100
	$(NH_4)_2SO_4$ precipitate	5	1.8	36
	TPP	20	7	96
pH 4.2	Homogenate	2.4	1	100
	TPP	9.7	4	58

3.13.6 Purification and characterisation of type IV collagenase from human leucocytes

From the results reported in the previous section, it is evident that TPP was superior to ammonium sulfate precipitation for the crude fractionation of type IV collagenase from human leucocytes. Type IV collagenase was precipitated into the third phase during TPP at pH 5.5, which is close to the pI of 5.2 reported for the enzyme (Garbisa *et al.*, 1986) and which agrees with the finding of Pike and Dennison (1989a) that proteins precipitate most readily into the third phase at or below their pI. A redissolved TPP-15-30% $(NH_4)_2SO_4$ cut, prepared in pH 5.5 buffer, was further purified on the anti-COL476-490 peptide antibody immunoaffinity column (Fig. 6). The bound peak, eluted from the immunoaffinity column with sodium thiocyanate, showed very little activity (Table 8) in the soluble ^{14}C-type IV collagen assay, despite dialysis immediately after elution. Enzyme activity could, however, be demonstrated on a gelatin containing zymogram (see later, Fig. 9). Residual sodium thiocyanate may have interfered with the soluble ^{14}C-type IV collagen assay. Höyhtÿa *et al.* (1988), who used the same chaotropic agent to elute type IV collagenase from an anti-whole type IV collagenase immunoaffinity column, only demonstrated enzyme activity in a gelatin-containing zymogram. Alternatively, subjecting the enzyme to SDS-PAGE in the gelatin-containing gel, could have been sufficient to activate the enzyme, since it has been reported that type IV collagenase is activated by SDS (Mackay *et al.*. 1990). The SDS may dissociate TIMP from the enzyme (Wilhelm *et al.*, 1989).

Table 8. Purification of human leucocyte type IV collagenase.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	1392	3173	2.3	1	100
TPP	78	1387	18	8	44
Anti-peptide immunoaffinity chromatography	0.83	145	175.7	76	4.6

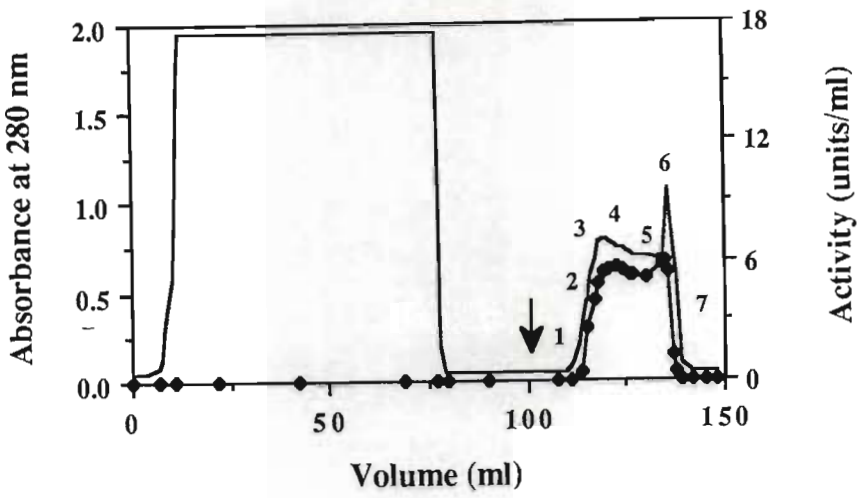

Figure 6. Immunoaffinity purification of human leucocyte type IV collagenase on the anti-type IV collagenase peptide antibody column.
Column, 1.8 x 3.6 cm (9.2 ml bed volume); buffer, 50 mM Tris-HCl, 1 M NaCl, 10 mM $CaCl_2$, 0.5% (m/v) Brij-35, pH 7.4, followed at ↓ by the same buffer containing 3.5 M Na-thiocyanate. Flow rate, 25 ml/h (10 cm/h); fractions, 3 ml (7 min). (—), A_{280}; (◆), enzyme activity (units/ml) from the ^{14}C-type IV collagen assay and 1-7 = fractions analysed by reducing SDS-PAGE (see Fig. 7).

The low enzyme activity measured following immunoaffinity purification, had a profound affect on the specific activity and percentage recovery of the enzyme, giving much lower values than those reported by Murphy *et al.* (1982, 1989a) and Hibbs *et al.* (1985). The percentage recoveries of enzyme in these purification procedures were 60 and 80% (1.68 and 2.85 mg) respectively, from human leucocytes (Murphy *et al.*, 1982; Hibbs *et al.*, 1985) and 15% (3.2 mg) from porcine leucocytes (Murphy *et al.*, 1989a), compared to the 4.5% (0.83 mg) in this study. Murphy *et al.* (1982) obtained a higher yield of human type IV collagenase by subcellular fractionation of neutrophil homogenates, separating specific granules and C-particles from serine proteinase-containing azurophil granules by rate zonal sedimentation, the facilities for which were not available in our laboratory. Because starting material, specifically enriched with the type IV collagenase-containing granules, could not be prepared in this study, leucocytes were stored in, and subsequently extracted with, buffer containing the serine proteinase inhibitor PMSF. Alternatively, Hibbs *et al.* (1985) and Murphy *et al.* (1989a) purified type IV collagenase from PMNL culture media, but the prohibitively high cost of cell culture on the scale required made this approach impractical in our laboratory. The simplicity of the two-step purification method used in this study makes it possible, however, to purify the same amount of type IV collagenase within one or two cycles of the procedure.

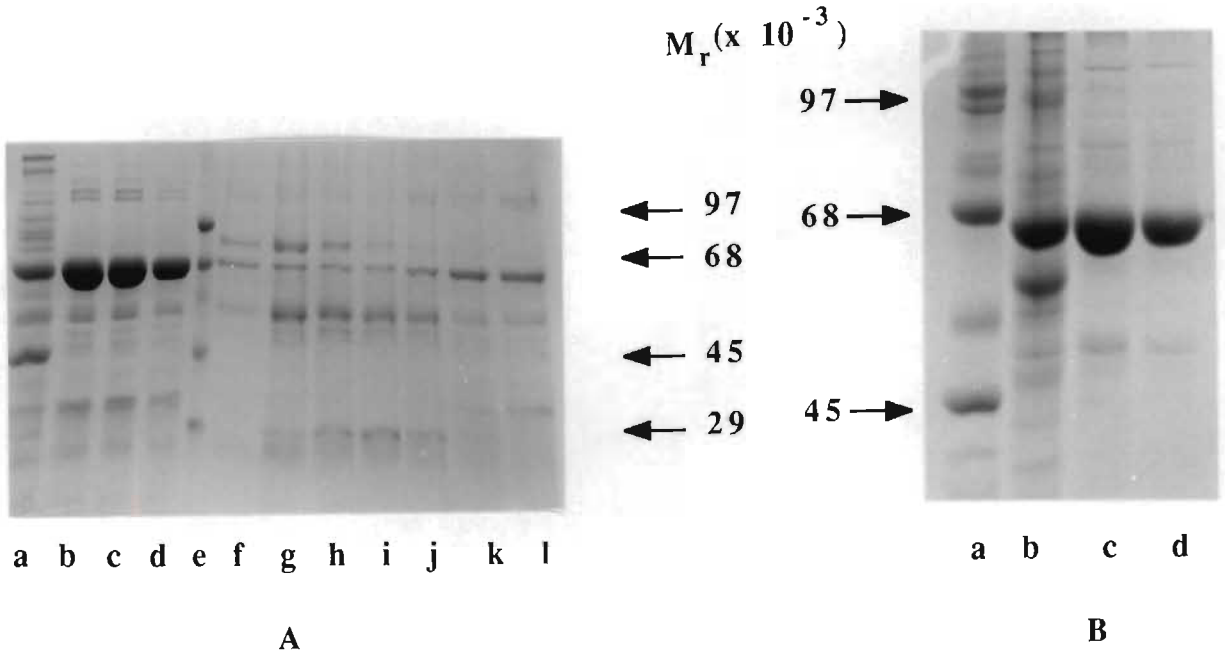

Figure 7. Reducing SDS-PAGE of fractions obtained during the isolation of type IV collagenase from human leucocytes.
Fractions were reduced and loaded onto a 7.5% gel. (**A**) Crude fractionation and immunoaffinity chromatography fractions. (a) Leucocyte homogenate; TPP-15-30% $(NH_4)_2SO_4$ cut, (b) and (c) 1/10 dilution; (d) 1/20 dilution; (e) MW markers (phosphorylase b, M_r 97 400; BSA, M_r 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 29 000); affinity eluate fractions (10 µg), (f) 1; (g) 2; (h) 3; (i) 4; (j) 5; (k) 6 and (l) 7 (See Fig. 6). (**B**) Crude fractionation and molecular exclusion chromatography fractions. (a) MW markers, as above (without carbonic anhydrase, M_r 29 000); (b) TPP-15-30% $(NH_4)_2SO_4$ cut (1/40 dilution); type IV collagenase, (c) 25 µg and (d) 15 µg.

Although the results presented in Table 8 suggest that substantial losses were incurred in the immunoaffinity chromatography step, following TPP, this step contributed usefully to the purification procedure, as shown in Fig. 7A (lanes a-d). Furthermore, SDS-PAGE (Fig. 7A, lanes k-l) of the immunoaffinity eluate indicated that type IV collagenase was purified to near homogeneity in this step (as evidenced by a major band at M_r 72 000 and minor bands at M_r 50 000 and 30 000 - see below). Sephadex G-100 chromatography succeeded in removal of most of the contaminating IgG leaching from the immunoaffinity column (Fig. 7B). In order to determine whether the TPP process could have introduced anomalous interaction with the affinity ligand, and hence a loss in enzyme activity, both an unfractionated leucocyte homogenate sample and a fraction obtained by 25-60% ammonium sulfate precipitation were applied to the immunoaffinity column. In both cases the affinity eluates had very low activity in the soluble ^{14}C-type IV collagen assay, but showed activity in a gelatin zymogram, suggesting that TPP is not uniquely damaging.

The low activity of the isolated enzyme in the soluble ^{14}C-type IV collagen assay, together with the presence of an M_r 30 000 band in reducing SDS-PAGE (Fig. 7A) and in non-reducing gelatin-substrate SDS-PAGE (Fig. 9), led to the hypothesis that the enzyme is, at least partly, complexed with the naturally occurring TIMP, which has an M_r of 30 000 (Welgus *et al.*, 1979; Murphy *et al.*, 1981). Identification of the M_r 30 000 band as TIMP is circumstantial, however, since the purification of the inhibitor for antibody production to test this hypothesis, did not fall within the scope of this study. This approach was, however, successfully applied to the identification of the cathepsin L/cystatin complex (Chapter 4).

The low yield, and hence activity, obtained from the anti-COL476-490 peptide antibody immunoaffinity column may also be explained in terms of the low enzyme binding capacity of the immunoaffinity column, resulting from antibody specificity and orientation. Only a small percentage of the antibodies produced in the polyclonal response to the COL476-490 peptide-KLH conjugate may be directed against the correct conformation of the peptide, i.e. the conformation it adopts in the native type IV collagenase. A low percentage of these specific antibodies would in turn be correctly orientated on the solid support for antigen (type IV collagenase) binding, after conjugation to the column. Hill *et al.* (1989) estimated that only 10% of the antigen binding sites remain available after covalent immobilisation to a matrix. The small amount of COL476-490 peptide available, precluded the preparation of a peptide affinity column for the purification of the anti-peptide antibodies. Removal of anti-KLH antibodies provided a partial compromise. Since the covalent bonds formed between CNBr-activated matrix and ligand (antibody) are relatively labile (Hill *et al.*, 1989), it is also often found that antibody leaches from the column, as evidenced by the presence of the M_r 50 000 IgG heavy chain band observed in some eluates. Immunoaffinity purification procedures often suffer from the disadvantage that the most specific antibodies bind antigen with a very high affinity, which is not readily reversible and which would lead to a low antigen recovery. Anti-peptide antibodies, however, usually bind to protein with a lower affinity constant than monoclonal antibodies (Muller, 1988b) and would thus constitute superior immunoaffinity ligands for protein purification.

The presence of an M_r 92 000 band in the first fractions eluted, from the anti-COL476-490 peptide immunoaffinity column (Fig. 6), with sodium thiocyanate, may be explained in terms of the degree of sequence homology found between the 92 and 72 kDa forms of type IV collagenase, in the peptide recognised by the anti-peptide antibody (Fig. 8).

72 kDa type IV collagenase:	M G P L L V A T F W P E L P E
92 kDa type IV collagenase:	Q G P F L I A D K W P A L P R
Fibroblast collagenase:	E L N F I S V - F W Q L P N G
Neutrophil collagenase:	E M N F I S L - F W P S L P T
Stromelysin:	E L H F I S S - F W P S L P S

Figure 8. Structural relationship between the human 72 and 92 kDa type IV collagenase sequences recognised by anti-COL476-490 peptide antibodies.
Identical amino acid residues are underlined (Wilhelm *et al.*, 1989). The corresponding sequences from the interstitial (fibroblast and neutrophil) collagenases (Hasty *et al.*, 1990) and stromelysin (Wilhelm *et al.*, 1989) are included for comparison. Anti-COL476-490 peptide antibodies are directed against the sequence in the 72 kDa type IV collagenase form.

Since there is 53% sequence homology between peptide COL476-490 in the 72 kDa form and the corresponding peptide, residues 563-577, in the 92 kDa form of type IV collagenase, it is conceivable that the anti-COL476-490 peptide antibodies might recognise this sequence in both forms of the enzyme. Furthermore, conserved residues include three Pro-residues whose presence may contribute to conformational homology between the two peptide sequences and therefore aid in antibody recognition. The sequence of the 92 kDa form of type IV collagenase (Wilhelm *et al.*, 1989), was only elucidated after the preparation of the anti-COL476-490 peptide antibodies and the immunoaffinity purification of type IV collagenase.

A zymogram of the leucocyte sample before immunoaffinity purification [TPP-15-30% $(NH_4)_2SO_4$-cut] (Fig. 9, lane a), as well as a purified enzyme sample (Fig. 9, lanes b-d), indicated gelatinolytic activity at M_r 84 000 and 66 000, the active forms of the 92 and 72 kDa type IV collagenases respectively (Wilhelm *et al.*, 1989; Collier *et al.*, 1988).

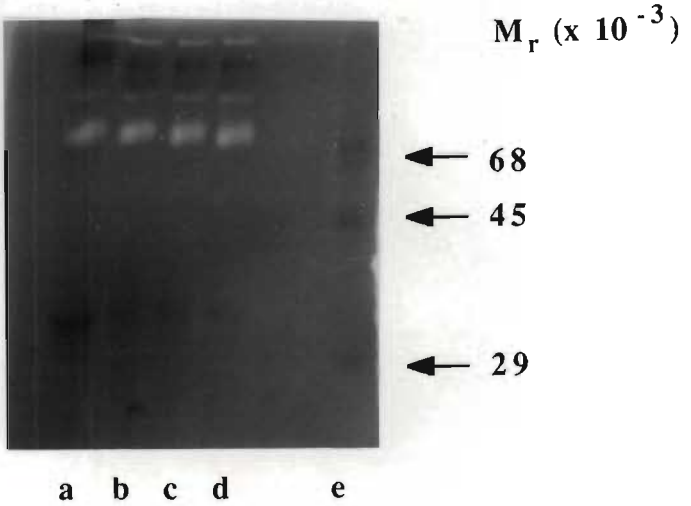

Figure 9. Gelatin-substrate SDS-PAGE of immunoaffinity purified type IV collagenase.
Non-reduced samples were loaded onto a 7.5% gelatin-containing gel, and following electrophoresis, the proteinases were renatured and the gel treated as described in section 2.6. (a) TPP-15-30% $(NH_4)_2SO_4$ cut (1/20 dilution); (b) to (d) pooled affinity eluted type IV collagenase fractions and (e) MW markers as in Fig. 7A.

Western blot analysis of a TPP sample showed targeting of only the 72 kDa form of type IV collagenase, and not the 92 kDa form (Fig. 10, lane a). Following immunoaffinity purification, very slight targeting of the 92 kDa was observed (Fig. 10, lane b). This may indicate that the anti-COL476-490 peptide antibody recognises mainly the native form of the 92 kDa type IV collagenase, since it binds to the enzyme during immunoaffinity chromatography, but does not bind the denatured, immunoblotted form to the same degree. These anti-peptide antibodies seem, however, to recognise both native and denatured forms of the 72 kDa form, as evidenced by binding in immunoaffinity chromatography and on a Western blot. This differential binding may also be ascribed to the degree of homology in the peptide sequence recognised by the anti-peptide antibodies.

The purified enzyme was identified as a metalloproteinase, requiring Ca^{2+}-ions for activity, as shown by its behaviour in the presence of a number of enzyme inhibitors (Table 9). The general metal chelator, EDTA, and the calcium ion chelator, EGTA, as well as 1,10-phenanthroline, completely inhibited the enzyme. The inhibition by EDTA could be reversed by the addition of APMA. The sulfhydryl alkylating agent, iodoacetate, activated the enzyme, while 62% inhibition was observed in the presence of cysteine. The serine proteinase inhibitor, PMSF, had no effect on the activity of the enzyme.

a b

Figure 10. Targeting of type IV collagenase by anti-peptide COL476-490 antibodies on a Western blot. Fractions were reduced and subjected to electrophoresis on a 7.5% gel, electroblotted onto nitrocellulose and probed with anti-KLH-purified anti-COL476-490 IgG and visualised using sheep anti-rabbit IgG-HRPO conjugate as described in section 2.8. (a) TPP-15-30% $(NH_4)_2SO_4$-cut (25 µg), and (b) immunoaffinity purified type IV collagenase (10 µg).

Table 9. Effect of inhibitors and activators on the isolated human type IV collagenase.

Compound	Concentration (mM, final in assay mixture)	Activity (% of control)
APMA	1	100
APMA	0	10
EDTA	10	5
EGTA	5	0
APMA + EDTA	1+10	100
1,10 phenanthroline	5	0
Cysteine	10	38
PMSF	2	100
Iodoacetate	1	131

3.14 Discussion

In this chapter the purification of human type IV collagenase from leucocytes using anti-peptide antibody immunoaffinity chromatography was described. The amino acid sequence used to generate the anti-peptide antibodies was identical to a sequence in the 72 kDa type IV collagenase present in human A2058 melanoma cells derived from brain metastases (Höyhtyä *et al.*, 1988), and the enzyme isolated from leucocytes may, therefore, be the normal equivalent of the enzyme expressed by tumour cells. Both PMNLs and

tumour cells need to migrate through connective tissue barriers, especially the basement membrane of the capillary endothelium, and thus require specific type IV collagenase degrading enzymes. These anti-peptide antibodies are, therefore, very likely to cross-react with tumour cell type IV collagenase and may thus find application in immunolocalisation and tumour invasion model studies. The potential usefulness of these anti-type IV collagenase peptide antibodies for immunocytochemical detection of the enzyme, was also indicated by their specificity in recognising the enzyme in crude leucocyte samples, as judged by the binding of the enzyme to the immunoaffinity column.

Anti-peptide antibodies have also contributed to the elucidation of the activation mechanism of the matrix metalloproteinase gene family (Stetler-Stevenson *et al.*, 1989; Brown *et al.*, 1990). By using anti-peptide antibodies against the N-terminal sequence of the 72 kDa-type IV collagenase (residues 1-17) as well as against an internal sequence (residues 472-490), Stetler-Stevenson *et al.* (1989) have demonstrated that the organomercurial induced activation of the latent proenzyme is accompanied by the removal of an N-terminal peptide fragment. Maximum collagenolytic activity was, however, obtained shortly after addition of APMA and before full conversion to the stable lower M_r form occurred. This indicated that a conformational rearrangement was probably induced in the zymogen by APMA, which resulted in an active, but unstable, intermediary form of the enzyme. Amino acid sequencing, and activation studies with highly purified type IV procollagenase showed that organomercurial activation leads to the autoproteolytic removal of an 80-residue fragment from the N-terminus of the latent 72 kDa proenzyme to form the stable, active 62 kDa enzyme. This site of autoproteolysis is the same as that occurring in other members of the matrix metalloproteinases, i.e. prostromelysin (Whitham *et al.*, 1986) and interstitial collagenase (Murphy *et al.*, 1987). It was also found that all three enzymes contain a highly conserved region immediately upstream from the activation locus, consisting of the sequence PRCGVPDV (Whitham *et al.*, 1986). The unpaired Cys-73 present in this sequence plays an important role in the activation mechanism of the proenzyme as will be discussed later.

It has been reported that the purification of type IV collagenase from human PMNLs is technically difficult, because the enzyme is present in relatively low amounts and is susceptible to serine proteinases, which are also present in PMNLs (Murphy *et al.*, 1980, 1982; Callaway *et al.*, 1986; Hibbs and Bainton, 1989). In those studies, these difficulties were largely overcome by subcellular fractionation of PMNLs or by growing the cells in culture. The enzyme has been found to be readily and specifically secreted in response to phorbol 12-myristate 13-acetate (PMA) treatment (Hibbs *et al.*, 1985), which generated culture supernatants rich in type IV collagenase, but largely devoid of serine proteinases and

non-secretory PMNL proteins. Subcellular fractionation of PMNLs ensured the separation of type IV collagenase-containing specific granules and C-particles from the serine proteinase-containing azurophil granules (Murphy *et al.*, 1980, 1982). The facilities for large scale centrifugal subcellular fractionation and large scale cell culture were not available in the present study and alternative approaches were consequently investigated.

Separation of PMNLs from mononuclear leucocytes on discontinuous Ficoll-Hypaque density gradients (English and Andersen, 1974), was reported to be suitable where relatively small numbers of PMNLs were required for cell culture (Murphy *et al.*, 1989a). This technique was not practical, however, for the large volumes of buffy coat layers which needed to be processed to obtain a suitable amount of enzyme-containing starting material. Leucocytes were furthermore found to be far more fragile than reported in the literature (Murphy *et al.* , 1980; Engelbrecht *et al.*, 1982; Callaway *et al.*, 1986). Conditions were therefore optimised for the rapid separation of leucocytes from erythrocytes and plasma, before lysis of leucocytes could lead to a loss in enzyme. Freezing at -70°C, followed by thawing, was found to be sufficient for leucocyte lysis. Since the duration of the separation process seemed to be important for maintaining the morphological integrity of leucocytes, obtaining leucocytes by leucopheresis of regular blood donors (Baugh and Travis, 1976), could have been advantageous, but was not practical in this study.

Because of the relatively low amount of type IV collagenase in the starting material, the possible improvement of the crude fractionation step was investigated. Three-phase partitioning (Pike and Dennison, 1989a) was found to be superior to conventional ammonium sulfate precipitation. Although TPP had to be carried out in the absence of Triton X-100, it was found that freezing and thawing of the leucocyte preparation seemed to be sufficient to release the enzyme from the specific granules and C-particles. Replacing ammonium sulfate precipitation with TPP, therefore, held much promise for a good yield of type IV collagenase, despite the absence of enrichment procedures such as subcellular fractionation of leucocytes. A much lower yield of enzyme was, however, obtained compared to that reported in the literature. This was shown not to be due to TPP, but rather to be the result of some interference in the soluble ^{14}C-type IV collagen assay, because an even lower yield of active enzyme was obtained when ammonium sulfate precipitation was used instead of TPP.

Despite the low activity in the soluble ^{14}C-type IV collagen assay, immunoaffinity purified type IV collagenase did, however, show gelatinolytic activity at 66 kDa with an additional major active polypeptide at M_r 84 000 upon SDS-PAGE in a gelatin-containing zymogram, consistent with the observations of Vartio and Baumann (1989) on plasma and

Murphy *et al.* (1989a) in studies on human and porcine PMNLs. The 72-kDa type IV collagenase could possibly have been purified as a complex with TIMP-2, with which it has been shown to form a very stable, non-covalent complex (Goldberg *et al.*, 1989) and activity in gelatin zymograms may, therefore, be as a result of the separation of TIMP from type IV collagenase, effected in the presence of 0.1 % SDS (Goldberg *et al.*, 1989). Low levels of activity measured in the soluble ^{14}C-type IV collagen assay, despite the presence of TIMP-2 and the absence of SDS, is consistent with the partially active TIMP-2/72-kDa type IV collagenase complexes, resulting from APMA activation, reported by Goldberg *et al.* (1989) in the conditioned medium of SV-40 transformed fibroblasts. Activation by APMA did not dissociate TIMP from these complexes, and the presence of TIMP slowed, but did not prevent, the activation of type IV collagenase by APMA (Murphy *et al.*, 1989). Further addition of stoichiometric amounts of TIMP did, however, inhibit the enzyme completely.

Work from the laboratory of Wilhelm *et al.* (1989) indicated that TIMP co-immunoprecipitated with type IV collagenase when a monospecific antibody, directed against the enzyme, and which does not recognise TIMP, was used. The TIMP-type IV collagenase complex, isolated in the present study, could thus conceivably have co-purified on the anti-peptide antibody immunoaffinity column. Recognition of such a complex by the anti-peptide antibody immunoaffinity column would only be possible if binding of the TIMP-2 molecule does not obscure amino acid residues 476-490 in the enzyme. Howard and Banda (1991) have recently distinguished two distinct TIMP-2 binding sites on 72 kDa type IV collagenase. In addition to binding to the active site, TIMP-2 was found to bind to a stabilisation site situated in the C-terminus, spanning the region beginning at amino acid residue 414. The partially active TIMP-72 kDa type IV collagenase complexes, reported by Goldberg *et al.* (1989), may have had TIMP-2 bound to the stabilisation site, and complete inhibition caused by further addition of TIMP-2, may have been due to binding to the active site. Howard and Banda (1991) have indeed found that the binding of TIMP-2 to the stabilisation site has a K_d of 0.42 nM, compared to 0.72 nM for the active site, indicating that binding may take place in this order. Binding of TIMP-2 to the stabilisation site, rather than the active site, may also explain why TIMP-2/72 kDa type IV collagenase complexes can be activated by APMA, while TIMP-2 remains bound to the enzyme (Goldberg *et al.*, 1989; Murphy *et al.*, 1989a).

It is unlikely that TIMP-2 was bound at the stabilisation site of type IV collagenase purified in this study, since the anti-peptide antibody immunoaffinity purification step should recognise amino acid residues 476-490, which would have been masked by TIMP-2 bound in this region. Active site bound TIMP-2 would, however, not have interfered with binding

to the immunoaffinity matrix. Binding of TIMP-1 to the active site cannot be ruled out, since both TIMP-1 and -2 have been found to bind to the active site of type IV collagenase (Goldberg *et al.*, 1989). The exclusivity of binding of TIMP-2 to the 72 kDa form (Goldberg *et al.*, 1989), seems to refer to binding to the stabilisation site on the 72 kDa form.

The 72 kDa form of type IV collagenase has previously mainly been purified from mononuclear leucocytes (Garbisa *et al.*, 1986) and connective tissue cells (Collier *et al.*, 1988), while the 92 kDa form was obtained from PMNLs (Murphy *et al.*, 1982; 1989a). Murphy *et al.* (1989a,b) have, however, observed additional lower levels of the 66 kDa active type IV collagense on gelatin zymograms of PMNLs, while the 72 kDa type IV collagenase, purified by Höyhtyä *et al.* (1988; 1990) from A2058 melanoma cells on type IV collagen-Sepharose, was contaminated by small amounts of the 92 kDa-form. Both enzyme forms may, therefore, be present in one and the same cell type. Since there was little possibility of contamination of PMNLs by monocytes in the present study, PMNLs were probably the source of the 72 kDa type IV collagenase. This study illustrates, therefore, that PMNLs provide a suitable and readily available source of the human enzyme.

Peptide selection for anti-peptide antibody production for immunoaffinity purification of type IV collagenase was based on the amino acid sequences known at the time (Collier *et al.*, 1988). The peptide COL476-490 was chosen because it appeared to have little sequence homology with interstitial fibroblast and neutrophil collagenase and stromelysin, MMPs also present in candidate sources for type IV collagenase purification (Fig. 8). When the amino acid sequence of the 92 kDa type IV collagenase was later elucidated (Wilhelm *et al.*, 1989), it showed 53% sequence homology with the 72 kDa form in the peptide selected, which was significantly higher than the 20% for interstitial neutrophil and 33% for fibroblast collagenase. Wilhelm *et al.* (1989) were the first group to show that the two forms of type IV collagenase, i.e. the 72 and 92 kDa forms, are genetically distinct, although they share substrate specificity and physiochemical properties. Comparison of the amino acid sequences of the 72 kDa (Collier *et al.*, 1988) and 92 kDa (Wilhelm *et al.*, 1989) forms of type IV collagenase, revealed the presence of a 54 residue long proline rich domain in the 92 kDa form, which could provide a candidate peptide for the specific purification of the 92 kDa form by anti-peptide antibody immunoaffinity purification.

An apparently curious property of the 72-kDa type IV collagenase isolated in this study was its activation by iodoacetate in a 16 h ^{14}C-type IV collagen assay. Although Uitto *et al.* (1980) found that human leucocyte collagenase was rapidly activated by another alkylating agent, NEM, this activation was lost after six hours, and the activity returned to levels equal

to those in the absence of NEM. Murphy *et al.* (1980), on the other hand, reported 50% inhibition of the 92 kDa type IV collagenase by NEM. Van Wart and Birkedal-Hansen (1990) made the observation that the apparently disparate group of compounds capable of activating the metalloproteinases, can be divided into four groups, including proteinases (e.g. trypsin and plasmin), conformational perturbants (e.g. the detergent SDS and the chaotropic agent NaSCN), reversible sulfhydryl-group modifiers (e.g. organomercurials) and irreversible sulfhydryl-group modifiers (e.g. oxidants such as HOCl and NaOCl and alkylating agents such as NEM). It has long been poorly understood how the matrix metalloproteinases could be activated by multiple means, until the involvement of an inaccessible Cys-73 present in the conserved propeptide region was identified (Springman *et al.*, 1990). In the latent enzyme the active site Zn atom is coordinated to Cys-73 and two His-residues present in the highly conserved active site region, VAAGHEFGHALG, thus blocking the active site. Activation results in the dissociation of Cys-73 from the intramolecular Zn-coordination complex (Van Wart and Birkedal-Hansen, 1990). This cysteine switch mechanism leads to a conformational rearrangement in the enzyme, thus exposing the active site and leading to the attainment of proteolytic activity. The active enzyme will then undergo autoproteolytic cleavage of the N-terminal propeptide region, resulting in the fully active, stable metalloproteinase. Although possible physiological activation mechanisms, such as proteolytic activation by trypsin (Liotta *et al.*, 1981a), plasmin, via plasminogen activator (Werb *et al.*, 1977) and stromelysin (Murphy *et al.*, 1987), as well as non-proteolytic activation by HOCl, produced oxidatively by neutrophils during the respiratory burst (Weiss, 1989), have been proposed, the *in vivo* activation mechanism for type IV collagenase has not been elucidated.

In the present study it was shown that the anti-peptide antibody approach seems to be viable for the producion of specific antibodies, capable of recognising both the native and denatured forms of type IV collagenase, and which may, therefore, find application in immunoaffinity enzyme purification, tumour invasion studies and immunocytochemical localisation of the enzyme in tumour tissue. With the elucidation of the amino acid sequences of all the members of the MMP gene family, it should now be possible to prepare anti-peptide antibodies capable of distinguishing between the different members. This would be useful in determining the relative contributions of these enzymes to extacellular matrix proteolysis during tumour invasion. On the other hand, anti-peptide antibodies against the active site sequence, VAAHEFGHAMGLEHS, shared by all the members of the matrix metalloproteinase family, could provide a general immunoinhibitory agent for these enzymes in pathological processes where a cascade of ECM degrading MMPs may be involved.

CHAPTER 4

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

4.1 Introduction

The initial objective of the study reported in this chapter was the identification of additional immunoinhibitory epitopes in cathepsin L for the production of anti-peptide antibodies to complement the polyclonal and anti-peptide antibodies produced in a previous study in this laboratory (Pike, 1990), and possibly to improve on their level of immuno-inhibition. Such immunoinhibitory antibodies could conceivably be used as a means of passive immune-therapy, or alternatively, peptides, corresponding to these immuno-inhibitory epitopes, could possibly be employed as vaccines to elicit immunoinhibitory antibodies to arrest tumour invasion (Dennison, 1989).

In order to map the active site of cathepsin L for the identification of possible immunoinhibitory epitopes, both a suitable source for the purification of large quantities of cathepsin L, and the primary structure of the enzyme are required. Since it has been found that sheep liver contains relatively large amounts of cathepsin L which is enzymologically and immunologically similar to the human enzyme, and that the yields are higher than from other species' liver (Mason, 1986), this tissue provided the most suitable and convenient source of a model for human cathepsin L. When this study was initiated, the complete amino acid sequences of only mouse (Portnoy *et al.*, 1986), chicken (Wada *et al.*, 1987; Dufour *et al.*, 1987), rat (Ishidoh *et al.*, 1987) and human (Ritonja *et al.*, 1988) cathepsin L had been determined, but no sequence information was available for sheep cathepsin L. At the outset the aim of this study was, therefore, to isolate sufficient quantities of cathepsin L from sheep liver to allow for primary sequence determination. Once the sequence is known, peptides could be synthesised, and tested for their ability to compete with whole cathepsin L for binding to immunoinhibitory anti-whole cathepsin L antibodies. Thus, inhibition of immunoinhibition would identify a peptide as a candidate immunoinhibitory epitope; an approach which was successfully used with a synthetic peptide for the localisation of such an epitope in cathepsin L (Chapter 5).

Cathepsin L was purified from sheep liver using the rapid, high yield method developed in this laboratory by Pike and Dennison (1989b), which involved homogenisation of thawed liver, autolysis at pH 4.2 and a novel fractionation procedure, three-phase

partitioning of the autolysate (Pike and Dennison, 1989a). This was followed by adsorption of cathepsin L to a strong cation exchanger, S-Sepharose, even at pH values close to its pI, the latter being an unique property of cathepsin L amongst the cathepsins. Since the sample obtained from crude purification by TPP was relatively salt-free, it could be directly applied to the cation exchanger, thus decreasing the length of time cathepsin L was in contact with other proteinases. Two consecutive cation-exchange steps at pH 5.5 and 4.5 on S-Sepharose, were initially used. However, analysis of the pH 4.5 S-Sepharose-fraction on MEC and reducing SDS-PAGE, revealed the presence, in addition to free M_r 26 000 single-chain cathepsin L, of an M_r 37 000 complex of cathepsin L and an M_r 14 000 component, which was later tentatively identified as the endogenous cysteine proteinase inhibitor, stefin B (Pike, 1990). The original purification method (Pike and Dennison, 1989b) was, therefore, modified by replacing the second cation exchange step at pH 4.5 with MEC on Sephadex G-75, which separated the M_r 37 000 cathepsin L/stefin B complex from free M_r 26 000 cathepsin L. The M_r 37 000 complex was, surprisingly, found to be active against both azocasein and Z-Phe-Arg-NHMec substrates (Pike, 1990).

In order to improve the yield of free enzyme for the purposes of this study, the complex, which contains a large proportion of cathepsin L, was initially studied in an attempt to identify means of separating free cathepsin L from this complex. During the course of these studies it was found that a significant proportion of cathepsin L was bound to stefin B in an unusual manner, to form complexes of different M_r. This interaction was distinct from the reversible, tight binding, non-covalent inhibitory complexes normally formed between cysteine proteinases and cystatins (Anastasi *et al.*, 1983; Katunuma *et al.*, 1983; Nicklin and Barrett, 1984). From results obtained in the ensuing collaboration with Dr. Robert Pike in this laboratory on the characterisation of these novel cathepsin L/stefin B complexes, and the finding that these complexes were stable and more active than free cathepsin L at neutral pH (Pike, 1990; Dennison *et al.*, 1992), it became evident that the formation of these novel, proteolytically active complexes could be relevant in the context of tumour invasion. The direction of the present study was, therefore, changed to focus on the properties of these unusual complexes in their own right and to determine whether similar complexes might occur in higher primates, which would add to their significance in tumour invasion.

In order to find unequivocal proof that the complexing cystatin was stefin B, and to characterise the unusual mode of binding between the cathepsin L and stefin B, it became necessary to determine the primary structure of the sheep liver cystatin as well as that of cathepsin L. Furthermore, sequencing might provide confirmatory evidence of the single-chain nature of the cathepsin L, purified using the rapid isolation method of Pike and

Dennison (1989b), and which may have a bearing on the formation of these unusual complexes. Approaches leading to an increase in the yield of free cathepsin L were, therefore, further pursued. In addition to the possible dissociation of the cathepsin L/stefin B complexes, strategies to prevent their formation were investigated and purification from alternative organs was also investigated.

Stefin B, which is present in the cytosol of liver tissue and spleen (Kominami *et al.*, 1982; Järvinen and Rinne, 1982; Green *et al.*, 1984) and, stefin A, which has been isolated from blood PMNLs (Brzin *et al.*, 1983), belong to the Type 1 cystatin, or stefin, family of endogenous cysteine proteinase inhibitors, a subgroup of the cystatin superfamily (Barrett, 1987). Human stefin A has been characterised as an acidic protein with pI-values in the 4.5 - 5.0 range (Brzin *et al.*, 1983) and was found to be immunologically identical to the human liver cysteine proteinase inhibitor A (CPI-A) (Green *et al.*, 1984). Whereas stefin A contains no cysteine residues (Machleidt *et al.*, 1983), stefin B, which is a more neutral protein with pI-values in the 5.9 - 6.5 range (Lenarcic *et al.*, 1986), is found in both monomer and dimer forms by virtue of the presence of a cysteine residue in position 3 (Ritonja *et al.*, 1985). The absence of disulfide bonds in stefins distinguishes them from members of the Type 2 cystatins, or cystatin family, which contain two disulfide loops towards the C-terminus. This family includes chicken egg-white (CEW) cystatin (Fossum and Whitaker, 1968), human cerebrospinal fluid and serum cystatin C (Barrett *et al.*, 1984) and human saliva cystatin S (Isemura *et al.*, 1984). Type 3 cystatins, the kininogen family, are high MW cysteine proteinase inhibitors found in plasma (Barrett *et al.* 1986). This is the only glycosylated cystatin family and each member consists of three copies of Type 2 cystatin sequences with additional disulfide loops. They comprise three distinct groups of proteins, high M_r kininogens (H-kininogens) with an M_r of 120 000 (Gounaris *et al.*, 1984), low M_r kininogens (L-kininogens) with an M_r of 68 000 (Pagano *et al.*, 1984) and T-kininogens (Barrett, 1987). All members of the cystatin superfamily inhibit most of the cysteine proteinases of the papain superfamily. Thus, cathepsin L is strongly inhibited by stefin A and B (Green *et al.*, 1984; Kominami *et al.*, 1982), cystatin C (Barrett *et al.*, 1984), L- and H-kininogens (Barrett, 1987) and T-kininogen (Moreau *et al.*, 1989).

Although cathepsins and stefins normally occur in separate subcellular compartments, freezing of liver tissue causes rupturing of the lysosomes and, upon subsequent thawing and homogenisation of the tissue, lysosomal cathepsin L may complex with cytosolic stefin B. This was circumvented in the purification methods of Kirschke *et al.* (1977a) and Dufour *et al.* (1987) by isolation of cathepsin L from lysosomes, from rat and chicken livers, respectively. Since the isolation of lysosomes requires the use of fresh liver tissue and is also technically demanding, the discovery by Mason *et al.* (1985) that cathepsin L from

frozen human liver could be activated by a process known as "autolysis", provided a practical alternative. Autolysis involves acidification of the liver homogenate to pH 4.2 and incubation at RT, a procedure which is thought to disrupt enzyme/inhibitor complexes and/or activate latent enzymes (Mason *et al.*, 1985). The mechanism of autolysis is not known, but it does not seem to be of a proteolytic nature, because neither Matsumoto *et al.* (1983), nor Mason *et al.* (1985) could inhibit the activation process by using inhibitors of most known classes of proteolytic enzymes. In the context of this study; particularly in an attempt to increase the yield of free cathepsin L for sequencing purposes, it was considered important, however, to establish whether the formation of proteolytically active, covalent complexes could be prevented by avoiding autolysis, or alternatively, whether adjustment of the pH of isolated complexes to 4.2 could lead to their dissociation.

Tissue destruction occurs in pathologies such as invasive cancer, and this could provide a mechanism whereby the lysosomal cathepsin L could encounter the cytoplasmic stefin B. In this way, proteolytically active cathepsin L/stefin B complexes may be formed in cancer tissue and, consequently, proteolytically active complexes of cystatins with proteinases, such as cathepsin L, might be relevant to invasive cancer. It was, therefore, considered to be of interest to determine whether similar complexes could be demonstrated in human tissue.

Fresh human tissue, especially liver is not readily available in South Africa, but, fortuitously, liver tissue from another higher primate, the baboon (*Papio ursinus*), became available as a by-product of an unrelated research project. Consequently the isolation of cathepsin L/stefin B complexes from baboon liver was attempted as a preliminary study to ascertain whether the expense of going to a laboratory in the United States of America, where human liver tissue was available, was warranted. In the light of supportive evidence for the presence of such complexes in baboon liver homogenates, which manifested properties with potential physiological significance, pursuing this option was considered justified and consequently cathepsin L was also studied in human liver tissue.

4.2 Purification of free and stefin B-complexed cathepsin L from sheep liver and kidney

4.2.1 Reagents

Extraction solution [1% (m/v) NaCl, 0.1% (m/v) Na_2EDTA, 2% (v/v) n-butanol]. NaCl (10 g) and Na_2EDTA (1.0 g) were dissolved in 950 ml cold dist.H_2O, n-butanol (20 ml) was added, thoroughly mixed in and the solution was made up to 1 litre.

Homogenisation buffer [100 mM Na-acetate, 1% (m/v) NaCl, 0.1% (m/v) Na_2EDTA, 2% (v/v) n-butanol, pH 4.5]. Glacial acetic acid (5.72 ml), NaCl (10 g) and Na_2EDTA (1.0 g) were dissolved in 950 ml cold dist.H_2O, n-butanol (20 ml) was added, the solution was titrated to pH 4.5 with NaOH, and made up to 1 litre.

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 of 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 70 ml of the supplied hydrated gel in 140 ml of buffer A, and packing the resulting slurry into a glass column under gravity. The column bed was initially regenerated with two column volumes of 1 M NaCl in Buffer A. The gel was equilibrated with five column volumes of 200 mM NaCl in Buffer A, before use. In between purification procedures, the column was routinely regenerated with one column volume of 1 M NaCl in Buffer A.

Sheep liver and kidney. Fresh sheep livers and kidneys were obtained from Cato Ridge Abattoir, cut into strips and frozen at -70°C for at least 3 days before use. Organs were generally not stored for longer than 3 weeks before use.

Sheep liver and kidney pH 5.0 homogenates. Fresh sheep livers and kidneys were cut into small pieces, mixed 1:2 with homogenisation buffer, and homogenised in a Waring blendor for 2.5 min, immediately following organ collection. The resulting homogenates were frozen at -70°C for at least three days before use. It was empirically established that a pH of 4.5 was required in the homogenisation buffer to bring the final pH of the homogenate to 5.0.

4.2.2 Procedure

Cathepsin L was isolated essentially as described by Pike and Dennison (1989b), with the modifications described by Pike *et al.* (1992). Frozen liver or kidney was thawed, cut into small pieces and mixed 1:2 with extraction solution, and homogenised in a Waring blendor for 2.5 min. The resulting homogenate or thawed pH 5.0 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 for 16 h at RT, to effect activation of the enzyme. The mixture was centrifuged (6 000 x g, 20 min, 4°C) and the supernatant was collected for TPP. Three phase partitioning was effected by adding and mixing in 30% (v/v) of t-butanol (in this step, initially, the supernatant was at 4°C and the t-butanol was at 25°C; subsequent steps were

done at 4°C). Ammonium sulfate [20% (w/v), based on the volume of the pH 4.2 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 TPP supernatant and subnatant were poured away from the precipitate, which was discarded. A further amount of $(NH_4)_2SO_4$ was added, to bring the solution to 30% (m/v) $(NH_4)_2SO_4$, and well stirred. Upon complete dissolution of the salt, the mixture was again centrifuged (6 000 x g, 15 min, 4°C), and the interfacial precipitate was collected for further purification.

The precipitate from TPP was redissolved, in one fifth of the pH 4.2 supernatant volume, in buffer A. The pH was readjusted to 5.5 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 (2.5 x 14 cm = 70 ml), equilibrated with buffer A containing 200 mM NaCl, and eluted with a 200-600 mM NaCl gradient in buffer A. Fractions corresponding to the peak of activity, eluted at about 400 mM NaCl, were pooled and concentrated by dialysis against sucrose, before chromatography on a calibrated column of Sephadex G-75 (2.5 x 90 cm = 450 ml), equilibrated with buffer A containing 200 mM NaCl. Peaks with activity against azocasein (Section 4.6.1) or Z-Phe-Arg-NHMec (Section 4.6.2) (K_{av} = 0.27 and 0.43) were collected for further study.

4.3 Purification of cathepsin L from baboon and human liver

4.3.1 Reagents

Baboon liver. This was obtained from Dr Jean Petit, Department of Dentistry, University of Durban-Westville, Durban.

Human liver. This was obtained from Dr Hillary Newland, Department of Pathology, Athens Regional Medical Centre, Athens, Ga, USA, immediately following surgery, and frozen at -20°C. Before use, the liver was tested for both HIV and hepatitis B infection and was found to be negative.

4.3.2 Procedure

The purification of baboon and human liver cathepsin L was carried out as described for the sheep liver and kidney cathepsin L (Section 4.2).

4.4 Isolation of stefin B from sheep liver

4.4.1 Preparation of carboxymethyl-papain-Sepharose column

Carboxymethyl (CM)-papain was prepared and coupled to CNBr-activated Sepharose 4B according to Anastasi *et al.* (1983).

4.4.1.1 Reagents

CNBr-activated Sepharose 4B. This was prepared as described in Section 2.10.

Activation buffer (2 mM cysteine.HCl, 1 mM Na_2EDTA, 100 mM NaH_2PO_4, pH 6.0). Cysteine.HCl (0.04 g), Na_2EDTA (0.037 g) and NaH_2PO_4 (1.38 g) were dissolved in 90 ml of dist.H_2O, titrated to pH 6.0 with NaOH, and made up to 100 ml.

4.4.1.2 Procedure

The papain preparation contained 83% protein, and 60 mg was, therefore, weighed out to give 50 mg of protein. This was activated in 7.5 ml of activation buffer for 10 min at RT. Following this 10 mM iodoacetate (500 µl) was added and allowed to react with the activated papain for 15 min to form CM-papain. This solution was dialysed overnight against 10 volumes of coupling medium (Section 2.10.1).

CM-Papain in coupling medium (25 ml) was added to the CNBr-activated Sepharose 4B (25 g wet weight, also in 25 ml of coupling buffer). Coupling was facilitated by end-over-end mixing on a Heidolph mixer overnight at 4°C. The gel was treated for 1 h at RT with 100 mM glycine, to block excess activated groups, and washed with two volumes of cold 100 mM Na-citrate, pH 3.0, and two volumes of 100 mM Na-phosphate, pH 11.0. Conjugation efficiency was determined from measurements of the A_{280} of the CM-papain solution before and after conjugation and was found to be about 94%.

The CM-Papain-Sepharose was finally washed with loading buffer (Section 4.4.2.1) and packed in a column (2.5 x 5 cm = 25 ml). Weakly bound ligand was

eluted with one column volume of elution buffer (Section 4.4.2.1), before the column was equilibrated with 10 column volumes of loading buffer, at a linear flow rate of 10 cm/h.

4.4.2 Purification of stefin B from sheep liver

4.4.2.1 Reagents

Loading buffer [50 mM $NaH_2PO_4.H_2O$, 0.5 M NaCl, 0.1% (m/v) Brij 35, 0.02% (m/v) NaN_3, pH 6.5]. $NaH_2PO_4.H_2O$ (6.9 g), NaCl (29.22 g), NaN_3 (0.2 g) and Brij 35 (1 g) were dissolved in 950 ml of dist.H_2O, titrated to pH 6.5 with NaOH, and made up to 1 litre.

Elution buffer [50 mM $K_2HPO_4.3H_2O$, 0.5 M NaCl, 0.1% (m/v) Brij 35, 0.02% (m/v) NaN_3, pH 11.5]. $K_2HPO_4.3H_2O$ (5.71 g), NaCl (14.61 g), NaN_3 (0.1 g) and Brij 35 (0.5 g) were dissolved in 450 ml of dist.H_2O, titrated to pH 6.5 with NaOH, and made up to 500 ml.

4.4.2.2 Procedure

A cystatin fraction was isolated from sheep liver as described by Green *et al.* (1984), for the isolation of the inhibitor from human liver. Frozen sheep liver (450 g) was thawed, cut into small pieces, mixed 1:2 with 1% (m/v) NaCl/2% (v/v) n-butanol/3mM Na_2EDTA and homogenised for 2.5 min, at 4°C, in a Waring blendor. This homogenate was centrifuged (2 000 x g, 30 min, 4°C) and the resulting supernatant was filtered through butter muslin under vacuum. The supernatant was adjusted to pH 11.0 by the addition of 3 M NaOH, with stirring, to denature cysteine proteinases complexed to the inhibitor. This solution was left to stir for 2 h at 4°C, and the pH was adjusted to 6.5 by the gradual addition of 2 M HCl. Precipitated protein was removed by centrifugation (4 000 x g, 30 min, 4°C).

The supernatant (at 4°C) was placed in an ice/NaCl bath and an equal volume of acetone at -20°C was run in, with stirring, over a period of 10 min. The mixture was centrifuged (4 000 x g, 30 min, 4°C) and the precipitate was discarded. Acetone was added to the supernatant, at the same volume as before, with stirring, over 10 min. The mixture was centrifuged as before (4 000 x g, 30 min, 4°C) and the precipitate was collected. The pellet was extracted overnight at 4°C, with stirring, in 75 ml of loading buffer. The undissolved material was removed by centrifugation (10 000 x g, 30 min, 4°C).

The redissolved protein was applied to a CM-papain Sepharose column (2.5 x 5 cm = 25 ml) equilibrated in loading buffer. The column was washed until the A_{280} reached baseline, whereupon the bound protein was eluted using elution buffer. The single peak of protein collected was adjusted to pH 6.5, concentrated by dialysis against sucrose in M_r 12 000 cut-off dialysis tubing, and further fractionated on Sephadex G-75 (2.5 x 90 cm = 450 ml), equilibrated with buffer A (Section 4.2.1) containing 200 mM NaCl and 1 mM DTT. The DTT reduces stefin B dimers and ensures elution of stefin B in a single peak. Fractions were assayed, using Z-Phe–Arg–NHMec, for their inhibitory activity against 10 μM papain; the active fraction at M_r 12 - 14 000 was collected, and dialysed against loading buffer and concentrated by dialysis against sucrose in M_r 12 000 cut-off dialysis tubing.

4.5 Production of anti-sheep liver cathepsin L and stefin B antibodies

Antibodies against sheep liver cathepsin L were raised in chickens by intramuscular injections, at two sites in the breast muscle, with a total of 50 μg cathepsin L emulsified in Freund's complete adjuvant in a 1:1 (v/v) ratio. Two further weekly inoculations, in the same manner and at the same dose, were administered in Freund's incomplete adjuvant, followed by two inoculations at two week intervals, and finally at monthly intervals. Eggs were collected continuously throughout the immunisation period. Chicken anti-sheep liver cathepsin L (IgY) antibodies were purified from egg yolks by precipitation with PEG (Section 2.7.3).

Antibodies were raised against sheep liver stefin B in rabbits by immunising rabbits subcutaneously at 5-6 sites on the back, with a total of 100 μg of sheep liver stefin B emulsified in a 1:1 ratio with Freund's complete adjuvant. Further inoculations were administered, in the same manner, in Freund's incomplete adjuvant, using the same dose, after two weeks and then at 4-week intervals. Blood was collected from the marginal ear vein at 3 and 8 weeks and rabbit anti-sheep liver stefin B (IgG) antibodies were purified from the serum by precipitation with PEG (Section 2.7.2).

Chicken and rabbit antibodies were characterised by Western blotting (Section 2.8) against crude and purified cathepsin L and stefin B fractions. The crude fraction, containing both cathepsin L and stefin B, was obtained by a modification of the cathepsin L purification procedure (Section 4.2) in which all of the proteins bound to S-Sepharose were eluted with 1 M NaCl.

4.6 Enzyme Assays

Cathepsin L is the most effective lysosomal endoproteinase against protein substrates and digests azocasein (Barrett and Kirschke, 1981), collagen (Kirschke *et al.*, 1982) and elastin (Mason *et al.*, 1984) several fold faster than the related cathepsins, B and H. The proteolytic activity of cathepsin L is usually assayed with azocasein in the presence of 3 M urea, which not only sensitises azocasein to proteolysis, but also enhances the specificity of the assay for cathepsin L, which is stable to 3 M urea, at pH 5, whereas cathepsin B is inactivated (Kirschke *et al.*, 1982). Pepstatin is also included to inhibit any residual cathepsin D activity.

Although there is no specific synthetic substrate for cathepsin L, which requires a hydrophobic group in position P_2 (Schechter and Berger, 1967; Katunuma *et al.*, 1983; Mason *et al.*, 1984), Z-Phe-Arg-NHMec, which is also hydrolysed by cathepsin B, has been found to be a very suitable and sensitive substrate for cathepsin L (Barrett and Kirschke, 1981).

In the present study the azocasein assay was used routinely, while the synthetic substrate was used in cases where a higher degree of sensitivity was required.

4.6.1 Azocasein assay

Studies by Langner *et al.* (1973) on the preparation and properties of azocasein, showed that it is a suitable substrate for activity measurements of proteolytic enzymes. Azocasein is formed by derivatisation of the tyrosine and histidine residues in casein, by coupling with diazotised sulphanilic acid or sulphanilimide in an alkaline medium. Proteolytic degradation of azocasein results in the release of peptides which are soluble in TCA, while the larger undegraded fragments are precipitated by TCA. The soluble fragments have an intensely yellow colour, by virtue of the attached azo-groups, which can be quantified by measuring their A_{366}. Although the absorption maximum of azocasein is at 334 nm, the measurement at 366 nm, introduced by Langner *et al.* (1973), because of the limitations of their spectrophotometer, has been widely adopted. Absorbance measurements at 366 nm are acceptable, however, since this gives 75% of the extinction at 334 nm. In the present study, azocasein was used as described by Barrett and Kirschke (1981).

4.6.1.1 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), $Na_2EDTA.2H_2O$ (0.185 g) and NaN_3 (0.1 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 5.0 with NaOH, pepstatin (500 µg) was added and the solution was made up to 500 ml. Shortly before the assay, cysteine.HCl (0.04 g) was added to 5 ml of the buffer.

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

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

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

4.6.1.2 Procedure

Sample (200 µl) was mixed with assay buffer containing cysteine (200 µl). After activation, for 5 min at 37°C, azocasein/urea solution (400 µl) was added. A sample (200 µl) of the mixture was immediately withdrawn and mixed with 5% TCA (1 ml) in a polyethylene microfuge tube. This served as the blank for the reaction. After a specified period of time (usually 30 min. or 2 h for dilute fractions from MEC), a further sample (200 µl) was withdrawn and mixed with TCA (1 ml). The samples in the 1.5 ml centrifuge tubes were centrifuged in a Sigma mini-centrifuge (12 000 x g, 5 min, RT). The A_{366} values of the supernatants were read using glass micro-cuvettes with blacked-out sides, which prevented transmission of light around the sample. Transmission of light around the sample was found to cause a non-linear response in the assay.

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

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

$$\% \text{ Hydrolysis } (\% \text{ H}) = \frac{A_{366} - 0.0223}{0.0436}$$

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

$$\text{Units} = \frac{(\% \text{ H} \times 10^{-2}) \times 2000 \text{ µg azocasein}}{\text{time}}$$

Hence

Units/ ml = 5 x units.

4.6.2 Synthetic substrate assay

The most commonly used synthetic substrate for cathepsin L is Z-Phe-Arg-NHMec which liberates the intensely fluorescent 7-amino-4-methylcoumarin group upon hydrolysis (Barrett and Kirschke, 1981). This substrate is, however, also cleaved by other proteinases such as cathepsin B and plasma and tissue kallikreins. The specificity of the hydrolysis of Z-Arg-Arg-NHMec by cathepsin B can be used to account for cathepsin B activity, whereas cathepsin L activity can be confirmed by the use of suitable inhibitors in the assay. The assays used in this study were carried out as described by Barrett and Kirschke (1981).

4.6.2.1 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.

Z-Arg-Arg-NHMec assay buffer [352 mM KH_2PO_4, 48 mM Na_2HPO_4, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 2.7 mM Cysteine.HCl, pH 6.0]. KH_2PO_4 (23.95 g), $Na_2HPO_4.2H_2O$ (4.27 g), $Na_2EDTA.2H_2O$ (0.75 g) and NaN_3 (0.1 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 500 ml. Prior to the assay, cysteine.HCl (0.0024 g) was added to 5 ml of buffer.

1 mM substrate stock solutions. Z-Phe-Arg-NHMec (1 mg) and Z-Arg-Arg-NHMec (1.1 mg) were dissolved in separate volumes of DMSO (1.5 ml) and stored at 4°C.

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

Brij 35 diluent. 0.1% (m/v) Brij 35 was used to dilute enzyme samples to the correct concentration for the assay.

Stopping reagent (100 mM monochloroacetate, 30 mM Na-acetate, 70 mM acetic acid, pH 4.3). Monochloroacetate (9.45 g), Na-acetate.$3H_2O$ (4.08 g) and glacial acetic acid (4 ml) were dissolved in 950 ml of dist.H_2O, titrated to pH 4.3 with NaOH and made up to 1 litre. Great care was taken with the highly corrosive monochloroacetate.

1 mM aminomethyl coumarin standard. 7-amino-4-methyl coumarin (1.8 mg) was dissolved in DMSO (10 ml). The standard was used as a 0.5 µM solution by diluting stock solution (5 µl) in a 1:1 mixture of the assay buffer and stopping reagent (10 ml).

4.6.2.2 Procedure

Usually 1-5 ng of cathepsin L, or 25-250 ng cathepsin B was used. The appropriate volume of enzyme solution was diluted to 500 µl with diluent, followed by the addition of assay buffer (250 µl). After a 1 min activation period at 30°C, substrate (250 µl) was added and incubated for 10 min at 30°C. Stopping reagent (1 ml) was added and the solution was briefly vortexed. A blank was prepared by adding stopping reagent before the enzyme. Fluorescence was read on a Hitachi F-2000 spectrofluorimeter with excitation at 370 nm and emission at 460 nm. Continuous assays in a temperature controlled

cell were also carried out in much the same way (i.e. by eliminating the addition of the stopping reagent) to assess the linearity of the reactions.

According to Barrett and Kirschke (1981), the reading given by the 0.5 µM standard can be taken to be equal to 1000 arbitrary enzyme units, which in turn is equal to 0.1 mU of enzyme activity. The reading given by a 0.5 µM standard was 10 400 and thus milliunits of activity in a sample could be calculated as follows:

$$\text{mUnits activity} = \frac{\text{Fluorescent intensity x 0.1mU}}{10\ 400}$$

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

4.7 Measurement of inhibition characteristics of free and stefin B-complexed cathepsin L

Inhibition assays, to characterise the activity of free and stefin B-complexed cathepsin L were carried out using the concentrations of inhibitors described by Kirschke *et al.* (1977a) and Mason *et al.* (1984). The irreversible inhibitors, iodoacetate, iodoacetamide, EDTA, E-64, and Z-Phe-Phe-CHN_2 were pre-incubated with the enzymes for 15 min at 30°C before the enzymes were assayed at pH 5.5 against Z-Phe-Arg-NHMec, while the reversible inhibitors leupeptin, PMSF, TLCK, TPCK, and pepstatin were assayed directly, without pre-incubation with the enzymes (Kirschke *et al.*, 1977a).

Active site titration of the different forms of cathepsin L with the inhibitor E-64 was done as described by Barrett and Kirschke (1981). Enzyme (25 µl) was incubated together with the inhibitor (25 µl of a 1-10 µM solution) for 30 min at 30°C in the presence of the pH 5.5 assay buffer, containing activator (50 µl). After 30 min the mixture was diluted to 1 ml by the addition of Brij 35 diluent, and aliquots (25 µl) were assayed for activity as described in section 4.6.2. For the titration of complex against azocasein using E-64, the above procedure was followed but the incubation mixture with E-64 was not diluted for the assay (Section 4.6.1).

In tests to determine the effect of increasing cysteine concentrations on the molar concentrations of active enzyme, equimolar amounts of the different forms of cathepsin L were titrated with E-64 as described above, except that the incubation was carried out in pH 5.5 assay buffer containing a range of cysteine concentrations (0-200 mM final concentration). The final assay of residual activity was carried out using the same cysteine concentration as used during the respective incubation steps.

The rates of inactivation of free and stefin B-complexed cathepsin L by E-64 and Z-Phe-Phe-CHN_2 were determined as described by Barrett *et al.* (1982). Free or stefin B-complexed cathepsin L (1×10^{-8} M) was mixed at zero time with a five fold excess of inhibitor (1×10^{-7} M) in the Brij 35 diluent (500 µl) and Z-Phe-Arg-NHMec buffer (500 µl) at 37°C, in the presence of 2 mM DTT. Samples (25 µl) were removed at 30, 60, 120, 180, 240 and 300s, and added to the Z-Phe-Arg-NHMec substrate assay mixures, and stopped time assays were carried out at 37°C for 10 min. The effect of each inhibitor, on both free or stefin B-complexed cathepsin L, was tested in triplicate for each time interval. Semilogarithmic plots of the mean fluorescence intensity values against time were constructed, analysed by linear regression, and the slopes of the regression lines were used to calculate the observed (pseudo first-order) rates of inactivation, k_{obs}, using the equation (Segel, 1976):

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

Inhibition of cathepsin L, and the isolated cathepsin L/stefin B complex, by sheep liver stefin B, was carried out at pH 6.0 with 50 nM active cathepsin L or complex. Stefin B samples in Brij-35 diluent (0-90 µl, i.e. 0-100 ng) were mixed with assay buffer, containing 8 mM DTT (50 µl), and made up to 140 µl with Brij 35 diluent. Free or isolated stefin B-complexed cathepsin L (20 µl) was added and the mixture was incubated for 10 min at 30°C. Assay buffer (without DTT) (4.0 ml) and Brij 35 diluent (8.8 ml) were mixed and 590 µl of this mixture was added to the incubation mixtures. After equilibration for 1 min, Z-Phe-Arg-NHMec (250 µl) was added and the residual activity measured in a stopped time assay (Section 4.6.2).

4.8 Measurement of the effect of pH and cysteine concentration on the activity of free and stefin B-complexed cathepsin L

The pH for optimum hydrolysis of Z-Phe-Arg-NHMec by free and stefin B-complexed cathepsin L was determined using the acetate-MES-Tris (AMT) buffers of constant ionic strength across a pH range, described by Ellis and Morrison (1982). These buffers were also used in assays to establish what effect cysteine concentration has on both forms of cathepsin L.

4.8.1 Reagents

Acetate-MES-Tris (AMT) buffers (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 solution was split into 10 aliquots (20 ml), each of which was titrated to pH values in the range of 4-8.5 using HCl or NaOH, and diluted to 25 ml.

4.8.2 Procedure

Standard stopped time assays (Section 4.6.2) were carried out at 30°C over the pH range 4-8.5, in AMT buffers containing 2 mM DTT (final concentration).

The activity of equimolar amounts of free and stefin B-complexed cathepsin L against Z-Phe-Arg-NHMec was determined at pH 5.5 and 7.0, in the presence of a range of cysteine concentrations between 0 and 800 mM, in standard 10 min stopped time assays. After the addition of the required amount of cysteine to each buffer solution, the pH was titrated back to 5.5 and 7.0 respectively, before use.

4.9 Results

4.9.1 Isolation of cathepsin L from sheep liver

Sephadex G-75 chromatography (Fig. 11) revealed that the active peak from S-Sepharose ion-exchange chromatography, at pH 5.5, could be resolved into two proteolytically active peaks, with M_r 37 000 and 26 000. Both peaks had activity against azocasein and Z-Phe-Arg-NHMec (Fig. 11), but were not active against Z-Arg-Arg-NHMec, the synthetic substrate for cathepsin B (results not shown). The activity of both was characterised as being that of cathepsin L, on the basis of the results obtained with a range of inhibitors and activators previously used to characterise cathepsin L (Kirschke *et al.*, 1977a; Mason *et al.*, 1984) (Table 10).

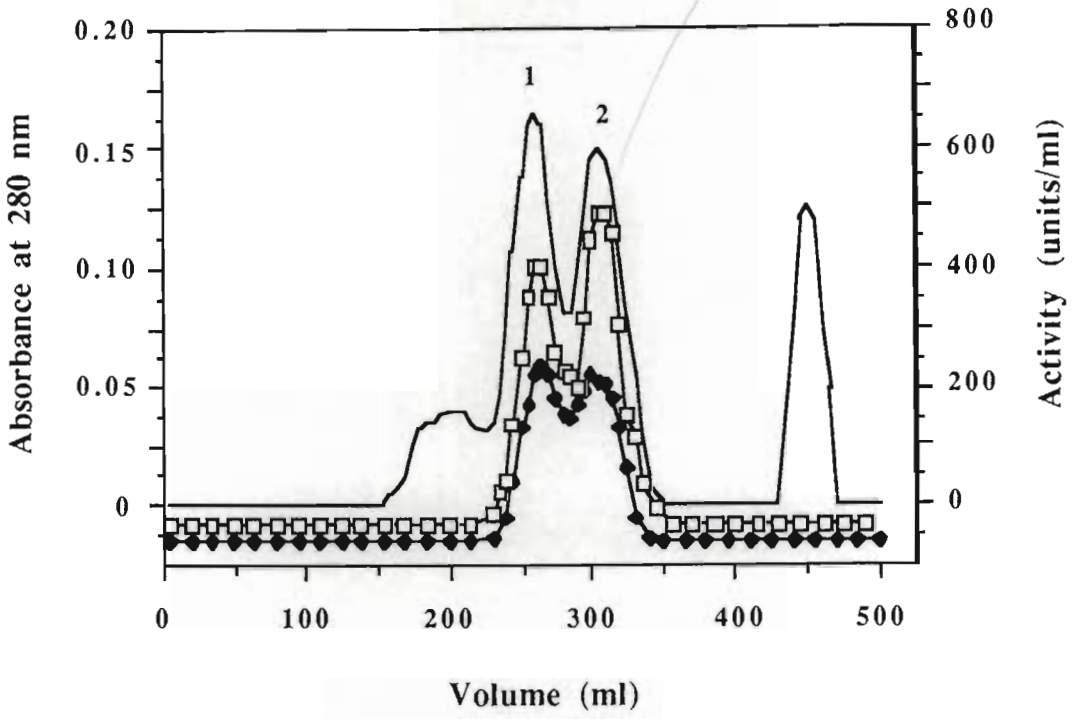

Figure 11. Molecular exclusion chromatography of the pH 5.5 S-Sepharose fraction from sheep liver on Sephadex G-75.
Column, 2.5 x 87.5 cm (430-ml bed volume); buffer, 20 mM Na-acetate, pH 5.5, containing 1 mM Na_2EDTA and 200mM NaCl; flow rate, 25 ml/h (5 cm/h); and fractions, 5 ml (12 min). Void volume was 150 ml. (—), A_{280}; (◆), enzyme activity (units/ml) from the assay against azocasein; and (□), from the assay against Z-Phe-Arg- NHMec. For clarity these traces have been displaced slightly downwards. 1, complexed cathepsin L; 2, free cathepsin L.

Table 10. The effect of inhibitors and the absence of DTT on free and complexed forms of sheep liver cathepsin L.

Inhibitor	Final concentration (μM)	% activity	
		Complexed (M_r 37 000 form)	Free (M_r 26 000 form)
E-64	1	3	6
Iodoacetamide	1000	10	15
Leupeptin	1	2	1
Pepstatin	1	98	96
PMSF	250	95	90
SBTI	1	80	71
Z-Phe-Phe-CHN_2	1	2	3
DTT	0	10	5

On SDS-PAGE, with reduction, the M_r 37 000 peak (Fig. 11, peak 1) separated into two components, of M_r 26 000 and M_r 14 000, while the M_r 26 000 peak (peak 2) yielded a single band of M_r 26 000 (Fig.12A). It therefore appeared that the M_r 37 000 form of cathepsin L comprised a complex of cathepsin L with an M_r 14 000 protein. On non-reducing SDS-PAGE peak 1 yielded bands of apparent relative molecular weights of 42 000, 37 000 and 26 000 with an additional faint band at M_r 68 000 (Fig. 12B). In some preparations where the M_r 26 000 band was more prominent, an additional band at M_r 14 000 was also visible. These results indicate that the proportion of the M_r 37 000 complex which does not dissociate without reduction, represented here by the M_r 68 000, 42 000 and 37 000 forms, is covalently bound to the M_r 14 000 moiety, while the remainder of the complex is non-covalently bound and does not require reduction to yield M_r 26 000 and 14 000 components.

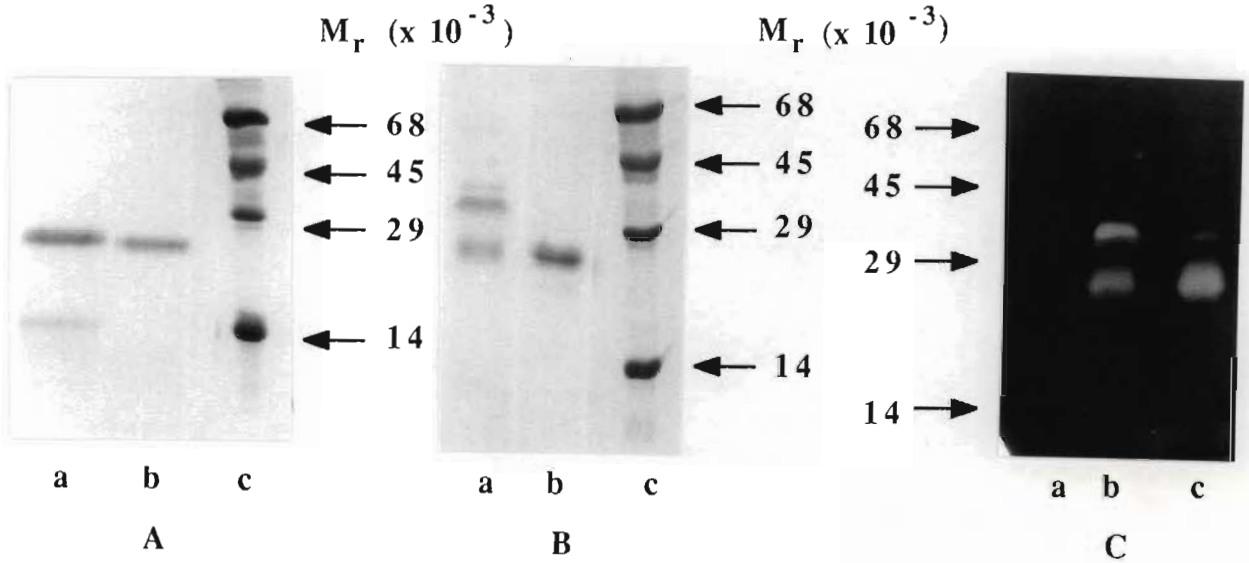

Figure 12: SDS-PAGE of free cathepsin L and isolated sheep liver cathepsin L complexes. (**A**) Samples (5 µg) were reduced and loaded onto a 12.5% gel. (a) Isolated cathepsin L complex; (b) free cathepsin L; and (c) MW markers (phosphorylase b, M_r 97 000; BSA, M_r 68 000; ovalbumin, M_r 45 000; carbonic anhydrase, M_r 29 000; lysozyme, M_r 14 000). (**B**) Non-reduced samples (2.5 µg) were loaded onto a 12.5% gel. (a) Isolated cathepsin L complex; (b) free cathepsin L; and (c) MW markers, as above. (**C**) Non-reduced samples (2.5 µg) were loaded onto a 12.5% gelatin-containing gel. (a) MW markers, as above; (b) isolated cathepsin L complex; and (c) free cathepsin L.

On SDS-PAGE without reduction peak 2 yielded a single band of M_r 26 000 (Fig. 12B). Since there was no difference in M_r on SDS-PAGE, with and without reduction, it suggests that the M_r 26 000 form of cathepsin L was isolated in a single-chain form by this purification procedure, which is in contrast to the two-chain cathepsin L isolated by Mason (1986) from sheep liver. The single-chain nature of the cathepsin L purified using the method of Pike and Dennison (1989b) was confirmed by amino sequence

determination (Section 4.9.11). Free M_r 26 000 single-chain cathepsin L was subsequently used to raise antibodies in chickens to assist in the characterisation of the higher M_r complexes.

To ascertain which of the different components of peak 1 (Fig. 11), revealed by SDS-PAGE without reduction, were proteolytically active, the fractions were examined by SDS-PAGE in a gel containing gelatin as a substrate (Section 2.6). This showed that the M_r 37 000 and 26 000 components were proteolytically active while the M_r 14 000 component was inactive (Fig. 12C). In some preparations, however, the M_r 26 000 component of peak 1 was inactive. The M_r 68 000 component was usually present in cathepsin L/stefin B complex fractions eluting immediately after the void volume and showed activity on gelatin-containing substrate gels. Gelatinolytic activity of the M_r 42 000 form was usually only evident in complexes formed *in vitro* (see later, Section 4.9.5). The single M_r 26 000 component of peak 2 was proteolytically active (Fig. 12C). The proteolytic activity of the components of peaks 1 and 2 on substrate containing SDS-PAGE could be extinguished by either Z-Phe-Phe-CHN_2, E-64 or leupeptin (results not shown) thus indicating that all of the activity is due to forms of cathepsin L.

Since the M_r of 14 000 of the complexing moiety resembles that of the cystatins, a group of natural inhibitors of cysteine proteinases, of which the type 1 cystatins or stefins have been isolated from human and rat liver (Green *et al.*, 1984; Wakamatsu *et al.*, 1984), it was thought that the M_r 37 000 form may consist of covalent and non-covalent complexes of cathepsin L and a stefin. In order to find direct proof for this hypothesis, specific antibodies were produced against the stefin isolated from sheep liver, and used in Western blots in parallel with anti-cathepsin L antibodies for the characterisation of the cathepsin L/stefin complexes. The stefin was identified as stefin B by determination of its amino acid sequence (Section 4.9.11).

4.9.2 Isolation and characterisation of sheep liver stefin B

A single peak, inhibitory toward papain, was eluted from the CM-papain column. Non-reducing SDS-PAGE of the affinity-purified stefin fraction showed a prominent band at M_r 14 000, as well as several contaminating higher M_r bands, of which a band at M_r 28 000 was the most prominent. Since stefin B occurs in both monomer and dimer forms (Ritonja *et al.*, 1985) and the monomer could additionally form mixed disulfides with glutathione (Wakamatsu *et al.*, 1984), further purification by MEC was done in the presence of 1 mM DTT, which ensured that all the forms of stefin B eluted in a single peak, at an M_r of 14 000, well resolved from contaminants eluting at the void volume (V_o) (Fig. 13). This approach obviated the need for further sub-fractionation on a strong anion

exchanger (see below) and resulted in the purification of a homogenous stefin B preparation, for amino acid sequencing. Stefin B absorbs poorly at 280 nm, as reflected by the relative sizes of the stefin B and V_0 peaks, which contain the same amount of protein, since it contains only 3 Tyr-, 7 Phe- and no Trp-residues (Section 4.9.11), the respective phenolic and indolic groups of which contribute largely to the absorption of proteins at 280 nm (Dunn, 1989). Stefin B showed a single band at M_r 14 000 on reducing SDS-PAGE and an additional band at M_r 28 000 under non-reducing conditions (Fig. 14), which may be attributed to the dimer form of the inhibitor. It was found that the relative amounts of the M_r 28 000 dimer present varied from liver to liver.

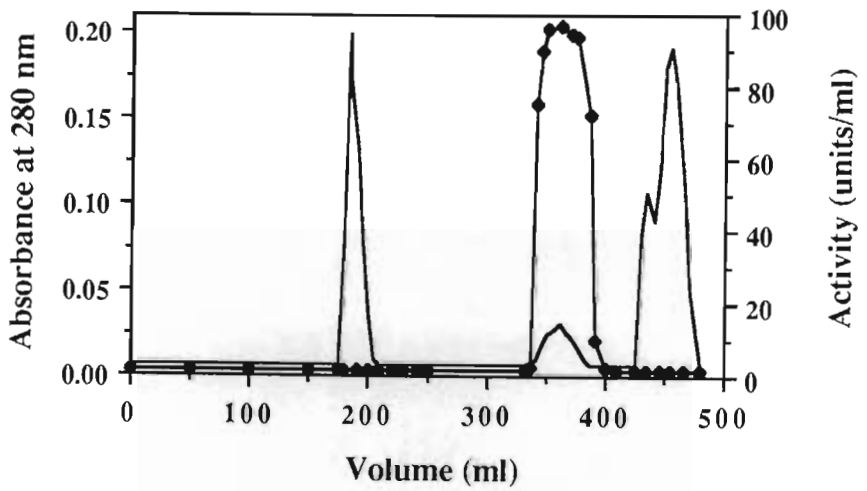

Figure 13. Molecular exclusion chromatography of the CM-papain affinity eluate, containing stefin B from sheep liver, on Sephadex G-75.
Column, 2.5 x 87.5 cm (430 ml bed volume); buffer, 20 mM Na-acetate, pH 5.5, containing 1 mM Na_2EDTA, 200mM NaCl and 1 mM DTT; flow rate, 25 ml/h (5 cm/h); and fractions, 5 ml (12 min). Void volume was 150 ml. (—), A_{280}; (◆), inhibitor activity, expressed as the percentage inhibition of 10 μM papain activity against Z-Phe-Arg- NHMec.

The types of cystatin present in sheep liver were characterised in a previous study by sub-fractionation of the CM-papain-fraction on a Q-Sepharose anion-exchanger, followed by reducing and non-reducing SDS-PAGE and PAGE in the presence and absence of DTT (Pike, 1990). By analogy with the results of Wakamatsu *et al.* (1984), it was concluded that all the forms of cystatin in sheep liver are probably of the stefin B-type, containing reactive cysteine residues, to which various substituents may be attached. In the present study the cystatin in sheep liver was unequivocally identified as stefin B, based on the amino acid sequence determined in a collaboration with Dr Anka Ritonja of the Stefan Institute, Ljubljana, Slovenia (Section 4.9.11).

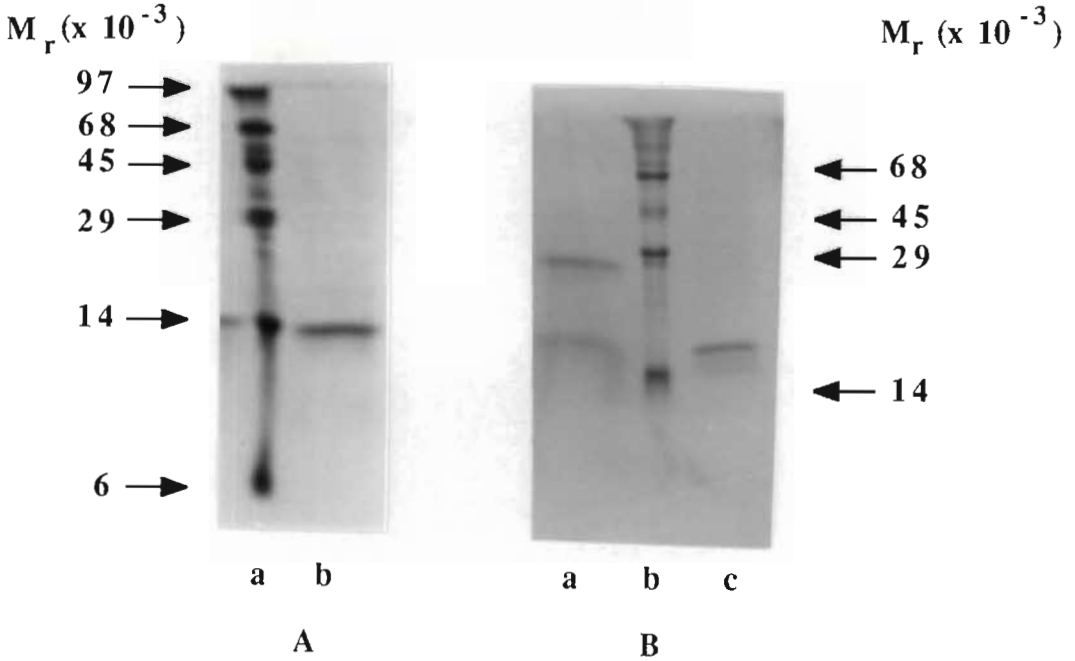

Figure 14. SDS-PAGE of stefin B isolated from sheep liver.
(**A**) Reducing 15% SDS-PAGE of (a) MW markers, as in Fig. 12 (with insulin M_r 6 000); and (b) stefin B (5 µg). (**B**) Non-reduced samples (2.5 µg) were loaded onto a 15% gel. (a) stefin B sample dialysed against DTT-free buffer; (b) MW markers, as in Fig. 12; and (c) stefin B containing 1 mM DTT.

When tested for its capacity to inhibit purified cathepsin L and isolated stefin B-complexed cathepsin L, the isolated sheep liver stefin B was found to inhibit both forms of the enzyme in a dose-dependent manner (Fig. 15).

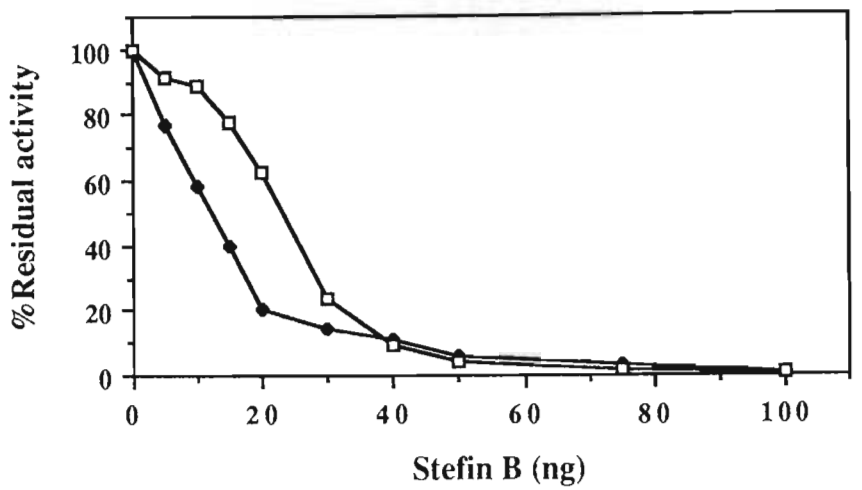

Figure 15. Inhibition, of isolated stefin B-complexed and free forms of sheep liver cathepsin L, by sheep liver stefin B.
Cathepsin L (50 nM active enzyme) and stefin B-complexed cathepsin L (50 nM active enzyme) were incubated with increasing amounts of stefin B, at pH 6.0, and residual activity was assayed using Z-Phe-Arg-NHMec. (□), cathepsin L; and (■), isolated stefin B-complexed cathepsin L.

4.9.3 Characterisation of anti-cathepsin L and anti-stefin B antibodies

The specificity of the antibodies raised against free M_r 26 000 cathepsin L and stefin B, in chickens and rabbits respectively, for the characterisation of the cathepsin L/stefin B complexes, was tested against a crude S-Sepharose ion-exchange fraction from sheep liver. The chicken anti-cathepsin L IgY antibody preparation was specific for cathepsin L and did not target any other components in the crude extract and also did not target any stefin B (Fig. 16). Similarly, the rabbit anti-stefin B IgG antibody preparation was specific for stefin B and did not target any other components of the crude extract and did not target purified cathepsin L (Fig. 16).

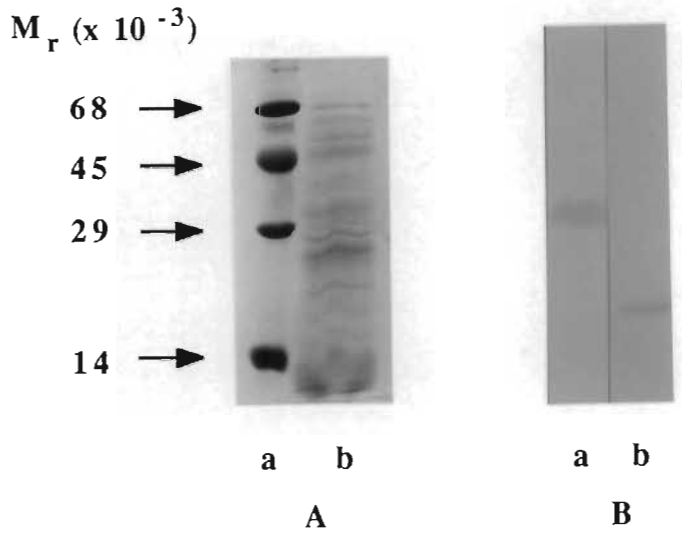

Figure 16. Specificity of anti-cathepsin L and anti-stefin B antibodies.
(**A**) Reducing SDS-PAGE of (a) MW markers as in Fig. 12; and (b) the crude fraction eluted from S-Sepharose (25 µg). (**B**) Western blots of the crude fraction eluted from S-Sepharose, probed with (a) chicken anti-cathepsin L IgY (5 µg/ml); and (b) rabbit anti-stefin B IgG (50 µg/ml).

4.9.4 Characterisation of the stefin B/cathepsin L complexes

Western blotting, after reducing SDS-PAGE, with the monospecific antibodies raised against free sheep liver cathepsin L and sheep liver stefin B (Section 4.9.3), revealed that the M_r 26 000 component of peaks 1 and 2 (Section 4.9.1; Fig. 11) reacted with anti-cathepsin L antibodies and the M_r 14 000 component of peak 1 reacted with anti-stefin B antibodies (Fig. 17A and B). Western blotting, after non-reducing SDS-PAGE, using the same monospecific antibodies, revealed that the M_r 68 000, 42 000 and 37 000 components of peak 1 contained both cathepsin L and stefin B (Fig. 17C and D) and, therefore, represent complexes of cathepsin L and stefin B. By summation of the molecular weights, it may be deduced that the M_r 37 000 and 42 000 forms of the complex probably consist of cathepsin L bonded to one stefin B molecule, and may be conformational variants of one another, while the M_r 68 000 form may comprise two cathepsin L molecules and one stefin B molecule. The SDS-stability of these novel cathepsin L/stefin B complexes is unusual since Anastasi *et al.* (1983) and Wakamatsu *et al.* (1984) showed that complexes between chicken egg white cystatin and papain dissociate on non-reducing SDS-PAGE.

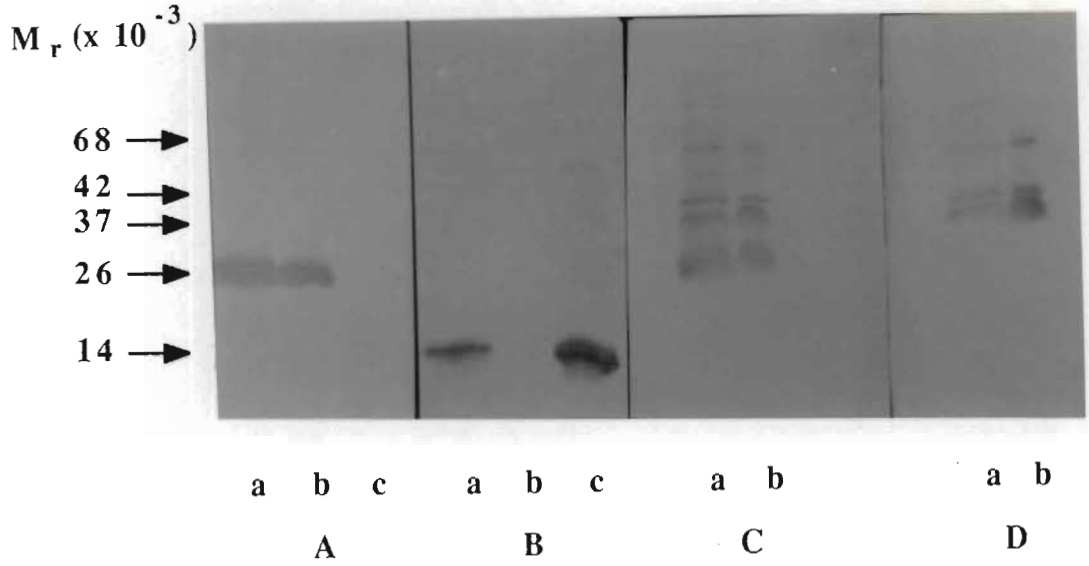

Figure 17. Western blots of free and stefin B-complexed forms of sheep liver cathepsin L. Reduced samples (5 µg), (a) isolated stefin B-complexed cathepsin L; (b) free cathepsin L; and (c) sheep liver stefin B were separated by SDS-PAGE and probed with (**A**) chicken anti-cathepsin L IgY (5 µg/ml) and (**B**) rabbit anti-stefin B IgG (50 µg/ml). Non-reduced samples, (a) the crude fraction eluted from S-Sepharose (25 µg), and (b) isolated stefin B-complexed cathepsin L (2.5µg) were separated by SDS-PAGE and probed with (**C**) chicken anti-cathepsin L IgY (5 µg/ml) and (**D**) rabbit anti-stefin B IgG (50 µg/ml). Indicated molecular weights were determined by SDS-PAGE.

In order to determine whether the M_r 26 000 component, observed in non-reducing SDS-PAGE of the complex samples, was due to contamination by free cathepsin L, resulting from incomplete separation on MEC, the complex fraction was re-chromatographed on Sephadex G-75. Only a single peak eluted at M_r 37 000, which showed the same pattern on non-reducing SDS-PAGE as before. It seems, therefore, that the M_r 37 000 stefin B/cathepsin L complex consists of a mixture of the known non-covalent complex, which dissociates in the presence of SDS, and a novel covalent complex which does not.

Further evidence for this was found by active site titration of free and stefin B-complexed cathepsin L with E-64, which confirmed earlier results of Pike (1990). Using Z-Phe-Arg-NHMec as a substrate, active site titration with E-64 (Barrett and Kirschke, 1981) revealed that the free enzyme fraction titrated completely with E-64, and that approximately 30% of the protein represented active enzyme (Fig. 18), consistent with the amount of active enzyme reported by Mason *et al.* (1985). By contrast, only 60% of the stefin B-complexed cathepsin L activity titrated with E-64, when assayed with Z-Phe-Arg-NHMec (Fig. 18). With azocasein as substrate, however, the activity of the complex titrated completely with E-64 (Fig. 18). These results also suggest the presence of two forms of complex, the known dissociable complex and the novel covalent complex.

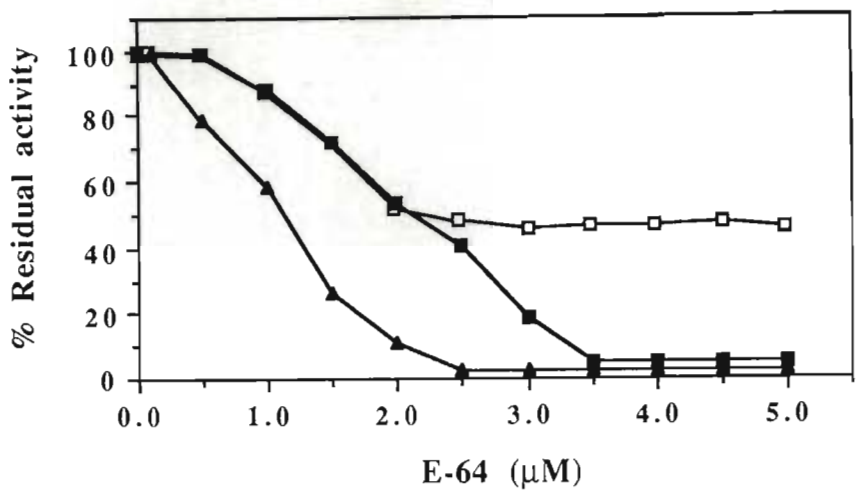

Figure 18. Active site-titration of isolated stefin B-complexed and free forms of sheep liver cathepsin L, using E-64 in assays against Z-Phe-Arg-NHMec and azocasein substrates.
The titrations were carried out as described in section 4.7 on 6.75 μM of isolated stefin B-complexed cathepsin L and 7.6 μM of free cathepsin L. (❑) Isolated stefin B-complexed cathepsin L; and (▲) free cathepsin L, both against Z-Phe-Arg-NHMec. (■) Isolated stefin B-complexed cathepsin L against azocasein.

Chicken egg white cystatin has been found to bind to papain in spite of the presence of E-64, but prior binding of the cystatin to the papain precludes E-64 binding to the enzyme (Nicklin and Barrett, 1984). At the concentrations at which the complex was incubated with E-64, the normal cathepsin L/sheep liver stefin B complex might not have dissociated sufficiently to allow binding of the E-64. Subsequent to this incubation, the mixture is diluted 1000-fold for the assay with the sensitive synthetic substrate, and thus the (non-titratable) further 40% of activity against this substrate could be due to dissociation of the normal enzyme/stefin B complex at this high dilution. This residual 40% activity could be extinguished with Z-Phe-Phe-CHN_2 after dilution for the assay with Z-Phe-Arg-NHMec, however, suggesting that it too is due to cathepsin L. Active site titration of the complex, using azocasein as the substrate, supports this interpretation, since the complex titrated completely in this assay, in which no dilution of the complex takes place. In the absence of dilution, the normally bound stefin B-complexed cathepsin L would not dissociate and would thus not show activity, while the abnormally bound stefin B-complexed cathepsin L would be available for E-64 titration and can, therefore, be titrated to completion.

The effect of pH on the complex was studied in relation to the pH 4.2 activation (autolysis) step used during the purification procedure, which is thought to disrupt enzyme/inhibitor complexes (Mason *et al.*, 1985). Incubation of the isolated complex at pH 4.2 for different periods of time, converted the M_r 37 000 complex to an M_r 45 000 form on non-reducing SDS-PAGE, while the M_r 68 000 complex disappeared and the

M_r 42 000 complex and free M_r 26 000 cathepsin L remained unchanged (Fig. 19). These changes occurred after short incubation periods, of the order of 5 min at pH 4.2. Gelatin substrate-containing SDS-PAGE showed that the M_r 45 000 and 42 000 complexed forms and the free M_r 26 000 form of cathepsin L were inactive at pH 4.2 (Section 4.9.6; Fig. 27B). Incubation at pH 4.2, therefore, does not disrupt the covalent complex, but seems to induce a rapid destabilising conformational change in the M_r 37 000 complex, evidenced by the change in M_r to 45 000 and a loss in proteolytic activity. This conformational change may also occur in the homogenate during the pH 4.2 autolysis step but may be reversible, leading to reversion back to the active M_r 37 000 form upon an increase in pH to 5.5 following the TPP step in the purification procedure. Evidence that this may indeed be the case was found in results of non-reducing SDS-PAGE of isolated complexes incubated at different pH-values (Section 4.9.6; Fig. 27B), where it was illustrated that the M_r 37 000 complex only became apparent at pH 5.5 and above, whereas below this pH only M_r 45 000 and 42 000 complexes were visible.

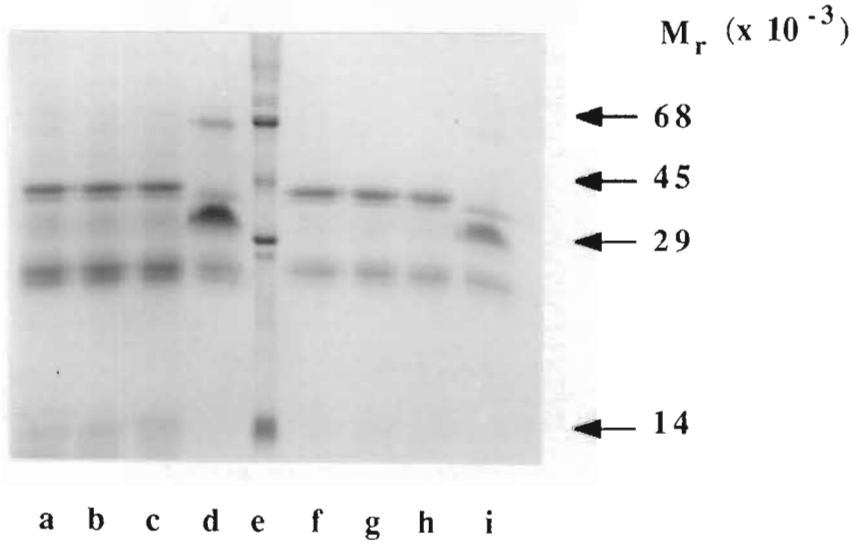

Figure 19. Non-reducing SDS-PAGE of the cathepsin L/stefin B complexes, treated at pH 4.2. Isolated complex samples (5 µg) were incubated at pH 4.2 and 37°C, for (a) 5 min; (b) 30 min; (c) 60 min; or (d) untreated; (e) MW markers as in Fig. 12; and (f), (g), (h), and (i) are 2.5 µg of the isolated complex, treated in the same order as (a) to (d).

Since the autolysis step did not dissociate either the non-covalently bound or the novel, covalently bound stefin B/cathepsin L complexes, studies were undertaken to determine whether the autolysis step was instrumental in the formation of the novel complexes. Elimination of the autolysis step from the purification procedure did not prevent complex formation, but instead caused a two-fold increase in the specific activity of stefin B-complexed cathepsin L isolated (Table 11). These seemingly contradictory results pertaining to the acidification of the homogenate (which causes an increase in the amount of proteolytically active complex formed) and that of the isolated complex (which causes its

inactivation), may indicate that active, covalent complexes are formed during later steps in the purification process, or that the isolated complexes are more sensitive to pH, especially in the presence of SDS.

Table 11. Comparison of the purification of free and stefin B-complexed cathepsin L from autolysed and non-autolysed sheep liver homogenates.

Preparation	Specific activity (units/mg)[a]	
	Autolysed homogenate	Non-autolysed homogenate
Cathepsin L/stefin B complex	1674	3050
Free cathepsin L	2782	2527
$\frac{\text{Cathepsin L/stefin B complex}}{\text{Free cathepsin L}}$	0.6	1.2

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

A further concern was that the TPP step, unique to the purification procedure used in this study (Pike and Dennison, 1989b), could be responsible for producing the active M_r 37 000 stefin B/cathepsin L complex. Underpinning this concern was the observation that fractionation of plasma proteins with TPP led to the formation of albumin dimers (Forsyth, Pike and Dennison, unpublished results). Substitution of ammonium sulphate fractionation [commonly used for cathepsin L isolation (e.g. Mason, 1986)] for the TPP step, was found to have no effect on the proportions of the M_r 37 000 and 26 000 forms of the enzyme isolated, and the M_r 37 000 complex had exactly the same characteristics, i.e. comprising a mixture of covalently and non-covalently bound cathepsin L and stefin B, active against both protein and synthetic substrates (result not shown). These novel, covalent complexes were, therefore, probably not artifacts of the unique purification procedure used in this study.

4.9.5 Formation of stefin B/cathepsin L complexes *in vitro*

Gelatin substrate-containing SDS-PAGE revealed that co-incubation of purified, active sheep liver cathepsin L and stefin B resulted in the formation of proteolytically active complexes, of Mr 37 000, 42 000 and 68 000 at pH 5.5 and above, but not below this value (Fig. 20). Parallel SDS-PAGE, in the absence of gelatin, showed that no inactive complexes were formed below pH 5.5 either, and pH 5.5, therefore, seems

to be pivotal in the formation of the complexes. Complexes formed very rapidly *in vitro* at pH 6.0, and could be detected in less than 1 min after incubation of the stefin B with cathepsin L, and complete complex formation was attained at about 10 min (Fig. 21). The activity of the complexes formed *in vitro* could be extinguished by incubation with either Z-Phe-Phe-CHN_2, E-64 or leupeptin thus indicating that the activity is due to cathepsin L (results not shown). A difference between the isolated complexes and those formed *in vitro* is that the M_r 42 000 complex was proteolytically active in the latter (Fig. 20), but not in the former (Fig. 12C). Both isolated and *in vitro* formed M_r 37 000 complexes manifested significantly higher gelatinolytic activity than what would be expected from the intensity of the corresponding Coomassie blue stained bands, which suggests that the interaction of stefin B with cathepsin L may have an activating effect. Further evidence for this phenomenon was obtained from studies on the activation of cathepsin L by elevated levels of cysteine (Section 4.9.8).

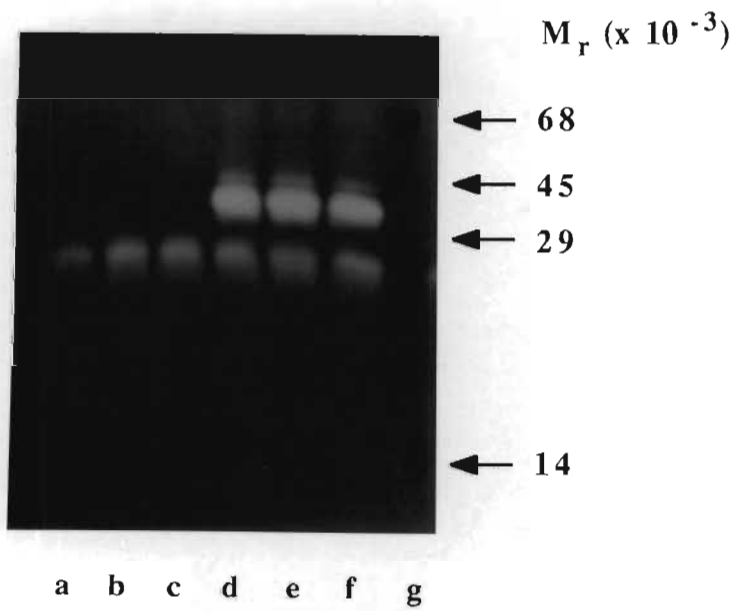

Figure 20. Gelatin substrate SDS-PAGE, showing the effect of pH on the *in vitro* formation of covalent complexes between isolated sheep liver cathepsin L and stefin B fractions.
Sheep liver cathepsin L (2.5 µg) and stefin B (2.5 µg) were incubated together for 1 h at 37°C in 100 mM buffers of the desired pH, and 2.5 µg of protein was electrophoresed on a 12.5% gelatin substrate gel. Cathepsin L and stefin B incubated at (a) pH 4.2; (b) pH 4.5; (c) pH 5.0; (d) pH 5.5; (e) pH 6.0; and (f) pH 6.5; and (g) MW markers, as in Fig. 12.

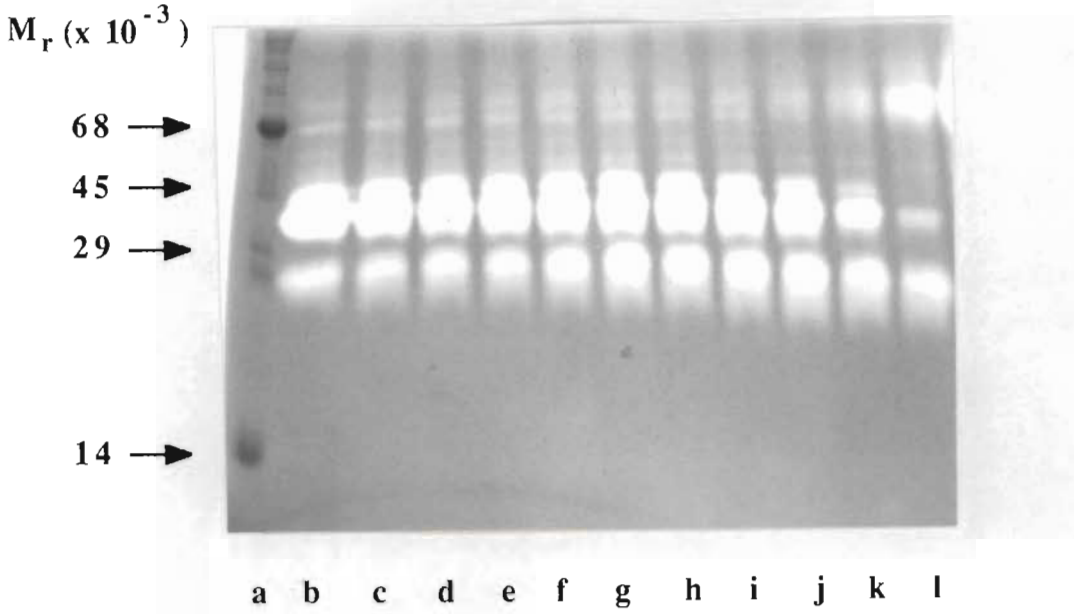

Figure 21. *In vitro* covalent complex formation between sheep liver cathepsin L and stefin B over time. Sheep liver stefin B (2.5 µg) was incubated with cathepsin L (2.5 µg) at 37°C in 100 mM MES-buffer, pH 6.0 for different periods of time, and 2.5 µg of protein was electrophoresed on a 12.5% gelatin substrate gel. (a) MW markers, as in Fig. 12; incubation for (b) 120 min; (c) 90 min; (d) 60 min; (e) 45 min; (f) 30 min; (g) 20 min; (h) 15 min; (i) 10 min; (j) 5 min; (k) 1 min; and (l) 0 min.

Incubation of different molar ratios of cathepsin L:stefin B during *in vitro* complex formation, followed by analyses on SDS-PAGE, both in the presence and absence of gelatin, further confirmed the important role played by pH in complex formation *in vitro* (Fig. 22). No complexes were formed at pH 5.0 at any of the molar ratios tested, but at pH 5.5 complexes were formed at equimolar and 1:2 ratios of cathepsin L:stefin B, while no complexes were formed at ratios of two molecules of cathepsin L to one molecule of stefin B. The complexes formed at equimolar ratios at pH 5.5 could only be seen on the more sensitive gelatin-containing substrate gels. At pH 6.0 active complexes formed at equimolar, 1:2 and 2:1 ratios of cathepsin L:stefin B. Complex formation is, therefore, primarily pH-dependent and is probably promoted by an excess of stefin B.

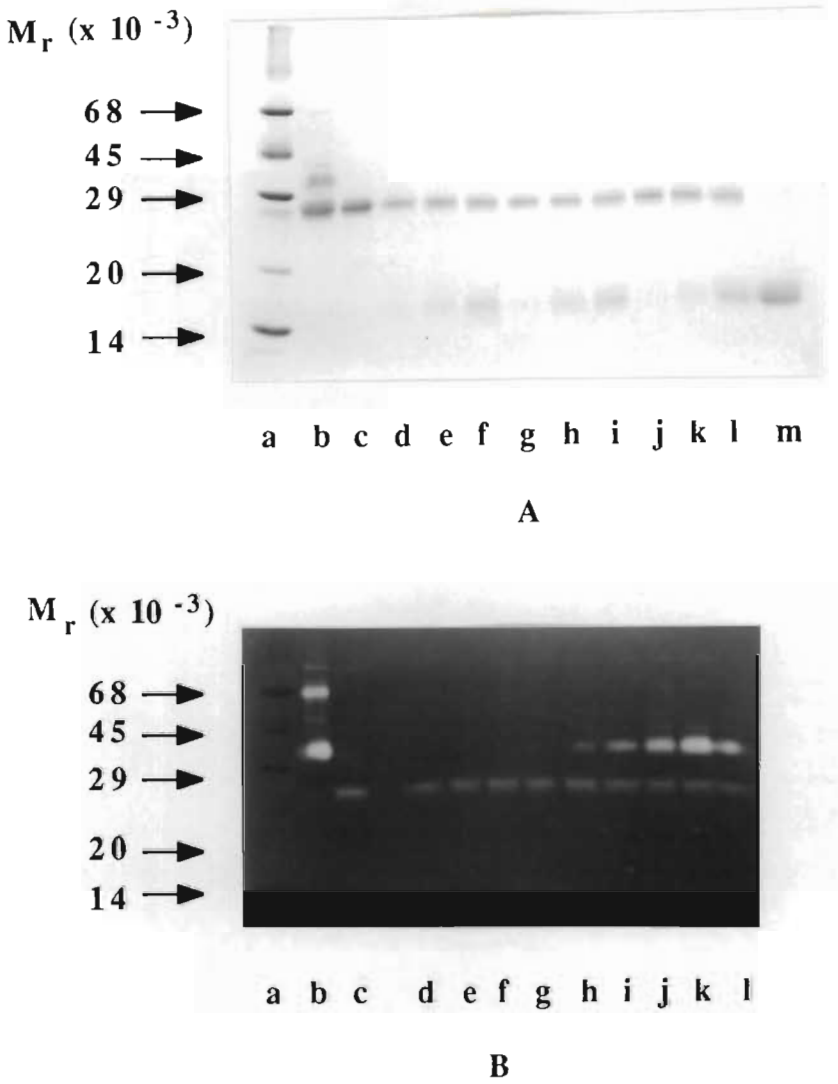

Figure 22. Non-reducing SDS-PAGE of different molar ratios of sheep liver cathepsin L and stefin B incubated together for *in vitro* covalent complex formation.
Sheep liver cathepsin L and stefin B were incubated for 30 min at 37°C in 100 mM buffers of the desired pH, and 2.5 µg of protein was electrophoresed on a 12.5% gel in the (**A**) absence, and (**B**) presence of copolymerised gelatin. (a) MW markers, as in Fig. 12 (with SBTI M_r 20 000); (b) isolated cathepsin L/stefin B complex; (c) free cathepsin L; molar ratios of cathepsin L:stefin B of (d) 2:1; (e) 1:1; (f) 1:2, incubated at pH 5.0; (g), (h), (i) the same sequence of molar ratios of cathepsin L:stefin B incubated at pH 5.5; and (j), (k), (l) incubated at pH 6.0; and (m) stefin B [not present in (**B**)].

On account of the observations that cathepsin L and stefin B are covalently bound in the isolated complexes, the effect of DTT on *in vitro* complex formation was also investigated at the different pH-values, since it was thought that this reducing agent might prevent formation of covalent disulfide bonds. Complex formation followed the same pH-dependent pattern in the presence of DTT as it did in its absence, shown previously in Fig. 20, with complex formation only evident at and above pH 5.5. The formation of the M_r 42 000 and 37 000 complexes at pH 5.5 and 6.0 were slightly enhanced by 10 mM DTT, while the formation of the M_r 68 000 complex was prevented. A typical result is given in Fig. 23 for complex formation at pH 6.0, in the absence (Lane d) and presence

(Lane h) of DTT. This increased activity of the M_r 37 000 and 42 000 complexes in the presence of DTT may be the result of the cathepsin L and stefin B molecules, formerly constituting a loosely bound M_r 68 000 complex, re-associating to form lower M_r complexes. Incubation with DTT probably activated cathepsin L, thereby promoting complex formation and, together with the observation that denaturation of cathepsin L in 0.1% SDS, prior to incubation with stefin B, prevented complex formation, provides further evidence that active constituents may be required for complex formation.

The pH-dependent formation of novel, proteolytically active, covalent complexes between purified, active cathepsin L and stefin B could thus be demonstrated *in vitro*. It appears to be a rapid process which is enhanced by low levels of reducing agent and requires equimolar amounts of the reactants, or an excess of stefin B, for its rapid formation.

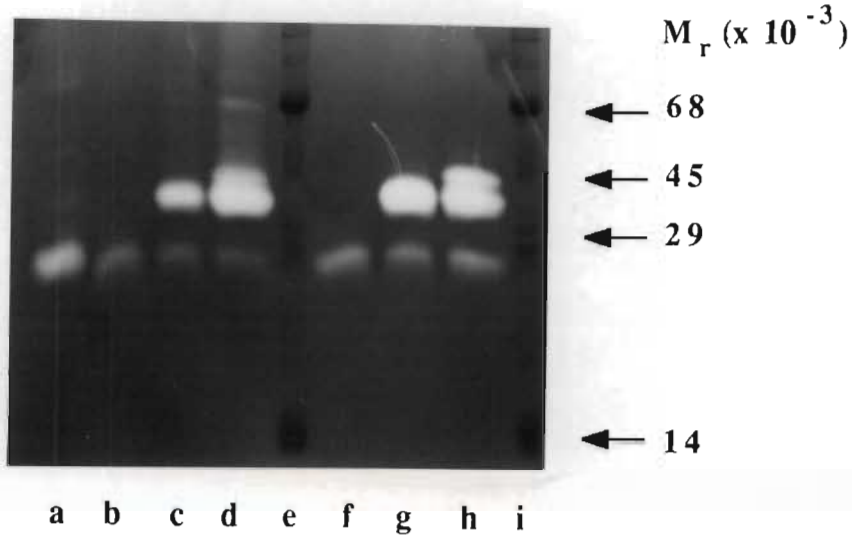

Figure 23. Gelatin substrate SDS-PAGE demonstrating the effect of alkylation of stefin B on the *in vitro* formation of covalent cathepsin L/stefin B complexes.
Cathepsin L (2.5 µg) and stefin B (2.5 µg) were incubated in 100 mM MES-buffer, pH 6.0, for 30 min at 37°C before (except where stated otherwise) 2.5 µg protein was loaded onto a 12.5% gelatin substrate gel. (a) Free cathepsin L (5 µg); free cathepsin L (2.5 µg); free cathepsin L incubated in the absence of DTT, (b) without stefin B; and with (c) carboxyamidomethylated stefin B; and (d) untreated stefin B; (e) MW markers, as in Fig. 12; (f), (g) and (h) free cathepsin L incubated in the presence of DTT in the same sequence as (b), (c) and (d).

4.9.6 Studies on the nature of the bond formed between cathepsin L and stefin B in the novel complexes

From the results of reducing and non-reducing SDS-PAGE of the isolated complexes, it became evident that the M_r 68 000, 42 000 and 37 000 complexes are formed by SDS-stable, covalent bonds between cathepsin L and stefin B. Futhermore, analyses by gelatin-containing substrate gel electrophoresis, showed that the isolated M_r 37 000 complexes were stable, when tested by preincubating in concentrations of 2-mercaptoethanol up to 1.4 M or DTT up to 0.5 M (result not shown), but could be dissociated by boiling in 1.4 M 2-mercaptoethanol, as carried out routinely for reducing SDS-PAGE (Fig. 12). However, boiling in 0.1 or 0.2% SDS without 2-mercaptoethanol did not dissociate the complex. Low levels of DTT (1 mM) were sufficient to reduce the M_r 68 000 complex, however, which seems to represent a loosely bound form of the cathepsin L/stefin B complex. It therefore appeared that the covalent bonds between cathepsin L and stefin B in these novel complexes may be disulfide bonds. It was consequently thought that alkylation of either stefin B or cathepsin L might prevent the formation of these complexes.

Alkylation of stefin B with iodoacetamide, prevented the formation of the M_r 42 000 complex, but not the M_r 37 000 complex *in vitro* in the absence of DTT, while the formation of both M_r 68 000 and 42 000 complexes were prevented in the presence of DTT (Fig. 23). Incubation of cathepsin L with Z-Phe-Phe-CHN_2 and E-64, which alkylate the active site Cys-residue of cysteine proteinases, prior to the addition of stefin B during *in vitro* complex formation at pH 6.0, also prevented the formation of complexes at M_r 42 000, but not at M_r 37 000 (Fig. 24). These results indicate that free cysteine residues in cathepsin L and stefin B are required, probably to form a disulfide bond, to account for the formation of the M_r 42 000 complex *in vitro*. The inability to prevent the formation of the M_r 37 000 complex by alkylation suggests that the covalent bond in this complex could possibly be due to the formation of a thioester bond, which would only require one free Cys-residue, contributed by either of the two participating components. Also, it was shown that no proteolytically active, covalent complexes were formed *in vitro*, either at pH 5.0 or 6.0, between cathepsin L and members of the cystatin superfamily lacking a free sulfhydryl group, e.g. chicken egg white cystatin and recombinant human cystatin C (Fig. 25). Baboon stefin A, which presumably has no free Cys-residues, also did not form novel covalent complexes, whereas baboon stefin B and cathepsin L did form such complexes (see Section 4.9.12, Fig. 35).

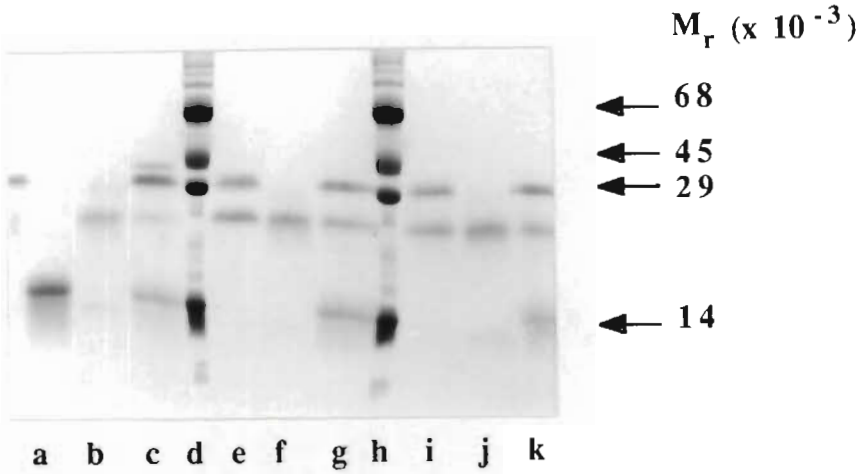

Figure 24. Non-reducing SDS-PAGE showing the effect of alkylation of the active site Cys-residue of cathepsin L, on the *in vitro* formation of covalent cathepsin L/stefin B complexes.
Samples (2.5 µg) were loaded onto a 12.5% gel. (a) Stefin B; (b) free cathepsin L; (c) *in vitro* formed complex following incubation of cathepsin L (2.5 µg) and stefin B (2.5 µg) in 100 mM MES-buffer, pH 6.0, containing 8 mM DTT; (d) MW markers, as in Fig. 12; incubation in the presence of Z-Phe-Phe-CHN_2 (10 µM) of (e) isolated cathepsin L/stefin B complex; (f) free cathepsin L; and (g) free cathepsin L (2.5 µg) prior to incubation with stefin B (2.5 µg) for *in vitro* complex formation; (h) MW markers as above; (i), (j) and (k), incubation in the presence of E-64 (10 µM) in the same sequence as (e), (f) and (g).

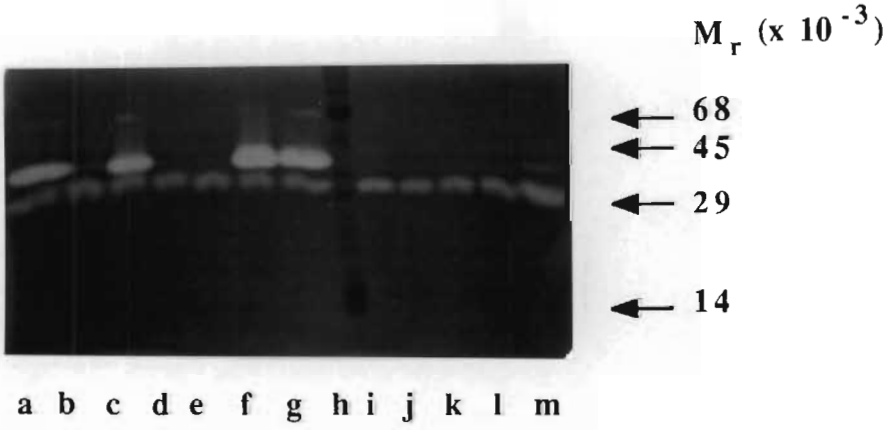

Figure 25. Gelatin substrate SDS-PAGE of sheep liver cathepsin L incubated with types 1 and 2 cystatins.
Sheep liver cathepsin L (2.5 µg) was incubated with the respective cystatins (2.5 µg) for 30 min at 37°C in 100 mM buffers of pH 6.0 or 5.0, and 2.5 µg protein was electrophoresed on a 12.5% gelatin substrate gel. (a) Isolated sheep cathepsin L/stefin B complex; (b) free sheep cathepsin L; sheep cathepsin L incubated at pH 6.0 with (c) sheep stefin B; (d) chicken egg white cystatin; (e) recombinant human cystatin C; sheep liver stefin B, in the (f) presence; and (g) absence of 1 mM DTT; (h) MW markers as in Fig. 12; (i), (j), (k), (l) and (m), sheep cathepsin L incubated at pH 5.0 in the same sequence as (c), (d), (e), (f) and (g).

In order to address the question of whether the M_r 68 000 and 42 000 complexes were disulfide linked, and the M_r 37 000 complex was due to thioester bond formation, a means of differentiating between the two types of covalent bond was required. Fenton and Fahey (1986) showed that it is possible to differentiate between disulfides and thioesters by selective cleavage of thioesters with 200 mM hydroxylamine at pH 7.0. Other nucleophiles, such as methylamine and ammonium acetate have also been used in studies on the internal thioester bond in complement component C3 (Pangburn, 1992) and α_2-macroglobulin (α_2M) (Howard, 1981). Analysis on non-reducing SDS-PAGE, following incubation of isolated complexes with hydroxylamine, methylamine and ammonium acetate (Fig. 26), showed that none of these reagents had any effect on any of the complexes at pH 6.0 (lanes j to n), whereas at pH 5.0 both the M_r 37 000 and 42 000 bands disappeared following treatment with hydroxylamine (lane d). When incubated in the presence of ammonium acetate at pH 5.0, the M_r 37 000 band disappeared while the M_r 42 000 band remained (Fig. 26 lanes f to h). However, a control sample, incubated in pH 5.0 buffer alone, showed a similar result (lane c) and indicated that pH may be playing a more important role than the nucleophiles *per se*.

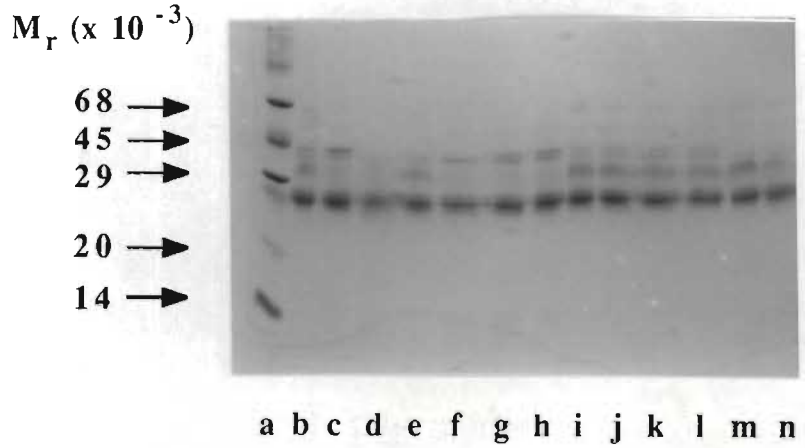

Figure 26. Non-reducing SDS-PAGE of isolated covalent cathepsin L/stefin B complexes treated with thioester-bond-reactive nucleophiles.
Samples (5 µg) were incubated with an equal volume of 100 mM buffers of pH 5.0 or 6.0 containing the respective nucleophiles, to give a final concentration of the latter as indicated in brackets. Following incubation for 30 min at 37°C, 2.5 µg protein was loaded onto a 12.5% gel. (a) MW markers, as in Fig. 22; isolated cathepsin L/stefin B complex (b) not incubated; incubated at pH 5.0 (c) alone; with (d) hydroxylamine (200 mM); (e) methylamine (200 mM); ammonium acetate (f) (200 mM); (g) (100 mM); (h) (50 mM); isolated cathepsin L/stefin B complex incubated at pH 6.0 (i) alone; (j), (k), (l), (m), (n) with nucleophiles in the same sequence as (d), (e), (f), (g) and (h).

This was confirmed by incubation of the complexes at pH 4.2, 4.5, 5.0, 5.5, 6.0 and 7.0 in the presence and absence of ammonium acetate, which showed the disappearance of the M_r 37 000 band at, and below pH 5.0, regardless of the presence or absence of the nucleophile, while the M_r 42 000 complex was unaffected throughout (Fig. 27A). Gelatin-substrate containing SDS-PAGE showed activity of the M_r 37 000 complex at, and above pH 5.5, in both the absence and presence of ammonium acetate, as well as an additional band of activity at M_r 68 000 (Fig. 27B). This preparation of isolated complex showed no activity at M_r 26 000, at any of the pH-values tested.

In spite of circumstantial evidence indicating that the M_r 37 000 complex might possibly be due to thioester bond formation, treatment of isolated complexes with nucleophiles did apparently not seem to affect the M_r 37 000 complexes, at and above pH 5.5, as evidenced by SDS-PAGE. If the M_r 37 000 complex was, however, to consist of a mixture of disulfide and thioester linked stefin B/cathepsin L complexes, it would explain both the formation of the M_r 37 000 complex despite alkylation of the constituents, and the effect of the nucleophiles. Cleavage of the thioester linked portion would then probably not be distinguishable on SDS-PAGE, which is not a sufficiently quantitative method of analysis to show a small decrease in the intensity of the M_r 37 000 band. The free sulfhydryl group which appears following cleavage of thioester bonds by nucleophiles (Pangburn, 1992), could possibly react rapidly with a reactive sulfhydryl during incubation prior to electrophoresis, and form a disulfide-linked M_r 37 000 complex. Conversely, removal of excess amines could lead to reformation of the thioester bonds, as was shown for complement component C3 (Pangburn, 1992). This may occur during electrophoresis, which is conducted well below the pKa of the primary amines, and will result in cathodal migration of excess amines. Treatment of isolated complexes with radiolabeled nucleophiles, followed by separation of the labeled cleavage products on MEC, may give more conclusive results as to the type of covalent bond involved. The presence of a radiolabeled γ-glutamylmethylamide in either cathepsin L or stefin B may also indicate which of the two molecules contribute the cysteinyl sulfhydryl and which the glutaminyl γ-carbonyl for thioester formation.

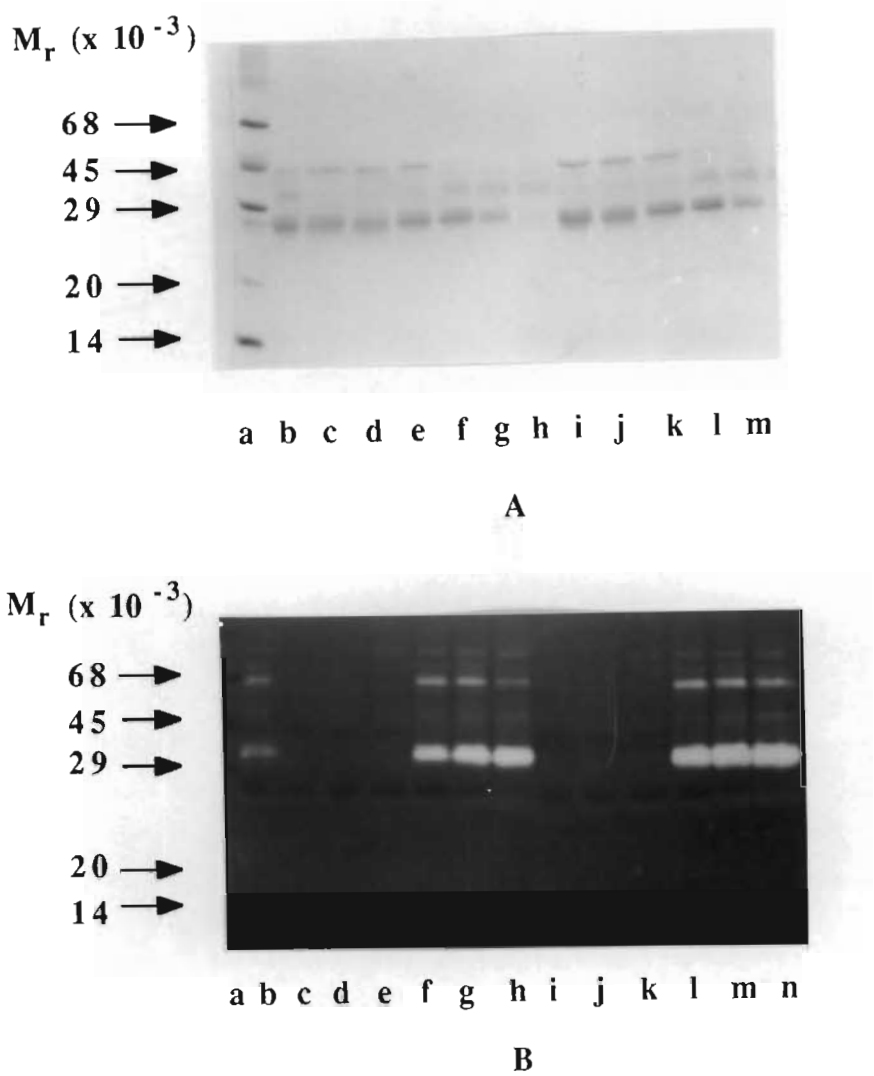

Figure 27. Non-reducing SDS-PAGE demonstrating the effect of pH and ammonium acetate on the isolated complexes.
Covalent complexes (5 μg) were incubated with 100 mM buffers of the desired pH, in the presence or absence of ammonium acetate, for 30 min at 37°C, and 2.5 μg protein was electrophoresed on 12.5% SDS-PAGE gels, (A) without, and (B) with copolymerised gelatin. (a) MW markers, as in Fig. 22; isolated covalent complex (b) not incubated; incubated with ammonium acetate at (c) pH 4.2; (d) pH 4.5; (e) pH 5.0; (f) pH 5.5; (g) pH 6.0; (h) pH 7.0; (i), (j), (k), (l), (m) incubated without ammonium acetate in the same sequence as (c), (d), (e), (f) and (g). In (B), (n) same as (h).

4.9.7 Rates of inactivation of free and stefin B-complexed cathepsin L

In order to investigate the mode of interaction between cathepsin L and stefin B, resulting in the formation of the novel proteolytically active complexes, the rates of inactivation of free and stefin B-complexed cathepsin L by the two structurally different active site titratants, the dipeptidyl diazomethyl ketone, Z-Phe-Phe-CHN_2, and the dipeptidylepoxide, E-64, were compared. The different active site binding pocket requirements of these inhibitors could influence their association with free and stefin B-

complexed cathepsin L and hence their rates of inactivation of the two forms of cathepsin L, which in turn could be illustrative of the mode of interaction between stefin B and cathepsin L.

The rate of inactivation of free cathepsin L by Z-Phe-Phe-CHN_2 was four times, and that of stefin B-complexed cathepsin L two and a half times, faster than that by E-64 (Table 12). Both inhibitors showed a higher rate of inactivation of the free, than the stefin B-complexed enzyme; three times in the case of Z-Phe-Phe-CHN_2 and two times for E-64.

The higher rates of inactivation of both free and stefin B-complexed cathepsin L by Z-Phe-Phe-CHN_2 than by E-64, may be a result of differences in structure and thus mode and/or position of binding of the inhibitors to the active site of cathepsin L. Initially it was thought that E-64 binds in the S'-subsites of cysteine proteinases, while the dipeptidyldiazomethyl ketone inhibitors bind in the S-subsites (Barrett *et al.*, 1982). Studies on crystal structures of papain–E-64 complexes (Varughese *et al.*, 1989) showed, however, that both types of inhibitors bind to the S-subsites of the enzyme and that the interactions utilised by the two inhibitors are very similar. The major difference is that the Phe-side chain of Z-Phe-Phe-CHN_2 is able to extend much further into the hydrophobic pocket of the S_2-subsite than the Leu-side chain of E-64, which may contribute to the higher rate of inactivation by Z-Phe-Phe-CHN_2, found in this, and other (Barrett *et al.*, 1982; Mason *et al.*, 1985; Mason, 1986) studies.

The higher rate of inactivation of free cathepsin L than the stefin B-complexed enzyme, provides further evidence that a proportion of the complex fraction could be complexed with stefin B in the usual non-covalent manner, and is thus initially unavailable for E-64 binding. During the course of the assay, stefin B could dissociate from this proportion, allowing binding by the inhibitors and subsequent inactivation. In other words, both free and covalently complexed cathepsin L would probably immediately start undergoing inactivation, but full inactivation of the mixed complex fraction would only be reached once dissociation of the non-titratable proportion has taken place.

Table 12. Rates of inactivation of free and stefin B-complexed cathepsin L by E-64 and Z-Phe-Phe-CHN_2

Inhibitor	k_{obs} (x 10^3) (min^{-1})		Ratio of rate of inactivation		
	Free	Complex	Free / Complex	[a]C–Z-Phe-Phe-CHN_2 / C–E-64	[b]F–Z-Phe-Phe-CHN_2 / F–E-64
E-64	2.302	1.101	2.1	2.5	
Z–Phe-Phe-CHN_2	8.970	2.760	3.3		3.9

[a] C = stefin B-complexed cathepsin L; [b] F = free cathepsin L

4.9.8 The effect of cysteine concentration on the activity of free and stefin B-complexed cathepsin L

The effect of cysteine concentration, on the activity of free and stefin B-complexed cathepsin L at pH 5.0 and 7.0, is shown in Fig. 28. Both forms of cathepsin L showed a similar increase in activity with an increase in cysteine concentration at pH 5.0 and 7.0, but whereas stefin B-complexed cathepsin L attained full activity in 50 mM cysteine, free cathepsin L required three times this amount of cysteine in order to be fully activated. These results, therefore, suggest that complexing of cathepsin L with stefin B has a similar activating effect as high cysteine concentrations. Also, free, single-chain cathepsin L apparently requires much higher levels of cysteine for complete activation, than has previously been reported to be the case for single- and two-chain forms of cathepsin L (Dufour *et al.*, 1987; Barrett and Kirschke, 1981). Furthermore, it was shown in a parallel study on the effect of pH on the activity of the two forms of cathepsin L (in the presence of 150 mM cysteine) that at equimolar concentrations, stefin B-complexed cathepsin L has approximately four-fold greater activity than the free enzyme (Dennison *et al.*, 1992). It, therefore, appears that in complexing with single-chain cathepsin L in the novel, covalent mode, stefin B acts as an activator.

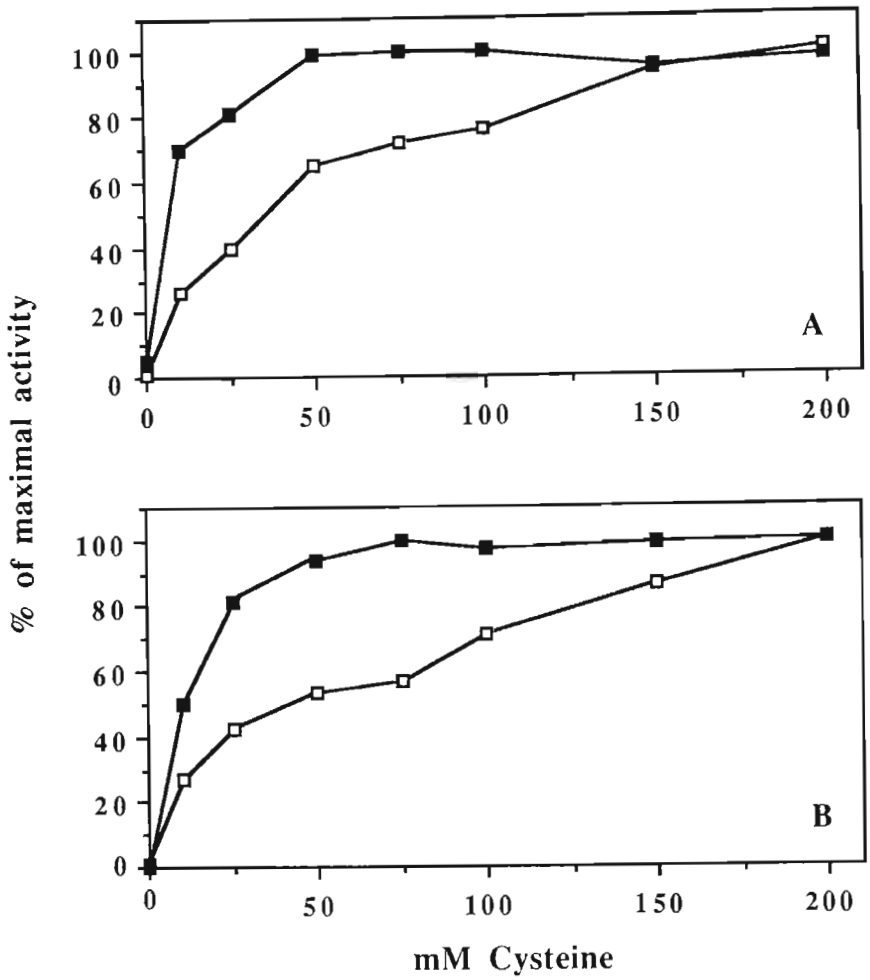

Figure 28. The effect of cysteine concentration on the activity of free and stefin B-complexed forms of sheep liver cathepsin L.
Z-Phe-Arg-NHMec hydrolysis of equimolar amounts of free and stefin B-complexed cathepsin L was measured in standard 10 min assays using AMT buffers of pH 5.5 (**A**) and 7.0 (**B**), as described in section 4.8.1, containing a range of cysteine concentrations. (❑), free cathepsin L; (■), stefin B-complexed cathepsin L. Each point represents the mean of five determinations.

4.9.9 The effect of increasing cysteine concentration on active site titration of free and stefin B-complexed cathepsin L

As a result of the significant increase in the activity of free, and to a lesser extent stefin B-complexed cathepsin L, in the presence of elevated levels of cysteine, it was of interest to ascertain whether this increase in activity would reflect an increase in the molar concentration of active enzyme measured by active site titration. This was found to be the case (Fig. 29). The maximum effect on the amount of active stefin B-complexed cathepsin L, i.e. a 10% increase, was reached at 50 mM cysteine, with no further increase at higher cysteine concentrations. The molar concentration of active free cathepsin L also increased with an increase in cysteine concentration, but required 150 mM cysteine to reach the level attained by the complexed form at 50 mM, after which the molar concentration of

active enzyme remained the same. The free enzyme showed a 23% increase in molar concentration of active enzyme at 150 mM, over that measured in the presence of 10 mM cysteine, the level of reducing agent commonly used in active site titrations (Barrett and Kirschke, 1981). It is unlikely that the increase in the amount of active cathepsin L could be due to quenching of E-64 by excess free cysteine, because it has been shown that active site cysteine-specific epoxide and diazomethane inhibitors do not react with free cysteine (Barrett *et al.*, 1982).

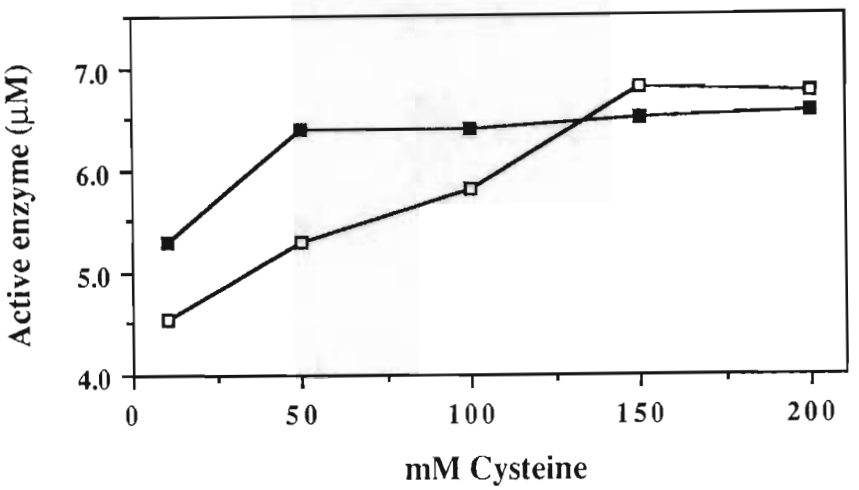

Figure 29. The effect of increasing cysteine concentration on the molar concentration of active free and stefin B-complexed cathepsin L, measured by active site titration with E-64.
Titrations against Z-Phe-Arg-NHMec were carried out on equimolar amounts of free and stefin-B complexed cathepsin L as described in section 4.7. (❑), Free cathepsin L; (■), stefin B-complexed cathepsin L.

4.9.10 Comparison of the relative yields of free and stefin B-complexed cathepsin L purified from different organs and by homogenisation at different pH-values

In an attempt to increase the yield of free sheep cathepsin L for amino acid sequence determination, alternative organ sources for purification purposes, as well as strategies aimed at the prevention of covalent cathepsin L/stefin B complex formation, were investigated. Since a comparison of various rat tissue extracts for their cathepsin L content showed that kidney had three to five times more cathepsin L than liver (Bando *et al.*, 1986; Katunuma and Kominami, 1986), the purification of cathepsin L from sheep kidney was compared to that from sheep liver, the routinely used source of cathepsin L in this study. A further comparison between the organs was based on the finding in this study that the covalent cathepsin L/stefin B complexes did not form *in vitro* below pH 5.5 (Section 4.9.5; Fig. 20). Kidney and liver tissue was, therefore, homogenised at pH 4.5 (which titrates to pH 5.0 in the homogenate), immediately following organ collection and before freezing at

-70°C (which causes a breakdown of the subcellular boundaries separating cathepsin L and stefin B) to increase the probability of preventing covalent complex formation.

Using the homogenisation procedure described by Barrett and Kirschke (1981) and adopted by Pike and Dennison (1989b), cathepsin L was also purified from sheep kidney in a free, as well as a covalently complexed form (Fig. 30). The covalent complex was also proteolytically active as evidenced by activity against azocasein and gelatin (result not shown). The complexing moiety in kidney was also identified as stefin B by Western blotting (result not shown). More stefin B-complexed cathepsin L than free enzyme was purified from both sources, giving a ratio of free to complexed cathepsin L of 0.6 in liver and 0.33 in kidney (Tables 13 and 14). Unlike the single-chain liver cathepsin L, which shows a single band at M_r 26 000 under both reducing and non-reducing SDS-PAGE conditions, the kidney cathepsin L preparation possibly contains an additional small proportion of two-chain enzyme, as evidenced by the presence of an M_r 20 000 component in reducing SDS-PAGE. Comparable amounts of free enzyme per gram of wet organ mass were purified from liver (2.2 μg/g) and kidney (2.5 μg/g), but a significantly higher proportion of cathepsin L was purified in the stefin B-complexed form from kidney (5.9 μg/g compared to 2.2 μg/g from liver). In terms of specific activity, however, 3.6 times more free, and two times more stefin B-complexed cathepsin L was purified from liver than from kidney.

Purification of cathepsin L from thawed pH 5.0 liver and kidney homogenates, prepared immediately upon organ collection, resulted in a three-fold increase in specific activity of free over complexed enzyme, in both liver and kidney. On comparing specific activities, liver yielded three times more free, and two times more stefin B-complexed enzyme than kidney. In addition to an increase in ratio of free over complexed cathepsin L, purification from pH 5.0 homogenates resulted in higher yields of both forms of enzyme. This isolation method resulted in the purification of 3.6 μg cathepsin L per gram wet tissue, which is 1.5 times higher than the 2.4 μg/g purified by Mason (1986) from the same source.

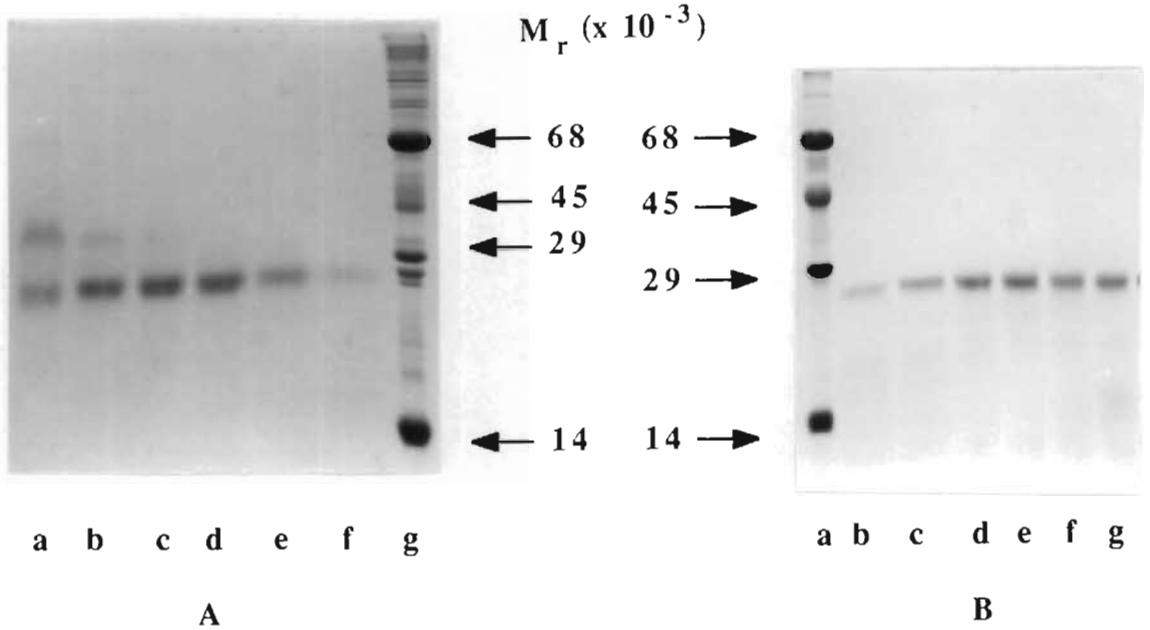

Figure 30. SDS-PAGE of free and stefin-B complexed sheep kidney cathepsin L
(**A**) Non-reduced samples were loaded onto a 12.5% gel. Stefin B-complexed kidney cathepsin L (a) 3 µg; (b) 2.5 µg; (c) 2 µg; free kidney cathepsin L (d) 2.5 µg; (e) 1 µg; (f) 0.5 µg; (g) MW markers as in Fig. 12. (**B**) Samples were reduced and loaded onto a 12.5% gel. (a) MW markers, as in Fig. 12; free kidney cathepsin L (b) 1 µg; (c) 2.5 µg; (d) 5 µg; (e) 10 µg; stefin B-complexed kidney cathepsin L (f) 5 µg; and (g) 7.5 µg.

The increase in total activity of the supernatants obtained following autolysis at pH 4.2 compared to that of both liver and kidney homogenates (Tables 13 and 14), could be due to the removal of cystatins binding in the non-covalent, inhibiting mode, as suggested by Mason *et al.* (1985) as a possible explanation for the inactivity observed in human liver homogenates prior to pH 4.2 autolysis. Homogenisation in the pH 4.5 buffered extraction solution could possibly have had the same effect, as evidenced by the higher total activity in these homogenates than their pH 4.2 autolysates.

Although complex formation was not prevented by maintaining the pH below 5.5, the marked increase in the ratio of free to complexed enzyme purified, especially from liver pH 5.0 homogenates, made this the organ and homogenisation method of choice for the purification of sufficiently large amounts of cathepsin L for amino acid sequencing.

Table 13. Comparison of the purification of free and stefin B-complexed cathepsin L from sheep liver using different homogenisation media.

Step	Vol. (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Purification (fold)	Yield (%)
Liver homogenate pH 4.2	767	16950	14042	0.83	1	100
supernatant	619	4023	16923	4.21	5	120
TPP	140	665	12310	18.51	22	88
S-Sepharose	42	5.20	5449	1047	1267	38
MEC (complex)	32	0.74	1519	2039	2466	11
MEC (free)	41	0.76	913	1224	1480	7
pH 5.0 liver homogenate pH 4.2	850	17167	15841	0.92	1	100
supernatant	820	8468	12320	1.45	1.6	78
TPP	170	2118	14962	7.06	7.6	95
S-Sepharose	42	4.80	11764	2451	2334	74
MEC (complex)	42	1.32	2210	1674	181	14
MEC (free)	59	1.34	3728	2782	3014	23

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

Table 14. Comparison of the purification of free and stefin B-complexed cathepsin L from sheep kidney using different homogenisation media.

Step	Vol. (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Purification (fold)	Yield (%)
Kidney homogenate	650	34375	18198	0.53	1	100
pH 4.2 supernatant	520	4768	40604	8.51	16	223
TPP	115	768	20290	26.42	50	112
S-Sepharose	48	10	7412	741.2	1401	41
MEC (complex)	41	1.62	1654	1020	1927	10
MEC (free)	36	0.69	230	336	634	1.3
pH 5.0 kidney homogenate	700	57764	36647	0.63	1	100
pH 4.2 supernatant	647	2621	28313	10.8	17	77
TPP	140	543	16356	30.2	48	45
S-Sepharose	50	6.5	5365	825	1302	15
MEC (complex)	40	1.02	955	936	1477	2.6
MEC (free)	36	0.81	819	1009	1591	2.9

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

4.9.11 Primary structures of sheep liver cathepsin L and stefin B

The purification methods of Pike and Dennison (1989b) and Green *et al.* (1984), for the purification of cathepsin L and stefin B respectively, modified as described in Sections 4.2 and 4.4, could be repeated reproducibly to isolate the required amounts of pure protein for amino acid sequence determination. This was done in a collaboration with Dr Anka Ritonja (Department of Biochemistry, Jozef Stefan Institute, Ljubljana, Slovenia). The primary structures of sheep liver cathepsin L and stefin B, are shown in Figs. 31 and 32, compared with related cysteine proteinases and cystatins.

The single-chain nature of sheep liver cathepsin L was confirmed during amino acid sequencing since a single N-terminal sequence was obtained following reduction and S-carboxymethylation (Dr Anka Ritonja, personal communication). As a result of this, residues 166-185 (papain numbering, Fig. 31), which would have yielded the additional N-terminus of the light chain in two-chain cathepsin L, have not been sequenced at the time of writing. The light chain of 42 residues, which results from cleavage between residues Thr-175 and Asn-176 (cathepsin L numbering, as this sequence forms part of the sequence not present in papain between papain residues 167 and 168) is usually sequenced directly by automated solid-phase Edman degradation (Ritonja *et al.*, 1988).

Sheep cathepsin L shows 85% sequence homology with the human enzyme, which corroborates the immunological cross-reactivity between the two enzymes (Pike, 1990 and Chapter 5) and also confirms the thesis of Mason (1986) that sheep cathepsin L would be a suitable model for human cathepsin L, based on enzymological and immunological similarities between the enzymes.

Amino acid sequencing of the cystatin from sheep liver, which forms covalent complexes with cathepsin L, provided unequivocal proof that it is stefin B (Fig. 32). Sheep stefin B is identical to bovine thymus stefin B, except that Gly-5 and Thr-6 (stefin B numbering) are replaced by Ala and Pro in sheep stefin B, as is the case in human stefin B (Krizaj *et al.*, 1992). In both sheep and bovine stefin B, Val-47, in the highly conserved QVVAG region, is replaced by a Leu-residue. The N-terminus of sheep stefin B, like those of bovine (Krizaj *et al.*, 1992) and human spleen (Lenarcic *et al.*, 1986), is blocked by N-acetylation of the Met-residue, consistent with the co-translational modification associated with cytoplasmically localised proteins (Bradshaw, 1989).

```
                                10                  20                  30
Sh cath L  : V P K S V D W R K K G Y V T P V K N Q G Q C G S C W A F S A
Hu cath L  : A P R S V D W R E K G Y V T P V K N Q G Q C G S C W A F S A
Papain     : I P E Y V D W R Q K G A V T P V K N Q G S C G S C W A F S A

                        40                  50
Sh cath L  : T G A L E G Q M F R K T G K L V S L S E Q N L V D C S R P Q
Hu cath L  : T G A L P G Q M F R K T G R L I S L S E Q N L V D C S G P Q
Papain     : V V T I D G I I K I R T G N L N Q Y S E Q E L L D C D R - -

              60                  70                  80
Sh cath L  : G N Q G C N G G L M D N A F Q Y I K E N G G L D S E E S Y P
Hu cath L  : G N E G C N G G L M D Y A F Q Y V Q D N G G L D S E E S Y P
Papain     : R S Y G C N G G Y P W S A L Q L V A Q Y - G I H Y R N T P Y

            90                  100                 110
Sh cath L  : Y L A T D E S C N Y K P E X S A A K D T G F V D I P Q Q E -
Hu cath L  : Y E A T E E S C K Y N P K Y S V A X D T G F V D I P K Q E -
Papain     : Y E G V Q R Y C R - S R E K G P Y - - A A K T D G V R Q V Q

                        120                 130                 140
Sh cath L  : - - - - - - R A F M K A V A T V G P I S V A I D A G H S S F Q
Hu cath L  : - - - - - - K A L M K A V A T V G P I S V A I D A G H E S F L
Papain     : P Y N Q G - A L L Y S I A N - Q P V S V V L Q A A G K D F Q

                                150                 160
Sh cath L  : F Y K S G I Y Y D P D C S S K D L D H G V L V V G Y X X
Hu cath L  : F Y K E G I Y F E P N C S S E D M D H G V L V V G Y G F
Papain     : L Y R G G I F - V G P C G - N K V D H A V A A V G Y N -

                        170                 180                 190
Sh cath L  : X X X X X X X X X X X X X X X X X X X X X X Y N K M A K D Q
Hu cath L  : E S T N N K Y W L V K N S W G E E W G M G G Y V K M A K D R
Papain     : - - - - P N Y I L I K N S W G T G W G E N G Y I R I K R G T

                        200                 210
Sh cath L  : N - - - N H C G I A T A A.S Y P T V
Hu cath L  : R - - - N H C G I A S A A S Y P T V
Papain     : G N S Y G V C G L Y T S S F Y P V K N
```

Figure 31. Alignment of the sequences of sheep cathepsin L (Sh cath L) with those of human cathepsin L (Hu cath L; Ritonja *et al.*, 1988) and papain (Dufour, 1988). The residue numbers are those of papain (Dufour, 1988). Non-identical residues in sheep and human cathepsin L are underlined. Residues 166-185 in sheep cathepsin L which have not yet been determined are indicated by (X). The active site Cys-25, and the sequence around the active site His-159, referred to in Chapter 5, are indicated in bold.

```
                                                  10                  20
S stef B  :                 acM M C G A P S A T Q P A T A E T Q A I A D K V K S
H stef B  :                 acM M C G A P S A T Q P A T A E T Q H I A D Q V R S
H stef A  :                   M I P G A L S E A K P A T P E I Q E I V D Q V R P
H cyst C  : S S P G K P P R L V G G P M D A S V E E E G V R R A L D F A V G
Cew cyst  :     S E D R S R L L G A P V P V D E N D E G L Q R A L Q F A M A

                      30                  40                  50
S stef B  : Q L E E K E N K K F - - P V F K A L E F K S Q L V A G K N Y F
H stef B  : Q L E E K Y N K K F - - P V F K A V S F K S Q V V A G T N Y F
H stef A  : Q L E E K T N E T Y - - G K L E A V Q Y K T Q V V A G T N Y Y
H cyst C  : E Y N K A S N D M Y H S R A L Q V V R A R K Q I V A G V N Y F
Cew cyst  : E Y N R A S N D K Y S S R V V R V I S A K R Q L V S G I K Y I

                      60                  70
S stef B  : I K V Q V D E D D F V H I R - - - - - - - V F E S L P H E N K - -
H stef B  : I K V H V G D E D F V H L R - - - - - - - V F Q S L P H E N K - -
H stef A  : I K V R A G D N D Y M H L K - - - - - - - V F K S L P G Q N E - -
H cyst C  : L D V E L G R T T C T K T Q P N L D N C - F H D Q P H L K R K A
Cew cyst  : L Q V E I G R T T C P K S S G D L Q S C E F H D E P E M A K Y T

              80                  90
S stef B  : P V A L T S Y Q T N K G R H D E L T Y F
H stef B  : P L T L S N Y Q T N K A K H D E L T Y F
H stef A  : D L V L T G Y Q V D K N K D D E L T G F
H cyst C  : F C S F Q I Y A V P W Q G T M T L S K S T C Q D A
Cew cyst  : T C T F V V Y S I P W L N Q I K L L E S K C Q
```

Figure 32. Alignment of the amino acid sequences of sheep stefin B with those of human stefin B (Ritonja *et al.*, 1985), human stefin A (Machleidt *et al.*, 1983), human cystatin C (Turk *et al.*, 1986) and chicken egg white (Cew) cystatin (Turk *et al.*, 1983). Numbering according to the stefins. Non-identical residues in sheep and human stefin B are underlined. Residues in bold are referred to in the Discussion (Section 4.10).
(S = sheep; H = human;stef = stefin; cyst = cystatin).

4.9.12 Isolation and characterisation of free and stefin B-complexed cathepsin L from baboon (*Papio ursinus*) liver

Cathepsin L was purified from baboon (*Papio ursinus*) liver to determine whether the novel proteolytically active, covalent complexes isolated from sheep liver may also be isolated from the tissues of higher primates, before embarking on a similar study using human liver. Baboon liver also yielded cathepsin L as M_r 26 000 and 37 000 forms, active against both azocasein and Z-Phe-Arg-NHMec (Fig. 33). The M_r 26 000 component showed a higher mobility in SDS-PAGE under non-reducing, than reducing conditions, which may be ascribed to glycosylation of the enzyme, or a more tightly folded conformation which, upon reduction adopts a more extended conformation and manifests a higher M_r (Fig. 34). Since no decrease in M_r was observed under reducing, compared to non-reducing SDS-PAGE conditions, the M_r 26 000 form of baboon cathepsin L probably represents a single-chain form, comparable to the sheep enzyme. Non-reducing SDS-PAGE showed that a proportion of the M_r 37 000 MEC peak, which separates into M_r 26 000 and 14 000 components under reducing conditions, does not dissociate without reduction, as manifested by the presence of bands of M_r of 37 000 and 26 000. (The M_r 14 000 band is not always visible on reducing SDS-PAGE).

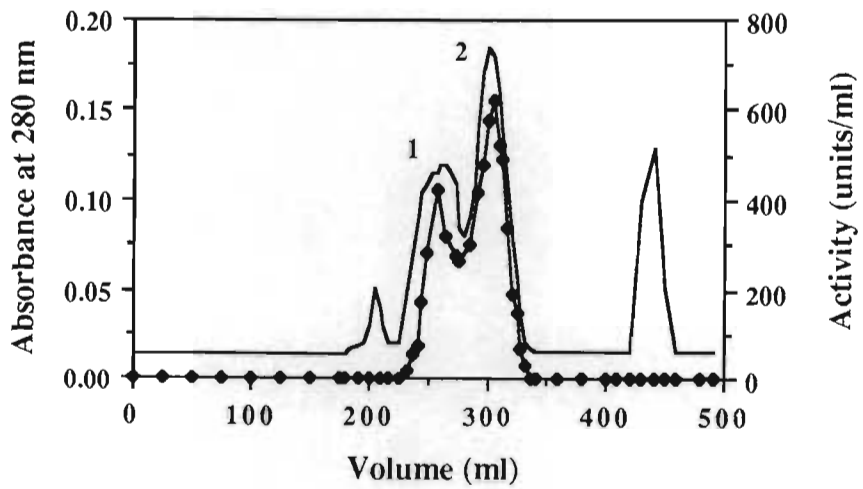

Figure 33. Molecular exclusion chromatography of the pH 5.5 S-Sepharose fraction from baboon liver on Sephadex G-75.
Column, 2.5 x 87.5 cm (430-ml bed volume); buffer, 20 mM Na-acetate, pH 5.5, containing 1 mM Na_2EDTA and 200mM NaCl; flow rate, 25 ml/h (5 cm/h); and fractions, 5 ml (12 min). Void volume was 150 ml. (—), A_{280}; (◆), enzyme activity (units/ml) from the assay against Z-Phe-Arg- NHMec. 1, complexed cathepsin L; 2, free cathepsin L.

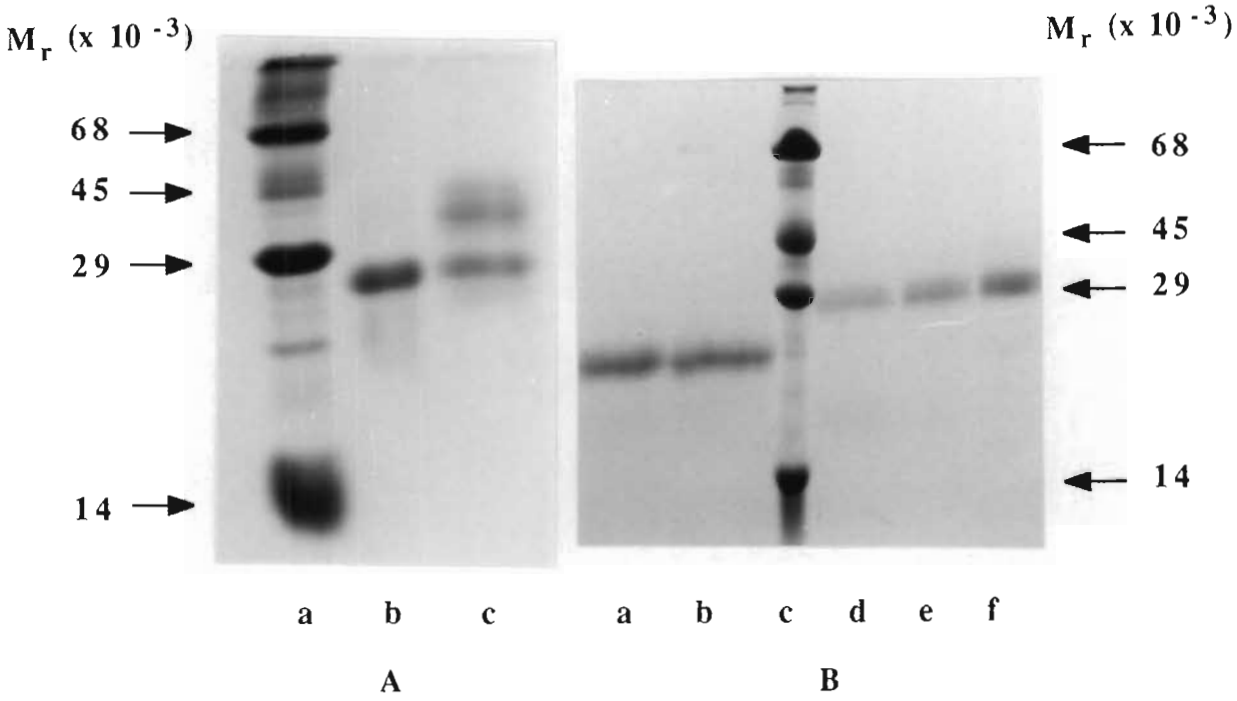

Figure 34. SDS-PAGE of free and stefin B-complexed baboon liver cathepsin L.
(**A**) Non-reduced samples (2.5 µg) were loaded onto a 12.5% gel. (a) MW markers, as in Fig. 12; (b) free baboon cathepsin L; (c) stefin B-complexed baboon cathepsin L. (**B**) Samples were loaded onto a 12.5% gel. (a), (b) Non-reduced free baboon cathepsin L (2.5 µg); (c) reduced MW markers, as in Fig. 12; reduced free baboon cathepsin L (d) 2.5 µg; (e) 5 µg; and (f) reduced stefin B-complexed baboon cathepsin L (5 µg).

Gelatin-substrate containing SDS-PAGE revealed that these M_r 37 000 and 26 000 components were proteolytically active (Fig. 35). The M_r 26 000 MEC peak showed additional activity at M_r 20 000 on gelatin-substrate containing SDS-PAGE, not visible on non-reducing SDS-PAGE in the absence of gelatin. By analogy with the sheep liver situation, the M_r 42 000 and 37 000 components were identified as complexes between cathepsin L and a cystatin, probably stefin B. The latter could not be proved unequivocally, as the antibodies to sheep liver stefin B did not cross-react with the baboon inhibitor. Proteolytically active complexes could, however, form spontaneously *in vitro* between baboon cathepsin L and stefin B isolated from baboon liver, while virtually no complex formation took place with baboon stefin A (Fig. 36). The baboon stefins were classified on the basis of their pI values and mobility on PAGE in the presence and absence of DTT (Dennehy, Coetzer and Dennison, unpublished results).

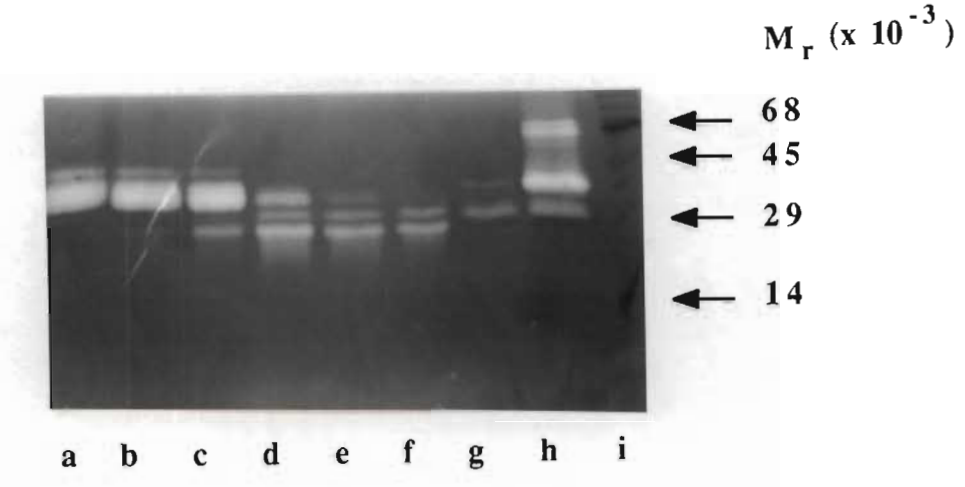

Figure 35. Gelatin-containing substrate SDS-PAGE of free and stefin B-complexed baboon cathepsin L. Samples (2.5 µg) from peaks 1 and 2 from Fig. 33 were electrophoresed on a 12.5% gelatin substrate gel. Stefin B-complexed baboon cathepsin L (a) and (b) from peak 1; (c) and (d) eluted intermediary to peaks 1 and 2; (e) and (f) free baboon cathepsin L from peak 2; (g) free sheep cathepsin L; (h) stefin B-complexed sheep cathepsin L; and (i) MW markers as in Fig. 12.

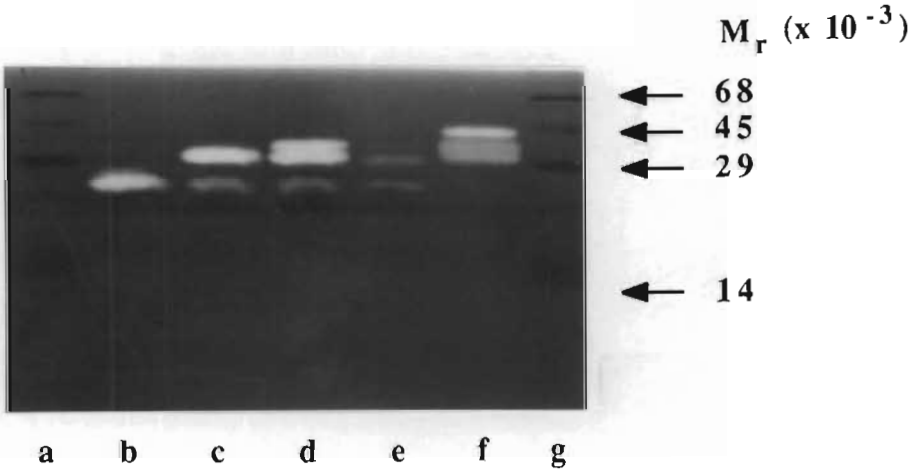

Figure 36. Gelatin-containing substrate SDS-PAGE of baboon cathepsin L incubated with baboon stefins A and B.
Non-reduced samples were loaded onto a 12.5% gelatin substrate gel. (a) and (g) MW markers, as in Fig. 12; (b) free baboon cathepsin L; free baboon cathepsin L (2.5 µg) incubated for 30 min at 37°C in 100 mM MES buffer, pH 6.0, with (c) baboon stefin B isoform subfraction; (d) baboon stefin B subfraction; (e) baboon stefin A subfraction; and (f) isolated stefin B-complexed baboon cathepsin L alone.

In contrast to sheep liver, the baboon organ yielded more free (1.26 µg) than complexed cathepsin L (1 µg) per gram of wet tissue weight (Table 15), which is about one third of the yield from sheep liver for both forms of enzyme. Free baboon cathepsin L was purified 2 500-fold, on the basis of specific activity against azocasein, but only 1 350-fold, on the basis of specific activity against Z-Phe-Arg-NHMec (Table 15). This corresponds to a yield of 2.6% based on total azocasein activity and 1.8% on that of Z-Phe-Arg-NHMec;

the underestimate of the latter being partly due to hydrolysis of the synthetic substrate by cathepsin B, present in the homogenate (Barrett and Kirschke, 1981).

Table 15. Purification of free- and stefin B complexed cathepsin L from baboon liver. Comparison of activity against azocasein and Z-Phe-Arg-NHMec.

Step	Vol. (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Purification (fold)	Yield (%)
Homogenate pH 4.2	943	36588	21291	0.58	1	100
supernatant	752	4083	22844	5.5	5	107
TPP	208	580	11356	19.6	34	53
S-Sepharose	38	5	2848	560	972	13
MEC (complex)	30	0.4	449	1119	1923	2.1
MEC (free)	40	0.5	559	1479	2541	2.6

Step	Vol. (ml)	Total protein (mg)	Total activity (mU)[b]	Specific activity (mU/mg)	Purification (fold)	Yield (%)
Homogenate pH 4.2	943	36588	62460	1.7	1	100
supernatant	752	4083	45580	11.2	6.5	73
TPP	208	580	17216	29.7	17.4	27
S-Sepharose	38	5	5774	1148	672	9
MEC (complex)	30	0.4	910	2275	1133	1.4
MEC (free)	40	0.5	1133	2312	1354	1.8

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

[b] 1 mU milli-(unit) of activity = activity against Z-Phe-Arg-NHMec as defined by Barrett and Kirshcke (1981)

Whereas the increase in total activity of the supernatants, obtained following autolysis at pH 4.2, over that of the initial homogenates, was ascribed to the dissociation during autolysis of cystatins bound in the non-covalent inhibiting mode in the case of sheep liver and kidney, non-covalently bound cystatins would have dissociated during assays against the sensitive synthetic substrate used for the comparative baboon cathepsin L purification table. The relatively high total activity measured in the homogenate in this case could be due to additional hydrolysis by cathepsin B (Barrett and Kirscke, 1981). This possibly indicates that the azocasein assay which may be conducted in the presence of urea, to inactivate cathepsin B (Kirschke *et al.*, 1982), may be more useful for the purpose of constructing purification tables for cathepsin L, despite its known limitations (Mason *et al.*, 1982).

Both M_r 26 000 and 37 000 components were further characterised as cathepsin L, on the basis of their behaviour with a range of inhibitors and the activator, DTT (Table 16). The high level of inhibition by Z-Phe-Phe-CHN_2 is especially noteworthy in relation to the pH optimum of Z-Phe-Arg-NHMec hydrolysis of 7.0 (see later), which raises the possibility of the baboon enzyme being cathepsin S. Cathepsin S does not, however, react with Z-Phe-Phe-CHN_2 (Kirschke *et al.*, 1984), nor does it bind strongly to cation exchangers (Maciewicz and Etherington, 1988), and it is usually purified from spleen (Kirschke *et al.*, 1984; Ritonja *et al.*, 1991).

Table 16. The effect of inhibitors and the absence of DTT on free and stefin B-complexed forms of baboon liver cathepsin L.

Inhibitor	Final concentration (μM)	% activity	
		Complexed	Free
Bestatin	5	94	93
E-64	1	1.8	2.9
Iodoacetamide	1000	51	14
Iodoacetate	1000	2.5	3.5
Leupeptin	1	0.4	0.25
Pepstatin	1	80	90
PMSF	250	78	82
SBTI	1	139	42
TLCK	0.2	0.5	0
TPCK	1000	9.7	16
Z-Phe-Phe-CHN_2	1	0.9	1
DTT	0	0.8	0.4

Both forms of baboon cathepsin L showed a similar increase in activity, with an increase in cysteine concentration, as the sheep enzyme did (Section 4.9.9) and also consistent with the situation in the sheep system that complexing of stefin B with cathepsin L had a similar activating effect as increased cysteine concentrations (Fig. 37).

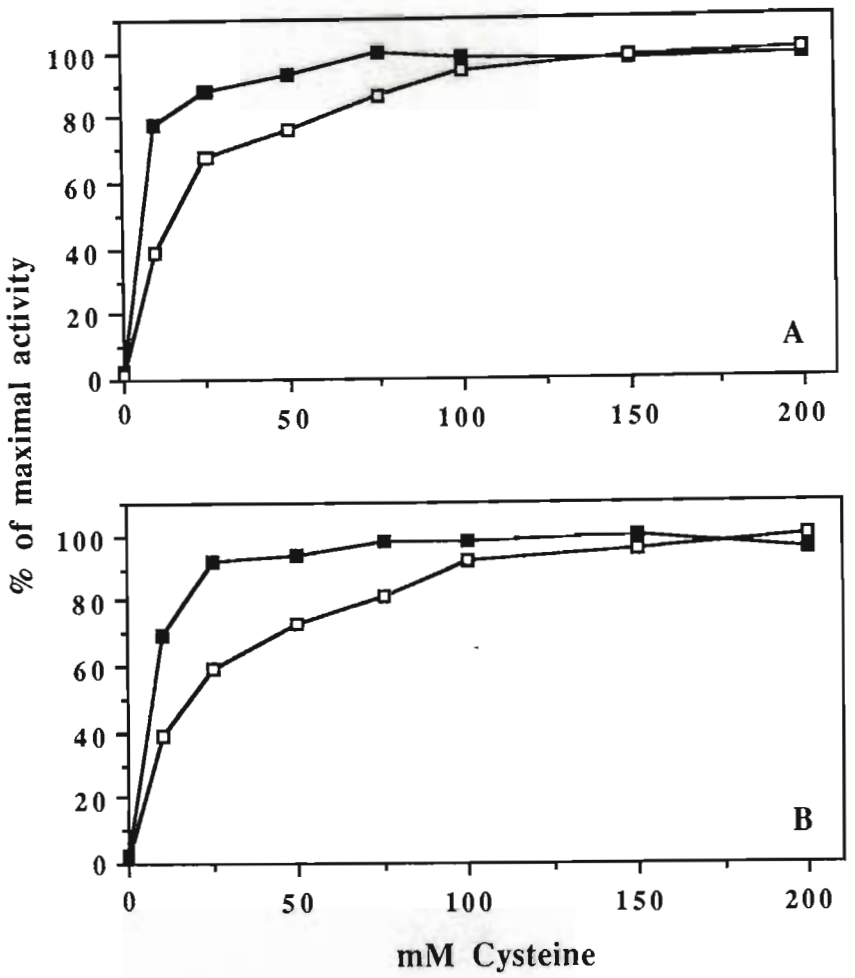

Figure 37. The effect of cysteine concentration on the activity of free and stefin B-complexed forms of baboon liver cathepsin L.
Z-Phe-Arg-NHMec hydrolysis of equimolar amounts of free and stefin B-complexed baboon cathepsin L was measured in standard 10 min assays using AMT buffers of pH 5.5 (**A**) and 7.0 (**B**), as described in section 4.8.1, containing a range of cysteine concentrations. (❑), free cathepsin L; (■), stefin B-complexed cathepsin L. Each point represents the mean of five determinations.

The free and complexed single-chain baboon cathepsin L have very similar pH profiles (Fig. 38) with pH optima of 7.0. Both forms retained almost full activity at pH 7.5; 75% in the case of the free enzyme and 83% by the complexed enzyme. Free and stefin B-complexed single-chain sheep cathepsin L manifested pH-optima of 6.0, with 60% activity remaining at pH 7.0 (Dennison *et al.*, 1992). A similar pH-optimum of 6.5, with 80% activity remaining at pH 7.5, was described for recombinant processed single-chain human cathepsin L (Smith and Gottesman, 1989), and a pH-optimum of 7.0, with 70% activity remaining at pH 7.5 for cystatin-complexed single-chain human spleen cathepsin L (Pike, 1990). These pH-optima differ considerably from the values of 5.5, with very little activity remaining at pH 7.0 and above, reported for Z-Phe-Arg-NHMec hydrolysis by two-chain human cathepsin L (Mason *et al.*, 1985). This decline in activity above pH 6.0 was ascribed to the instability of the enzyme, as measurements of substrate hydrolysis during the

first 30 s of incubation resulted in the detection of *ca.* 70% activity at pH 8.0 and 75% at pH 7.5 (Mason *et al.*, 1985).

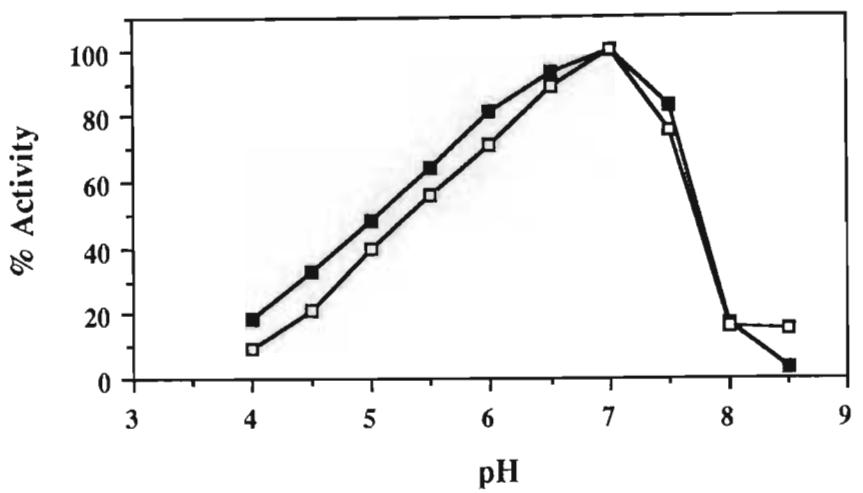

Figure 38. The effect of pH on the hydrolysis of Z-Phe-Arg-NHMec by free and stefin B-complexed forms of baboon liver cathepsin L.
Z-Phe-Arg-NHMec hydrolysis of equimolar amounts of free and stefin B-complexed cathepsin L was measured in standard 10 min assays using the range of AMT buffers described in section 4.8.1, containing 8 mM DTT. (❑), free cathepsin L; (■), stefin B-complexed cathepsin L. Each point represents the mean of five determinations.

The free and complexed forms of the baboon cathepsin L, therefore, seems to have a higher stability around neutral pH, since the activities were retained during the course of the assay. Studies on the stability of the different forms of sheep liver cathepsin L, based on half-life measurements at the different pHs, showed that the enzyme's behaviour in the pH-optimum assay gives a satisfactory reflection of its stability at those pH-values (Dennison *et al.*, 1992). This unusual activity and inferred stability shown by baboon cathepsin L at neutral pH, which seems to correlate with the single-chain form of the enzyme, raises the possibility that both free and stefin B-complexed forms of cathepsin L would be capable of digesting extracellular matrix components at physiological pH and could, therefore, play a role in tumour invasion.

Following the successful demonstration that the novel, proteolytically active covalent complexes are not restricted to sheep, but also occur in higher primates, the isolation of similar complexes in human liver was attempted.

4.9.13 Isolation of free and stefin B-complexed cathepsin L from human liver

Cathepsin L isolated from human liver eluted at positions corresponding to M_r 37 000 and 26 000 during MEC on Sephadex G-75 (Fig. 39). A proportion of the M_r 37 000 form was stable under non-reducing SDS-PAGE conditions (Fig. 40B), and by analogy with a similar situation found in sheep and baboon liver, this M_r 37 000 form was identified as a complex between cathepsin L and a cystatin, probably stefin B. Once again the complexing moiety could not be identified unequivocally as stefin B, because the antibodies to sheep stefin B did not cross-react with human stefin B. The free M_r 26 000 form of cathepsin L migrated to the same position in SDS-PAGE under both reducing and non-reducing conditions, indicating the single-chain nature of the enzyme (Fig. 40A and B). Both free and stefin B-complexed human cathepsin L showed activity against Z-Phe-Arg-NHMec and proteolytic activity against azocasein (Fig. 39) and gelatin, the latter on gelatin-substrate SDS-PAGE (Fig. 40C). The isolation of single chain cathepsin L and the novel proteolytically active cathepsin L/stefin B complexes from human liver, therefore, means that these complexes may indeed be relevant in human tumour invasion.

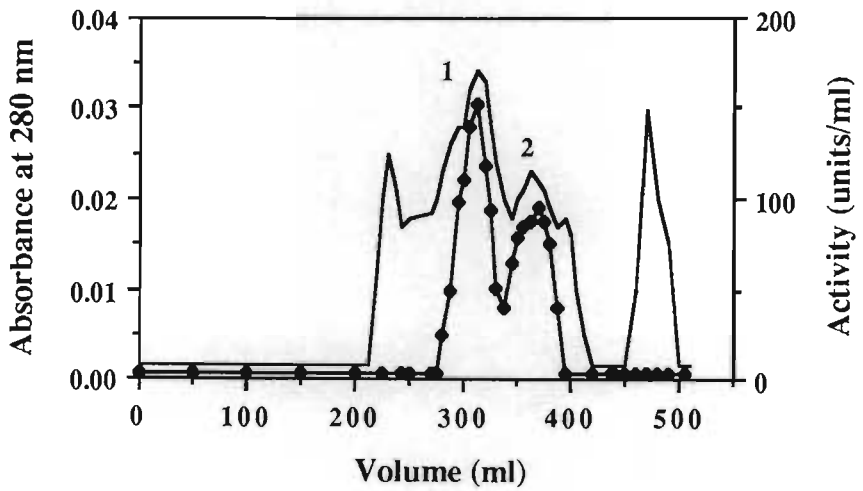

Figure 39. Molecular exclusion chromatography of the pH 5.5 S-Sepharose fraction from human liver on Sephadex G-75.
Column, 2.5 x 90 cm (450 ml bed volume); buffer, 20 mM Na-acetate, pH 5.5, containing 1 mM Na_2EDTA and 200mM NaCl; flow rate, 25 ml/h (5 cm/h); and fractions, 5 ml (12 min). Void volume was 150 ml. (—), A_{280}; (◆), enzyme activity (units/ml) from the assay against azocasein. 1, complexed cathepsin L; 2, free cathepsin L.

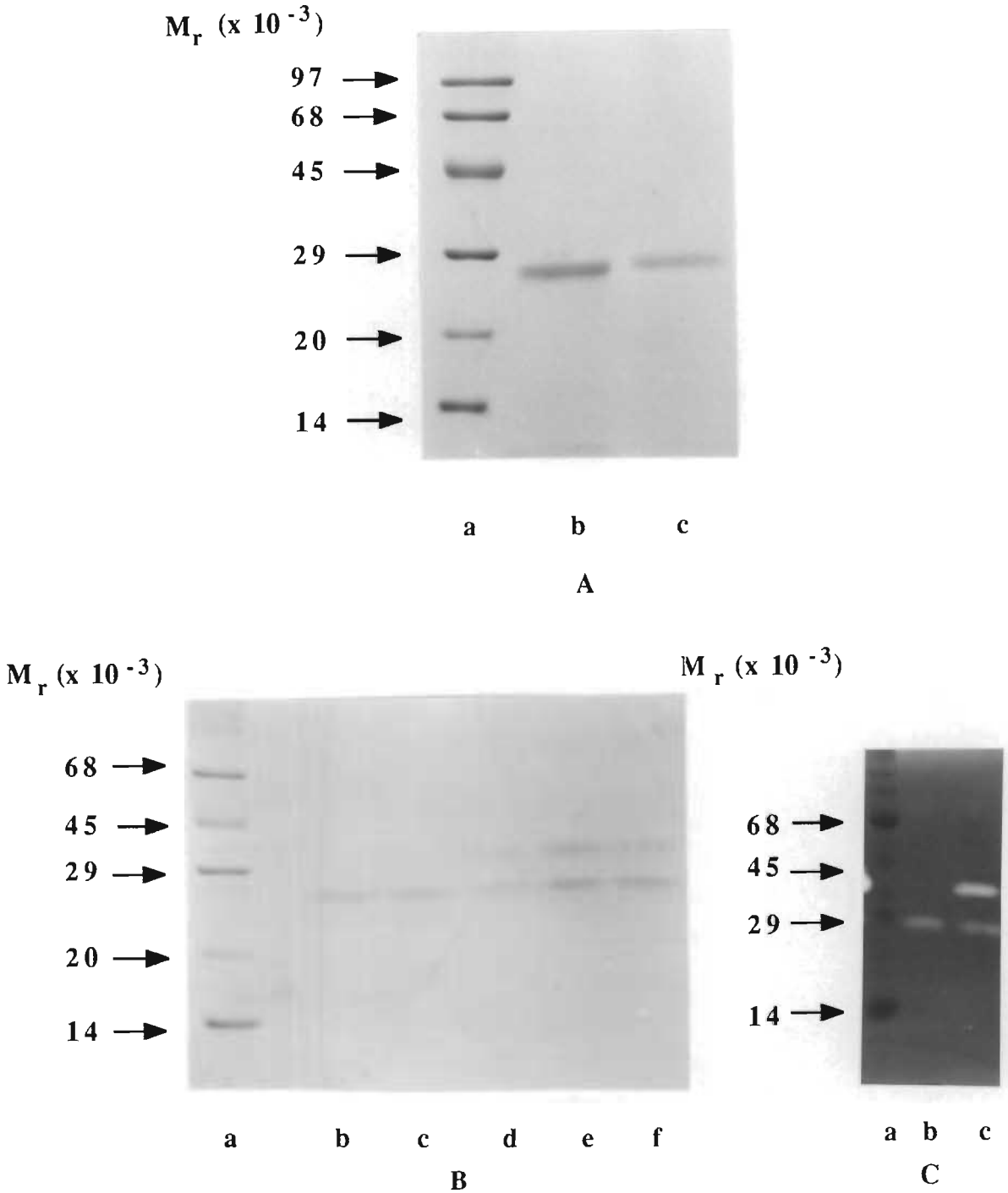

Figure 40. SDS-PAGE of free and stefin B-complexed forms of human liver cathepsin L.
(**A**) Samples (10 µg) were reduced and loaded onto a 12.5% gel. (a) MW markers, as in Fig. 22 (with phosphorylase b, M_r 97 000); (b) free human cathepsin L; and (c) stefin B-complexed human cathepsin L. (**B**) Non-reduced samples were electrophoresed on a 12.5% gel. (a) MW markers, as in Fig. 22 ; (b) and (c) free human cathepsin L (1.5 µg); stefin B-complexed human cathepsin L, (d) 1 µg; (e) and (f) 1.5 µg. (**C**) Non-reduced samples (2 µg) were loaded onto a 12.5% gelatin substrate gel. (a) MW markers, as in Fig. 12; (b) free human cathepsin L; (c) stefin B-complexed human cathepsin L.

4.10 Discussion

This chapter was concerned with the isolation of single-chain cathepsin L from sheep liver for amino sequence determination, and the characterisation of the novel proteolytically active, covalent cathepsin L/stefin B complexes which are isolated in conjunction with the free enzyme. Characterisation was facilitated by the elucidation of the amino acid sequences of sheep cathepsin L and stefin B, which are reported here for the first time. Single-chain forms of cathepsin L, as well as similar novel cathepsin L/stefin B complexes, were also isolated from the liver of higher primates, suggesting potential roles for these forms of cathepsin L in the digestion of the ECM during tumour invasion.

The single-chain form of cathepsin L isolated from sheep, baboon and human liver using the method of Pike and Dennison (1989b) contrasts with the two-chain form isolated from sheep (Mason, 1986) and human liver (Mason *et al.*, 1985). On account of conflicting results obtained in various isolation procedures from different sources, there is some doubt as to whether cathepsin L exists *in vivo* in a two-chain form. Whereas Dufour *et al.* (1987) isolated single-chain M_r 27 000 cathepsin L from chicken liver lysosomes using a rapid procedure, Wada and Tanabe (1986) isolated a two-chain M_r 28 000 and 5 000 form from chicken liver homogenates using a longer procedure. However, isolation of cathepsin L directly from rat liver lysosomes yielded only a two-chain form (Kirschke *et al.*, 1977a). A mixture of single- (M_r 30 000) and two-chain (M_r 25 000 and 5 000) cathepsin L was isolated from rat kidney homogenates using a fairly rapid procedure (Bando *et al.*, 1986), but only the presence of the single-chain form could be demonstrated by Western blots in the homogenates in the same study. Dalert-Fumeron *et al.* (1991) also isolated mixtures of the two forms of cathepsin L from human liver using a three-step HPLC isolation method. The two-chain nature of the cathepsin L, purified from human kidney and bovine spleen, was confirmed during amino acid sequence determination (Ritonja *et al.*, 1988; 1991) and similarly two-chain forms were also purified from rabbit (Mason *et al.*, 1984), human (Mason *et al.*, 1985) and bovine liver (Mason, 1986), which contrasts with the single band of M_r 30 000 obtained for human liver cathepsin L under reducing SDS-PAGE conditions reported by Pagano and Engler (1982). Interestingly, the only other report of single-chain cathepsin L was the recombinant human cathepsin L, expressed in *Escherichia coli* (Smith and Gottesman, 1989).

Pulse-chase labelling and immunoprecipitation techniques, used in studies on the intracellular processing of cathepsin L in various cell types, showed that this takes place in the lysosomes, but there was some discrepancy regarding the class of proteinase involved. Cathepsin D was shown to be the posttranslational processing proteinase for procathepsin L

in rat hepatocytes, converting the M_r 39 000 pro-enzyme to the mature M_r 30 000 single-chain form (Nishimura *et al.*, 1988b; 1989). The conversion of the single- to the two-chain form appeared to be a rapid process, but the processing proteinase was not identified in this case (Nishimura *et al.*, 1988a). Processing of the M_r 39 000 procathepsin L to a single-chain M_r 32 500 mature form in rat fibroblasts could also be accelerated by cathepsin D (Wiederanders and Kirschke, 1989). No further processing to a two-chain form was observed in the presence of cathepsin D, nor could processing to the two-chain form be brought about by cathepsins B, H or L.

In mouse NIH3T3 fibroblasts, intracellular processing of procathepsin L to the M_r 29 000 single-chain form was mainly catalysed by cathepsin D, while subsequent processing to the M_r 20 000 two-chain form appeared to be mostly cathepsin L dependent (Salminen and Gottesman, 1990). In rat macrophages, however, a metalloendopeptidase was implicated in the conversion of the M_r 39 000 procathepsin L to the M_r 29 000 single-chain form, while a cysteine proteinase was found to be responsible for further conversion to the two-chain form (Hara *et al.*, 1988). This is consistent with the two-chain form of chicken liver cathepsin L purified by Wada and Tanabe (1986), despite the inclusion of pepstatin in the homogenate. Although there is conflicting evidence on the class of processing proteinase involved in the conversion of procathepsin L to the mature single- and/or two-chain forms, it seems to be cell type dependent and cathepsin D appears to emerge as the most likely candidate for processing to the single-chain form in hepatocytes and fibroblasts, while further processing to the two-chain form could be the result of autocatalysis (Salminen and Gottesman, 1990).

Although it has been postulated (Mason *et al.*, 1985; Wiederanders and Kirschke, 1989) that the two-chain form of cathepsin L results from autolytic cleavage during the autolysis step, used in the isolation of human (Mason *et al.*, 1985), sheep and bovine (Mason, 1986), as well as chicken cathepsin L (Wada and Tanabe, 1986), two-chain cathepsin L was isolated directly from the lysosomes (Kirschke *et al.*, 1977a), while single-chain cathepsin L resulted from the method used in the present study, which does involve autolysis. Initially it was thought that the two-chain form of the enzyme, isolated by other workers in the field, could be the result of using *post mortem* tissue, especially since only single-chain cathepsin L could be purified from sheep liver in the present study, even when using the purification method of Mason *et al.* (1985) as a comparison, but this was negated by the fact that the two-chain form was isolated directly from lysosomes (Kirschke *et al.*, 1977a) and that single-chain cathepsin L was isolated in the present study from *post mortem* human liver tissue.

The isolation of single-chain cathepsin L seems to correlate to a larger extent with the length of a purification procedure, especially the time taken before cathepsin L is separated from other proteinases. Both the isolation method developed in this laboratory by Pike and Dennison (1989b), which was used in this study, and the procedure used by Dufour *et al.* (1987), are rapid purification methods, distinguished by chromatography on a strong cation exchanger early in the isolation method, which selects for cathepsin L and separates it from other proteinases, thus possibly preventing further processing. The latter appears to happen during the lengthy chromatography steps employed in other isolation methods, which often include a weak cation exchanger during the initial steps. As will be discussed below, the single-chain nature of cathepsin L, purified in the present study, may be instrumental in the formation of novel complexes with stefin B.

The results of Western blots (Fig. 17) and substrate SDS-PAGE analysis (Fig. 12C and 20), provide evidence that sheep liver stefin B, the identity of which was confirmed by amino acid sequencing, is apparently able to complex with single-chain cathepsin L in at least two modalities - the established inhibiting mode and an alternative non-inhibiting mode. Unequivocal evidence for the existence of the non-inhibitory binding mode is provided by the purification of proteolytically active cathepsin L/stefin B complexes, from sheep liver, and by the demonstration that similar complexes can be formed *in vitro* from purified, active constituents.

The isolated complexes were active against proteins, such as azocasein, as well as against Z-Phe-Arg-NHMec, while those formed *in vitro* were only tested against gelatin, but were able to digest this substrate. In terms of a model of tight binding, reversible inhibition, activity against the synthetic substrate is not unexpected since the assay is very sensitive and a high dilution of the enzyme is required; at this dilution, non-covalent enzyme/inhibitor complexes may dissociate. Activity against protein substrates is not expected, however, and cannot be explained in this way, as assays using protein substrates require higher concentrations of enzyme, at which enzyme/inhibitor complexes would not be expected to dissociate. A different model is, therefore, required to explain the active complexes.

A possible explanation is that covalent complex formation may require denatured or inactivated forms of stefin B, in a reaction of the form

$$E + I^* \rightarrow EI^*,$$

where I* represents denatured or inactivated stefin B and EI* represents active complex. The results of the isolation study, and those presented in Fig. 20, however, argue against such an explanation. Of the myriad of molecules present in a homogenate, it is apparently only stefin B that complexes with cathepsin L, in the novel, covalent manner reported here, which suggests that the reaction is highly specific and is, therefore, unlikely to be due to disabled stefin B. Also, although cystatins are particularly stable molecules, their denaturation would more likely occur at pH extremes. On the other hand, inactivation, especially by proteolysis by residual cathepsin L, would be expected to approximately follow the pH activity curve of cathepsin L. The results presented in Fig. 20 show that neither of these obtains; complexes do not form below pH 5.5, but form readily above this pH. It was also found that no covalent complexes formed *in vitro* with SDS-denatured constituents, indicating that the conformation of the components is important for the formation of the novel, covalent complex.

Sheep liver stefin B is able to inhibit both free cathepsin L and the isolated complex (Fig. 15), the latter suggesting that cathepsin L can perhaps interact with two stefin B molecules, in two different ways, including the possibility that the covalently bound stefin B does not occlude the active site. Although the inhibition of sheep liver cathepsin L by sheep liver stefin B has not been subject to kinetic analysis and the K_i values are not known, the inhibition and complex-formation reactions, in their simplest form and in qualitative terms may be proposed to be of the form

$$E + I \rightleftharpoons EI \rightleftharpoons EI^* + I \rightarrow EI^*I$$

a model which was derived from computer simulation studies using theoretical sets of data (Dennison, unpublished results). Several pieces of evidence are consistent with the isolated complex fraction (peak 1, Fig. 11) comprising a mixture of forms of complex; one in which the two molecules are associated in the usual non-covalent, reversible, inhibiting way (EI), another in which the molecules are more firmly bound, but in a non-inhibiting manner (EI*), and, perhaps, a third (EI*I) comprised of both forms of interaction. For example, it has previously been shown that complexes of chicken egg white cystatin with papain dissociate on SDS-PAGE, without reduction (Anastasi *et al.*, 1983; Wakamatsu *et al.*, 1984). In the

case of the active complex eluted from Sephadex G-75 in the M_r 37 000 region, however, on non-reducing SDS-PAGE, M_r 42 000, 37 000, 26 000 (and 14 000) components were found (Fig. 12). This suggests that a proportion of the complex fraction (the M_r 42 000 and 37 000 constituents) does not dissociate without reduction (by boiling in 1.4 M 2-mercaptoethanol), and may represent conformational variants of complexes consisting of cathepsin L bound to a single stefin B molecule (EI*), while another proportion (the M_r 26 000 and 14 000 constituents) does dissociate. Binding of stefin B in the usual inhibiting mode to the two forms of cathepsin L would, therefore, represent EI, in the case of binding to free cathepsin L, and EI*I when bound to the novel cathepsin L/stefin B complex.

The results of E-64 active site titrations (Fig. 18) are also consistent with a model of mixed forms of complex. The proportion of the complex fraction, active against Z-Phe-Arg-NHMec, but inaccessible to E-64, could be complexed with the stefin B in a normal manner, and is thus unavailable for E-64 binding. The E-64 results, therefore, suggest that the inhibitor may be normally complexed to about 40% of the active enzyme in the complex, and abnormally complexed to 60% of the active enzyme in the complex. The effect of Z-Phe-Phe-CHN_2, suggests that all of the proteinase activity is due to cathepsin L.

An indication that the novel, proteolytically active, covalent complexes between cathepsin L and the endogenous inhibitor, stefin B, are not restricted to sheep and may, therefore, be relevant in the context of tumour invasion, was given by the purification of similar complexes from the liver of a higher primate, the baboon (*Papio ursinus*), and the illustration that these complexes form spontaneously *in vitro* from purified, active, baboon cathepsin L and stefin B. Subsequently it was demonstrated that similar proteolytically active covalent complexes may be isolated from human liver homogenates. The isolation of single-chain cathepsin L from baboon and human, consistent with that from sheep liver, gives further support to the contention that the single-chain form of the enzyme may be a prerequisite for covalent complex formation (Dennison *et al.*, 1992).

Formation of the novel, covalent complexes seems to be organ, rather than species specific. Whereas active, covalent cathepsin L/stefin B complexes were purified in the present study from sheep, baboon and human liver, as well as sheep kidney, only inactive, non-covalent complexes could be isolated from both sheep and human spleen (Pike, 1990). This is in agreement with studies by Mason *et al.* (1989), using the radiolabelled active site probe, Z-[^{125}I]Tyr-Ala-CHN_2, which showed that active cathepsin L was mainly found in human liver and kidney, with only very small amounts in spleen.

An indication of the possible involvement of a cysteine residue in the covalent bond between cathepsin L and stefin B, stems from the observation that this bond is only broken by reduction, with boiling, prior to SDS-PAGE. Disulfide links usually form more readily at higher pH, however, and pH 5.5 (see Fig. 20) is lower than the pH usually required (Creighton, 1984), but it has been found that disulfides may form in a relatively reducing environment, provided the conformation of the protein ensures that the sulfhydryl groups are in favourable proximity for disulfide formation (Creighton, 1984). The cysteine residue at position 3 in stefin B could conceivably participate in a link of some sort. The fact that the M_r 37 000 and 42 000 complexes are proteolytically active, and are not dissociated by activating levels of reducing agent, indicates that the linkage is unlikely to be through the active site Cys-25 of cathepsin L. The inactive M_r 45 000 variant of the M_r 37 000 complex induced by incubation at pH 4.2 could, however, involve the active site cysteine. Two questions thus arose, firstly, whether there is a plausible mechanism by which a disulfide link could be formed between cathepsin L and stefin B, and secondly, whether the Cys-3–residue in stefin B could become optimally orientated for the proposed covalent complex formation during its usual association with cathepsin L.

An intermolecular disulfide bridge could possibly be formed between stefin B and cathepsin L by thiol-disulfide interchange, in a manner similar to the intrachain mechanism proposed for papain activation (Fig. 41) (Brocklehurst and Kierstan, 1973). Extrapolations between papain and cathepsin L are possible because of the high degree of similarity in structure and catalytic properties (Dufour, 1988; Dufour *et al.*, 1987). The active site Cys-25 in inactive papain is disulfide bonded to a neighbouring cysteine residue, Cys-22 or Cys-63, and upon activation by a reducing agent, undergoes thiol-disulfide interchange which results in the formation of a disulfide bond between Cys-22 and Cys-63, while a Cys-25 thiolate–His-159 imidazolium ion pair is formed. Activation of alkylated papain with DTT, showed furthermore that the formation of a disulfide bond between Cys-22 and Cys-63 is not essential for activity (Brocklehurst and Kierstan, 1973). Binding of stefin B to either of these Cys-residues would, therefore, not interfere with enzyme activity, provided stefin B does not occlude the active site cleft.

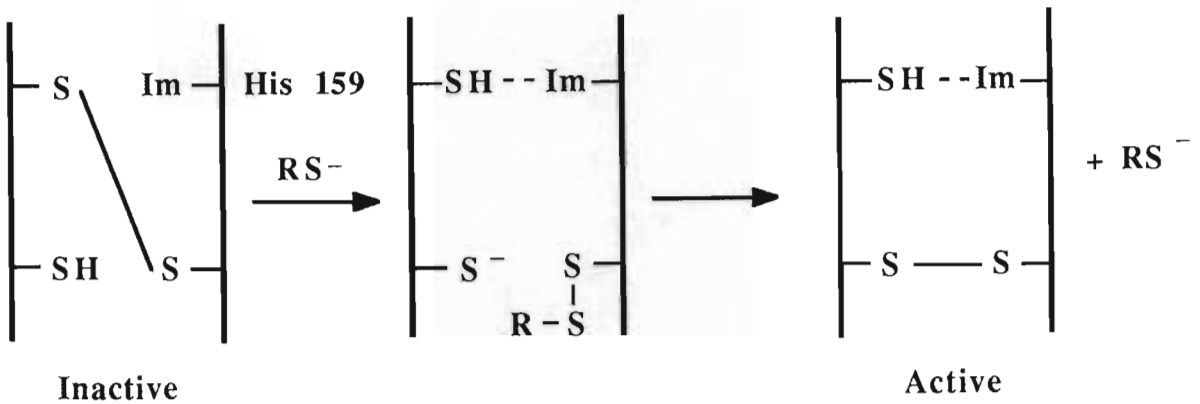

Figure 41. Activation of papain by intrachain thiol-disulfide interchange, according to Brocklehurst and Kierstan (1973).
An active site Cys-25 thiolate–His-159 imidazolium ion pair (–SH- -Im–) is formed following formation of a disulfide bond between Cys-22 and Cys-63. Either of these Cys-residues may be disulfide bonded to the active-site Cys-25, before activation by a reducing agent, RS^-.

Two observations; firstly that, at equimolar concentrations, the stefin B-complexed cathepsin L has approximately four-fold greater activity than the free enzyme (Dennison *et al.*, 1992), and, secondly, that complexing of stefin B with cathepsin L has a similar activating effect as the addition of high levels of cysteine, suggest that bound in this alternate manner, stefin B may possibly act as an activator, and may aid the reducing agent in the thiol-disulfide interchange activation mechanism, by increasing the availibility of the active site Cys-25 for activation. Further evidence for this possible activating function of covalently bound stefin B was found in the increase in the molar concentration of active free cathepsin L, measured by active site titration, achieved in the presence of high levels of cysteine. Similar proportions of active stefin B-complexed cathepsin L were measured at low cysteine concentrations.

According to the inhibitory mechanism of interaction between stefin B and papain, established by Stubbs *et al.* (1990) from X-ray crystallography studies on such complexes, the N-terminal trunk and two hairpin loops of stefin B form a hydrophobic wedge which is complementary to, and slots into the active site groove of papain (Fig. 42). The main interactions are provided by the N-terminal trunk, which incidently contains a free Cys-residue at position 3, and the first hairpin loop which contains the highly conserved QVVAG sequence, with minor contributions by the second hairpin loop. Additional interaction is provided by the carboxy terminus of stefin B.

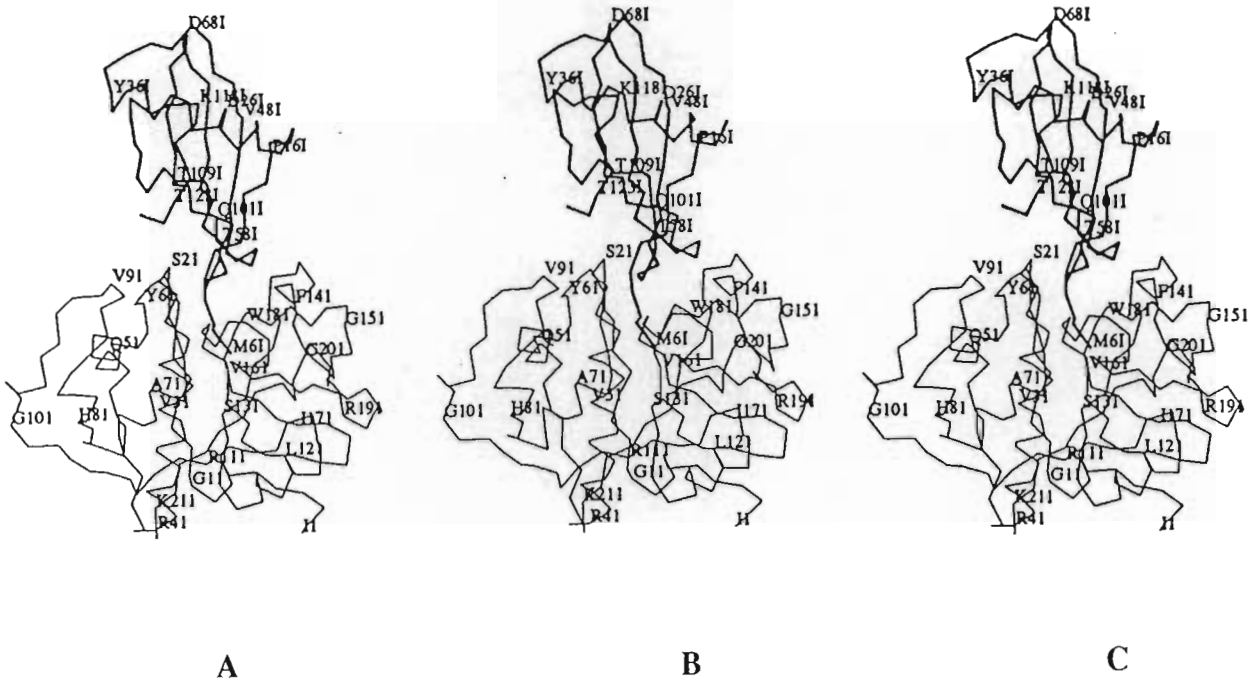

Figure 42. An α-carbon trace of the complex formed between human stefin B (bold connections) and papain (thin connections), adapted from Stubbs *et al.* (1990).
Every tenth residue is labelled; the suffix "I" serves to distinguish the inhibitor residues from those of the enzyme. The view is from the "front", looking along the active site cleft with the N-terminal trunk closest to the viewer. The complex may be visualised three dimensionally by viewing traces A and B with a stereoscope, or by stereo viewing traces B and C with unaided eyes using proximal convergence (Wood *et al.*, 1981).

Although it was initially thought that the QVVAG sequence was essential for inhibitory activity (Teno *et al.*, 1987), it was shown that substitution of Val-47 by hydrophobic residues had little effect on the inhibitory activity of stefin B on papain (Jerala *et al.*, 1990). The QLVAG sequence variant found in bovine and sheep stefin B, therefore, probably has little effect on the K_i of normal inhibitory interaction. Examination of the three-dimensional structure of the inhibitory complex reveals that the free Cys-3 residue in the trunk of stefin B will be close to Cys-residues 22, 25 and 63 and could, therefore, conceivably become covalently bonded to cathepsin L during the normal association/dissociation it undergoes as a reversible inhibitor. This covalent bond would not have formed in the crystallised complex of recombinant human stefin B and papain, prepared by Stubbs *et al.* (1990), because they used the mutant in which Cys-3 was replaced by Ser to prevent dimerisation of the stefin B.

The presence of a Pro-residue in position 6 of sheep and human stefin B (Fig. 32), both of which form unusual covalent complexes with cathepsin L, could conceivably contribute to the flexibility of the Cys-3 containing part of the N-terminal trunk, thereby enhancing the probability of thiol-disulfide interchange resulting in covalent complex formation. Since the Pro-residue in position 6 is replaced by Thr in bovine stefin B (Krizaj

et al., 1992), it would be interesting to determine whether proteolytically active, covalent cathepsin L/stefin B complexes could be isolated from bovine liver and whether bovine stefin B is able to form similar complexes with sheep cathepsin L *in vitro*.

Another candidate disulfide for intermolecular thiol-disulfide interchange between stefin B and cathepsin L is the labile bond between Cys-residues 56 and 95 (papain numbering), described by Shapira and Arnon (1969) in papain. This bond is more labile to reduction in 8 M urea than any of the other disulfides in papain and is not essential for activity, since the partially reduced enzyme retains activity. Although this labile disulfide bond is further away from the docking position of the Cys-3 containing trunk of stefin B, it is found in a part of the enzyme which differs most between papain and cathepsin L. Dufour (1988) showed that the main conformational differences between papain and the other cysteine proteinases are found in the middle region of the molecule, between residues 71 and 117. There is an additional α-helix in cathepsin L, spanning residues 85-108 (Dufour, 1988), which may have an important bearing on the tertiary structure of cathepsin L, bringing the labile disulfide Cys-56-95 closer to Cys-3 in the stefin B molecule.

Docking of stefin B in the active site groove of cathepsin L, presents a further possibility for Cys-3 of stefin B to undergo thiol-disulfide interchange with the disulfide between Cys-residues 153 and 200 (papain numbering) for proteolytically active, covalent complex formation. However, this disulfide bond between Cys-residues 153 and 200 has been shown by deletion analysis to be a critical structural feature, essential for enzyme activity (Smith and Gottesman, 1989). This disulfide bridge probably stabilises the active site conformation since the Cys-153 forms part of the sequence which contains the active site His-159. In the two-chain version of cathepsin L, where the peptide bond between Thr-175 and Gln-176 had been hydrolysed, resulting in a flexible light chain only attached by the aforementioned disulfide bond, disruption of this disulfide bond, by thiol-disulfide interchange with stefin B, might lead to loss of the light chain, with consequent loss of activity. A single-chain enzyme might, therefore, be a prerequisite for the formation of catalytically active cathepsin L/stefin B complexes. Additionally, the fact that the free single-chain enzyme itself apparently requires much higher levels of cysteine, for complete activation, than has previously been reported to be the case for single- and two-chain forms of cathepsin L (Dufour *et al.*, 1987; Barrett and Kirschke, 1981), suggests that the single-chain sheep enzyme might be able to tolerate higher levels of cysteine, or that two-chain forms of cathepsin L might be susceptible to inactivation by high cysteine concentration and/or covalent bonding to stefin B.

The reactivity of stefin B for covalent complex formation with cathepsin L could possibly be regulated by formation of a mixed disulfide with glutathione, as was demonstrated for a form of rat stefin B (Wakamatsu *et al.*, 1984). This reaction may be catalysed by the glutathione specific thiol transferase found in the hepatic cytosol (Mannervik and Axelsson, 1975). Depending on the cytosolic ratio of reduced to oxidised glutathione, Cys-3 on stefin B may be available to a lesser or greater extent for covalent complex formation. It has been shown that the levels of glutathione-protein mixed disulfides vary diurnally with the feeding cycle in rat hepatic tissue (Isaacs and Binkley, 1977) and this may explain the liver to liver variation in the amounts of covalent cathepsin L/stefin B complex isolated, as well as stefin B monomer/dimer ratios. Oxidised glutathione may also act as the electron acceptor necessary for disulfide formation between cathepsin L and stefin B and the redox potential in the cytosolic environment following homogenisation of liver tissue could, therefore, play an important role in the relative amounts of covalent complex formed.

In the context of tumour invasion, PMNLs and macrophages, drawn to the inflammatory site, would generate elevated levels of oxidised glutathione as a result of increased respiration during phagocytosis (Tschesche and Macartney, 1981). These changes in oxygen metabolism, known as the "respiratory burst" of phagocytic cells, are coupled to an increased glucose catabolism through the hexose monophosphate shunt and to an increased production of superoxide anions, and ultimately hydrogen peroxide in the cytosol. Glutathione peroxidase or myeloperoxidase then catalyses the formation of oxidised glutathione from hydrogen peroxide and reduced glutathione (Weiss, 1989). It is thus plausible that the formation of cathepsin L/stefin B complexes may occur via thiol-disulfide interchange, coupled to the glutathione cycle, in highly inflamed pathological tissue where the subcellular compartmentation might have broken down.

Studies on the *in vitro* formation of covalent stefin B/cathepsin L complexes and the characterisation of isolated complexes, suggest that different types of covalent bonds may be involved in their formation. Whereas cathepsin L and stefin B are probably linked by disulfide bonds in the M_r 68 000 and 42 000 complexes, the M_r 37 000 complex could possibly comprise a mixture of complexes formed by disulfide and thioester bonds. The thioester bond could be formed between the stefin B glutaminyl γ-carbonyl (Gln-10) and a cathepsin L cysteinyl sulfhydryl, since no covalent complexes were formed *in vitro* between cathepsin L and the type 2 cystatins, recombinant cystatin C or chicken egg white cystatin, which in addition to all lacking free sulfhydryl-groups, also do not posess a Gln-residue close to the N-terminus (Fig. 32). Additionally, carboxyamidomethylation of stefin B did not prevent the formation of the M_r 37 000 complex, which decreases the probability of involvement of the stefin B Cys-3. The suggestion that the cathepsin L may contribute the

Cys- component of a thioester bond is supported by the observation that prior incubation of cathepsin L with E-64 had no apparent effect on the level of M_r 37 000 complex formed *in vitro*, and Varughese (1989) has shown that Gln-19 in papain is hydrogen bonded to the C-1 carboxylic acid oxygen of E-64 so that thioester formation via a cathepsin L glutaminyl γ-carbonyl is not very likely. Despite thioesters being high energy bonds (Torchinsky, 1981), direct thioester formation driven by conformational folding energy has been been suggested by Feinman (1983), for α_2M. Conformation of the cathepsin L molecule may have a bearing on covalent complex formation, since the phenomenon is strongly pH dependent. It is interesting to note that Iijima *et al.* (1984) reported the existence of protein factors in liver homogenates which catalyse thioester formation.

The possible presence of factors in liver homogenates which promote thioester (Iijima *et al.*,1984) or disulfide bond formation (Goldberger *et al.* 1964; Hillson *et al.*, 1984), warrants further investigation in the context of the covalent stefin B/cathepsin L complex isolated in this study. It was shown that active, covalent stefin B/cathepsin L complexes formed *in vitro* only at pH 5.5 and above, and since the pH of the homogenate is usually at *ca.* pH 6.0, conditions conducive to complex formation exist during homogenisation of liver tissue, which may be further promoted by the presence of factors which catalyse covalent complex formation. By maintaining the pH of liver and kidney homogenates below 5.5, the level of active, covalent stefin B/cathepsin L complex isolated was decreased significantly, but its formation was not prevented. This suggests that the novel complexes are not only formed very early in the purification process, but are also formed despite the low pH, which gives further support to the proposed role of factors promoting complex formation in liver homogenates, overiding a purely pH-dependent mechanism.

These proposed modes of interaction between cathepsin L and stefin B, leading to the formation of the novel covalent complexes, must largely remain speculative, however, until the three-dimensional structure of such complexes have been resolved by X-ray crystallography. Nevertheless, it was shown in the present study that the single-chain nature of cathepsin L, which was confirmed by amino acid sequencing, may be the key to the formation of these novel, proteolytically active, covalent complexes, thus providing a possible mechanism by which cathepsin L could be active despite the presence of stefin B. Although these studies were conducted on constituents isolated from sheep liver, this was a convenient and uniquely readily available source of cathepsin L, which Mason (1986) has suggested is a suitable model for the human enzyme. Proof for the validity of extrapolations between sheep and human cathepsin L was given in the present study by showing that the primary structures of the sheep and human enzymes are 85% homologous and that the novel

complexes may also be isolated from liver homogenates of higher primates. The higher pH-optima described for the free and stefin B-complexed forms of single-chain sheep cathepsin L (Dennison *et al.*, 1992) were also features of both forms of single-chain baboon cathepsin L, thus suggesting that single-chain cathepsin L *per se*, or complexed to stefin B may be active extracellularly to effect ECM degradation during tumour invasion.

CHAPTER 5

IMMUNOINHIBITORY POLYCLONAL AND ANTI-PEPTIDE ANTIBODIES AGAINST CATHEPSINS L AND H

5.1 Introduction

Antibodies directed at a specific epitope in a protein may, by virtue of their targeting specificity, be used to modulate the function of that protein, or to identify it in a mixture of antigens. Protein antigens would, however, elicit polyclonal antibodies, derived from a heterogenous population of B-cell clones, each responding to a distinct epitope. Polyclonal antibody specificity would, therefore, be an average characteristic of all the antibodies in that preparation and may be governed by the immunodominant epitopes stimulated. Monoclonal antibodies, in contrast, are the homogenous products of a single B-cell clone, selected for its targeting specificity of a particular epitope (Köhler and Milstein, 1975). Specificity intermediary to that of polyclonal and monoclonal antibodies is provided by anti-peptide antibodies, since they are raised against an appropriate peptide sequence selected from the protein antigen, but their production is not subjected to *in vitro* clonal selection. These anti-peptide antibodies are often able to cross-react with the corresponding whole native protein and thus provide a population of antibodies of predetermined specificity (Lerner, 1984).

Specific immunoinhibition of cathepsins L and H by active site-directed antibodies, compared to the relatively non-specific inhibition provided by synthetic inhibitors to date (Kirschke and Shaw, 1981; Kirschke *et al.*, 1988), may find application in elucidating the enzymes' role in tumour invasion and may also be useful in tumour therapy. The identification of the immunoinhibitory epitopes of the enzymes is important, however, for the production of antibodies with high immunoinhibitory activity. Once such epitopes have been identified, anti-peptide antibodies which specifically target them in the native enzyme may be prepared, and these may constitute useful immunoinhibitory reagents.

Immunoinhibition of a number of enzymes has been studied, such as papain (Arnon and Shapira, 1967), cathepsin D (Dingle *et al.*, 1971; Weston and Poole, 1973), ß-lactamases (Richmond, 1977), gelatinase (Murphy *et al.*, 1989a), type IV collagenase (Höyhtyä *et al.*, 1990) and acetylcholinesterase (Olson *et al.*, 1990). "Inhibitory antibodies" against cathepsin D were used to investigate the role of the enzyme in cartilage breakdown (Dingle *et al.*, 1971), demonstrating the potential these agents have in elucidating the role of enzymes in biological functions. A distinction needs to be made, however, between true

immunoinhibition of enzyme activity and pseudoinhibition, due to immunoprecipitation. In immunoinhibition, it is envisaged that an antibody binds to an enzyme in such a way that the active site is rendered incompetent. In immunoprecipitation, optimal proportions of antibody and antigen (enzyme) interact to form a cross-linked network or lattice which precipitates. This process requires at least two epitopes, which are sufficiently separated, to ensure interaction with paratopes on two different antibody molecules (Clark, 1986). Anti-peptide antibodies (and monoclonal antibodies), directed at a single epitope could, therefore, not be immunoprecipitating. Immunoprecipitation may therefore not necessarily involve the active site, and enzyme activity may be impaired simply by the enzyme being locked in an insoluble complex, which is removed from solution (Arnon and Shapira, 1967; Dingle *et al.*, 1971). Two populations of anti-papain antibodies have been distinguished, those which both immunoinhibit and precipitate the enzyme, and those which only precipitate the enzyme (Arnon and Shapira, 1967).

Antibodies against cathepsin L have previously been raised in rabbits and sheep (Mason, 1986), as is common practice. Due to the greater evolutionary distance between mammals and birds, however, antibodies raised in chickens might respond to different epitopes on the enzyme compared to those raised in mammals, such as the rabbit. Chicken IgY antibodies, which can be extracted from the egg yolk (Polson *et al.*, 1980; 1985), may, therefore, have novel immunoinhibitory properties compared to their rabbit counterparts, thus imparting new information about those epitopes of the enzyme which elicit the formation of immunoinhibitory antibodies.

Immunoinhibitory epitopes may be localised by testing the ability of synthetic peptides, corresponding to sequences in the active site of the enzyme, to compete with the native enzyme for binding to immunoinhibitory polyclonal antibodies. Such inhibitory peptides may then be used to produce immunoinhibitory anti-peptide antibodies. In the absence of such immunoinhibitory polyclonal antibody probes, the selection of appropriate peptides for immunoinhibitory anti-peptide antibody production may be accomplished by examination of the tertiary structure of the enzyme's active site (Pike, 1990), and by using prediction algorithms derived from the observed correlation between the location of continuous epitopes and structural parameters such as hydrophilicity (Hopp and Woods, 1981; 1983) and segmental mobility (Westhof *et al.*, 1984).

Although the tertiary structures of cathepsins L and H are not yet available, these enzymes show a 45% overall sequence homology with papain (Ritonja *et al.*, 1988), and there is also a high degree of similarity in secondary structure between cathepsin L and papain (Dufour, 1988). The published three-dimensional structure of papain (Drenth *et al.*,

1971) could, therefore, be used with some degree of confidence as a model for the tertiary structures of these mammalian cysteine proteinases, to aid in the selection of appropriate target peptides for the production of immunoinhibitory anti-peptide antibodies. In addition to binding to a peptide in the active site of the enzyme, thereby occluding the active site from substrate binding, the anti-cathepsin L and H peptide antibodies should also be able to discriminate between the different cysteine cathepsins to enhance their usefulness in tumour invasion studies. If the respective anti-peptide antibodies could also target the individual enzymes across species, their versatility would be significantly enhanced.

This chapter reports on the production of polyclonal antibodies in rabbits and chickens against cathepsin L and a comparison of their titre, specificity, immunoinhibitory properties and use as probes for the localisation of an immunoinhibitory epitope in cathepsin L. The production of anti-cathepsin L and H peptide antibodies, using different modes of presentation of the peptides to the immune systems of both chickens and rabbits, is compared. The characterisation and future applications of the resulting anti-peptide antibodies, targeting sequences in the active sites of these enzymes, will be discussed.

5.2 Selection and synthesis of peptides

Inspection of the active site of papain, in the diagrammatic representation of its three-dimensional structure (Fig. 43; Drenth *et al.*, 1971), leads to the identification of three candidate regions for peptide selection. These are a "hinge" region (residues 9-19), an α-helix (residues 20-35) which includes the active site Cys-25, and a loop of amino acids (residues 149-162) containing the active site His-159 (Table 16). A peptide from the hinge-like sequence would be unsuitable for cathepsins L and H, because of the 55% sequence homology between cathepsins L, H and S in this peptide. There is thus an increased probability that anti-peptide antibodies to this region will cross-react with these different cysteine proteinases. The active-site cysteinyl region of the four human cathepsins is highly conserved and shows 47-82% identity; the highest (82%) between cathepsins L and H. A linear peptide from this region will also possibly rarely adopt an α-helical conformation, mimicking the corresponding sequence in the native protein. The loop region around the active site His-159 showed the most promise, because the sequence was quite unique to cathepsin L when compared to cathepsins H, B and S (Table 16; Fig. 44), and only showed between 8 and 38% homology; the lowest between cathepsins L and B and the highest when cathepsin L was compared to cathepsin S, and 23% sequence homology with cathepsin H. The presence of the active-site histidine in this sequence was also thought to increase the probability that antibodies targeting this region might be inhibitory.

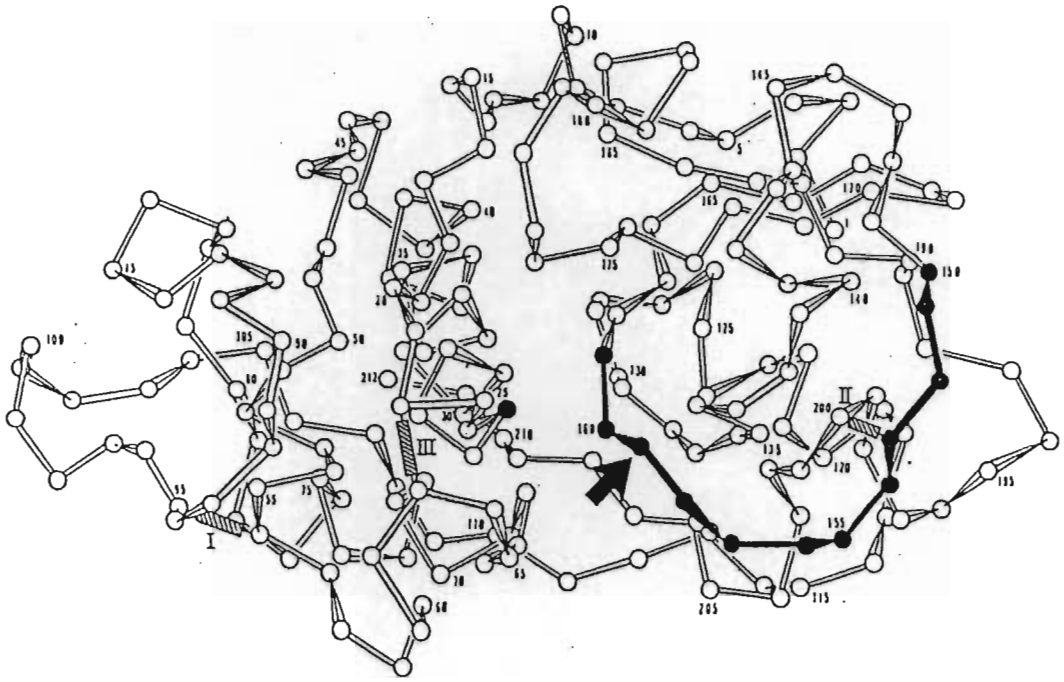

Figure 43. Representation of the three-dimensional structure of papain according to Drenth *et al.* (1971). The peptide corresponding to those selected for cathepsins L and H is indicated by the dark shaded region, as is the active-site Cys-residue at position 25. The arrow indicates the active-site His-residue at position 159.

The amino acid sequence, L153-165 (Fig. 44), thus selected for cathepsin L (Pike, 1990), corresponds to residues 153-165 in the sequence of human cathepsin L (Ritonja *et al.*, 1988); in papain the comparable residues are 150-161 (Dufour, 1988). The sequence is largely hydrophilic, but has a cluster of hydrophobic residues towards its C-terminus and is most mobile at the N-terminus (Fig. 45A). This sequence is relatively conserved between species (Fig. 46) and, therefore, the anti-peptide antibodies produced against this sequence have the potential of targeting cathepsin L across species.

Human cathepsin L:	E	P	D	C	S	-	-	S	E	D	M	D	**H**	G	V
Human cathepsin H:	S	T	S	C	H	K	T	P	D	K	V	N	**H**	A	V
Human cathepsin B:	H	V	T	G	E	-	-	-	M	M	G	G	**H**	A	I
Human cathepsin S:	E	P	-	S	C	-	-	T	Q	N	V	N	**H**	G	V

Figure 44. Amino acid sequence homology of the active-site histidyl-regions in human cathepsins L and H, selected for anti-peptide antibody production, compared to the corresponding sequences in cathepsins B and S (Wiederanders *et al.*, 1992). The active-site His-residue is indicated in bold. In the text the human cathepsin L peptide is referred to as L153-165 and the human cathepsin H peptide as H157-168.

Table 17. Homology of amino acid sequences of human cathepsins L, H, B and S.

Cathepsins compared	Residues in papain[a]		
	9-19 (Hinge-like region)	20-35 (Active-site Cys-region)	149-162[b] (Active-site His-region)
Cath L/cath H	67	82	23
Cath L/cath S	67	81	38
Cath L/cath B	27	64	8
Cath H/cath S	55	75	31
Cath H/cath B	55	53	15

[a] Identity (%) was calculated from the sequence alignment for human cathepsins L, H, B and S (Wiederanders *et al.*, 1992); [b]Sequence alignment shown in Fig. 44.

Peptide H157-168 (Fig. 44), chosen for cathepsin H corresponds to the active-site associated residues 157-168 in human cathepsin H (Ritonja *et al.*, 1988); the corresponding sequence in papain is between residues 153 and 161. This peptide shows a similar hydrophilicity pattern as peptide L153-165, while the segmental mobility increases towards the C-terminus (Fig. 45B). Only the amino acid sequences of human (Ritonja *et al.*, 1988) and rat (Takio *et al.*, 1983) cathepsin H have been determined and these are identical in the peptide H157-168 region, which suggests that anti-peptide antibodies against this sequence could potentially cross-react with cathepsin H across species.

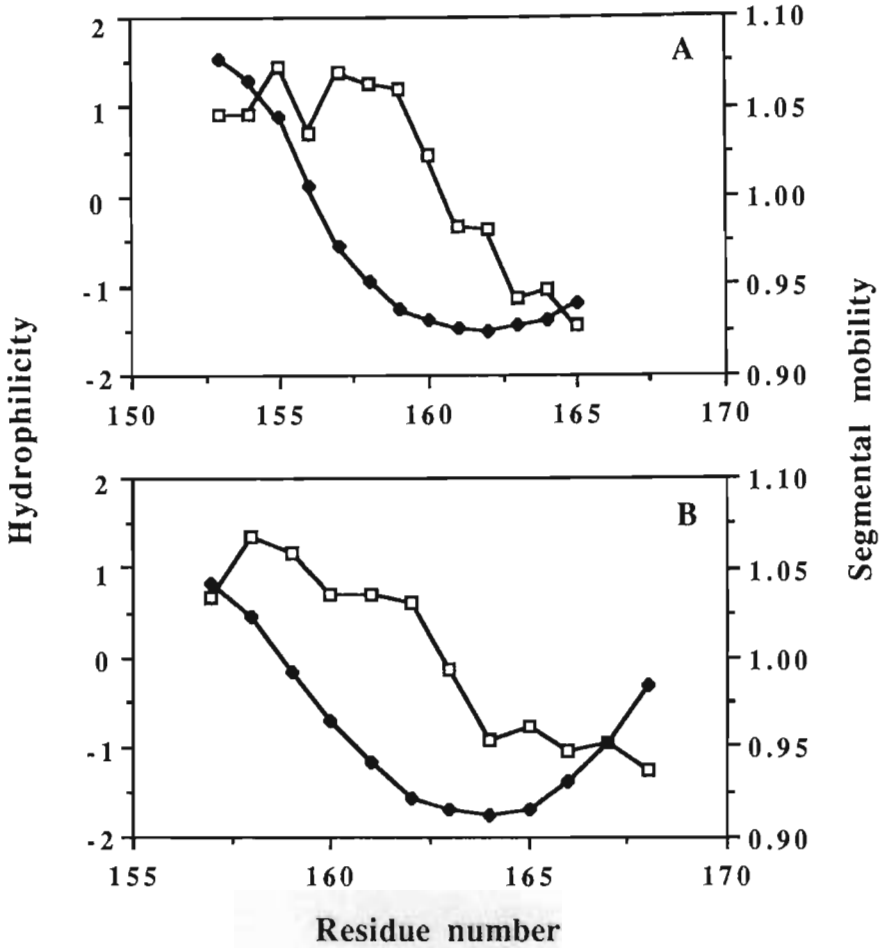

Figure 45. Hydrophilicity and segmental mobility profiles of the peptides selected from human cathepsins L and H.
(**A**) Peptide L153-165 from cathepsin L; and (**B**) peptide H157-168 from cathepsin H. (❑), hydrophilicity, calculated according to Hopp and Woods (1981; 1983); and (◆), segmental mobility, calculated according to Westhof *et al.* (1984).

Human cathepsin L:	E	P	D	C	S	S	E	D	M	D	**H**	G	V
Sheep cathepsin L:	D	P	D	C	S	S	K	D	L	D	**H**	G	V
Mouse cathepsin L:	E	P	N	C	S	S	K	N	L	D	**H**	G	V
Rat cathepsin L:	E	P	N	C	S	S	K	D	L	D	**H**	G	V
Chicken cathepsin L:	E	P	D	C	S	S	E	D	L	D	**H**	G	V

Figure 46. Amino acid sequence homology of the active-site associated peptide selected from human cathepsin L (L153-165) for anti-peptide antibody production, with the same sequence in sheep, mouse (Portnoy *et al.*, 1986), rat (Ishidoh *et al.*, 1987) and chicken (Dufour *et al.*, 1987) cathepsin L. Identical or similar residues are underlined.

In addition to the hydrophilicity and segmental mobility properties, both peptides L153-165 and H157-168 meet further criteria for peptide immunogenicity (Shinnick *et al.*, 1983; Van Regenmortel, 1988), i.e. they are more than ten residues in length, accessible in the native protein, and contain a Pro-residue which may contribute to the peptide's ability to adopt the same conformation as the corresponding peptide in the native protein.

On account of the higher degree of hydrophilicity and segmental mobility shown by the N-termini of peptides L153-165 and H157-168, conjugation of these peptides to carrier proteins, to enhance their immunogenicity, would usually be effected through their C-termini. These peptides were, however, coupled via their N-termini to ensure the accessibility of the active site-associated His-residue. The sequence H157-168 was also injected into experimental animals in the less frequently used unconjugated, free form.

Peptides L153-165 and H157-168 were custom synthesised by Multiple Peptide Systems, San Diego, Ca. The selected peptide L153-165 was modified, before synthesis, by the substitution of the Cys-residue with α-amino butyric acid, to prevent dimerisation of the peptide, and conjugation via the cysteinyl sulfhydryl (Muller, 1988a). The N-terminal Cys-residue of peptide H157-168 was, however not modified, because it was required for conjugation using the *m*-maleimido benzoyl-N-hydroxysuccinimide ester (MBS) method (Kitagawa and Aikawa, 1976). The C-terminal glutamate residue of both peptides were converted to the corresponding amides, thus more effectively mimicking the peptide conformation in the native protein, since the amidation renders the C-terminal residue uncharged, and bonded as it would be in a peptide bond in the native protein. This modification also ensures a higher yield from peptide synthesis (Multiple Peptide Systems technical bulletin).

5.3 Conjugation of peptides to carrier proteins

5.3.1 Conjugation of peptide L153-165 to KLH using glutaraldehyde

Peptide L153-165 was conjugated to KLH via the bifunctional agent glutaraldehyde, according to the method of Bulinski and Gunderson (1986), but using 1% glutaraldehyde which results in more stable conjugates (Briand *et al.*, 1985). The pH of 5.5 used by Bulinski and Gunderson (1986) was also used in this study since it was thought to mimic the lysosomal environment of cysteine proteinases and may, therefore, aid in retaining the native conformation of the cathepsin L peptide.

5.3.1.1 Reagent

Conjugation buffer [200 mM Na-acetate buffer, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (11.43 ml) and NaN_3 (0.2 g) were dissolved in 850 ml of dist.H_2O, titrated to pH 5.5 with NaOH, and made up to 1 litre.

5.3.1.2 Procedure

KLH (7.4 mg, 0.037 µmoles, assuming an M_r of 200 000) was dissolved in conjugation buffer (1 ml) and dialysed against the same buffer for 16 h at 4°C, before the solution was centrifuged (10 000 x g, 10 min, 4°C), to remove undissolved particulate material. Peptide (5 mg, 3.7 µmoles, M_r 1 349) was dissolved in conjugation buffer (100 µl) and added to the KLH solution. Glutaraldehyde [80 µl of a 25% solution (Merck, E.M Grade, d = 1.06)] was added dropwise, with stirring, to the KLH-peptide mixture at room temperature over a period of 5 min. The reaction was allowed to proceed overnight with stirring, because longer reaction periods were found to be required at pH 5.5 than at pH 7.2 as used for COL476-490 (Section 3.6) (Avrameas and Ternynck, 1969). Longer reaction periods are necessary at pH 5.5 because glutaraldehyde reacts with uncharged -NH_2-groups which are more prevalent at higher pH. The reaction was stopped by the addition of $NaBH_4$ (10 mg/ml) and incubation for 1 h at 4°C. Following this, conjugated and free peptide were separated by MEC and the amount of peptide conjugated to carrier protein was estimated as described in Section 5.3.2.

5.3.2 Estimation of coupling yield obtained with glutaraldehyde

The peptide-KLH conjugate mixture (2 ml) was applied to a Sephadex G-100 (superfine) column (1.8 x 20 cm = 56 ml) and eluted at a flow rate of 12.7 ml/h (5 cm/h). The elution profile was determined using the micro-biuret assay (Section 3.3.1) for which a standard curve was constructed using a 1 mg/ml peptide L153-165 solution, diluted to five levels from 0-500 µg. Linear regression analysis of the calibration curve gave the equation:

$$A_{310} = -0.0011 + 0.000246[\text{peptide concentration (mg/ml)}] \quad (R = 1.00)$$

The free peptide peak was pooled and quantitated by the same method. The coupling yield was estimated by subtracting the amount of free peptide from the original amount used for conjugation, and expressing this as a percentage of the total amount. This

figure was used to determine the amount of conjugate needed to immunise chickens at the correct dose.

5.3.3 Conjugation of peptide H157-168 to KLH and ovalbumin using MBS

The peptide, H157-168, selected from cathepsin H, was conjugated to KLH or ovalbumin via the peptide's N-terminal Cys-residue using the hetero-bifunctional reagent MBS (Kitagawa and Aikawa, 1976), and according to the method described by Muller (1988a). The glutaraldehyde conjugation method was not suitable as a result of the presence of the ε-amino group of the Lys-residue in the peptide (Avrameas and Ternynck, 1969), which would prevent exclusive conjugation via the N-terminus.

5.3.3.1 Reagents

Ellman's reagent [50 mM phosphate buffer, pH 7.0, 10 mM DTNB, 10% (v/v) methanol]. $NaH_2PO_4.2H_2O$ (0.69 g) was dissolved in 90 ml of dist.H_2O, titrated to pH 7.0 with NaOH, and made up to 100 ml. DTNB (40 mg) was dissolved in methanol (100 µl) and diluted to 10 ml with phosphate buffer.

Reducing buffer [100 mM Tris-HCl, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 8.0]. Tris (1.21 g), Na_2EDTA (0.037 g), and NaN_3 (0.02 g) were dissolved in 90 ml of dist.H_2O, titrated to pH 8.0 with HCl, and made up to 100 ml.

MEC buffer [100 mM phosphate buffer, 0.02% (m/v) NaN_3, pH 7.0]. $NaH_2PO_4.2H_2O$ (6.9 g) and NaN_3 (0.1 g) were dissolved in 400 ml of dist.H_2O, titrated to pH 7.0 with NaOH, and made up to 500 ml.

5.3.3.2 Procedure

Carrier protein [KLH (18.5 mg, 0.0925 µmoles, assuming an M_r of 200 000) or ovalbumin (4.16 mg, 0.0925 µmoles, M_r 45 000)] was dissolved in PBS (2 ml) and dialysed against the same buffer for 16 h at 4°C, and (in the case of the KLH solution) centrifuged (10 000 x g, 10 min, 4°C) to remove undissolved particulate material. Peptide [5 mg, 3.7 µmoles, M_r 1 349, to give a molar ratio of peptide to activated carrier (see below) of 40:1] was dissolved in reducing buffer (1 ml) and 10 mM DTT, in the same buffer (1 ml), was added with stirring and reduction was effected by incubation at 37°C for 1.5 h. Reduced peptide was separated from unreacted DTT by MEC on a Sephadex G-10 column (1 x 13 cm = 10.2 ml) equilibrated with MEC buffer and eluted at a linear flow rate of

10 cm/h. Fractions (500 µl) were collected and the elution profile was determined by the addition of 10 mM Ellman's reagent [5, 5'-dithiobis-(2-nitrobenzoic acid); DTNB] (10 µl) to an equal sample volume from each fraction. A yellow colour was indicative of the elution of the reduced peptide peak, which was resolved from the unreacted DTT peak, which gave an intensely yellow colour. Peptide samples taken before reduction and after separation of reduced peptide from excess DTT, were titrated with Ellmans's reagent for the estimation of free sulfhydryls available for conjugation to activated carrier (Section 5.3.4).

MBS [5 mg/ml dissolved in dry dimethylformamide (DMF)] was added to the carrier protein [KLH (232 µl) or ovalbumin (279 µl)] to give a final molar ratio of carrier protein to MBS of 1:40. The acylation of carrier protein amino groups via the N-hydroxysuccinimide ester was allowed to proceed for 30 min at RT. Excess MBS was removed by MEC on a Sephadex G-25 column (1 x 20 cm = 15.7 ml), equilibrated with MEC buffer and eluted at a linear flow rate of 10 cm/h. The A_{280} was monitored and the fractions containing the activated carrier protein were immediately allowed to react with reduced peptide for 3 h at RT with stirring. Unbound peptide was removed by dialysis against four changes of PBS for 16 h at 4°C and the coupling yield was determined (Section 5.3.4).

5.3.4 Estimation of coupling yield obtained with MBS

The coupling yield was estimated by determining the maleimide content of the activated carrier before and after conjugation (Kitagawa and Aikawa, 1976). This is accomplished by allowing a known amount of excess sulfhydryl containing reagent, such as 2-mercaptoethanol, to react with the activated carrier, following which the amount of unbound sulfhydryl reagent is quantitated by titration with Ellman's reagent (Glazer *et al.*, 1975). This allows calculation of the amount of sulfhydryl reagent bound, and hence the number of *m*-maleimido benzoyl-groups available on the carrier protein.

5.3.4.1 Reagents

Ellman's reagent. See section 5.3.3.1

Sample diluent for Ellman's test [100 mM Tris-HCl, 10 mM Na_2EDTA, 1 % (m/v) SDS, 0.02% (m/v) NaN_3, pH 8]. Tris (1.21 g), Na_2EDTA (0.37 g), SDS (1 g) and NaN_3 (0.02 g) were dissolved in 90 ml of dist.H_2O, titrated to pH 8 with HCl, and made up to 100 ml.

25 mM 2-Mercaptoethanol. 2-Mercaptoethanol (17.5 µl) was diluted in MEC buffer (Section 5.2.3.1) (10 ml).

5.3.4.2 Procedure

An aliquot (30 µl) of activated carrier protein, separated from unreacted MBS on MEC (Section 5.3.3.2), was added to 25 mM 2-mercaptoethanol (4 µl; 100 nmoles) and allowed to react for 3h at RT. A control was prepared by replacing the activated carrier with reducing buffer (Section 5.3.3.1). Following incubation, free sulfhydryls were estimated by titration with Ellman's reagent. Sample diluent (2.5 ml) was placed in two matched quartz cuvettes and the sample [25 mM 2-mercaptoethanol-containing reaction mixture (4 µl) or peptide (8 µl); 0.03 µmoles] was added to one of the cuvettes and mixed in, while the other cuvette, containing sample diluent, served as the reference. The cuvettes were placed in a Hitachi 220 spectrophotometer and Ellman's reagent was rapidly added to each. Colour development was allowed to take place for 1.5 min before the A_{412} was read. An extinction coefficient of 13 600 $M^{-1}cm^{-1}$ for the released 2-nitro-5-thiobenzoate anion (Glazer *et al.*, 1975) was used to determine the number of free sulfhydryls, and hence the number of activated MB-groups on the carrier protein or the percentage of peptide reduction.

5.4 Production of antibodies in chickens and rabbits

Polyclonal antibodies against free cathepsin L, isolated from sheep liver as outlined in section 4.2, were raised in chickens as described in section 4.5. Rabbits were inoculated subcutaneously at 4-6 sites on the back, with the same dose of cathepsin L as the chickens, i.e. a total of 50 µg per animal, emulsified in a 1:1 (v/v) ratio with Freund's complete adjuvant. Further inoculations were administered, in the same manner, in Freund's incomplete adjuvant, using the same dose of 50 µg, at week two, followed by monthly boosters. Blood was collected from the marginal ear vein of rabbits at 3 and 8 weeks, and by non-lethal cardiac puncture at 12 weeks. Serum was separated from the blood clots and IgG isolated, as described in section 2.7, and stored at -20°C.

Anti-peptide antibodies against the human cathepsin L peptide, L153-165, conjugated to KLH, were raised in chickens in order to complement the anti-peptide L153-165 antibodies produced in rabbits in a previous study (Pike, 1990). Chickens were inoculated intramuscularly, at two sites in their large breast muscles, with a total of 200 µg peptide conjugate, emulsified with Freund's complete adjuvant in a 1:1 (v/v) ratio. Three further inoculations (at the same dose) were administered in Freund's incomplete adjuvant in the

same manner at two-week intervals, followed by monthly boosters. Eggs were collected daily throughout the immunisation period and stored at 4°C.

Anti-peptide antibodies against the human cathepsin H peptide, H157-168, conjugated to KLH and ovalbumin respectively, were raised in both chickens and rabbits, using 200 µg of conjugate per inoculation and the same immunisation protocol as outlined above for peptide L153-165 in chickens and for cathepsin L in rabbits. Free peptide was also used to raise antibodies in both chickens and rabbits, using essentially the same immunisation protocols as described above. Peptide H157-168 [200 µg dissolved in 100 mM phosphate buffer, pH 6.0 (500 µl)] was emulsified with an equal volume of Freund's complete adjuvant prior to inoculation, throughout the rabbit inoculation protocol (Muller, 1988b), while Freund's incomplete adjuvant was used for every alternative inoculation in chickens, since it was found previously that continual use of Freund's complete adjuvant causes an oedematous reaction in chickens (Coetzer, unpublished observation).

The progress of the chickens' and rabbits' response to the immunogens was followed by ELISA analyses of egg yolk extracts and serum samples respectively, before IgY or IgG was isolated (Section 2.7) from pools of egg yolks or sera of maximum titre. Yolk extracts were prepared by mixing the yolk, separated from the egg white, with two volumes of 100 mM phosphate buffer, pH 7.6, followed by incubation overnight at 4°C. Titres obtained in ELISAs using the supernatants of the yolk extracts, were in good agreement with values obtained using IgY isolated from the egg yolks (Coetzer, 1985). This presented a satisfactory method for selecting eggs for IgY isolation, especially where the progress of antibody production over several weeks, using different methods for immunogen presentation, was compared.

5.5 Immunoinhibition assays

5.5.1 Reagents

Cathepsins. Sheep liver cathepsin L was purified as described in section 4.2, while human kidney cathepsin L was obtained from Novabiochem, U.K. Human liver cathepsin H was obtained from Athens Research and Technology, Ga, USA.

1 mM Arg-NHMec-substrate stock solution. Arg-NHMec (1.15 mg) was dissolved in DMSO (1.5 ml) and stored at 4°C.

20 µM Arg-NHMec-substrate solution. Arg-NHMec-substrate stock solution (100 µl) was diluted to 5 ml with dist.H_2O.

Arg-NHMec assay buffer [200 mM KH_2PO_4, 200 mM $NaHPO_4$, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 40 mM cysteine.HCl, pH 6.8]. KH_2PO_4 (13.61 g), $NaHPO_4$ (14.2 g), $Na_2EDTA.2H_2O$ (0.75 g) and NaN_3 (0.1 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 6.8 with NaOH and made up to 500 ml. Prior to the assay, cysteine.HCl (0.04 g) was added to 5 ml of buffer.

All other reagents were as described in section 4.6.2.

5.5.2 Procedure

Assays for the immunoinhibition of cathepsins L and H were carried out using the synthetic substrates Z-Phe-Arg-NHMec and Arg-NHMec respectively, as described by Barrett and Kirschke (1981). Initially, immunoinhibition of cathepsins L or H by antibody preparations was assayed by monitoring the cleavage of the synthetic substrate Z-Phe-Arg-NHMec or Arg-NHMec on a continuous basis. Enzyme (5 ng sheep or 25 ng human cathepsin L, or 250 ng human cathepsin H) was diluted in 0.1% Brij 35 (500 µl), and to this was added the relevant IgY or IgG preparation at 1 mg/ml in 400 mM phosphate buffer, pH 6.0, containing 4 mM EDTA and 0.1% (v/v) Tween 20 (500 µl). This mixture was incubated at 30°C for 15 min, before an aliquot (500 µl) was removed and activated by the above buffer, containing 8 mM DTT (250 µl), for 1 min. Substrate, Z-Phe-Arg-NHMec or Arg-NHMec, at 40 µM (250 µl) was added, and the increase in fluorescence was monitored for 5 min in a temperature controlled cell in a Hitachi Model F-2000 spectrofluorimeter, with excitation at 370 nm and emission at 460 nm. The slope of the linear increase in activity, in the presence of the different IgY or IgG preparations, was calculated, and the percentage inhibition was expressed in comparison to the slope in the presence of non-immune IgY or IgG at the same concentration, to account for the non-specific competitive inhibition by the presence of antibodies. Intrinsic activity against Z-Phe-Arg-NHMec, but not against Arg-NHMec, in rabbit IgG fractions, probably due to serum kallikrein contamination (Colman and Bagdasarian, 1976), was inhibited by SBTI (40 µg/ml), which had no effect on cathepsin L, and by subtracting the residual activity in

the antibody preparations from the measured cathepsin L activity; a procedure which was not necessary for IgY fractions. Continuous monitoring of substrate hydrolysis established, using minimal amounts of enzyme, whether a particular antibody preparation was immunoinhibitory and whether optimal conditions prevailed for the duration of the assay, such as the amount of substrate available.

For the measurement of the extent of immunoinhibition across a range of concentrations of antibody, continuous monitoring was less useful, because of the duration of each assay. Stopped time assays were, therefore, carried out over a range of antibody concentrations in a similar manner to that described above, except that the enzyme activity was stopped by the addition of the monochloroacetate reagent, as described in section 4.6.2. The inhibition of enzyme activity by the antibodies was expressed as a percentage of the activity in the presence of non-immune chicken IgY or rabbit IgG, with the subtraction of the intrinsic Z-Phe-Arg-NHMec activity of IgG fractions as described above.

5.6 Inhibition of immunoinhibition, by an active site-associated peptide sequence

Chicken anti-cathepsin L IgY (125 µg/ml final concentration; 500 µl) was incubated with increasing levels of peptide L153-165 (representing increasing molar ratios of peptide to cathepsin L) (100 µl) at 30°C for 15 min, before cathepsin L (5 ng) was added and further incubated at 30°C for 15 min. Following this, the extent of immunoinhibition was determined by monitoring the cleavage of substrate on a continuous basis as described above (Section 5.5). In control experiments non-immune IgY was substituted for immune IgY.

5.7 Results

5.7.1 Production and characterisation of polyclonal anti-sheep cathepsin L antibodies

Antibodies were readily raised against sheep cathepsin L, using both a conventional (rabbit) and a more novel (chicken) route of antibody production. Chicken serum antibodies are passed on to the egg yolk to confer passive immunity to the developing chick. Consequently the egg yolks of immunised chickens constitute a convenient source of antibody from which the chicken egg yolk immunoglobulin (IgY) is easily isolated by PEG precipitation (Polson *et al.*, 1980; 1985). The increase in antibody titre, which is expressed here as the concentration of antibody at which A_{405}-values are still significantly higher than equivalent non-immune controls, was followed for IgY over the course of the inoculation

period (Fig. 47). The chicken antibody titre showed a large increase from four to six weeks, with a further small increase from six to eight weeks, and a large increase from eight to twelve weeks, reaching a titre of at least 4 µg/ml (Fig. 47A).

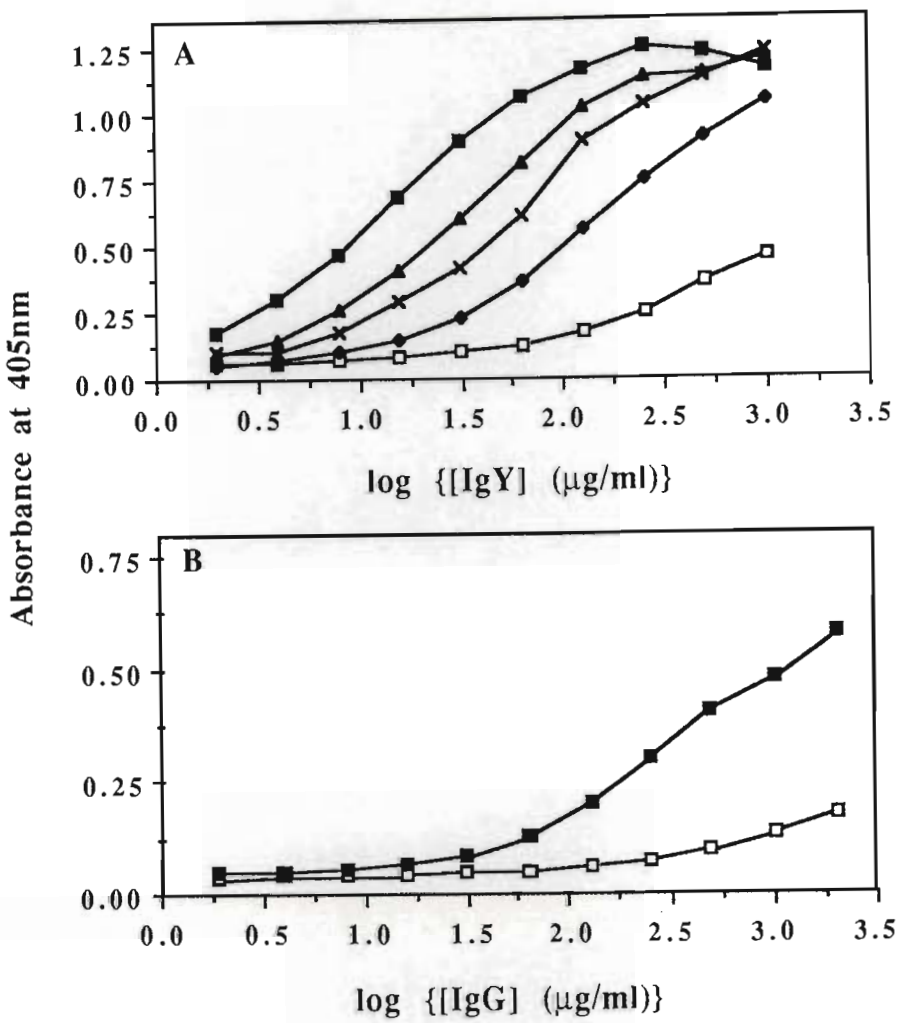

Figure 47. ELISA of the binding of polyclonal antibodies to sheep cathepsin L.
Sheep cathepsin L was coated at 1 µg/ml to microtitre plates and incubated with serial two-fold dilutions of, (**A**), chicken anti-sheep cathepsin L collected after 4 (◆), 6 (×), 8 (▲) and 12 weeks (■) and non-immune IgY (❑); and, **B**) rabbit anti-sheep cathepsin L IgG (■) and non-immune IgG (❑). Binding was visualised by incubation with HRPO-linked secondary antibodies as described in (14) and in materials and methods. Each point is the mean absorbance at 405 nm of duplicate samples.

A similar study of the timing of the antibody response against cathepsin L in rabbits was not done, since this has already been established (Mason, 1986). The titre of the rabbit antibody was about 30 µg/ml, which is about a ten-fold lower dilution than that of the chicken antibody, since the latter still had not titrated to completion at the high dilution of 4 µg/ml (Fig. 47B). The chickens and rabbits used in each of the studies responded similarly and the results given in Fig. 47 are typical of each response. Chicken anti-sheep cathepsin L antibodies target cathepsin L across species, as evidenced by the strong reaction

with human cathepsin L in ELISAs (Fig. 54). This high degree of cross-reactivity may be attributed to the fact that the amino acid sequences of sheep and human cathepsin L are 85% homologous (Section 4.9.11).

The specificities of both rabbit and chicken antibodies were tested in a Western blot against crude ion-exchange fractions from sheep liver, obtained by a modification of the cathepsin L purification procedure in which all of the proteins bound to S-Sepharose were eluted with 1M NaCl (Fig. 48). Only cathepsin L was targeted in the mixture of proteins, by all antibody preparations.

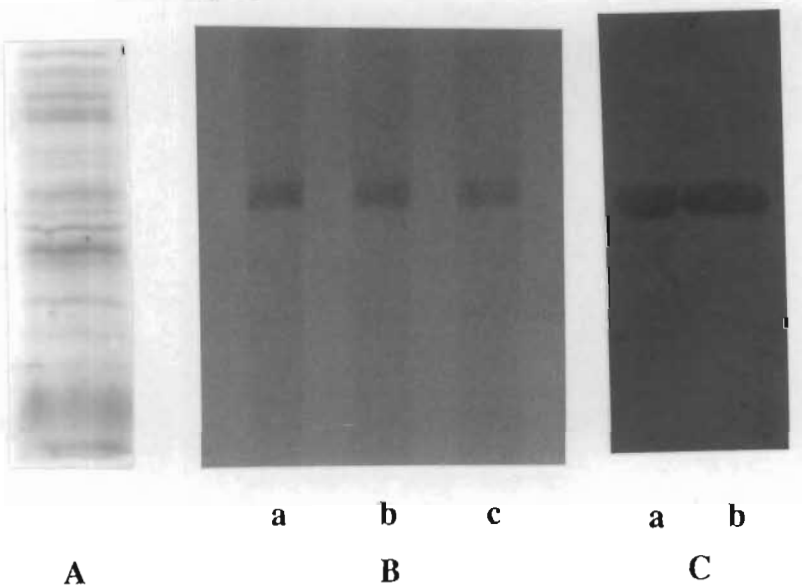

Figure 48. Targeting of sheep cathepsin L in a Western blot.
(**A**) 12.5% SDS-PAGE of a crude S-Sepharose ion-exchange fraction from sheep liver. This fraction was electroblotted onto nitrocellulose and probed with, (**B**): (a) Rabbit anti-sheep cathepsin L IgG, at (a) 0.2 mg/ml; (b) 0.125 mg/ml; and (c) 0.100 mg/ml visualised using sheep anti-rabbit-HRPO conjugate, and (**C**): chicken anti-sheep cathepsin L at (a) 5 µg/ml and (b) 10 µg/ml, visualised using rabbit anti-chicken-HRPO conjugate as described in section 2.8.

A comparison of the immunoinhibitory properties of the rabbit and the chicken anti-cathepsin L antibodies showed a distinct difference between the two preparations. Immunoactivation, rather than immunoinhibition, of the hydrolysis of the synthetic substrate Z-Phe-Arg-NHMec was found with rabbit anti-cathepsin L IgG, since the activity in the presence of the immune IgG was greater than that in the presence of the non-immune IgG (Fig. 49A). Contrary to rabbit IgG, chicken-anti sheep cathepsin L IgY was found to inhibit cathepsin L, since the activity in the presence of the immune IgY was about half of that of the non-immune IgY (Fig. 49B).

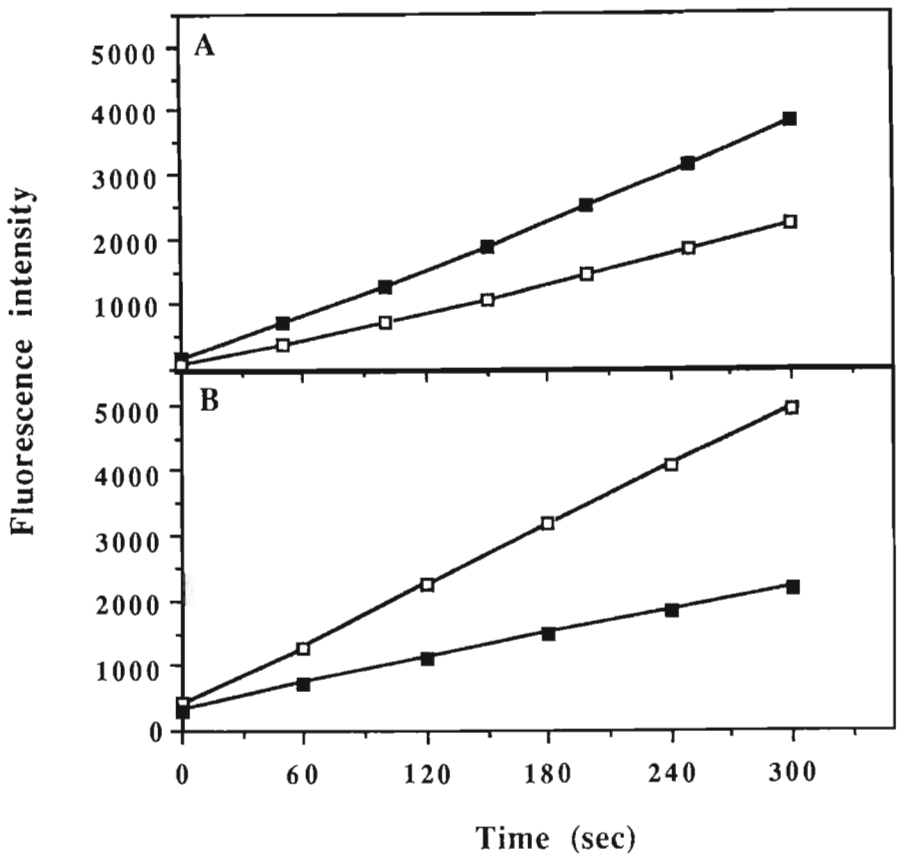

Figure 49. Immunoinhibition of sheep cathepsin L by rabbit and chicken anti-sheep cathepsin L antibodies.
Sheep cathepsin L (5ng) was incubated with, (A) rabbit anti-cathepsin L IgG (■) or non-immune IgG (❑) (0.5 mg/ml final concentration); or, (B) chicken anti-cathepsin L IgY (■) or non-immune IgY (❑) (0.5 mg/ml final concentration) for 15 min at 30°C, before being assayed on a continuous basis for 5 min against the fluorogenic substrate Z-Phe-Arg-NHMec.

Immunoinhibition tests were carried out across a range of IgY concentrations (Fig. 50) and revealed that the antibodies inhibited the enzyme weakly up to concentrations of 31.25 μg/ml, after which the inhibition increased up to 45% at 500 μg/ml. The high dilution necessary in the assay with the sensitive synthetic enzyme substrate possibly favours dissociation of the antigen-antibody complex, and may explain why a relatively high concentration of antibody is required to effect immunoinhibition.

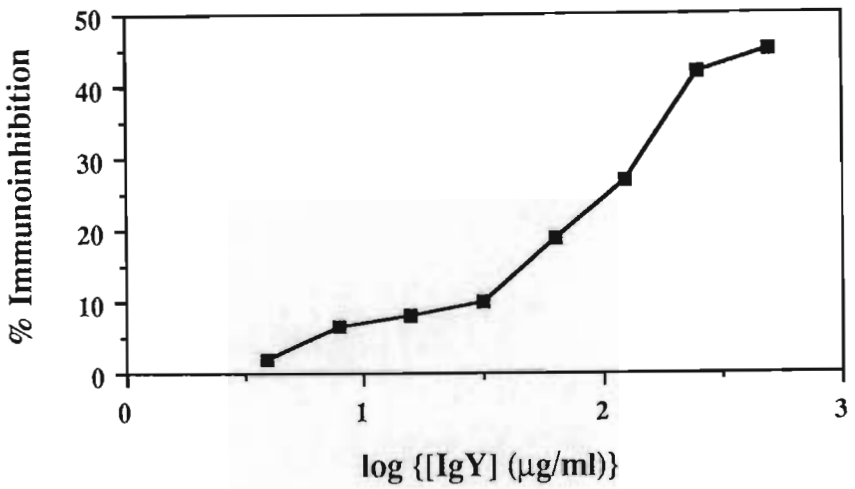

Figure 50. Immunoinhibition of sheep cathepsin L by chicken anti-cathepsin L antibodies.
Sheep cathepsin L (5 ng) was incubated with decreasing amounts of chicken anti-cathepsin L IgY (■) and the cleavage of Z-Phe-Arg-NHMec was monitored in a stopped time assay. The percentage inhibition was calculated relative to control assays with non-immune IgY.

5.7.2 Localisation of an immunoinhibitory epitope in cathepsin L

5.7.2.1 Targeting of an active site-associated peptide by chicken and rabbit polyclonal antibodies

Immunoinhibitory anti-peptide antibodies against cathepsin L were previously produced by the inoculation of rabbits with peptide L153-165 from the active site of cathepsin L (Pike, 1990). As an extension of that study, the ability of the chicken and rabbit polyclonal antibodies to target this peptide was, therefore, of interest, in order to determine whether this peptide was a naturally occurring immunoinhibitory epitope. Figure 51 shows that, while the immunoinhibitory chicken polyclonal antibody targeted the peptide immobilised on an ELISA plate fairly strongly (Fig. 51A), the non-inhibitory rabbit polyclonal antibody showed no binding to the peptide at all (Fig. 51B). No targeting of the peptide was observed with any of the rabbit antibody preparations, whereas all of the chicken antibody preparations showed comparable targeting of the peptide. The correlation between the binding of the active site-associated peptide [known to generate immunoinhibitory antibodies (Pike, 1990)] by the chicken antibodies, and the inhibitory properties of these antibodies, suggests that they may exert at least part of their inhibition through the binding of this peptide in the native protein. This suggests that the peptide may be a natural epitope for chicken antibody production.

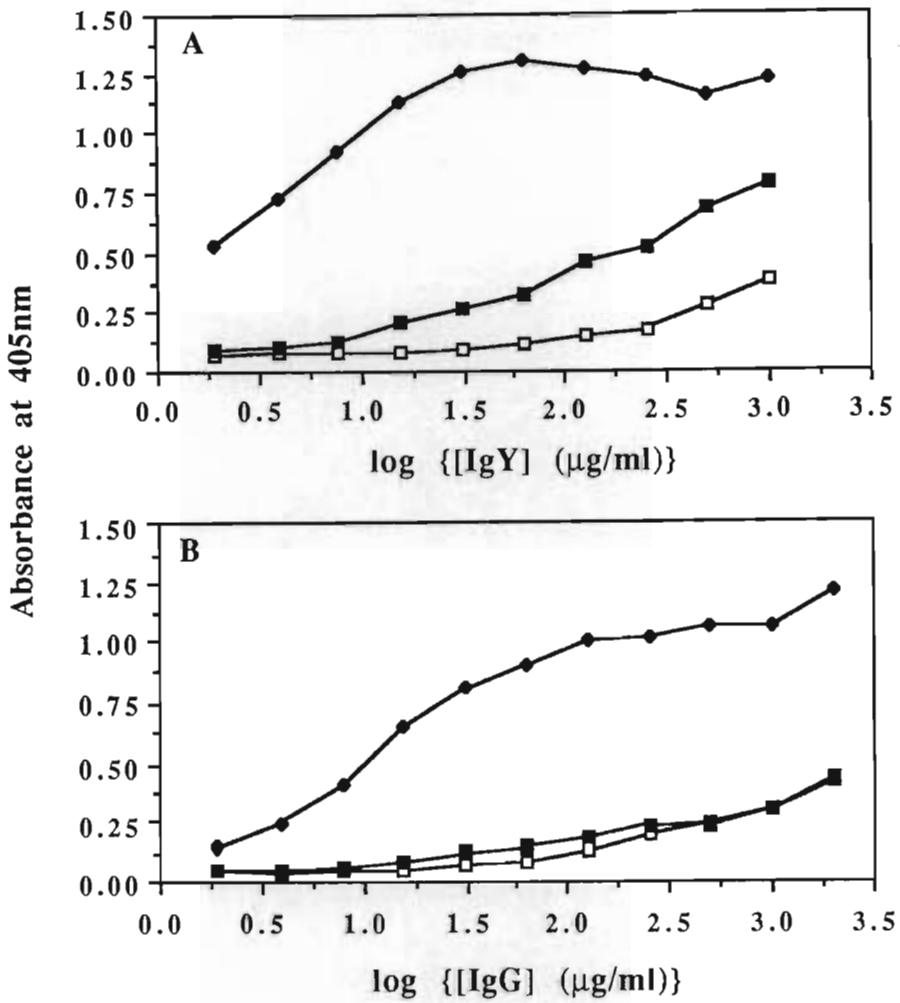

Figure 51. Binding of chicken and rabbit anti-cathepsin L antibodies to an active site-associated synthetic peptide in an ELISA.
The cathepsin L active site-associated synthetic peptide was coated to microtitre plates at 1 µg/ml and incubated with serial two-fold dilutions of, (A) chicken, and (B) rabbit antibodies. Chicken anti-whole cathepsin L (■), chicken anti-L153-165 peptide IgY (◆) and non-immune IgY (❑) were visualised using rabbit anti-chicken-HRPO conjugate. Rabbit anti-whole cathepsin L IgG (■), rabbit anti-L153-165 peptide IgG (◆) and non-immune rabbit IgG (❑) were visualised using sheep anti-rabbit-HRPO conjugate.

5.7.2.2 Inhibition of immunoinhibition, by the active site-associated peptide

In order to determine whether binding of the active site-associated peptide in the ELISA format was relevant in terms of the manner in which the chicken anti-cathepsin L antibodies exerted their immunoinhibitory effect, the inhibition by the free peptide of this immunoinhibition was investigated. Since all the chicken antibody preparations showed very similar immunoinhibitory characteristics and targeted the peptide in an ELISA to the same extent, antibodies from one chicken were used for this investigation. The limited amount of synthetic peptide available further necessitated the use of only representative antibody samples. A typical result is shown in Fig. 52. Prior

incubation of the IgY with the active site-associated peptide, reduced immunoinhibition of native cathepsin L by up to 35% (Fig. 52), showing that binding of the peptide in the whole enzyme is, at least in part, responsible for immunoinhibition. Since there is often only a small degree of antigenic cross-reactivity between peptides and antibodies, raised against the corresponding whole protein, large molar excesses of peptide to the enzyme are required to show binding of the peptide (Van Regenmortel, 1988a). Cross-reactivity, as evidenced by this reduction in immunoinhibition, could be illustrated using peptide:cathepsin L molar ratios of 500 000:1 to 50 000:1. It is furthermore envisaged that the linear peptide could adopt a number of conformations in solution, and a molar excess of peptide over antibody might therefore be required to maximise the probability of it adopting the same conformation that it has in native cathepsin L, to ensure optimal epitope-paratope interaction. The peptide: cathepsin L molar ratios (shown above), which caused a reduction in immunoinhibition, corresponded to peptide:antibody ratios of 125:1 to 1.25:1. These results complement those obtained in the ELISA (Section 5.7.2.1), and provide further evidence that this peptide, in the enzyme, may be part of an immunoinhibitory epitope, which is immunogenic in chickens.

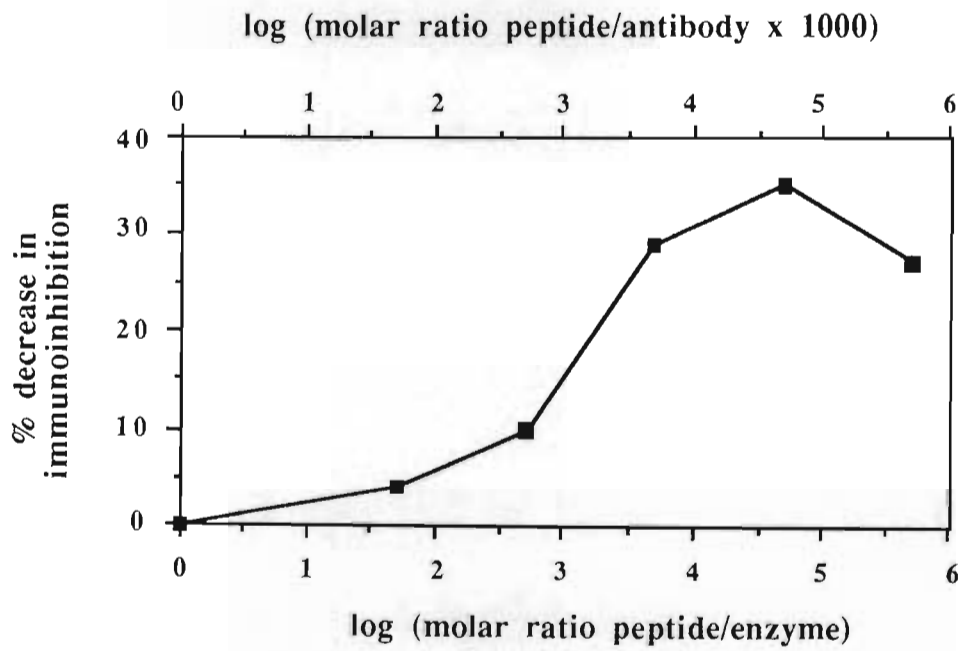

Figure 52. Inhibition, by an active site-associated peptide, of the immunoinhibition by chicken anti-cathepsin L IgY.
Chicken anti-cathepsin L (0.125 mg/ml final concentration) was incubated with increasing amounts of synthetic peptide (15 min, 30°C), before cathepsin L (5 ng) was added, incubated for a further 15 min at 30°C and the cleavage of Z-Phe-Arg-NHMec monitored on a continuous basis. In control assays immune IgY was replaced by non-immune IgY. Inhibition by the peptide of immunoinhibition by chicken anti-cathepsin L IgY was calculated relative to assays in the absence of the peptide.

5.7.3 Production and characterisation of anti-peptide antibodies raised in chickens against the human cathepsin L active-site peptide

Following conjugation of peptide L153-165 to KLH, the coupling yield was estimated using the micro-biuret method, which is based on the formation of a copper coordination complex with peptide nitrogen atoms and is, therefore, most suitable for peptides. Although the peptide showed absorption maxima at 225 and 280 nm (Pike, 1990), its absorbance was masked by that of glutaraldehyde at these wavelengths. Quantification of residual free peptide separated from conjugate by MEC, showed that 45% of peptide L153-165 was conjugated to KLH, which is within the range of 35-50% reported by Bulinski and Gundersen (1986). The coupling yield was used to determine the amount of immunogen required for the inoculation of chickens at the required dose.

The progress of chicken anti-peptide L153-165 antibody production over time was monitored by an ELISA, in which peptide was coated directly to the wells of microtitre plates (Fig. 53). The chickens responded very similarly during the course of the immunisation programme and the response by only one chicken is, therefore, shown. Antibody production reached a maximum at six weeks with a gradual decline at eight and twelve weeks. The antibody did not show any appreciable non-specific binding to unrelated peptides, COL476-490, B13-22 or D112-122 from type IV collagenase and cathepsins B and D respectively (Coetzer *et al.*, 1991), coated to ELISA plates, and values below those of non-immune IgY were obtained (results not shown). The use of peptide coated microtitre plates possibly enhances the specificity of the ELISA, in that only peptide-specific antibodies would be targeted and not CAMOR antibodies (Briand *et al.*, 1985), which would give false positive results. It also obviates the need for the preparation of a separate conjugate, using a different carrier and conjugation method, for coating the peptide to the ELISA plate, to account for the presence of anti-carrier antibodies. Although some workers are sceptical regarding the use of peptides coated directly to ELISA plates, this approach has been successfully used in several studies to detect anti-peptide antibodies produced against a wide range of peptides ranging between 10 and 20 residues (Tanaka *et al.*, 1985; Davies *et al.*, 1987; Moroder *et al.*, 1992).

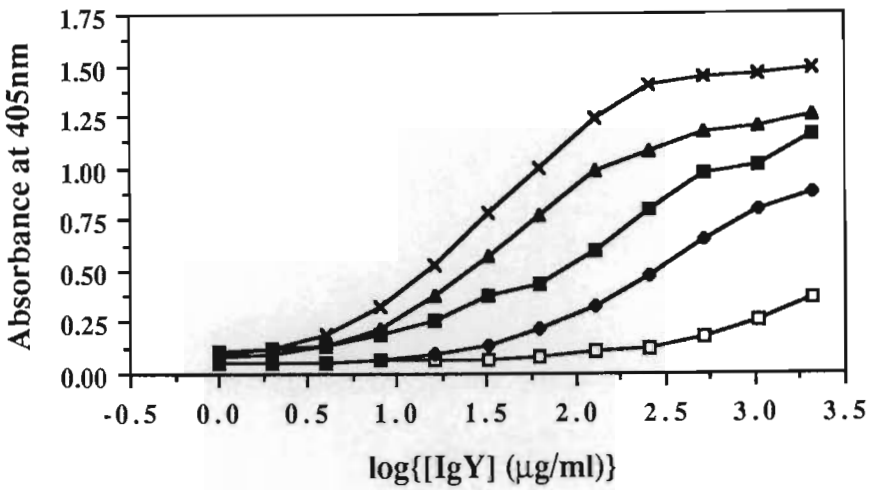

Figure 53. Progress of immunisation with peptide L153-165 conjugate as determined by ELISA. Human cathepsin L peptide L153-165 was coated at 1 µg/ml to microtitre plates and incubated with serial two-fold dilutions of chicken anti-human cathepsin L peptide L153-165 IgY collected after 4 (◆), 6 (×), 8 (▲) and 12 weeks (■) and non-immune IgY (❑). Binding was visualised by incubation with rabbit anti-chicken HRPO-linked secondary antibodies as described in section 2.9. Each point is the mean absorbance at 405 nm of duplicate samples.

Chicken anti-peptide L153-165 IgY recognises whole human cathepsin L in an ELISA and the antibodies titrated to at least 0.5 µg/ml (Fig. 54). These antibodies also target cathepsin L across species, as shown by the strong reaction with whole sheep cathepsin L in the same ELISA. Although it is generally recognised that proteins become at least partially denatured upon adsorption to the wells of ELISA-plates (Van Regenmortel, 1988a,b), the native conformation of cathepsin L seems to have been maintained to some degree by coating at 4°C in a pH 6.0 buffer, since a lower level of binding by anti-peptide antibodies to human cathepsin L, in particular, was observed using the regular cathepsin L coating conditions which involves incubation at 37°C for three hours prior to the 4°C incubation step (result not shown).

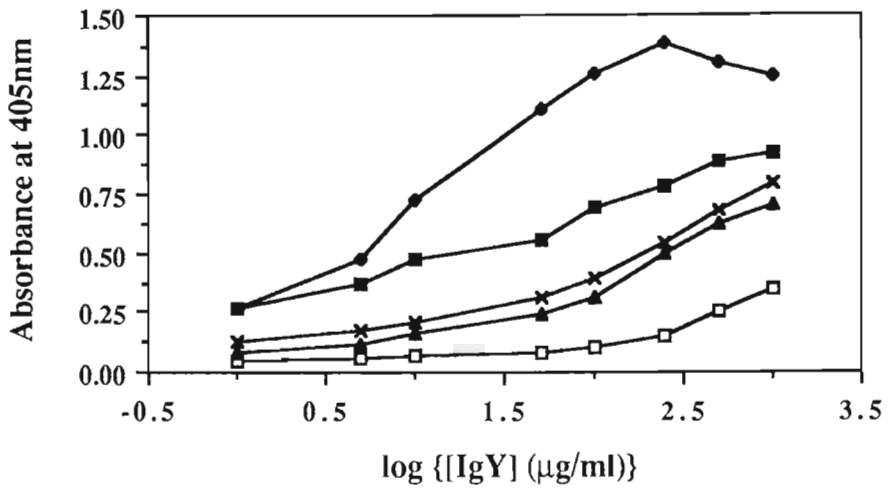

Figure 54. ELISA of the binding of anti-human cathepsin L peptide antibodies to whole human and sheep cathepsin L.
Human cathepsin L was coated at 1 μg/ml to microtitre plates and incubated with serial two-fold dilutions of, (■), chicken anti-human cathepsin L peptide IgY and chicken anti-whole sheep cathepsin L IgY (▲). Sheep cathepsin L was coated at 1 μg/ml to microtitre plates and incubated with serial two-fold dilutions of, (×), chicken anti-human peptide IgY and chicken anti-whole sheep cathepsin L IgY (◆). Non-immune IgY (❑). Binding was visualised by incubation with HRPO-linked secondary antibodies as described in section 2.9. Each point is the mean absorbance at 405 nm of duplicate samples.

The reaction of whole human cathepsin L with the chicken anti-human cathepsin L peptide IgY was stronger than that with the control chicken anti-whole sheep cathepsin L IgY, while stronger targeting of sheep cathepsin L was shown by the control chicken anti-whole sheep cathepsin L IgY, than by the chicken anti-human cathepsin L peptide IgY (Fig. 54). The presence of anti-KLH antibodies in the chicken anti-peptide L153-165 IgY preparation did not seem to interfere in the ELISA, as similar results were obtained with preparations from which anti-KLH antibodies were removed by affinity chromatography (Section 3.9).

Chicken anti-peptide L153-165 IgY also targeted SDS-denatured human and sheep cathepsin L, electroblotted onto nitrocellulose in a Western blot (Fig. 55). In contrast to the ELISA, it was necessary to absorb the anti-KLH antibodies from the chicken anti-peptide L153-165 IgY for use in Western blots, since, as found previously (Coetzer *et al.*, 1991), a significant level of non-specific binding resulted from the presence of antibodies to this carrier protein.

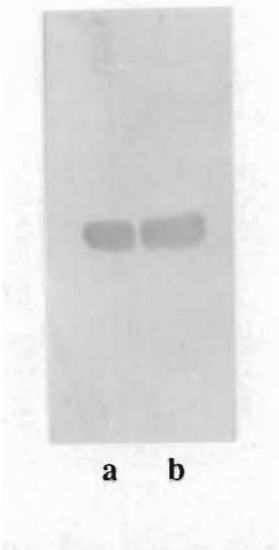

Figure 55. Targeting of human and sheep cathepsin L by chicken anti-human cathepsin L peptide antibodies in a Western blot.
(a) Human cathepsin L (10 µg); and sheep cathepsin L (10 µg) were electrophoresed on a 12.5% SDS-PAGE gel, with reduction, electroblotted onto nitrocellulose and probed with 100 µg/ml anti-KLH purified chicken anti-human cathepsin L peptide IgY. Targeting was visualised using rabbit anti-chicken-HRPO conjugate as described in section 2.8.

On the strength of the indications by ELISA and Western blot results that the chicken anti-peptide L153-165 IgY targets whole human cathepsin L, immunoinhibition assays were conducted. The hydrolysis of Z-Phe-Arg-NHMec by human cathepsin L was inhibited by chicken anti-peptide L153-165 IgY, and tests over a range of IgY concentrations showed 66% immunoinhibition at the highest antibody concentration with a gradual decrease in the level of inhibition at lower concentrations of IgY (Fig. 56). These results also give evidence that the chicken anti-cathepsin L peptide antibodies target the native, enzymically active form of cathepsin L.

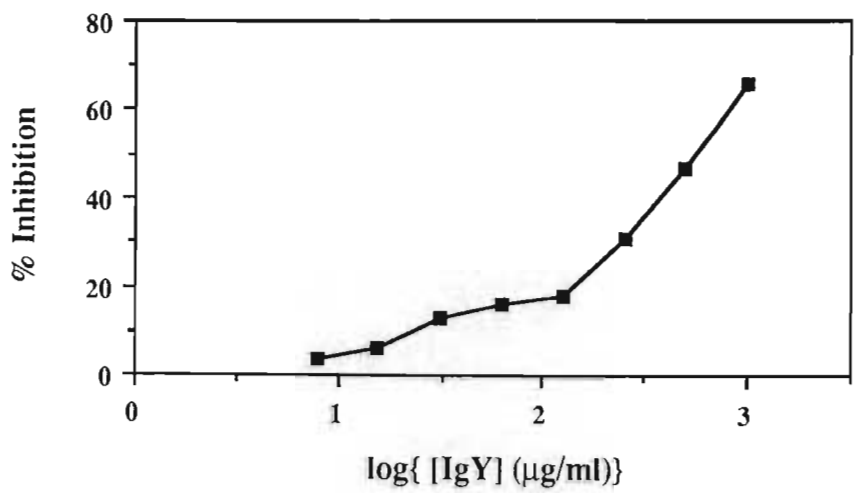

Figure 56. Immunoinhibition of human cathepsin L by chicken anti-human cathepsin L peptide antibodies. Human cathepsin L (5 ng) was incubated with decreasing amounts of human anti-cathepsin L IgY (■) and the cleavage of Z-Phe-Arg-NHMec was monitored in a stopped time assay. The percentage inhibition was calculated relative to control assays with non-immune IgY.

The lower level of immunoinhibition of cathepsin L by the chicken than the 85% by the rabbit anti-peptide L153-165 antibodies, raised in a previous study (Pike, 1990), may be ascribed to the presence of a significantly higher level of anti-KLH antibodies in the chicken IgY preparation, since haemocyanin from *Jasus lalandii* has been found to be extremely immunogenic in chickens (Polson *et al.*, 1980). Improved immunoinhibition may, therefore, be obtained by removal of anti-KLH antibodies from this preparation, or alternatively by adsorbing out cathepsin L specific antibodies using immobilised cathepsin L. The presence of anti-carrier antibodies may be eliminated altogether by raising antibodies against free, unconjugated peptide as discussed below.

5.7.4 Production and characterisation of anti-peptide antibodies raised in chickens and rabbits against the active-site peptide in human cathepsin H

Chickens produced anti-peptide antibodies when immunised with free, unconjugated peptide H157-168, selected from the active site of human cathepsin H, as evidenced by targeting of the peptide coated directly to the multititre plate wells in an ELISA (Fig. 57A). Anti-peptide antibody production peaked in both chickens at six weeks, with a gradual, small decline in antibody production over time.

Before conjugation, 85% of peptide H157-168 was reduced and, since the number of activated groups available on both carrier proteins for coupling was in excess over the number of free sulfhydryls on the peptide, the residual number of activated groups following conjugation was thought to be an adequate estimate of the coupling efficiency to determine the dose of conjugate for the immunisation protocol. It was thus found that 46% of peptide H157-168 was coupled to KLH and 44% to ovalbumin, which falls within the range of 30-50% coupling yield reported by Muller (1988a).

By testing different peptides, it was found in this laboratory, that these "estimation by difference" methods of measuring coupling efficiency (Coetzer *et al.*, 1991; Sections 3.7; 5.3.2; 5.3.4) provided a satisfactory alternative to amino acid analysis of carrier protein before and after conjugation (Briand *et al.*, 1985), for estimating the amount of immunogen to be used for the inoculation of experimental animals. As a result of the indeterminate molecular size of KLH, and the presence of impurities in commercially available preparations, the amino acid analysis method is especially unsuitable for this carrier protein. Since the objective of this study was the production of protein-reactive anti-peptide antibodies, using well documented conjugation methods, rather than the comparison of different conjugation methods *per se*, the estimate of the amount of peptide coupled to the

carrier proteins seems to have been adequate for successfully eliciting immune responses against the different peptides.

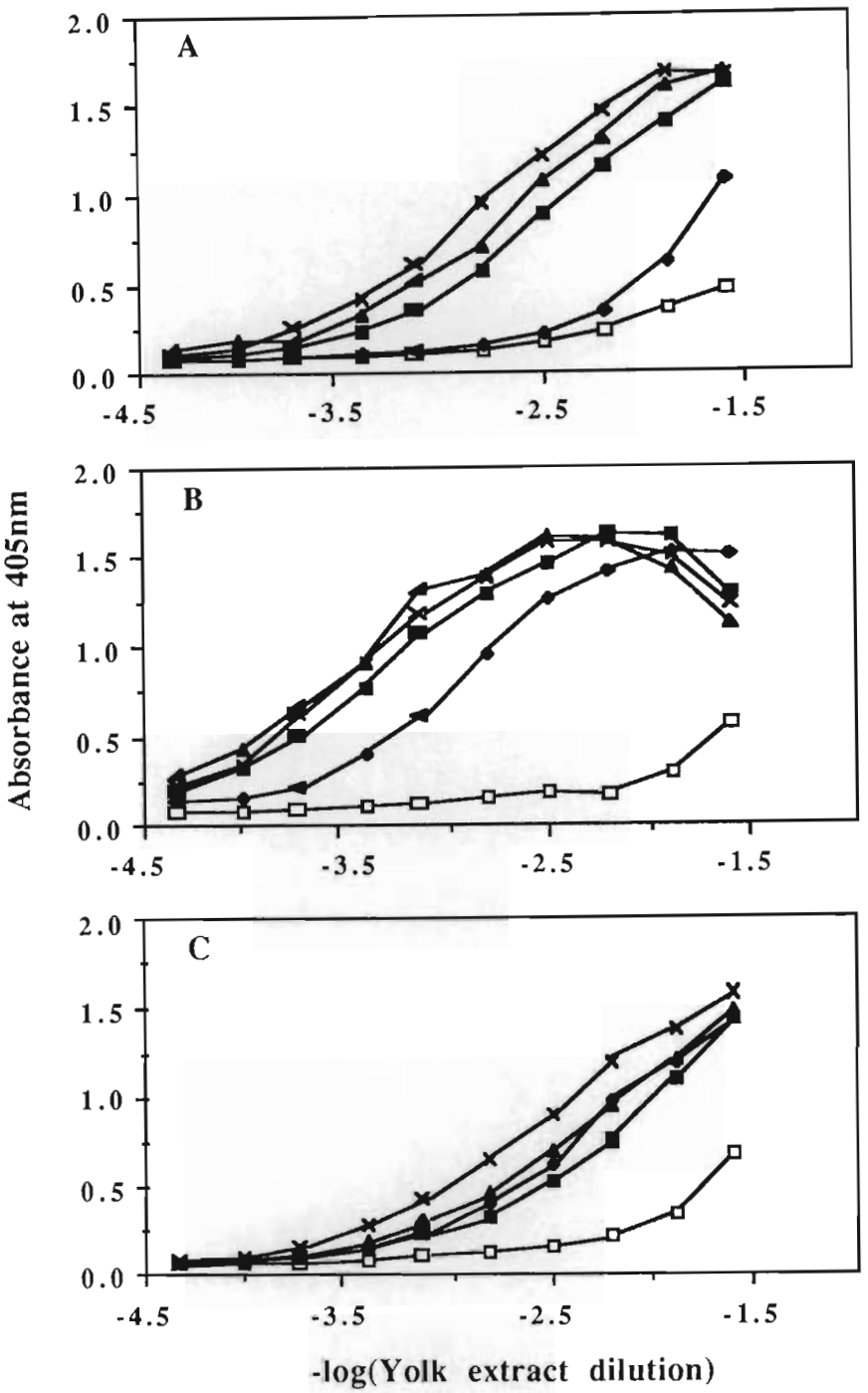

Figure 57. ELISA of progress of immunisation of chickens with free and conjugated cathepsin H peptide H153-168.
Human cathepsin H peptide H157-168 was coated at 1 µg/ml to microtitre plates and incubated with serial two-fold dilutions of chicken anti-human cathepsin H peptide L157-168 yolk extract from eggs collected after 4 (◆), 6 (×), 8 (▲) and 12 weeks (■) and non-immune egg yolk extract (❑). Immunisation with (A) free peptide; (B) KLH-conjugated peptide; and (C) ovalbumin-conjugated peptide. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked secondary antibodies as described in section 2.9. Each point is the mean absorbance at 405 nm of duplicate samples.

Anti-peptide H157-168 antibodies were generally produced more rapidly when the peptide was conjugated to either KLH or ovalbumin, compared to free peptide (Fig. 57B and C), with antibody production almost reaching peak levels after three weeks. These peak levels were, however, comparable to those reached in response to immunisation with free peptide. Inoculation with the KLH-conjugate resulted in anti-peptide antibody production reaching a peak at six weeks, which was maintained at 8 weeks, before dropping slightly at twelve weeks. Anti-peptide antibody production with the ovalbumin-conjugate also peaked at six weeks, before showing a gradual, small decline in antibody production over time (Fig. 57C). Eggs from each chicken, corresponding to the peaks of antibody production against free and conjugated peptide, indicated by the ELISA results using yolk extracts, were used for IgY isolation for further testing against whole cathepsin H in ELISAs, Western blots and immunoinhibition assays.

Inoculation of rabbits with free peptide H157-168 also resulted in the formation of anti-peptide antibodies which targeted the peptide strongly in an ELISA (Fig. 58A). There was very little difference in peptide targeting by antibodies produced after eight and twelve weeks, with serum from one rabbit showing a slightly stronger reaction after eight weeks than twelve weeks, while a negligible increase in antibody production occurred at twelve weeks in the other rabbit. Very little antibody was produced after three weeks against free peptide.

Similarly to chickens, anti-peptide H157-168 antibodies were produced more rapidly in rabbits when the peptide was conjugated to either KLH or ovalbumin, as evidenced by a very significant difference in binding to the peptide by serum collected only three weeks after the first inoculation and the non-immune serum control (Figs. 58B and C). There was only a small further increase in anti-peptide antibody production after eight weeks, to equal the peak levels reached by inoculation with free peptide, followed by a slight decrease after twelve weeks. Sera obtained from each rabbit at eight and twelve weeks was, therefore, pooled separately for the isolation of IgG for further evaluation of the individual anti-peptide antibody preparations.

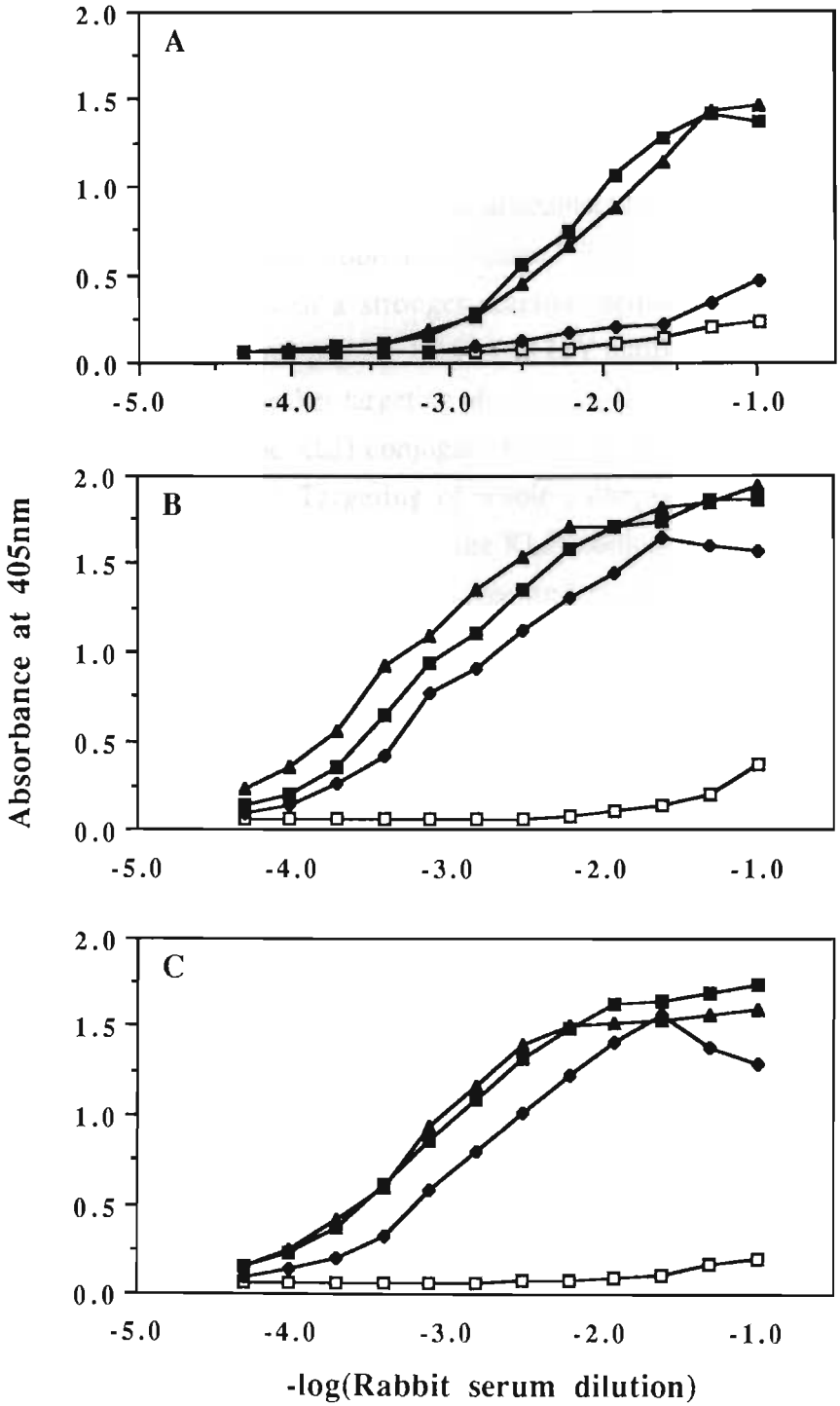

Figure 58. ELISA of progress of immunisation of rabbits with free and conjugated cathepsin H peptide H153-168.
Human cathepsin H peptide H157-168 was coated at 1 μg/ml to microtitre plates and incubated with serial two-fold dilutions of rabbit anti-human cathepsin H peptide L157-168 serum collected after 4 (◆), 6 (×), 8 (▲) and 12 weeks (■) and non-immune rabbit serum (❑). Immunisation with (A) free peptide; (B) KLH-conjugated peptide; and (C) ovalbumin-conjugated peptide. Binding was visualised by incubation with sheep anti-rabbit HRPO-linked secondary antibodies as described in section 2.9. Each point is the mean absorbance at 405 nm of duplicate samples.

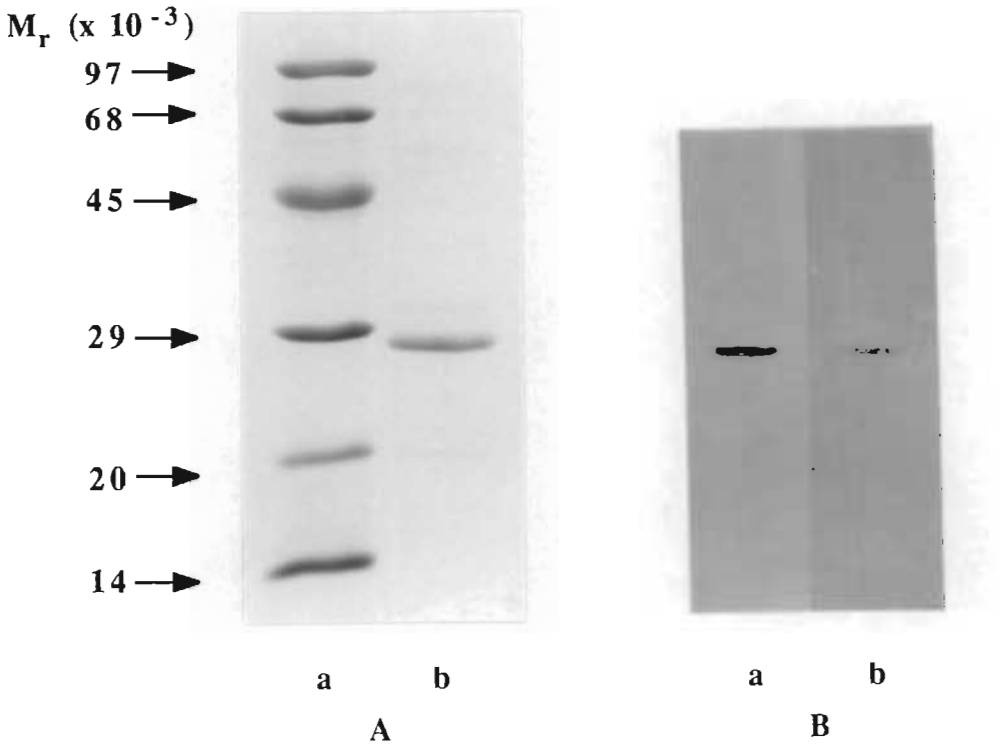

Figure 60. Targeting of human and sheep cathepsin L by chicken anti-human cathepsin L peptide antibodies in a Western blot.
(A) 12.5% SDS-PAGE of (a) MW markers as in Fig. 22; (b) human cathepsin H (2 µg). This sample was electroblotted onto nitrocellulose and probed in (B) with, (a) 150 µg/ml chicken anti-human cathepsin H peptide-ovalbumin conjugate IgY; and (b) 500 µg/ml rabbit anti-human cathepsin H peptide-ovalbumin conjugate IgG. Targeting was visualised using HRPO conjugates as described in section 2.8.

Various levels of immunoinhibition of cathepsin H were observed with all the anti-peptide H157-168 antibody preparations raised in both chickens and rabbits, using free and carrier-conjugated peptide (Table 18). The chicken anti-cathepsin H peptide antibodies were more immunoinhibitory than their rabbit counterparts, which is consistent with the reactivity of these antibodies in the ELISAs, where the native conformation of the enzyme was probably largely maintained by coating at pH 6 and 4°C. Anti-peptide antibodies raised against the free peptide showed the highest level of immunoinhibition in both chickens and rabbits, while the anti-peptide antibodies raised against the KLH conjugated-peptide had the least effect on the activity of cathepsin H. As a result of the relative insensitivity of the Arg-NHMec assay for cathepsin H, where 250 ng of enzyme was required per assay, which is in good agreement with the 40-400 ng recommended by Barrett and Kirschke (1981), and the limited amount of enzyme available, immunoinhibition could only be tested with replicates of a limited number of antibody concentrations. In the case of the chicken anti-peptide antibodies, raised against free and ovalbumin conjugated peptide H157-168, immunoinhibition seemed to follow a prozone effect, where lower levels of immunoinhibition were observed at higher antibody concentrations, which could suggest that

even higher levels of immunoinhibition could be achieved at lower antibody concentrations than those used within the constraints of these tests.

Table 18. A comparison of the immunoinhibition of cathepsin H by chicken and rabbit anti-peptide antibodies raised with different peptide immunogens.

Antibody	Immunogen	[Final antibody] (mg/ml)	% Immunoinhibition
Chicken IgY	Free H157-168	0.25	44
	H157-168-ovalbumin	0.25	42
	H157-168-KLH	0.5	36
Rabbit IgG	Free H157-168	1.0	29
	H157-168-ovalbumin	1.0	23
	H157-168-KLH	1.0	19

5.8 Discussion

In this chapter the production and characterisation of chicken and rabbit polyclonal and anti-peptide antibodies, potentially useful in studies elucidating the involvement of cathepsins L and H in tumour invasion, were described. In this regard, firstly, the novel targeting properties of the polyclonal chicken anti-cathepsin L antibodies were exploited in the localisation of an immunoinhibitory epitope in cathepsin L, laying the groundwork for defining additional epitopes of this nature for the production of immunoinhibitory anti-peptide antibodies. Secondly, the immunoinhibitory and selective targeting properties of the anti-peptide antibodies, directed at active site-associated peptides similarly positioned in cathepsins L and H, but essentially distinct in amino acid composition, was examined with a view to providing specific agents for use in *in vitro* tumour invasion model studies and tumour immunotherapy.

In an attempt to expand on the immunoinhibition of cathepsin L previously obtained with rabbit anti-peptide antibodies (Pike, 1990), differences between chicken and rabbit polyclonal antibodies were investigated. The hypothesis that sheep cathepsin L may be more

immunogenic in chickens than in rabbits, due to a greater evolutionary distance from the sheep, seems to be borne out by the results of the ELISA tests, since the chicken antibodies had approximately 10-fold higher titres than their rabbit counterparts. The evolutionary distance between avian and mammalian species has also been successfully exploited by other workers in the production of chicken anti-rat and human insulin and IGF-1 receptor antibodies, after attempts to produce antibodies in rabbits had failed (Song, 1985; Stuart *et al.*, 1988).

It has been reported that smaller molecules ($M_r < 70\ 000$) are poorly immunogenic in chickens (Polson *et al.*, 1980). In contrast to these results, it was found in the present study that antibodies could be readily produced in chickens against the M_r 26 000 cathepsin L and the unconjugated M_r 1 349 cathepsin H peptide (see below). This contrast might, in part, be due to the method of testing, i.e. immunoprecipitation in a gel matrix, used by these workers, since it was found that antibodies against cathepsin L, from both rabbits and chickens, do not precipitate the antigen in Ouchterlony tests. This is in contrast to strong reactions obtained in tests such as ELISA or Western blotting. Thus previous results obtained by Polson *et al.* (1980) might be due to their antibodies being poorly immunoprecipitating, rather than low titre antibodies *per se*.

Binding of an antibody to an enzyme may have one of three outcomes with respect to enzyme activity: firstly, it may have no effect; secondly, it may lead to immunoinhibition of the enzyme, either as a result of a distortion of the structure of the enzyme, or by impairing its flexibility, or by binding to an amino acid sequence in the active site, or by occluding the active site. Thirdly, it may lead to immunoactivation (Richmond, 1977; Höyhtyä *et al.*, 1990), possibly by changing the conformation of the enzyme, thereby enhancing substrate binding.

The chicken polyclonal antibodies seem to target different epitopes from the rabbit polyclonal antibodies, reflected, in this instance, by their immunoinhibitory characteristics, compared to the rabbit polyclonal antibodies, which showed immunoactivation of the enzyme. A decrease in the activity of an enzyme after incubation with an antibody, particularly using polyclonal antibodies, as opposed to anti-peptide or monoclonal antibodies, might be the result of either immunoprecipitation of the enzyme, or immunoinhibition due to specific targeting of an epitope in the active site of the enzyme. In the case of the results presented here, the decrease in activity brought about by the chicken polyclonal antibodies is most likely due to immunoinhibition, since, as mentioned above, they do not immunoprecipitate the enzyme in an Ouchterlony test. The amount of enzyme used to test for immunoinhibition in the synthetic substrate assay is probably also too small

to generate the lattice necessary for immunoprecipitation (Richmond, 1977) and the molar excess of antibody over enzyme used in this assay will also not favour the formation of insoluble complexes. Immunoinhibition, independent of immunoprecipitation has been reported for a monospecific antiserum to acetyl-CoA carboxylase (Mayer and Walker, 1987) and mouse liver lactate dehydrogenase (Ng and Gregory, 1969).

The immunoinhibitory ability of the chicken polyclonal antibodies seems to be derived, at least in part, by their ability to target the active site-associated peptide. This is illustrated by the binding of chicken anti-sheep cathepsin L IgY to the peptide L153-165 in an ELISA and, arguably more conclusively, by inhibition of the immunoinhibition by prior incubation of the antibodies with the peptide. This suggests that this peptide is not only able to elicit immunoinhibitory antibodies by "artificially" inducing an immune response via the anti-peptide antibodies approach, but is also a natural, immunogenic epitope for the chicken.

Although complete inhibition of the immunoinhibition was not obtained with the active site-associated peptide, the degree of blocking of the paratope by the peptide suggests that the immunoinhibitory characteristics of the chicken polyclonal antibody is, in part, due to targeting of this sequence in the whole native cathepsin L. Most epitopes are discontinuous, rather than continuous (Van Regenmortel, 1986) and these results may indicate that this linear active site-associated peptide sequence forms part of a larger discontinuous epitope, hence showing only partial inhibition of the immunoinhibition. Further studies, using overlapping peptides of decreasing length, are required to elucidate the precise immunoinhibitory targets of the chicken polyclonal antibodies on the enzyme. This information could then assist in the production of further anti-peptide antibodies which may have superior immunoinhibitory properties than those raised in this, and a previous study (Pike, 1990).

The immunogenicity of cathepsin L, observed in the present study, may be rationalised in the context of results obtained in studies on the proteolytic enzymes involved in antigen processing during an immune response, and the influence specific proteolytic processing has on the integrity of T-cell epitopes. In contrast to B-cells which recognise native antigen molecules free in solution by means of surface Ig-receptors, T-cell receptors on helper T-cells recognise processed (i.e. partially degraded or denatured) antigen, associated with class II major histocompatibility complex (MHC) encoded molecules, on the surfaces of antigen presenting cells (APCs) (Roitt *et al.*, 1989a). Helper T-cells, stimulated by antigen presentation, secrete interleukin 2 which stimulates B-cells to proliferate and differentiate into antibody producing plasma cells (Roitt *et al.*, 1989b).

Studies using class-specific proteinase inhibitors have implicated the lysosomal cathepsins B, L and D in the proteolytic processing of antigens (Buus and Werdelin, 1986; Puri and Factorovich, 1988; Takahashi *et al.*, 1988). Although Takahashi *et al.* (1988) concluded that cathepsin B, rather than cathepsin L, was responsible for the processing of myoglobin, Buus and Werdelin (1986) showed that degradation and presentation of the synthetic antigen dinitrophenyl poly-L-lysine was inhibited by Z-Phe-Ala-CHN_2, which has a 2 000-fold higher affinity for cathepsin L than cathepsin B (Kirschke and Shaw, 1981). Since there is some evidence that cathepsin L may be involved in antigen processing, it also creates the possibility that there might be some degree of prejudice against producing antibodies against an enzyme with such a key immunological function. This may explain the need to exploit the evolutionary distance presented by the avian immune system in order to produce immunoinhibitory polyclonal antibodies against mammalian cathepsin L. The necessity for this approach seems to be obviated by the use of synthetic peptide conjugates which seem to be sufficiently foreign to another mammalian species' immune system to be able to stimulate the production of protein-reactive anti-peptide antibodies, as was illustrated by the production of immunoinhibitory anti-human cathepsin L peptide antibodies raised in rabbits (Pike, 1990).

Although proteolytic processing is required for the production of peptides necessary for MHC molecule assembly and subsequent presentation to T-cells (Lotteau *et al.*, 1990), it may also destroy potential T-cell epitopes, so that no response is mounted against those epitopes. Sequences in proteins which are resistant to proteolytic attack, may, therefore, represent T-cell epitopes. Mouritsen *et al.* (1991) located distinct Cys-residue containing amino acid combinations in twelve large protein antigens which constitute poor substrates for cathepsins B, L and D. Interestingly, in the context of the present study, the amino acid sequence EPNC, which constitutes the N-terminal portion of the active-site sequence shown in the present study to form part of a natural epitope in cathepsin L, has been identified as a cathepsin L insensitive sequence (Mouritsen *et al.*, 1991). This may imply that this sequence in the sheep cathepsin L immunogen may be resistant to hydrolysis by the chicken cathepsin L involved in antigen processing. This gives further support to the contention that this sequence in cathepsin L probably constitutes a natural T-cell epitope, which is not processed, especially in the avian immune system, before interacting with a class II MHC molecule for presentation to the relevant helper T-cell receptor and subsequent stimulation of B-cells for antibody production..

The avian immune system was also successful in producing anti-peptide antibodies reactive with native cathepsins L and H. Evidence for the recognition of the native enzymes was given by the results of the liquid-phase enzyme immunoinhibition assays, where the

native conformation of the enzyme is maintained, as evidenced by full activity in the absence of immune antibodies. Claims of a high frequency of raising anti-peptide antibodies capable of recognising the native protein are often found in the literature, but they are mostly based on immunoassays known to alter the conformation of the native protein, such as Western and dot blots, or immunoprecipitation of radiolabeled proteins (Wilczynska *et al.*, 1992; Kennedy *et al.*, 1987). Anti-peptide antibodies tend to cross-react very strongly with the denatured form of the corresponding whole protein, which is probably conformationally less restrictive and would, therefore, show a greater resemblance to the conformation of the free peptide than to that in the native protein (Van Regenmortel, 1988).

Although the primary objective of raising anti-peptide antibodies was the production of native protein-reactive immunoinhibitory agents, the chicken anti-human cathepsin L and H peptide antibodies proved to be versatile reagents, additionally capable of recognising the partially denatured enzymes in ELISAs and their fully denatured forms in Western blots. These chicken anti-peptide antibodies may, therefore, find application in studies requiring targeting of the full range of enzyme conformations. These would include inhibition of native enzyme in *in vitro* tumour invasion studies, their quantitation by ELISA in tumour homogenates and targeting in immunocytochemistry where fixation may denature the enzymes to some degree.

Chicken anti-cathepsin H peptide antibodies appeared to have superior immunoinhibitory, as well as ELISA and Western blot targeting properties, than their rabbit counterparts, consistent with the polyclonal anti-whole cathepsin L antibodies. Despite the fact that the chicken anti-cathepsin L peptide antibodies did not show the same level of immunoinhibition as the corresponding rabbit anti-peptide antibodies, raised in a previous study (Pike, 1990) (65% as opposed to 85%), their apparent superior targeting of whole cathepsin L in an ELISA holds much promise for immunoaffinity purification of human and sheep cathepsin L, by exploiting features of the peptide they target (see below). Furthermore, sufficient quantities of antibody for large scale immunoaffinity purification could be more readily obtained from chickens than from rabbits, since a single egg yolk yields 75 mg of IgY antibody, while 15 ml of blood is required to isolate the same amount of IgG.

It was shown in Chapter 3 that anti-peptide antibodies could be used for immunoaffinity isolation of type IV collagenase and, likewise, the anti-human cathepsin L and H peptide antibodies could be employed in the purification of the corresponding enzymes. Most immunoaffinity chromatography elution steps make use of relatively harsh procedures such as lowering the pH to 2.4 or desorption with chaotropic agents such as

SCN^-, which may have detrimental effects on bioactive molecules, such as proteolytic enzymes. However, both anti-cathepsin L and H peptide antibodies, produced in this study, potentially have properties which could allow the use of a subtle pH change for the elution of the corresponding enzyme. The binding affinity (for the corresponding peptide) of anti-peptide antibodies raised againt peptide sequences containing His-residues decreases sharply at around pH 5-6, which may be attributed to the pKa of 6.0 for these residues (Muller *et al.*, 1985; Kondo *et al.*, 1989; Sada *et al.*, 1990). Peptides L153-165 and H157-168 contain one and two His-residues respectively, and following adsorption of crude fractions containing these enzymes to the corresponding anti-peptide antibody immunoaffinity columns at pH 6.0-6.5, elution of the respective enzymes could, conceivably, be effected by decreasing the pH to 5.0, or even 4.5 in the case of cathepsin L. Studies to explore this apparoach are underway, but fall outside the scope of this thesis.

Although it is not common practice to raise anti-peptide antibodies against free, i.e. unconjugated peptide, because it is commonly believed that free small peptides are not immunogenic, cathepsin H-reactive anti-peptide antibodies were successfully raised in both chickens and rabbits against the twelve-residue, cathepsin H peptide, H157-168. The anti-cathepsin L peptide antibodies raised in this study against conjugated peptide, were produced before those against cathepsin H, and prior to appreciating the immunogenicity of free peptides (Dr Sylvianne Muller, CNRS, Strasbourg, personal communication). The free peptide route of anti-peptide antibody production is now also being used for cathepsin L, but these studies fall outside the timeframe of this thesis. The absence of anti-carrier and CAMOR antibodies in these preparations has obvious advantages for the various immunological applications envisaged. A number of studies adopting the free peptide approach, in addition to the conjugated route, for anti-peptide antibody production have, however, been reported in the literature (Atassi and Webster, 1983; Atassi, 1984; Muller *et al.*, 1986; 1988), and Muller (1988b) suggested an empirical approach using free, conjugated and cyclised peptides for raising anti-peptide antibodies. Although Mariani *et al.* (1987) concluded that low affinity anti-peptide antibodies are raised against free peptides, since they found that the affinity of monoclonal antibodies, raised against a nine-residue synthetic peptide, for the native protein could be enhanced by conjugation to a carrier protein, this might have been a feature of monoclonal anti-peptide antibodies, the length and/or the electrostatic charge of the synthetic peptide. Kondo *et al.* (1990) showed that the affinity of antibodies raised against peptides with charged groups, is affected strongly by pH and ionic strength, and peptide composition may, therefore, be more important than peptide length in determining anti-peptide antibody affinity.

The selection of immunogenic peptides for the production of anti-peptide antibodies with predetermined specificity is usually aided by prediction algorithms which identify the positions of continuous epitopes in proteins. There have, however, been conflicting claims in the literature as to which algorithm, based on parameters such as hydrophilicity, segmental mobility and accessibility of short sequences of polypeptide chains (Hopp and Woods, 1981; 1983; Westhof *et al.*, 1983; Parker *et al.*, 1986), is most successful in predicting the position of potential epitopes. Hopp (1986) showed that the hydrophilicity prediction method (Hopp and Woods, 1981; 1983) performed better than any of the other methods tested, whereas a comparison of the ability of different methods to correctly predict antigenic residues in proteins, known to be antigenic, has shown that none of the most commonly used prediction methods was consistently superior to all the others (Van Regenmortel and Daney de Marcillac, 1988).

Although only a small number of peptides were tested in this laboratory, it became evident that the use of these prediction algorithms, based on primary structure, is probably less important than a study of the tertiary structure of the specific protein, or a suitable model for the class of protein, for the selection of suitable peptides for the production of protein-reactive anti-peptide antibodies. This was illustrated by the cathepsin B peptide B13-22 which, despite being on a peak of both hydrophilicity and segmental mobility, resulted in the production of anti-peptide antibodies which failed to recognise the whole proteinase in either a competition, or direct binding ELISA (Coetzer *et al.*, 1991). Subsequent elucidation of the tertiary structure of cathepsin B (Musil *et al.*, 1991) revealed that it differs significantly from that of papain, which was used, in conjunction with the epitope prediction algorithms, as a model for cathepsin peptide selection in this study, and that peptide B13-22 was in fact not readily accessible. In the case of cathepsins L and H, however, the three-dimensional structure of papain indicated that the hydrophilic N-termini of peptides L153-165 and H157-168, which also showed a high degree of segmental mobility, correspond to a peptide located on the surface of the molecule and is, therefore, readily accessible. Consistent with amino acid sequencing (Ritonja *et al.*, 1988) and structural studies (Dufour, 1988), papain seems, therefore, a most suitable model of the three-dimensional structures of cathepsins L and H for the successful selection of accessible, active-site associated peptides.

Immunoinhibitory polyclonal and particularly anti-peptide antibodies may have advantages over synthetic cysteine proteinase inhibitors for use in studies on proteinase function in ECM degradation in disease states such as tumour invasion, emphysema and arthritis since, as was shown in this, and a previous study (Pike, 1990), antibodies may distinguish between individual cathepsins. Synthetic proteinase inhibitors are usually class specific, since they are constructed to fit into the active site cleft of the enzyme, the

conformation of which is shared by the individual members of a class of proteinases. The highest degree of discrimination between cathepsins L and B is provided by Z-Phe-Tyr(O-t-But)-CHN_2 and Z-Phe-Phe-CHN_2 for which cathepsin L shows a higher susceptibility than cathepsin B (Kirschke *et al.*, 1988), and Z-Ala-His-CHN_2 which is more effective in inactivating cathepsin B than L (Angliker *et al.*, 1991). It was shown in this study that the active site sequence previously selected for the production of immunoinhibitory rabbit anti-cathepsin L antibodies (Pike, 1990) is not only a natural epitope in chickens for the production of immunoinhibitory polyclonal antibodies, but that targeting of the corresponding peptide in cathepsin H by anti-peptide antibodies also leads to the selective inhibition of cathepsin H. By exploiting the differences in amino acid sequence in the vicinity of the active site His-residue in cysteine proteinases, selective anti-peptide antibody inhibitors may, therefore, be produced for the different cysteine proteinases.

The immunoinhibitory polyclonal and anti-peptide antibodies produced in this study using the novel chicken route facilitated the localisation of an immunoinhibitory epitope in cathepsin L and provided a panel of antibodies with unique characteristics which may find application in localisation of cathepsin L and H in tumour and other pathological tissues, *in vitro* tumour invasion studies and following structural manipulation (see General Discussion), possibly also in tumour immunotherapy.

CHAPTER 6

GENERAL DISCUSSION

Proteolytic degradation of the ECM barriers, particularly basement membranes, is a prerequisite for tumour invasion and other tissue destructive pathologies, such as emphysema and rheumatoid arthritis (Tryggvason *et al.*, 1987). The structural complexity of the ECM, which consists mainly of interstitial and basement membrane collagen, associated with elastin, fibronectin, laminin and proteoglycans, necessitates coordinated hydrolysis by a panel of proteinases with a wide range of catalytic specificities (Kühn, 1986; Martin *et al.*, 1988; Barrett, 1980). Collagenolytic proteinases would play a key role in dissolution of the ECM during tumour invasion, and in this regard the basement membrane degrading metalloproteinases, type IV collagenases, and the cysteine proteinases, cathepsins L and H, have been strongly implicated (Liotta *et al.*, 1980; Maciewicz *et al.*, 1989; Guinec *et al.*, 1990; Gabrijelcic *et al.*, 1992).

The 72 and 92 kDa, genetically distinct, molecular forms of type IV collagenase, both of which have been correlated with the metastatic phenotype of *ras*-transformed cells, have similar substrate specificity and selectively cleave type IV collagen tetramers to effect disruption of basement membranes (Fessler *et al.*, 1984; Garbisa *et al.*, 1987; Murphy *et al.*, 1989a; Wilhelm *et al.*, 1989). Type IV collagenases also digest denatured fibrillar collagen, i.e. gelatin, as well as type V collagen which anchors the interstitial collagen stroma to the basement membranes, thus potentially providing a passageway for metastasising tumour cells (Liotta *et al.*, 1980; Okada *et al.*, 1990). This is supported by a substantial body of evidence which has shown a positive correlation between augmented type IV collagenolytic activity and invasion by a variety of rodent and human tumours (Liotta *et al.*, 1979, 1980; Turpeenniemi-Hujanen *et al.*, 1985; Nakajima *et al.*, 1987; Yamagata *et al.*, 1988; Brown *et al.*, 1990; Lyons *et al.*, 1991). Furthermore, type IV collagenase synthesis is not only under control of the *ras*-oncogene, but is also regulated by growth factors and tumour promoters (Garbisa *et al.*, 1987; Wilhelm *et al.*, 1989; Brown *et al.*, 1990).

Cathepsins L and H may also be instrumental in degradation of the basement membrane by virtue of their strong hydrolytic activity against type IV collagenase, laminin and fibronectin (Baricos *et al.*, 1988; Maciewicz *et al.*, 1989; Guinec *et al.*, 1990). Interstitial fibrillar, bone and cartilage collagens, elastin, proteoglycan aggregates and link protein have also been shown to be degraded by cathepsin L (Kirschke *et al.*, 1982; Mason *et al.*, 1986; Maciewicz *et al.*, 1990; Nguyen *et al.*, 1990). In addition to lysosomal degradation of small phagocytosed collagen fragments liberated by collagenases, cathepsin L has also been found extracellularly where ECM degradation is initiated (Maciewicz *et al.*,

1989). The latent pro-cathepsin L may be secreted into an acidic microenvironment at the host: tumour interface, similar to the "extracellular lysosomes" demonstrated at the ECM attachment site of osteoclasts and macrophages (Baron *et al.*, 1985; Silver *et al.*, 1988). Secretion of the cathepsin L precursor, formerly known as MEP, by tumour virus transformed cell lines, has been shown to be the result of over-expression of the enzyme and reduced affinity for MPRs, which target enzymes to lysosomes (Gottesman, 1987; Mason *et al.*, 1986; 1987; Dong *et al.*, 1990). Increased cathepsin L synthesis is also transcriptionally regulated by tumour promoters, growth factors and the *ras*- and *fos*-oncogenes (Gottesman and Sobel, 1980; Nilsen-Hamilton *et al.*, 1981; Frick *et al.*, 1985; Denhardt *et al.*, 1987). Elevated levels of cathepsins L and H have also been demonstrated in metastatic colorectal tumours, melanomas and breast carcinomas, providing further evidence for the involvement of these cathepsins in tumour invasion (Maciewicz *et al.*, 1989; Sloane *et al.*, 1991; Gabrijelcic *et al.*, 1992).

The majority of these findings, showing a positive correlation between collagenolytic proteinase activity and tumour invasion, have been based on tumour cell culture studies, which only give an indication of the cells' propensity for proteinase production and secretion. These studies also give only a partial indication of the imbalance between proteinases and their endogenous inhibitors in facilitating the invasive process. Immunolocalisation studies using highly specific antibodies, and *in vitro* invasion model studies with specific inhibitory agents would give more direct evidence of the functional role of these enzymes in promoting tumour cell migration *in vivo*. This approach could also distinguish between a host or tumour cell source of the collagenolytic enzymes, since tumour tissue is often invaded by macrophages, which have been shown to contain both type IV collagenase and cathepsins L and H (Garbisa *et al.*, 1986; Bando *et al.*, 1986).

Only a limited number of immunolocalisation studies in tumour tissue, and *in vitro* invasion model studies have been reported for type IV collagenase. Immunostaining for type IV collagenase was demonstrated in invasive breast carcinomas and their resulting lymph node metastases (Barsky *et al.*, 1983; Monteagudo *et al.*, 1990) and, at the electron microscope level, in specific granules of PMNLs (Hibbs and Bainton, 1989). The invasion of tumour cells through a reconstituted basement membrane *in vitro* has been used by Reich *et al.* (1988) and Höyhtyä *et al.* (1990) to study the effect of inhibitory agents of type IV collagenase on invasion. Immunolocalisation studies of cathepsins L and H focussed mainly on their distribution in different non-malignant organs and lysosomal subpopulations (Rinne *et al.*, 1986; Katunuma and Komanimi, 1986), as well as pathologies such as muscular dystrophy and rheumatoid arthritis (Kominami *et al.*, 1987; Trabandt *et al.*, 1990). Plasma membrane localisation of cathepsin L has, however, been demonstrated in colon adenoma

and carcinoma cells, and co-localisation of cathepsin L and p21 in endosome-like cytoplasmic vesicles in *ras*-transformed cells (Maciewicz *et al.* 1989; Hiwasa *et al.*, 1991). Cooperation between cathepsin L and a metalloproteinase was shown to be required for penetration of a human amnion basement membrane by murine melanoma and mammary carcinoma cells (Yagel *et al.*, 1989).

Immunolocalisation of type IV collagenases and cathepsins L and H in human tumour tissue is, therefore, a fairly uncharted area, and complemented by *in vitro* invasion model studies, may provide a means of reaching the ultimate goals of the present study, i.e. elucidation of the relative contributions of the collagenolytic proteinases to tumour invasion, and consequently the development of means of inhibiting the invasive behaviour of tumour cells, and thus metastasis. However, a prerequisite for both techniques, namely antibodies with specific targeting, and/or selective immunoinhibitory properties had to first be provided.

The preparation of specific antibodies against type IV collagenase for immunolocalisation studies in human tumours, required the purification of type IV collagenase from a suitable human source, which was provided by PMNLs, isolated from the buffy coat layers from blood of regular blood donors. In contrast to the lengthy purification methods described before for type IV collagenase (Hibbs *et al.*, 1985; Collier *et al.*, 1988; Murphy *et al.*, 1989a), a markedly simplified procedure was developed in the present study, employing crude fractionation by TPP (Pike and Dennison, 1989a), followed by immunoaffinity chromatography using anti-type IV collagenase peptide antibodies (Coetzer *et al.*, 1991). The peptide selected for anti-type IV collagenase peptide antibody production was based on the primary structure of the 72 kDa form of the enzyme, the only type IV collagenase amino acid sequence known at the time this study was initiated, and the form of the enzyme implicated in tumour invasion (Collier *et al.*, 1988). This sequence showed very little homology with those of interstitial collagenase and stromelysin (Höyhtyä *et al.*, 1988), and the corresponding anti-peptide antibodies were successfully applied to the purification of type IV collagenase from PMNLs.

Despite reports of only low levels of the 72 kDa form relative to that of the 92 kDa type IV collagenase in PMNLs (Murphy *et al.*, 1989a,b), the results presented here indicate that these phagocytic cells may constitute an equally suitable source of the 72 kDa form of the enzyme. Since the 72 kDa enzyme, purified here, shares characteristics with that of the enzyme purified from metastatic murine melanoma cells (Liotta *et al.*, 1980; Höyhtyä *et al.*, 1988), it may represent the normal human equivalent of the murine tumour enzyme. The functional similarity between PMNLs, which extravasate from the circulation through the basement membrane of the capillary endothelium to enter tissue at an inflammatory site, and

invading tumour cells, gives further support to the potential targeting of the enzyme in tumour tissue by antibodies raised against the 72 kDa PMNL type IV collagenase.

The initial objective was to raise polyclonal antibodies against the purified type IV collagenase for use in immunolocalisation studies, but the necessity for this was obviated by the targeting specificity manifested by the anti-type IV collagenase peptide antibodies during the course of the enzyme purification procedure. These anti-peptide antibodies recognised both native 72 kDa type IV collagenase, as evidenced by the proteolytic activity retained by the immunoaffinity purified enzyme, and the SDS-denatured enzyme in Western blots. This holds much promise for immunocytochemical studies, where the antigen may be denatured to varying degrees by the tissue preparation and/or fixation procedures. However, the small size and linear nature of epitopes targeted by anti-peptide antibodies reduces the probability of denaturation of the epitope during tissue processing.

These anti-72 kDa type IV collagenase peptide antibodies did not recognise the 92 kDa form in Western blots of PMNL homogenates, despite this being the predominant type IV collagenase in PMNLs (Murphy *et al.*, 1989a,b), possibly because there is only 53% sequence homology between the target sequences in the two forms of type IV collagenase (Wilhelm *et al.*, 1989). The degree of homology between the two peptides allowed a small degree of cross-reactivity, in that a very small proportion of the 92 kDa form became loosely bound to the immunoaffinity matrix. However, since immunolabelling requires high affinity antibodies, the 92 kDa form of the enzyme would most probably not be detected in tissue sections, allowing the exclusive recognition of the 72 kDa form of the enzyme by these anti-peptide antibodies.

In order to discriminate between the role of the two forms of type IV collagenase in tumour invasion, the 72 kDa specific antibodies developed in the present study may be complemented by anti-peptide antibodies specifically recognising the 92 kDa type IV collagenase, which could be produced against a sequence from the 54 amino acid residue domain unique to the 92 kDa enzyme form. The enzyme purification approach developed in the present study may then also be applied to that of the 92 kDa form, to allow enzymological studies.

In contrast to the sequence spanning the active site His-residue in cysteine proteinases (Wiederanders *et al.*, 1992), the zinc-binding region in the active site of metalloproteinases is conserved and anti-peptide antibodies directed against this sequence would probably only provide a general metalloproteinase inhibitor. This may, however, find application in *in vitro* tumour invasion studies where the relative contribution by the different classes of

proteinases needs to be established. Following identification of the active-site sequence targeted by immunoinhibitory poly- or monoclonal anti-type IV collagenase antibodies, such as those raised by Höyhtyä *et al.* (1990), anti-peptide antibodies with superior immunoinhibitory specificity may be produced for use in tumour invasion studies and ultimately for immunotherapy to block the invasive process.

Following the successful production of specific anti-72 kDa type IV collagenase peptide antibodies, which promise to fill an important niche in addressing key questions regarding collagenolytic degradation of the basement membrane, during tumourigenic and other pathological invasive processes, the focus was moved to the production of immunoinhibitory anti-peptide antibodies for a study of, and possible blocking of collagenolysis of the ECM by cathepsins L and H in these pathologies. In an endeavour to identify the exact minimal epitopes in the active site of cathepsin L, which could elicit immunoinhibitory anti-peptide antibodies with a lower effective dose than those raised previously (Pike 1990), the primary structure, and sufficient quantities of cathepsin L to allow exploratory work, were needed. Furthermore, with an ultimate application in human malignancies envisaged, human cathepsin L was required. In the light of the difficulty in obtaining HIV-negative human liver and kidney tissue, the expression of enzymatically active recombinant human cathepsin L in *E. coli* (Smith and Gottesman, 1989), could potentially provide an alternative source of the enzyme. However, the enzyme is not secreted, and purification from bacterial lysates, which requires solubilisation and renaturation of insoluble recombinant enzyme aggregates, is not conducive to very high recoveries. Alternative expression systems, such as the high yield baculovirus expression system which produces recombinant protein in cultures of insect cells (Page and Murphy, 1990), are therefore, now being developed in our laboratory. In this study, however, sheep livers were used as a source of cathepsin L, since Mason (1986) found that sheep cathepsin L represents, by virtue of enzymological and immunological homology, a suitable model for human cathepsin L. Sheep livers were found to be a most convenient and readily available tissue source, a situation possibly unique to South Africa, Australia and New Zealand. Indications are that the opposite situation probably prevails in the Northen Hemisphere, and consequently the amino acid sequence of sheep cathepsin L was not available at the outset of this study. The unique availability of sheep liver was thus further exploited, in that the first goal of this part of the present study became the purification of sufficient quantities of sheep cathepsin L for amino acid sequencing.

The rapid, high yield method of Pike and Dennison (1989b), developed in this laboratory, was employed to this end. This procedure allows the purification of single-chain cathepsin L, which contrasts with the two-chain form isolated from sheep liver (Mason

1986) and human liver and kidney (Mason *et al.*, 1985; Ritonja *et al.*, 1988), and which may be an artifact of the lengthy purification methods used. A further proportion of single-chain cathepsin L was found to be uniquely complexed to an endogenous cysteine proteinase inhibitor, a cystatin, in such a way that proteolytic activity is retained (Pike, 1990). In the present study the cystatin was unequivocally identified as stefin B by amino acid sequencing. Studies undertaken to improve the yield of free enzyme for sequencing purposes, revealed that cathepsin L was bound in two modalities to stefin B, i.e. in the established tight-binding, non-covalent, inhibitory way, described for cysteine proteinases and cystatins (Anastasi *et al.*, 1983; Nicklin and Barrett, 1984), and in a novel, covalent, non-inhibiting manner. It was also shown in the present study that similar proteolytically active, covalent complexes form spontaneously from purified, active constituents in a pH-dependent manner at pH 5.5 and above (Pike *et al.*, 1992). This sharp pH dependence of complex-formation was exploited in significantly improving the yield of free cathepsin L by maintaining the pH of liver homogenates at 5.0. In this way sufficient material was obtained to enable the determination of the primary structure. The amino acid sequence provided confirmatory evidence of the single-chain nature of the cathepsin L isolated in this laboratory. Subsequent studies on the mode of interaction between cathepsin L and stefin B suggested that a single-chain form of cathepsin L may be a prerequisite for formation of the novel, covalent complexes.

Stefin B could possibly become covalently linked to cathepsin L by thiol-disulfide interchange between a characteristic free, reactive Cys-residue in the N-terminal trunk of the inhibitor and the labile intramolecular disulfide bridges in papain-like cysteine proteinases, usually involved in thiol-disulfide interchange during enzyme activation (Brocklehurst and Kierstan, 1973). Docking of the N-terminal trunk of stefin B in the active site groove of cathepsin L during their usual inhibitory interaction, may, by analogy with a similar association between papain and stefin B, elucidated by X-ray crystallography (Stubbs *et al.*, 1990), conceivably result in optimal orientation of the Cys-residue for thiol-disulfide interchange with any one of the three disulfide bridges in cathepsin L. Thiol-disulfide interchange between stefin B and the disulfide bridge which stabilises the cathepsin L active site (Smith and Gottesman, 1989), would in the case of two-chain cathepsin L lead to the loss of the light chain, and consequently a loss of activity. Since most studies have reported the purification of two-chain cathepsin L, this may explain why these novel covalent complexes have not been described before.

Further indications are that a proportion of the covalent complex may comprise thioester bonds, possibly between a stefin B glutaminyl γ-carbonyl and a cathepsin L sulfhydryl group. These suggested modes of interaction between cathepsin L and stefin B

are merely speculative and await elucidation by X-ray crystallography. However, evidence for binding of stefin B to cathepsin L in a manner distinct from the usual inhibiting mode, provides a possible mechanism by which cathepsin L could be active despite the presence of inhibitory amounts of stefin B. Although the presence of these novel, covalent complexes has not been demonstrated *in vivo*, and their physiological role remains speculative, factors present in liver homogenates promoting disulfide and thioester bond formation (Iijima *et al.*, 1984; Hillson *et al.*, 1984), may be of particular relevance to covalent cathepsin L/stefin B complex formation in pathologies where the integrity of the subcellular compartments have been breached. Initially inappropriate cathepsin L activity may be controlled by stefin B, since the K_i-value for inhibition measured *in vitro* suggests a functional role *in vivo*, both intra- and extracellularly (Bieth, 1980; Barrett *et al.*, 1986). Overexpression and secretion of cathepsin L may subsequently cause an imbalance with cysteine proteinase inhibitors, as illustrated in breast tumours (Lah *et al.*, 1992), thus favouring proteolysis. If, additionally, the formation of covalent, proteolytically active cathepsin L/stefin B complexes are promoted over time, the initial level of control would be further diminished. Since, bound in this novel way, stefin B may act as an activator, ECM degradation could be promoted and tumour invasion thereby facilitated.

These findings in the sheep system, that proteolytically active, covalent cathepsin L/stefin B complexes could conceivably form at extra-lysosomal pH-values, following dissolution of the subcellular compartments, together with their stability and increased activity at neutral pH (Dennison *et al.*, 1992), suggest that covalent complexes may be involved in extracellular degradation of ECM components during tumour invasion. Their potential involvement in tumour invasion would, however, be strengthened if similar complexes could be demonstrated in human tissue. The only fairly readily available human tissue, spleen, was, however, not suitable, since both human and sheep spleen cathepsin L were complexed to stefin B only in the usual, non-covalent, inhibiting manner in this tissue (Pike, 1990). Novel, covalent complex formation appears to be an organ specific phenomenon, since they were purified from sheep liver and kidney homogenates in the present study. Liver tissue from a higher primate, the baboon, presented a most suitable compromise, since liver was used previously to purify human cathepsin L (Mason *et al.*, 1985).

Not only were proteolytically active, covalent cathepsin L/stefin B complexes isolated form baboon liver homogenates, but this tissue also yielded free single-chain cathepsin L. Both the free and stefin B-complexed cathepsin L manifested similar neutral pH-optima, with substantial activity retained at physiological pH, consistent with the corresponding forms of the sheep enzyme. These observations suggest, firstly, that the phenomenon of novel,

covalent, proteolytically active complexes of cathepsin L and stefin B is not resticted to sheep, secondly, that a single-chain form of the enzyme may indeed be required for covalent complex formation and, thirdly, that extrapolations may most probably be made between cathepsin L from sheep and higher primates. This was further underscored by the subsequent purification of single-chain cathepsin L and proteolytically active, covalent cathepsin L/stefin B complexes from human liver, and the demonstration of 85% sequence homology between human and sheep cathepsin L. Cathepsin L may, therefore, either by virtue of its single-chain nature, or complexed in this novel manner to stefin B, initiate degradation of the ECM in the extracellular milieu, to facilitate tumour cell invasion, or tissue destruction in pathologies such as rheumatoid arthritis or emphysema. Both cathepsin L and stefin B have indeed been shown to be secreted by macrophages and tumour cells and stefin B was also found extracellularly in urine and blood plasma (Hopsu-Harvu *et al.*, 1984; Abrahamson *et al.*, 1986; Reilly *et al.*, 1989; Kolár *et al.*, 1989).

Whereas powerful cathepsin L activity on components of the ECM is well-documented (Kirschke *et al.*, 1982; Mason *et al.*, 1986; Maciewicz *et al.*, 1989; Nguyen *et al.*, 1990), the question persists as to how a cysteine proteinase with a typical acidic pH-optimum could be active extracellularly, or alternatively when bound to the plasma membrane (Mason *et al.*, 1985; Maciewicz *et al.*, 1989). The proposed acidic pericellular microenvironment demonstrated for osteoclasts and macrophages (Baron *et al.*, 1985; Silver *et al.*, 1988) and proposed for the tumour cell: host interface, has not been demonstrated *in vivo*. The answer may thus be found in single-chain cathepsin L being the physiological relevant form of the enzyme, displaying stability and increased activity under these conditions, either *per se* or complexed to stefin B, in the novel manner described here.

Although results presented here suggest the possible involvement of these proteolytically active, covalent stefin B/cathepsin L complexes in tumour invasion, the question arises as to what their normal physiological function might be, if any. Binding of proteinase inhibitors to their target proteinases do not only regulate proteolytic activity, but also act as signals for receptor interaction or clearance from the extra-or intravascular space (Bode and Huber, 1992). The retention of proteolytic activity, following stefin B binding to cathepsin L, may provide a mechanism by which cathepsin L/stefin B complexes may be recognised by the general proteinase inhibitor, α_2M which functions as a molecular trap of active proteinases (Barrett and Starkey, 1973). The α_2M-proteinase complexes thus formed, usually bind to α_2M receptors on hepatocytes, monocyte/macrophages and fibroblasts and are cleared from circulation (Sottrup-Jensen and Birkedal-Hansen, 1989). An exposed peptide stretch, the bait region, is presented by α_2M to proteinases and, upon cleavage, α_2M undergoes conformational changes, the internal thioester bond is activated and a

cysteinyl sulfhydryl becomes available for covalent binding to the activating proteinase (Sottrup-Jensen, 1989). Bait region cleavage also results in exposure of α_2M binding sites recognised by receptors on several cell types (Kaplan and Nielsen, 1979).

Three possible cleavage sites for papain have been identified in the α_2M bait region (Travis and Salvesen, 1983), while direct evidence for covalent binding of cathepsin L to α_2M, following bait region cleavage, was provided by Mason (1989). Furthermore, proteinases which show a broad substrate specificity, such as cathepsin L, react more readily with α_2M (Sottrup-Jensen and Birkedal-Hansen, 1989). Although proteinases bound to α_2M usually only retain activity against low M_r synthetic substrates and inhibitors (Sottrup-Jensen and Birkedal-Hansen, 1989), the M_r 21 000 SBTI was not occluded from α_2M-trypsin complexes (Travis and Salvesen, 1983). Binding of cathepsin L/stefin B complexes could thus conceivably be accommodated. Complexing of cathepsin L to stefin B may function in carrying cathepsin L from the extravascular to the intravascular space, as was suggested for the cathepsin L/cystatin C and cathepsin L/L-kininogen complexes in human liver (Pagano *et al.*, 1984). These active complexes may then bind to α_2M and the α_2M-cathepsin L/stefin B complexes may be endocytosed by macrophages, following binding to α_2M-receptors on these cells.

Immunolocalisation studies, using antibodies which specifically recognise the covalent complex, may be used to substantiate the proposed physiological functions of the novel covalent complexes under normal and/or pathological conditions. In this regard, anti-peptide antibodies against a suitable peptide spanning the covalent link region may recognise the complex, but not the complexing moieties, such as those anti-peptide antibodies reactive with ubiquitin/histone complexes, but not the free constituents (Dr. Sylvianne Muller, CNRS, Strasbourg, personal communication). Alternatively, the same species from which the mixture of covalent and non-covalent cathepsin L/stefin B complexes had been isolated, may be immunised with this mixture of complexes. Antibodies may then be produced exclusively against the immunologically foreign covalent complex. The immunogenicity of the covalent complex may, therefore, give an indication whether such complexes are expressed physiologically and consequently regarded by the immune system as being self components.

Anti-cathepsin L/stefin B complex antibodies, together with the polyclonal chicken anti-cathepsin L and rabbit anti-stefin B antibodies produced in the present study, may provide suitable probes for immunolocalisation of free and stefin B-complexed cathepsin L as well as stefin B in tumour tissue, and thereby possibly elucidate their relative contributions to the complex process of ECM degradation which facilitates tumour cell

migration. Although it was shown in the present study that there is a high degree of sequence homology between both human and sheep cathepsin L and stefin B, targeting by these antibodies in human tumour tissue would have to rely on their cross-reactivity across species. This is not ideal since immunolabelling requires high affinity antibodies. Anti-peptide antibodies directed against a human cathepsin sequence might, therefore, better provide the required immunospecific reagents. The specificity and high titre of the chicken anti-human cathepsin L peptide antibodies produced in this study, thus show some promise for immunolocalisation studies.

The novel targeting characteristics of the polyclonal chicken anti-sheep cathepsin L antibodies, which endowed them with immunoinhibitory properties, seem to be derived from their ability to target a peptide located in the active site of cathepsin L (Coetzer *et al.*, 1992). The evolutionary distance between the avian and mammalian immune systems appears to result in the recognition of different epitopes on mammalian immunogens, evidenced by the targeting specificity of noninhibitory polyclonal rabbit anti-sheep cathepsin L antibodies. Versatile chicken anti-human cathepsin H peptide antibodies with superior immunoinhibitory and targeting properties than the corresponding rabbit anti-peptide antibodies have also been raised. Similarly to the immunoinhibitory chicken anti-human cathepsin L peptide antibodies, these anti-peptide antibodies may be used as specific inhibitory agents in *in vivo* tumour invasion model studies, or to quantify the respective enzymes in tumour tissue homogenates by ELISA, or for immunolocalisation studies and for immunoaffinity purification of the corresponding enzymes. This study has, therefore, firstly, confirmed the finding by Polson *et al.* (1980) that chickens provide a most suitable, high yield system for the production of antibodies, which are readily extractable from the egg yolks, and, secondly, expanded on the range of immunogenic antigens for chickens, by showing that antibodies could be elicited against a free M_r 1 300 cathepsin H peptide. Additionally, the chicken antibodies were generally of higher titre than the corresponding rabbit antibodies, which would ensure antigen targeting at lower concentrations, hence avoiding non-specific protein-protein interaction. Chicken antibodies may, therefore, provide the preferred immunological tools for a study of collagenolytic proteinases in ECM degradative pathologies.

In addition to identifying the precise immunoinhibitory targets of the polyclonal chicken anti-cathepsin L antibodies for the production of more effective immunoinhibitory anti-peptide antibodies, the present polyclonal and anti-peptide antibodies may be manipulated to increase their sensitivity as immunoinhibitory reagents for use in *in vitro* tumour invasion studies and in tumour immunotherapy. Affinity purification of cathepsin L specific antibodies could result in preparations of immunoinhibitory anti-peptide antibodies

with specificity approaching that of monoclonal antibodies. These immunoinhibitory antibody enriched preparations may then be used to prepare progressively smaller active site binding units in an attempt to construct the most effective immunoinhibitory agent. Selective cleavage by pepsin and papain would yield bivalent [$F(ab')_2$] and monovalent antibody binding fragments (Fab), respectively, which would retain the complete binding specificity of both immunoglobulin heavy and light chain complementarity determining regions (CDRs). Higher antigen binding specificity may be provided by smaller fragments, which either consist of only the variable domain of the heavy and light chains (Fv fragments), or the variable domain of the heavy chain alone (V_H-domains) (Ward *et al.*, 1989).

Effective immunoinhibition may, however, require binding by both heavy and light chain CDRs to active site residues, and recombinant single chain variable regions (scFvs) may provide an immunoinhibitory agent with the required binding affinity and size. These scFvs consist of light and heavy chain antibody variable regions, linked by a 15-25 residue peptide from the C-terminus of the one chain, to the N-terminus of the other polypeptide chain (Huston *et al.*, 1988). Antibody binding would be preserved since the framework variable regions of different antibody molecules fold into almost identical β-sheet domains with the hypervariable sequences in exposed positions at the ends of the β-strands (Roitt *et al.*, 1989a). Variable region cDNAs may be cloned from hybridoma cells and used to construct scFvs genes which include appropriate restriction sites to insert oligonucleotides coding for linker sequences, and may be expressed in *E. coli* (Winter and Milstein, 1991; Bird and Walker, 1991). Immunoinhibition may be improved even further by introducing subtle variations in the variable domain pairings which may lead to the production of higher affinity scFvs. Phage display libraries are constructed in which scFv genes, coding for different combinations of variable heavy and light immunoglobulin chains, are expressed on the surface of a bacteriophage, fused with a minor coat protein of the phage (Clackson *et al.*, 1991). The scFvs with the highest affinity may then be selected by fractionation of the pool of phages by chromatography on a cathepsin L affinity column.

The use of whole antibodies for tumour therapy may, however, have advantages over these small antigen binding antibody fragments in that they are more slowly cleared from the bloodstream and tissues, thereby potentially showing an enhanced effectiveness in reaching the tumour invasion front or areas of overexpression of cathepsins associated with disease states. The effector functions of the Fc region of the antibody molecules, such as complement protein and phagocyte binding, would be essential to activate the complement cascade, which would mediate the inflammatory response and, also recruit macrophages and PMNLs to the site of tissue destruction to stimulate further cell-mediated responses. Monoclonal anti-human cathepsins L and H peptide antibodies, which have proved to have

immunoinhibitory potential, may provide more specific therapeutic reagents in that antibody producing clones may be selected which show the highest level of immunoinhibition. Since the product of each clone will have a single specificity this approach would ensure the use of significantly lower doses of antibody to effect immunoinhibition. Both xenogeneic mouse monoclonal antibodies and the chicken polyclonal and anti-peptide immunoinhibitory antibodies produced in this study would not be directly suitable for tumour immunotherapy in humans in their present form, but the CDRs of these antibodies may be inserted into human variable region genes by site directed mutagenesis to form humanised antibodies (Jones *et al.*, 1986).

This study endeavoured to make progress towards the ultimate goals of elucidating the relative contributions by the different collagenolytic proteinases, type IV collagenase and cathepsins L and H, to ECM degradation and consequently tumour invasion, and to develop suitable inhibitory agents to block this process. Progress in this direction was made by showing that single-chain cathepsin L might be the physiologically relevant form of the enzyme in higher primates, on the strength of the increased activity and the stability of the enzyme at neutral pH, which may increase its potential to participate in the collagenolysis of the ECM that is the hallmark of tumour invasion. Furthermore, the discovery that the proteolytically active complexes between cathepsin L and its natural inhibitor, stefin B, form spontaneously by covalent interaction under physiological conditions provides a possible mechanism by which cathepsin L might retain activity extracellularly to facilitate invasion. This probability was underscored by isolating similar complexes, manifesting stability and higher activity at neutral pH, from liver tissue homogenates of higher primates, especially man. These studies are expected to be complemented by immunolocalisation and *in vitro* tumour invasion model studies using the panel of highly specific and versatile antibodies against type IV collagenase and cathepsins L and H produced in the present study. Also, the inhibitory anti-peptide antibodies may open up new possibilities for therapeutic intervention aimed at reducing or preventing tumour invasion and metastatic spread.

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

Specific recognition and inhibition of the enzymes

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

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

Introduction

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

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

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

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

Materials and methods

Reagents

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

Selection of peptides

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

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

TABLE I

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

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

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

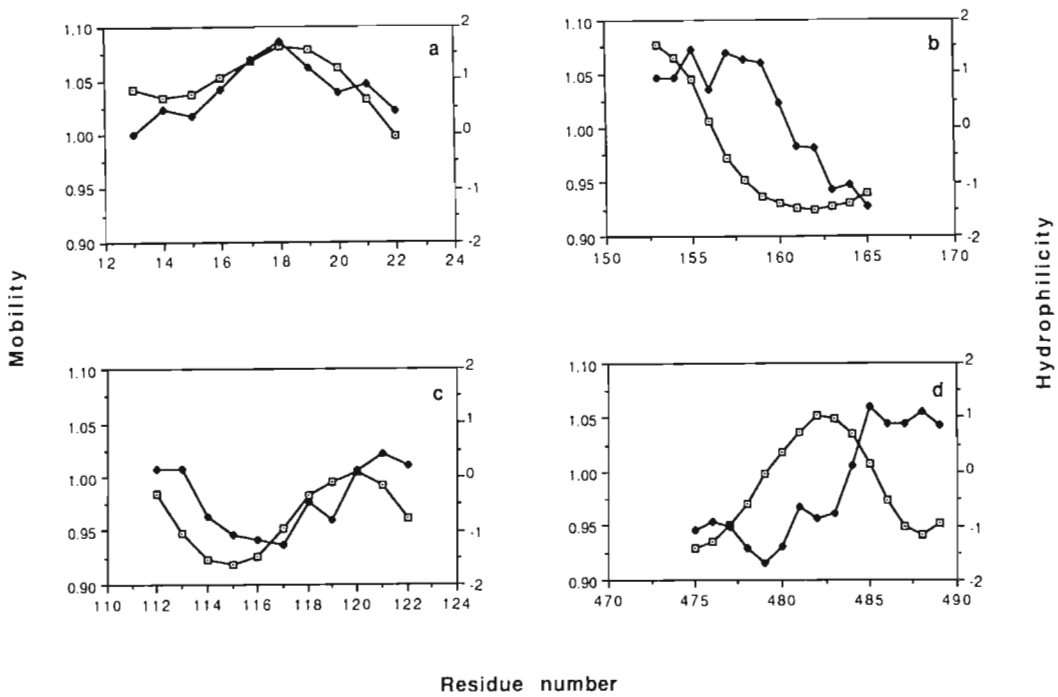

Fig. 1. Hydrophilicity and segmental mobility profiles of the selected peptides. ◆, hydrophilicity, calculated according to Hopp and Woods (1981, 1983); □, segmental mobility, calculated according to Westhof et al., (1986). Profiles indicated are for the peptides: (*a*) B13–22; (*b*) L153–165; (*c*)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

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

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

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

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

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

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

Synthesis of peptides

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

Conjugation

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

TABLE II

INOCULATION PROTOCOL

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

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

Inoculation protocol

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

ELISA for anti-peptide antibodies

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

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

ELISA for immobilized enzyme

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

Competition ELISA for native enzyme

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

Removal of anti-KLH antibodies

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

Immunoblotting

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

Immunoinhibition assays

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

Results

Anti-peptide antibody production

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

Recognition of enzymes coated to ELISA plates

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

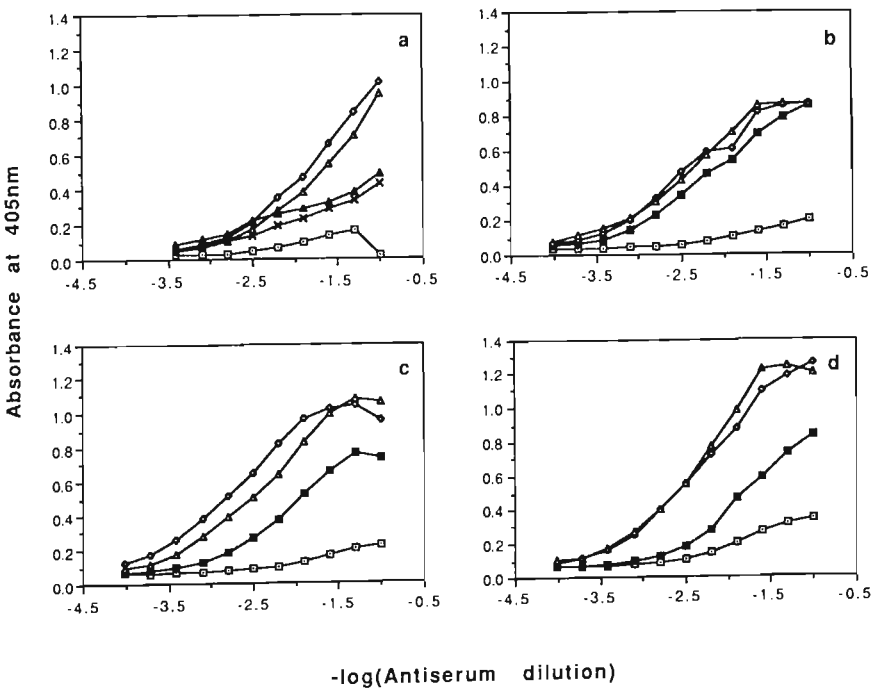

Fig. 2. Progress of immunisation with peptide conjugates as determined by ELISA. Peptides were coated to microtitre plates, (*a*) B13–22; (*b*) L153–165; (*c*) D112–122; and (*d*) COL467–490 and incubated with serial two-fold dilutions of antisera collected after 3 (■), 8 (◇), 12 (△), 30 (▲) and 32 weeks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

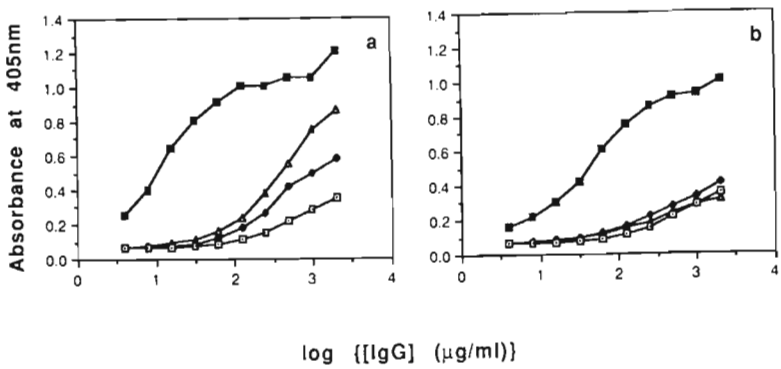

Fig. 3. ELISA of binding of anti-peptide antibodies to whole immobilised cathepsin L. Cross-reaction of anti-L153–165 antibodies with human (◆) and sheep (△) cathepsin L, and peptide L153–165 (■). Normal rabbit IgG (□). Experimental procedure as in Fig. 2 and in the materials and methods section. (*a*)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

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

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

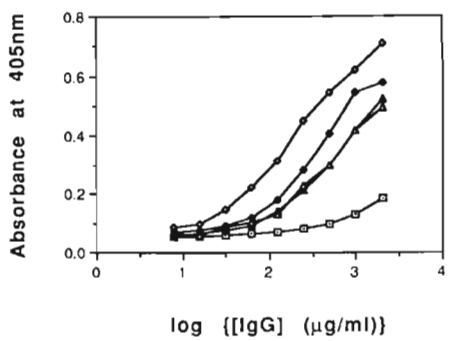

Fig. 4. ELISA of binding of anti-peptide antibodies to whole immobilised cathepsin D. Cross-reaction of anti-D112–122 antibodies with human (◆), porcine (△) and bovine (▲) cathepsin D, and peptide D112–122 (◇). Normal rabbit IgG (□). Experimental procedure as in Fig. 2 and in the materials and methods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

Specificity of anti-peptide antibodies: Western blot analyses

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

Recognition of native enzymes

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

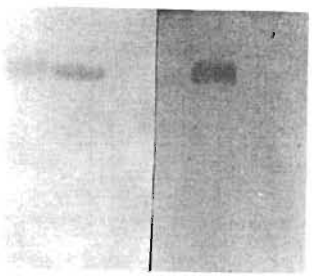

Fig. 5. Targeting of cathepsin L and type IV collagenase by anti-peptide antibodies on Western blots. *A*: samples ((1) sheep cathepsin L; (2) human cathepsin L; (3) human cathepsin B) were subjected to 12.5% reducing SDS-PAGE, electroblotted onto nitrocellulose and then incubated with anti-KLH-purified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

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

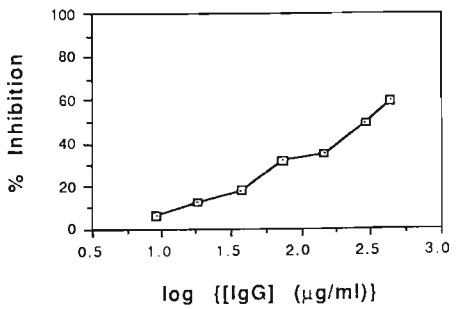

Fig. 6. Competition ELISA for native cathepsin D. The ability of cathepsin D to inhibit the binding of anti-D112–122 antibodies to immobilised D112–122 was measured by pre-incubating various amounts of IgG with different levels of enzyme before transfer of the incubation mixture to peptide coated plates.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

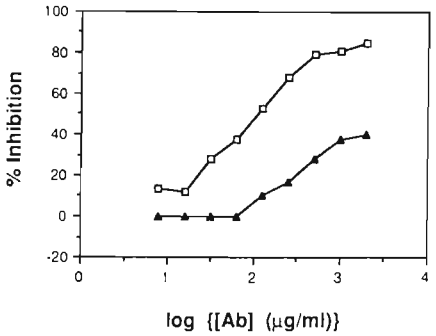

Fig. 7. Immunoinhibition of human and sheep cathepsin L by anti-L153–165 antibodies. Stopped time assays were carried out using human (□) and sheep (△) cathepsin L as described in the materials and methods section and the percentage inhibition calculated relative to control assays with normal rabbit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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements

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Proteolytically Active Complexes of Cathepsin L and a Cysteine Proteinase Inhibitor; Purification and Demonstration of Their Formation *in Vitro*

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Proteolytically active complexes of the proteinase cathepsin L, with an endogenous inhibitor of cysteine proteinases, were purified from sheep liver. The complexes were active against the synthetic substrate Z-Phe–Arg–NHMec and also the proteins azocasein and gelatin. The composition of the complexes was demonstrated by Western blotting, after reducing and nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis with monospecific antibodies raised against purified sheep liver cathepsin L and purified sheep liver cysteine proteinase inhibitor (probably stefin B). Similar complexes could be formed *in vitro*, by coincubation of purified sheep liver cathepsin L with the purified sheep liver cystatin at a pH of 5.5 or higher. © 1992 Academic Press, Inc.

Cathepsin L (E.C. 3.4.22.15) is an endoproteinase, most commonly found in lysosomes, and has been isolated in single (1) and two-chain (2) M_r 26,000–29,000 forms, active against both protein substrates such as azocasein and the synthetic substrate Z-Phe–Arg–NHMec.[2] Cystatins, which are found in extralysosomal locations, are commonly known as cysteine proteinase inhibitors that form tight binding, noncovalent complexes with these proteinases (3). The interactions of cystatins with cathepsin L, and other proteinases, are of interest for several reasons, including their possible relevance to pathologies such as invasive cancer. In such cases, proteinases, secreted by transformed cells or released as a consequence of tissue destruction, would be expected to encounter the cystatins, which may form a primary defense against proteolysis of tissue.

We have recently reported on the isolation of cathepsin L from sheep liver in a single-chain form (4). As reported here, we have subsequently found that a further proportion of this sheep liver enzyme could be purified in the form of complexes with a sheep liver cystatin. These complexes were found to be active against azocasein and gelatin, as well as Z-Phe–Arg–NHMec. This suggests that the cystatin is apparently able to interact with cathepsin L in a noninhibiting mode, in addition to the well-established inhibiting mode.

The noninhibiting interaction may represent a possible mechanism whereby cathepsin L may be active extracellularly, despite the presence of cystatins, in situations such as tumor invasion in which the enzyme has been strongly implicated (5, 6). A preliminary report on these findings has been given elsewhere (7).

EXPERIMENTAL PROCEDURES

Materials. Z-Phe–Arg–NHMec and Z-Arg–Arg–NHMec were purchased from Cambridge Research Biochemicals, United Kingdom. Azocasein, DTT, E-64, leupeptin, 2× crystallized papain, pepstatin, PMSF, SBTI, and M_r 12,000 cut-off dialysis tubing were from the Sigma Chemical Co., St. Louis, Missouri. Z-Phe–Phe–CHN_2 was from Enzyme Systems Products, Livermore, California. Amido black 10B and Coomassie blue R-250 dyes were from Merck.

Purification of free and cystatin-complexed cathepsin L. The isolation procedure was a modification of that previously described for sheep liver cathepsin L (4). The procedure involves homogenization of thawed liver, autolysis at pH 4.2, three-phase partitioning, and chromatography on S-Sepharose at pH 5.5. The active fraction from S-Sepharose chromatography at pH 5.5 was concentrated by dialysis against sucrose and chromatographed on Sephadex G-75, instead of being rechromatographed on S-Sepharose at pH 4.5, as described previously (4). Peaks with activity against azocasein and Z-Phe–Arg–NHMec were collected for further study.

Enzyme and protein assays. Fractions were tested for activity against the synthetic substrates Z-Phe–Arg–NHMec and Z-Arg–Arg–NHMec, and against azocasein (8). For samples with low levels of azocasein-digesting activity, the incubation time was extended to 2 h.

Inhibition assays, to characterize the activity, were carried out at the concentrations previously described (9, 10), by incubating samples with

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[2] Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; IgG, immunoglobulin; NHMec, 7-(4-methyl)coumarylamide; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; Z-, carbobenzoxy.

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the respective inhibitors for 15 min, before assaying with Z-Phe–Arg–NHMec. Activity against Z-Arg–Arg–NHMec was also measured, to eliminate cathepsin B-like activity.

The inhibitor E-64 was used for active site titration of the different forms of the sheep liver cathepsin L (8). Inhibition of cathepsin L, and isolated cathepsin L/cystatin complex, by the sheep liver cystatin (0–100 ng) was carried out at pH 6.0 with 50 nM active cathepsin L or complex, in the presence of 2 mM DTT (11). Protein concentration was determined using the Bradford dye binding method as modified by Read and Northcote (12).

Isolation of sheep liver cystatin. Sheep liver cystatins were isolated, through the CM-papain stage, as described for human liver (13), and further fractionated on Sephadex G-75, in the presence of 1 mM DTT. Fractions were assayed, using Z-Phe–Arg–NHMec, for their inhibitory activity against 10 μM papain; the active fraction, at M_r 12,000–14,000, was collected and concentrated by dialysis against sucrose in M_r 12,000 cut-off tubing. The concentrate was used to raise anti-cystatin antibodies, as described below.

Characterization of sheep liver cystatin. The cystatin fraction isolated from sheep liver, through the CM-papain stage, was used to characterize the types of cystatin present. This fraction was desalted on Sephadex G-25 (2.5 × 40 cm), previously equilibrated in 20 mM ethanolamine, pH 9.5, and applied to Q-Sepharose (1 × 25 cm), equilibrated with the same buffer. Subfractionation on Q-Sepharose was effected as described for cystatins from human urine (14). Bound cystatin fractions were eluted with two gradients: one from 0–0.2 M NaCl, followed by another from 0.2–1 M NaCl, both in 5 column volumes of ethanolamine buffer. The fractions which manifested inhibitory activity against papain were pooled, concentrated against sucrose in M_r 12,000 cut-off dialysis tubing, and analyzed by reducing and nonreducing SDS–PAGE, and by continuous PAGE.

Formation of a cathepsin L/cystatin complex in vitro. Sheep liver cathepsin L and sheep liver cystatin, purified as described above, were incubated together, at a 1:2 molar ratio, under defined conditions, at 37°C, for specified times. Formation of the complex was detected by nonreducing SDS–PAGE in a gelatin-containing substrate gel, as described below.

Antibodies. Chicken anti-sheep cathepsin L (IgY) antibodies were raised against free sheep liver cathepsin L, isolated as described above. IgY antibodies were purified from egg yolks by precipitation with polyethylene glycol (15). Rabbit anti-sheep liver cystatin antibodies were raised against the purified cystatin and IgG was purified from serum by precipitation with 14% (w/v) polyethylene glycol (16). Antibodies were characterized by Western blotting against crude and purified cathepsin L and cystatin fractions. The crude fraction, containing both cathepsin L and cystatins, was obtained by a modification of the catbepsin L purification procedure in which all of the proteins bound to S-Sepharose were eluted with 1 M NaCl.

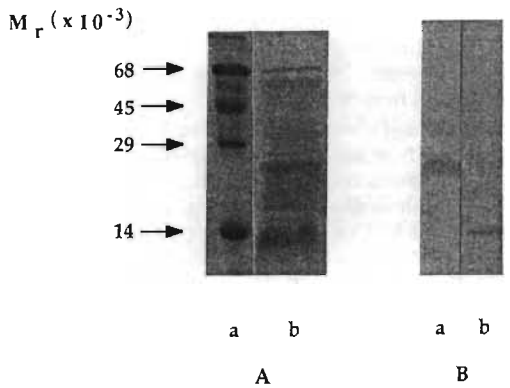

FIG. 1. Specificity of anti-cathepsin L and anti-cystatin antibodies. (A) Reducing SDS–PAGE of (a) MW markers and (b) the crude fraction eluted from S-Sepharose (25 μg). (B) Western blots of the crude fraction eluted from S-Sepharose, probed with (a) chicken anti-cathepsin L IgY (5 μg/ml) and (b) rabbit anti-cystatin IgG (50 μg/ml).

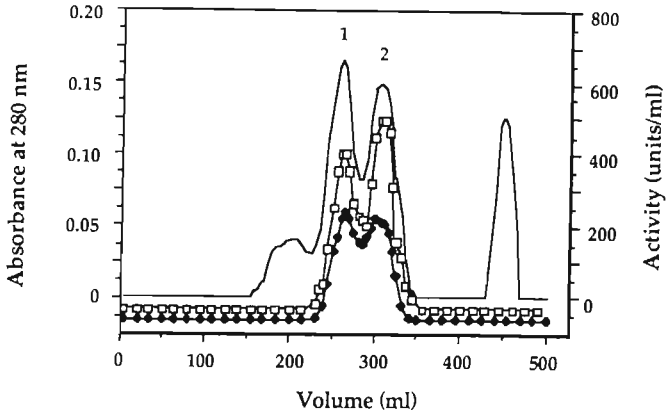

FIG. 2. Molecular exclusion chromatography of the pH 5.5 S-Sepharose fraction on Sephadex G-75. Column, 2.5 × 87.5 cm (430-ml bed volume); buffer, 20 mM Na-acetate, pH 5.5, containing 1 mM Na_2-EDTA and 200 mM NaCl; flow rate, 25 ml/h (5 cm/h); and fractions, 5 ml (12 min). Void volume was 150 ml. (—) A_{280}; (♦) enzyme activity (units/ml) from the assay against azocasein; and (□) enzyme activity (units/ml) from the assay against Z-Phe–Arg–NHMec. 1, cystatin-complexed cathepsin L; 2, free cathepsin L.

Electrophoresis and Western blotting. SDS–PAGE was carried out according to Laemmli (17). For nonreducing SDS–PAGE, the sample was incubated at 37°C, unless otherwise stated, in treatment buffer, without reducing agent. Gels were stained in 0.125% Coomassie blue R-250 in 50% methanol:10% acetic acid and destained in 50% methanol: 10% acetic acid, followed by 5% methanol:7% acetic acid. Protein fractions were electroblotted onto nitrocellulose and detected as described previously (18).

Gelatin substrate SDS–PAGE (19) was carried out, on 12.5% gels containing 0.1% (w/v) gelatin, followed by incubation, for 3 h at 37°C, in 100 mM acetate buffer, pH 5.0, containing 1 mM Na_2-EDTA, 1 μg/ml pepstatin, and 40 mM cysteine. Gels were stained in 0.1% amido black as described by Heussen and Dowdle (19). Cathepsin L activity was demonstrated by extinguishing the activity with either Z-Phe–Phe–CHN_2 or E-64, added either before electrophoresis or in the assay buffer used after electrophoresis. The reversible inhibitor, leupeptin, was used similarly but only after electrophoresis. Continuous PAGE was carried out using the Laemmli system (17) except that no stacking gel was cast.

RESULTS

Characterization of Anti-Cathepsin L and Anti-Cystatin Antibodies

The anti-cathepsin L antibody preparation was specific for cathepsin L and did not target any other components in the crude extract, including cystatin (Fig. 1). Similarly, the anti-cystatin antibody preparation was specific for cystatin and did not target any other components of the crude extract, including cathepsin L (Fig. 1).

Isolation and Characterization of Free and Cystatin-Complexed Cathepsin L

Sephadex G-75 chromatography (Fig. 2) revealed that the active peak from ion-exchange chromatography, at

TABLE I

The Effect of Inhibitors and the Absence of DTT on Free and Cystatin-Complexed Forms of Sheep Liver Cathepsin L

Inhibitor	Final concentration (μM)	% activity	
		Complexed	Free
Leupeptin	1	2	1
Z-Phe-Phe-CHN_2	1	2	2.3
E-64	1	3	6
SBTI	1	80	71
PMSF	250	95	90
-DTT	—	9	5

pH 5.5, could be resolved into two proteolytically active peaks, with M_r 37,000 and 26,000. Both peaks had activity against azocasein and Z-Phe-Arg-NHMec (Fig. 2), but were not active against Z-Arg-Arg-NHMec (results not shown). The activity of both was characterized as being that of cathepsin L, on the basis of the results obtained with a range of inhibitors and activators at concentrations previously used to characterize cathepsin L (9, 10) (Table I).

Substitution of ammonium sulfate fractionation (commonly used for cathepsin L isolation [e.g., Ref. (2)]) for the three-phase partitioning step, unique to the purification procedure used here (4), was found to have no effect on the proportions of complexed and free enzyme isolated, and the complexes had exactly the same characteristics.

On SDS-PAGE, with reduction, the M_r 37,000 peak (Fig. 2, peak 1) separated into two components, of M_r 26,000 and 14,000, while the M_r 26,000 peak (peak 2) yielded a single band of M_r 26,000 (Fig. 3A). On nonreducing SDS-PAGE, peak 1 yielded bands of apparent M_r 42,000, 37,000 and 26,000, with an additional faint band at M_r 68,000 (Fig. 3B). In some preparations, where the M_r 26,000 band was more prominent, an additional band at M_r 14,000 was also visible. Peak 2 yielded a single band of M_r 26,000 (Fig. 3B). Similar results were obtained whether or not the samples were boiled in SDS, without reduction, before the electrophoresis step.

To ascertain which of the different components of peak 1, revealed by nonreducing SDS-PAGE, were proteolytically active, the fractions were examined by SDS-PAGE in a gel containing gelatin as a substrate. This showed that the M_r 37,000 and 26,000 components were proteolytically active (Fig. 3C). The single M_r 26,000 component of peak 2 was proteolytically active (Fig. 3C). The proteolytic activity of the components of peaks 1 and 2 could be extinguished by Z-Phe-Phe-CHN_2, E-64, or leupeptin (results not shown), thus indicating that the activity is due to forms of cathepsin L. Analyzed by substrate gel electrophoresis, the isolated complexes were stable, when tested by preincubating in concentrations of mercaptoethanol up to 1.4 M or DTT up to 0.5 M (result not shown), but could be dissociated by boiling in 1.4 M mercaptoethanol, as carried out routinely for reducing SDS-PAGE (Fig. 3A).

Western blotting, after reducing SDS-PAGE, with monospecific antibodies raised against free sheep liver cathepsin L and sheep liver cystatin, revealed that the M_r 26,000 component of peaks 1 and 2 (from Fig. 2) reacted with anti-cathepsin L antibodies and the M_r 14,000 component of peak 1 reacted with anti-cystatin antibodies (Figs. 4A and 4B). Western blotting after nonreducing SDS-PAGE, using the same monospecific antibodies, revealed that the M_r 68,000, 42,000, and 37,000 component(s) of peak 1 contained both cathepsin L and cystatin (Figs. 4C and 4D).

Using Z-Phe-Arg-NHMec as a substrate, active site titration with E-64 (8) revealed that the free enzyme fraction titrated completely with E-64 and that approximately 30% of the protein represented active enzyme (Fig. 5). By contrast, only 60% of the cystatin-complexed cathepsin L activity titrated with E-64 when assayed with Z-Phe-

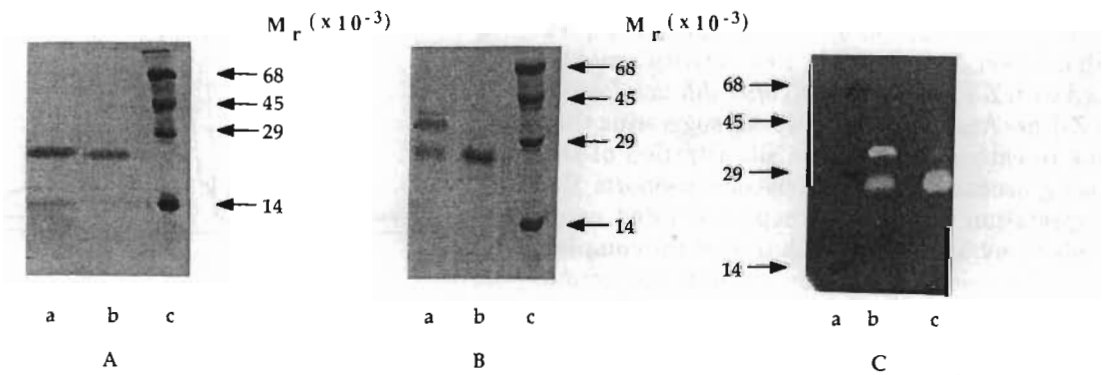

FIG. 3. SDS-PAGE and substrate SDS-PAGE of free and isolated cystatin-complexed sheep liver cathepsin L. (A) Samples (5 μg) were reduced and loaded onto a 12.5% gel. (a) Isolated cystatin-complexed cathepsin L; (b) free cathepsin L; and (c) MW markers (phosphorylase b, M_r 97,000; BSA, M_r 68,000; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 29,000; lysozyme, M_r 14,000). (B) Nonreduced samples (2.5 μg) were loaded onto a 12.5% gel. (a) isolated cystatin-complexed cathepsin L; (b) free cathepsin L; and (c) MW markers, as above. (C) Nonreduced samples (2.5 μg) were loaded onto a 12.5% gelatin-containing gel. (a) MW markers, as above; (b) isolated cystatin-complexed cathepsin L; and (c) free cathepsin L.

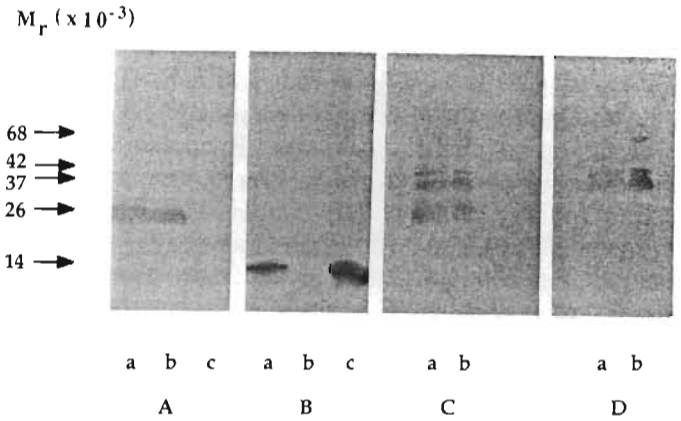

FIG. 4. Western blots of free and cystatin-complexed forms of sheep liver cathepsin L. Reduced samples (5 μg), (a) isolated cystatin-complexed cathepsin L; (b) free cathepsin L; and (c) sheep liver cystatin were separated by SDS–PAGE and probed with (A) chicken anti-cathepsin L IgY (5 μg/ml) and (B) rabbit anti-cystatin IgG (50 μg/ml). Nonreduced samples, (a) the crude fraction eluted from S-Sepharose (25 μg), and (b) isolated cystatin-complexed cathepsin L (2.5 μg) were separated by SDS–PAGE and probed with (C) chicken anti-cathepsin L IgY (5 μg/ml) and (D) rabbit anti-cystatin IgG (50 μg/ml). Indicated molecular weights were determined by SDS–PAGE.

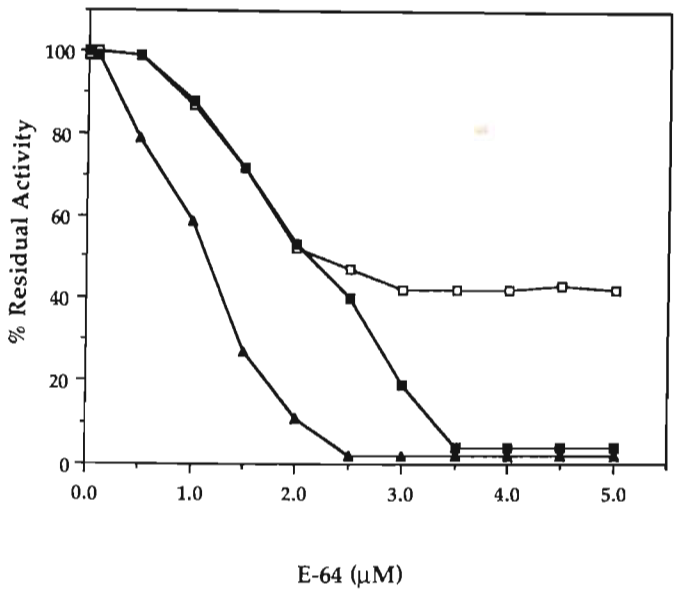

FIG. 5. Active site titration of isolated cystatin-complexed and free forms of sheep liver cathepsin L, using E-64 in assays against Z-Phe–Arg–NHMec and azocasein substrates. The titrations were carried out according to Barrett and Kirschke (8) on 6.75 μM of isolated cystatin-complexed cathepsin L and 7.6 μM of free cathepsin L. (□) Isolated cystatin-complexed cathepsin L and (▲) free cathepsin L, both against Z-Phe–Arg–NHMec. (■) Isolated cystatin-complexed cathepsin L against azocasein.

Arg–NHMec (Fig. 5). With azocasein as a substrate, however, the activity of the complex titrated completely with E-64 (Fig. 5).

We interpret these results to suggest the presence of two forms of complex, the known dissociable complex and the novel (covalent) complex reported here. Chicken egg white cystatin has been found to bind to papain in spite of the presence of E-64, but prior binding of the cystatin to the papain precludes E-64 binding to the enzyme (20). At the concentrations at which the complex was incubated with E-64, the normal cathepsin L/sheep liver cystatin complex might not have dissociated sufficiently to allow binding of the E-64. Subsequent to this incubation, the mixture is diluted 1000-fold for the assay with the sensitive synthetic substrate, and thus the (nontitratable) further 40% of activity against this substrate could be due to dissociation of the normal enzyme/cystatin complex at this high dilution. This residual 40% activity could be extinguished with Z-Phe–Phe–CHN_2 *after dilution* for the assay with Z-Phe–Arg–NHMec, however, suggesting that it too is due to cathepsin L. Active site titration of the complex using azocasein as the substrate supports the above interpretation, since the complex titrated completely in this assay, in which no dilution of the complex takes place. In the absence of dilution, the normally bound cystatin-complexed cathepsin L would not dissociate and would thus not show activity, while the abnormally bound cystatin-complexed cathepsin L would be available for E-64 titration and can, therefore, be titrated to completion.

Isolation and Characterization of Sheep Liver Cystatin

A single peak, inhibitory toward papain, was eluted from the CM-papain column. Subfractionation of this cystatin fraction on the Q-Sepharose anion-exchanger yielded three inhibitory subfractions, eluted in the first gradient, and another inhibitory peak, not completely resolved from a noninhibitory protein peak, eluted in the second gradient (Fig. 6).

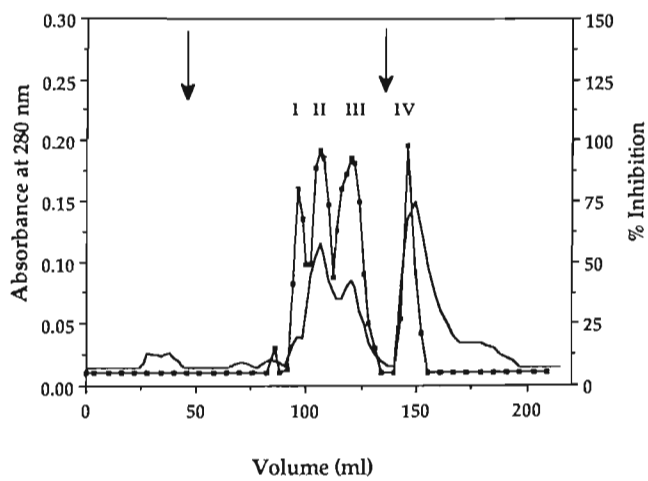

FIG. 6. Anion-exchange chromatography of the sheep liver cystatin fraction on Q-Sepharose. Column, 1 × 25 cm (20-ml bed volume); buffer, 20 mM ethanolamine, pH 9.5, followed by a 0–0.2 M NaCl gradient, in 5 column volumes, in the same buffer, followed by a 5-column-volume gradient (0.2–1 M NaCl). The volumes at which the gradients were started are indicated by ↓. Flow rate, 10 ml/h (13 cm/h); fractions, 2 ml (12 min). (—) A_{280} and (■) inhibitor activity, expressed as the percentage inhibition of 10 μM papain activity against Z-Phe–Arg–NHMec.

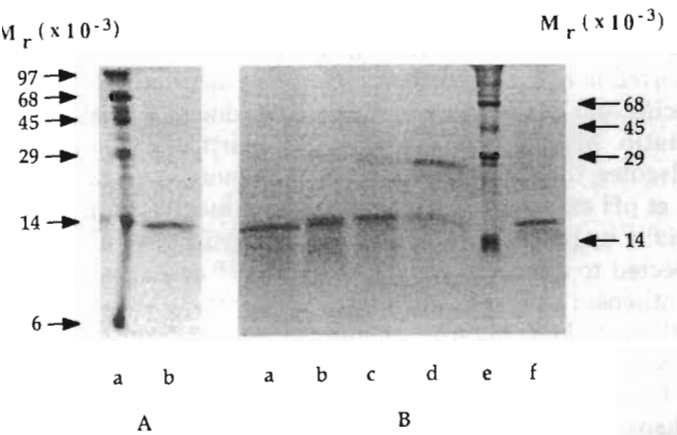

FIG. 7. SDS-PAGE of subfractions from anion-exchange chromatography of the sheep liver cystatin fraction. (A) Reducing 15% SDS-PAGE of (a) MW markers, as in Fig. 3 (with insulin, M_r 6000) and (b) the sheep liver cystatin fraction (5 μg). (B) Nonreduced samples (2.5 μg) were loaded onto a 15% gel. (a) Subfraction I; (b) subfraction II; (c) subfraction intermediate to subfractions II and III; (d) subfraction III; (e) nonreduced MW markers, as in Fig. 3; and (f) subfraction IV.

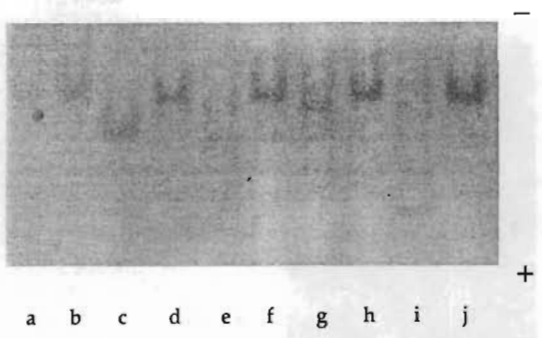

FIG. 8. Continuous PAGE of sheep liver cystatin subfractions. Each subfraction (2 μg) was loaded onto continuous 7.5% PAGE. Each subfraction was also treated with 1 mM dithiothreitol for 10 min at RT and loaded in alternate lanes (b, d, f, h and j). (a and b) Subfraction I; (c and d) subfraction II; (e and f) subfraction intermediate to subfractions II and III; (g and h) subfraction III; (i and j) subfraction IV.

The subfractions, analyzed by reducing SDS-PAGE, all manifested a common size of M_r 14,000 (Fig. 7A). Without reduction, however, subfraction I ran at M_r 14,000, subfractions II and IV ran at a slightly higher MW, and the major band in subfraction III ran at M_r 28,000, suggesting that it may be a dimer (Fig. 7B).

On continuous PAGE (Fig. 8), with and without DTT treatment, the subfractions I–III behaved in a manner consistent with the results of Wakamatsu *et al.* (21). DTT treatment caused all three subfractions to run with the same mobility as subfraction I, while without this treatment subfractions II and III had different mobilities. The result for subfraction IV was different from that of Wakamatsu *et al.* (21), however, in that DTT treatment also converted it to run similarly to subfraction I. By analogy with the results of Wakamatsu *et al.* (21), the results reported here suggest that all the forms of cystatin in sheep liver are of the stefin B-type: subfraction I may exist in a reduced state, while subfractions II, III, and IV may contain reactive cysteine residues, to which various substituents may be attached. Upon reduction, the substituents are removed and the common stefin B molecule becomes evident. Further evidence is needed, however, before the cystatin can be unequivocally identified as stefin B.

Based on this information, an abbreviated procedure for the isolation of sheep liver cystatin was devised, employing chromatography on Sephadex G-75, in the presence of 1 mM DTT, after chromatography on CM-papain. When tested for its capacity to inhibit purified cathepsin L, and isolated cystatin-complexed cathepsin L, the isolated sheep liver cystatin was found to inhibit both forms of the enzyme in a dose-dependent manner (Fig. 9).

Formation of Cystatin/Cathepsin L Complexes in Vitro

Gelatin substrate-containing SDS-PAGE revealed that coincubation of purified cathepsin L with the sheep liver cystatin resulted in the formation of complexes, of M_r 37,000, 42,000, and 68,000, at pH 5.5 and above, but not below this value (Fig. 10), and pH 5.5 therefore seems to be pivotal in the formation of the complexes. A difference between the isolated complexes and those formed *in vitro* is that the M_r 42,000 component was proteolytically active in the latter (Fig. 10), but not in the former (Fig. 3C). The activity of the complexes formed *in vitro* could be extinguished by incubation with either Z-Phe-Phe-CHN_2, E-64, or leupeptin (results not shown). Complexes formed *in vitro*, at pH 6, could be detected in less than 1 min after incubation of the cystatin with cathepsin L, and complete complex formation was attained after about 10 min (result not shown).

DISCUSSION

The results of Western blots (Fig. 4) and substrate SDS-PAGE analysis (Figs. 3C and 10), provide evidence

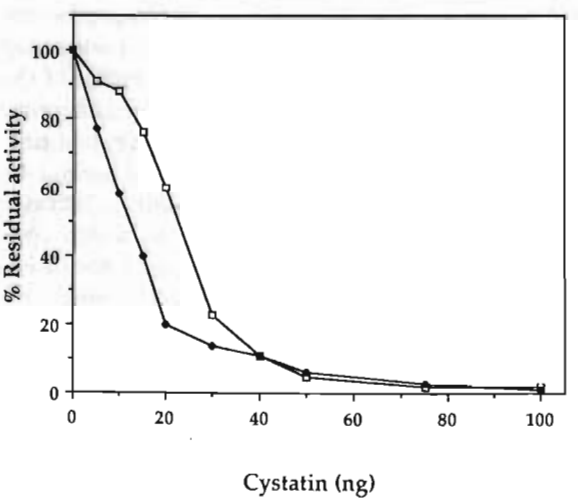

FIG. 9. Inhibition of isolated cystatin-complexed and free forms of sheep liver cathepsin L by sheep liver cystatin. Cathepsin L (50 nM active enzyme) and cystatin-complexed cathepsin L (50 nM active enzyme) were incubated with increasing amounts of cystatin, at pH 6.0, and residual activity was assayed using Z-Phe-Arg-NHMec. (□) Cathepsin L and (♦) isolated cystatin-complexed cathepsin L.

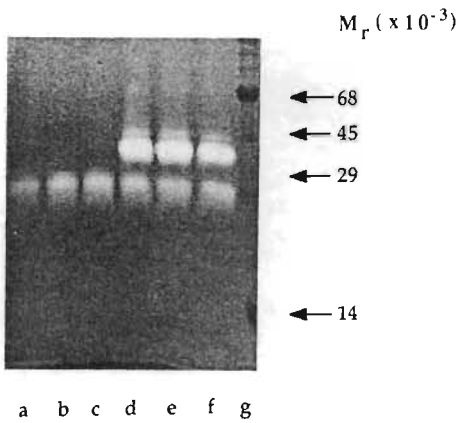

FIG. 10. Gelatin substrate SDS–PAGE, showing the effect of pH on the *in vitro* formation of covalent complexes between isolated sheep liver cystatin and cathepsin L fractions. Sheep liver cystatin fraction (2.5 μg) and cathepsin L (2.5 μg) were incubated together for 1 h at 37°C in 100 mM buffers of the desired pH, and 2.5 μg of protein was electrophoresed on a 12.5% gelatin substrate gel. Cystatin and cathepsin L incubated at (a) pH 4.2; (b) pH 4.5; (c) pH 5.0; (d) pH 5.5; (e) pH 6.0; (f) pH 6.5; and (g) MW markers, as in Fig. 3.

that a sheep liver cysteine proteinase inhibitor, probably stefin B, is apparently able to complex with cathepsin L in at least two modalities—the established inhibiting mode and an alternative noninhibiting mode. Unequivocal evidence for the existence of the noninhibitory binding mode is provided by the purification of proteolytically active cathepsin L/stefin B complexes, from sheep liver, and by the demonstration that similar complexes can be formed *in vitro* from purified, active constituents.

The isolated complexes were active against proteins, such as azocasein and gelatin, as well as against Z-Phe–Arg–NHMec, while those formed *in vitro* were only tested against gelatin, but were able to digest this substrate. In terms of a model of tight-binding, reversible inhibition, activity against the synthetic substrate is not unexpected since the assay is very sensitive and a high dilution of the enzyme is required; at this dilution, noncovalent enzyme/inhibitor complexes may dissociate. Activity against protein substrates is not expected, however, and cannot be explained in this way, as assays using protein substrates require higher concentrations of enzyme, at which concentrations enzyme/inhibitor complexes would not be expected to dissociate. A different model is, therefore, required to explain the active complexes.

A possible explanation is that (covalent) complex formation may require denatured or inactivated forms of the cystatin, in a reaction of the form

$$E + I^* \rightarrow EI^*,$$

where I^* represents denatured or inactivated cystatin and EI^* represents active complex. The results of the isolation study, and those presented in Fig. 10, however, argue against such an explanation. Of the myriad of molecules present in a homogenate, it is apparently only a cystatin that complexes with cathepsin L, in the novel manner reported here, which suggests that the reaction is highly specific and is, therefore, unlikely to be due to a disabled cystatin. Also, although cystatins are particularly stable molecules, their denaturation would be more likely to occur at pH extremes. On the other hand, inactivation, especially by proteolysis by residual cathepsin L, would be expected to approximately follow the pH activity curve of cathepsin L. The results presented in Fig. 10 show that neither of these obtains; complexes do not form below pH 5.5, but form readily above this pH.

The sheep liver cystatin is able to inhibit both free cathepsin L and the isolated complex (Fig. 9), the latter suggesting that cathepsin L can perhaps interact with two cystatin molecules, in two different ways. Although the inhibition of sheep liver cathepsin L by sheep liver cystatin has not been subject to kinetic analysis and the K_i values are not known, we envisage the inhibition and complex-formation reactions, in their simplest form and in qualitative terms, to be as shown in Fig. 11.

Several pieces of evidence presented here are consistent with the isolated complex fraction (peak 1, Fig. 1) comprising a mixture of forms of complex; one in which the two molecules are associated in the usual noncovalent, reversible, inhibiting way (*EI*), another in which the molecules are more firmly bound, but in a noninhibiting manner (*EI**), and, perhaps, a third (*EI*I*) comprising both forms of interaction. For example, it has previously been shown that complexes of chicken egg white cystatin with papain dissociate on SDS–PAGE, without reduction (11, 21). In the case of the active complex eluted from Sephadex G-75 in the M_r 37,000 region, however, on nonreducing SDS–PAGE, M_r 42,000, 37,000, and 26,000 (and 14,000) components were found (Fig. 3B). This suggests that a proportion of the complex fraction (the M_r 42,000 and 37,000 constituents) does not dissociate without re-

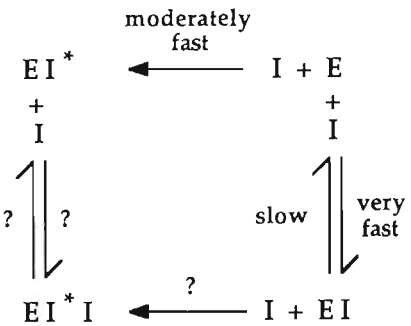

FIG. 11. A model of the reactions envisaged to be involved in the formation of active and inactive cathepsin L/cystatin complexes. E, cathepsin L; I, cystatin; EI*, active complex of cathepsin L with one molecule of cystatin; EI, inactive complex of cathepsin L with one molecule of cystatin; and EI*I, inactive complex of cathepsin L with two molecules of cystatin. The formation of EI* is thought to involve a reaction which is not easily reversed, since the link forming EI* is resistant to boiling in SDS.

duction by boiling in 1.4 M mercaptoethanol, while another proportion (the M_r 26,000 and 14,000 constituents) does dissociate. By summation of the molecular weights, it may be deduced that the M_r 37,000 and 42,000 forms probably consist of cathepsin L bonded to one cystatin molecule and may be conformational variants of one another. An additional, minor, component of M_r 68,000 was also found; this comprises cathepsin L and cystatin molecules (Figs. 4C and 4D), but the proportions are unknown.

The results of E-64 active site titration (Fig. 5) are also consistent with a model of mixed forms of complex. The proportion of the complex fraction, active against Z-Phe–Arg–NHMec, but inaccessible to E-64, could be complexed with the cystatin in a normal manner and is thus unavailable for E-64 binding. The E-64 results therefore suggest that the inhibitor may be normally complexed to about 40% of the active enzyme in the complex and abnormally complexed to 60% of the active enzyme in the complex. The effect of Z-Phe–Phe–CHN_2 suggests that all of the proteinase activity is due to cathepsin L.

Cathepsin L and cystatins normally occur in separate cellular compartments and we imagine that the fact that complexes of the two molecules can be purified from liver is a consequence of homogenization of the tissue. The consequent coexistence of the two molecules in the same solution presumably enables complexes to form; this may occur at any stage in the purification when conditions favor complex formation. In one sense, therefore, the complexes reported here may be considered as artifacts of tissue homogenization. On the other hand, tissue destruction also occurs in pathologies, such as invasive cancer, and proteolytically active complexes of cystatins with proteinases, such as cathepsin L, might be relevant to invasive cancer, especially if similar complexes can be demonstrated in human tissue. In a study to be reported elsewhere, we have recently isolated similar active complexes from the liver of the baboon (*Papio ursinus*), a higher primate, which suggests that formation of proteolytically active cystatin/cathepsin L complexes is not limited to sheep. The only comparable study, of which we are aware, is that of Wakamatsu *et al.* (21), who visualized the binding of rat liver stefin B to rat cathepsin H, on PAGE, but these authors did not observe any active complexes.

A question which is beyond the scope of the present study is that concerning the nature of the bond between the cystatin and cathepsin L. The fact that the bond may be broken by reduction, with boiling, prior to SDS–PAGE, points to the possibility of cysteine involvement. Disulfide links usually form more readily at higher pH, however, and pH 5.5 (Fig. 10) is lower than the pH usually required. The cysteine residue commonly at position 3 in stefin B (3) could conceivably participate in a link of some sort. The fact that the complexes are proteolytically active, and are not dissociated by activating levels of reducing agent, indicates that the linkage cannot be through the active site cysteine of cathepsin L. More detailed knowledge of the structure of both sheep liver cathepsin L and sheep stefin B is required before this question can be properly answered.

ACKNOWLEDGMENTS

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Characterisation of the Activity and Stability of Single-chain Cathepsin L and of Proteolytically Active Cathepsin L/ Cystatin Complexes

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Summary

The activity of single-chain cathepsin L was found to be markedly dependent on cysteine concentration, while a covalent, proteolytically active cathepsin L/ cystatin complex was less cysteine-dependent. Cysteine levels and ionic strength did not affect the stability of either enzyme form and both enzyme forms were found to be stable for significant periods of time at or near physiological pH.

Introduction

We have previously reported [1,2] on the isolation of proteolytically active complexes of single-chain cathepsin L with a cystatin, thought to be stefin B, and on the formation of similar complexes *in vitro*, from purified constituents. These complexes can only be disrupted by boiling in sodium dodecyl sulfate in the presence of a reducing agent and in these proteolytically active complexes the enzyme and cystatin may, therefore, be covalently bound [2].

We report here on the characterisation of the activity and stability of the single-chain sheep liver enzyme and of a proteolytically active complex fraction. Two-chain forms of cathepsin L [3] and single-chain chicken cathepsin L [4] have previously been characterised in this respect, while the activity and stability of cathepsin L/cystatin complexes have not previously been documented. Because cathepsin L has been implicated in cancer [5,6], the activity of this enzyme under extracellular conditions of pH, ionic strength and redox potential is of some interest, in the context of invasion.

Abbreviations: NHMec, 7-(4-methyl)coumarylamide; Z-, benzyloxycarbonyl.

Methods

Single-chain sheep liver cathepsin L, and a proteolytically active fraction of sheep liver cathepsin L complexed with a sheep liver cystatin, were isolated as described previously [7], through the pH 5.5 step on S-Sepharose. The active peak was subsequently separated on Sephadex G-75, the first included peak being the complex fraction and the second being free cathepsin L.

Acetate-MES-Tris buffer systems, of defined ionic strength (0.1, unless stated otherwise), described by Ellis & Morrison [8] were used in the assays for activity and stability. Enzyme activity was measured at 30°C according to Barrett & Kirschke [9]; unless stated otherwise, all other assays were done at 37°C. As a measure of stability, the half-life of the enzyme was determined by continuously monitoring the fluorogenic products produced, from Z-Phe-Arg-NHMec, by an enzyme sample incubated in a defined buffer containing a thiol activator. (Activity could also be estimated from the initial slope of the same progress curve.) For determination of the half-life, the period of measurement was usually 30 min. As a null hypothesis it was assumed that the loss of activity was a first-order process. In each case, therefore, the logarithm of the activity (i.e. the logarithm of the slope of the plot of fluorescence intensity vs time) was plotted against time, and the slopes of the regression lines were used to calculate K_{obs} and hence $T_{1/2}$ values, where;

$$K_{obs} = \text{Slope} \times -2.3$$
$$T_{1/2} = 0.693/ K_{obs}.$$

Statistical measures of significant differences may be done at the level of the slopes of the semi-log plots. Error bars were determined from 95% confidence limit upper and lower values of the slope of the semi-log plot, calculated as slope ± (1.96 x standard error of the slope). The program "Statgraphics" [10] was used to calculate the standard error of the slope. Upper and lower values of K_{obs} and hence of $T_{1/2}$ were calculated from upper and lower values of the slope.

Note that the error bars are asymmetrically distributed, being larger on the "upper" side. For this measure of the half-life to be useable it is important that substrate is never limiting and that there is both measurable activity, initially, and a measurable loss of activity over the period of measurement. To meet these conditions, it may be necessary to change the enzyme concentration over a wide range, as the conditions are altered: the effect which this change in concentration itself might have on the half-life is accepted as a limitation of the method. If the enzyme is unusually stable, under the conditions used, the progress curve will tend to a straight line (which corresponds to infinite stability). Occasionally, the 95% confidence limit encompasses the possibility that the enzyme is infinitely stable - in this case, the upper limit of the slope of the semi-log plot will be >0, and the calculated value of the upper limit of $T_{1/2}$ will be less than the value of $T_{1/2}$! Such a "nonsensical" result should, of course, be interpreted to indicate that the upper limit represents "infinite" stability.

Results and Discussion

The effect of cysteine concentration, on the activity of free and complexed cathepsin L, is shown in Figure 1. The results suggest that complexing of the enzyme with the cystatin has an effect similar to that of high cysteine concentrations. Also, the free single-chain enzyme itself apparently requires much higher levels of cysteine, for complete activation, than has previously been reported to be the case for single- and two-chain forms of cathepsin L [4,9]. Alternatively, this may indicate that the single-chain sheep enzyme might be able to tolerate higher levels of cysteine.

The complex can be disrupted by reducing SDS-PAGE [2] which suggests the involvement of a cysteine residue (or residues) in the linkage between the enzyme and the cystatin. Perhaps the formation of this link requires the disruption of an intramolecular disulfide bond in the enzyme; an effect which can be mimicked by a high cysteine concentration. We can envisage that, in the two-chain version of the enzyme, disruption of this disulfide bond might lead to

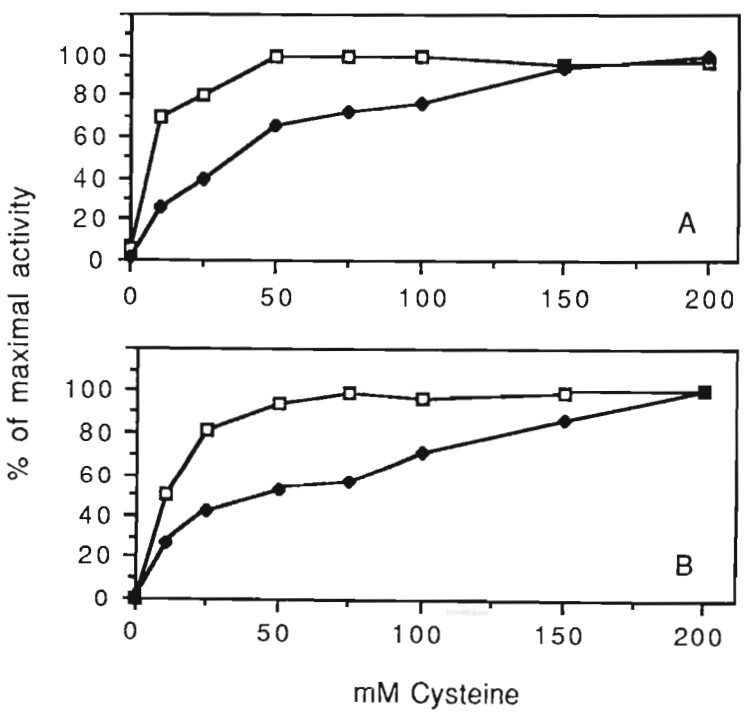

Figure 1. The effect of cysteine concentration on the activity of single-chain sheep liver cathepsin L.
◆, free cathepsin L; ❑, cystatin-complexed cathepsin L. **A**, pH 7.0; **B**, pH 5.5.

loss of the light chain, with consequent loss of activity. A single-chain enzyme might, therefore, be a prerequisite for the formation of catalytically active enzyme/cystatin complexes. In other words, two-chain forms of cathepsin L might be susceptible to inactivation by high cysteine concentrations and/or covalent bonding to the cystatin.

As shown in Figure 2, the single-chain form apparently suffers a slight decrease in stability with increasing cysteine concentration, but there is less of a differential in stability between the two forms, compared to the difference in activity.

The results in Figure 1 suggest that 150 mM cysteine is required for full activation and this concentration was consequently used in determination of the pH activity curves shown in Figure 3. Qualitatively similar results were obtained using 50 mM cysteine, however, and the pH optima were the same (results not shown). Note that, at equimolar concentrations, the cystatin-complexed cathepsin L has approximately four-fold greater activity than the free

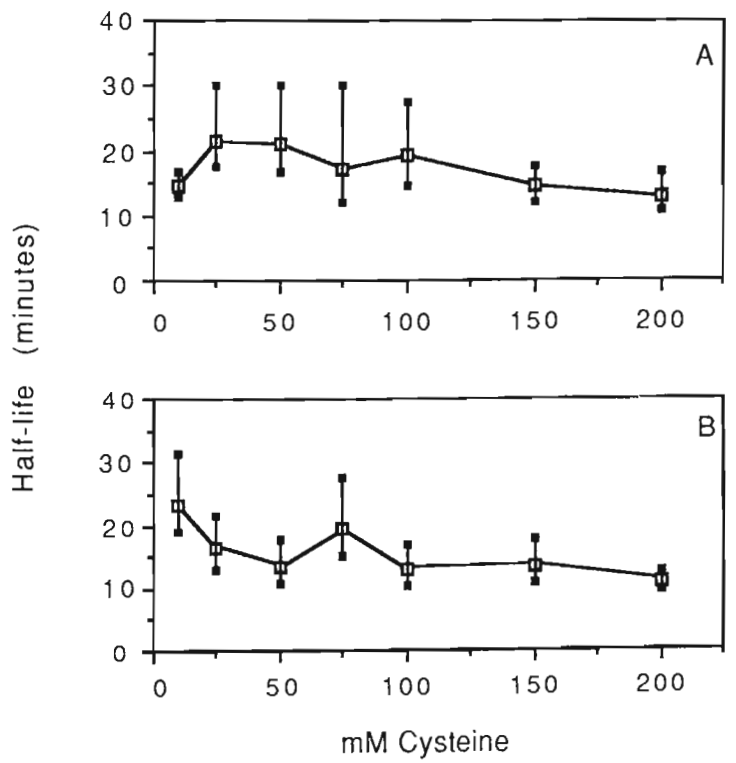

Figure 2. The effect of cysteine concentration, at pH 7.0, upon the stability of single-chain sheep liver cathepsin L.
A, free cathepsin L; **B**, cystatin-complexed cathepsin L.

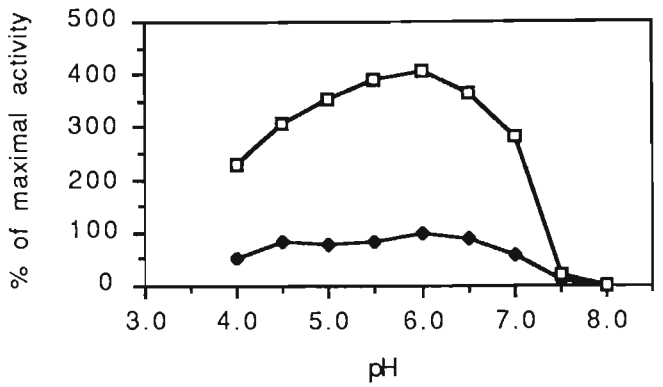

Figure 3. The effect of pH on the activity of sheep liver cathepsin L.
◆, free cathepsin L; ❑, cystatin-complexed cathepsin L. Ionic strength, 0.1.
The results are normalised to equimolar concentrations of the two forms of cathepsin L and the activities are expressed as % of the maximal activity of free cathepsin L.

enzyme (Fig. 3), suggesting that, bound in this alternate manner, the cystatin is acting as an **activator**.

Both the free and complexed enzyme have an apparent optimum at pH 6.0 and both have significant activity at pH 7.0 (*ca.* 60% of their respective maximal activities) (Fig. 3). Stability determinations suggest that both forms of the enzyme are maximally stable at *ca.* pH 5.5-6.0 (Fig. 4).

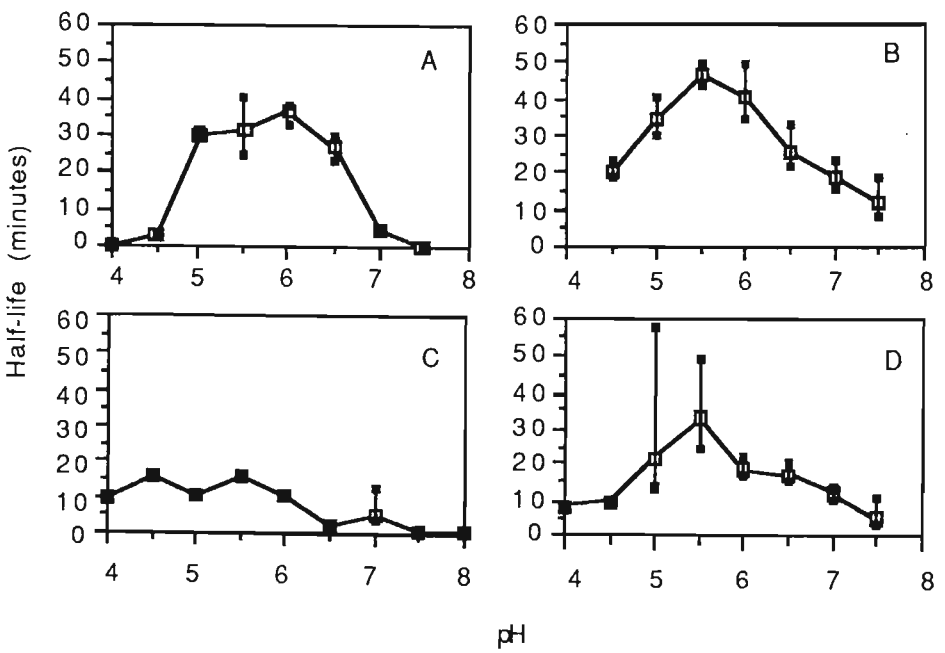

Figure 4. The effect of pH on the stability of single-chain sheep liver cathepsin L.
Free cathepsin L, at (**A**) 50 mM cysteine and (**B**) 150 mM cysteine;
Cystatin-complexed cathepsin L, at (C) 50 mM cysteine and (D) 150mM cysteine.

In general, the activity of both the free and complexed forms of the enzyme declined with an increase in ionic strength of the buffer, an effect which was more pronounced at pH 7.0, than at pH 5.5 (Fig. 5). Nevertheless, significant activity remains at physiological ionic strengths (I = *ca.* 0.15). Ionic strength does not appear to have a significant effect upon the stability of either form of the enzyme (result not shown).

Previous measures of the activity and stability of cathepsin L have suggested that this enzyme is insufficiently active and insufficiently stable, at physiological pH values, to have significant activity in extracellular locations. These previous measurements have been made on two-chain enzymes, which, as we have previously argued [7], may be artifacts of the isolation methods used. The single-chain enzyme, studied here, appears to have significant activity at pH 7.0 and, very significantly, appears to be activated by its interaction with a cystatin (thought to be stefin B [2]), which it might only encounter as a result of tissue destruction. This suggests that cathepsin L may, in fact, have significant activity in the extracellular milieu, and might be further activated by tissue destruction.

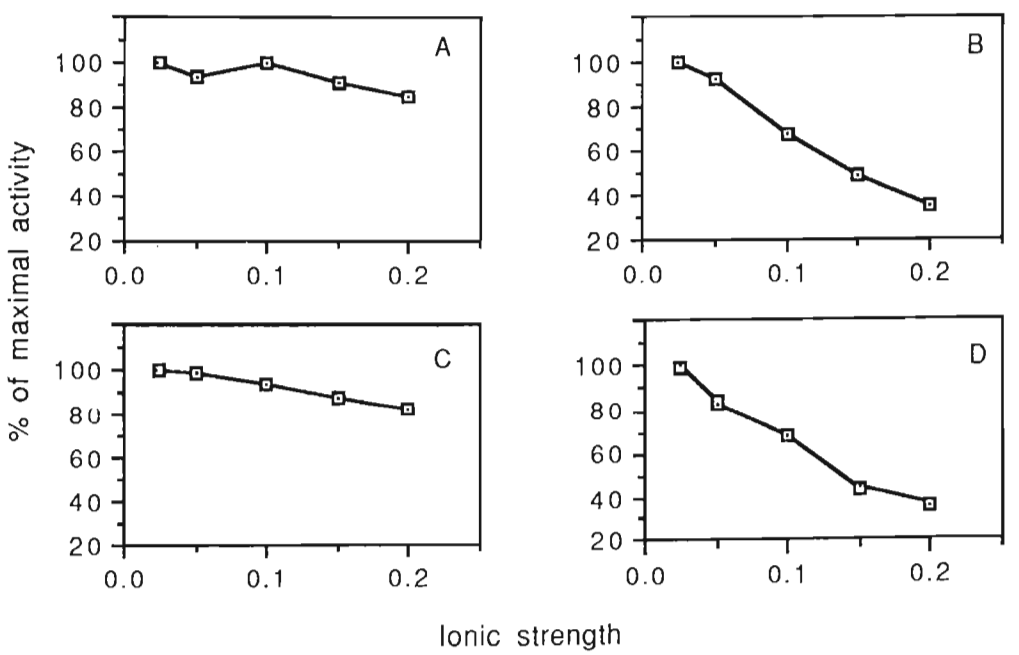

Figure 5. The effect of ionic strength on the activity of single-chain sheep liver cathepsin L.
Free cathepsin L at (**A**) pH 5.5 and (**B**) pH 7.0;
Cystatin-complexed cathepsin L at (**C**) pH 5.5 and (**D**) pH 7.0. All at 50 mM cysteine.

Methods used previously for the measurement of the pH stability of cathepsin L have involved incubation of the enzyme at different pH values for 1h, before measurement of the residual activity at pH 5.5 [3], or at the pH of incubation [11]. While arguments can be made for both of these assays, they do tend to underestimate the stability under circumstances where the half-life is significantly less than the incubation period. The measure of stability employed in this study does not suffer from this particular limitation and its use has suggested that single-chain sheep cathepsin L might be more stable at physiological pH than previously surmised.

The activity and stability of the free single-chain form of the enzyme and of the enzyme/cystatin complexes, therefore, appear to be such that they could have significant activity in the extracellular milieu. Such properties are not inconsistent with a possible role of similar enzymes and enzyme complexes in tumor invasion, in which cathepsin L has been implicated.

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LOCALIZATION OF AN IMMUNOINHIBITORY EPITOPE OF THE CYSTEINE PROTEINASE, CATHEPSIN L.

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ABSTRACT

Antibodies, raised in chickens (IgY) and rabbits (IgG) against the lysosomal proteinase cathepsin L, targeted the enzyme in an ELISA and Western blot. In contrast to the rabbit IgG, the chicken IgY was immunoinhibitory towards cathepsin L. An epitope that elicits immunoinhibitory antibodies has been localized to an active site-associated peptide sequence. The corresponding free peptide, coated down in an ELISA, is recognised by the chicken IgY, but not the rabbit IgG. This peptide was able to inhibit the immunoinhibition of cathepsin L by chicken anti-cathepsin L IgY, suggesting its complete or partial identity with an immunogenic epitope for chickens in whole cathepsin L.

INTRODUCTION

The lysosomal cysteine proteinase cathepsin L has been implicated in processes such as tumour invasion and metastasis (1). Specific immunoinhibition of the enzyme by active site-directed antibodies, compared to the relatively non-specific inhibition provided by synthetic inhibitors to date (2), may find application in elucidating the enzyme's role in tumour invasion and in tumour therapy (3). The identification of the immunoinhibitory epitopes (defined as epitopes capable of eliciting immunoinhibitory antibodies) of the enzyme is important for the production of antibodies with high immunoinhibitory activity.

Immunoinhibition of a number of enzymes has been studied, such as papain (4), cathepsin D (5, 6), ß-lactamases (7), gelatinase (8) and acetylcholinesterase (9). "Inhibitory antibodies"

against cathepsin D were used to investigate the role of the enzyme in cartilage breakdown (5), demonstrating the potential these agents have in elucidating the role of enzymes in biological functions. A distinction needs to be made, however, between true immunoinhibition of enzyme activity and pseudoinhibition, due to immunoprecipitation. In immunoinhibition, it is envisaged that an antibody binds to an enzyme in such a way that the active site is rendered incompetent. In immunoprecipitation, optimal proportions of antibody and antigen (enzyme) interact to form a cross-linked network or lattice which precipitates. This process requires at least two epitopes, which are sufficiently separated, to ensure interaction with paratopes on two different antibody molecules (8). Immunoprecipitation may therefore not necessarily involve the active site, and enzyme activity may be impaired simply by the enzyme being locked in an insoluble complex, which is removed from solution (4, 5). Two populations of anti-papain antibodies have been distinguished, those which both immunoinhibit and precipitate the enzyme and those which only precipitate the enzyme (4).

Antibodies against cathepsin L have previously been raised in rabbits and sheep (11), as is common practice. Due to the greater evolutionary distance between mammals and birds, however, antibodies raised in chickens might respond to different epitopes on the enzyme compared to those raised in mammals, such as the rabbit. Chicken IgY antibodies, which can be extracted from the egg yolk (12, 13), may, therefore, have novel immunoinhibitory properties compared to their rabbit counterparts, thus imparting new information about those epitopes of the enzyme which elicit the formation of immunoinhibitory antibodies.

We report here on raising polyclonal antibodies in rabbits and chickens, and compare the titer, specificity and immunoinhibitory properties of the antibody preparations. Previous studies on the immunoinhibition of cathepsin L (3, 14) have focused on the use of anti-peptide antibodies against an active site-associated peptide (L153-165) from cathepsin L, which effectively immunoinhibited the enzyme. In this study we discuss the finding that immunoinhibitory chicken polyclonal antibodies seem to target this same active site-associated peptide, suggesting that it may constitute the whole, or a part, of an epitope that elicits the formation of immunoinhibitory antibodies.

MATERIALS AND METHODS

Purification of cathepsin L

Cathepsin L was purified from sheep liver essentially as described previously (15), except that chromatography on S-Sepharose at pH 4.5 was replaced by chromatography on Sephadex G-75.

Cathepsin L active site-associated peptide sequence

An active site-associated peptide sequence, designated L153-165, was chosen for cathepsin L (14) by consideration of the published 3-dimensional structure of the analogous enzyme, papain (16). The amino acid sequence, E-P-D-C-S-S-E-D-M-D-H-G-V, corresponds to residues 153-165 in the amino acid sequence of human cathepsin L; in papain the comparable residues are 150-161 (17). There are marked differences in the sequences in this region between cathepsins B, H and L (17), while this sequence is relatively conserved between species (17, 18, 19). The peptide was custom synthesised by Multiple Peptide Systems, San Diego, California.

Inoculation protocol

Two rabbits and two chickens were inoculated with sheep cathepsin L (50 μg), emulsified in a 1:1 ratio with Freund's complete adjuvant. Further inoculations were administered in Freund's incomplete adjuvant at week 2, followed by monthly boosters (9). Rabbits were inoculated subcutaneously on the back and chickens intramuscularly in the large breast muscle. In a repeat study a further two rabbits and two chickens were inoculated using the same protocol.

Isolation of antibodies

Rabbit IgG was isolated from serum (20) and IgY from egg yolks (13).

ELISA and Western blot for characterisation of antibody

The ELISA and Western blot for immobilised whole enzyme were as described before (14), except that rabbit anti-IgY-horse radish peroxidase (HRPO) conjugate (21) was used at a 1/750 dilution to detect IgY. In the Western blot, antibodies were characterised against a crude cathepsin L fraction, obtained by a modification of the cathepsin L purification procedure in which all of the proteins bound to S-Sepharose were eluted with 1M NaCl. ELISAs, to test targeting of the active site-associated peptide sequence from cathepsin L (L153-165), by rabbit and chicken antibody preparations, were carried out as described previously for anti-peptide antibodies (14).

Immunoinhibition assays

Immunoinhibition of cathepsin L by antibody preparations was assayed by monitoring the cleavage of the synthetic substrate Z-Phe-Arg-NHMec, either on a continuous basis or by stopped time assays, as described previously (14). Intrinsic proteolytic activity in rabbit IgG fractions was inhibited by soya bean trypsin inhibitor (40 μg/ml): this was not necessary for IgY fractions.

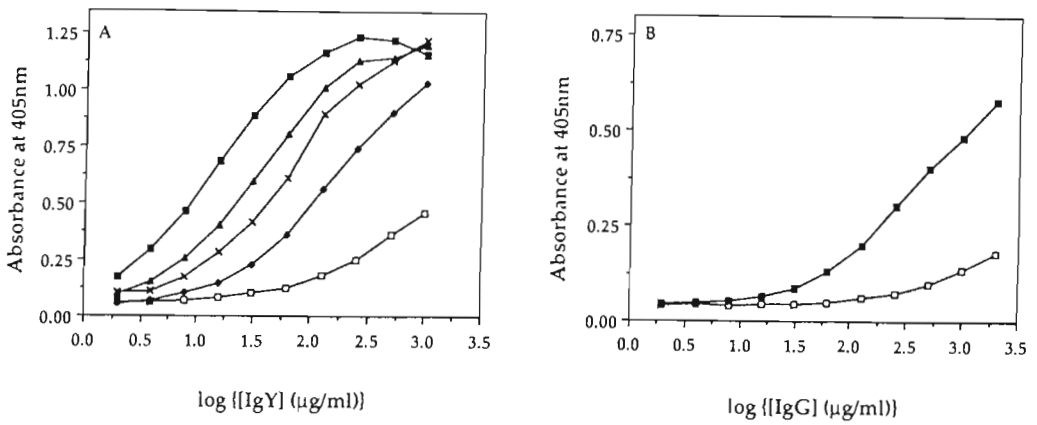

FIGURE 1

ELISA of the binding of polyclonal antibodies to sheep cathepsin L.
Sheep cathepsin L was coated at 1 µg/ml to microtiter plates and incubated with serial two-fold dilutions of, **A**), chicken anti-sheep cathepsin L collected after 4 (◆), 6 (X), 8 (▲) and 12 weeks (■) and non-immune IgY (❑); and, **B**) rabbit anti-sheep cathepsin L IgG (■) and non-immune IgG (❑). Binding was visualised by incubation with HRPO-linked secondary antibodies as described in (14) and in materials and methods. Each point is the mean absorbance at 405 nm of duplicate samples.

Inhibition of immunoinhibition, by an active site-associated peptide sequence

Chicken anti-cathepsin L IgY (125 µg/ml final concentration) was incubated with increasing molar ratios of peptide L153-165 to cathepsin L at 30°C for 15 min, before cathepsin L (5 ng) was added and further incubated at 30°C for 15 min. Following this, the extent of immunoinhibition was determined by monitoring the cleavage of substrate on a continuous basis as described above. In control experiments non-immune IgY was substituted for immune IgY.

RESULTS

Characterisation of polyclonal anti-sheep cathepsin L antibodies

Antibody production

Antibodies were readily raised against sheep cathepsin L in both rabbits and chickens. The increase in antibody titer, which is expressed here as the concentration of antibody at which A_{405}-values are still significantly higher than equivalent non-immune controls, was followed for IgY over the course of the inoculation period (Fig. 1). The chicken antibody titer showed

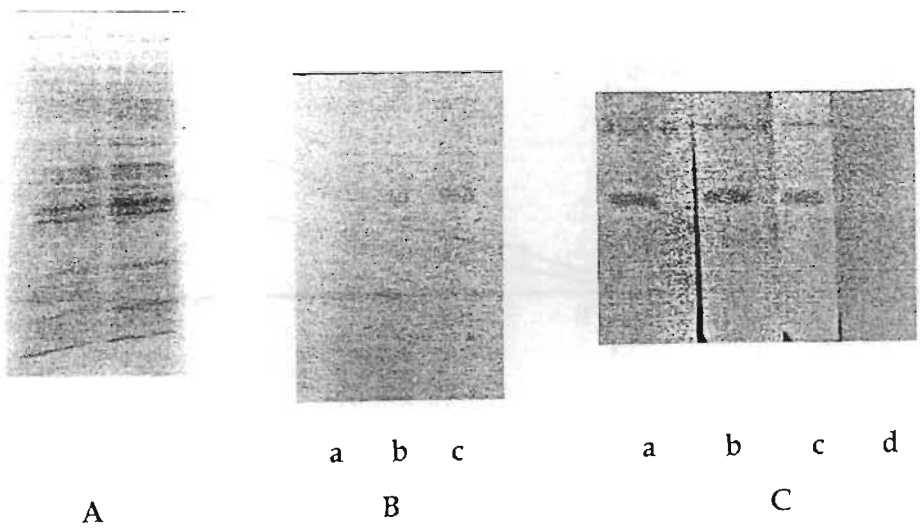

FIGURE 2

Targeting of sheep cathepsin L in a Western blot.
A) 12.5% SDS-PAGE of a crude S-Sepharose ion-exchange fraction from sheep liver. This fraction was electroblotted onto nitrocellulose and probed with, **B**: a) non-immune rabbit IgG (0.2 mg/ml); rabbit anti-sheep cathepsin L IgG, at b) 0.2 mg/ml; and c) 0.125 mg/ml, visualised using sheep anti-rabbit-HRPO conjugate, and **C**: chicken anti-sheep cathepsin L at a) 30 μg/ml; b) 15 μg/ml; c) 7.5 μg/ml and d) non-immune IgY at 30 μg/ml, visualised using rabbit anti-chicken-HRPO conjugate.

a large increase from 4 to 6 weeks, with a small further increase from 6 to 8 weeks, and a large increase from 8 to 12 weeks, reaching a titer of at least 4 μg/ml (Fig. 1A). A similar study of the timing of the antibody response against cathepsin L in rabbits was not done, since this is known (11). The titer of the rabbit antibody was about 30 μg/ml (Fig. 1B). The two chickens and rabbits used in each of the studies responded similarly and the results given in Fig. 1 are typical of each response.

Antibody specificity

The specificities of both rabbit and chicken antibodies were tested in a Western blot against crude ion-exchange samples from sheep liver (Fig. 2). Only cathepsin L was targeted in the mixture of proteins, by all antibody preparations.

Immunoinhibition

Immunoactivation, rather than immunoinhibition, of the hydrolysis of the synthetic substrate Z-Phe-Arg-NHMec was found with rabbit anti-cathepsin L IgG, since the activity in the

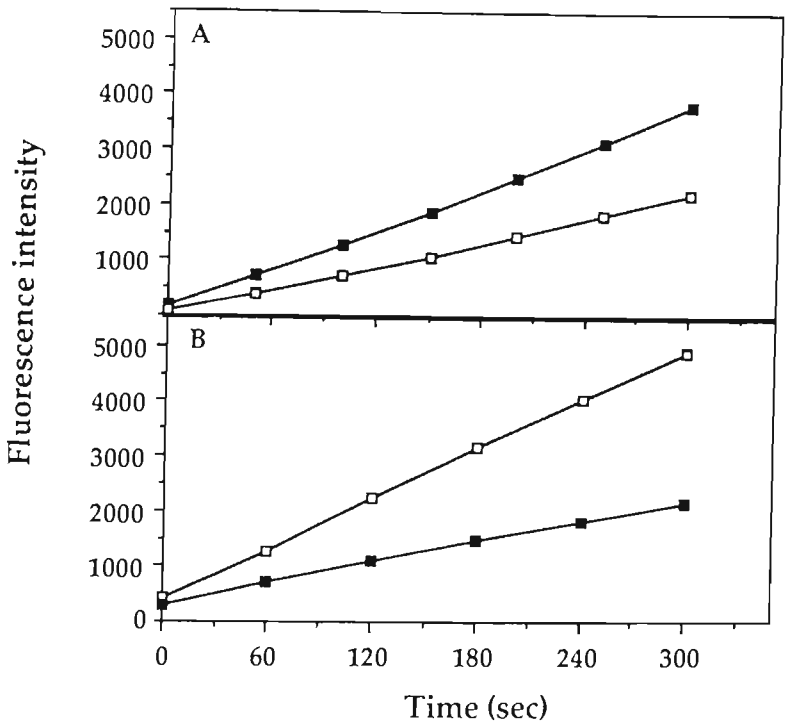

FIGURE 3

Immunoinhibition of sheep cathepsin L by rabbit and chicken anti-sheep cathepsin L antibodies.
Sheep cathepsin L (5ng) was incubated with, **A**) rabbit anti-cathepsin L IgG (■) or non-immune IgG (❑) (0.5 mg/ml final concentration); or, **B**) chicken anti-cathepsin L IgY (■) or non-immune IgY (❑) (0.5 mg/ml final concentration) for 15 min at 30°C, before being assayed on a continuous basis for 5 min against the fluorogenic substrate Z-Phe-Arg-NHMec.

presence of the immune IgG was greater than that in the presence of the non-immune IgG (Fig. 3A). A similar result was obtained with the rabbit antibodies raised in the repeat inoculation procedure. Contrary to rabbit IgG, chicken-anti sheep cathepsin L IgY was found to inhibit cathepsin L, since the activity in the presence of the immune IgY was about half of that of the non-immune IgY (Fig. 3B).

Immunoinhibition tests across a range of IgY concentrations (Fig. 4), revealed that the antibodies inhibited the enzyme weakly up to concentrations of 31.25 µg/ml, after which the inhibition increased up to 45% at 500 µg/ml. Inhibition of cathepsin L by chicken-anti sheep cathepsin L IgY could be demonstrated for all four chickens used in the two studies and representative results are given in Figs. 3B and 4.

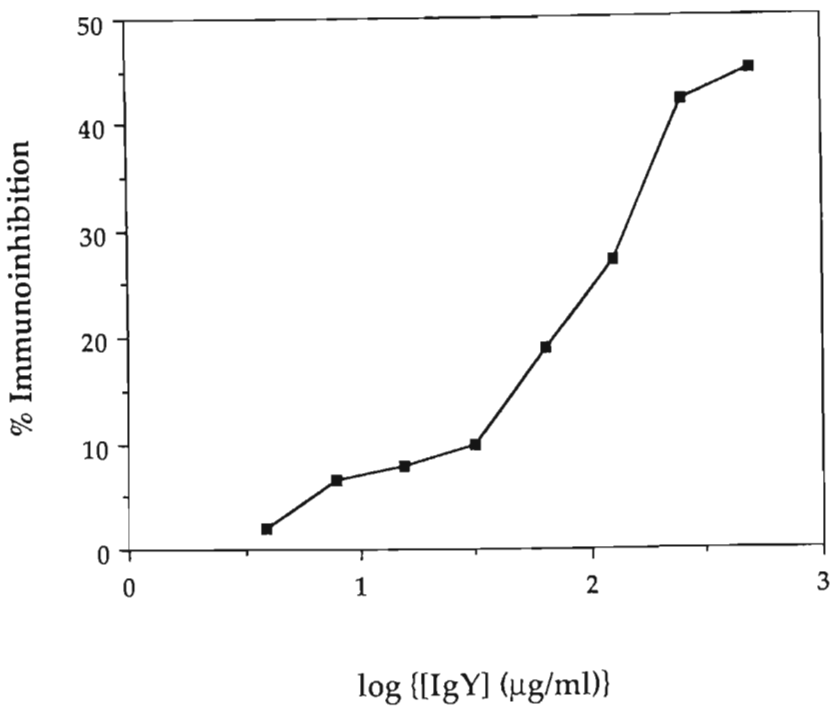

FIGURE 4

Immunoinhibition of sheep cathepsin L by chicken anti-cathepsin L antibodies.
Sheep cathepsin L (5ng) was incubated with decreasing amounts of chicken anti-cathepsin L IgY (■) and the cleavage of Z-Phe-Arg-NHMec was monitored in a stopped time assay. The percentage inhibition was calculated relative to control assays with non-immune IgY.

Targeting of active site-associated peptide by chicken and rabbit polyclonal antibodies

Immunoinhibitory anti-peptide antibodies against cathepsin L were previously produced by the inoculation of rabbits with peptide L153-165 from the active site of cathepsin L (14). The ability of the chicken and rabbit polyclonal antibodies to target this peptide was, therefore, of interest, in order to determine whether this peptide was a naturally occurring immunoinhibitory epitope. Figure 5 shows that, while the immunoinhibitory chicken antibody targeted the peptide immobilised on an ELISA plate fairly strongly (Fig. 5A), the non-inhibitory rabbit antibody showed no binding to the peptide at all (Fig. 5B). No targeting of the peptide was observed with any of the rabbit antibody preparations, whereas the four chicken antibody preparations showed comparable targeting of the peptide. The correlation between the binding of the active site-associated peptide [known to generate immunoinhibitory antibodies (14)] by the chicken antibodies, and the inhibitory properties of these antibodies, suggests that they may exert at least part of their inhibition through the binding of this peptide

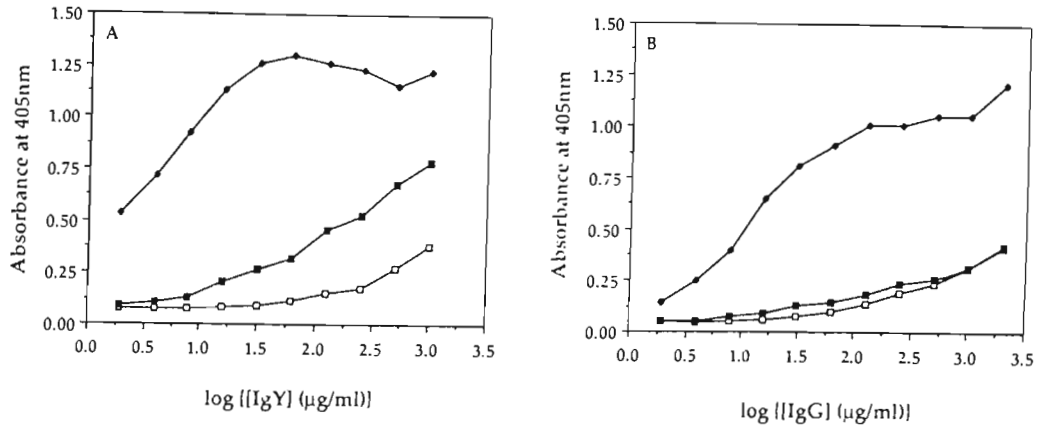

FIGURE 5

Binding of chicken and rabbit anti-cathepsin L antibodies to an active site-associated synthetic peptide in an ELISA.
The cathepsin L active site-associated synthetic peptide was coated to microtiter plates at 1 µg/ml and incubated with serial two-fold dilutions of, **A**) rabbit, and **B**) chicken antibodies. Rabbit anti-whole cathepsin L IgG (■), rabbit anti-L153-165 peptide IgG (◆) and non-immune rabbit IgG (❑) were visualised using sheep anti-rabbit-HRPO conjugate. Chicken anti-whole cathepsin L (■), chicken anti-L153-165 peptide IgY (◆) and non-immune IgY (❑) were visualised using rabbit anti-chicken-HRPO conjugate.

in the native protein. This suggests that the peptide may be a natural epitope for chicken antibody production.

Inhibition of immunoinhibition, by the active site-associated peptide

In order to determine whether binding of the active site-associated peptide in the ELISA format was relevant in terms of the manner in which the chicken anti-cathepsin L antibodies exerted their immunoinhibitory effect, the inhibition by the free peptide of this immunoinhibition was investigated. Since all four chicken antibody preparations, raised in separate inoculation studies, showed very similar immunoinhibitory characteristics and targeted the peptide in an ELISA to the same extent, antibodies, from one chicken from each inoculation study, were used for this investigation. The limited amount of synthetic peptide available further necessitated the use of only representative antibody samples. A typical result is shown in Fig 6. Prior incubation of the IgY with the active site-associated peptide, reduced immunoinhibition of native cathepsin L by up to 35% (Fig. 6), showing that binding of the peptide in the whole enzyme is, at least in part, reponsible for immunoinhibition. These results complement those obtained in the ELISA, and provide further evidence that this

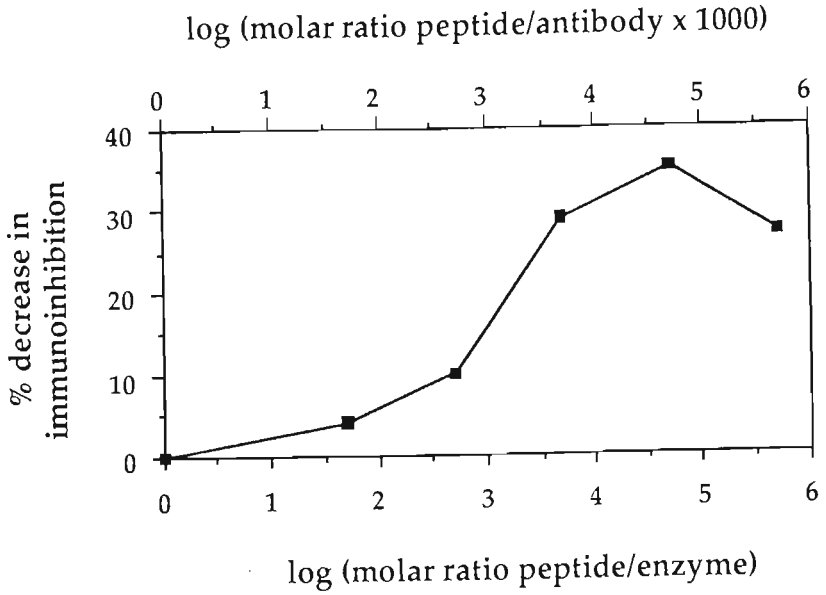

FIGURE 6

Inhibition, by an active site-associated peptide, of the immunoinhibition by chicken anti-cathepsin L IgY.
Chicken anti-cathepsin L (0.125 mg/ml final concentration) was incubated with increasing amounts of synthetic peptide (15 min, 30°C), before cathepsin L (5 ng) was added, incubated for a further 15 min at 30°C and the cleavage of Z-Phe-Arg-NHMec monitored on a continuous basis. In control assays immune IgY was replaced by non-immune IgY. Inhibition by the peptide of immunoinhibition by chicken anti-cathepsin L IgY was calculated relative to assays in the absence of the peptide.

peptide, in the enzyme, may be part of an immunoinhibitory epitope, which is immunogenic in chickens.

DISCUSSION

In an attempt to expand on the immunoinhibition of cathepsin L previously obtained with anti-peptide antibodies (14), the difference between chicken and rabbit polyclonal antibodies, with regard to their targeting of the enzyme, was investigated. The hypothesis that sheep cathepsin L may be more immunogenic in chickens than in rabbits, due to a greater evolutionary distance from the sheep, seems to be borne out in the results of the ELISA tests, since the chicken antibodies had approximately 10-fold higher titers than their rabbit counterparts. These results contrast with the studies of Polson *et al.* (12), in which a poor antibody response was found to smaller molecules (<70,000kDa) in chickens. This contrast

might, in part, be due to their method of testing, i.e. immunoprecipitation in a gel matrix, since we have found that antibodies against cathepsin L, from both rabbits and chickens, do not precipitate the antigen in Ouchterlony tests. This is in contrast to strong reactions obtained in more recently developed tests such as ELISA or Western blotting. Thus previous results obtained by Polson *et al.* (12) might be due to their antibodies being poorly immunoprecipitating, rather than low titer antibodies *per se*.

Binding of an antibody to an enzyme may have one of three outcomes with respect to enzyme activity: firstly, it may have no effect; secondly, it may lead to immunoinhibition of the enzyme, either as a result of a distortion of the structure of the enzyme, or by impairing its flexibility, or by binding to an amino acid sequence in the active site, or by occluding the active site. Thirdly, it may lead to immunoactivation (7), possibly by changing the conformation of the enzyme, thereby enhancing substrate binding.

The chicken antibodies seem to target different epitopes from the rabbit antibodies, reflected, in this instance, by their immunoinhibitory characteristics compared to the rabbit antibodies, which showed immunoactivation of the enzyme. A decrease in the activity of an enzyme after incubation with an antibody, particularly using polyclonal antibodies, as opposed to peptide or monoclonal antibodies, might be the result of either immunoprecipitation of the enzyme, or immunoinhibition due to specific targeting of an epitope in the active site of the enzyme. In the case of the results presented here, the decrease in activity brought about by the chicken antibodies is most likely due to immunoinhibition, since, as mentioned above, they do not immunoprecipitate the enzyme in an Ouchterlony test. The amount of enzyme used to test for immunoinhibition in the synthetic substrate assay is probably also too small to generate the lattice necessary for immunoprecipitation (7) and the molar excess of antibody over enzyme used in this assay will also not favour the formation of insoluble complexes. Immunoinhibition, independent of immunoprecipitation has been reported for a monospecific antiserum to acetyl-CoA carboxylase (22) and mouse liver lactate dehydrogenase (23).

The immunoinhibitory ability of the chicken antibodies seems to be derived, at least in part, by their ability to target the active site-associated peptide. This is illustrated by the binding of chicken anti-sheep cathepsin L IgY to the peptide L153-165 in an ELISA and, arguably, more conclusively by inhibition of the immunoinhibition by prior incubation of the antibodies with the peptide. This suggests that this peptide is not only able to elicit immunoinhibitory antibodies by "artificially" inducing an immune response via the anti-peptide antibodies approach, but is also a natural, immunogenic epitope for the chicken. Since there is often only a small degree of antigenic cross-reactivity between peptides and antibodies, raised against the

corresponding whole protein, large molar excesses of peptide to the enzyme are required to show binding of the peptide (24). Cross-reactivity, as evidenced by a reduction in immunoinhibition of up to 35%, could be illustrated using peptide: cathepsin L molar ratios of 500 000: 1 to 50 000: 1. It is furthermore envisaged that the linear peptide could adopt a number of conformations in solution, and a molar excess of peptide over antibody might therefore be required to maximise the probability of it adopting the same conformation that it has in native cathepsin L, to ensure optimal epitope-paratope interaction. The peptide: cathepsin L molar ratios (shown above), which caused a reduction in immunoinhibition, corresponded to peptide: antibody ratios of 125: 1 to 1.25: 1.

Although complete inhibition of the immunoinhibition was not obtained with the active site-associated peptide, the degree of blocking of the paratope by the peptide suggests that the immunoinhibitory characteristics of the chicken antibody is, in part, due to targeting of this sequence in the whole native cathepsin L. Most epitopes are discontinuous, rather than continuous (25) and these results may indicate that this linear active site-associated peptide sequence forms an integral part of a larger discontinous epitope, hence showing only partial inhibition of the immunoinhibition. Further studies are required to elucidate the precise immunoinhibitory targets of the chicken antibodies on the enzyme.

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