

Fasciola hepatica secretion of a novel cathepsin L proteinase

Thesis

Presented for the Degree of

Doctor of Philosophy

by Andrew Dowd B Sc , M Sc

under the supervision of John P Dalton, B Sc , Ph D

School of Biological Sciences

Dublin City University

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Andrew Dowd

Andrew Dowd

Date: 17/8/94

Date: 17/8/94

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To my parents and family

Table of Contents

ABSTRACT	1
ABBREVIATIONS	2
1 0 INTRODUCTION	4
1 1 Proteinases	5
1 1 1 Serine proteinases	5
1 1 2 Aspartic proteinases	7
1 1 3 Metalloproteinases	7
1 1 4 Cysteine proteinases	7
1 2 Mammalian cathepsins	8
1 2 1 Cathepsin B	15
1 2 2 Cathepsin D	18
1 2 3 Cathepsin E	19
1.2 4 Cathepsin H	20
1 2 5 Cathepsin J	21
1 2 6 Cathepsin L	22
1 2 7 Cathepsin M	25
1 2 8 Cathepsin N	26
1 2 9 Cathepsin S	27
1 2 10 Cathepsin T	29
1 3 Parasitic cathepsins	29
2 0 MATERIALS AND METHODS	41
2 1 Materials	42
2 2 Source of <i>Fasciola hepatica</i> cysteine proteinases	44
2 3 Enzymatic assays with fluorogenic substrates	44
2.4 Purification of <i>Fasciola hepatica</i> cysteine proteinase	45

2 5	SDS-polyacrylamide gel electrophoresis (SDS-PAGE)	46
2 6	Zymography	46
2 7	Protein concentration	46
2 8	Amino-terminal sequence analysis	46
2 9	Kinetic studies	47
2 10	Active site titration of cathepsins L1 and L2	47
2 11	Direct fluorogenic substrate analysis in polyacrylamide gels	47
2 12	Nitration of cysteine proteinases with tetranitromethane	48
2 13	Preparation of antibody to cathepsin L2	48
2 14	Preparation of mature <i>Fasciola hepatica</i> extract	49
2 15	Immunoblotting analysis	49
2 16	<i>In vitro</i> clotting assay	49
2 17	Electrophoretic analysis and immunoblotting of fibrin clots	50
2 18	Fibrin plate assay	50
2 19	Studies of fibrin clots with prior addition of fibrin anti-polymerants	51
2 20	Studies of enzyme activity with prior addition of fibrin anti-polymerants and proteinase inhibitors	51
2 21	Positioning of the cleavage site of thrombin and cathepsin L2 on fibrinogen	52
3 0	RESULTS PART 1 Purification and characterisation of cathepsin L2 from adult <i>Fasciola hepatica</i>	53
3 1	Purification of cathepsin L2	54
3 2	N-terminal sequence analysis of cathepsin L2	55
3.3	Immunoblotting studies	56

4 0	RESULTS PART 2 Comparison of <i>Fasciola hepatica</i> cathepsin L1 and cathepsin L2 activities	63
4 1	Kinetic studies of cathepsin L1 and L2	64
4 2	Active site titration of cathepsin L1 and L2	65
4 3	Direct fluorogenic substrate PAGE analysis	65
4 4	Nitration of cathepsin Ls with tetranitromethane	66
5 0	RESULTS PART 3 Fibrin clot formation by <i>Fasciola hepatica</i> cathepsin L2 comparison with the clot produced by bovine thrombin	70
5 1	Fibrin clot formation by cathepsin L2	71
5 2	Electrophoretic analysis of fibrin clots	72
5 3	Positioning of the cleavage sites of thrombin and cathepsin L2 on fibrinogen	73
6 0	DISCUSSION	83
7 0	BIBLIOGRAPHY	110
	APPENDICES	132

ABSTRACT

A 29.5 kDa cysteine proteinase was purified from medium in which mature *Fasciola hepatica* parasites were maintained. The N-terminal sequence (14 amino acid residues) of the purified protein is homologous to known cathepsin L proteinases, including a 27 kDa cathepsin L proteinase, also secreted by this parasite, which had been isolated previously in our laboratory (Smith *et al* , Mol Biochem Parasitol.,62, 1-8, 1993). The N-terminal sequences of the 29.5 and 27 kDa cathepsin L proteinases differ only in residue seven (arginine and proline, respectively). Immunoblot studies, using antiserum that reacts with both cathepsin Ls, rule out the possibility of both enzymes arising from a higher molecular sized parent molecule. The reaction kinetics of the two *F. hepatica* cathepsin Ls on a variety of peptide substrates revealed that the two enzymes differ in their substrate specificity. Five peptide substrates that are cleaved with high affinity by the 29.5 kDa cathepsin L isolated in this study are not cleaved by the previously purified 27 kDa cathepsin L. The protein modifying reagent, tetranitromethane, affected the 29.5 kDa cathepsin L proteinase only, causing inactivation of the enzyme and changing its migration in polyacrylamide gels. Our studies suggest that the two *F. hepatica* cysteine proteinases represent two distinct subclasses within the cathepsin L class. The 29.5 kDa cathepsin L can cleave fibrinogen and produce a fibrin clot *in vitro*. This is the first demonstration of a cysteine proteinase with the ability to clot fibrinogen. The mechanism of clot formation of the cathepsin L was compared to bovine thrombin. SDS-PAGE analysis of clots showed differences in enzyme activities between the two proteinases. Cathepsin L was also not inhibited by the thrombin specific inhibitor hirudin. Studies using peptides which prevent fibrin polymerisation showed that the fibrin clot formed by cathepsin L polymerises in a different manner to the clot formed by thrombin. Clotting time assays in the presence of anti-polymerisation peptides and SDS-PAGE analysis of these clots showed that the cathepsin L fibrinogen cleavage site was located to the C-terminal of the thrombin cleavage site. Therefore *F. hepatica* cathepsin L forms a fibrin clot by a novel mechanism and may represent a means whereby *F. hepatica* may prevent excessive blood loss while migrating through host tissue.

List of Abbreviations

AFC	7-amino-4-trifluoromethylcoumarin
BCA	bicinchoninic acid
Boc	t-butyloxycarbonyl
Bz	benzoyl
CBZ	benzyloxy carbonyl
CHN ₂	diazomethyl ketone
cDNA	complementary DNA
DEAE	diethylaminoethyl
E-64	L-3-carboxy-2,3 <i>trans</i> epoxypropionyleucyl amido (4-guanidino) butane
E-64-d	ethyl (2S,3S)-3-(s)-3-methyl-2-(-3-methylbutyl-carbamoyl oxirane-2-carboxylate)
EDTA	ethylenediaminetetraacetic acid
<i>g</i>	acceleration due to gravity
HEPES	N-[2-hydroxyethyl]piperazine N'[2-ethane sulphonic acid]
HPLC	high performance liquid chromatography
k_{cat}	first order rate constant (turnover number)
K_m	Michaelis-Menten term ($(k_{-1} + k_2) / k_1$)
MBNA	4-methoxy- β -naphthylamide
NNap	2-naphthylamine
NHMec	7-amido-4-methyl-coumarin
NIH	National Institutes of Health
PBS	phosphate buffered saline
PAGE	polyacrylamide gel electrophoresis

PMSF	phenylmethanesulphonyl fluoride
pI	isoelectric point
pro-UPA	urokinase-type plasminogen activator
RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulphate
Suc	succinyl
TAT	tyrosine aminotransferase
TIMP	tissue inhibitor of metalloproteinases
TNM	tetranitromethane
Tris	tris-(hydroxy-methyl)-methylamine(2-amino hydroxymethyl) propane-1,3-diol
TWEEN 20	polyoxymethylenesorbitan monolaurate
Tos	tosyl
QAE	quaternary aminoethyl
Z	benzyloxy carbonyl

CHAPTER 1

INTRODUCTION

1.1 PROTEINASES

Proteolytic cleavage of peptide bonds is one of the most important enzymatic modifications of proteins. Investigations of kinetics, substrate specificity, inhibition, amino acid composition and X-ray structural data have led to the identification of the components of their active sites, and from these studies the mechanism of action of the best characterised proteinases were deduced. As a result it became evident that proteinases can be classified into families, members of each family having similar structures and mechanisms of action. Four mechanistic classes are recognised by the International Union of Biochemistry, and within these classes, six families of proteinases are recognised to date. These classes are as follows: serine proteinases I and II, cysteine proteinases, aspartic proteinases, and the metalloproteinases I and II (Neurath, 1989).

1.1.1 SERINE PROTEINASES

The serine proteinases include two distinct families: the mammalian serine proteinases (e.g. chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4) and elastase (EC 3.4.21.11)) and the bacterial serine proteinases (e.g. subtilisin (EC 3.4.21.14)). Serine proteinases contain an essential serine residue at the catalytic site. The hydroxyl group of this serine attacks the carbonyl group of a peptide bond to form a tetrahedral intermediate prior to hydrolysis (Figure 1.1). This intermediate is stabilised by hydrogen bonding of the oxygen anion. Deacylation of the intermediate is catalysed by the addition of a proton from the imidazole group of a histidine. These amino acids are essential for hydrolysis.

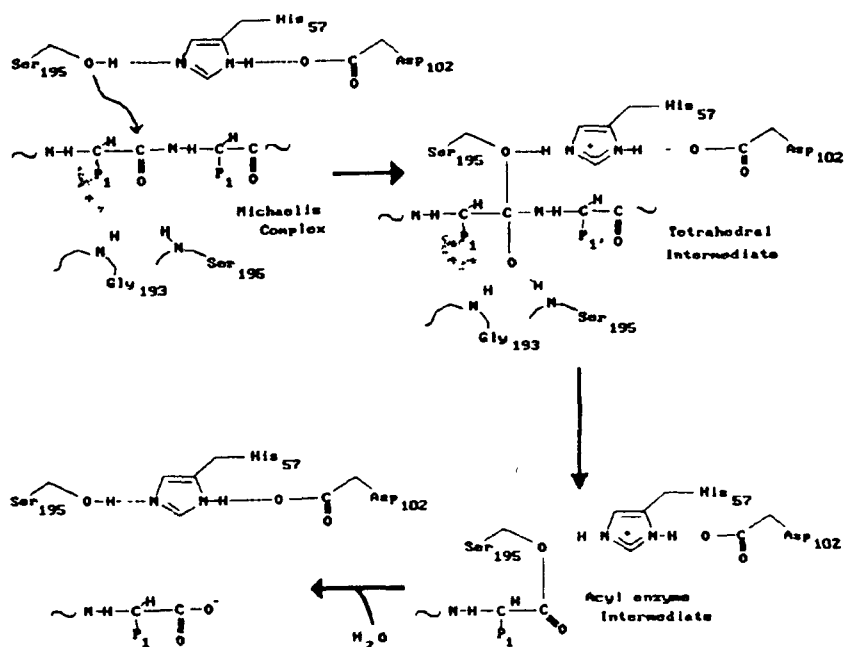


Figure 1.1

Schematic representation of the steps involved in catalysis by the serine protease type of enzyme.

The reaction proceeds through formation of a tetrahedral intermediate followed by loss of the right-hand half of the substrate to give an acyl enzyme intermediate. Breakdown of that intermediate occurs by enzyme-catalysed attack of water to generate the product acid (Taken from Dunn, 1989).

1.1.2 ASPARTIC PROTEINASES

The major intracellular mammalian aspartic proteinases are cathepsin D and rennin. The precise mechanism of action of aspartic proteinases is not clear, but may be a simple acid-base type of mechanism at acidic pH involving the carboxyl side chains of the two essential aspartic acid residues (Figure 1.2). A potent inhibitor of aspartic proteinases is pepstatin, which is a hexapeptide that in the transition state resembles normal substrates.

1.1.3 METALLOPROTEINASES

This class of enzymes includes the matrix metalloproteinases that are inhibited by a protein called TIMP (tissue inhibitor of metalloproteinases) and plasma membrane-bound metalloproteinases such as endopeptidase 24.11 (enkephalinase) and meprin, for which no natural proteinase inhibitors are known. Metalloproteinases coordinate a zinc ion with the aid of glutamic acid and histidine residues. It has been proposed that the zinc ion acts as an electrophile to enhance the reactivity of the carbonyl group of the amino acid whose peptide bond is to be hydrolysed to facilitate nucleophilic attack by a water molecule (Figure 1.3).

1.1.4 CYSTEINE PROTEINASES

The major mammalian cysteine proteinases are the cytoplasmic calpains and the lysosomal cysteine proteinases, e.g. cathepsins B, H and L (c.f. part 1.2). The calpains are calcium dependent proteinases that consist

of two subunits of 80 kDa and 30 kDa. One portion of the larger subunit carries a sequence that shows a small degree of identity with papain, but the degree of identity is insufficient to show whether or not they are evolutionally related. All cysteine proteinases have a catalytic triad consisting of essential cysteine, histidine and asparagine residues. The mechanism of action is similar to that of serine proteinases except that the sulphonium ion of the cysteine provides the nucleophilic attack on the carbonyl group of the peptide bond (Figure 1.4). Both calpain and the lysosomal cysteine proteinases are inhibited by the class-specific inhibitor E-64, and the more general inhibitor, leupeptin.

1.2 MAMMALIAN CATHEPSINS

The mammalian cathepsins are lysosomal proteinases and are present in all cell types with the exception of enucleated red blood cells. These enzymes are cysteine proteinases with two exceptions, cathepsins D and E which belong to the aspartic class. The mammalian cathepsins are mainly involved in protein turnover in tissues and may be the most active proteinases in the body, even more active than the digestive pancreatic serine proteinases (Bond and Butler, 1987). Of the lysosomal enzymes, the aspartic proteinase, cathepsin D, seems to have rather a restricted activity on proteins, whereas the cysteine proteinases have broad activity (Bond and Butler, 1987).

The amino acid sequences of some of the cysteinyl cathepsins have been elucidated and it is now known that these enzymes have evolved from the same ancestor as the plant cysteine proteinase, papain and are grouped

together into the papain superfamily (Rawlings and Barrett, 1993, Neurath, 1989, Bond and Butler, 1987) The sequences of these enzymes are divided into three regions, two of which are highly conserved These two highly conserved regions are the amino-terminal or cysteinyl active site region and the carboxy terminal or histidine active site region The amino terminal region contains the cysteine rich site with the amino acid sequence, NH₂-C-G-S-C-W-COOH This five amino acid motif is conserved among all members of the papain superfamily The third or central region is not as conserved as the other two regions

Despite some similarities, these enzymes may be differentiated and assigned to a particular class Table 1.1 shows all the different types of cathepsins characterised to date compared according to their type of activities, substrate specificities, molecular weights, isoelectric points and pH optima There are ten different classes of lysosomal cathepsin enzyme characterised to date (cathepsins B, D, E, H, J, L, M, N, S and T) The best characterised cathepsins are cathepsin B, H, L and S Cathepsins J, M, N and T are the least well characterised enzymes and they have as yet not been assigned enzyme classification (EC) numbers

Point mutation studies of cloned lysosomal cysteine proteinases showed that the substrate specificity of these enzymes resides in the S₂ subsites of their active sites (Schechter and Berger nomenclature, Schechter and Berger, 1967, Bromme *et al* , 1994) For example, a single amino acid substitution (glycine to alanine) within position 133 of the S₂ site caused the specificity of cathepsin S to change to cathepsin L- activity (Bromme *et al.*, 1994) Another amino acid substitution (phenylalanine to glutamate) in position 205 changed the specificity from cathepsin S-like to B-like (Bromme

et al , 1994)

In the sections below the properties of each class of these enzymes will be discussed

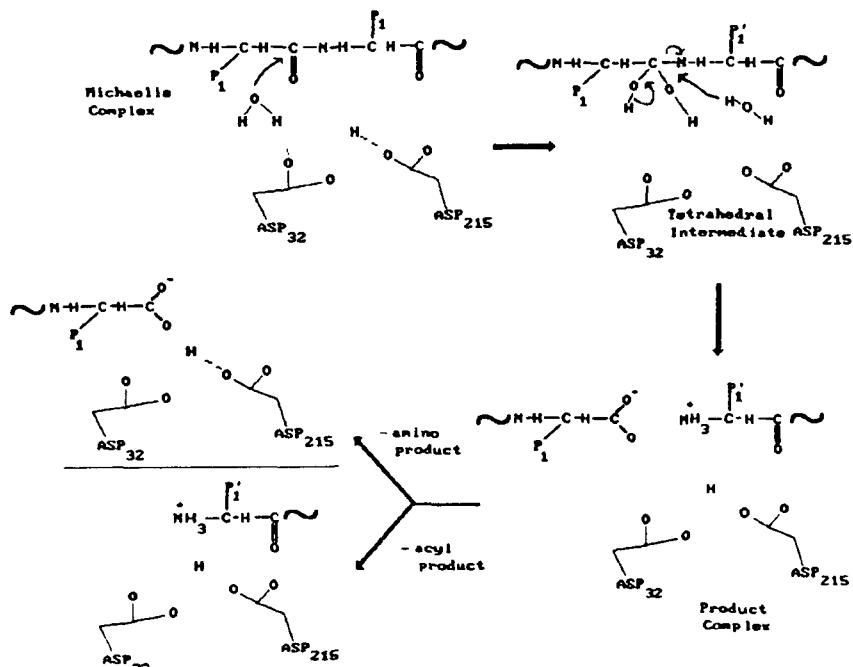


Figure 1.2

Schematic representation of the general acid-general base catalytic mechanism of the aspartic peptidase type of enzyme

Breakdown of the tetrahedral intermediate gives a product complex containing both halves of the substrate, and this scheme indicates that dissociation of either can follow to give an acyl product complex or an amino product complex. (Taken from Dunn, 1989)

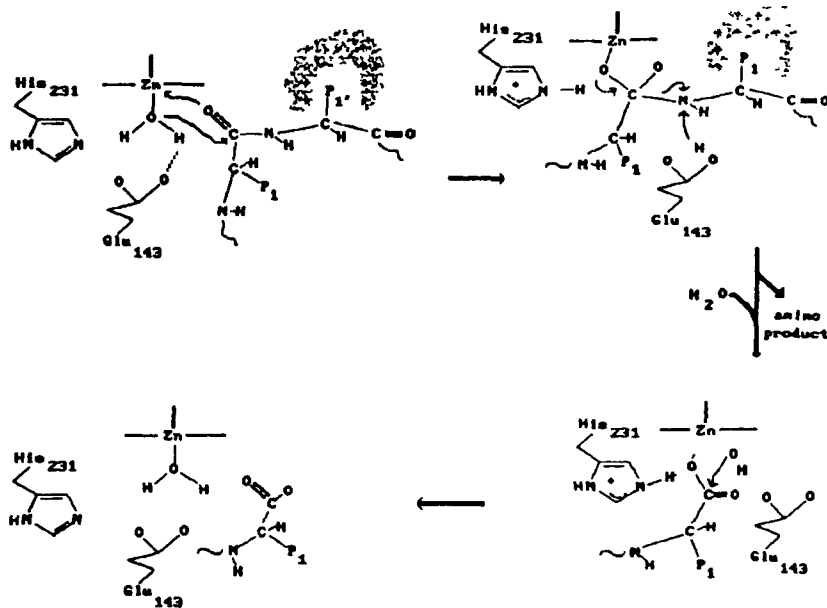


Figure 1.3

Schematic representation of the catalysis of peptide bond cleavage carried out by a member of the metallo-peptidase class of enzyme (Taken from Dunn, 1989)

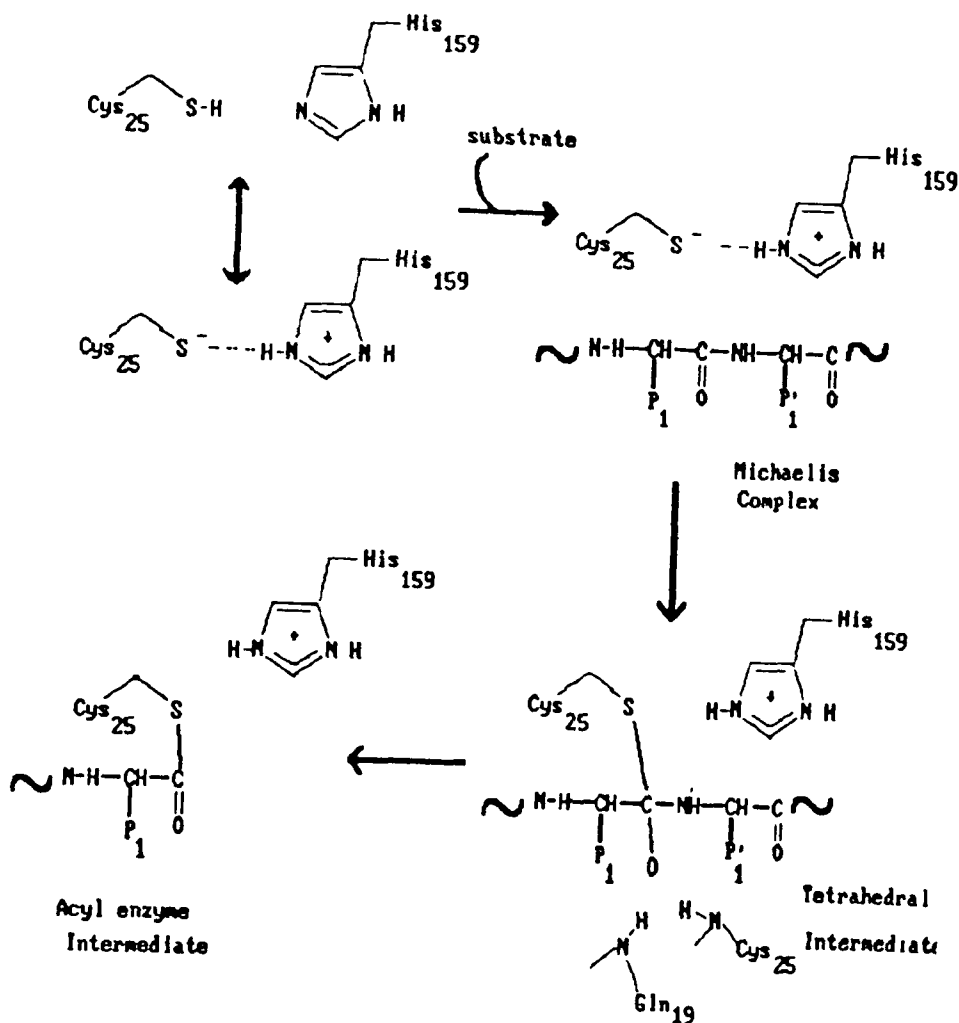


Figure 1.4

Schematic representation of the steps involved in catalysis by the cysteine peptidase type of enzyme

The catalytic Cys is involved in a tautomeric equilibrium between the neutral and zwitterionic forms. It is believed that the anionic sulphur is involved in direct nucleophilic attack on the substrate carbonyl. This scheme represents only the reaction through the acyl enzyme intermediate. Breakdown of that again involves enzyme-catalysed attack of water (Taken from Dunn, 1989)

TABLE 1.1

The mammalian cathepsins The properties of each type of enzyme is summarised below

Class	Type of activity	Preferred substrate	pH optimum	pI	Mol. Wt (kDa)
B	cys	Z-arg-arg-NHMec	6	5-5.2	30*
D	asp	haemoglobin	3-5	5.5-7.5	50*
E	asp	albumin	2.5	n.d.	100
H	cys	H-arg-NHMec	6.8	6-7	28*
J	cys	gly-arg-NNap	5-7.5	5.7	160
L	cys	Z-phe-arg-NHMec	4.5-6	5-6.3	30*
M	cys	aldolase	5-7	n.d.	30
N	cys	collagen	3.5	6.2	34
S	cys	Z-val-val-arg-NHMec	6-7.5	7	24
T	cys	TAT, azocasein	6	n.d.	35

* Denotes molecular weight of heavy plus light chains

n d Not determined

1.2.1 CATHEPSIN B (EC 3.4.22.1)

Cathepsin B is the most abundant lysosomal cysteine proteinase present in mammalian tissues (Barrett and Kirschke, 1981) Cathepsin B has been purified from human liver (Barrett, 1973), calf liver (Snellman, 1969), bovine spleen (Etherington, 1974), rabbit skeletal muscle (Okitani *et al* , 1988) and rabbit spleen (Maciewicz and Etherington, 1988)

The cDNA encoding human and mouse cathepsins B has been cloned and sequenced (Chan *et al* , 1987) It was found that the cDNA sequences of procathepsins B of mouse, human and rat were relatively highly conserved with a minimum of 68% sequence identity (Chan *et al* , 1987) The amino acid sequences of cathepsin B, H, L and papain were compared by Dufour (1988) It was observed that rat cathepsin B was 31.5% homologous to rat cathepsin H, 32% homologous to avian cathepsin L and 30% homologous to papain (Dufour, 1988)

From the results of protein purification and cDNA cloning studies it is now known that mammalian cathepsin B is composed of two chains, a heavy chain of 25 kDa and a light chain of 5 kDa (Mason, 1991). A single-chain enzymatically active enzyme of 29 kDa can coexist with the two chain molecule (Barrett and Kirschke, 1981) Human cathepsin B has different isoforms with pI values in the pH 4.5-5.5 range The predominant isoform has a pI of 5.0-5.2 (Barrett, 1977) Cathepsin B is a glycoprotein but it does not bind Concanavalin A-Sepharose (Barrett and Kirschke, 1981) Cathepsin B was found from amino acid sequencing to have one potential glycosylation site (Meloun *et al* , 1988)

Recent studies of rat cathepsin B show that it is synthesised as a

precursor of 39 kDa and then processed to a single chain form of 29 kDa and eventually processed to the stable two chain form (Hara *et al* , 1988, Nishimura *et al* , 1988, Mach *et al* , 1993, 1994a,1994b) It was originally thought that cathepsin D catalysed the conversion of cathepsin B to the mature form (Nishimura *et al* , 1988) Hara *et al* , (1988) found that the processing and activation of cathepsin B to the single chain form was halted by metalloproteinase inhibitors and the conversion of the single chain to the two chain form was stopped by the cysteine proteinase inhibitor E-64-d Experiments by Rowan *et al* (1992) using cloned procathepsin B expressed in yeast showed that cathepsins D and L as well as mature cathepsin B can produce a processed (single-chain) form of the enzyme from its proenzyme More recent experiments on recombinant cathepsin B found that its proenzyme form was dependent on the processed active enzyme for maturation and activation (Mach *et al* , 1993, 1994a, 1994b)

The usual assay for cathepsin B is the synthetic fluorogenic peptidyl substrate Z-arg-arg-NHMec because the enzyme is active towards synthetic substrates containing arginine in the P₁ position (Barrett and Kirschke, 1981) Cathepsin B cleaves protein substrates *in vitro* such as fibrinogen (Gabrijelcic *et al* , 1988), collagen (Etherington, 1974), haemoglobin, azocasein, proteoglycans, gelatin, IgG and bacterial cell wall proteins (Barrett, 1977). The pH optimum for cathepsin B on most substrates is pH 6 (Barrett, 1972, Snellman, 1969) Cathepsin B is inactivated at alkaline pHs (Barrett and Kirschke, 1981)

From studies of its enzyme activity it was found that cathepsin B has the unusual property among the cathepsin enzymes of acting both as a peptidyl dipeptidase and an endopeptidase enzyme depending on its

substrate For example, human cathepsin B digests the B chain of insulin at 10 endoproteolytic points of cleavage within the polypeptide but cleaves dipeptides from the C-terminus of both glucagon and rabbit muscle fructose-1,6-bisphosphate aldolase (Bond and Butler, 1987). Koga *et al* (1991) investigated the pH dependence of the cathepsin B exo- and endopeptidase activities and found that the exopeptidase activity showed a broad substrate specificity and pH optimum at pH 4-6 while the endopeptidase activity had a sharp pH optimum at pH 6 and showed a preference for substrates with basic amino acid residues in their P₁ sites

Cathepsin B plays a role in both normal metabolic processes For example cathepsin B has been found to process human prorennin to its active form (Wang *et al* , 1991). Renin is the rate limiting enzyme in the generation of angiotensin II which is a major determinant of blood pressure and intravascular volume Therefore cathepsin B in processing of prorennin plays a role in blood pressure and intravascular volume regulation (Wang *et al* , 1991).

Thyroid hormones play an important role in the regulation of metabolism The thyroid hormones form at discrete sites within the prohormone thyroglobulin (Dunn *et al* , 1991) Cathepsin B along with cathepsins D and L process thyroglobulin to release the thyroid hormones by a complex synergistic process (Dunn *et al* , 1991)

Cathepsin B is also implicated in tumour growth and invasion Cathepsin B-like enzymes are released extracellularly by neoplastic epithelial cells (Olstein and Liener, 1983) Sloane *et al* (1982) have reported that a close correlation exists between the cathepsin B activity of variants of a murine melanoma tumour and their metastatic potential Kobayashi *et al.*,

(1991) demonstrated that cathepsin B activated the tumour cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (pro-uPA). The presence of uPA activity is associated with the invasive potential of tumour cells.

1.2.2 CATHEPSIN D (EC 3.4.23.5)

Cathepsin D, is a lysosomal aspartic proteinase. This enzyme was purified from both porcine and bovine spleens by conventional purification methods (Takahashi and Tang, 1981).

Cathepsin D can be separated by isoelectric focusing into five iso-enzymes (I to V) with pIs ranging from pH 5.5-7.5. Iso-enzymes I-IV are two chain enzymes of 50 kDa (35 kDa heavy chain, 15 kDa light chain). Iso-enzyme V is a 100 kDa protein known as high molecular weight (HMW) cathepsin D. Bovine cathepsin D has two iso-enzymes (A and B) separable by isoelectric focusing. Isoenzyme A has a pI of 6.49 and isoenzyme B has a pI of 6.04 (Takahashi and Tang, 1981). Both A and B iso-enzymes are two chain proteins of 46 kDa (34 kDa heavy chain, 12 kDa light chain).

Cathepsin D is a glycoprotein that binds to the lectin affinity resin Concanavalin A-Sepharose (Takahashi and Tang, 1981).

The proteolytic activity of cathepsin D is monitored by the cleavage of acid denatured haemoglobin at pH 3.2 (Takahashi and Tang, 1981, Bond and Butler, 1987). Cathepsin D is also assayed using the synthetic renin substrate, tetradecapeptide (TDP) $\text{NH}_2\text{-asp-arg-val-tyr-ile-his-pro-phe-his-leu-leu-val-tyr-ser-COOH}$. Angiotensin I (N-terminal, 10 residues of TDP) is released and measured by radioimmunoassay (Takahashi and Tang, 1981).

Cathepsin D may also be assayed using the pentapeptide substrate BL-arg-gly-phe-phe-pro-MBNA in a coupled assay where cathepsin D cleaves the peptide substrate to release phe-pro-MBNA which is subsequently cleaved by dipeptidylaminopeptidase II, to release the chromophore MBNA (Ryvnyak *et al* , 1990)

Cathepsin D plays a role in the processing of mature acid phosphatase in the lysosomes (Tanaka *et al* , 1990) Alkaline phosphatase is transported from the site of its synthesis in the endoplasmic reticulum via the Golgi complex to lysosomes as a membrane-bound enzyme , and is released from the lysosomal membranes into the lysosomal matrix in soluble form due to the action of the proteinase (Tanaka *et al* , 1990)

Cathepsin D is implicated in liver cirrhosis because it has the ability to cleave collagen and other components of the extracellular matrix (Ryvnyak *et al* 1991) Using electron histochemistry, Ryvnyak *et al* (1991) found that cathepsin D was secreted by liver cells into the intracellular space during the course of this disease

1.2.3 CATHEPSIN E (EC 3.4.23.34)

Little information is known about cathepsin E. It is similar to cathepsin D in that it is a lysosomal aspartic proteinase It has a molecular weight of 100 kDa and a pH optimum of 2.5 Its usual assay substrate is albumin (Bond and Butler, 1987) Cathepsin E is found in bone marrow polymorphonuclear leukocytes and macrophages It has a high molecular weight for a lysosomal cathepsin enzyme and may be dimeric (Bond and Butler, 1987)

1.2.4 CATHEPSIN H (EC 3.4.22.16)

The lysosomal cysteine proteinase cathepsin H, like cathepsins B, L and S has been extensively studied. Cathepsin H has been purified from human liver (Barrett and Kirschke, 1981) and human kidney (Popovic *et al.*, 1993) using conventional chromatographic methods.

Rat kidney cathepsin H was cloned and the sequence of its cDNA was determined (Ishidoh *et al.*, 1987a). From the deduced amino acid sequence, cathepsin H was 44% homologous to cathepsin L and 30% homologous to papain. Certain residues in the pro-peptide region of cathepsin H were sequenced and found to be highly homologous to the sequences of other cysteine proteinases (Ishidoh *et al.*, 1987a).

Cathepsin H is synthesised as a high molecular weight form (procathepsin H) of 41 kDa, transported to the lysosomes and subsequently processed by proteolytic cleavage to a single chain molecule of 28 kDa (Hara *et al.*, 1988). It may be eventually processed to the two chain form with a heavy chain of 23 kDa and a light chain of 5 kDa (Hara *et al.*, 1988). Cathepsin H is a glycosylated protein as it binds Concanavalin A-Sepharose (Barrett and Kirschke, 1981). From the results of molecular cloning of cathepsin H cDNA it was found that it possesses two potential glycosylation sites (Ishidoh *et al.*, 1987a).

Cathepsin H isolated using conventional chromatographic methods was found to have a molecular weight of 28 kDa. Human liver cathepsin H has two isoforms of pI 6.0 and 6.4 (Barrett and Kirschke, 1981). Affinity purified human kidney cathepsin H has two isoforms of pI 6.1 and 6.3 with molecular weights of 30 kDa (Popovic *et al.*, 1993). Rabbit lung cathepsin H

has several isoforms in the range 5.8-6.5 and the rat liver enzyme has a pI of 7.1 (Barrett and Kirschke, 1981). In order to assay for cathepsin H, either the unblocked fluorogenic peptide arg-NHMeC or the blocked fluorogenic substrate Z-arg-NHMeC may be used because cathepsin H has the unusual property in that it can act both as an endopeptidase and an aminopeptidase *in vitro* (Barrett and Kirschke, 1981). When the enzyme is tested against synthetic substrates it has a pH optimum of pH 6.8 but is more active at pH 5-6 against azocasein (Barrett and Kirschke, 1981).

1.2.5 CATHEPSIN J

Cathepsin J is a high molecular weight (160 kDa) lysosomal cysteine proteinase (Nikawa *et al* , 1992) consisting of two subunits α and β . The α subunit is a glycoprotein of 19-24 kDa and the β subunit, which is also a glycoprotein, has a molecular weight of 17 kDa.

Cathepsin J was purified to homogeneity from the mitochondrial-lysosomal fraction of rat liver by acid treatment, ammonium sulphate precipitation, cation exchange chromatography, lectin affinity chromatography, organomercurial agarose affinity chromatography, anion exchange HPLC and size exclusion HPLC (Nikawa *et al* , 1992). Isoelectric focusing of purified cathepsin J following treatment with sialidase to remove carbohydrate groups resulted in the protein focusing as two bands with pI values of 5.7 and 5.8.

Following purification the N-terminal amino acid sequence of both subunits of cathepsin J were determined. The N-terminal amino acid

sequence of the α subunit was very similar to the sequences of rat cathepsins B, H and L. The sequence of the β subunit was not similar to other cathepsins.

Cathepsin J is stable at slightly acid pHs but has a broad pH range of 5 to 7.5 showing especially high activity at pH 7.5. It cleaves the endopeptidase fluorogenic substrate Z-phe-arg-NHMec in preference to Z-arg-arg-NHMec and arg-NHMec. It also cleaves the exopeptidase substrates gly-arg-NHnap, ala-ala-NHnap and gly-phe-NHnap. Cathepsin J, therefore exhibits dipeptidylaminopeptidase activity as well as endopeptidase activity.

Antiserum against rat liver dipeptidylaminopeptidase I reacted with rat cathepsin J. The cysteine proteinase specific inhibitor E-64 was not as effective at inhibiting both cathepsin J and dipeptidylpeptidase I compared to cathepsins B, H and L. Therefore, cathepsin J shares some similarities to dipeptidylaminopeptidase I (Nikawa *et al*, 1992).

1.2.6 CATHEPSIN L (EC 3.4.22.15)

Cathepsin L is one of the most powerful lysosomal proteinases when assayed against protein substrates (Bond and Butler, 1987). It cleaves extracellular matrix proteins such as collagen and elastin, even more efficiently than collagenase and neutrophil elastase (Mason, 1991). It also cleaves azocasein, insulin B-chain, glucagon, histones and haemoglobin (Barrett and Kirschke, 1981, Kirschke *et al*, 1982, Kargel *et al.*, 1980).

Cathepsin L has been extensively purified and characterised from a wide variety of mammalian sources such as human liver (Mason *et al*,

1985), rat liver (Kirschke *et al* , 1977, Bando *et al* , 1986, Kirschke *et al* , 1982), rabbit liver (Mason *et al* , 1984), mouse fibroblasts (Mason *et al* , 1987) and chicken liver (Dufour *et al* , 1987) The pro-form of mouse fibroblast cathepsin L has also been purified (Gal and Gottesman, 1986)

The cDNA encoding rat cathepsin L (Ishidoh *et al.*, 1987b), mouse pro-cathepsin L (Troen *et al* , 1987, Portnoy *et al* , 1986), human pro-cathepsin L (Gal and Gottesman, 1988) and human cathepsin L (Chauhan *et al* , 1993) have been isolated and sequenced

It was found that rat cathepsin L shared 45 and 25% amino acid identity with rat cathepsins H and B respectively which was in agreement with the previous results of homology using direct amino acid sequencing (Ishidoh *et al* , 1987b)

Gal and Gottesman (1988) cloned and sequenced the cDNA encoding the major excreted protein from malignantly transformed mouse fibroblasts They found that it was the pro-form of cathepsin L as it exhibited 98% N-terminal amino acid sequence similarity with human cathepsin L directly sequenced from purified protein by Mason *et al* (1986) Sequencing analysis of the cDNA encoding the major excreted protein (MEP) of malignantly transformed mouse fibroblasts (Troen *et al.*, 1987) and a mouse cysteine proteinase (Portnoy *et al* , 1986) identified the protein as the same molecule, mouse pro-cathepsin L It was found that this protein was 48% homologous to rat cathepsin H (Portnoy *et al* , 1986) and 80% homologous to human cathepsin L (Troen *et al* , 1987)

Chauhan *et al* (1993) cloned human cathepsin L and found that there were two forms of this protein expressed from two distinct mRNAs in certain cancer cell lines These two mRNAs were encoded for by a single gene

which they mapped to chromosome 9q21-22

Recombinant human cathepsin L has been expressed in the bacterium *Escherichia coli* (Smith and Gottesman, 1989). Deletion mutation experiments demonstrated that the enzyme needs the pro-peptide region for proper folding of the expressed protein and that the disulphide bond between the heavy and light chain of cathepsin L was found to be necessary for enzyme activity.

Cathepsin L is synthesised in the rough endoplasmic reticulum as a pre-pro-form, subsequently transported to the Golgi stack as an inactive pro-form of 39 kDa and finally translocated to the lysosomes of the rat liver (Nishimura *et al*, 1988) or macrophages (Hara *et al*, 1988) where it is processed to the single chain active form of 29 kDa. It is thought that cathepsin D (Nishimura *et al*, 1988) or a metal chelator sensitive enzyme (Hara *et al*, 1988) are responsible for removal of the pro-peptide. Cathepsin L is finally processed to the two chain form of the enzyme by the lysosomal cysteine proteinases (Hara *et al*, 1988). The two chain enzyme is composed of a heavy chain of 25 kDa and a light chain of 5 kDa (Mason *et al*, 1986), or 19.1 kDa and 4.8 kDa when the molecular weight values of the carbohydrate groups are omitted from the calculations (Wada *et al*, 1987).

From the results of purification studies cathepsin L has a pI of 5.8-6.1 (Barrett and Kirschke, 1981). Mason *et al* (1984) found multiple forms of rabbit cathepsin L with pIs from 5.5-9. Multiple forms of human cathepsin L also exist with pIs from 5.7-6.3 (Mason *et al*, 1985).

Cathepsin L is a glycoprotein with an affinity for concanavalin A-Sepharose (Barrett and Kirschke, 1981). Cloning studies showed that cathepsin L has two potential N-glycosylation sites in its heavy chain.

(Ishidoh *et al* ,1987b)

Enzyme kinetic studies of purified cathepsin L using synthetic fluorogenic substrates show that it has a preference for hydrophobic amino acid residues in the P₂ position (Barrett and Kirschke, 1981) Cathepsin L cleaves the synthetic substrate Z-phe-arg-NHMec but not the cathepsin B specific substrate Z-arg-arg-NHMec or the cathepsin H specific substrate arg-NHMec (Mason *et al* , 1984,1985, Barrett and Kirschke, 1981) The diazomethylketone inhibitor Z-phe-phe-CHN₂ is specific for cathepsin L (Bond and Butler, 1987, Barrett and Kirschke, 1981)

Cathepsin L is known as the most unstable lysosomal cysteine proteinase at neutral or alkaline pH (Barrett and Kirschke, 1981, Turk *et al* , 1993, Mason *et al* , 1985) The pH optimum for cathepsin L on fluorogenic peptide substrates is in the pH 4.5-6.0 range (Dufour *et al* , 1987, Mason *et al* , 1984,1985, Kirschke *et al* , 1977)

Cathepsin L may play a role in disease states such as cancer. The secretion and expression of cathepsin L has been shown to be induced by malignant transformation, growth factors and tumour promoters (Chauhan *et al* , 1993) Cathepsin L, if secreted may help tumour cells to metastasise as it cleaves a wide variety of protein substrates (Mason, 1991) Secreted cathepsin L may further cause extracellular matrix degradation by its activation of α_1 -proteinase inhibitor which normally regulates neutrophil elastase (Mason, 1991)

1.2.7 CATHEPSIN M

Cathepsin M is a lysosomal cysteine proteinase which can be

distinguished from the other cysteine proteinases on the basis of its substrate specificity and sensitivity to inhibitors such as antipain, elastinal, chymostatin and leupeptin (Pontremoli *et al* , 1982, Bond and Butler, 1987) Cathepsin M was purified from the soluble fractions of rabbit liver lysosomes using a combination of gel filtration chromatography and cation exchange chromatography (Pontremoli *et al* , 1982)

Cathepsin M has a molecular weight of approximately 30 kDa and is active in the pH 5-7 range Cathepsin M catalyses the limited modification and inactivation of fructose 1,6-bisphosphate aldolase (Pontremoli *et al* , 1982)

A high proportion of cathepsin M (50%) is associated with lysosomal membranes which distinguishes this enzyme from the other lysosomal cysteine proteinases which are 'soluble' within the lysosome (Pontremoli *et al* , 1982)

1.2.8 CATHEPSIN N

Cathepsin N is a lysosomal cysteine proteinase of 34 kDa which is similar in activity to cathepsin L (Bond and Butler, 1987) This enzyme cleaves N-terminal peptides of native collagen and is sometimes termed 'collagenolytic cathepsin' (Bond and Butler, 1987)

Cathepsin N was separated from cathepsins B, H, L and S present in Triton X-100 extracts of rabbit spleens using ammonium sulphate precipitation, ion-exchange chromatography, gel-filtration chromatography, hydrophobic interaction chromatography and isoelectric focusing (Maciewicz and Etherington, 1988)

Cathepsin N was found to cleave Z-phe-arg-NHMe better than Z-arg-

arg-NHMec and arg-NHMec but it could be distinguished from cathepsin L on the basis of Bz-phe-val-arg-NHMec cleavage. Cathepsin N cleaves Bz-phe-val-arg-NHMec slightly better than Z-phe-arg-NHMec while cathepsin L cleaves Z-phe-arg-NHMec 20 times better than Bz-phe-val-arg-NHMec (Maciewicz and Etherington, 1988). Cathepsin N has a pH optimum of 3.5 and a pI of 6.2.

Cathepsin N cleaves the collagen α chain with a similar activity to cathepsin L. Both cathepsins S and B do not cleave collagen as well as cathepsins L and N (Maciewicz and Etherington, 1988). In terms of protein hydrolysis, cathepsin N may be distinguished from cathepsin L as it has little activity against azocasein (Bond and Butler, 1987).

1.2.9 CATHEPSIN S (EC 3.4.22.27)

Cathepsin S shares characteristics in common with cathepsin L such as its high endopeptidase activity with native protein substrates (Bond and Butler, 1987, Wiederanders *et al.*, 1992).

Cathepsin S was isolated from bovine (Kirschke *et al.*, 1986) and rabbit spleens (Maciewicz and Etherington, 1988) using conventional chromatographic methods.

The amino acid sequence of bovine spleen cathepsin S was determined using a combination of direct peptide sequencing and cDNA cloning (Wiederanders *et al.*, 1991). Amino acid sequence identities of bovine cathepsin S to human cathepsins L, H and B are 56%, 47% and 31% respectively. Cathepsin S also shares 41% identity with papain.

Human cathepsin S cDNA has been cloned and sequenced. The deduced cDNA sequence of the cathepsin S cDNA shares 85% similarity

with bovine cathepsin S, 57% with human cathepsin L, 41% with cathepsin H and 31% with cathepsin B (Wiederanders *et al*, 1992)

Although cathepsins L and S are closely related molecules, they can be distinguished from each other on the basis of their enzyme activities. Cathepsin S is stable at pH 7 while cathepsin L is not (Kirschke *et al*, 1989, Bromme *et al*, 1989). Cathepsin L is more sensitive than cathepsin S to inhibition by the peptide inhibitor, Z-phe-phe-CHN₂ (Bond and Butler, 1987). Cathepsin S is more sensitive to the inhibitor Z-phe-ala-CHN₂ than cathepsins L or B (Bromme *et al*, 1989). Cathepsins L and S can be distinguished from each other from their different ratios of cleavage of Z-phe-arg-NHMec and Bz-phe-val-arg-NHMec (Bromme *et al*, 1989).

Purified cathepsin S is a single chain protein unlike cathepsins B, H and L (Wiederanders *et al*, 1991). Bovine spleen cathepsin S has a molecular weight of 24 kDa, a pI of 7.0 and a pH optimum on synthetic substrates of pH 6-7 (Kirschke *et al*, 1989). Rabbit spleen cathepsin S is a protein of 30 kDa with a pI of 6.8 (Maciewicz and Ethenngton, 1988). Experiments using lectin affinity chromatography showed that cathepsin S is glycosylated as the protein was retained on these columns (Kirschke *et al*, 1989).

A cDNA encoding human lysosomal cathepsin S has been expressed in yeast (Bromme *et al*, 1993). The recombinant enzyme was processed *in vitro* to yield an active mature enzyme of 24 kDa. The purified enzyme had a pH optimum of 6.5 and an isoelectric point of between pH 8.3 and 8.6 which was about 1.5 pH units higher than for the bovine enzyme. Kinetic substrate data revealed a preference for smaller amino acid residues in the binding subsites S₂ and S₃ of cathepsin S. Like the bovine enzyme, recombinant cathepsin S is characterised by a broader range of pH stability (pH 5-7.5).

than cathepsins B or L

1.2.10 CATHEPSIN T

Cathepsin T from rat liver is a lysosomal cysteine proteinase of 35 kDa and pH optimum of 6 (Bond and Butler, 1987) It can be distinguished from the other lysosomal cysteine proteinases by its ability to act on tyrosine aminotransferase (Bond and Butler, 1987)

1.3 PARASITIC CATHEPSINS

The presence of cathepsin-like proteinases have been recorded for many parasites The best characterised of these enzymes have been studied in trematodes such as *Fasciola hepatica* and *Schistosoma mansoni* and the protozoan parasites from the *Trypanosoma* spp However, other parasites possess these enzymes

Mammalian cathepsin enzymes have been shown to degrade molecules which make up connective tissue, haemoglobin, fibrinogen and immunoglobulins, especially when secreted in disease states such as cancer Therefore, it is possible that similar molecules are involved in the pathogenesis of a number of parasitic diseases Parasite proteinases have been demonstrated to facilitate invasion of host tissues, allow parasites to digest host proteins, help them to evade the host immune response, and prevent blood coagulation (McKerrow, 1989) Therefore these proteins are important molecules in the study of parasitic diseases and have been proposed as potential targets for immunotherapeutic and chemotherapeutic reagents (McKerrow, 1989)

The best studied parasitic cysteine proteinases have been purified to

homogeneity and have been characterised by enzymology studies and protein sequencing. These proteinases have been shown to share enzymatic characteristics and sequence homologies with the mammalian cathepsins. The majority of these proteinases share similarities with either cathepsins B or L.

In the following pages some of the better characterised parasitic cathepsin-like cysteine proteinases are listed. However, it can be seen that there is less information about these molecules in comparison to their mammalian counterparts.

Parasite cathepsin B-like enzymes

The best characterised parasitic cathepsin B-like enzyme has been studied from the trematode *Schistosoma mansoni*, the causative agent of Bilharzia in humans. This molecule of 31 kDa (Sm31) was shown to be the 'haemoglobinase' previously described from adult *S. mansoni* (Felleisen and Klinkert, 1990, Gotz and Klinkert, 1993).

Lindquist *et al* (1986) purified a cysteine proteinase from *S. mansoni* adult worms using a combination of gel filtration chromatography and chromatofocusing. They found that the enzyme had different isoforms of 30-35 kDa depending on the preparation. The enzyme degraded haemoglobin and had a preference for the cathepsin B peptide substrate Boc-arg-arg-NHMec.

Chappell and Dresden (1987) purified a cysteine proteinase from media in which adult *S. mansoni* worms were incubated. The enzyme was isolated using a combination of gel filtration and immunoaffinity chromatography steps. It was found to have a molecular weight of 32 kDa.

and to cleave the cathepsin B fluorogenic substrate CBZ-arg-arg-AFC. The proteinase cleaved haemoglobin released from red blood cells and was activated by reduced glutathione present in sera and red blood cells (Chappell *et al* (1987)).

This *S. mansoni* cathepsin B-like enzyme described above has been cloned and its complete nucleotide sequence elucidated (Klinkert *et al* 1989). The inferred amino acid sequence showed extensive homology to mouse, rat and human liver cathepsins B.

McGinty *et al*, (1993) showed, using novel active-site affinity labels, that adult and juvenile forms of the trematode parasite *F. hepatica*, the causative agent of liver fluke disease in mammals, possess cathepsin B-like enzymes of 25-26 kDa. They also found that these *F. hepatica* proteinases were stable under alkaline conditions, a characteristic not shared with mammalian enzymes.

Heussler and Dobbelaere (1994) confirmed the presence of *F. hepatica* cathepsin B-like enzymes using cDNA cloning studies. They found that this organism possesses two cysteine proteinases which are related to mammalian cathepsins B.

The swine parasitic nematode, *Strongyloides ransomi* was shown to have two cysteine proteinases which were extracted from the larval stage of the parasite at acid pH. The pH optima of these enzymes was 4.8 and their molecular weights were 28 kDa and 32 kDa. These enzymes were cathepsin B-like as they cleaved the substrate Z-arg-arg-NHMec (Dresden *et al*, 1985).

The nematode *Haemonchus contortus*, which is one of the most pathogenic endoparasites of sheep, has a cysteine proteinase termed AC-1

which has been cloned and expressed from adult worms (Cox *et al* , 1990) From the deduced amino acid sequence of almost full-length cDNAs of the proteinase, the enzyme was found to have a molecular weight of 35 kDa It was also determined that the proteinase displayed a 42% identity to human lysosomal cathepsin B which was more homologous to it than either cathepsins H or L Further studies showed that *H contortus* had 5 cysteine proteinases AC-1 to AC-5 which are all cathepsin B-like (Pratt *et al.*, 1990, Pratt *et al* , 1992)

The human protozoan parasite, *Entamoeba histolytica* causes dysentery, ulceration of the colon and invasion of the intestinal wall followed by metastasis to other organs This organism produces a cathepsin B-like enzyme (Lushbaugh *et al* , 1985) This proteinase was originally isolated from freeze-thaw extracts of *E histolytica* using a combination of ion-exchange chromatography, organomercurial agarose affinity chromatography and size exclusion chromatography (Lushbaugh *et al* , 1985) The purified enzyme had a molecular weight of 16 kDa and cleaved the fluorogenic cathepsin B substrate Z-arg-arg-AFC

Luaces and Barrett (1988) purified a cysteine proteinase from *E. histolytica* to homogeneity using a single step affinity chromatography method with an immobilised dipeptide The pure enzyme had a final molecular weight of 26 kDa It was claimed that this enzyme despite cleavage of Z-arg-arg-NHMec in preference to Z-phe-arg-NHMec was not cathepsin B-like due to kinetic and N-terminal sequence differences between it and mammalian cathepsin Bs and its stability at alkaline pHs For this reason, this proteinase was subsequently called 'histolysin'

A cysteine proteinase isolated by Otte and Werries (1989) from *E.*

histolytica homogenate using a combination of gel filtration chromatography, ion exchange chromatography and organomercurial agarose affinity chromatography, was found to be the same enzyme as that isolated by Luaces and Barrett (1988)

Molecular genetic studies of 'histolysin' were performed by Eakin *et al.*, (1990) A 450 base pair fragment representing approximately 70% of the coding region of the enzyme was sequenced. It was found that the predicted amino acid sequence of the enzyme was 45% homologous to chicken cathepsin L and 30 - 40% homologous to other eukaryotic cathepsins B or L which demonstrated that 'histolysin' belongs to the cathepsin family of cysteine proteinases. This enzyme was found in endosome-like vesicles found abundantly in amoeba cytoplasm which was analogous to the targeting of mammalian cathepsins L and B to lysosomes for intracellular protein degradation (McKerrow *et al.* 1991)

Parasite cathepsin Ds

A cathepsin D-like proteinase has been purified and characterised from the parasitic nematode, *Dirofilaria immitis* (Swamy and Jaffe, 1983). This enzyme, referred to as Fp-II had a molecular weight of 48 kDa and was purified using ion-exchange chromatography, ammonium sulphate precipitation and pepstatin agarose affinity chromatography. The enzyme was active in the 2.6-3.4 pH range and was highly sensitive to pepstatin.

Bogtish and Kirschke (1987), demonstrated the possibility that the blood fluke, *Schistosoma japonicum* possess a cathepsin D-like proteinase by immuno-cytochemistry using antiserum raised to bovine cathepsin D as a probe for the enzyme.

A cathepsin D-like molecule was isolated from the avian malaria parasite, *Plasmodium lophurae*. A combination of cation-exchange chromatography, affinity chromatography on pepstatin agarose and gel filtration chromatography were used to purify this molecule from haemoglobin-free parasite lysate (Sherman and Tanigoshi, 1983). The purified enzyme had a pH optimum of 3.5 against haemoglobin, a molecular weight of 32 kDa and a pI of 4.3. This enzyme digested membrane proteins and proteins from the cytoskeleton such as bands 1 and 2 (spectrin), bands 2.1-2.6 (spectrin-binding proteins) and band 3 (Sherman and Tanigoshi, 1983).

Parasite cathepsin Ls

Cathepsin L-like enzymes are present in the trematode parasite *Fasciola hepatica*. These secreted liver fluke cysteine proteinases were originally shown to cleave IgG in a similar way to cathepsin B or papain (Chapman and Mitchell, 1982, Smith *et al* , 1993b, Carmona *et al* , 1993).

Smith *et al* (1993a) purified and characterised a cathepsin L-like enzyme from culture medium in which adult *F. hepatica* were incubated. They isolated the enzyme by using gel filtration and ion-exchange chromatography and demonstrated that the enzyme had a molecular weight of 27 kDa. The enzyme was N-terminally sequenced and was found to share considerable homology with mammalian cathepsins L. Antisera raised against the pure protein showed that the enzyme was located within vesicles of the gut epithelial cells of the liver fluke. The same cathepsin L-like enzyme also degraded host IgG molecules (Smith *et al* 1993b, Carmona *et al* , 1993) and the proteinase prevented the antibody-mediated attachment of

eosinophils to *F hepatica* larvae (Carmona *et al* , 1993)

A cysteine proteinase was isolated from adult *F hepatica* homogenates using ion-exchange chromatography and molecular sieve HPLC (Rege *et al* , 1989) The molecular weight of the purified enzyme was 14.5 kDa The enzyme had a pH optimum at pH 6 and it cleaved CBZ-phe-arg-AFC in preference to CBZ-arg-arg-AFC and CBZ-arg-AFC which indicated that the enzyme had cathepsin L-like rather than cathepsin H- or B-like activity

Yamasaki *et al* (1989) isolated a cysteine proteinase of 27 kDa by ammonium sulphate precipitation, gel filtration and affinity chromatography on activated thiol-Sepharose A monoclonal antibody was raised against the purified protein and immunohistochemical studies using this monoclonal antibody located the enzyme to intestinal epithelial cells suggesting that the enzyme was secreted from the epithelial cells into the intestinal lumen to act as a digestive proteolytic enzyme The enzyme was subsequently cloned and was found to have a molecular weight, from the deduced amino acid sequence of 24.4 kDa (Yamasaki and Aoki, 1993) The amino acid sequence of the enzyme showed 50, 44 and 27% homology to mammalian cathepsins L, H, and B respectively

Cathepsin L-like enzymes (5 in total) from *F hepatica* were cloned by Heussler and Dobbelaere (1994) One of these enzymes (called Fcp1) had a deduced molecular weight of 30 kDa From the amino acid sequence of this molecule, it was found that it shared homologies with human cathepsin L

Wijffels *et al* (1994) isolated cathepsin L-like isoenzymes from *F hepatica* using a single step gel filtration protocol They found that the major

isoenzyme had a molecular weight of 28 kDa and a pH optimum of 7.45. This proteinase cleaved Z-phe-arg-NHMec but did not cleave Z-arg-NHMec. It was subsequently cloned and from the deduced amino acid sequence of its cDNA, the proteinase was found to be similar to enzymes belonging to the cathepsin L subfamily but was less homologous to cysteine proteinases from *Schistosoma*, nematode cathepsins and the mammalian cathepsin B subfamily.

Apart from the well characterised cathepsin B enzyme, adult *Schistosoma mansoni* were also found to possess a cathepsin L-like enzyme (Smith *et al.*, 1994). Using fluorogenic substrates, they demonstrated that the enzyme had a preference for Z-phe-arg-NHMec over Z-arg-arg-NHMec and Bz-arg-NHMec. Further evidence demonstrating that the enzyme was cathepsin L-like came from the deduced amino acid sequence of its cDNA. The cDNA encoded a polypeptide of 24 kDa which was only 26% homologous to *S. mansoni* cathepsin B but 46-47% homologous to cathepsins L from rat, mouse and chicken (Smith *et al.*, 1994).

A neutral cysteine proteinase was characterised from the larvae of the helminth parasite *Paragonimus westermani*, the causative agent of the lung disease paragonimiasis in humans (Yamakami and Hamajima, 1989, Hamajima *et al.*, 1994). This enzyme was purified by a single step chromatography on a gel filtration column but the enzyme was fractionated not by molecular size but by its electrostatic behaviour on this chromatographic support (Yamakami and Hamajima, 1989). The enzyme had a molecular weight of 22 kDa and preferentially cleaved the fluorogenic substrate Boc-val-leu-lys-NHMec. The enzyme was shown to have immunosuppressive properties and the deduced amino acid sequence from cDNA

cloning studies showed that the enzyme was 20% homologous to cathepsin Ls, cathepsin Hs, chymopapain and papain precursor. The percentage homologies were highest among the N-terminal regions. The amino acid composition of this proteinase was shown to be most similar to cathepsin L (Hamajima *et al*, 1994)

The dog hookworm *Ancylostoma caninum* is the causative agent of the allergic condition eosinophilic enteritis in humans. This organism was shown by studies with the fluorogenic substrates Z-phe-arg-NHMec, Z-arg-arg-NHMec and Z-arg-NHMec to secrete a cathepsin L-like cysteine proteinase (Dowd *et al.*, 1994)

The protozoan parasites of the *Trypanosoma* spp which cause Chaga's disease and sleeping sickness in humans possess cysteine proteinases which are similar to mammalian cathepsins L. The best characterized cysteine proteinase from trypanosomes is a molecule called cruzipain and was isolated from *Trypanosoma cruzi*

Cruzipain has been purified to homogeneity from cell free extracts by a procedure involving ammonium sulphate precipitation, gel filtration, and ion-exchange chromatography (Cazzulo *et al*, 1989)

The purified protein had a molecular weight of 60 kDa. This protein was sequenced and was found to be homologous to cathepsin L (65% identity in 32 amino acids). The enzyme was located in lysosomes (Bontempi *et al*, 1989) and degraded IgG *in vitro* (Bontempi and Cazzulo, 1990)

Murta *et al*, (1990) found that an antigen of 57 kDa from *T. cruzi* was a cysteine proteinase closely related to cathepsin L using N-terminal sequencing and enzyme assays with the fluorogenic substrate Z-phe-arg-

NHMec

The complete sequence of the gene encoding cruzipain was elucidated (Eakin *et al* , 1992), and the deduced amino acid of the cDNA encoding the proteinase was determined. The sequence of the protein was found to be most closely related to the sequence for *T brucei* cysteine proteinase (59.3%) and mouse cathepsin L (42.2%). The deduced molecular weight of the protein was found to be 36 kDa which was considerably different to the molecular weight of 57-60 kDa found by Cazzulo *et al* (1989) and Murta *et al* (1990) for the same molecule. Carbohydrate residues which were resistant to enzymatic hydrolysis were put forward as an explanation for the differences in molecular weights between the cloned molecule and the native protein (Eakin *et al* , 1992).

The African Trypanosome, *Trypanosoma congolense* was shown to possess a cathepsin L-like cysteine proteinase termed 'trypanopain-Tc' (Mbawa *et al* , 1992). This enzyme was purified from lysosomal extracts using activated thiol-Sepharose affinity chromatography and gel filtration. The enzyme had a molecular weight of 31-32 kDa by SDS-PAGE. The proteinase hydrolysed the cathepsin L substrate Z-phe-arg-NHMec but did not hydrolyse cathepsin H or B substrates. The enzyme was also shown to cleave fibrinogen, serum albumin and trypanosome-variant surface glycoprotein *in vitro*.

A cysteine proteinase from *Trypanosoma brucei* has also been characterised and has been found to be cathepsin L-like (Mottram *et al* , 1989, Robertson *et al* , 1990). From cloning and sequence analysis the deduced amino acid sequence of the *T brucei* cysteine proteinase shared 48% homology in the central region to human cathepsin L. This homology was

higher than either cathepsins B or H (Mottram *et al* , 1989) The cysteine proteinase from *T brucei* was also analysed using fluorogenic substrate SDS-PAGE and it was shown that the enzyme had inhibitor and substrate profiles similar to mammalian cathepsins L (Robertson *et al* , 1990)

Malaria parasites belong to the genus *Plasmodium* and produce cathepsin L-like enzymes (Rosenthal *et al* , 1993, Rosenthal *et al* , 1989, Rosenthal *et al* , 1988) A *Plasmodium vinckei* cathepsin L-like proteinase, which may be a haemoglobin degrading enzyme, was found to have a molecular weight of 27 kDa on zymograms containing gelatin Its pH activity optimum was found to be from pH 4-6 (Rosenthal *et al* ; 1993)

Plasmodium falciparum also produces a cathepsin L-like enzyme which may potentially degrade haemoglobin (Rosenthal *et al* , 1989) From studies with fluorogenic substrates of extracts of trophozoites it was shown that the *P falciparum* enzyme had a pH optimum in the 5.5-6.0 range and retained 50% of its activity at pH 7 (Rosenthal *et al* , 1989) From the results of enzyme kinetic studies it was shown that the enzyme favoured Z-phe-arg-NHMec over Z-arg-arg-NHMec and Z-arg-NHMec showing cathepsin L-like substrate specificity

Studies of the tick-transmitted, cattle protozoan parasite, *Theileria parva* by molecular genetic studies and fluorogenic studies of parasite lysates (Nene *et al* , 1990), showed that this organism possessed a cysteine proteinase of 23.5 kDa which may be related to lysosomal cathepsins L found in other protozoan parasites (Lonsdale-Eccles and Grab, 1987, Rosenthal *et al* , 1989)

In conclusion, cathepsin-like proteinases have a wide distribution in the animal kingdom Because of their implied roles in the pathogenesis of

disease, be that cancer or infectious diseases, these enzymes are of great medical importance. The present study describes the isolation and characterisation of a cathepsin L proteinase from *Fasciola hepatica*, a parasitic helminth of mammals.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Bachem

H-gly-his-arg-pro-OH

H-gly-pro-arg-OH

H-gly-pro-arg-pro-OH

H-leu-val-tyr-NHMec

Suc-leu-leu-val-tyr-NHMec

Tos-gly-pro-lys-NHMec

Z-phe-ala-CHN₂

Z-phe-arg-NHMec

Cambridge Research Biochemicals

Z-arg-arg-NHMec

Gibco Life Technologies Ltd.

RPMI-1640 (10X) without L-glutamine and Na₂HCO₃

ICN Biomedicals Ltd.

Bovine fibrinogen (95% clottable)

Bovine thrombin (1147 NIH units/ml)

Pharmacia LKB Biotechnology

QAE Sephadex A50

Sephacryl S200HR

Pierce Chemical Co.

BCA protein assay kit

Promega

Anti-rabbit IgG (Fc) alkaline phosphatase conjugate

Agarose (molecular biology grade)

University of Cambridge protein sequencing facility

Polyvinylidene difluoride (PVDF, Problott)

Schleicher and Schuell

Nitrocellulose (0.45 µm pore size)

Sigma Chemical Co.

Boc-val-leu-lys-NHMec

Boc-val-pro-arg-NHMec

Bz-arg-NHMec

Bz-phe-val-arg-NHMec

dithiothreitol (Cleland's reagent)

E-64

Gentamicin

Freund's Complete and Incomplete Adjuvants

Gelatin

HEPES

Hirudin (650 U/mg)

Suc-ala-phe-lys-NHMec

tetranitromethane (TNM)

Tos-gly-pro-arg-NHMec

2.2 SOURCE OF *FASCIOLA HEPATICA* CYSTEINE PROTEINASES

Mature *F hepatica* parasites were removed from the bile ducts of infected bovine livers obtained at a local abattoir. The parasites were washed six times in phosphate buffered saline (PBS), pH 7.3, and maintained for 16 hours in RPMI-1640, pH 7.3, containing 2% glucose, 30 mM HEPES and 25 mg/ml gentamicin at 37°C. Following the incubation period, the culture medium was removed, centrifuged at 14,900 x *g* for 30 min and the supernatant stored at -20°C (Dalton and Heffernan, 1989).

2.3 ENZYMATIC ASSAYS WITH FLUOROGENIC SUBSTRATES

Proteinase activity was measured fluorometrically using peptide-NHMec as substrate. Each substrate was stored as a 1 mg 100 µl⁻¹ stock solution in dimethyl-formamide. Assays were carried out using a final concentration of 10 µM substrate in 0.1 M glycine-HCl, pH 7, containing 0.5 mM dithiothreitol, in a volume of 1 ml. The mixtures were incubated at 37 °C for 30 min before stopping the reaction by the addition of 200 µl of 1.7 M acetic acid. The amount of 7-amino-4-methylcoumarin (NHMeC) released was measured using a Perkin-Elmer fluorescence spectrophotometer with excitation set at 370 nm and emission at 440 nm. One unit of enzyme activity was defined as that amount which catalysed the release of one µmole of NHMeC per min at

37°C

2.4 PURIFICATION OF *FASCIOLA HEPATICA* CYSTEINE PROTEINASE

Five hundred ml of culture medium in which mature *F hepatica* were maintained were thawed and concentrated to a volume of 10 ml in an Amicon 8400 concentrator using an Amicon YM3 membrane (3 kDa molecular weight cut-off, Amicon, WI), and applied to a Sephacryl S200HR gel filtration column (2.6 cm X 74.5 cm) equilibrated in 0.1 M Tris-HCl, pH 7, at 4°C. The column was eluted with 0.1 M Tris-HCl, pH 7, and 5 ml fractions were collected. Each fraction was assayed for cathepsin L activity using the fluorogenic substrate Tos-gly-pro-arg-NHMec at a final concentration of 10 µM in 0.1 M glycine-HCl, pH 7.

The Sephacryl S200 fractions containing cathepsin L activity were pooled and applied to a 50 ml QAE Sephadex column (2.5 cm X 100 cm) equilibrated in 0.1 M Tris-HCl, pH 7. The QAE Sephadex column was washed with 300 ml of 0.1 M Tris-HCl, pH 7, and then proteins eluted with 150 ml of 75 mM NaCl in 0.1 M Tris-HCl, pH 7, and then 250 ml of 400 mM NaCl in 0.1 M Tris-HCl, pH 7. Five ml fractions (180 fractions total) were collected and assayed for cathepsin L activity using the fluorogenic substrate Tos-gly-pro-arg-NHMec. The cathepsin L activity was pooled and then concentrated to 20 ml on an Amicon 8400 concentrator using a YM3 membrane. The concentrate was then diluted with distilled water to a volume of 100 ml and re-concentrated to a final volume of 10 ml to have a NaCl concentration of approximately 80 mM. The concentrated cathepsin L was stored as 1 ml aliquots at -80°C.

2.5 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Homogeneity of the purified cathepsin L2 was determined by denaturing SDS-PAGE gels containing 12% polyacrylamide using the buffers and methods described by Laemmli (1970)

2.6 ZYMOGRAPHY

Zymography was performed, both in the presence and absence of SDS, using 12% PAGE gels according to the method of Dalton and Heffernan (1989)

2.7 PROTEIN CONCENTRATION

Protein concentration was measured using a BCA protein assay kit in microtitre plates according to the method of Redinbaugh and Turley (1986) Bovine serum albumin was used as a protein standard.

2.8 AMINO-TERMINAL SEQUENCE ANALYSIS

Forty μ l (40 μ g) of concentrated culture medium in which mature *F. hepatica* were maintained were applied to a 12% SDS-PAGE gel and electrophoresed as described above After electrophoresis, the gel was incubated for 30 min in transfer buffer (25 mM Tris, 190 mM glycine and 10% (v/v) methanol) A PVDF membrane was immersed in methanol for 10 sec and then equilibrated in transfer buffer for 5 min The electrophoretically separated proteins were transferred to the PVDF membrane using a semi-dry electroblotting apparatus (Atto Corp , Tokyo, Japan) according to the

manufacturers instructions. The membrane was stained in 0.1% Coomassie blue R in 50% methanol, 1% acetic acid for 5 min and destained for 5 min with 5 changes in 50% methanol, 10% acetic acid and then air-dried. The protein of interest was sequenced on an Applied Biosystems 477A protein sequencer at the Biochemistry Department, University of Cambridge, UK.

2.9 KINETIC STUDIES

The kinetic constants of the two purified *F. hepatica* cathepsin L proteinases were determined for 11 different substrates: Z-phe-arg-NHMec, Bz-phe-val-arg-NHMec, Suc-leu-leu-val-tyr-NHMec, H-leu-val-tyr-NHMec, Tos-gly-pro-lys-NHMec, Tos-gly-pro-arg-NHMec, Boc-val-pro-arg-NHMec, Z-arg-arg-NHMec, Boc-arg-NHMec, Suc-ala-phe-lys-NHMec and Bz-val-leu-lys-NHMec. The kinetic constants, k_{cat} and K_m , were obtained by non-linear regression analysis by using the program Enzfitter (Leatherbarrow, 1987).

2.10 ACTIVE SITE TITRATION OF CATHEPSINS L1 AND L2

Active site titration using the cysteine proteinase inhibitor L-*trans*-epoxysuccinyl-leucylamido-(4-guanido)-butane (E-64) was performed according to the method of Barrett *et al.* (1982) using the fluorogenic substrate Z-phe-arg-NHMec.

2.11 DIRECT FLUOROGENIC SUBSTRATE ANALYSIS IN POLYACRYLAMIDE GELS

Culture medium in which mature *F. hepatica* were maintained was electrophoretically separated in non-denaturing 12% polyacrylamide gels. Following electrophoresis the substrate specificities of the two cathepsin L

proteinases was examined by incubating replicate gels in different fluorogenic substrates. This method was performed as described by Robertson *et al* (1990) with the exception that SDS was not included in the gels. The gels were first washed twice at room temperature in 0.1 M glycine, pH 7, before being transferred to 0.1 M glycine, pH 7, containing 10 mM cysteine and 10 μ M of one of the following substrates: Z-phe-arg-NHMec, Boc-val-leu-lys-NHMec, Tos-gly-pro-arg-NHMec and Tos-gly-pro-lys-NHMec. The gels were then incubated at 37°C for 15 min. Fluorescent bands locating the substrate cleaving enzymes within the gel were detected using a UVP Chromatovue model TM-20 UV-Transilluminator and recorded using a Polaroid camera fitted with a Kodak Wratten gelatin filter (model 2E).

2.12 NITRATION OF CYSTEINE PROTEINASES WITH TETRANITROMETHANE (TNM)

Proteins in the culture medium in which mature *F. hepatica* were maintained and purified cathepsin L were modified by nitration with tetranitromethane (TNM) (Riordan and Vallee, 1972). The samples (<20 μ g) were incubated with varying concentrations of TNM (0.5 mM - 4.0 mM) in PBS, pH 7.3, and 10% ethanol, for 1 hour at room temperature. The effect of nitration on the activity of the cysteine proteinases was analysed by zymography in native non-SDS polyacrylamide gels.

2.13 PREPARATION OF ANTIBODY TO CATHEPSIN L2

Polyclonal antiserum against cathepsin L purified in this study was prepared in New Zealand white rabbits. An initial immunisation of 100 μ g of purified

cathepsin L in Freund's Complete Adjuvant was followed by 4 immunisations, 4 weeks apart, of 100 µg in Freund's Incomplete Adjuvant

2.14 PREPARATION OF MATURE *FASCIOLA HEPATICA* EXTRACT

Mature *F hepatica* were homogenised in 0.1 M Tris-HCl, pH 7, or in the same buffer containing 1 mM EDTA, 5 µg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF) and 4 mg/ml iodoacetamide, in a glass-glass hand homogeniser. Extracts were centrifuged for 10 min at 14,900 x *g* and the supernatant used in immunoblotting experiments.

2.15 IMMUNOBLOTTING ANALYSIS

Mature *F hepatica* extracts, culture medium in which parasites were maintained and the two purified cathepsin L proteinases were separated by reducing SDS-PAGE and electrophoretically transferred to nitrocellulose paper using a semi-dry electroblotting system (Smith *et al* , 1993a). Following blocking in 0.5% bovine serum albumin and 0.1% Tween 20 the nitrocellulose membrane was incubated in anti-cathepsin L2 or non-immune rabbit serum. Bound immunoglobulin was visualised using alkaline phosphatase-conjugated anti-rabbit serum. Nitro-blue tetrazolium and 5-bromo-4-chloro-indolyl phosphate prepared in dimethylformamide were used as substrates for alkaline phosphatase (Smith *et al* , 1993a).

2.16 *IN VITRO* CLOTTING ASSAY

F hepatica cathepsin L2 (2.3×10^{-3} units) or bovine thrombin (5.7×10^{-3} NIH units) was added to 100 µl of a 20 mg ml⁻¹ solution of bovine fibrinogen in

phosphate buffered saline (PBS) in 12x75 mm test tubes. Ten μl of 1 mM dithiothreitol in PBS was added to the tubes and the volume then adjusted to 150 μl with PBS. The tubes were incubated at 37°C and the time taken for stable fibrin clots to form was recorded.

2.17 ELECTROPHORETIC ANALYSIS AND IMMUNOBLOTTING OF FIBRIN CLOTS

Fibrin clots were centrifuged at 15,000 x *g* for 1 min. The supernatant was removed and clot was washed 5 times by adding 1 ml of PBS to the tubes followed by centrifugation at 15,000 x *g* for 1 min. The clot was finally resuspended in 100 μl reducing SDS-PAGE sample buffer and boiled for 2 min. Reducing 10% SDS-PAGE was performed according to the method of Laemmli (1970).

Immunoblotting studies of fibrin clots were performed as described in section 2.15.

2.18 FIBRIN PLATE ASSAY

Ten ml of 1% agarose, warmed to 50°C, was mixed with 9.5 ml of a 0.4% (w/v) fibrinogen solution (in PBS containing 100 μM dithiothreitol) which was also heated to 50°C. Three ml of the fibrinogen-agarose mixture was applied to a glass microscope slide (76 x 26 mm) and allowed to solidify. Wells of 4mm diameter were cut into the fibrin-agarose plate. Aliquots (10, 15 or 20 μl) of cathepsin L or thrombin were added to the wells in the fibrin-agarose plate. The plates were then incubated for 5h at 37°C in a humid chamber.

Following incubation the diameters of the 'hazy' rings, formed due to the polymerisation of fibrin, were measured using a vernier callipers. Results were also photographically recorded on Polaroid film. In the inhibition studies, hirudin (1 unit ml^{-1}) and Z-phe-ala-CHN₂ ($600 \text{ } \mu\text{M}$) were added to the cathepsin L and thrombin samples prior to the addition of these to the fibrin-agarose plate wells.

2.19 STUDIES OF FIBRIN CLOTS WITH PRIOR ADDITION OF FIBRIN ANTI-POLYMERANTS

The fibrin-anti-polymerants, H-gly-pro-arg-OH, H-gly-his-arg-pro-OH and H-gly-pro-arg-pro-OH were added to test tubes, at a final concentration of $75 \text{ } \mu\text{M}$, containing fibrinogen ($100 \text{ } \mu\text{l}$, 20 mg ml^{-1}) and assayed for *in vitro* clotting ability as described in section 2.16.

2.20 STUDIES OF ENZYME ACTIVITY WITH PRIOR ADDITION OF FIBRIN-ANTI-POLYMERANTS AND PROTEINASE INHIBITORS

The fibrin anti-polymerants, H-gly-pro-arg-OH, H-gly-his-arg-pro-OH and H-gly-pro-arg-pro-OH and the cathepsin specific inhibitor Z-phe-ala-CHN₂ were added to enzyme assay mixtures at final concentrations of 75 and $300 \text{ } \mu\text{M}$. The thrombin-specific inhibitor, hirudin, was added to enzyme assay mixtures at a final concentration of 1 unit ml^{-1} . Proteinase activity was assessed using the fluorogenic substrate Tos-gly-pro-arg-NHMec as described in section 2.3.

2.21 POSITIONING OF THE CLEAVAGE SITE OF THROMBIN AND CATHEPSIN L2 ON FIBRINOGEN

Fibrinogen was incubated with thrombin (5.7×10^{-3} NIH units) and 0, 200, 250, 300, 350 or 400 μM of the fibrinogen anti-polymerant, H-gly-pro-arg-pro-OH, and incubated for 100 min at 37°C as described in section 2.16. Thrombin or cathepsin L2 was subsequently added to replicate sets of assay tubes and the time taken for clot formation was recorded.

After 60 minutes incubation, reducing SDS-PAGE sample buffer was added to the assay tubes containing 0, 200, 300 and 400 μM anti-polymerant. The samples were boiled and then analysed on 10% SDS-PAGE gels as described in section 2.5.

CHAPTER 3
RESULTS PART 1
Purification and characterisation of cathepsin L2
from adult *Fasciola hepatica*

3.1 PURIFICATION OF CATHEPSIN L2

F hepatica cysteine proteinase was purified by gel filtration on Sephadex S200 (Figure 3 1) followed by ion exchange chromatography on QAE Sephadex (Figure 3 2) The proteinase eluted from the gel filtration column in a broad protein peak together with another cysteine proteinase that has been isolated and characterised as a cathepsin L-like proteinase by Smith *et al* (1993a), however, the two proteinases were separated by ion exchange chromatography on QAE Sephadex as the proteinase described by Smith *et al* (1993a) did not bind to the column and was therefore carried in the column run-through The other cysteine proteinase activity binds to the column and was subsequently eluted with 400 mM NaCl

Analysis of the cysteine proteinase in the protein peak eluted at 400 mM NaCl by zymography in the presence of SDS revealed that the enzyme migrates as four bands, four similarly migrating bands are also observed in the total culture medium in which mature *F hepatica* were maintained (Figure 3 3A) In contrast, when the proteinase is analysed by zymography in the absence of SDS it migrates more slowly and as a single proteolytic band (Figure 3 3B) SDS-PAGE analysis, under reducing conditions revealed that the proteinase is homogeneous and migrates as a single band of 29.5 kDa on a reducing SDS-PAGE gel (Figure 3 3C) The cysteine proteinase was purified 150-fold from the *F hepatica* culture medium by the two column procedure (Table 3 1) The cysteine proteinase represents a major protein in the culture medium (Figure 3 3C) which would account for the relative ease by which the protein can be isolated The other major protein observed in the culture medium at 27 kDa represents the cathepsin L-like proteinase isolated by Smith *et al* (1993a) The proteinase isolated in this study had a final

specific activity of 1 941 units mg⁻¹

3.2 N-TERMINAL SEQUENCE ANALYSIS OF CATHEPSIN L2

The first 14 N-terminal amino acids of the purified cysteine proteinase were determined. The N-terminal sequence, aligned with the N-terminal sequences determined for *F hepatica* cathepsin L isolated by Smith *et al* (1993a), is shown in Figure 3.4. Homologous amino acids are indicated by dots. The N-terminal sequence of the cysteine proteinase isolated in the present study is almost identical to that of the cathepsin L isolated by Smith *et al* (1994a). One amino acid difference was observed, an arginine in position 7 in the cysteine proteinase isolated in this study is substituted for a proline in the cathepsin L sequence (Smith *et al*, 1993a).

The cysteine proteinase sequence is 47% homologous to the N-terminal region of chicken liver cathepsin L (Dufour *et al*, 1987), rat liver cathepsin L (Ishidoh *et al*, 1987b) and human liver cathepsin L (Mason *et al*, 1986) and 40% homologous to the *Trypanosoma cruzi* cathepsin L-like proteinase (Cazzulo *et al*, 1989). The sequence is only 20% homologous to the cathepsin B of the related parasitic trematode, *Schistosoma mansoni* (Feilleisen and Klinkert, 1990). A feature that appears to be peculiar to the presently described *F hepatica* cysteine proteinase and the cathepsin L isolated by Smith *et al* (1993a) is the presence of an extra alanine residue at the beginning of the N-terminal sequence.

The N-terminal sequences of the *F hepatica* cathepsins are 47% homologous to that of the bovine cathepsin L (Turk *et al*, 1985) and to a recently described cathepsin S (Wiederanders *et al*, 1991). Our enzyme kinetic studies (see below) are consistent with the classification of the two *F*

hepatica cysteine proteinases as cathepsin Ls. To distinguish the two *F hepatica* cathepsin L proteinases, they have been designated cathepsin L1 (27 kDa) (Smith *et al* , 1993a) and cathepsin L2 (29.5 kDa)

3.3 IMMUNOBLOTTING STUDIES

Aqueous extracts of mature *F hepatica* were prepared in the presence and absence of the proteinase inhibitors EDTA, leupeptin, phenylmethylsulfonylfluoride (PMSF) and iodoacetamide. When these extracts were analysed and compared by reducing SDS-PAGE, it was observed that many proteins were degraded when the proteinase inhibitors were omitted (Figure 3.5, panel A, compare lanes 1 and 2). Other samples of these extracts were transferred, following reducing SDS-PAGE, to nitrocellulose filters which were subsequently probed with anti-cathepsin L2 serum. The anti-cathepsin L2 antibodies were reactive with two proteins corresponding to cathepsin L1 (27 kDa) and cathepsin L2 (29.5 kDa), the enzymes were not susceptible to proteolytic degradation in the extracts which did not contain proteinase inhibitors (Figure 3.5 panel B, compare lanes 1 and 2). When the mature *F hepatica* culture medium, and cathepsin L1 and cathepsin L2 purified from this medium, were similarly analysed by immunoblotting it was observed that both cathepsin L1 and cathepsin L2 were secreted as molecules of similar molecular size to the enzymes in the extracts of whole parasites (Figure 3.5, panel C).

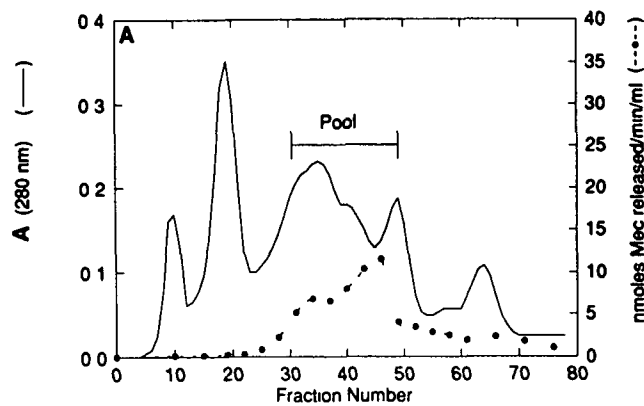


Figure 3.1

***Sephacryl S200HR* chromatography of cathepsin L2**

The culture medium in which mature *F. hepatica* were maintained (340 mg) was concentrated to 10 ml and applied to a *Sephacryl S200HR* column (2.6 cm x 74.5 cm). The mobile phase was 0.1 M Tris-HCl, pH 7. Protein elution from the column was monitored by absorbance at 280 nm using a flow-through spectrophotometer. Cysteine proteinase activity in collected fractions was assayed using the fluorogenic substrate Tos-gly-pro-arg-NHMec. Fractions containing Tos-gly-pro-arg-NHMec were pooled (solid bar) and applied to a QAE-Sephadex column.

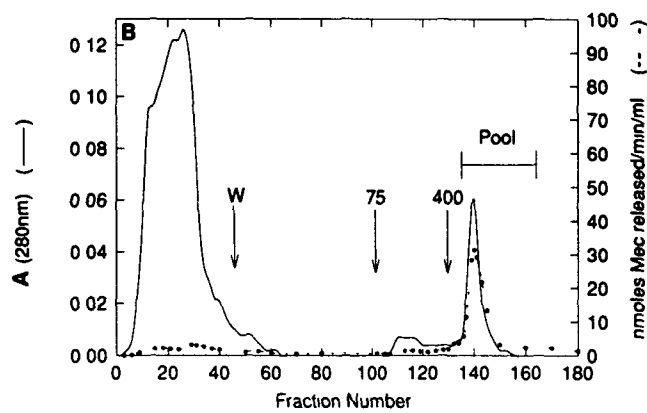


Figure 3.2

QAE-Sephadex chromatography of cathepsin L2.

The S200HR pooled fractions (16 mg protein) were applied to a QAE-Sephadex column (2.5cm x 10cm) equilibrated in 0.1M Tris-HCl, pH 7. The column was washed with the same buffer and the cysteine proteinase was eluted with 400mM NaCl in 0.1M Tris-HCl, pH 7. Protein content was monitored at 280 nm. Enzyme activity in collected fractions was assayed using the fluorogenic substrate Tos-gly-pro-arg-NHMeC and fractions containing activity were pooled (solid bar).

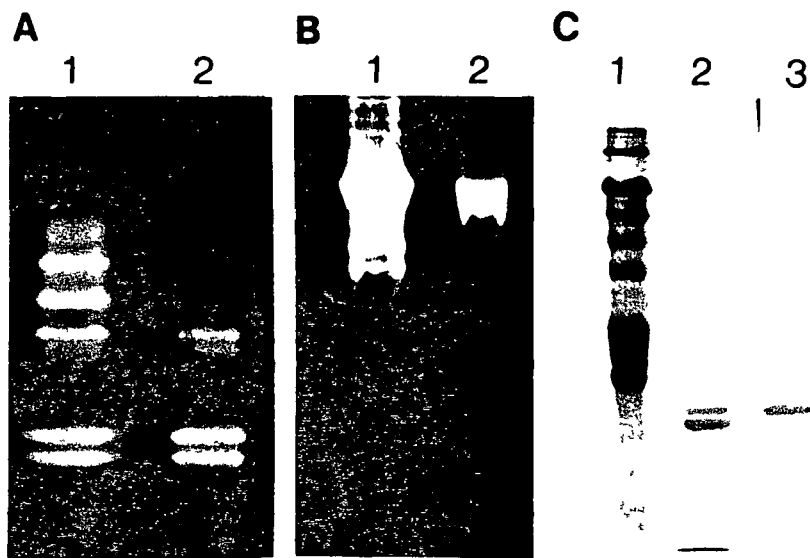


Figure 3.3

Analysis of purified cathepsin L2 by zymography and reducing SDS-PAGE.

A Zymograms performed in the presence of SDS. Culture medium (20 μ g) in which mature *F. hepatica* were maintained (Lane 1) and purified cysteine proteinase (2.5 μ g) were analysed by zymograms containing 0.1% SDS.

B. Zymograms performed in the absence of SDS. Culture medium in which mature *F. hepatica* were maintained (Lane 1) and purified cysteine proteinase were analysed by zymograms in the absence of SDS.

C. Reducing SDS-PAGE analysis of purified cathepsin L2. Culture medium in which mature *F. hepatica* were maintained (Lane 2) and purified cysteine proteinase (Lane 3) were analysed by SDS-PAGE under reducing conditions. Lane 1, prestained molecular size markers (α_2 macroglobulin, 211 kDa; beta-galactosidase, 119 kDa, fructose-6-phosphate kinase, 98 kDa; pyruvate kinase, 80.6 kDa, fumarase, 64.4 kDa; lactic dehydrogenase, 44.6 kDa; and triosephosphate isomerase, 38.9 kDa).

	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
<i>E. hepatica</i> (cathepsin L2)	A	V	P	D	K	I	D	R	R	E	S	.	.	G	Y	V	.
<i>E. hepatica</i> (cathepsin L1)	A	V	P	D	K	I	D	P	R	E	S	.	.	G	Y	V	T
Chicken liver (cathepsin L)	.	A	P	R	S	V	D	W	R	E	K	.	.	G	Y	V	T
Rat liver (cathepsin L)	.	I	P	R	S	V	D	W	R	E	K	.	.	G	Y	V	T
Human liver (cathepsin L)	.	A	P	R	S	V	D	W	R	E	K	.	.	G	Y	V	T
Bovine liver (cathepsin L)	.	L	P	D	S	V	D	W	R	E	K	.	.	G	G	V	T
Bovine spleen (cathepsin S)	.	L	P	D	S	M	D	W	R	E	K	.	.	G	C	V	T
<i>T. cruzi</i> (cruzipain)	.	A	P	A	A	V	D	W	R	A	R	.	.	G	A	V	T
<i>S. mansoni</i> (cathepsin B)	.	I	P	S	N	F	D	S	R	K	K	W	P	G	C	K	S

Figure 3.4

N-terminal amino acid sequence of cathepsin L2.

The determined sequence (14 amino acids) is shown compared to the N-terminal sequence of *F. hepatica* cathepsin L1 (Smith *et al.*, 1994a) and cathepsin L from chicken liver (Dufour *et al.*, 1987), rat liver (Ishidoh *et al.*, 1987b) and human liver (Mason *et al.*, 1986). Also included for comparison are the N-terminal sequences of bovine liver cathepsin L (Turk *et al.*, 1985), bovine spleen cathepsin S (Wiederanders *et al.*, 1991), *Trypanosoma cruzi* cathepsin L-like proteinase (cruzipain) (Cazzulo *et al.*, 1989) and *Schistosoma mansoni* cathepsin B (Feilleisen and Klinkert, 1990). Homologous residues are indicated by dots.

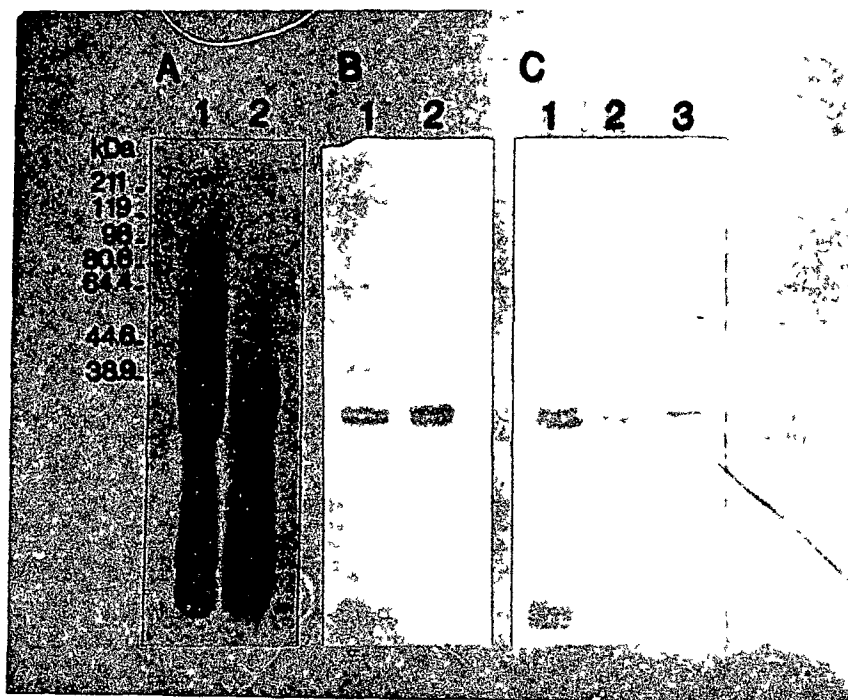


Figure 3.5

Immunoblotting experiment with anti-cathepsin L2.

A. Reducing SDS-PAGE analysis of PBS extracts of whole mature *F. hepatica* parasites. Parasites were extracted in the presence (lane 1) and in the absence (lane 2) of proteinase inhibitors. Gels were stained with Coomassie blue R.

B. Immunoblot analysis of parasite extracts. Mature *F. hepatica* proteins (120 µg) extracted in the presence (lane 1) and absence (lane 2) of proteinase inhibitors were electrophoretically separated and transferred to nitrocellulose. Nitrocellulose filters were then probed with rabbit antiserum prepared against cathepsin L2. No bands were visualised when control rabbit serum was used to probe duplicate blots (not shown).

C. Immunoblot analysis of culture medium and purified cathepsin L1 and cathepsin L2. Medium in which mature *F. hepatica* were maintained (10 µg) (lane 1), purified cathepsin L1 (1 µg) (lane 2) and purified cathepsin L2 (1 µg) (lane 3) were electrophoretically separated and transferred to nitrocellulose. Filters were then probed with rabbit antiserum prepared against the purified cathepsin L2. No bands were visualised when control rabbit serum was used to probe duplicate blots. The molecular size markers (pre-stained) were: alpha₂ macroglobulin, 211 kDa; beta-galactosidase, 119 kDa; fructose-6-phosphate kinase, 98 kDa; pyruvate kinase, 80.6 kDa; fumarase, 64.4 kDa; lactic dehydrogenase, 44.6 kDa, and triosephosphate isomerase, 38.9 kDa.

Table 3.1*Purification of cathepsin L2*

Step	Protein (mg)	Specific Activity (units)	Total Activity (units mg ⁻¹)	Recovery (%)	Purif- ication (-fold)
Culture Medium	341.7	4.44	0.013	-	1
S200	16.4	1.68	0.102	38	7.85
QAE400	0.68	1.32	1.941	29.7	149

* Enzyme activity was measured using the substrate
Tos-gly-pro-arg-NHMeC

CHAPTER 4
RESULTS PART 2
Comparison of *Fasciola hepatica*
cathepsin L1 and L2 activities

4.1 KINETIC STUDIES OF CATHEPSINS L1 AND L2.

Enzyme kinetic studies were carried out using purified *F hepatica* cathepsin L1 and cathepsin L2. Kinetic constants were obtained for eleven different fluorogenic peptide substrates including those that are classically used for the characterisation of cathepsin proteinases, namely, Z-phe-arg-NHMec, Z-arg-arg-NHMec and Bz-arg-NHMec (Table 4.1 and Barrett and Kirschke, 1981). Both the *F hepatica* cathepsins L1 and L2 have a high affinity (k_{cat} / K_m) for the substrate Z-phe-arg-NHMec and low affinity for the substrates Z-arg-arg-NHMec and Bz-arg-NHMec. The preference for a hydrophobic phenylalanine in the P₂ position is consistent with the classification of both *F hepatica* proteinases as cathepsins L (Barrett and Kirschke, 1981) or cathepsins S (Bromme *et al*, 1989, Bromme *et al*, 1993). Both enzymes, however, have a three-fold greater affinity for the substrate Boc-val-leu-lys-NHMec over the substrate Z-phe-arg-NHMec, the cathepsin L2 has over twice the affinity for these two substrates when compared to the cathepsin L1.

We analysed the activity of both cathepsin Ls on a range of fluorogenic peptide substrates in an effort to find substrates that could differentiate between the two enzymes. We arrived at five substrates, namely Bz-phe-val-arg-NHMec, H-leu-val-tyr-NHMec, Boc-val-pro-arg-NHMec, Tos-gly-pro-arg-NHMec and Tos-gly-pro-lys-NHMec, that are cleaved by the *F hepatica* cathepsin L2 with much greater affinities ($38.6 - 113.3 \text{ mM}^{-1} \text{ s}^{-1}$) than by the cathepsin L1 ($0.3 - 3.9 \text{ mM}^{-1} \text{ s}^{-1}$). For this reason one of these substrates, Tos-gly-pro-arg-NHMec, was chosen in this study to monitor the purification of the cathepsin L2 (c.f. Results part 3.1).

Bromme *et al* (1989) showed that bovine cathepsin L had a ten-fold

preference for the substrate Z-phe-arg-NHMec over the substrate Z-phe-val-arg-NHMec whereas bovine cathepsin S had a five-fold preference for Z-phe-val-arg-NHMec over Z-phe-arg-NHMec. The *F. hepatica* cathepsin L1 and cathepsin L2 have a preference for the substrate Z-phe-arg-NHMec over the substrate Z-phe-val-arg-NHMec (25 fold and three-fold, respectively) suggesting that both enzymes are cathepsin L-like rather than cathepsin S-like proteinases.

4.2 ACTIVE SITE TITRATION OF CATHEPSINS L1 AND L2

Active site titration of cathepsins L1 and L2 using the cysteine proteinase inhibitor E-64 (Barrett *et al* , 1982) were performed. There is approximately 10 μ M active cathepsin L1 and approximately 1.0 μ M active cathepsin L2 following purification which is 80% of the cathepsin L1 and 40% the cathepsin L2 molecules present based on calculated molarities.

4.3 DIRECT FLUOROGENIC SUBSTRATE PAGE ANALYSIS

The substrate specificity of the *F. hepatica* cathepsin L1 and cathepsin L2 were also compared by electrophoretically separating the proteins in *F. hepatica* culture medium by native polyacrylamide gel electrophoresis and then immersing replicate gels in different fluorogenic substrates. In native gels the cathepsin L1 migrates as multiple bands whilst the cathepsin L2 focuses as a single slower migrating band (Smith *et al* , 1993a) (Figure 4.1). Bands representing both enzymes are visualised with the substrates Z-phe-arg-NHMec and Boc-val-leu-lys-NHMec, although the sensitivity by which the bands are detected is greater with the latter substrate, an observation that is consistent with the results of our kinetic data. Also consistent with our

kinetic data was the preferential detection of the cathepsin L2 within these gels when the substrates Tos-gly-pro-lys-NHMec and Tos-gly-pro-arg-NHMec are used (Figure 4 1)

4.4 NITRATION OF CATHEPSIN LS WITH TETRANITROMETHANE (TNM).

TNM completely inactivates purified *F hepatica* cathepsin L2 at concentrations greater than 4 mM. When purified cathepsin L2 was incubated with various concentrations of TNM, ranging from 0.5 mM to 4 mM, the intensity of the cathepsin L2 band in zymograms gradually decreased (Figure 4 2A). However, corresponding with the decrease in the intensity of this band was the appearance of several faster migrating bands, these bands became inactivated at TNM concentrations of 4 mM (Figure 4 2A). When the total culture medium in which adult flukes were incubated was treated with various concentrations of TNM we again observed the disappearance of the cathepsin L2 band and the corresponding appearance of the faster migrating bands (Figure 4 2B). In contrast, the bands representing cathepsin L1 were not affected by TNM even at concentrations of 4 mM (Figure 4 2B). The concentration of ethanol (10% v/v) used to dissolve the TNM did not inactivate cathepsin L1 or cathepsin L2 (Figures 4 2A and 4 2B, lanes 2).

Table 4.1

Reaction kinetics for the F hepatica cathepsins L1 and L2 on peptide substrates

Substrate	CATHEPSIN L1			CATHEPSIN L2		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{mM}^{-1}\text{s}^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{mM}^{-1}\text{s}^{-1}$)
z-arg-NHMec	20.4	0.04	2.2	14.1	0.09	6.4
z-arg-arg-NHMec	65.6	0.002	0.03	9.1	0.02	2.2
z-phe-arg-NHMec	14.7	1.08	73.6	4.0	0.56	140.0
Bz-phe-val-arg-NHMec	9.3	0.03	3.0	1.2	0.06	45.8
H-leu-val-tyr-NHMec	5.4	0.02	3.9	3.8	0.21	55.5
S-leu-leu-val-tyr-NHMec	38.5	0.01	0.3	23.3	0.08	3.3
Boc-val-pro-arg-NHMec	43.6	0.02	0.5	24.8	1.19	48.0
Tos-gly-pro-arg-NHMec	26.0	0.03	1.2	17.1	1.93	113.3
Tos-gly-pro-lys-NHMec	106.9	0.03	0.3	35.5	1.37	38.6
Suc-ala-phe-lys-NHMec	65.3	0.05	0.8	41.2	0.14	3.4
Boc-val-leu-lys-NHMec	34.7	7.90	227.7	7.3	3.75	510.9

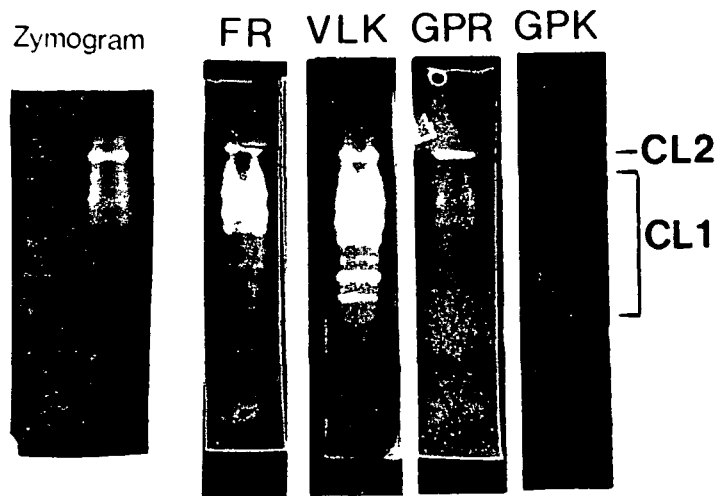


Figure 4.1

Direct visualisation of cathepsin L1 and cathepsin L2 proteinases in polyacrylamide gels.

Aliquots of culture medium in which mature *F. hepatica* were maintained (20 μ g) were applied to several lanes of a gelatin-containing 12% polyacrylamide gel (without SDS). Following electrophoresis the gel was divided into 1cm strips that corresponded to the lanes and the strips washed in 0.1 M glycine, pH 7.0. One strip was processed as for the zymograms. Other strips were incubated in 10 μ M of fluorogenic peptide substrate. The substrates used were, Z-phe-arg-NHMec (FR); Boc-val-leu-lys-NHMec (VLK), Tos-gly-pro-arg-NHMec (GPR) and Tos-gly-pro-lys-NHMec (GPK). After 10 mins the gel strips were removed, transilluminated and photographed.

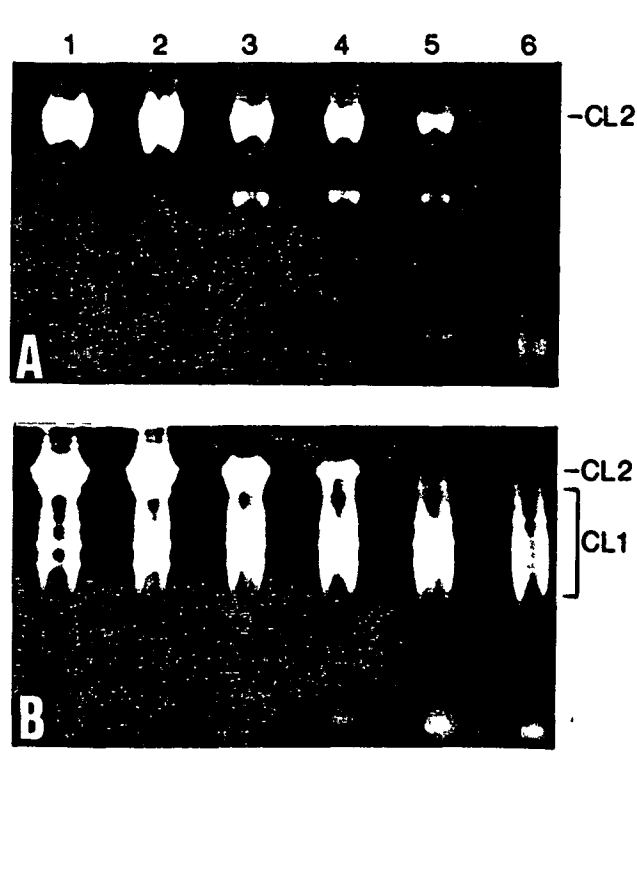


Figure 4.2

Nitration of cathepsin L2 proteinase with tetranitromethane (TNM)

A Purified cathepsin L2 (1 µg) incubated for 1 hr in various concentrations of TNM in PBS, pH 7.3. Following this incubation the mixtures were analysed by zymography. Enzyme preparations were incubated with PBS (control, lane 1), 10% ethanol (control, lane 2), 0.5 mM TNM (lane 3), 1.0 mM TNM (lane 4), 2.0 mM TNM (lane 5), and 4.0 mM TNM (lane 6) CL2, cathepsin L2

B Medium in which mature *F. hepatica* were maintained (20 µg) incubated for 1 hr in various concentrations of TNM in PBS, pH 7.3. Following this incubation the mixtures were analysed by zymography. Enzyme preparations were incubated with PBS (control, lane 1), 10% ethanol (control, lane 2), 0.5 mM TNM (lane 3), 1.0 mM TNM (lane 4), 2.0 mM TNM (lane 5), and 4.0 mM TNM (lane 6) CL2, cathepsin L2, CL1, cathepsin L1

CHAPTER 5

RESULTS PART 3

**Fibrin clot formation by *Fasciola hepatica* cathepsin L2:
comparison with the clot produced by bovine thrombin**

5.1 FIBRIN CLOT FORMATION BY CATHEPSIN L2

In a previous section of this Thesis (Results part 3.1) it was shown that *Fasciola hepatica* cathepsin L2 cleaved the protein substrate gelatin. Fibrinogen was included in a range of macromolecules that we examined for susceptibility to digestion by this enzyme. We noticed, however, that when the cathepsin L2 was added to a fibrinogen solution a rigid clot formed. This observation prompted us to examine more closely the characteristics of this clotting property of the enzyme and to compare it to that of blood clotting agent thrombin (Factor IIa). Since cathepsin L2 is a cysteine proteinase all reactions were carried out in the presence of the reducing agent dithiothreitol. When 2.3×10^{-3} units of cathepsin L2 was added to 100 μl of a 20 mg ml^{-1} fibrinogen solution, clots were observed within 25 min at 37°C . Clots formed within 20 min when 5.7×10^{-3} NIH units of thrombin were added to fibrinogen solutions (Table 5.1). Therefore the clotting ability of the cathepsin L2 enzyme is equivalent to 2.85 NIH units/ unit of Tos-gly-pro-arg-NHMec cleaving activity.

Fibrinogen-agarose gels, set on microscope slides, were used to visualise the clot formation and to quantitate the clotting efficiency of the cathepsin L2 using thrombin as a standard. Clots formed around the wells to which the enzymes were added and appeared as 'hazy' rings. The diameter of which can be measured and compared to the standard (Figure 5.1A). Using this assay it was determined that 2.3×10^{-3} units of cathepsin L (measured using the fluorogenic substrate Tos-gly-pro-arg-NHMec) had the equivalent clotting efficiency as 1.9×10^{-4} NIH units of thrombin.

Inhibitors were added with cathepsin L2 or thrombin to the wells to demonstrate that the clots formed were indeed a result of enzyme activity. Hirudin, an inhibitor of thrombin, prevented the formation of clotting zones by thrombin but did not affect the cathepsin L2 clotting activity. In contrast, the cathepsin L inhibitor, Z-phe-ala-CHN₂, inhibited the clotting activity of the cathepsin L2 but not of thrombin (Figure 5 1B).

Two observations indicate that the clots produced by thrombin and cathepsin L2 differ physically. Firstly, the hazy zone produced on the fibrinogen-agarose plates by cathepsin L2 appears more ring-like than that produced by thrombin (Figure 5 1A). Secondly, the cathepsin L2 clot is less stable. We observed that on vigorous vortexing, the matrix of the cathepsin L2 clot appeared to collapse and form a precipitate in the tube, thrombin clots treated in the same manner remained gel-like.

5.2 ELECTROPHORETIC ANALYSIS OF FIBRIN CLOTS

Fibrin clots formed by cathepsin L and thrombin were solubilised in SDS-PAGE reducing sample buffer and analysed by SDS-PAGE. Under reducing conditions fibrinogen migrates as three bands that represent the α (66 kDa), β (52 kDa) and γ (46.2 kDa) subunits (Figure 5 2A, lane 1). These three polypeptides were also observed when the thrombin clots were analysed under similar conditions, however, the α and β polypeptides migrated slightly faster due to the cleavage of the fibrinopeptides (16 and 14 amino acids, respectively) from these polypeptides by thrombin. The γ polypeptide is not cleaved by thrombin (Figure 5 2A, lane 3). The pattern observed for the cathepsin L2 produced clot is remarkably dissimilar to that of the thrombin

clot, the α and β polypeptides are not observed and the γ polypeptide migrates slightly faster. However, several polypeptides of higher molecular size are observed two of which, of 120 kDa and 100 kDa, are the most predominant. A faster migrating polypeptide of 25 kDa is also visualised on these gels (Figure 5 2A, lane 2)

To demonstrate that the polypeptides observed in the cathepsin L2 produced clots were derived from fibrinogen, the clots were separated by reducing SDS-PAGE, transferred to a nitrocellulose filter and subsequently probed with anti-human fibrinogen antibodies. This antiserum contained antibodies reactive with the α , β and γ polypeptides of fibrinogen as seen when thrombin clots are probed (Figure 5 2B, lane 2). The antiserum also contained antibodies reactive with the 120, 100 and 25 kDa polypeptides observed in the cathepsin L2 produced clots (Figure 5 2B, lane 1). Normal human serum did not contain antibodies reactive with any of the above polypeptides (Figure 5 2B, Lane 3 and 4)

5.3 POSITIONING OF THE CLEAVAGE SITES OF THROMBIN AND CATHEPSIN L2 ON FIBRINOGEN

We have previously shown that *F. hepatica* cathepsin L2 can cleave some peptide bonds that are also cleaved by thrombin, for example both enzymes have a high affinity for Tos-gly-pro-arg-NHMeC (Results parts 4 1 and 4 2, Lotterberg *et al*, 1981). We used three synthetic peptides to examine whether the cleavage site on fibrinogen for both enzymes were the same. The peptide H-gly-pro-arg-pro-OH corresponds to the terminal sequence of the α -chain after removal of the fibrinopeptide A, and prevents the

polymerisation of the fibrin monomers H-gly-pro-arg-OH is a derivative of this above peptide but does not prevent polymerisation. The peptide H-gly-his-arg-pro-OH corresponds to the terminal sequence of the β -chain after removal of the fibrinopeptide B but is not an inhibitor of fibrin polymerisation (Doolittle, 1981)

Using the fluorogenic peptide Tos-gly-pro-arg-NHMec as a substrate for cathepsin L2 we demonstrated that the peptides H-gly-pro-arg-pro-OH and H-gly-pro-arg-OH are competitive inhibitors of cathepsin L2 (Table 5 2). At a 20-fold molar concentration of these peptides (300 μ M) over substrate a >50% inhibition of cathepsin L activity was observed, however, a five-fold excess of peptide (75 μ M) resulted in less than 10% inhibition. H-gly-his-arg-pro-AMC had no effect on cathepsin L2 activity. None of the three fibrin-antipolymerants significantly inhibited thrombin activity, even at a 40 fold excess over substrate (Table 5 2)

The effect of these peptides, at a concentration that do not inhibit enzyme activity (75 μ M), on the clotting time of thrombin and cathepsin L2 was examined. The clotting time for thrombin in the presence of H-gly-pro-arg-pro-OH was >9 hr compared to 20 min in the absence of the anti-polymerant (Table 5 1). Neither H-gly-pro-arg-OH or H-gly-his-arg-pro-OH significantly affected the clotting time of thrombin. In contrast, the clotting time for cathepsin L2 was not affected by any of the three peptides (Table 5 1)

Hirudin inhibited the activity of thrombin on the substrate Tos-gly-pro-arg-NHMec and its ability to polymerise fibrinogen, but had no effect on cathepsin L2 activity. Z-phe-ala-CHN₂ inhibited cathepsin L2 activity on the fluorogenic substrate and fibrinogen but showed no effect on thrombin (Tables 5 1 and 5 2)

The above data suggested that the cathepsin L cleavage site on fibrinogen does indeed differ to that of thrombin. We examined if the fibrinogen cleavage site of the cathepsin L2 remained following the removal of fibrinopeptides A and B by thrombin. Thrombin was incubated with a fibrinogen solution ($100 \mu\text{l}$, 20 mg ml^{-1}) for 100 min in the presence of the various concentrations of the anti-polymerant H-gly-pro-arg-pro-OH. This incubation period would allow the removal of the fibrinopeptides but would not allow the formation of fibrin polymers. Cathepsin L2 was then added and to the tubes and the mixture examined for clot formation. Thrombin was added to a second series of tubes. The addition of cathepsin L2 resulted in the formation of clot within 10 -20 min in tubes preincubated with thrombin and 200 - 300 μM anti-polymerant. Tubes containing 350 and 400 μM anti-polymerant formed incomplete clots (semi-clots) within 60 min, however, at these concentrations of anti-polymerant it was expected that some inhibition of the cathepsin L2 would occur (Table 5.3; see also Table 5.2). Whilst the addition of more thrombin to tubes preincubated with thrombin and anti-polymerant also produced clots, these were much slower to form (Table 5.3).

At the end of the above experiment reducing SDS sample buffer was added to the tubes, the mixture boiled and the analysed by reducing SDS-PAGE. Samples from tubes that were pre-incubated with thrombin and anti-polymerant (200, 300 and 400 μM) and then additional thrombin added showed similar electrophoretic pattern to tubes containing no anti-polymerant (Figure 5.3), when compared to the untreated fibrinogen it can be seen that the fibrinopeptides have been cleaved by thrombin. In contrast, the electrophoretic profile of samples from tubes in which cathepsin L2 was

added following pre-incubation with thrombin and anti-polymerant was similar to the profile obtained for clots produced by cathepsin L2 alone (Figure 5 3, see also Figure 5 2) The fibrinogen α and β -chains disappear, although in these experiments it can be seen that the digests did not go to completion most likely because of the inhibitory affects of the anti-polymerant The fibrinogen γ -chain migrates slightly faster, as the concentration of the anti-polymerant in the mixtures decreases the more digestion of this polypeptide is observed and concurrent with this is an increase of the 25 kDa band

Table 5.1.*Fibrin-clotting times for thrombin and F hepatica cathepsin L2*

PEPTIDE	CLOTTING TIME (min)*		
	thrombin	cathepsin L2	
control	20	25	
Hirudin	1 unit ml ⁻¹	>540	25
Z-phe-ala-CHN ₂	75 μM	30	>540
H-gly-pro-arg-pro-OH	75 μM	>540	40
H-gly-pro-arg-OH	75 μM	35	35
H-gly-his-arg-pro-OH	75 μM	30	30

* Thrombin (5.7×10^{-3} NIH units) and cathepsin L2 (2.3×10^{-3} units) were added to 100μl fibrinogen (20 mg ml^{-1}) as described in Materials and Methods. The assay mixture was incubated at 37°C and the time taken to form a solid clot recorded.

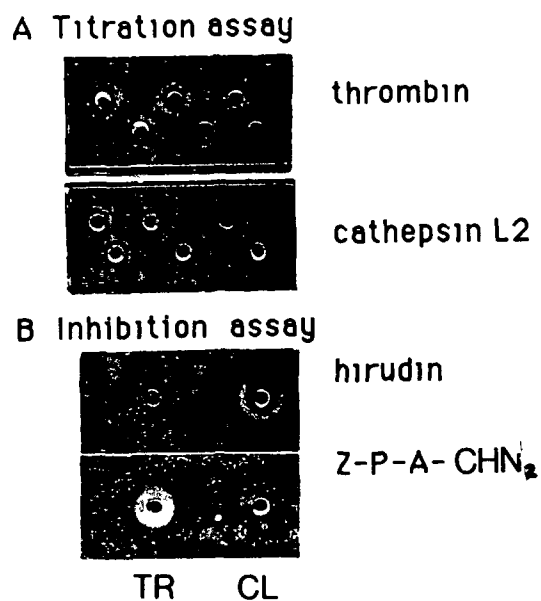


Figure 5.1

Fibrinogen-agarose plate assay of clots produced by cathepsin L2 and thrombin.

A Titration of thrombin (NIH units $\times 10^{-3}$, from left to right, 5.7, 2.97, 1.4, 0.7, 0.36 and 0.0) and cathepsin L2 (units $\times 10^{-3}$, from left to right, 2.3, 2.0, 1.85, 1.409, and 0.45)

B Inhibition assay of cathepsin L2 (2.3×10^{-3} units) and thrombin (5.7×10^{-3} NIH units) by the thrombin-specific inhibitor hirudin (3 units) and the cathepsin L specific inhibitor Z-phe-ala-CHN₂ (Z-F-A-CHN₂, 600 μ M)

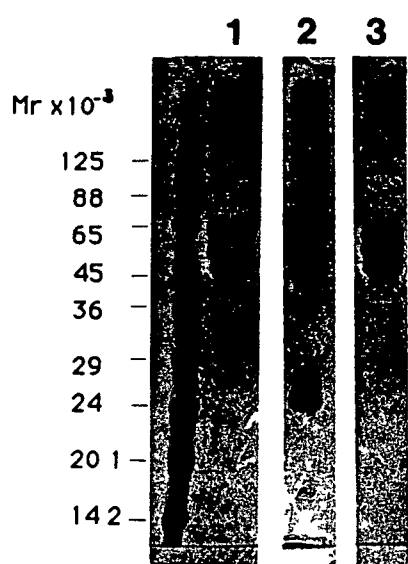
Figure 5.2

Electrophoretic analysis of fibrin clots

A SDS-PAGE of fibrinogen and fibrin clots produced by thrombin and cathepsin L2. 2 μl of bovine fibrinogen (20 mg ml^{-1}) without added enzyme (lane 1), 4 μl of the reaction mixture of bovine fibrinogen ($100 \mu\text{l}$, 20 mg ml^{-1}) incubated in the presence of purified cathepsin L2 (2.3×10^{-3} units) (lane 2) and 4 μl of the reaction mixture of bovine fibrinogen ($100 \mu\text{l}$, 20 mg ml^{-1}) incubated in the presence of thrombin (5.7×10^{-3} units) (lane 3)

B Immunoblotting analysis of fibrinogen clots. 1 μl of the reaction mixture of bovine fibrinogen ($100 \mu\text{l}$, 20 mg ml^{-1}) incubated in the presence of cathepsin L2 (2.3×10^{-3} units) and probed with anti-fibrinogen antibodies (lane 1), 1 μl of the reaction mixture of bovine fibrinogen ($100 \mu\text{l}$, 20 mg ml^{-1}) incubated in the presence of thrombin (5.7×10^{-3} NIH units) and probed with anti-fibrinogen antibodies (lane 2), 1 μl of the reaction mixture of bovine fibrinogen ($100 \mu\text{l}$, 20 mg ml^{-1}) incubated in the presence of cathepsin L2 (2.3×10^{-3} units) and probed with normal human serum (lane 3), and 1 μl of the reaction mixture of bovine fibrinogen ($100 \mu\text{l}$, 20 mg ml^{-1}) incubated in the presence of thrombin (5.7×10^{-3} units) and probed with normal human serum (lane 4). The molecular weight markers were β -galactosidase (125 kDa), Fructose-6-phosphate kinase (88 kDa), pyruvate kinase (65 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.2 kDa)

A.



B.

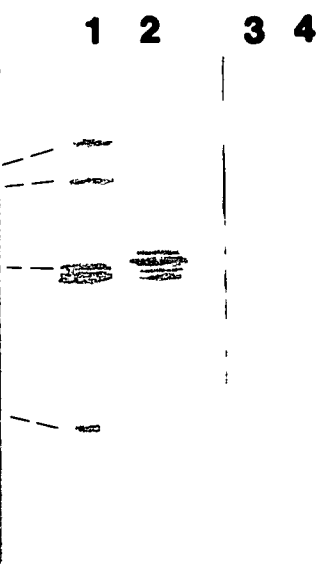


Table 5.2.

*The effect of anti-polymerants on the Tos-gly-pro-arg-NHMec cleaving ability of thrombin (5.7×10^{-3} NIH units) and *F. hepatica* cathepsin L2 (0.5×10^{-3} units)*

PEPTIDE	Inhibition*	
	(% activity remaining)	
	thrombin	cathepsin L2
control	100	100
Hirudin 1 unit ml ⁻¹	8	97
Z-phe-ala-CHN ₂		
75 μM	96	12
300 μM	72	6
H-gly-pro-arg-pro-OH		
75 μM	98	96
300 μM	80	42
H-gly-pro-arg-OH		
75 μM	96	93
300 μM	82	43
H-gly-his-arg-pro-OH		
75 μM	110	102
300 μM	98	99

* data represents mean of results from three separate assays

Table 5.3.

Fibrin-clotting times for thrombin and cathepsin L2 following a pre-incubation of fibrinogen with thrombin and anti-polymerant

Time		H-gly-pro-arg-pro-OH (μM) *					
		0	200	250	300	350	400
0'	+ thrombin*	+	-	-	-	-	-
	+ cathep.L	+	-	-	-	-	-
10'	+ thrombin	+	-	-	-	-	-
	+ cathep L	+	+/-	-	-	-	-
20'	+ thrombin	+	+	-	-	-	-
	+ cathep. L	+	+	+	+	-	-
30'	+ thrombin	+	+	-	-	-	-
	+ cathep L	+	+	+	+	-	-
60'	+ thrombin	+	+	+	+/-	-	-
	+ cathep L	+	+	+	+	+/-	+/-

Fibrinogen ($100 \mu\text{l}$, 20 mg ml^{-1}) was pre-incubated with thrombin (5.7×10^{-3} NIH units) in the presence various concentrations of the anti-polymerant H-gly-pro-arg-pro-OH for 100 min at 37°C . Thrombin (5.7×10^{-3} NIH units) or cathepsin L2 (2.3×10^{-3} units) was then added and the time taken for clot formation recorded. Arbitrary scales are, no clot -, semi-clot +/- and clot +

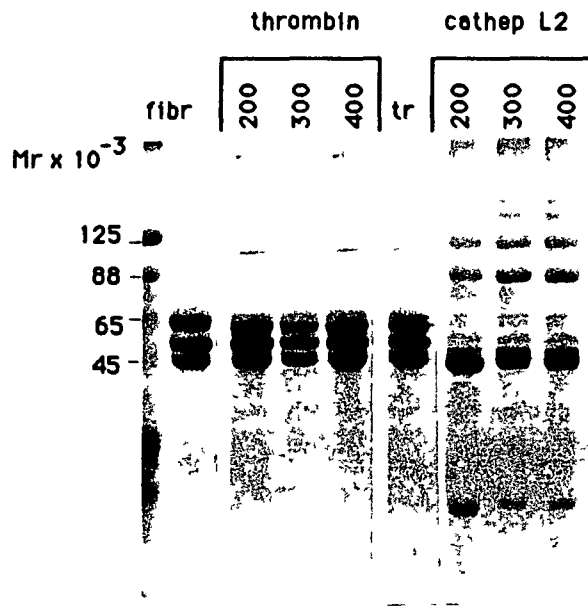


Figure 5.3

Electrophoretic analysis of fibrin clots formed subsequent to pre-incubation of fibrinogen with thrombin and fibrin anti-polymerants

Fibr, 2 μ l of Fibrinogen (20 mg ml⁻¹) without added enzyme, thrombin 200, 300 and 400; 4 μ l of the reaction mixture of fibrinogen (100 μ l, 20 mg ml⁻¹) pre-incubated with 200 μ M, 300 μ M and 400 μ M of anti-polymerant and 5.7 $\times 10^{-3}$ NIH units of thrombin with subsequent application of 5.7 $\times 10^{-3}$ NIH units of thrombin. Tr, 4 μ l of the reaction mixture of fibrinogen (100 μ l, 20 mg ml⁻¹) incubated with 5.7 $\times 10^{-3}$ NIH units of thrombin. Cath L2 200, 300 and 400; 4 μ l of the reaction mixture of fibrinogen (100 μ l, 20 mg ml⁻¹) pre-incubated with 200 μ M, 300 μ M and 400 μ M of anti-polymerant and 5.7 $\times 10^{-3}$ NIH units of thrombin and subsequent application of 2.3 $\times 10^{-3}$ units of cathepsin L2. The molecular weight markers were as follows. β -galactosidase (125 kDa), Fructose-6-phosphate kinase (88 kDa), pyruvate kinase (65 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.2 kDa)

CHAPTER 6
DISCUSSION

Characterisation of a second Fasciola hepatica cathepsin L

The first trematode cathepsin L to be characterised was isolated by Smith *et al* (1993a) from medium in which the mammalian parasite, *Fasciola hepatica*, was cultured. The proteinase was classified as a cathepsin L because of its location in vesicles, N-terminal sequence of its first 20 amino acids and its preference for cleaving fluorogenic peptide substrates with hydrophobic amino acids in the P₂ position (Schechter and Berger nomenclature, Schechter and Berger, 1967). Dalton and Heffernan (1989), however, had shown that multiple cysteine proteinases were secreted into culture medium by the parasite. They speculated that the parasite used these proteinases for feeding and migration as they were shown to be present in immature and adult parasites. Eleven bands of proteolytic activity were observed by Dalton and Heffernan (1989) all of which were subsequently shown to be enhanced by reducing reagents and inhibited by cysteine proteinase specific inhibitors. This thesis describes the isolation, to homogeneity, of a second cysteine proteinase secreted into culture medium by adult *F hepatica* parasites. N-terminal sequence data and experiments on the substrate specificity of the enzyme reveal that this cysteine proteinase is also a cathepsin L. For the purpose of clarity, the cathepsin L isolated by Smith *et al* (1993a) is referred to as cathepsin L1 and the proteinase described in this thesis designated cathepsin L2. Cathepsin L2 proteinase has the unique property among parasitic proteinases of cleaving fibrinogen in such a manner as to form a fibrin clot which is different to the type of fibrin clot produced by the serum proteinase thrombin (factor II_a). This novel property of cathepsin L2 is described in this

thesis also

Purification of a second cysteine proteinase from adult Fasciola hepatica culture medium

A two-step purification protocol was designed to a) separate the cysteine proteinase fraction from other proteins and peptides present in adult *F hepatica* culture medium by using gel-filtration chromatography and b) separate the two cysteine proteinases, present in the cysteine proteinase pool from the initial gel-filtration step, from each other by using anion-exchange chromatography. The presence of the second cysteine proteinase which has a substrate specificity distinct to the *F hepatica* cysteine proteinase described by Smith *et al* (1993a) was specifically measured using the fluorogenic peptide substrate Tos-gly-pro-arg-NHMec (see below). Both the gel-filtration and ion-exchange columns were equilibrated in 0.1 M Tris buffer, pH 7. Therefore, gel-filtration as the first step of the purification procedure was designed to achieve both separation of the proteins present in *F hepatica* culture medium according to molecular weight, and equilibration of the separated proteins into the buffer used for the subsequent anion-exchange-chromatography step. This makes the purification procedure simpler and more rapid by avoiding extra concentration and buffer exchange steps which may also adversely affect proteinase activity.

A single peak of cysteine proteinase activity, was observed following gel-filtration chromatography of the *F hepatica* culture medium. The cysteine proteinase specifically measured using the substrate Tos-gly-pro-

arg-NHMec, has a molecular weight of approximately 30 kDa according to the results of its migration on the gel-filtration column. The results of protein estimation from the BCA assay show that 5% of the protein applied to the gel-filtration column is recovered in the peak containing the cysteine proteinase activity. When protein content is estimated by the U V absorbance profile of the material eluted from the gel-filtration column, it is evident that a higher proportion (approximately 25%) of the total protein applied to the column is recovered. This shows that the BCA assay gives lower readings for the amount of protein present during purification than protein measurement by U V absorbance.

Anion-exchange chromatography using QAE-Sephadex resin followed gel-filtration in the purification procedure. QAE-Sephadex binds proteins with a net negative charge at pH 7. The pool of cysteine proteinase activity from the gel-filtration stage was applied directly to the QAE-Sephadex column. The cysteine proteinase described by Smith *et al* (1993a) does not bind to QAE-Sephadex at pH 7 and is therefore eluted in the flow-through fraction. The QAE-Sephadex column was subsequently washed with 5 column volumes of 0.1M Tris pH 7 to remove any traces of the proteinase described by Smith *et al* (1994a).

The cysteine proteinase described in this thesis binds to QAE-Sephadex at pH 7. Before elution with 400 mM NaCl, the QAE-Sephadex column was washed with 75 mM NaCl. A small amount of protein which did not contain cysteine proteinase activity was eluted with 75 mM NaCl. Cysteine proteinase was subsequently eluted from the QAE-Sephadex resin with 400 mM NaCl. As the cysteine proteinase was only eluted by the higher salt concentration it is evident that it binds firmly to QAE-Sephadex resin at

pH 7 Therefore QAE-Sephadex chromatography at pH 7 distinguishes between two cysteine proteinases present in *F hepatica* culture medium and is a good method to separate both of these enzymes from each other

The two column purification procedure described in this thesis results in a 149-fold of cysteine proteinase with approximately 30% recovery of active enzyme A two step purification procedure using similar chromatographic procedures to those described above was used by Smith *et al* (1993a) to purify the cysteine proteinase with cathepsin L-like properties from medium in which adult *F hepatica* were incubated Smith *et al.* (1993a) achieved a recovery of 12% active enzyme following purification which is lower than the recovery achieved for the cysteine proteinase described in this work This difference in fold-purifications could be due to the possible lower abundance of the present cysteine proteinase in the culture medium compared to the cysteine proteinase described by Smith *et al* , (1993a) Alternatively the proteinase described by Smith *et al* , (1993a) may be less stable during purification than the cysteine proteinase described in this work.

Reducing SDS-PAGE of the pool from the QAE-Sephadex chromatography shows a single band of protein at 29.5 kDa Reducing SDS-PAGE results of total adult *F hepatica* culture medium, shows a second major protein present which has a molecular weight of 27 kDa The results of the purification of a previously described cysteine proteinase from adult *F hepatica* culture medium show that the 27 kDa protein band corresponds to the enzyme isolated by Smith *et al* (1993a)

Zymograms containing SDS show that the lower proteolytic bands present in *F hepatica* culture medium can be separated from the upper

proteolytic bands. The upper proteolytic bands are due to the single cysteine proteinase of 27 kDa described by Smith *et al* (1993a). Zymography without SDS show that these 'lower bands' are due to a single proteinase. Therefore, both zymography and reducing SDS-PAGE revealed that the two-step purification procedure used in this work separates the two cysteine proteinases and results in an homogeneous preparation of a second *F hepatica* cysteine proteinase.

The procedure used in this thesis to purify the second *F hepatica* cysteine proteinase is different to the procedures utilised by other investigators to purify *F hepatica* cysteine proteinases. Rege *et al* (1989), purified a cathepsin L-like enzyme of 14.5 kDa from homogenates of *F hepatica* using MonoS cation-exchange chromatography and molecular sieve HPLC. Both columns were prepared at pH 5.0. Unlike this study and that of Smith *et al* (1993a), Rege *et al*, (1989) did not find more than one form of cysteine proteinase enzyme.

Yamasaki *et al* (1989) did not use ion-exchange chromatography to purify a cysteine proteinase enzyme. They achieved substantial purification using gel-filtration and affinity chromatography using activated thiol-Sepharose. Activated thiol-Sepharose selects for proteins with exposed cysteine residues (DeMartino, 1989). As both cysteine proteinases isolated from adult *F hepatica* culture medium have cysteine active site residues, the procedure of Yamasaki *et al* (1989) would not have separated the cysteine proteinases. Therefore their purified preparation may have contained both cysteine proteinases.

Wijffels *et al*. (1994) purified cysteine proteinases from media in which adult *F hepatica* were incubated using a single size exclusion

chromatography step They pooled fractions containing a 26 kDa species (as assessed by the results of non-reducing SDS-PAGE) for further analyses This protein was subsequently analysed by Western blot and reducing SDS-PAGE and was found to have heterogeneity Reducing SDS-PAGE of the pooled fraction showed a major protein band of 28 kDa with less intensely staining bands migrating at lower molecular weights It was also found by using 2-dimensional PAGE that the cysteine proteinase resolved into 3 major peaks of differing pIs This indicates that Wijffels *et al* (1994) also observed more than one type of *F hepatica* cysteine proteinase but they did not attempt further purification of these proteins

The above investigators purified cysteine proteinases from adult *F hepatica* homogenates (Yamasaki *et al* , 1989, Rege *et al* , 1989) or *F hepatica* culture medium (Wijffels *et al* ,1994) None of these studies resulted in the isolation of more than a single cysteine proteinase although it is evident that more than a single cysteine proteinase is present in *F hepatica* (Heussler and Dobbelaere, 1994) The results in this thesis describe the separation for the first time of two cysteine proteinases from adult *F hepatica* culture medium by column chromatographic methods As both of these enzymes were isolated to homogeneity it was therefore possible to compare these enzymes on the basis of their enzyme activities, N-terminal sequences and electrophoretic profiles

Comparison of cathepsins L1 and L2

The cysteine proteinases present in *F hepatica* culture medium can be resolved into two fractions by QAE-Sephadex chromatography The

differences in binding of these two enzymes to QAE-Sephadex ion-exchange resins demonstrates that there is a major charge difference in the molecules at pH 7.0. From the results of reducing SDS-PAGE, the cysteine proteinase isolated by Smith *et al* (1994a) (cathepsin L1) is 27 kDa and the cysteine proteinase isolated in this thesis (cathepsin L2) is 29.5 kDa. Since the two molecules differ in molecular size, this charge difference may reside in the amino acids present in the extra 2.5 kDa of cathepsin L2 but not present in cathepsin L1.

The data published by Smith *et al* (1993a) and that presented here demonstrate that adult *F. hepatica* secrete two cathepsin L proteinases. An immediate explanation for the presence of two cathepsins L in the *F. hepatica* culture medium is that cathepsin L1 (27 kDa) is a processed form of cathepsin L2 (29.5 kDa) or both are processed from a higher molecular sized parent form. However, a comparison of the N-terminal sequence of the two enzymes reveals that this is not the case. The enzymes have different amino acid residues at position seven from the N-terminal region, which is the most highly conserved region in cathepsin Ls (Dufour *et al.*, 1987, Mason *et al*, 1986). Cathepsin L2 has an arginine residue at this position whereas cathepsin L1 has a proline residue.

From the results of the sequences of the first 14 amino acids of cathepsin L1 (Smith *et al*, 1993a) and cathepsin L2 both enzymes are highly similar (47%) to mammalian and avian cathepsins L enzymes (Ishidoh *et al*, 1987b, Mason *et al*, 1986, Turk *et al*, 1985, Dufour *et al*, 1987). In contrast, cathepsins L1 and L2 are not so similar to cathepsin B from the closely related trematode parasite *Schistosoma mansoni* (20%) (Feilleisen and Klinkert, 1990). The two cathepsins L from *F. hepatica* show more identity to

a cathepsin L-like enzyme (cruzain or cruzipain) (Cazzulo *et al* , 1989) from the protozoan parasite *Trypanosoma cruzi* (40%) than to the *S. mansoni* cathepsin B. Interestingly, cathepsin L1 and L2 are more similar to mammalian cathepsins L than they are to the parasite cathepsin L-like enzyme (47% vs 40% respectively). The cathepsins L1 and L2 sequences are equally similar to mammalian cathepsin S (Wiederanders *et al* , 1991) and mammalian cathepsins L (47%). However, enzyme kinetic studies show that both *F. hepatica* cysteine proteinases are cathepsin L-like and not cathepsin S-like (see below).

Further evidence to demonstrate that cathepsin L1 was not a processed form of cathepsin L2 was obtained by immunoblotting experiments. Adult *F. hepatica* extract homogenised with or without proteinase inhibitors, medium in which adult *F. hepatica* were incubated, purified cathepsin L1 and purified cathepsin L2 were separated by reducing SDS-PAGE, transferred to nitrocellulose membranes and probed with antiserum which recognised both cathepsin L1 and L2. The enzymes present in adult worm extract are of the same molecular size (29.5 kDa and 27 kDa) as the proteinases secreted into the culture medium and the purified cathepsins L1 and L2. The results of the immunoblotting experiments were the same both in the presence and absence of proteinase inhibitors. No pro-form of cathepsin L1 or L2 was detected in the adult worm extracts either in the presence or absence of proteinase inhibitors. This indicates that the pro-form of cathepsin L1 and L2 is converted to the mature enzyme very rapidly. Furthermore, if cathepsin L1 was the mature processed form of cathepsin L2 then it would be expected that cathepsin L1 alone would be detected in the *F. hepatica* culture medium. Mammalian cathepsin L is

synthesised as an inactive pro- form of 39 kDa and this molecule is subsequently processed by proteolysis to the mature form of 30 kDa (Nishimura *et al* , 1988, Hara *et al* , 1988, Rowan *et al* , 1992) The difference in molecular weight between the pro- and mature form of mammalian cathepsin L is 9 kDa which is considerably larger than the 2.5 kDa difference between *F hepatica* cathepsin L1 and L2 which further supports the evidence that cathepsin L1 is not the processed form of cathepsin L2

Zymography in the presence of SDS was one method used to detect and compare proteolytic activities of cathepsins L1 and L2. Gelatin was co-polymerised into an SDS-PAGE gel as the protein substrate for zymography. Following separation of the proteins by SDS-PAGE, the gel was washed and then incubated for a sufficient length of time to allow digestion of the gelatin in the gel by the proteinase enzymes. Following the incubation step, the gelatin in the PAGE gel was stained with coomassie blue dye. Proteinase activity was visualised as clear bands where the gelatin was digested by the proteinases. The samples were not denatured by boiling or reduced with mercaptoethanol in order that enzymatic activity was retained.

When the proteinase activity in *F hepatica* culture medium was visualised by zymography with incorporated gelatin as the protein substrate, 11 distinct proteinase bands are present (Dalton and Heffernan, 1989). These proteinase bands were divided into two groups on the basis of their pH optima for cleavage of gelatin. Group 1 with a pH optimum of 3-4.5 contains 5 bands in the 60 to 88 kDa range and Group 2 with a pH optimum of 4.5 to 8, contains 6 bands in the 27.5 to 46 kDa range. Subsequent protein purification studies by Smith *et al* (1993 a,b) showed that the Group 1

proteinases are due to a single proteolytic enzyme, cathepsin L1. The results of this study show that the Group 2 enzymes correspond to single enzyme, cathepsin L2.

The proteinases present in adult *F. hepatica* culture medium were also visualised by zymography without SDS. This technique visualises proteinases following electrophoretic separation based on both size and charge (Dowd *et al* , 1994, Smith *et al* , 1994). When *F. hepatica* culture medium containing both cathepsins L1 and L2 is subject to zymography without SDS, cathepsin L1 migrates as multiple bands while cathepsin L2 is visualised as a single band.

Studies using the protein modifying reagent, TNM, also demonstrated a structural difference between the two *F. hepatica* cathepsins L. Under mild conditions, as employed in this study, TNM is highly specific for tyrosyl modification (Eyzaguirre, 1987). Under these conditions, nitration of tyrosine residues by TNM produces an ionisable chromophore, 3-Nitro-tyrosine (Eyzaguirre, 1987). However, under more vigorous conditions (high molar concentrations or reactions carried out at high pH values) other residues such as histidine, tryptophan and methionine are modified (Riordan and Vallee, 1972). These residues must not be accessible to TNM on the cathepsin L1 molecule since this enzyme was not inactivated by this reagent. In contrast, nitration of cathepsin L2 modification resulted in inactivation of the enzyme. The cathepsin L2 is 2.5 kDa larger than the cathepsin L1, the part of the cathepsin L2 that make up this additional 2.5 kDa may contain the residues that are susceptible to modification by TNM.

The inactivation of cathepsin L2 by TNM was visualised by zymography without SDS for the reasons outlined above. Following incubation with

increasing concentrations of TNM the proteolytic band representing the cathepsin L2 gradually disappeared. Accompanying this disappearance was the gradual appearance of several faster migrating bands. The faster migrating bands may be various modified forms of the enzyme each with a different number of residues modified. Alternatively, modification of the cathepsin L2 with TNM may cause the unfolding of the molecule which then becomes vulnerable to auto-digestion, if this were the case the faster migrating bands may be active fragments of the enzyme.

Cathepsins L are proteinases that have a high affinity for the peptide substrate Z-phe-arg-NHMec and have little affinity for the substrates Z-arg-arg-NHMec and Bz-arg-NHMec (Barrett and Kirschke, 1981). The *F hepatica* cysteine proteinases studied by Smith *et al.* (1993a) and the cysteine proteinase studied in this thesis favour Z-phe-arg-NHMec over Z-arg-arg-NHMec and Z-arg-NHMec which demonstrates that both enzymes belong to the cathepsin L class (Barrett and Kirschke, 1981). This confirms the results of N-terminal amino acid sequencing which demonstrated that both the first 14 N-terminal amino acids of cathepsins L1 and L2 share substantial homology with the first 14 N-terminal amino acids of mammalian cathepsins L. It is unlikely that *F hepatica* would secrete two proteinases of the same specificities especially as they both are the most abundant proteins secreted by the parasite according to the results of reducing SDS-PAGE. Therefore, the possibility that cathepsin L1 and L2 differ in their substrate specificities was investigated using a bank of 11 commercially available fluorogenic substrates.

The mammalian cathepsin S proteinase, shares similar properties to cathepsin L (Bond and Butler, 1987, Kirschke *et al.*, 1986, Bromme *et al.*,

1989) From the results of the N-terminal sequences of their first 14 amino acids the *F hepatica* cysteine proteinases are equally homologous to mammalian cathepsin S and cathepsin L. Therefore the possibility of the two *F hepatica* cysteine proteinases being cathepsins S rather than cathepsins L was investigated. Cathepsin S and cathepsin L can be distinguished by a comparison of their affinities for the fluorogenic substrates Z-phe-arg-NHMec and Z-phe-val-arg-NHMec (Bromme *et al* , 1989). Cathepsin S has a five-fold higher affinity for Z-phe-val-arg-NHMec than for Z-phe-arg-NHMec. Cathepsin L has a higher affinity for Z-phe-arg-NHMec over Z-phe-val-arg-NHMec. Both cathepsin L1 and L2 cleave Bz-phe-val-arg-NHMec with less affinity than they cleave Z-phe-arg-NHMec confirming that neither cathepsin L1 nor L2 could be classified as cathepsins S (Bromme *et al* , 1989).

Both cathepsins L1 and L2 had an approximately 3-times greater affinity for Boc-val-leu-lys-NHMec than for Z-phe-arg-NHMec. Few cathepsin Ls have a higher affinity for this substrate than for Z-phe-arg-NHMec except for the cathepsin L-like enzyme from the trematode parasite, *Paragonimus westerami* (Yamakami and Hamajima, 1989). The relative efficiency of Boc-val-leu-lys-NHMec over Z-phe-arg-NHMec as a substrate for assaying cathepsins L1 and L2 may be explained by the presence of leucine in the P₂ position. Leucine is similar to phenylalanine in being a hydrophobic amino acid but its R-group is aliphatic rather than aromatic. Therefore the S₂ pocket of the *Fasciola hepatica* cathepsins L1 and L2 active sites may accommodate aliphatic hydrophobic amino acids in preference to aromatic hydrophobic amino acids.

Cathepsins L1 and L2 also have a preference for glycine over valine in the P₃ position of peptide substrates since they cleave Tos-gly-pro-arg-

NHMec in preference to Boc-val-pro-arg-NHMec. As glycine is a non-polar amino acid but smaller than valine, the S_3 sites of both cathepsins L1 and L2 seem to have a preference for small non-polar amino acid residues.

The tetrapeptide Suc-leu-leu-val-tyr-NHMec is far less readily hydrolysed than the tri-peptide H-leu-val-tyr-NHMec by cathepsins L1 and L2. This result may indicate that tetrapeptides do not fit into the active sites of cathepsins L1 and L2 as readily as di- and tri-peptides.

The peptide substrates Boc-val-pro-arg-NHMec, Tos-gly-pro-arg-NHMec and Tos-gly-pro-lys-NHMec were cleaved with much higher affinity (approximately 100-fold) by cathepsin L2 than by cathepsin L1. These substrates have a proline residue in the P_2 position. Proline is an imino acid because its side chain is bonded to the nitrogen as well as to the α -carbon of the central compound. This restricts the geometry of the peptide in which it is incorporated. Because the presence of a proline residue in a peptide may alter the structure of the peptide due to the absence of a free amino group, the shape of the cathepsin L2 active site may differ to the shape of the cathepsin L1 active site.

The cleavage of peptide substrates by cathepsins L1 and L2 may provide clues to the types of protein substrates which may be cleaved *in vivo* by these enzymes. For example, the $\alpha 1$ and $\alpha 2$ chains of tropocollagen in type I collagen, the most common collagen found in tendons, bone, skin, ligaments, cornea and internal organs, is composed of a regularly repeating sequence in which glycine is found at every third residue (Rawn, 1989). This sequence can be written $(\text{gly-X-Y})_n$, where the Xs and Ys are often proline and hydroxyproline. Cathepsin L2 therefore, may specifically recognise this

sequence and may be more efficient at cutting type I collagen than cathepsin L1. Cathepsin L2 may play a more important role than cathepsin L1 in the migration of the parasite as it would need to be able to breakdown internal organs to achieve its task of migrating from the duodenum to the liver. Studies on the cleavage of collagens by cathepsins L1 and L2 are in progress (Drs C. Carmona and J. Dalton, personal communication).

Direct visualisation of cathepsin L1 and L2 activity in zymograms (without SDS) was possible by incubation of gels in peptide fluorogenic substrates. When the zymogram was developed for Z-phe-arg-NHMec and Boc-val-leu-lys-NHMec cleaving activities, both cathepsins L1 and L2 are shown to be equally active on these substrates. The fluorogenic peptide substrate, Boc-val-leu-lys-NHMec is preferentially cleaved by both cathepsin L1 and L2. When the zymograms are incubated in the fluorogenic substrates Tos-gly-pro-arg-NHMec and Tos-gly-pro-lys-NHMec, cathepsin L2 cleaves both of these substrates better than cathepsin L1. Zymography in the presence of fluorogenic peptide substrates support the enzyme kinetics studies and further demonstrate the different substrate specificities of cathepsin L1 and L2.

To summarise, having the two *F. hepatica* cysteine proteinases in homogeneous form provided the opportunity to compare their reaction kinetics on a range of peptide substrates and to N-terminally sequence each enzyme. These studies classified the two *F. hepatica* cysteine proteinases as cathepsin L-like. From the enzyme kinetics studies it is clear that the two cathepsin Ls have different substrate specificities and represent distinct subclasses of cathepsin L. The two cathepsins L also differ in their susceptibility to modification by TNM.

Comparison of *F hepatica* cathepsins L to cathepsins L from other parasites

Until recently it was thought that parasitic trematodes produce cathepsin B-like and not cathepsin L-like proteinases (British Society of Parasitology Proteinase workshop, Spring meeting 1992, York, England) The first cathepsin L produced by a parasitic trematode was isolated from *F hepatica* by Smith *et al* (1993a) This study describes the isolation of a second *F hepatica* cathepsin L Since the paper of Smith *et al* (1993a) cathepsin L proteinases from other trematodes have been described

The blood fluke, *Schistosoma mansoni* is the causative agent of the major human disease Bilharzia which occurs in tropical areas This trematode parasite is closely related to the liver fluke, *F hepatica* *S mansoni* has been shown to possess a cysteine proteinase called Sm31 which is classed as a cathepsin B on the basis of its hydrolysis of the fluorogenic substrate Z-arg-arg-NHMec (Gotz and Klinkert, 1993) and because the sequence of a cDNA transcript encoding Sm31 shows homology with cathepsin B genes (Klinkert *et al.*, 1989) Sm31 is an important diagnostic antigen and may function as a haemoglobinase (Gotz and Klinkert, 1993, Klinkert *et al* , 1987,1989, Ruppel *et al* , 1985, Davis *et al* , 1987) Because *F hepatica* was shown to have a cathepsin L, the possibility that *S mansoni* may also have a cathepsin L was investigated Fluorogenic substrate assays and molecular cloning studies demonstrated that *S. mansoni* indeed express a cathepsin L enzyme (Smith *et al* , 1994) From the deduced amino acid sequence of the cDNA encoding the *S mansoni* cathepsin L the enzyme has a molecular weight of 24 kDa The *S mansoni* cathepsin L gene exists as a single copy (Smith *et al* , 1994) This

is in contrast to *F hepatica* which has multiple copies of its cathepsin L gene (Heussler and Dobbelaere, 1994) The *S mansoni* cathepsin L proteinase was suggested to be involved in haemoglobin digestion in conjunction to the cathepsin B proteinase (Smith *et al* 1994)

Paragonimus westermani is a medically important trematode parasite which causes human paragonimiasis Paragonimiasis a disease of the lungs which may be associated with tumour formation (Smyth, 1979)

Paragonimus westermani produces a cysteine proteinase termed neutral thiol proteinase (NTP) (Yamakami and Hamajima, 1989, Hamajima *et al* , 1994) NTP is an immunosuppressive agent (Hamajima *et al.*, 1994) and has been shown to share homologies with cathepsin L enzyme using molecular cloning studies (Hamajima *et al* , 1994) NTP cleaves the fluorogenic substrate Boc-val-leu-lys-NHMec in preference to Z-phe-arg-NHMec (Yamakami and Hamajima, 1989) This substrate preference is similar to that described here for *F hepatica* cathepsins L1 and L2

The zoonotic hookworm, *Ancylostoma caninum* is a nematode parasite which is mainly an infection of dogs Recently, *A caninum* has been shown to be the causative agent of a medical condition called eosinophilic enteritis (EE) of humans resident in the tropical regions of Australia (Prociv and Croese, 1990) EE is probably caused by the induction of allergic responses to the secretions of *A caninum* *A caninum* is known to secrete a metalloproteinases which has elastinolytic activity and inhibits fibrin clot formation (Hotez and Cerami, 1983) However, *A caninum* also secretes a cysteine proteinase that cleaves Z-phe-arg-NHMec in preference to Z-arg-arg-NHMec and Z-arg-NHMec These results indicate that *A caninum* cysteine proteinase may have a cathepsin L-like specificity similar

to that found for the *F hepatica* cathepsins L1 and L2. The *A caninum* cathepsin L may be involved in the induction of the allergic reactions observed in EE (Dowd *et al* , 1994)

The African trypanosomes *Trypanosoma congolense* and *Trypanosoma brucei* are parasites which cause the usually fatal disease known as sleeping sickness. These protozoan parasites infect humans and domestic animals such as sheep and cattle. Both of these parasites produce cathepsin L-like enzymes (Mbawa *et al* , 1992, Mottram *et al* , 1989, Robertson *et al* ,1990). The cathepsin L-like proteinase produced by *T congolense* has a molecular weight of 31-32 kDa. From cloning studies, the cathepsin L-like enzyme from *T brucei* was found to have a molecular weight of 23.1 kDa. The *T congolense* cathepsin L-like enzyme cleaves Z-phe-arg-NHMec with a K_m which is similar to the K_m of the *F hepatica* cathepsins L1 and L2 for Z-phe-arg-NHMec (Mbawa *et al* , 1992). *T brucei* cathepsin L-like proteinase has a similar fluorogenic peptide substrate specificity as *F hepatica* cathepsins L1 and L2 (Robertson *et al* ,1990). *T congolense* cathepsin L-like enzyme cleaves fibrinogen but the cleavage pattern is different to the cleavage pattern obtained using *F hepatica* cathepsin L (see below)

Trypanosoma cruzi , is a protozoan parasite found in South America, is the causative agent of Chaga's disease. The cysteine proteinase from *T. cruzi* (called cruzipain or cruzain) has been well characterised (Cazzulo *et al* , 1989, 1990, Bontempi *et al* , 1989, Murta *et al.*, 1990, Bontempi and Cazzulo, 1990, Eakin *et al* , 1992). Cruzain cleaves the fluorogenic substrate Z-phe-arg-NHMec with a K_m similar to the K_m s found for the *F hepatica* cathepsins L1 and L2. The N-terminal sequence (14 amino acids)

of cruzain is 40% similar to the amino acid sequence of *F hepatica* cathepsins L1 and L2. Furthermore, cruzain degrades IgG in a similar manner to the *F hepatica* cathepsin L1 (Smith *et al* , 1993b, Carmona *et al* , 1993, Bontempi and Cazzulo, 1990)

The protozoan parasite, *Theileria parva* is the tick-bourne causative agent of an acute and usually fatal disease in cattle. *T parva* produces a cathepsin L-like proteinase which has been cloned and sequenced (Nene *et al* , 1990). From the deduced amino acid sequence of its cDNA sequence the *T parva* cysteine proteinase was found to have a molecular weight of 23.5 kDa. The *T parva* cysteine proteinase exhibits a similar substrate preference to *F hepatica* cathepsins L1 and L2 as it cleaves Z-phe-arg-NHMec in preference to Z-arg-arg-NHMec and Z-arg-NHMec (Nene *et al* , 1990). The *T parva* cathepsin L-like enzyme therefore shares a similar molecular weight and N-terminal sequence to *F hepatica* cathepsins L1 and L2.

Protozoan parasites belonging to the genus *Plasmodium* are the causative agents of malaria the most important human parasitic disease. *P falciparum* and *P vinckei* are both known to produce cathepsin L-like enzymes which may play a role in the digestion of haemoglobin (Rosenthal *et al* , 1988 ,1989 ,1993). The *P vinckei* cysteine proteinase has a similar molecular weight (28 kDa) to *F hepatica* cathepsins L1 and L2 and shares a similar substrate specificity (Rosenthal *et al* , 1993). *Plasmodium falciparum* also possess a cathepsin L-like cysteine proteinase which has a similar peptide substrate hydrolysis profile to *F hepatica* cathepsins L1 and L2 (Rosenthal *et al* , 1989).

Cathepsin L2 converts soluble fibrinogen into an insoluble fibrin clot by a novel mechanism

From a survey of the literature it was found that the blood clotting enzyme thrombin (factor II_a, EC 3.4.21.5) cleaves peptide substrates with arginine in the P₁ position and glycine in the P₂ position. This substrate specificity is similar to that demonstrated here for cathepsin L2 (Lottenberg *et al*, 1981). Thrombin is a serine proteinase that plays a central role in blood clotting by cleaving soluble fibrinogen to form an insoluble fibrin clot (NG *et al*, 1993, Doolittle, 1981). When cathepsin L2 is added to bovine fibrinogen at a concentration of 20 mg ml⁻¹ an insoluble clot forms. This is the first demonstration of the clotting of fibrinogen by a cysteine proteinase. Apart from thrombin and cathepsin L2, only the snake venom serine proteinases cause fibrinogen to clot (Shieh *et al*, 1985, Pirkle *et al*, 1986, Fand *et al*, 1989, Dyr *et al*, 1989, Nishida *et al*, 1994).

Having demonstrated that cathepsin L2 can cause fibrin to clot its mechanism of clot formation was compared to that of thrombin. Hirudin, a protein of 65/66 amino acid residues, is an extremely tight binding and selective inhibitor of thrombin. It inhibits the activity of thrombin by mimicking the binding of thrombin to its natural substrate, fibrinogen. i.e. the tail of hirudin binds to the fibrinogen-binding exosite of thrombin (Bode and Huber, 1992). As cathepsin L2 was not inhibited by hirudin, this result shows that the 3-dimensional structure of the active site of cathepsin L2 is different to the structure of the active site of thrombin.

The cathepsin B and L specific peptide inhibitor Z-phe-ala-CHN₂ was included in the fluorogenic assay to determine whether it interfered with the

cleavage of Tos-gly-pro-arg-NHMec by cathepsin L2 and thrombin (Shaw and Dean, 1980) Z-phe-ala-CH₂ completely inhibits cathepsin L2 This result shows that the P₁ alanine and the P₂ phenylalanine group of Z-phe-ala-CHN₂ binds to the S₁ and S₂ subsites in cathepsin L2 respectively, and the CHN₂ group of this molecule binds covalently to the active site cysteine residue However, Z-phe-ala-CHN₂ does not inhibit thrombin indicating that phenylalanine and alanine do not bind to the S₂ and S₁ binding sites of this enzyme Therefore the studies using Z-phe-ala-CHN₂ show that thrombin and cathepsin L2 differ in the structures of their active sites

Further evidence to show that cathepsin L2 and thrombin differ in the structures of their active sites comes from studies using fibrin-anti-polymerants (Laudano and Doolittle, 1980) The two fibrinopeptides, H-gly-pro-arg-pro-OH and H-gly-pro-arg-OH decreased the activity of cathepsin L2 on Tos-gly-pro-arg-NHMec by 60% Under the same conditions thrombin activity was inhibited by only 20% These results show that H-gly-pro-arg-OH and H-gly-pro-arg-pro-OH are competitive inhibitors of cathepsin L2 but not of thrombin

In order to visualise the clotting of fibrinogen *in vitro*, a fibrinogen plate assay was developed In this assay fibrinogen was incorporated into agar and poured onto glass slides Wells for were cut into the solidified agar and sample was subsequently applied to each well The slides were left to incubate overnight in a moist chamber to prevent drying and following incubation clotting was observed as opaque zones of precipitated fibrin against a clear background of soluble fibrinogen When thrombin was added to the plate assays an opaque zone due to clotted fibrin was visible Likewise when cathepsin L2 was added to the plate assays an opaque zone

of clotted fibrin was visible. In appearance the two types of fibrin zones are different as the ring produced by thrombin is more opaque and appears as a circle while the zone produced by cathepsin L2 is "ring-like" i.e. the centre is clear while the outside is opaque. This observation is consistent with a difference in the clotting mechanism of the two enzymes.

Fibrin plate assays were performed with the inhibitors hirudin and Z-phe-ala-CHN₂. The results of the fibrin plate assays with inhibitors were similar to those obtained for the fluorogenic substrate inhibition assays i.e. hirudin inhibited thrombin but not cathepsin L2 whilst Z-phe-ala-CHN₂ inhibits cathepsin L2 but not thrombin (see above).

The differences in the clotting mechanisms of cathepsin L2 and thrombin was further assessed by tube clotting assays. The ability of an enzyme to form an insoluble fibrin clot in a test tube is measured by the time taken to achieve a solid fibrin clot (Hougie, 1977). The clotting assay was used to compare the times of clot formation and hence relative clotting ability by cathepsin L2 and thrombin. The inhibitors hirudin and Z-phe-ala-CHN₂ were added to clotting time assays to assess the mechanism of action of both cathepsin L2 and thrombin. The inclusion of hirudin and Z-phe-ala-CHN₂ inhibited the activities of thrombin and cathepsin L2 respectively as demonstrated by the increase in clotting times obtained. This result confirms the results of the peptide substrate assays and the results of the fibrinogen plate assays.

The clots formed in *in vitro* clotting assays were analysed using reducing SDS-PAGE gels. The clots were centrifuged, the supernatant removed and the pellets were washed a number of times with PBS. Reducing sample buffer was added and the clots were denatured by boiling.

SDS PAGE analysis of solubilised thrombin and cathepsin L2 clots indicated that their subunit structures differed. In the thrombin produced clots the α , β and γ subunits of fibrin are still visible. The α and β subunits migrate at slightly lower molecular weights due to the loss of the A and B fibrinopeptides. SDS PAGE analysis of the cathepsin L2 produced clots revealed that the α and β subunits disappear but the γ subunit remains intact. However, proteins of 100, 125 and 25 kDa are visible.

When solubilised cathepsin L2 and thrombin clots were transferred to nitrocellulose strips and probed with specific anti-fibrinogen antibodies, the 100, 125 and 25 kDa bands were visualised. Taken together, these data demonstrate that cathepsin L2 cleaves fibrinogen in a different manner to thrombin and produces a novel type of fibrin clot. The cathepsin L2 clot is composed of fibrin subunits which have different molecular weights to the fibrin monomers produced by thrombin cleavage. The 100 and 125 kDa molecules may be produced by the polymerisation of the α and β polypeptides and/or fragments of these. This polymerisation reaction is covalent because it is not destabilised by reduction with mercaptoethanol and denaturation by boiling in the presence of SDS.

Clotting time assays were performed with the fibrin anti-polymerants H-gly-pro-arg-pro-OH, H-gly-pro-arg-OH and H-gly-his-arg-pro-OH (Laudano and Doolittle, 1980). The fibrin anti-polymerants H-gly-pro-arg-pro-OH and H-gly-pro-arg-OH correspond to the N-terminal end of the α subunit of fibrin that is exposed following the removal of the A fibrinopeptide by thrombin (Iwanga *et al.*, 1967, Cottrell and Doolittle, 1976).

H-gly-pro-arg-pro-OH is a more effective anti-polymerant of thrombin

than H-gly-pro-arg-OH. These fibrin anti-polymerants block polymerisation by binding to sites where the fibrin monomers normally assemble (Laudano and Doolittle, 1980). In mammals the removal of the A fibrinopeptides are more important for clot assembly than the removal of the B fibrinopeptides (Laudano and Doolittle, 1980).

The anti-polymerant H-gly-his-arg-pro-OH mimics the N-terminal end of the β subunit exposed after cleavage of the B fibrinopeptide (Iwanga *et al*, 1967, Cottrell and Doolittle, 1976). The β chain specific fibrin anti-polymerants do not prevent fibrin polymerisation of mammalian fibrinogen (Laudano and Doolittle, 1980). Only in the more primitive lamprey fibrinogen does the β chain specific synthetic fibrin anti-polymerant inhibit clotting, which is consistent with the observation that clotting can occur in this animal upon the removal of the B fibrinopeptides alone (Laudano and Doolittle, 1980).

The anti-polymerant H-gly-pro-arg-pro-OH increased the clotting time of thrombin. The other anti-polymerants H-gly-pro-arg-OH and H-gly-his-arg-pro-OH did not increase clotting times. In contrast, none of the fibrin anti-polymerants adversely affected the clotting ability of cathepsin L2 to any significant degree. These results suggest that the cleavage of fibrinogen by cathepsin L2 and thrombin must be at different sites in the molecule.

The cathepsin L2 cleavage site of fibrinogen was examined using clotting time assays. Fibrinogen was first pre-incubated with various amounts of the fibrin anti-polymerant H-gly-pro-arg-pro-OH. Thrombin was then added to allow the removal of the fibrinopeptides A and B. Therefore fibrin was formed by the removal of the fibrinopeptides from fibrinogen but, due to

the presence of the fibrin anti-polymerant, no clots were formed. Thrombin or cathepsin L2 were then added to the tubes. Thrombin caused clotting only in those tubes with a low concentration of anti-polymerant (200 μM). When cathepsin L2 was added, complete clot formation was seen in tubes containing 200, 250 and 300 μM but not 350 and 400 μM of fibrin anti-polymerant. The absence of clot formation in the tubes with 350 and 400 mM H-gly-pro-arg-pro-OH was not due to its anti-polymerant activity but to its competitive inhibition of enzyme activity (see above). Assembly of fibrin monomers generated by cathepsin L2 is unaffected by prior cleavage of fibrinogen by thrombin indicating that cathepsin L2 cleaves fibrinogen towards the C-terminal end of fibrinogen than thrombin.

SDS-PAGE analysis of cathepsin L2 clots formed following pre-incubation of fibrinogen with thrombin resulted in the same molecular structures as incubation of fibrinogen with cathepsin L2 alone. Cathepsin L2 cleaves fibrinogen to produce an identical type of clot irrespective of the presence of the A and B fibrinopeptides in the fibrinogen molecule. The results of SDS-PAGE analysis therefore, confirm that the location of the cathepsin L2 fibrinogen cleavage site is further to the C-terminal than the thrombin cleavage site.

Thrombin and the snake venom proteinases, and now *F. hepatica* cathepsin L2, are the only enzymes described to date which cleave fibrinogen to form a fibrin clot (Shieh *et al*, 1985, Pirkle *et al*, 1986, Farid *et al*, 1989, Dyr *et al*, 1989, Nishida *et al*, 1994). The snake venom enzymes share more similarities to thrombin than they do to cathepsin L2. Firstly, they belong to the same proteinase class (serine) as thrombin and share N-

terminal amino acid sequence similarities (Pirkle *et al* , 1986, Nishida *et al* , 1994, Farid *et al* , 1989) Secondly, the snake venom enzymes cleave fibrinogen at the same site as thrombin releasing both fibrinopeptides A and B (Pirkle *et al.*, 1986, Dyr *et al* , 1989) Some snake venom enzymes differ to thrombin in that they release only a single fibrinopeptide (e g bothrombin and the *Trimeresurus flavoviridis* coagulant enzyme (Nishida *et al* , 1994, Shieh *et al* , 1985)) As cathepsin L2 cleaves fibrinogen further to the C-terminal of the thrombin cleavage site, it must also cleave fibrinogen at a different site to the snake venom proteinases Finally, the SDS-PAGE profiles of the clots produced by the snake venom enzymes differ to the profiles of the clots produced by cathepsin L2 (Farid *et al* , 1989, Pirkle *et al* , 1986) The cleavage of fibrinogen by the snake venom proteases do not produce the higher molecular weight (100 and 125 kDa) or lower molecular weight (25 kDa) fibrin polypeptides characteristic of fibrinogen cleavage by cathepsin L2 In conclusion, *F hepatica* cathepsin L2 cleaves fibrinogen to form a fibrin clot in a novel manner

F hepatica is a blood feeder throughout the part of the life cycle that exists in the mammalian host Whilst in the bile ducts the parasite gains access to the blood primarily by puncturing the bile duct wall It is possible that cathepsin L2 plays some role in this feeding habit Possibly, after gaining access to the blood, the parasite plugs the wound to prevent excessive bleeding The parasite could then repeatedly feed at this access point Cathepsin L2 may also be used by the parasite to plug blood vessels which it severs while migrating through the gut wall and liver tissue, again the purpose being to prevent excessive blood loss from the host tissues

CONCLUSIONS

A cysteine proteinase was isolated to homogeneity from medium in which adult *F hepatica* were incubated using gel filtration and anion-exchange chromatography

The cysteine proteinase was characterised, by N-terminal amino acid sequencing and enzyme kinetic studies with fluorogenic substrates, as a cathepsin L

This cysteine proteinase is the second cathepsin L enzyme isolated from *F hepatica* culture medium and is termed cathepsin L2. Cathepsin L2 and the previously isolated *F hepatica* cysteine proteinase (cathepsin L1, Smith *et al* 1994) were differentiated on the basis of substrate specificities and chemical modification studies with the nitrating reagent TNM

Cathepsin L2 (29.5 kDa) is a larger molecule than cathepsin L1 (27 kDa) and was shown by immunoblotting studies to be a distinct enzyme and not the pro-form of cathepsin L1

Cathepsin L2 causes fibrinogen to clot *in vitro*. This clotting activity is the first described for a cysteine proteinase, and is shared only with the serine proteinase thrombin and snake venom thrombin-like enzymes

Characterisation of the fibrin clot formed by the cathepsin L2 by reducing SDS-PAGE showed that the molecular interactions between the fibrin subunits is different to those within a thrombin produced clot. Cathepsin L2 cleaves fibrinogen further to the C-terminal than the thrombin cleavage site and the polypeptides formed by cathepsin L2 cleavage of fibrinogen may be cross-linked by covalent interactions

CHAPTER 7
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APPENDICES

CURRICULUM VITAE

July 1994

Personal Details

Name	Andrew John Dowd
Business Address	School of Biological Sciences Dublin City University Dublin 9 Rep. of Ireland Ph 353-1-7045556 353-1-7045112
Home Address	91 Ferguson Road Drumcondra Dublin 9 Ireland Ph 353-1-376557
Date of Birth.	1 April 1966
Place of Birth	Dublin
Nationality	Irish
Marital Status	Single
Health.	Excellent

Education

- 1 B Sc (Biotechnology) (Honours 2 1) (1984 - 1988)
Dublin City University
Glasnevin
Dublin 9
Ireland
2. M Sc. (1988 - 1991)
Dublin City University/ Trinity College
Dublin, Ireland

M.Sc. thesis entitled " Studies of the bovine progesterone receptor"

3 Ph D candidate (1991-1994)
Dublin City University
Glasnevin
Dublin 9
Ireland

Project title "*Fasciola hepatica*: secretion of a novel cathepsin L proteinase"

Achievements

Gold medal for graduating 1st in my B Sc Biotechnology year (1988).

Scholarships

Australian-European Scholarship for Ireland from the Australian Vice-Chancellor's Committee in order to study for a year in the laboratory of Dr Paul Brindley in the Queensland Institute of Medical Research in Brisbane. May 1993-April 1994

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1. Dowd, A J., Smith, A M., McGonigle, S and Dalton, J P., (1994), Purification and characterisation of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica*, Eur J. Biochem (in press)
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Referees

1. Dr John Dalton
Senior Lecturer
School of Biological Sciences
Dublin City University
Glasnevin
Dublin 9
Ireland
Ph 353-1-7045407
Fax 353-1-7045412

2. Dr Paul Brindley
Senior Research Fellow
Molecular Parasitology Unit
Queensland Institute of Medical Research
300 Herston Road
Brisbane
Queensland, 4029
Ph 61-7-3620413
Fax 61-7-7045412

**SECRETION OF CYSTEINE PROTEINASE ACTIVITY BY THE ZONOTIC
HOOKWORM *ANCYLOSTOMA CANINUM***

**Andrew J. Dowd^{1,2}, John P. Dalton², Alex C. Loukas^{1,3}, Paul Prociv³,
and Paul J. Brindley¹**

¹Molecular Parasitology Unit, and Tropical Health Program, Queensland Institute of Medical Research, Brisbane, Queensland, Australia, ²School of Biological Sciences, Dublin City University, Ireland, and ³Department of Parasitology, University of Queensland, St Lucia, Queensland, Australia

Running title Cysteine proteinase secreted by hookworms

Correspondence address Paul J Brindley, Queensland Institute of Medical Research, The Bancroft Centre, 300 Herston Road, Brisbane, Queensland 4029, Australia Phone 61-7-362 0413, fax 61-7-362 0104, e-mail paulB@qimr.edu.au

ABSTRACT The zoonotic hookworm, *Ancylostoma caninum*, probably induces human eosinophilic enteritis by inducing allergic responses to its secretions. This species is already known to secrete metalloproteinases, but in other parasites cysteine proteinases are involved in pathogenesis. We studied somatic extracts of *A. caninum* adults and infective larvae and adult excretory/secretory (ES) antigens for cysteine proteinase activity using fluorogenic peptide substrates and by gelatin and fluorogenic substrate polyacrylamide gel electrophoresis. Proteolytic activity was observed against the cathepsins L and B-specific substrate Z-phe-arg-AMC, against the plasmin substrate Boc-val-leu-lys-AMC, and against gelatin. The Z-phe-arg-AMC-hydrolyzing activity in ES and in adult extracts was enhanced up to 15-fold by the reducing agent dithiothreitol (DTT) but was totally blocked by specific inhibitors of cysteine proteinases including the peptidyl diazomethyl ketone Z-phe-ala-CHN₂, E-64, leupeptin and N-ethylmaleimide. In like fashion, gelatinolytic activity in ES detected using substrate gels was enhanced by the addition of reducing agents and inhibited by Z-phe-ala-CHN₂ and E-64. The DTT-enhanced, Z-phe-arg-AMC hydrolyzing activity in ES was active over a wide pH range from pH 5 through pH 9. Similar cysteine proteinase activity to that detected in ES was present in extracts of adult and infective larvae of *A. caninum*. Because the substrate Z-phe-arg-AMC was specifically hydrolyzed, and because this hydrolysis was totally blocked by cysteine proteinase-specific inhibitors, ES and tissue extracts of *A. caninum* clearly possess cysteine proteinase activity. Furthermore, since the specific activity of Z-phe-arg-AMC-cleaving enzyme was up to five-fold higher in ES than in soluble extracts of parasites, the cysteine proteinase is actively secreted by adult hookworms. This is the first demonstration of cysteine proteinases from

hookworms and enlarges the range of potential allergens which may induce human eosinophilic enteritis

INTRODUCTION

It is now established beyond doubt that the canine hookworm *Ancylostoma caninum* can develop in the human gut, and in some cases cause eosinophilic enteritis (EE), a segmental inflammatory lesion of the gastrointestinal tract¹⁻³ Sera from many Australian patients with EE contain high titres of specific IgG and IgE antibodies to excretory/secretory (ES) antigens of adult hookworms³ Given that proteinases released by infectious organisms, including parasitic nematodes have been implicated in pathogenic immune reactions,^{4,5} and that allergic mechanisms are involved in EE,⁶ it is reasonable to assume that proteinases released by the dog hookworm may invoke hypersensitivity reactions underlying this disease in some cases

Hotez and Cerami described the presence of two proteinase activities in extracts of adult *A. caninum* and recorded that one of these (36 kDa) but not the other (40 kDa) was present in the excretory/secretory (ES) products released *in vitro* by these worms⁷ Characterization of the 36 kDa enzyme revealed it to be a metallo-proteinase that had elastolytic activity, and inhibited fibrin clot formation⁷ They postulated that it was involved in histolysis and prevented the coagulation of blood during feeding^{7,8} Subsequent studies demonstrated the presence of the metallo-proteinase in *A. caninum* larvae⁹ Preliminary findings indicated several bands of proteolytic activity other than those investigated by Hotez *et al.*⁷⁻⁹ and Loukas and coworkers³ In the present study, ES and soluble adult and larval extracts of *A. caninum* were analyzed for endoproteinase activities distinct from these metallo-enzymes We employed a panel of fluorogenic peptide and gelatin substrates, in test-tube and polyacrylamide gel electrophoretic assays, and class-

specific proteinase enhancers and inhibitors. We now report that adult and larval *A. caninum* possess cysteine proteinase activity, which is distinct from that described by Hotez and colleagues, and which is preferentially secreted by adult parasites.

MATERIALS AND METHODS

Parasites, preparation of antigen

Adult *A. caninum* hookworms were obtained at autopsy from the small intestine of naturally infected dogs, as previously described.¹⁰ ES was prepared by culturing adult hookworms in phosphate buffered saline, pH 7.3 (PBS) containing 1 mM disodium-ethylenediamine-tetraacetic acid (EDTA), 100 U ml⁻¹ penicillin, 100 µg streptomycin sulfate and 25 µg ml⁻¹ amphotericin (CSL, Parkville, Australia). EDTA was included in the culture medium in order to inactivate metallo-proteinase activity secreted by the parasites.^{3,8} The supernatant was filter-sterilized and concentrated as previously described.³

Tissue extracts were prepared from approximately 50 adult hookworms (of mixed sex), suspended in 500 µl PBS, by two freeze-thaw cycles (-70°C/ 4°C) followed by sonication (12 x 10 s bursts at duty cycle 10%, output 5, Branson Sonifier 250) (Branson Ultrasonics, Danbury, CT), after which the lysate was centrifuged at 14,000 x *g* for 30 min. Infective *A. caninum* larvae were recovered by an inverted gauze pad method from fecal/charcoal cultures of feces from naturally infected dogs. Approximately 2 x 10⁶ larvae were washed thoroughly in tap water and resuspended in 500 µl PBS. Soluble tissue extracts of larvae were

prepared by three freeze-thaw cycles, followed by sonication (5 x 5 s bursts at constant duty cycle, output 5, Branson Sonifier) and centrifugation as above. The supernatants were retained as adult (L5) and larval (L3) tissue extracts, respectively. Protein concentrations of ES and extracts were measured using a modified Lowry method (DC Protein Assay, Bio-Rad, Richmond, CA).

Enzyme assays

Proteinase activity was measured using the fluorogenic peptides, benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amido-4-methylcoumann (Z-phe-arg-AMC), benzyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methylcoumann (Z-arg-arg-AMC), benzoyl-L-arginine-7-amido-4-methylcoumarin (Bz-arg-AMC), tosyl-L-glycyl-L-prolyl-L-lysine-7-amido-4-methylcoumarin (Tos-gly-pro-lys-AMC), tosyl-L-glycyl-L-prolyl-L-arginine-7-amido-4-methylcoumarin (Tos-gly-pro-arg-AMC) and t-butyloxycarbonyl-L-valyl-L-leucyl-lysine-7-amido-4-methylcoumann (Boc-val-leu-lys-AMC) (Bachem, Bubendorf, Switzerland), as substrates. Peptide substrates were prepared as 10 mg ml⁻¹ stock solutions in dimethylformamide. ES and tissue extracts (0.3 µg - 18.6 µg) were incubated in PBS containing 10 µM fluorogenic substrate at 37°C for 30 minutes, after which the reaction was stopped by the addition of glacial acetic acid to 300 mM. The reducing agent dithiothreitol (DTT) (Boehringer Mannheim, Castle Hill, Australia) was included in some fluorometric assays at a concentration of 500 µM¹¹. The release of the fluorescent group 7-amino-4-methylcoumann (AMC) was measured using a fluorimeter (Kontron, model SFM 25, Milan, Italy) with excitation at 370 nm and emission at 440 nm. The amount of enzymatically-generated AMC was calculated from the standard

fluorescence intensity of authentic AMC (Sigma Chemical Co , St Louis, MO)

One unit (U) of enzyme activity was defined as that amount which catalyzed the release of one nmol AMC $\text{min}^{-1} \text{mg protein}^{-1}$ at 37°C.

Buffers for measuring pH/activity profile

The pH/activity profile of the Z-phe-arg-AMC-cleaving activity in ES (0.3 μg) was examined with the following buffers. Fifty mM glycine was adjusted to pH 2.8 - 3.6 with 200 mM HCl. Acetate buffer for measuring activity in the pH range 3.6 - 5.6 was adjusted by mixing 100 mM acetic acid and 100mM sodium acetate. Phosphate buffer for measuring activity in the pH range 5.9 - 7.3 was adjusted by mixing 100 mM monobasic sodium phosphate and 100 mM dibasic sodium phosphate. Fifty mM Tris for measuring activity in the pH range 7.6 - 8.8 was adjusted with 200 mM HCl. Fifty mM glycine was adjusted to pH 9.2 - 10.6 with 200 mM NaOH. The final salt concentration of each buffer was adjusted to 100 mM using 500 mM NaCl calculated using the Henderson-Hasselbach equation¹². Each buffer was supplemented with 500 μM DTT.

Fluorogenic- and gelatin-substrate polyacrylamide gels

Adult ES (1.25 μg -12.5 μg) was separated by non-denaturing, non-SDS, polyacrylamide gel electrophoresis using gels co-polymerized with the proteinase substrate gelatin at 0.1% (GS-PAGE).¹³ (GS-PAGE was employed without SDS or reducing agents in the sample or electrophoresis buffers because they may inhibit the hydrolysis of synthetic peptidyl AMC substrates [Dowd, unpublished, see also ref. 14]) Following electrophoresis, gels were washed for 1 h in PBS, pH 7.3

(three changes) For fluorogenic substrate analysis, GS-PAGE gels were immersed in 10 μ M fluorogenic substrate in PBS supplemented with the reducing agent cysteine at 10 mM for 30 min at 37°C. AMC-hydrolyzing activity was visualized by transilluminating the gels at 302 nm after which they were photographed using Polaroid 667 film. After fluorimetric analysis, gels were incubated in 10 mM cysteine in PBS at 37°C overnight, and stained subsequently with Coomassie Brilliant Blue R250 in order to detect gelatinolytic activity¹³. In addition, some samples were analyzed solely for gelatinolytic activity by GS-PAGE.

Inhibition analysis

Proteinase inhibitors were obtained from Sigma, *L-trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), N-ethylmaleimide (NEM), soybean trypsin inhibitor (SBTI), EDTA, phenylmethylsulfonyl fluoride (PMSF), leupeptin, and pepstatin A; or from Bachem, benzyloxycarbonyl-L-phenylalanyl-L-alanine-diazomethylketone (Z-phe-ala-CHN₂). Adult ES (0.3 μ g) was mixed with inhibitors at appropriate concentrations (Z-phe-ala-CHN₂, 500 nM, E-64, 14 μ M, NEM, 5 mM, leupeptin, 6 μ M; PMSF, 1 mM, SBTI, 5 μ M, EDTA, 10 mM, and pepstatin A, 70 μ M) after which proteolytic activities were analyzed by test-tube fluorometric analysis, as above. In addition, ES samples (1.25 μ g) separated by GS-PAGE were incubated overnight in 28 μ M E-64 or 1 μ M Z-phe-ala-CHN₂ in PBS supplemented with 10 mM cysteine.

RESULTS

Proteinase activity in *Ancylostoma caninum* adults and larvae

Extracts of *A. caninum* larvae and adults, and adult ES were assayed for cysteine proteinase activity using the fluorogenic peptide substrate Z-phe-arg-AMC. Proteinase activity capable of cleaving this substrate was detected in ES, and from extracts of larvae and adults. The proteinase activity in extracts of adult worms and ES was enhanced 9- to 15-fold in the presence of the reducing reagent DTT, and up to two-fold in larval extracts. By contrast, the Z-phe-arg-AMC hydrolysis detected in the presence of DTT was totally or substantially inhibited in all parasite fractions by the addition of 500 μ M Z-phe-ala-CHN₂, a specific inhibitor of cathepsin-like cysteine proteinases (Table 1)

Cysteine proteinase responsible for cleavage of Z-phe-arg-AMC

The proteinase activity responsible for the cleavage of Z-phe-arg-AMC was enhanced by the addition of the reducing agent DTT and blocked by the addition of Z-phe-ala-CHN₂ (see above). In order to determine the class(es) of endopeptidase responsible for the protease activity in ES, ES (0.3 μ g) was incubated with the fluorogenic substrate Z-phe-arg-AMC in the presence of class-specific proteinase inhibitors, and hydrolysis of AMC from the substrate measured by fluorescence spectrometry. Incubation of ES and Z-phe-arg-AMC with each of four specific inhibitors of cysteine proteinases (Z-phe-ala-CHN₂, E-64, NEM, and leupeptin) reduced enzyme activity by 94-100%. In contrast, inhibitors of aspartic (pepstatin A), serine (soybean trypsin inhibitor, and PMSF), and metallo-proteinases (EDTA)

had little or no inhibitory effect on the hydrolysis of Z-phe-arg-AMC (not shown)

pH/activity profile

The pH dependence of the *A. caninum* cysteine proteinase activity against the substrate Z-phe-arg-AMC was determined in various buffers in which the salt concentration of each buffer was adjusted to 100 mM to minimize salt concentration effects on proteinase activity. Potent Z-phe-arg-AMC hydrolyzing activity was observed from pH 5 through 9. In contrast, the activity fell rapidly at pHs below 5 and above 9 (Figure 1)

GS-PAGE analysis of *A. caninum* ES

A. caninum ES was analyzed under non-reducing conditions by GS-PAGE, conditions expected to separate proteins on the basis of molecular size and electric charge¹⁵. A single band of gelatinolytic activity was detected and this activity was substantially enhanced in the presence of cysteine. In contrast, the activity was blocked by the specific cysteine proteinase inhibitors, E-64 (28 μ M) and Z-phe-ala-CHN₂ (1 μ M) (Figure 2)

Substrate specificity of *A. caninum* ES cysteine proteinase

Having detected, measured, and inhibited the *A. caninum* proteinase activity in test-tube and gel assays, we attempted to visualize the fluorogenic activity in gels and to characterize its peptidyl substrate specificity. ES was subjected to GS-PAGE, after which replicate gel lanes were incubated in 10 μ M fluorogenic substrate in PBS supplemented with 10 mM cysteine. Vigorous AMC-hydrolyzing

activity co-migrating with the gelatinolytic activity was observed with the substrate Z-phe-arg-AMC, activity was also observed with Boc-val-leu-lys-AMC, whereas little or no activity was observed in these gels after incubation in the substrates Bz-arg-AMC (Figure 3), Tos-gly-pro-arg-AMC, and Tos gly-pro-lys-AMC, and Z-arg-arg-AMC (not shown).

In addition, ES was incubated in the presence of DTT with a panel of fluorescent peptide substrates in tube assays to further characterize the substrate specificity of the cysteine proteinase. The substrate Z-phe-arg-AMC was preferentially cleaved by the endopeptidase activity in ES. Boc-val-leu-lys-AMC was also cleaved, although to a lesser degree than Z-phe-arg-AMC. In contrast, little or no hydrolysis of Tos-gly-pro-arg-AMC, Tos-gly-pro-lys-AMC, and Z-arg-arg-AMC was detected (Table 2)

Preferential secretion of cysteine proteinase by adult hookworms

In order to determine whether the cysteine proteinase was preferentially secreted by adult hookworms, the specific activity of the cysteine proteinase in ES was compared with that from adult tissue extracts. The activity in ES was approximately five-fold higher than the proteinase activity in homogenates, indicating that the cysteine proteinase was preferentially secreted in ES by the adult hookworms (Table 1)

DISCUSSION

Proteinases are employed by parasitic worms in various pathologic processes including degradation of the extracellular dermal matrix during skin

penetration, hydrolysis of hemoglobin, inhibition of host protective immune responses including cleavage of immunoglobulin and complement components and, in addition, are known to invoke hypersensitivity reactions^{16,17} We have begun to investigate proteinases released by hookworms in order to determine their role in parasitism, and in particular to examine whether hookworm proteinases are associated with the allergic responses in humans manifested as eosinophilic enteritis This study demonstrates for the first time that the zoonotic hookworm *A caninum* synthesizes and secretes a cysteine (i e thiol dependent) proteinase activity capable of hydrolyzing gelatin and the synthetic peptide substrates Z-phe-arg-AMC and Boc-val-leu-lys-AMC.

The hydrolysis of Z-phe-arg-AMC was thiol-dependent because the activity was enhanced up to 15-fold by the addition of the thiol-containing reagents DTT and cysteine Since Z-phe-arg-AMC, a substrate normally used to assay the cysteine proteinases cathepsins B and L, was cleaved by the hookworm enzyme, whereas the cathepsin B-specific substrate Z-arg-arg-AMC and the cathepsin H substrate Bz-arg-AMC were not readily cleaved, the activity is cathepsin L-like¹⁸ The Z-phe-arg-AMC hydrolysis was blocked by the cysteine proteinase-specific inhibitors E-64 and Z-phe-ala-CHN₂, verifying that a cysteine proteinase in ES and tissue extracts was responsible for the Z-phe-arg-AMC hydrolysis^{19,20} Cysteine proteinases contain a thiol group which is essential for proteolytic activity and require reducing reagents *in vitro* to stabilize and/or enhance activity This essential thiol group is often found to be blocked under both natural and experimental conditions, but can be unblocked by reducing agents¹¹ NEM, an agent which alkylates thiol groups, inhibited the thiol-dependent hookworm

activity²¹ Leupeptin, a peptide aldehyde and a reversible inhibitor of cysteine proteinases, also inhibited the thiol-dependent proteolytic activity¹⁸ By contrast, specific inhibitors of serine, metallo-, and aspartic proteinases had no significant inhibitory effect on the Z-phe-arg-AMC cleaving activity in ES The cysteine proteinase appears to be actively secreted by adult *A. caninum* because the specific activity against the fluorogenic substrate Z-phe-arg-AMC was 5-fold higher in ES than in adult worm extracts

Using casein-agar gels, Hotez and Cerami⁷ detected metallo-proteinase activities in extracts of adult *Ancylostoma caninum* and observed that one (36 kDa) but not the other (40 kDa) was present in the ES released *in vitro* by hookworms. We strategically included the metal chelator, EDTA, in the hookworm culture media to inactivate these metallo-proteinases so that other activities masked or degraded by metallo-proteinases might be detected The thiol-dependent activity reported here appears to be distinct from the metallo-proteinase characterized by Hotez and co-workers^{4,7-9} In particular, the metallo-proteinase is inhibited by EDTA and 1,10 phenanthroline whereas cysteine proteinase inhibitors exert little or no inhibition⁸ By contrast, the Z-phe-arg-AMC hydrolyzing activity was not inhibited by EDTA Furthermore, the metallo-proteinase has a pH optimum of 10 and is inactive at neutral pH,⁸ that pH dependence is patently different to that of the cathepsin L-like activity reported here, since this activity was shown to be active over a broad pH range (pH 5-9) It is of interest to note that cysteine proteinases released by several other parasites are active at neutral and alkaline pH,^{22,23} although proteolytic activity at acid pH is usual for cathepsins B and L¹⁸

Proteinase activity in the hookworm ES and extracts was also shown to

cleave Boc-val-leu-lys-AMC, a substrate cleaved by plasmin *in vitro*. This suggests that the proteolytic activity may have anti-coagulant functions²⁴. The thrombin-like fluorogenic substrates Tos-gly-pro-lys-AMC and Tos-gly-pro-arg-AMC²⁴ were not readily hydrolyzed by the proteinase.

While a protein of apparent molecular weight 68 kDa appears to be the most specific diagnostic antigen for human infection with *A. caninum* on Western blots, the identity of the putative allergen(s) is not known³. Since the cysteine proteinase activity described here is apparently secreted by adult *A. caninum*, and since proteinases released by other parasitic helminths provoke IgE antibody responses and elicit immediate type hypersensitivity responses in infected humans and mice,^{17,25,26} the cathepsin L-like proteinase and the previously reported metallo-proteinases may play a role in the pathology of eosinophilic enteritis.

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Table 1 Enhancement and inhibition of cysteine proteinase activity of *Ancylostoma caninum* fractions The activity of each fraction was measured against the fluorogenic substrate Z-phe-arg-AMC.

Fraction	-DTT	+DTT	Enhancement	+DTT	Inhibition
	U*	U*	-fold	U*	%
L3 extract	0.5	0.8	1.6	0.2	75
L5** extract	7.7	66.4	9	7.4	89
L5 ES	21.3	330.5	15	0	100

* Enzyme activity units (U) are defined as nmoles AMC released $\text{min}^{-1} \text{mg}$ worm protein⁻¹. The cysteine proteinase-specific enhancer dithiothreitol (DTT) was used at a concentration of 500 μM and the cathepsin-specific inhibitor Z-phe-ala-CHN₂ at 500 nM.

** before *in vitro* culture

Table 2 Specific activities of proteinase from *Ancylostoma caninum* ES measured against fluorogenic peptide substrates

Substrate	Activity (U)*
Z-phe-arg-AMC	330.5
Z-arg-arg-AMC	1.1
Tos-gly-pro-arg-AMC	13.1
Tos-gly-pro-lys-AMC	6.3
Boc-val-leu-lys-AMC	48.5

* Units (U) of enzyme activity are defined as nmoles AMC released min^{-1} mg worm protein $^{-1}$

FIGURE LEGENDS

Figure 1. pH dependence of *Ancylostoma caninum* thiol-dependent cysteine proteinase activity. Adult *A. caninum* ES (0.3 μ g) was incubated in 50 mM glycine-HCl pH 2.8 - 3.6, 100 mM acetate pH 3.6 - 5.6, 100 mM sodium phosphate pH 5.9 - 7.3, 50 mM Tris-HCl pH 7.6 - 8.8 and 50 mM glycine-NaOH pH 9.2 - 10.6. Cysteine proteinase activity was measured against the cathepsins B- or L-like substrate Z-phe-arg-AMC in the presence of the reducing agent dithiothrietol (DTT).

Figure 2. Demonstration of proteinase activity in excretory/secretory products (ES) from adult *Ancylostoma caninum*. ES (1.25 μ g) was separated by electrophoresis through non-denaturing, non-SDS gels containing co-polymerized gelatin. After electrophoresis, gel strips were incubated in the absence (lane 1) or presence (lane 2) of cysteine and the cysteine proteinase inhibitors E-64 (lane 3) and Z-phe-ala-CHN₂ (lane 4). The results demonstrate that ES contains only one major proteinase activity capable of hydrolysis of the gelatin substrate, and that this activity is ascribable to a cysteine proteinase.

Figure 3. Substrate specificity of *Ancylostoma caninum* thiol-dependent, cysteine proteinase activity. Adult *A. caninum* ES (12.5 μ g) was separated by electrophoresis through non-denaturing, non-SDS gels containing co-polymerized gelatin. Panel A, cleavage of gelatin; panel B, cleavage of peptide fluorogenic substrates, Z-phe-arg-AMC (FR, lane 1), Boc-val-leu-lys-AMC (VLK, lane 2), and Bz-arg-AMC (R, lane 3). Together, the results show that the proteinase activity capable of cleaving the general purpose substrate gelatin also preferentially

cleaves the specific, cathepsin B- or L-like substrate Z-phe-arg-AMC

Fig 1

Figure 1

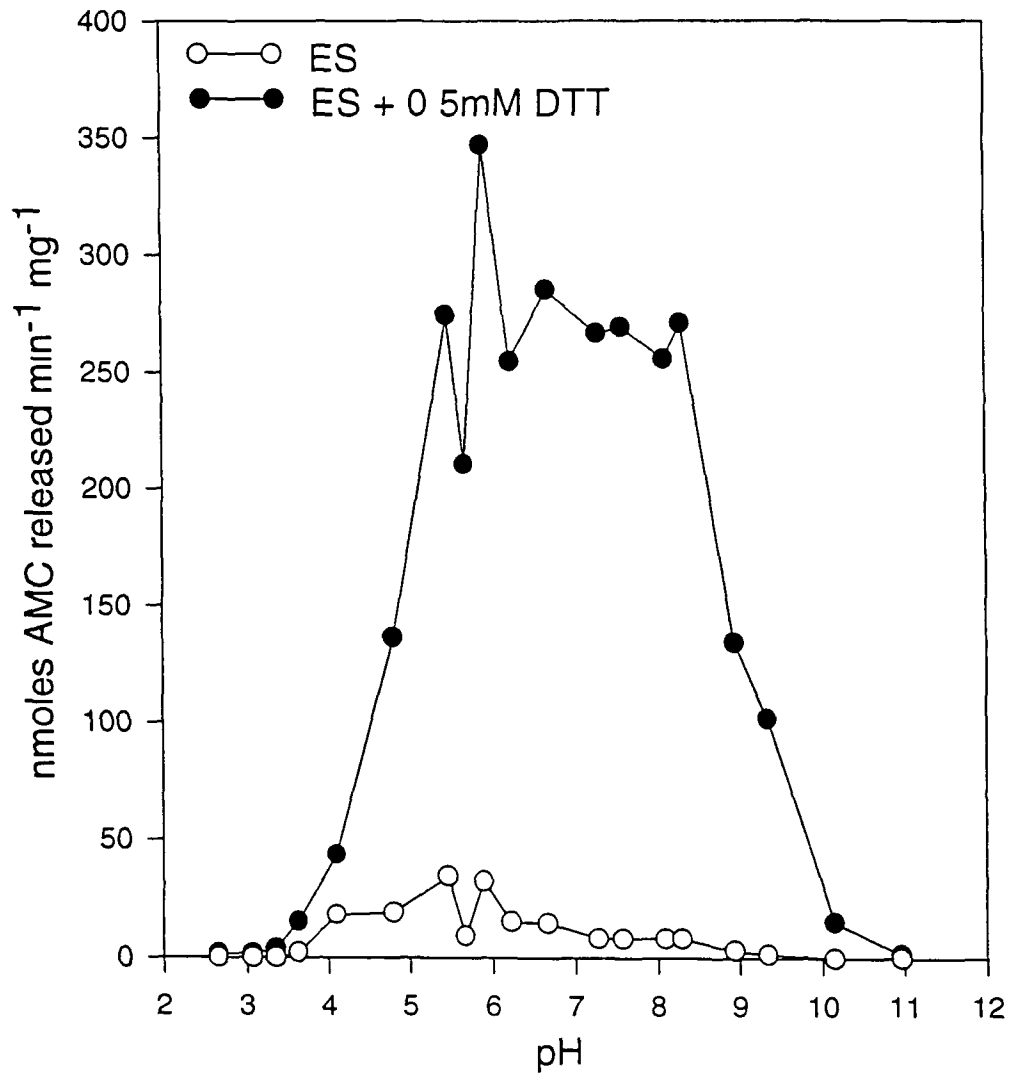


Figure 3

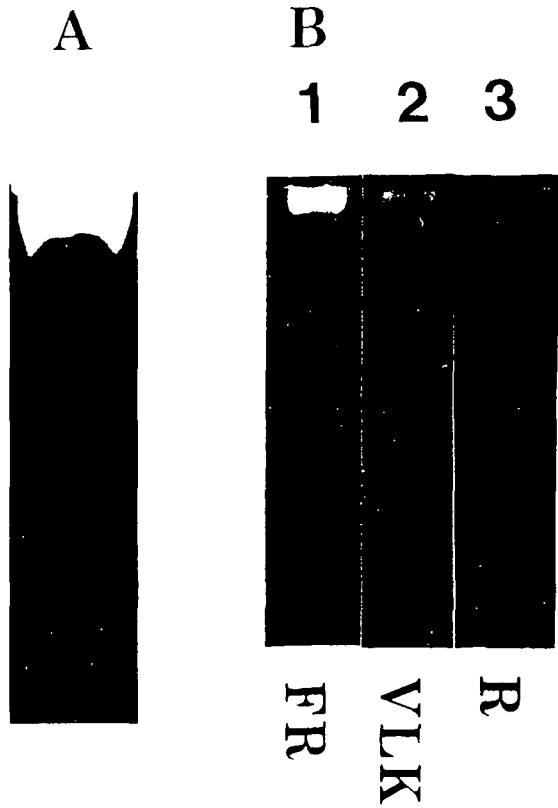


Figure 2

1 2 3 4

