Fasciola hepatica: Isolation and characterisation of a cathepsin L proteinase.

Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

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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: Angela Smith Date :_____

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Date:_____

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To my parents.

CONTENTS

ABSTRACT

Fasciola hepatica, a parasitic trematode, is the causative agent of liver fluke disease. It has been shown previously, that both the migratory and adult worm stage of the parasite secrete multiple cysteine proteinases when they are cultured overnight (Dalton & Heffernan, 1989). In this study, one of these proteinases has been purified by standard chromatographic techniques. The purified enzyme was characterised as a cathepsin L-like proteinase using synthetic substrates, inhibition studies, N-terminal sequencing and immunolocalisation studies. This is the first cathepsin L-like proteinase to be identified in a parasitic trematode. This cathepsin L-like proteinase is capable of cleaving immunoglobulin molecules, and is able to protect newly excysted juveniles from destruction by immune-effector cells when it is included in an eosinophil adherence assay. Antibodies to the purified proteinase are able to neutralise its proteolytic activity *in vitro*. A partial gene fragment encoding the cathepsin L-like proteinase has been obtained using PCR and subcloning techniques. The cathepsin L-like proteinase is present in all stages of *F. hepatica* and, hence, is considered an ideal target molecule at which to design a vaccine and/or drug, for use in the control of this agriculturally important parasitic disease.

ABBREVIATIONS

BCIP	5-bromo-5-chloro-3-indolyl phosphate
Bisacrylamide	*N, N*"-Methylene bisacrylamide
BSA	Bovine serum albumin
DMSO	Dimethyl sulphoxide
DPC	Diethylpyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid disodium salt
E-64	*trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Hepes	N-[2-hydroxyethyl] piperazine-N'[2-ethane sulphonic acid]
IPTG	Isopropyl-ß-thiogalactopyranoside
NBT	Nitro blue tetrazolium
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonyl fluoride
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	tris-(hydroxymethyl)-methylamine (2-amino-hydroxylmethyl) propane-1,3-diol
Z-F-A-CHN_2	*N*-benzyloxcarbonyl-L-phenylalanine-L-alanine-

	diazomethylketone
Z-F-R-AMC	*N*-benzyloxcarbonyl-L-phenylalanine-L-arginine-7-amino-4-methylcoumarin.HCl
Z-R-AMC	*N*-benzyloxcarbonyl-L-arginine-7-amino-4-methylcoumarin.HCl
Z-R-R-AMC	*N*-benzyloxcarbonyl-L-arginine-L-arginine-7-amino-4-methylcoumarin.HCl

CHAPTER ONE
INTRODUCTION

1.0 INTRODUCTION

In 1947, Professor Stoll drew attention to the worldwide presence of helminth parasites in his article "This wormy world". Helminth parasites infected 70 % of the then world population of approximately 2 billion (Stoll, 1947). Since that time, the prevalence of helminth infections has kept pace with the growth of the world population. If the trend continues till the year 2100, a predicted world population of 7-15 billion would harbour 5-10 billion helminth infections, unless special control measures are undertaken (Crompton, 1987).

The term "helminth" (derived from the Greek words *helmins* or *helminthos*), literally means "worm", zoologically speaking however, it has a more precise connotation and is currently restricted to members of the phyla Platyhelminthes, Nematoda and Acanthocephala (Smyth, 1976). The study of helminths is now regarded as being confined to the study of parasitic worms. Helminths typically parasitise vertebrates, although invertebrates act as intermediate hosts. The helminth diseases in man and domestic animals are caused by three groups of parasites belonging to the classes of *trematoda* (flatworms), *nematoda* (roundworms), and *cestoda* (tapeworms), and are distributed throughout the world (Singh & Sharma, 1991). There are approximately 200 recognised helminth parasites of man. Table 1.1 lists the parasites which are most common in humans.

For most helminth infections the relationship between between infection and disease is complex, and disease is not necessarily an automatic outcome of infection (Bundy *et al.,* 1992). Only a small proportion of those individuals with heavy infections are likely to develop overt disease. There is a low mortality/high morbidity rate associated with helminth infections, so although

Table 1.1

Parasitic helminth infections which are common to man, an example of a causative agent of each infection, and the numbers infected. Data obtained from Hopkins, (1992).

Parasite infection & example	Millions infected
Ascariasis *(Ascaris lumbricoides)*	1000
Hookworm *(Ancylostoma duodenale)*	900
Trichuriasis *(Trichuris trichiura)*	750
Schistosomiasis *(Schistosoma mansoni)*	250
Filariasis *(Wuchereria bancrofti)*	90
Taeniasis *(Taenia saginata)*	70
Onchocerciasis *(Onchocerca volvulus)*	30
Fascioliasis *(Fasciola hepatica)*	17
Trichinosis *(Trichinella spiralis)*	11

millions of people may be infected with helminths, relatively few will actually die as a result of infection, which seems to prevent well-focused investigation into their control and treatment (Parkhouse & Harrison, 1989). In fact it is estimated that at least one quarter of the worlds population is infected with helminthic parasites (Bundy, 1992), and about 150,000 die each year as a result of these infections (Bundy, 1990).

One feature in the evolution of some animals is the increasing complexity of their alimentary, respiratory and circulatory systems. The development of such systems was, of course, advantageous to these evolving organisms, but it was not without some inherent disadvantages. As each new organ system evolved,

especially those containing cavities or surfaces, it presented a habitat for potential parasites. These cavity containing organs appeared especially in vertebrates and every part of the vertebrate body capable of supporting parasitic life has been invaded (Smyth, 1976). A majority of helminths use the gastrointestinal tract as their favourite niche; however some parasites may also invade musculature, the blood circulatory system, and other parts of the body such as lungs, liver, lymphatics, and eyes, producing serious clinical complications.

Traditionally the control of helminth infections has relied heavily on the use of anthelmintic drugs, along with improvements in hygiene and reductions of vector populations. However, within a few years of their introduction, cases of resistance to anthelmintic drugs were reported (reviewed in Craig, 1993 and in Jackson, 1993). Resistance occurs when a portion of a population is able to tolerate doses of a compound that is effective against other populations (Craig, 1993). Resistance has been reported in many countries throughout the world, against anthelmintic drugs which are commonly used by the livestock industries (Jackson, 1993). More recently resistance to praziquantel treatment has been induced in laboratory mice infected with *Schistosoma mansoni* worms (Paul Brindley, personal communication).

The increasingly widespread problem of resistance to chemotherapeutic agents, has made the search for new ways of combating these helminth diseases even more important in terms of controlling helminthic infections. The successful eradication of all helminth diseases would involve more effective and economically viable drugs, with new modes of action, broad specificity and minimal toxicity to the host, combined with an immunisation program designed to enhance host resistance to reinfection. The final effective vaccines would be

multivalent with broad specificity. However to optimise the chances of success such vaccines would have to be closely modelled on each individual parasite life-cycle.

To invade the body of another species of animal, and to live and multiply in or on it, could not have been achieved without considerable morphological, physiological, biochemical and immunological adaptations by the parasite. Proteinases are enzymatic molecules which hydrolyse peptide bonds, and as such can be associated with all the adaptations which a parasite may have to undergo in order to survive in its parasitic environment. Proteinases are essential for life. The study of parasite antigens has focused mainly on surface molecules and secretions- both easily accessible targets. However it is unlikely that a parasite would express essential molecules on its surface. Indeed such an act would be suicidal, and would inevitably lead to the disappearance of the parasite through evolution. Excretory/secretory molecules have been shown to contain a variety of enzymatic activities. Some of these molecules may be essential to the survival of the parasite and would be suitable candidates for studies as targets for vaccine or rational drug design.

Table 1.2 lists the proteinases that are associated with helminths. In this report, we review the proteinases which have been well characterised but not extensively reviewed previously, and assess their potential as targets for immuno- or chemo- therapy in the eradication (full or partial) of helminthic and helminth associated diseases.

Table 1.2

A list of the proteinases activities which have been identified in helminth parasites.

Species	Class of proteinase	Ref.
Trematoda		
Schistosoma mansoni		
Egg	cysteine	Asch & Dresden, '79 Sung & Dresden, '86
Cercariae	serine	McKerrow & Doenhoff, '88 McKerrow *et al.*, '91
	47 kDa serine	Chavez-Olortegui *et al.*, '92
Schistosomula	cysteine	Zerda *et al.*, '88
Miracidia	cysteine	Yoshino *et al.*, '93
Adult	cysteine	Timms & Bueding, '59 Dresden & Deelder, '79 Chappell & Dresden, '86 Lindquist *et al.*, '86 Chappell *et al.*, '87 Chappell & Dresden, '87 Ruppel *et al.*, '85, '87 Davis *et al.*, '87 el Meanway *et al.*, '90 Klinkert *et al.*, '87, '88, '89 Felleisen *et al.*, '88 Felleisen & Klinkert, '90 Gotz & Klinkert '93 Smith *et al.*, '94b
	metalloproteinase	Auriault *et al.*, '81
	calpain	Andresen *et al.*, '91
	leucine aminopeptidase	Xu & Dresden, '86

Species	Class of proteinase	Ref.
S. mansoni (cont)		
Adult	dipeptidyl amino-peptidase I and II	Bogitsh & Dresden, '83
Fasciola hepatica		
NEJ	cysteine	Dalton & Heffernan, '89 Carmona *et al.*, '93 McGinty *et al.*, '93
	serine dipeptidylpeptidase	Carmona *et al.*, '94
Adults	cysteine	Howell, '66, '73 Simpkin *et al.*, '80 Chapman & Mitchell, '82 Dalton & Heffernan, '89 Rege *et al.*, '89a McGinty *et al.*, '93 Carmona *et al.*, '93 Smith *et al.*, '93a, '93b, '94a Dowd *et al.*, '94a Heussler & Dobbelaere, '94
	serine dipeptidylpeptidase	Carmona *et al.*, '94
Fasciola gigantica	cysteine	Fagbemi & Hillyer, '91
Fasciola sp.	cysteine	Aoki *et al.*, '83 Yamasaki *et al.*, '89, '92 Yamasaki & Aoki, '93
Paragonimus westermani	cysteine	Yamakami & Hamajima, '87, '89 and '90 Song & Dresden, '90

Species	Class of proteinase	Ref.
Nematoda		
Ancylostoma caninum		
	metalloproteinase	Hotez & Cerami, '83 Hotez *et al.*, '85, '90
	cysteine	Dowd *et al.*, '94b
Dictyocaulus viviparus		
	metalloproteinase	Britton *et al.*, '92
	cysteine & serine	
	cysteine	Rege *et al.*, '89b
Haemonchus contortus		
	metalloproteinase	Gamble *et al.*, '89
	cysteine	Cox *et al.*, '90 Pratt *et al.*, '90, '92a Knox & Jones, '90 Knox *et al.*, '93
Nippostrongylus brasiliensis		
	metalloproteinase	Healer *et al.*, '91
Necator americanus		
	invasive proteinase	Matthews, '82
Ostertagia ostertagi		
	cysteine	Pratt *et al.*, '92b
Strongyloides stercoralis		
	metalloproteinase	McKerrow *et al.*, '90
Ascaris suum		
	hemoglobinase	Maki *et al.*, '85
	serine	Knox & Kennedy, '88
Anisakis simplex		
	proteinase	Kennedy *et al.*, '88

Species	Class of proteinase	Ref.
Brugia malayi	proteinase	Petralanda *et al.*, '86
Brugia pahangi	metalloproteinase	Hong *et al.*, '93
Dirofilaria immitis	cysteine	Maki *et al.*, '85 Tamashiro *et al.*, '87
Onchocerca volvulus	proteinase	Petralanda *et al.*, '86
Cestoda		
Spirometra mansoni	cysteine	Song & Chappell, '93
Taenia solium	metalloproteinase aspartic cysteine	White *et al.*, '92

Schistosomes, or blood flukes, are the causative agent of the parasitic disease schistosomiasis, also known as Bilharzia, which afflicts more than 250 million people in tropical regions. There are three species of schistosome, *Schistosoma mansoni, Schistosoma japonicum* and *Schistosoma haematobium.* Proteinases of *S. japonicum* and *S. haematobium* are less well characterised than those of *S. mansoni.* For this reason, only the proteinases associated with *S. mansoni* will be dealt with in detail in this review.

Infection follows penetration of the skin by cercariae, the aquatic larvae. Cercariae develop in an intermediate host, the fresh-water snail, and find their human host by following a thermal gradient (Stirewalt, 1974). During human infection, cercariae transform into schistosomula which migrate to the lungs, and then to the liver, finally taking up residence in the vasculature of the intestines or bladder. Here adult females release numerous eggs each day, and can do so for many years. The eggs move through the intestinal wall and are liberated into the lumen of the bowel. Eggs are also carried with the circulation and are deposited in various body organs particularly in the liver. The hosts inflammatory response to the eggs causes the tissue pathology associated with schistosomiasis (Mahmoud & Wahals, 1990)

Proteinases are known to be secreted from *S. mansoni* parasites at several stages during migration in the mammalian host. Serine proteinases released by transforming cercariae and adult schistosomes are thought to be involved in a variety of functions including skin penetration and nutrition. These proteinases have been extensively reviewed previously (McKerrow & Doenhoff, 1988; McKerrow, 1989; McKerrow *et al.,* 1991) and for this reason will not be dealt with in this report.

Schistosomes feed on red blood cells, providing the parasites with the nutrients they require for growth and development. In the worm's digestive tract, ingested red blood cells are lysed and the hemoglobin released (Bogitsh, 1978). Proteolytic degradation of hemoglobin was first described by Timms and Bueding, (1959). They established that the proteinase was an acidic enzyme, found in highest concentration in female worms, which hydrolysed hemoglobin, but not natural blood proteins. Dresden & Deelder (1979), characterised the enzyme further by showing it was inactivated by inhibitors of thiol proteinases, but not by agents which inactivate serine, metallo, or carboxyl proteinases. Two forms of the cysteine proteinase have been purified from *S. mansoni* extracts. The more active form is capable of degrading hemoglobin, has a high specific activity on the synthetic substrate carbobenzoxy-arginyl-arginyl-7-amino-4-trifluoromethylcoumarin and is also highly immunogenic in infected animals (Chappell & Dresden, 1986a). Reduced glutathione, which in addition to the major constituent, hemoglobin, is also present in host red blood cells, has been shown to be effective in the activation of this proteinase (Chappell *et al.,* 1987). It is possible that the "hemoglobinase" is activated *in vivo* by this mechanism. Immunofluorescence studies using monoclonal antibodies have confirmed the gut localisation of the proteinase in adult worms (Chappell & Dresden, 1987). In addition there is strong evidence that this proteinase is also expressed at days 8-10 in *in vitro* cultured larvae, but it has not been detected in cercariae or eggs (Zerda *et al.,* 1988).

Ruppel *et al.*, (1985) demonstrated that natural infections in mice led to the early and predominant formation of antibodies against a 31 kDa protein of adult *S. mansoni*, the origin of which appeared to be the gut rather than the

tegument, suggesting that this protein may be present in the excretory/secretory products of worms. *S. mansoni* adult antigens were tested for cross-reactions with sera obtained from patients infected with *S. japonicum* using immunoblotting techniques. The sera consistently recognised a doublet of bands, which had molecular weights of approximately 31 and 32 kDa. Immunofluorescence assays performed with sera of *S. japonicum* patients confirmed the localisation of the Sm 31 and Sm 32 antigens to the gut of *S. mansoni* (Ruppel *et al.,* 1987).

Hence, the Sm 31 and Sm 32 antigens induced a strong and consistent antibody response in prepatent as well as long-standing infections of man and experimental animals. These antigens were considered to be potential targets for sero-diagnosis under field conditions, and to this end were expressed as fusion proteins with the ß-galactosidase gene of *Escherichia coli*. Using mouse and human infection sera, recombinant clones specific for a 31/32 kDa doublet were selected (Klinkert *et al.,* 1987). However, the fusion proteins were found to be unsuitable for use, as the ß-galactosidase protein cross reacted with anti-ß-galactosidase antibodies present in human sera. In order to be effective in immunodiagnosis the sera would have to be preadsorbed with *E. coli* extracts before use, making the employment of these recombinant antigens for routine diagnosis impractical.

To overcome this difficulty the Sm 31 and Sm 32 antigens were expressed as fusion proteins with the bacteriophage MS2 RNA polymerase (Klinkert *et al.,* 1988). However, other problems arose when trying to purify the fusion proteins free of contaminating *E. coli* antigens. These *E. coli* antigens were recognised by human infection sera and it was possible that if used, they would lead to false positives and hence, incorrect diagnosis. Infection sera did

not recognise the fusion proteins as well as it did native proteins, hence sensitivity was also quite low. Tests revealed that an array of epitopes was probably required for the reliable immunodiagnosis of schistosomiasis in the field (Klinkert *et al.,* 1988; Felleisen *et al.,* 1988). It was thought that the failure to reproduce the full reactivity of the native Sm 31 protein using fusion proteins was possibly due to conformational modifications in the antigenic sites of the recombinant molecules (Felleisen *et al.,* 1988). Alternative methods of expressing the molecules in their native conformation were sought. This would serve to develop the use of the antigens as diagnostic proteins and to characterise the molecules further.

Davis *et al.*, (1987) isolated cDNA clones encoding *S. mansoni* genes by immunologically screening an expression cDNA library with antisera raised against purified hemoglobinase. The recombinant fusion protein encoded by one cDNA clone exhibited the ability to degrade globin and was immunologically cross reactive with hemoglobinase isolated from adult worms. It was proposed that this molecule was responsible for hemoglobin digestion in the adult schistosome (Davis *et al.,* 1987). In 1989, Klinkert *et al.* published the primary structures of the Sm 31 and Sm 32 diagnostic proteins of *S. mansoni.* These sequences were derived from the nucleotide sequences of cDNA clones, isolated from a cDNA library which was screened with mouse and human infection sera. Both molecules were identified as acid proteinases. Based on the nucleotide and deduced amino acid sequence data, Sm 31 was found to be similar to the mammalian lysosomal enzyme, cathepsin B, and Sm 32 was found to be identical to the proposed schistosome "hemoglobinase" described by Davis *et al.,* (1987). This report by Klinkert *et al.,* (1989) seemed to provide conclusive evidence that the "hemoglobinase" and the cathepsin B-

like enzyme were two separate proteinases.

However, there was no evidence to prove that the Sm 32 molecule, "the hemoglobinase", was responsible for hemoglobin degradation. It had been shown that purified preparations of the "hemoglobinase" were found to be contaminated with *S. mansoni* cathepsin B activity (el Meanawy *et al.,* 1990), and also that the sequence of the Sm 32 molecule did not exhibit homology with published sequences of any other proteinases (Davis *et al.,* 1987). In contrast, since cathepsins had been implicated in the breakdown of hemoglobin as a source of nutrition in other parasites, Felleisen & Klinkert revised their original theory, that the cathepsin B proteinase and the hemoglobinase were separate enzymes, and suggested that the schistosome cathepsin B (Sm 31) was in fact responsible for the hemoglobinase activity of adult worms (Felleisen & Klinkert, 1990).

Purification of the Sm 31 and Sm 32 proteinases from the excretory/secretory products or extracts of adult *S. mansoni* worms was not possible due to the presence of contaminating proteins with similar physical properties (Chappell & Dresden, 1986b; Lindquist *et al.,* 1986). Expression of both molecules would provide the opportunity to characterise them further. Following its successful expression in insect cells, the Sm 31 molecule was shown to be capable of degrading hemoglobin (Gotz & Klinkert, 1993). Its substrate specificity, as well as its sensitivity to naturally occurring and synthetic inhibitors *in vitro,* proved it to have characteristic properties of the cysteine proteinase, cathepsin B. Hence, it was concluded that the Sm 31 molecule was a cathepsin B-like proteinase, and it was proposed that this proteinase was involved in hemoglobin degradation in the schistosome digestive tract (Gotz & Klinkert, 1993). The identification of the Sm 32 molecule,

the original "hemoglobinase", as a cysteine proteinase remains unverified, and its true function remains unknown (Gotz & Klinkert, 1993).

Whilst the recombinant Sm 31 molecule is capable of cleaving hemoglobin (Gotz & Klinkert, 1993), there has been no evidence to prove that the native proteinase is also capable of degrading hemoglobin *in vivo*. Furthermore, the participation of other proteinases in hemoglobin degradation has not been overruled (Bogitsh & Dresden, 1983; Kramer & Bogitsh, 1985; Bogitsh and Kirschner, 1987).

The work carried out, to date, on the Sm 31 molecule concentrated on recombinant fusion proteinases because of the problems encountered when purification of the native molecule was attempted. Using partially purified material from adult schistosomes, an enzyme was shown to be capable of degrading hemoglobin and synthetic peptides containing arginine (Dresden *et al.,* 1981). Sm 31 was believed to be the proteinase responsible for this activity, and it was thought that this cathepsin B proteinase was the principle enzyme responsible for proteolytic hydrolysis in adult worms.

Indeed it was considered, that helminths in general only synthesised cathepsin B-like proteinases. However, in a series of studies on the related parasitic trematode *Fasciola hepatica*, Smith *et al.,* (1993a, 1993b) and Carmona *et al.,* (1993) isolated and characterised a cathepsin L-like proteinase from medium in which adult and juvenile *F. hepatica* were maintained. This was the first trematode cathepsin L-like proteinase to be characterised and will be dealt with in more detail later. The identification of cathepsin L activity in *F. hepatica* led to a study on the proteolytic activities in *S. mansoni* and *S. japonicum*. A full length, cathepsin L cDNA clone has been isolated from an adult *S. mansoni* cDNA library, using PCR gene fragments

which encoded a cathepsin L-like proteinase, as a probe (Smith *et al.,* 1994b). Cathepsin L-like activity has been demonstrated as being dominant over cathepsin B-like activity in extracts of both adult *S. mansoni* and *S. japonicum* worms. The specific activity of the cathepsin L proteinase in these extracts was shown to be sixty-fold greater than that of the cathepsin B proteinase (Smith *et al.,* 1994b). These authors believe that it would not be impossible for the cathepsin L-like proteinase to play a greater role in hemoglobin digestion than cathepsin B.

So although it seemed that the roles of the Sm 31 and Sm 32 molecules had been resolved, in that the Sm 31 recombinant protein was proposed as a hemoglobinase, while there was little proof that the Sm 32 molecule was even a proteinase, now the proposed role of the Sm 31 proteinase as a major factor in hemoglobin degradation is questioned by the discovery of the more powerful and highly active cathepsin L-like proteinase in extracts of adult *S. mansoni* worms.

Fasciola hepatica, a parasitic trematode, related to *S. mansoni*, is the causative agent of liver fluke disease in mammals. The most common hosts for *F. hepatica* are agriculturally important animals such as cattle and sheep. Human fascioliasis has become an increasing problem in some tropical and developing countries (Apt *et al.,* 1992). Liver fluke infection occurs when the animal ingests vegetation contaminated with metacercarial cysts. The metacercaria excysts in the duodenum of the animal, migrates through the wall of the hosts digestive tract, and then enters the liver where it causes extensive damage over a 7-8 week period. The parasite then enters the immunologically safe environment of the bile ducts.

Howell, (1966) demonstrated that immature *F. hepatica* release enzymes *in vitro*, and postulated that *in vivo* these enzymes were involved in the penetration of the liver tissue. Locatelli & Berretta, (1969) showed that flukes can disrupt gelatin sheets *in vivo*, but are prevented from doing this when their pharynx is ligated, and concluded that the proteinases responsible for this activity reached "the outside" of the parasite as a result of regurgitation. In 1973, Howell localised the proteolytic activity involved in extracellular digestion to the gut cells, and confirmed the theory of Locatelli & Berretta, (1969).

Rupova & Keilova, (1979) and Simpkin *et al.,* (1980) described acidic proteinases in *F. hepatica*. However, no attempt was made to assign these enzymes to a particular class of proteinase. In 1982, Chapman & Mitchell described the presence of a thiol proteinase activity in immature and mature *F. hepatica* capable of cleaving immunoglobulin G into Fab and Fc fragments in a manner similar to the action of papain. They suggested that these enzymes may prevent antibody activating effector functions such as complement fixation in the vicinity of the migrating fluke affording them some protection from immune attack.

Rege *et al.,* (1989a) reported the purification of a cysteine class proteinase of 14.5 kDa from extracts of adult *F. hepatica* worms. Their preliminary data suggested that this enzyme was capable of digesting hemoglobin, collagen and immunoglobulin G. Also at this time, Dalton & Heffernan, (1989) observed that when immature and mature *F. hepatica* were maintained in culture for 16 hours they released proteolytic enzymes, and they speculated that these enzymes were important in the feeding and migration of the parasite. All of these enzymes were classified as cysteine proteinases due to their inactivation

by cysteine proteinase inhibitors and their enhanced activity in the presence of reducing agents. The proteinases were divided into two groups based on the pH range in which they were most active (Group 1, 60-90 kDa, pH 3.0-4.5; Group 2, 27.5-50 kDa, pH 4.5-8.0). In further studies it was shown that the Group 1 proteinases were capable of cleaving IgG molecules in a similar fashion to that reported in Chapman and Mitchell's earlier study (Smith *et al.,* 1993a). This immunoglobulin cleaving activity was classified as a cathepsin L-like proteinase based on inhibitor studies, which was in contrast to the cathepsin B-like activity described by Chapman & Mitchell, (1982). This was the first cathepsin L-like proteinase activity to be described for a parasitic trematode.

Smith *et al.,* (1993b) purified this 27 kDa proteinase to homogeneity, and demonstrated that it was one of the two major proteins released by adult *F. hepatica in vitro*. N-terminal sequencing analysis confirmed the identification of the proteinase as a cathepsin L-like enzyme and immunolocalisation studies at the light- and electron-microscope level revealed that these cathepsin L-like proteinases were concentrated in vesicles in the gut epithelial cells of adult *F. hepatica*. It was proposed that whilst the flukes migrated through the host liver, this proteinase was secreted to the exterior of the parasite, where it may play a role in immunoevasion by cleaving host immunoglobulin and thus preventing antibody-mediated immune effector cell attachment (Smith *et al.,* 1993b).

McGinty *et al.*, (1993) described the identification of E/S proteinases of adult and juvenile *F. hepatica* . They observed that a 25-26 kDa proteinase activity was a major released protein with a classical cysteine proteinase inhibitor profile. It was also capable of hydrolysing synthetic substrates, which indicated that it had a subsite specificity similar to that of the mammalian lysosomal

proteinase, cathepsin B. They speculated that this cathepsin B-like proteinase was identical to a 27 kDa cysteine proteinase which Yamasaki *et al.,* (1989) purified from the Japanese *Fasciola* spp. This 27 kDa proteinase was capable of degrading hemoglobin and was implicated in the feeding of the parasite (Yamasaki *et al.,* 1989). The performance of the proteinase when used in ELISAs suggested that it could be used as an important immunodiagnostic and prognostic tool (Yamasaki *et al.,* 1989). Immunocytochemical studies have since verified that the isolated enzyme is localised to the secretory granules of the intestinal epithelial cells, and suggest that it is secreted as a digestive enzyme into the intestinal lumen, where it may play an important role in the extracellular degradation of host proteins, including hemoglobin (Yamasaki *et al.*, 1989).

Recently Heussler & Dobbelaere, (1994) described the cloning of a proteinase gene family of *F. hepatica* by the polymerase chain reaction. Using degenerate oligonucleotide primers derived from conserved cysteine proteinase sequences, they amplified and isolated seven clones from cDNA prepared from RNA of adult worms. Five of these clones showed homology to cathepsin L type proteinases, while the remaining two clones were similar to cathepsin B type proteinases. One of the gene fragments, which was similar to cathepsin L-like proteinases was subcloned and expressed as a GST-fusion protein in *E. coli.* This fusion protein was purified and used to raise antibodies. Immunoblotting, with these antibodies, revealed a 30 kDa form of the proteinase, believed to represent the mature enzyme, in whole worm extract as well as in the excreted/secreted products of adult *F. hepatica*, and a 38 kDa parent form of the proteinase in the whole worm extracts only. These immunoblotting studies also indicated that the cathepsin L-like proteinase is

expressed or processed in a stage specific manner (Heussler & Dobbelaere, 1994).

Using information derived from the N-terminal sequence, which had been determined in an earlier study (Smith *et al.,* 1993b), a specific PCR oligonucleotide primer was designed and employed along with a generic cysteine proteinase primer (Eakin *et al.,* 1990), to amplify cathepsin L-like proteinase gene fragments, from cDNA isolated from adult *F. hepatica* worms (Smith *et al.,* 1994b). The sequences isolated by this technique were similar to the cysteine proteinase cDNA isolated from a cDNA library of *Fasciola* spp. by Yamasaki & Aoki, (1993), indicating that the proteinase purified in their earlier study was indeed a cathepsin L-like proteinase and not a cathepsin B-like proteinase as had been indicated by McGinty *et al.,* (1993).

A second cathepsin L-like (CL2) proteinase activity has been isolated from the E/S products of adult *F. hepatica* worms (Dowd *et al.,* 1994a). This proteinase has a molecular size of 29.5 kDa and shows a different substrate specificity to the cathepsin L-like proteinase, now termed CL1, isolated previously (Smith *et al.,* 1993a and 1993b). Using immunoblot techniques the possibility that both these proteinases arose from a larger sized parent molecule was ruled out. These two cathepsin L-like proteinases are the predominant molecules secreted by *F. hepatica* into the culture medium and represent greater than 80% of the secreted protein (Dowd *et al.,* 1994a). The ability of CL1 to cleave immunoglobulin *in vitro*, implicates that proteinase in immunoevasion mechanisms. It is likely that CL2, another dominant molecule, also has an important function, and plays an essential role in the survival of the parasite, possibly in feeding or in tissue penetration.

In a study by Carmona *et al.,* (1993) the role of the immunoglobulin cleaving, CL1 proteinase released *in vitro* by *F. hepatica* (Smith *et al.,* 1993a and 1993b) was investigated. They demonstrated that newly excysted juveniles, 3 week old, and 5 week old fluke E/S products also exhibited cathepsin L-like activity, and secreted an enzyme capable of cleaving immunoglobulin G. Using *in vitro* cell attachment assays they showed that the cathepsin L-like proteinase, purified from E/S products of adult *F. hepatica* worms can prevent the antibody-mediated attachment of eosinophils to newly excysted juveniles. They concluded that the cathepsin L-like proteinase was implicated in a key role in the immune evasion mechanism of *F. hepatica*, and regarded it as a potential target for vaccine and/or drug design (Carmona *et al.,* 1993).

Recently, this work has been followed by a study which looked at the ability of anti-cathepsin L antibodies to neutralise the activity of the cathepsin L1 proteinase. The ability of the enzyme to digest gelatin, in gelatin-substrate polyacrylamide gels, and immunoglobulin was inhibited by preincubation of the proteinase with antibodies, raised against the purified enzyme. The ability of these antibodies to neutralise the activity of the proteinase was also tested in an *in vitro* assay in which they were shown to interfere with the ability of the cathepsin L1 proteinase to prevent eosinophil attachment to juvenile *F. hepatica* (Smith *et al.,* 1994a). By indicating that antibodies, raised in response to immunisation with the CL1 molecule, were capable of neutralising the activity of the proteinase, and more importantly its immunoglobulin cleaving activity, this study confirmed the potential of the cathepsin L1 proteinase of both immature and mature *F. hepatica* worms as an ideal vaccine candidate.

In another study, Carmona *et al.,* (1994) have isolated and characterised a

dipeptidylpeptidase activity secreted by all stages of *F. hepatica* worms. It is classified as a serine proteinase of molecular weight greater than 200 kDa, and although similar in some properties to previously characterised dipeptidylpeptidases is different in its substrate preference and its susceptibility to inactivation by inhibitors. It is believed that the proteinase may function in the latter stages of the proteolytic digestion of host macromolecules, and could be essential for providing the fluke with dipeptides that could be absorbed through the intestine of the parasite (Carmona *et al.,* 1994). Dipeptidylpeptidase activities have been identified in the related trematodes *S. mansoni* and *S. japonicum* (Bogitsh & Dresden, 1983), but these enzymes have not been isolated and there is little known about them.

The nematodes, or "round worms", make up a large assemblage of worms of relatively simple structure with a widespread distribution, their cylindrical non-segmented bodies distinguishing them easily from other helminths. In vertebrates, they may parasitise the eye, mouth, tongue, alimentary canal, liver, lungs or body cavity, often causing destructive diseases and considerable hardship (Smyth, 1976). Hookworm infections are common in the rural population of the agrobased regions of the underdeveloped countries in the tropics, and are acquired by walking barefoot in damp soil contaminated with infective larvae (Singh & Sharma, 1991).

Ancylostoma caninum is a parasitic nematode which causes hookworm disease in dogs and humans. Its general symptoms are hyperchromic anaemia leading to general weakness, fatigue, and lack of physical and mental growth. The patient may also experience abdominal pain, constipation, anorexia and giddiness (Singh & Sharma, 1991). Adult parasites fastened to villi in the small

intestine of the host ingest host blood cells. The anaemia is a direct result of this blood loss (Roche & Layrisse, 1966).

In 1983, Hotez & Cerami described the release of a proteinase from adult *A. caninum*. This proteinase catalysed the hydrolysis of a number of plasma proteins and a peptide substrate known to be degraded by elastase. The purification of this proteinase was described by Hotez *et al.,* (1985), and consisted of three chromatographic steps in a defined order. It is believed that the adult worm probably uses this proteinase in three ways; (a) to degrade the bolus of intestinal mucosa lodged in the worms buccal capsule; (b) to destroy capillary walls and hence function as a hemorrhagic proteinase and (c) to function as an anticoagulant proteinase. The human (*Ancylostoma duodenale*) and zoonotic (*A. caninum*) hookworm larvae have been shown to exhibit histological similarities in the manner by which they migrate through connective tissue, and biochemical similarities in proteinase composition. Both *A. caninum* and *A. duodenale* larvae homogenates contain 38 and 68 kDa metalloproteinase activities (Hotez *et al.,* 1990).

The exact function of the larvae proteinase is unclear. McKerrow *et al.*, (1990) have shown that the metalloproteinase of *Strongyloides stercoralis* effects skin and connective tissue invasion. The *Strongyloides* proteinase is more enzymatically active than the hookworm metalloproteinase *in vitro*. It was thought that rather than representing a change in specificity, the difference in enzymatic activities of the two organisms reflected the kind of skin penetration that takes place in the two species. The *Strongyloides* entering the hosts body solely through skin penetration, whereas the *Ancylostoma* species are also orally infective (Hotez *et al.,* 1990).

The mechanics of skin penetration by the larval stages of nematode

infections are not very well understood, but it would seem that penetration does depend on parasite-derived proteinases (Matthews, 1982; McKerrow *et al.*, 1990). There is a controversy surrounding exsheathment and the role it plays in skin penetration in hookworm infections. Results by Hotez *et al.*, (1990) suggested that exsheathment, in *Ancylostoma* infections, occurs when the larvae encounter resistance to penetration i.e. unbroken skin, but when little resistance or broken skin was met the ensheathed larvae were able to achieve some degree of penetration. It was hypothesised that the timing of exsheathment may determine whether proteinases are released upon entry into the skin or at some later point in connective tissue migration (Hotez *et al.*, 1990).

Dowd *et al.*, (1994b) have shown that excretory/secretory products and soluble adult and larval extracts of *A. caninum* possess cysteine proteinase activity. This proteinase is actively secreted by adult parasites *in vitro*. Substrate specificity analysis revealed this enzyme to be cathepsin L-like in its proteinase activity. Cathepsin Ls play a role in lysosomal metabolism in mammalian cells (Barrett & Kirschke, 1980), this along with the mildly alkaline pH optimum of the enzyme reported in the study of Dowd *et al.*, (1994b) led them to suggest a role in feeding for the cathepsin L-like proteinase. The enzymes ability to degrade synthetic substrates in a manner similar to plasmin, also implicated the cathepsin L-like proteinase in anti-coagulant activities (Dowd *et al.*, 1994b).

Human eosinophilic enteritis has been shown to result from canine hookworm infection of the human gut (Prociv & Croese, 1990). The metalloproteinase isolated by Hotez *et al.*, (1990) was implicated as one of the causes of the pathogenicity of *Ancylostoma* infection because of its suggested

roles in capillary wall degradation and anti-coagulation. Dowd *et al.,* (1994b) have now described a cysteine proteinase activity, similar in properties to cathepsin L. Cathepsin L is one of the most powerful proteinases in the mammalian lysosome (Barrett & Kirschke, 1980). The identification of such a proteinase in the E/S products and in extracts of *A. caninum*, and the implication that this enzyme is also involved in anticoagulant activities, leads us to question the roles, combined or otherwise, of both these proteinases in the pathogenicity of hookworm infections.

Haemonchus contortus, another member of the nematode order *Strongylata*, is a highly pathogenic parasite that resides in the digestive tract of its host and feeds on host blood components. It is primarily a parasite of sheep although it also infects cattle, goats and other ruminants.

Gamble *et al.,* (1989) purified and characterised a proteinase which mediates the ecdysis of *H. contortus*. This enzyme, classified as a zinc metalloproteinase, was shown to hydrolyse a specific circular region of the second stage cuticle which results in the removal of the cuticular cap and allows the transition of the infective larvae from free living to parasitic environments.

In 1990, Cox *et al.* hypothesised that the blood feeding parasite, *H. contortus*, might possess an anticoagulant mechanism to prevent the hosts blood from clotting during feeding. They subsequently described a thiol proteinase activity that was able to degrade fibrinogen, and reported the primary structure of a 35 kDa cysteine proteinase that was believed to be responsible for the cleaving of fibrinogen *in vitro*. However, there is no evidence to prove that this is the case.

Results indicated that the cysteine proteinase was expressed primarily by blood-feeding *H. contortus* adult worms (Pratt *et al.,* 1990), but low levels of expression were also detected in all the blood-feeding stages with the exception of the L4 stage. This pattern of expression resembles that of the cathepsin B-like proteinase of *S. mansoni*, which is expressed by adult worms and late stage schistosomula but not by eggs or cercariae (Zerda *et al.,* 1988). The *H. contortus* proteinase was shown to be most closely related to cathepsin B-like proteinases, when compared to sequences of other known cysteine proteinases (42 % similarity to human and *S. mansoni* cathepsin B proteinases [Cox *et al.,* 1990; Pratt *et al.,* 1990]).

Further studies indicated that *H. contortus* adult worms express mRNAs for multiple cysteine proteinases. These are all closely related, in that they are similar to cathepsin B, but they are clearly distinct from one another (Pratt *et al.,* 1992a). This is different to the situation reported for both cathepsin B and cathepsin L-like proteinases of adult *S. mansoni* worms, which appear to be single copy genes (Klinkert *et al.,* 1989; Smith *et al.,* 1994b) as is the case for human cathepsins B and L (Chan *et al.,* 1986; Chauhan *et al.,* 1993). However, Heussler & Dobbelaere, (1994) have recently reported a gene family encoding cathepsins L and B in adult *F. hepatica*, and Eakin *et al.,* (1993) have also reported a gene family encoding cathepsin L-like proteinases in the protozoan parasite *Trypanosoma cruzi.*

Concurrent with the work described above Knox & Jones, (1990) also provided evidence that the excretory/secretory products of *H. contortus* possessed elastinolytic activity. They also postulated that this activity could act as an anticoagulant and hence be responsible for the continued bleeding from

damaged mucosal capillaries for extended lengths of time after the detachment of adult parasites. In a further study, they carried out extensive analysis to broaden the information available about these potentially important proteinases (Knox *et al.,* 1993). They confirmed the presence of several active proteinases of differing molecular size, inhibitor sensitivity and substrate specificity in extracts of adult parasites. The majority of these enzymes had an acidic pH optimum. Hemoglobin degradation appeared to be primarily catalysed by cysteine proteinases, and hence provides further evidence for the role of the cathepsin B-like proteinase of *H. contortus* in feeding (Knox *et al.,* 1993).

Vaccination of lambs with extracts of adult parasite enriched on the basis of fibrinogen-degrading activity confers significant protection against challenge infections with *H. contortus.* It was not known whether this protection was due to the neutralisation of the proteinase by antibodies, or to immunological reactions directed against other proteins in the extracts used to immunise the lambs (Cox *et al.,* 1990). Smith *et al.,* (1992) immunised lambs with gut antigen extracts, which contained a proteinase component, and they noticed a resistance to challenge infection. Sera from these lambs completely inhibited proteolytic activity of some of the cysteine proteinase activities. There was also an indication that parasites retrieved from immunised lambs had a modified proteinase expression, both in terms of total enzyme content and expression of differing molecular forms. It was thought that the modifications in proteinase expression may have a central role to play in parasite survival, in the face of host immune attack (Knox *et al.,* 1993).

In order to be considered as potential targets for either vaccine or drug design, proteinases must meet certain criteria. Proteinases are ubiquitous in all living organisms. There are many general proteinases, all of which perform similar functions, and hence, can be substituted for one another, making no one enzyme indispensable. Parasite proteinases being used as candidates for vaccines, or targets for drug inhibition must be indispensable to the parasite, performing a function which is solely that proteinase's responsibility. Cercariae of the parasitic trematode *S. mansoni* release a serine proteinase which is responsible for tissue invasion, inhibition of this proteinase, by synthetic inhibitors prevented the cercaria from penetrating the skin (reviewed in McKerrow & Doenhoff, 1988, McKerrow, 1989 and McKerrow *et al.,* 1991). The cathepsin L-like proteinase of *F. hepatica* is believed to play a role in protecting newly excysted juveniles from antibody-mediated eosinophil attachment and hence destruction (Carmona *et al.,* 1993).

It is critical that the proteinase in question is released early in the life cycle of the infectious stage of the parasite. Much of the physiological damage is caused by the migratory or burrowing stages of helminths. In order to decrease the effect of the parasite and to be of maximum benefit to the patient, it is crucial that the parasite is arrested at the earliest possible stage, be it by either vaccine and/or drug control. *S. mansoni* (reviewed in McKerrow & Doenhoff, 1988; McKerrow, 1989; McKerrow *et al.,* 1991), *F. hepatica* (Dalton & Heffernan, 1989; Carmona *et al.,*1993; McGinty *et al.,* 1993) and *A. caninum* (Hotez *et al.,* 1990) have all been shown to release proteinases in the early stages of their life cycles within their definitive hosts.

Specific drugs which may be administered and host antibodies which may be raised as a result of vaccination or drug use, must be able to reach the

target molecule and neutralise its activity. Proteinases of the helminth parasites *F. hepatica, Ascaris suum* and *Dictyocaulus viviparus,* have been shown to be inactivated by specific antibodies (Smith *et al.,* 1994a, Knox & Kennedy, 1988 and Britton *et al.,* 1992 respectively), the serine proteinase of *S. mansoni* cercariae is inactivated by specific synthetic inhibitors (Cohen *et al.,* 1991), and recently Klinkert *et al.,* (1994) and Wasilewski & McKerrow, (1994) have observed the ability of cysteine proteinase inhibitors to reduce the survival of *S. mansoni* in culture. Murine malaria caused by the protozoan parasite *Plasmodium vinckei* , has been cured using synthetic inhibitors of the cysteine proteinase found to be responsible for the digestion of host hemoglobin (Rosenthal *et al.,* 1993).

The proposed use of these antibodies and/or drugs in clinical situations raises the important point of specificity. Most parasite proteinases that have been characterised to date bear a significant degree of similarity to their mammalian homologues. This similarity is primarily around the active site residues of the proteinases. Antibodies/drugs which bind to and neutralise the active site of parasite proteinases may also exert the same effect on host proteinases. One answer, is the design of drugs or antibodies directed at dissimilar regions of the proteinases, binding of these molecules would have to alter the structure-function relationship of the proteinase, and in that way inactivate the proteinase. A second answer would be to control the dose level of the drug or vaccine, this would be effective providing that there was a critical low dosage, capable of inhibiting parasitic proteinases without exerting too many damaging side effects on both the host proteinases and the host itself.

Drugs and vaccines would ideally be easy to administer, and would not require careful monitoring in the case of every infected individual. The majority

of these parasitic infections are prevalent in underdeveloped countries where facilities are limited, and resources are very low, making such monitoring and frequent clinic/hospital visits impractical. However, helminth parasites do not replicate in their vertebrate hosts, and hence only a partial, nonsterilising, immunity or resistance is required. Complete sterilising immunity is not necessary. Ideally booster vaccine/drug doses would not be required as subsequent trickle infections would provide continuous restimulation of immunity. A cross reactive vaccine would be multiprotective to a host of helminthic diseases, and would be a solution to the problem of multiple infections which tends to occur with these parasites. It is unlikely that one antigen alone will confer adequate protection, and incorporation of both surface and secreted antigens into composite vaccines may prove to be more effective. Vaccination with a combination of three synthetic peptides into a polymeric synthetic hybrid peptide, SPf66, conferred protection on *Aotus* monkeys against *Plasmodium falciparum* malaria (Patarroyo, 1987). Protection has also been reported for this synthetic molecule in human trials (Patarroyo, 1988; Valero *et al.,* 1993).

One of the most fascinating aspects of parasites is their continued survival in the face of all of the defensive mechanisms at the disposal of the vertebrate host. Parasites do not passively submit to the onslaught of the hosts immune response, they suppress, subvert and evade that response in a wide variety of ways. The fact that parasites can themselves subvert host immune responses to their own advantage makes the task of vaccine development that much harder. Any induced immune response (be it vaccine or drug induced) in the host, must be capable of overcoming a sufficient number of the parasites immune evasion mechanisms in order that the parasite becomes susceptible

to the immune responses of the host and is destroyed. In some cases however, the pathogenicity of the helminth infection is a direct result of the hosts immune response, e.g. the hosts inflammatory response to the eggs causes the tissue pathology associated with schistosomiasis (Mahmoud & Wahals, 1990). Vaccination or drug use should avoid the exacerbation of any immune responses which are associated with the pathology of the diseases caused by parasitic helminths.

There is a need for less toxic compounds whose chemical synthesis is cheap enough to allow mass treatment in underdeveloped and developing countries. These synthetic compounds must also have desirable pharmacological properties including solubility, lack of systemic toxicity and high oral absorption. Although there are examples of synthetic inhibitors which inactivate proteinase activity *in vitro* (Cohen *et al.,* 1991; Klinkert *et al.,* 1994; Wasilewski & McKerrow, 1994) - or even in laboratory models such as the murine malaria model (Rosenthal *et al.,* 1993), there are few reports of inhibitors, natural or synthetic, being used to treat any illnesses. Once synthetic inhibitors have been developed for pharmacological use, these compounds will probably be more selective and less toxic than agents which are currently employed for treatment.

In order to fulfil all the criteria mentioned briefly above, it is clear that the full elucidation of the host-parasite relationship is required. The mechanisms controlling pathogenicity are for the large part unknown, as are those mechanisms responsible for immune evasion, and indeed even the basic functions of feeding and mobility are little understood. An overwhelming body of evidence shows that malnutrition, results in depressed immunological

competence and defective ability to combat infection (Wakelin, 1989). This affects the ability of the infected host to control infection and to respond to vaccine or drug therapy. Few people are aware of the socioeconomic impact of parasitic helminths in underdeveloped and developing countries. Improvements in nutrition, hygiene, education and medical aid as well as the understanding of the parasites interaction with its host and how we can effectively interfere with it, will help us to control, as Ken Mott of the WHO put it, "humanity's most widespread but hidden scourges" (Maurice, 1994).

CHAPTER TWO
MATERIALS and METHODS

2.1 MATERIALS

Aldrich Chemical Company

Ethidium bromide, Triton X-100.

Bachem

N-benzyloxcarbonyl-L-phenylalanine-L-arginine-7-amino-4-methylcoumarin.HCl (Z-F-R-AMC), *N*-benzyloxcarbonyl-L-phenylalanine-L-alanine-diazomethylketone (Z-F-A-CHN_2).

Bethesda Research Laboratories (BRL)

Ultra pure caesium chloride, mRNA isolation system.

Bio-rad Laboratories

Bradford reagent.

Biotrin Research

Protein-A agarose.

British Drug House

Acetic acid, acrylamide, bisacrylamide, 2-mercaptoethanol, sodium dodecyl sulphate (SDS).

Flow Laboratories

Foetal calf serum.

Fluka

Guanidinium thiocyanate.

Invitrogen

pCR II direct cloning vector.

Gibco

$NaHCO_3$, RPMI-1640.

Kodak

667 Polaroid film.

Labscan

Ethanol, chloroform, glutaraldehyde, methanol, propan-2-ol.

Nunc

24-well tissue culture plates, 96-multiwell plates.

Oxoid

Agar (technical grade), bacto-tryptone, bacto-yeast extract.

Pharmacia

QAE-Sephadex A-50, sephacryl S-200 HR.

Promega

Agarose (molecular biological grade), anti-rabbit IgG (Fc) alkaline

phosphatase conjugate, 5-bromo-4-chloro-3-indolyl-ß-galactoside (X-Gal), dNTPs, *Eco*RI, *Hin*dIII, isopropyl-ß-thiogalactopyranoside (IPTG), Riboclone cDNA synthesis kit, RNase ONE, Taq DNA polymerase, T4 DNA ligase, ØX174 digested with Hae III markers.

Reidel-de-Haen

Ammonium persulphate, ammonium acetate, bromophenol blue, citric acid, dimethyl formamide (DMF), di-sodium EDTA, di-sodium hydrogen phosphate, glucose, glycerol, glycine, hydrochloric acid, phenol, potassium dihydrogen phosphate, sodium acetate, sodium chloride, sodium dihydrogen phosphate, sucrose, tetramethylenediamine (TEMED), tris-(hydroxymethyl)-methylamine (2-amino-hydroxymethyl) propane-1,3-diol (Tris).

Schleicher & Schull

Nitrocellulose paper.

Sigma

Bovine serum albumin (BSA), 5-bromo-5-chloro-3-indolyl phosphate (BCIP), coomassie brilliant blue R, diethylpyrocarbonate (DPC), dithiothreitol (DTT), Freund's adjuvant (complete), Freund's adjuvant (incomplete), gelatin (type B: from bovine skin), gentamicin (10 mg ml^{-1}), 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (Hepes), horseradish peroxidase, IgG2a, lead citrate, lysozyme, methylene blue, nitro blue tetrazolium (NBT), papain, phenylmethylsulphonyl fluoride (PMSF), pre stained molecular weight markers, sacrosyl, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), tri-sodium citrate, Tween-20, uranyl acetate, xylene cyanol, *N*-

benzyloxcarbonyl-L-arginine-L-arginine-7-amino-4-methylcoumarin.HCl (Z-R-R-AMC), *N*-benzyloxcarbonyl-L-arginine-7-amino-4-methylcoumarin.HCl (Z-R-AMC).

University College Cork

New Zealand White rabbits.

Waters

TSK3000SW column

Whatman

Whatman No. 1 filter paper.

2.2 METHODS

2.2.1 Preparation of *in-vitro*-released products from adult *F. hepatica*.

Mature flukes were obtained from the infected livers of condemned animals at a local abattoir (Anglo-Irish Meat Processor's abattoir, Ballymun, Dublin). The flukes were washed 6 times in phosphate-buffered saline (PBS), pH 7.3, and then maintained (one mature fluke ml^{-1}) in RPMI-1640, pH 7.3, containing 2% glucose, 30 mM Hepes and 25 mg l^{-1} gentamicin overnight. The culture medium (excretory/secretory (E/S) products) was removed, centrifuged at 12,000 x *g* for 30 min, aliquoted and stored at -20°C.

2.2.2 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Samples were analysed by one dimensional SDS-PAGE according to the method of Laemmli, (1970), on gels containing 10% or 12% (w/v) acrylamide, 0.27% (w/v) bisacrylamide, 0.373 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate and 0.008% TEMED. The stacking gel contained 3% (w/v) acrylamide, 0.08% (w/v) bisacrylamide, 0.125 M Tris-HCl. pH 6.8, 0.075% (w/v) ammonium persulphate, 0.1% (w/v) SDS and 0.023% (w/v) TEMED. Samples were prepared in non-reducing sample buffer (0.12 M Tris-HCl, pH 6.8, 5% (w/v) SDS, 10% (w/v) glycerol and 0.01% (w/v) Bromophenol Blue) or reducing sample buffer (same as non-reducing buffer except that 5% 2-mercaptoethanol is included, and the samples are boiled in reducing sample buffer for 2 min). The sample and sample buffer were combined in a 1:1 ratio.

Gels were run in a vertical slab gel apparatus (Atto Corporation) in electrode

buffer containing 0.024 M Tris-HCl, 0.186 M glycine and 0.1% (w/v) SDS pH 8.3 at 25 mA, at room temperature. They were removed when the dye front was within approximately 1 cm of the bottom of the gel (approximately 3-4 h). The proteins were visualised by soaking the gel in a solution containing 0.1% (w/v) Coomassie Brilliant Blue R, 20% (v/v) methanol and 10% (v/v) acetic acid for 1 h at room temperature. Destaining was carried out in 20% (v/v) methanol, 10 % (V/V) acetic acid.

2.2.3 Gelatin-substrate gel analysis of fluke *in vitro* released products.

Gelatin-substrate PAGE (GS-PAGE) was carried out exactly as described by Dalton & Heffernan, (1989). Briefly, samples were mixed with non-reducing sample buffer (Section 2.2.2) applied to a 10% SDS-polyacrylamide gel containing 0.1% gelatin. After electrophoresis the gels were washed for 1 h in 0.1 M sodium citrate, pH 4.5, containing 2.5 % Triton X-100 with one change. The gels were then incubated in 0.1 M sodium citrate, pH 4.5 for 24 h at 37°C, and stained in Coomassie Brilliant Blue R solution as described in section 2.2.2.

2.2.4 Protein estimation.

A micro-Bradford assay was employed to determine the protein concentration of samples. Using a 96-well microtitre plate, standards (bovine serum albumin (BSA)) and samples were assayed by mixing 200 µl of 1 X Bradford (commercially obtained) reagent with 10 µl of test solution. The absorbance was read at 595 nm after 10 min incubation at room temperature, and the protein values of the samples extrapolated from the graph of the

protein values of the standards.

2.2.5 HPLC analysis of E/S products.

One hundred μg of mature fluke E/S products (Section 2.2.1) were subjected to molecular sieve HPLC on a TSK3000SW column. The mobile phase was 0.1 M potassium-phosphate, pH 7.0, the flow rate was 0.3 ml min^{-1} and the eluted proteins were monitored by absorbance at 280 nm using a sensitivity range of 0.05. The molecular sizes of proteins were determined by calibrating the column with the following proteins; IgG2a (150 kDa), bovine serum albumin (67 kDa), horseradish peroxidase (45 kDa) and lysozyme (14.3 kDa).

2.2.6 Assay for IgG2a cleaving activity.

Five μl of a purified IgG2a murine monoclonal antibody (a gift from Dr. E. Pearce. NIAID, National Institutes of Health, MD, U.S.A.) was mixed with 40 μl of adult *F. hepatica* E/S products (Section 2.2.1) or HPLC fractions (Section 2.2.5), 40 μl PBS, 1 μl 6 M 2-mercaptoethanol and 1 μl 100 mM EDTA, and incubated for 1 h at 37°C. Fragments derived from the proteolytically cleaved IgG2a were visualised by SDS-PAGE run under reducing conditions (Section 2.2.2).

2.2.7 Proteinase assays with synthetic fluorogenic peptide substrates.

The synthetic fluorogenic peptide benzyloxycarbonyl-L-phenylalanine-L-arginine-7-amido-4-methylcoumarin.HCl (Z-F-R-AMC) was used as a substrate to detect cathepsin L-like activity (Barrett & Kirschke, 1980). The assay mixture

(1 ml volume) contained 5 mM dithiothreitol (DTT) and 13 μM substrate in 0.1 M sodium citrate pH 4.5. Five to 20 μl samples were added to the mixture at 37°C for 1 h. The release of the fluorescent leaving group, 7-amino-4-methylcoumarin (AMC), was monitored in a Perkin-Elmer Luminescence Spectrometer model LS 50, at exciter and analyser wavelengths of 370 nm and 440 nm, respectively. Enzyme activity was expressed as μmol AMC released min^{-1} ml^{-1}, where one unit of activity releases 1 μmol of AMC min^{-1} at 37°C.

2.2.8 Fluorogenic visualisation of proteinases in SDS-PAGE.

Proteinases capable of cleaving various fluorogenic substrates were visualised directly in SDS-PAGE using the method described by Robertson *et al.,* (1990). Samples of E/S products (Section 2.2.1) and pooled HPLC fractions (Section 2.2.5) were applied to GS-PAGE gels (Section 2.2.3).

Following electrophoresis the gels were washed in 2.5% Triton X-100 in 0.1 M sodium acetate, pH 5.5, for 30 min at room temperature and then transferred to 0.1 M sodium acetate, pH 5.5, containing 1 mM DTT and 10 μM fluorogenic substrate. After an incubation time of between 10 and 30 min the proteinases could be visualised on a ChromatoVue model TL-23 UV Transilluminator and recorded immediately by photography. The fluorogenic substrates used were, Z-F-R-AMC, Z-R-R-AMC and Z-R-AMC.

2.2.9 Inhibition studies using diethylpyrocarbonate (DPC) and Z-F-A-CHN_2.

DPC was used to determine if it affected the activity of the adult fluke proteinase on the fluorogenic substrate Z-F-R-AMC. 2 μl of pooled enzyme

fractions from the HPLC column (Section 2.2.5) were incubated in various concentrations of DPC (0.35-50 mM) in 0.1 M sodium citrate, pH 4.5, for 5 min before addition to the enzyme assay mixture (Section 2.2.7). To test the inhibitory activity of DPC (25 mM) at different pHs the following buffers were used: 0.1 M sodium citrate, pH 3.0, 3.5, 4.0, 4.5 and pH 5.0, 0.1 M sodium phosphate, pH 6.0, and 0.1 M glycine, pH 7.0 and 8.0. The substrate Z-F-R-AMC was also prepared in these buffers.

DPC inhibition of the IgG2a cleaving ability of the adult fluke proteinases was also investigated. Twenty µl of pooled HPLC fractions (Section 2.2.5) were incubated in a final concentration of 200 mM DPC in PBS for 30 min at room temperature before addition to the IgG cleaving assay (Section 2.2.6). Adult fluke E/S products (Section 2.2.1), incubated with or without 200 mM DPC, for 10 min were analysed by GS-PAGE (Section 2.2.3) to determine which of the multiple proteolytic bands observed on these gels were inactivated by the inhibitor. Adult fluke E/S was incubated with 2, 10 and 20 µmol of the diazomethylketone, Z-F-A-CHN_2, a specific inhibitor of cathepsins B and L (Rosenthal *et al.,* 1989) for 10 min prior to addition of the E/S products to the fluorogenic assay and before analysis by GS-PAGE.

2.2.10 Purification of *F. hepatica* IgG cleaving cysteine proteinase.

E/S products (Section 2.2.1) (500 ml) were concentrated in an Amicon 8400 ultrafiltration unit (Danvers) with a YM3 membrane (3000 Da cut-off) to 10 ml and the sample applied to a 120 ml Sephacryl S-200 column (1.9 x 42 cm) equilibrated in 0.1 M Tris-HCl, pH 7.0. Fractions of 5 ml were collected after the void volume (110 ml) had been passed. The column eluate was monitored at 280 nm using a LKB Uvicord monitor. Fractions were analysed for enzymatic

activity using the synthetic substrate Z-F-R-AMC (Section 2.2.7) and by GS-PAGE (Section 2.2.3). Those fractions containing the Group 1 cysteine proteinases (Dalton & Heffernan, 1989) and having IgG cleaving activity were pooled and applied to a 50 ml QAE Sephadex column equilibrated in 0.1 M Tris-HCl, pH 7.0. The run through fraction (unbound proteins) (150 ml) was collected and concentrated in an Amicon ultrafiltration unit to a volume of 10 ml, dialysed against ultra-pure water and freeze dried. Purified enzyme was analysed by SDS-PAGE (Section 2.2.2) and GS-PAGE (Section 2.2.3). Protein concentration was determined by the micro-Bradford method (Section 2.2.4).

2.2.11 N-terminal sequence determination.

F. hepatica proteinase, purified as described in section 2.2.10 above, was sent to the Protein Sequencing Facility, Department of Biochemistry, Tennis Court Rd., Cambridge CB2 IQW, where the N-terminal sequence was determined using an Applied Biosystems 477A Protein sequencer.

2.2.12 Production of a polyclonal antiserum.

Polyclonal antisera against purified enzyme was prepared by injecting a New Zealand white rabbit subcutaneously 5 times, with 50 µg of purified enzyme (Section 2.2.10) per injection, in Freund's complete (initial injection) or incomplete (subsequent injections) adjuvant. Equal volumes of the antigen and Freund's complete or incomplete adjuvant were sonicated on ice to form an even emulsion. This was then injected at several points along the back of the rabbit. One week after the final injection the rabbit was sacrificed and the blood collected. The blood was left to clot overnight at 4°C and the serum was drawn off aliquoted and stored at -20°C. The IgG fraction of the serum was

purified using a protein-A column according to the procedure outlined in the Pharmacia manual.

2.2.13 Immunoblotting

Adult *F. hepatica* E/S products (Section 2.2.1) and purified enzyme (Section 2.2.10) were separated by SDS-PAGE (Section 2.2.2) and electrophoretically transferred to nitrocellulose paper using an Atto semi-dry blotting system. 1% foetal calf serum, and 0.5% Tween-20 in PBS was used to block non-specific binding sites. The nitrocellulose was incubated in anti-cathepsin L-like proteinase serum or normal rabbit serum (1 : 500 dilution), and the bound immunoglobulin was visualised using alkaline phosphatase-conjugated anti-rabbit IgG. Nitro blue tetrazolium (NBT) and 5-bromo-5-chloro-3-indolyl phosphate (BCIP) prepared in dimethyl formamide (DMF) were used as substrate.

2.2.14 Immunolocalisation studies.

This was carried out in collaboration with Dr. A. Trudgett's laboratory in the Medical Biology Centre, Queens University Belfast, Northern Ireland, according to the method described by Smith *et al.,* (1993b).

Immunolocalisation studies at light microscope level were carried out on 3-μm JB-4 plastic embedded adult fluke sections using FITC-conjugated goat anti-rabbit serum to detect bound antibody according to the procedure described previously (Hanna, 1980). Electron immunocytochemistry was performed using an indirect immunogold labelling method. Transverse slices 1 mm thick were cut from the mid-region of flukes (freshly obtained from the abattoir) so as to include gut and reproductive tissues. These were lightly fixed

in 2% double-distilled glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 3% sucrose, for 40 min at 4°C. The tissue slices were then washed in several changes of cold buffer, dehydrated in graded ethanol at -20°C, infiltrated overnight in LRGold resin, and polymerised under a quartz halogen visible light source, for 28 h at -20°C. Thin sections (60 nm-70 nm in thickness) were cut and mounted on bare 200 mesh nickel grids. Following incubation with normal goat serum for 30 min at room temperature, the sections were transferred to primary antibody, diluted 5000-fold with 20 mM Tris-HCl, pH 8.2, containing 0.1% (w/v) BSA and Tween 20, for 18 h at room temperature. After thorough washing in Tris/BSA the grids were transferred to the gold labelled solution, a 25 µl droplet of 15 nm gold-conjugated goat anti-rabbit IgG, for 1 h. Following this incubation the sections were buffer-washed, lightly fixed with 2% double-distilled glutaraldehyde (3 min), and finally washed with buffer and rinsed with distilled waster. The sections were double-stained with alcoholic, uranyl acetate (15 min) and aqueous lead citrate (8 min) and viewed in a JEOL 100CX transmission electron microscope.

2.2.15 Inhibition of proteinase activity using anti-cathepsin L-like proteinase antibody.

Purified anti-cathepsin L IgG (2-20 µg) (Section 2.2.12) was incubated with the purified proteinase (3 units) (Section 2.2.10) for 4 h at 4°C and then this was analysed by GS-PAGE (Section 2.2.3). Purified enzyme (6 units) was also incubated with anti-cathepsin L-like proteinase IgG (270 µg) or control IgG in a total volume of 700 µl. After 4 h at 4°C, seven µl of 6 M 2-mercaptoethanol and 7 µl of 100 mM EDTA were added to the reaction mixtures to activate the

enzyme. A 100 µl sample was removed from each reaction at this time (t_0), E-64 (0.5 µg) was added immediately to stop the enzyme reaction and it was stored at -20°C. The activated proteinase-antibody mixtures were transferred to 37°C, and a second sample from each reaction was removed after 30 min and treated as described above. The samples were analysed using reducing SDS-PAGE (Section 2.2.2). After staining the gels with Coomassie Brilliant Blue R they were analysed using a GS 300 Transmittance/Reflectance scanning densitometer (Hoefer Scientific Instruments).

Purified cathepsin L-like proteinase (6 units) can prevent antibody-mediated attachment of eosinophils to newly excysted juveniles (NEJ) in an *in vitro* assay (Carmona *et al.*, 1993.).

In collaboration with Dr. C. Carmona, we incubated anti-cathepsin L IgG (55 µg) and control IgG with cathepsin L-like proteinase for 30 min at 4°C prior to addition to the assay. The *in vitro* assay involves incubating the juvenile flukes with serum obtained from liver fluke infected rats (diluted 1: 100) or control serum and 8 x 10^6 eosinophils ml^{-1} in wells of a 24-well plate. After 2 h the juvenile flukes are transferred to a microscopic slide and examined at X 40 and X 100 magnifications. Individual flukes are examined and the number of bound eosinophils are assessed by counting. Due to their three dimensional structure and the difficulties associated with manipulating the NEJ to count the number of eosinophils bound to the entire surface of a single fluke, juvenile flukes with more than 20 eosinophils attached in the plain of the dissecting microscope are considered positive (Carmona *et al.*, 1993).

2.2.16 RNA isolation from adult *F. hepatica* worms.

RNA was isolated from adult flukes according to the method of Chomczynski

& Sacchi, (1987). All glassware, eppendorfs and pipette tips were treated as described (Maniatis *et al.,* 1982). Sterile, disposable plastic ware was used whenever possible and all chemicals were of the highest grade available. All solutions were treated with 0.1% DPC at 37°C overnight and autoclaved, unless otherwise stated.

The denaturing solution was 5 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sterile-filtered sacrosyl (w/v), 0.1 M 2-mercaptoethanol. Adult *F. hepatica* worms were isolated from the bile ducts of infected cattle and washed in PBS as described previously (Section 3.2.1). One g of tissue (approximately 10 flukes) was minced on ice and homogenised at room temperature with 10 ml of denaturing solution. 1 ml of 2 M sodium acetate pH 4.0, 10 ml of phenol (water saturated) and 2 ml of chloroform-isoamyl alcohol mixture (49:1) were added and the solution mixed by inversion after each addition. The final suspension was shaken vigorously for 10 s, cooled on ice for 15 min, and centrifuged at 10,000 x *g* for 20 min at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 10 ml of isopropanol and stored at -20°C for 1 h (at least) to precipitate RNA. Following centrifugation at 10,000 x *g* for 20 min, the resulting RNA pellet was dissolved in 3 ml of denaturing solution, and precipitated with 1 volume of isopropanol at -20°C for 1 h. The tubes were spun in a microcentrifuge at top speed for 10 min at 4°C, and the RNA pellet was washed in 75% ethanol, centrifuged, vacuum dried and stored under 100% ethanol at -70°C until required for use.

2.2.17 mRNA isolation.

mRNA was prepared from total RNA isolated as described above, by oligo-

dT cellulose chromatography. 3 ml of binding buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.3 M NaCl, 0.1 (w/v) SDS) was used to dissolve the RNA pellet (500 µg). This solution was heated in a 70°C water bath for 5 min and then chilled on ice for 5 min. The dissolved RNA was loaded onto an oligo-dT cellulose column (100 mg oligo-dT cellulose) under gravity flow and washed with 4 ml of binding buffer to elute non-messenger RNA. The mRNA was eluted with 1.5 ml of elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% (w/v) SDS), this was collected as one fraction and the column was regenerated using 4 ml of binding buffer. The RNA was heated at 70°C for 5 min, chilled on ice for 5 min and left at room temperature for 20 min. 90 µl of 5 M NaCl was added and then the solution was immediately reloaded onto the column which was subsequently washed with 4 ml of binding buffer. The mRNA was eluted into one fraction with 1.5 ml of elution buffer as buffer. 90 µl of NaCl and 3 ml of 100% ice cold ethanol were added and the tube was placed at -20°C overnight or until needed.

2.2.18 cDNA preparation.

cDNA was prepared from mRNA using the Promega Riboclone cDNA synthesis system. mRNA stored at -70°C was centrifuged at top speed (microcentrifuge), washed with 1 ml of 95% ethanol, respun and then vacuum dried for 15 min. The pellet was resuspended in 55 µl of DPC treated water and was used for cDNA synthesis.

In a sterile RNase-free microcentrifuge tube, the mRNA (5.5 µg) was added to the first strand primer (0.5 µg primer/µg mRNA) and was heated to 70°C for 5 min. This was cooled to room temperature and the tube was spun briefly to

collect the solution. First strand buffer and rRNasin ribonuclease inhibitor were added, the mixture heated to 42°C for 5 min and sodium pyrophosphate and AMV reverse transcriptase (also pre heated to 42°C) were added to a final volume of 100 µl. The contents of the tube were mixed gently by flicking and incubated at 42°C for 60 min and then stored on ice. The final reaction conditions for first strand synthesis are : 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 4 mM sodium pyrophosphate, 1 mM each dNTP, 1 U rRNasin ribonuclease inhibitor / µl reaction, 0.3 - 0.5 µg primer / µg mRNA and 15 U AMV reverse transcriptase / µg mRNA.

To the first strand reaction, second strand buffer, *E. coli* DNA polymerase I, *E. coli* RNase H, and DPC treated water were added to a final volume of 500 µl. The solution was mixed gently and incubated at 14°C for 2 h. The reaction was heated to 70°C for 10 min and the contents were collected at the bottom of the tube by brief centrifugation and then placed on ice. 2 U of T4 DNA polymerase/µg input mRNA were added to the reaction which was incubated at 37°C for 10 min. The reaction was stopped by adding 50 µl of 200 mM EDTA and then placed on ice. The final reaction conditions for second strand synthesis are as follows: 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 50 µg ml^{-1} BSA, 5 mM DTT, 8 U ml^{-1} RNase H, 230 U ml^{-1} *E. coli* DNA polymerase I; from the first strand reaction are : 0.2 mM each dNTP, 0.1 mM spermidine and 0.8 mM sodium pyrophosphate. The cDNA was extracted twice with an equal volume of TE-saturated phenol/chloroform, ethanol precipitated and stored at -70°C under 100% ethanol until required for use.

2.2.19 Construction of oligonucleotide primers.

Generic oligonucleotide primers for cysteine proteinases were prepared as described by Eakin *et al.,* (1990). The design of the primers used to amplify the cysteine proteinase gene fragments from *Fasciola hepatica* is based upon the conserved amino acid sequence in the region flanking the active site asparagine-175 residue of cysteine proteinases (Eakin *et al.*, 1990) (Figure 2.1). Mixed oligonucleotides were synthesised, using inosines in positions where all four bases were possible in the codon, in order to minimise the degeneracy of the DNA primer, but to maximise its ability to form stable hybrids with the target DNA. An oligonucleotide sense primer was also designed using the N-terminal sequence of the purified cathepsin L-like cysteine proteinase (Section 2.2.11). A codon bias towards *F. hepatica* was used for the sequence of this primer (Figure 2.1). The codon bias was worked out using published DNA sequence data and the method described by Hyde *et al.,* (1989).

The degeneracies of the oligonucleotides were 1024 for the cysteine (sense) primer, 384 for the asparagine (anti-sense) primer described by Eakin *et al.,* (1990), and 4 for the second sense primer which used the N-terminal sequence for its design. Recognition sites for the restriction endonucleases *Eco*RI and *Hin*dIII were added to the 5' ends of the forward and reverse primers respectively to allow for rapid subcloning in a known orientation for double stranded DNA sequencing. Three additional bases (ACA in the sense primers and TTA in the anti-sense primer) were added to the 5' ends to ensure polymerisation through the restriction sites. These three primers were used in combination with one another in polymerase chain reactions to amplify cysteine proteinase gene fragments from cDNA of *F. hepatica*. The oligonucleotides were synthesised by Oswell DNA Service, Edinburgh.

Asn 3' primer [Eakin *et al.*, 1990].

```
                    IGA
5' TTA AAG CTT CCA      RTT YTT IAC RAT CCA RTA 3'
                    RCT
     Hind III  W    S      N   K   V   I   W   Y
```

N-terminal *F. hepatica* cathepsin L primer.

```
5' ACA GAA TTC GGY TAT GTG ACT GGY GTG AAG G 3'
      EcoR1     G   Y   V   T   G   V   K
```

Figure 2.1

The oligonucleotide primers constructed in order to amplify the cathepsin L-like proteinase from F. hepatica, *where I= Inosine, R= A or G and Y= T or C. Details of their construction are given in Methods, section 2.2.19.*

2.2.20 Polymerase chain reaction (PCR).

A master mix containing 100 μM dNTPs, 2.5 U of Taq DNA polymerase (where one unit is defined as the amount of enzyme required to catalyse the incorporation of 10 nmol of dNTP into an acid-insoluble form in 30 min at 74°C), 5 mM $MgCl_2$, 10 X reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 (at 25°C) and 1.0% Triton X-100) and autoclaved ddH_2O to a final volume of 50 μl was prepared and added to 50 ng of cDNA from adult worms (section 2.2.18), and 100 nM of both primers in 0.5 ml eppendorf tubes. These were used in a polymerase chain reaction (PCR) which was carried out as follows : 40 cycles of denaturation at 94°C for 45 s, primer annealing at 40°C for 1 min and extension at 72°C for 2 min, with an initial denaturing step at 94°C for 2 min and a final extension time of 10 min at 72°C. Reactions were immediately chilled to 4°C. PCR gene fragments were visualised on 4% agarose-TAE gel electrophoresis.

2.2.21 Subcloning of PCR gene fragments.

The PCR amplified products were extracted twice with an equal volume of TE-saturated phenol/chloroform, precipitated with 100% ice cold ethanol, washed with 75% ethanol, and the pellet was resuspended in 9 μl of autoclaved ddH_2O. Amplified gene fragments were subcloned directly into the Invitrogen pCR II direct cloning vector. Extracted fragments were also double digested with 1 U μg^{-1} of each of the restriction enzymes *Hin*d III and *Eco*RI for 1 h at 37°C, and were then subcloned into Puc19 plasmid which had been similarly digested.

The ligation conditions were as follows 50 ng of vector was mixed with

approximately 1 μg of extracted PCR products, in the presence of 0.3 Weiss U of T4 DNA ligase (where 1 Weiss unit is equivalent to 1 ligation unit which catalyses greater than 95% ligation of 1μg of lambda/*Hin*d III fragments at 16°C in 20 min), 30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 5 mM ATP. The contents of the tube were mixed by tapping the side of the tube gently and the ligation was allowed to proceed overnight at 16°C. The ligase was heat inactivated by heating to 70°C for 10 min, extracted twice with TE-saturated phenol/chloroform, ethanol precipitated and resuspended in 10 μl of autoclaved ddH_2O.

Electro-competent cells (Dower *et al.*, 1988) were incubated on ice for at least 30 min with 5 μl of the extracted ligation reaction. The cells were then transformed by electroporation; the pulse generator was set to the 25 μF capacitor, 2.5 kV, and 200 Ω in parallel with the sample chamber, one pulse at these settings should result in a pulse of 12.5 kV cm^{-1} with a time constant of 4.5 to 5 ms. 500 μl of LB medium was added immediately following transformation and the cells were shaken gently at 37°C for 1 h to allow the cells to recover. The cells were concentrated by centrifuging the culture for 1 min in a microcentrifuge before plating out on an LB plate containing 50 μg ml^{-1} ampicillin, 0.5 mM IPTG, and 40 μg ml^{-1} X-Gal. The plates were incubated at 37°C overnight.

2.2.22 Screening of recombinant colonies.

Positive or recombinant colonies were picked from the LB plates and inoculated into 5 ml of LB containing 50 μg ml^{-1} ampicillin and grown overnight. Plasmid DNA from 0.5 ml of this mini-prep culture was isolated

according to the alkali-lysis method described by Maniatis *et al.*, (1982). The DNA was double digested with the restriction enzymes *Eco*RI and *Hin*dIII, and the inserts, if any, were visualised by electrophoresis on 2% agarose-TAE gels. Positive clones were taken to be those recombinants with inserts of the expected size i.e. approximately 500 bp.

2.2.23 Sequencing of PCR gene fragments.

Plasmid DNA from positive recombinants with inserts of the expected size was purified by centrifugation to equilibrium in caesium chloride-ethidium bromide gradients as described in Maniatis *et al.*, (1982). The DNA was sent to The Sequencing Facility in Durham University where it was sequenced commercially.

CHAPTER THREE
RESULTS

3.0 RESULTS

3.1 Characterisation of IgG cleaving enzyme in adult fluke E/S products.

The presence of an immunoglobulin cleaving enzyme was first indicated by Chapman & Mitchell, (1982). Such an enzyme/enzymes may play a significant role in the flukes immune evasion mechanism. Using comparisons to papain, HPLC analysis and various substrate hydrolysis techniques we have characterised the proteinase responsible for the IgG cleaving activity in E/S products.

3.1.1 Demonstration of IgG cleavage.

A murine monoclonal antibody IgG2a was incubated with a sample of adult *F. hepatica* E/S products, or with the cysteine proteinase papain, at 37°C for 1 h. SDS-PAGE analysis revealed that proteinases in the E/S products cleave the mouse IgG2a heavy chain into two fragments (Figure 3.1, lanes 2, 4 and 6). These fragments were similar in molecular size to the fragments produced by papain. Papain is a cysteine proteinase that cleaves the heavy chain of antibody molecules in the hinge region (Smyth & Utsumi, 1967). The hinge region of immunoglobulins is the site which is most susceptible to proteinase cleavage because it is not folded into domains like the rest of the molecule. Therefore, adult flukes secrete an enzyme that is capable of cleaving IgG2a close to the papain cleavage site, that is, within the hinge region of the antibody heavy chain. Papain appears to have an additional cleavage site to that of the *F. hepatica* E/S proteinase, note the additional bands at approximately 22 kDa when the IgG molecule is cleaved with papain (Figure 3.1, lanes 3 and 5).

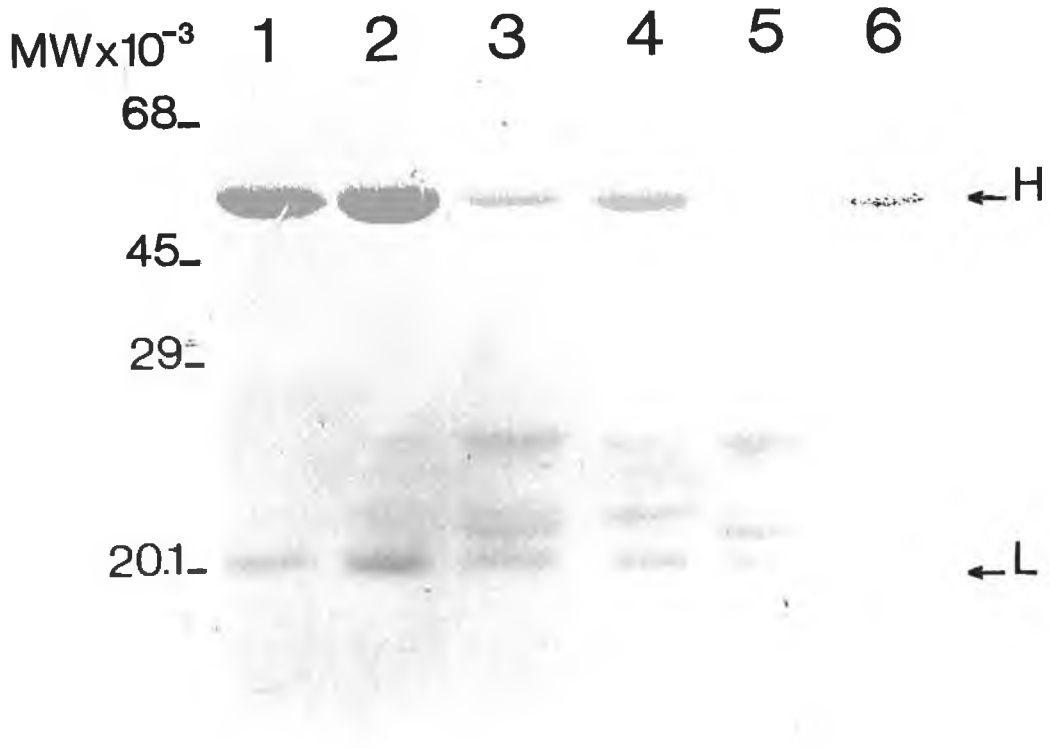


Figure 3.1

Cleavage of mouse monoclonal antibody IgG2a by papain and a cysteine proteinase in adult F. hepatica *E/S products. Fourteen µg of IgG2a were incubated with PBS (control, lane 1), adult fluke E/S products, 1.0, 2.0 and 5.0 µl (lanes 2, 4 and 6) and papain, 1.5 and 3 x 10^{-4} units (lanes 3 and 5 respectively). Cleavage fragments were analysed by 12% SDS-PAGE. H, IgG heavy chain; L, IgG light chain.*

3.1.2 HPLC analysis of E/S products.

To characterise the enzyme responsible for the IgG cleaving activity we subjected the adult fluke E/S products to size exclusion chromatography on HPLC. Analysis of the proteins eluted from HPLC yielded 3 major peaks of > 150 kDa (Peak I), 45 kDa (Peak II), and a broad peak ranging from 35 to <10 kDa (Peak III) (Figure 3.2A). A sample of each fraction was incubated with the IgG2a monoclonal antibody and the products of the reaction analysed by SDS-PAGE. The IgG2a cleaving enzyme was associated with Peak III (Figure 3.2B). We also tested each fraction for cathepsin proteinase activity using the synthetic fluorogenic peptide substrate Z-F-R-AMC. Cathepsin-like activity was also associated with Peak III (Figure 3.2A).

GS-PAGE analysis revealed that Peak III consisted of several enzymes with apparent molecular sizes ranging between 60 and 90 kDa (Figure 3.3A, lane 2). These enzymes correlated with the Group 1 cysteine proteinases described by Dalton & Heffernan, (1989).

3.1.3 Direct visualisation of proteinases in HPLC fractions.

In order to further characterise the proteinases in the apparent molecular size range 60-90 kDa we employed a method described by Robertson *et al.,* (1990), to directly visualise, using fluorogenic substrates different types of proteinases within polyacrylamide gels. GS-PAGE was carried out as normal, except that the gels were not incubated overnight, thereby avoiding digestion of the gelatin within the gel matrix by the proteinases. Instead they were washed immediately after electrophoresis was complete and then incubated for 10 to 30 min in fluorogenic substrate. Figure 3.3A shows GS-PAGE analysis of adult fluke E/S products and Peak III. Similar samples run on polyacrylamide

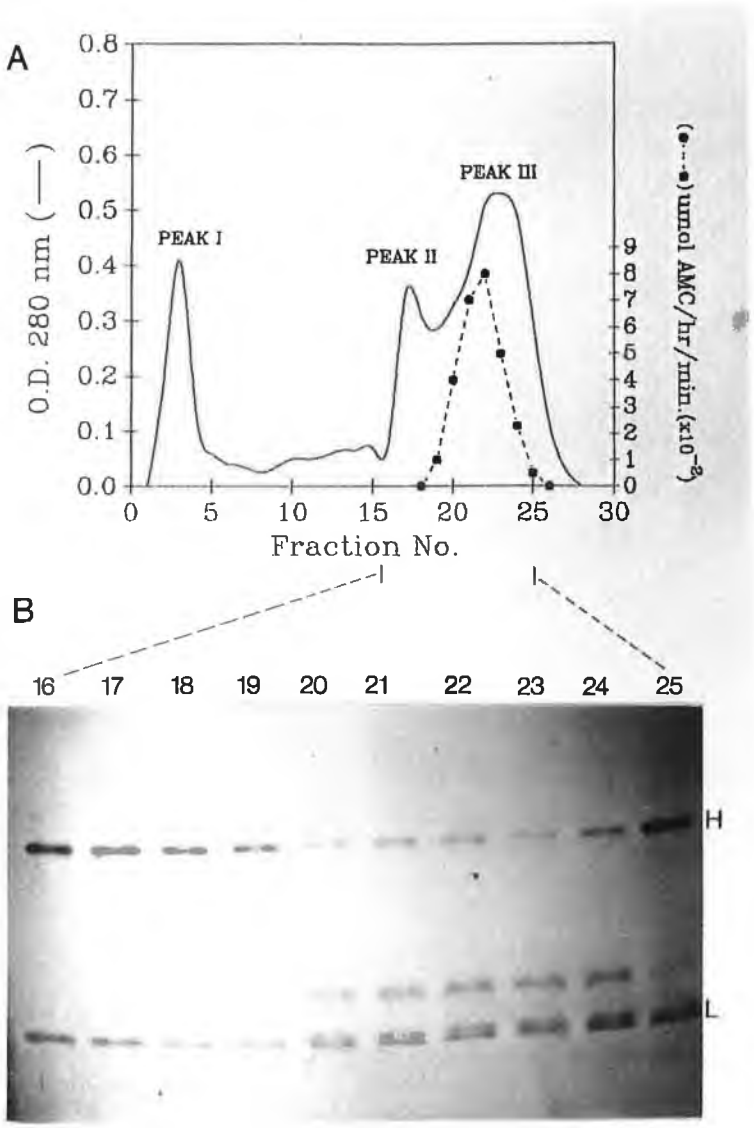


Figure 3.2

***A**. Size permeation HPLC analysis. Adult fluke E/S products (20 µg) were size separated on a TSK3000SW gel filtration column. Eluted proteins were monitored by absorbance at 280 nm. Each fraction (300 µl) was then assayed for cathepsin activity using the fluorogenic substrate Z-F-R-AMC.*

***B**. An aliquot (10 µl) of each fraction from HPLC size separation was mixed with monoclonal antibody IgG2a and the mixture was then analysed by 10% SDS-PAGE. L, IgG light chain, H, IgG heavy chain.*

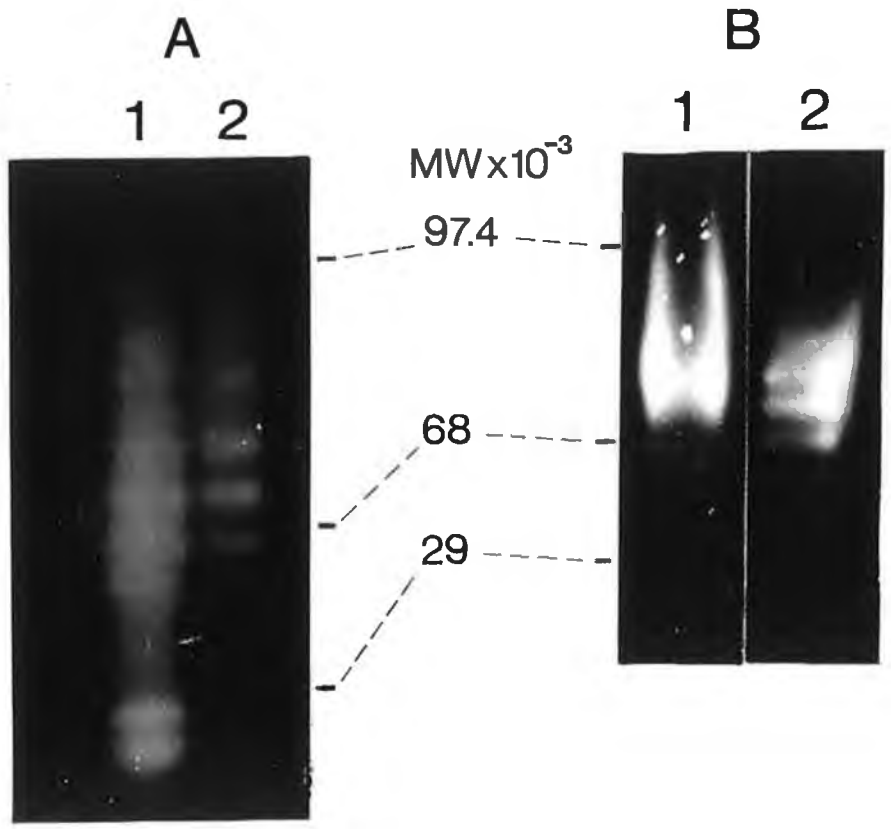


Figure 3.3

Analysis of cathepsin L-like proteinases of Peak III.

A. *Total adult fluke E/S products (lanes 1) and samples of HPLC Peak III (lanes 2) were analysed by GS-PAGE and proteinases visualised by staining with Coomassie Blue.*

B. *Proteinases reactive with specific peptide substrates were characterised by incubating gels, following electrophoresis, in the substrate Z-F-R-AMC for 10-30 min. Gels were then placed on a transilluminator and photographed.*

gels and overlayed with fluorogenic substrates revealed that all the enzymes in the 60-90 kDa size range cleaved the substrate Z-F-R-AMC (Figure 3.3B, lane 2). The enzymes were incapable of cleaving the fluorogenic substrates, Z-R-R-AMC and Z-R-AMC.

Each of the proteinases in the 60-90 kDa range showed identical substrate specificity. Of particular importance is the demonstration that these enzymes do not cleave the substrate Z-R-R-AMC, thus classifying these proteinases as cathepsin L-like rather than cathepsin B-like proteinases. The preference of this enzyme for the hydrophobic amino acid, phenylalanine, in the P_2 position of the substrate is typical of cathepsin proteinases.

3.2 Inhibition studies with DPC and Z-F-A-CHN_2.

3.2.1 Inhibition of the active site histidine residue with DPC.

Previous studies on cathepsin proteinases have shown that these enzymes contain a histidine residue in their active site (Barrett & Kirschke, 1980). To attribute the IgG2a cleaving activity in adult fluke E/S products to the cathepsin L-like enzyme, we carried out inhibition studies with DPC, a specific irreversible inhibitor of enzymes having histidines involved in their catalytic activity (Dickenson & Dickinson, 1975).

When DPC was incubated with samples from Peak III which contained the cathepsin L-like activity, it inhibited the enzymes' ability to cleave the substrate Z-F-R-AMC; greater than 30% inhibition of enzyme activity was observed at DPC concentrations of 1.5 mM and greater than 80% inhibition was observed at 12.5 mM DPC (Figure 3.4B). This study was carried out at pH 4.5, the optimal pH for Peak III activity (Figure 3.4A). Inhibition studies performed over a wide pH range, keeping the final DPC concentration at 25 mM, showed that

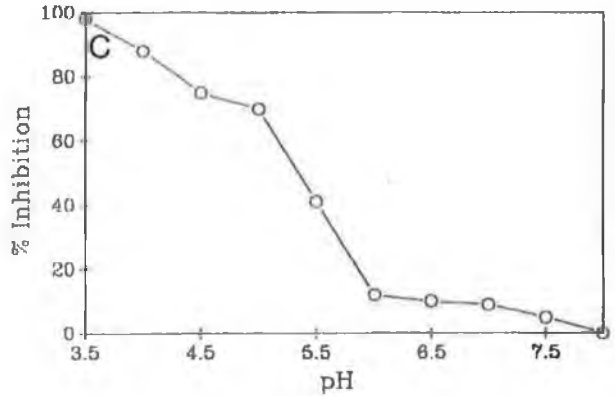
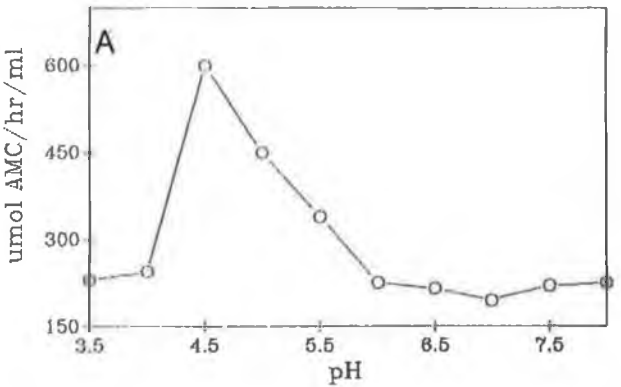


Figure 3.4


A
umol AMC/hr/ml
600
450
300
150
3.5
4.5
5.5
6.5
7.5
pH

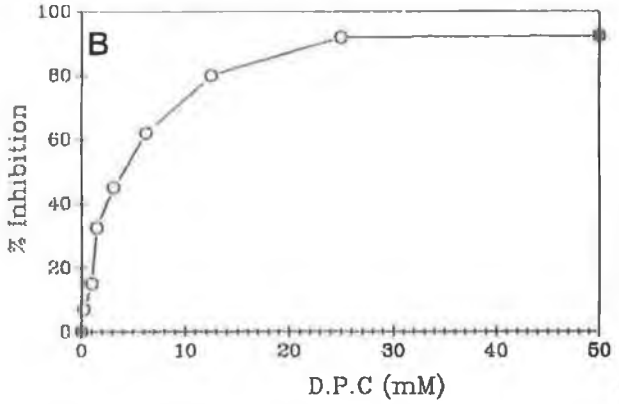

B
% Inhibition
100
80
60
40
20
0
0
10
20
30
40
50
D.P.C (mM)

Figure 3.4

***A**. Optimum pH for activity of the cathepsin L-like proteinase from HPLC Peak II was determined over a pH range of 3.5-8.0 using the fluorogenic substrate Z-F-R-AMC.*

***B**. Inhibition of cathepsin L-like activity by DPC. The ability of DPC to inhibit the activity of the HPLC Peak III proteinase on Z-F-R-AMC at pH 4.5 was tested over a final concentration range of 0.35-50 mM of inhibitor.*

***C**. DPC inhibition of cathepsin L-like activity from HPLC Peak III was investigated over a pH range of 3.5-8.0. The final concentration of inhibitor was 25 mM.*

this reagent was strongly inhibitory between pH 3.0 and 5.0 but had little effect on enzyme activity at pH 7.0 and none at pH 8.0 (Figure 3.4C).

DPC also inhibited the IgG2a cleaving ability of the enzymes in Peak III. This inactivation was not complete since the experiment was carried out at pH 7.3, a pH at which DPC is not very effective as an inhibitor (Figure 3.4C and Figure 3.5A).

To determine whether all the proteinases from 60 to 90 kDa were inhibited by DPC we incubated total adult fluke E/S products with DPC before application of samples to GS-PAGE. DPC inactivated only the proteinases in the apparent molecular size range 60-90 kDa in adult fluke E/S products (Figure 3.5B). This result indicates that all the cysteine proteinases between 60 and 90 kDa [Group 1 (Dalton & Heffernan, 1989)] have a histidine residue involved in their catalytic active site.

3.2.2 Inhibition with Z-F-A-CHN_2.

When samples of Peak III were mixed with 20 μmol of the specific inhibitor of cathepsin Ls and Bs, Z-F-A-CHN_2, prior to GS-PAGE analysis, all proteinases of 60-90 kDa were inhibited (Figure 3.5C, lane 2). This verifies that all the proteinases in the 60-90 kDa size range are cathepsin L-like cysteine proteinases.

3.3. Purification of the cathepsin L-like cysteine proteinase.

To characterise the cathepsin L-like proteinase further, larger quantities of purified enzyme were required. We used conventional purification techniques to obtain large amounts of purified proteinase from E/S products of *F. hepatica*.

The cathepsin L-like proteinase was purified to homogeneity from the *in*

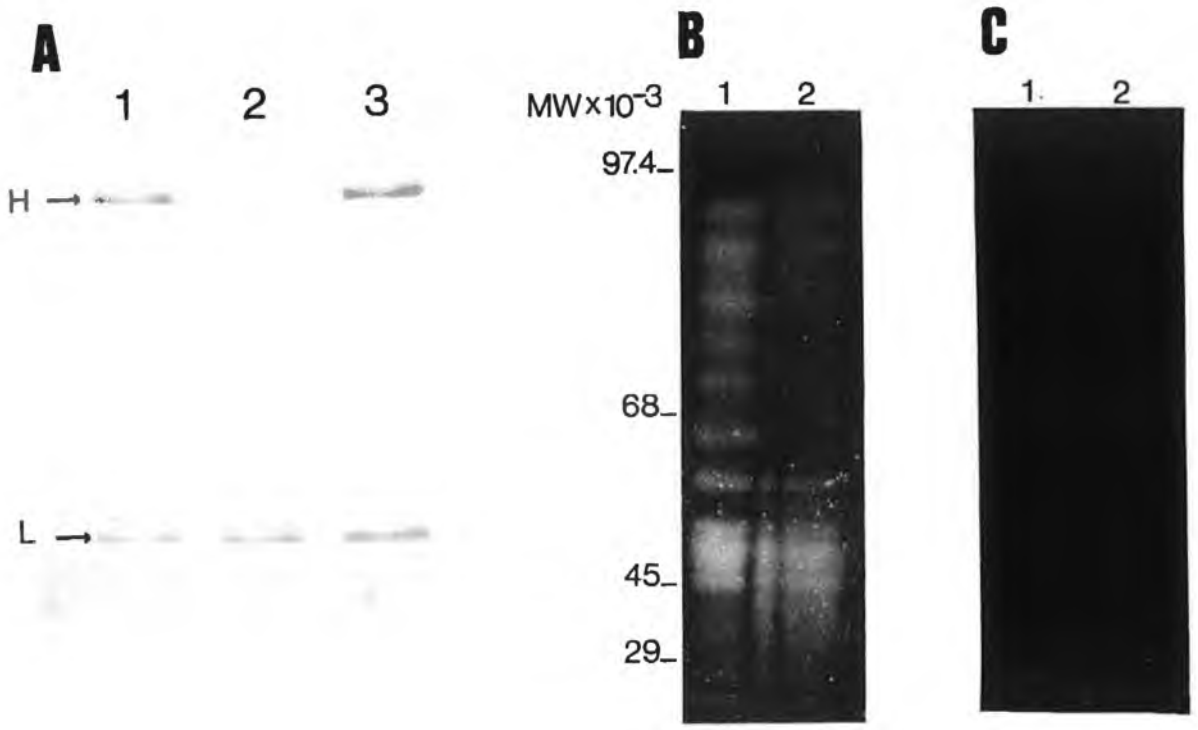


Figure 3.5

A. *DPC inhibition of IgG2a cleaving activity of the cathepsin L-like proteinase. Ten µg of IgG2a was incubated with PBS (lane 1), adult fluke E/S products (lane 2) and E/S products and 200 mM final concentration DPC (lane 3). H, Ig heavy chain; L, Ig light chain.*

B. *DPC inhibition of proteolytic bands in GS-PAGE. Adult fluke E/S products was incubated for 10 min with (lane 2) or without (lane 1) at a final concentration of 200 mM DPC.*

C. *Inhibition of the 60-90 kDa proteinases from HPLC Peak III with Z-F-A-CHN$_2$. Samples of Peak III were incubated with (lane 2) or without (lane 1) 20 µmol inhibitor prior to GS-PAGE analysis.*

vitro released products of adult flukes by a procedure involving two chromatographic columns. A large protein peak elutes at the void volume of the gel filtration column. A second large protein peak containing cathepsin L-like activity, determined using the synthetic peptide substrate Z-F-R-AMC, elutes in later fractions (Figure 3.6). These fractions contain both Group 1 and Group 2 cysteine proteinases (Dalton & Heffernan, 1989) as revealed by GS-PAGE (Figure 3.7A, lanes 1 and 2). When these fractions are pooled and passed over a QAE-Sephadex ion exchange column the Group 1 cysteine proteinases are separated from all other proteinases (Figure 3.7A, lanes 2 and 3); these proteinases do not bind to the ion exchange column at the running pH of 7.0 and are therefore collected in the unbound fraction. Analysis of this fraction by GS-PAGE reveals multiple proteinase bands in the apparent molecular size range 60-90 kDa (Figure 3.7A, lane 3). Coomassie blue staining of non-reducing SDS-PAGE also reveals multiple bands in the purified fraction (Figure 3.7B, lane 2); staining of reducing SDS-PAGE shows that these multiple bands are derived from a single protein of 27 kDa (Figure 3.7C, lane 2). The proteinase activity that remains bound to the column has been purified in our laboratory and has been reported recently by Dowd *et al.,* (1994a). The cathepsin L-like proteinase appears to be very labile throughout the purification, resulting in a low yield (12.1%) and purification (7.95-fold; Table 3.1). The final preparation has a specific activity of 69.2 U mg^{-1} and a K_m for the substrate Z-F-R-AMC of 14.7 μM.

3.4 N-terminal sequence determination.

In order to characterise the enzyme further, the purified proteinase was subjected to N-terminal sequencing (Figure 3.8). The amino acid sequence

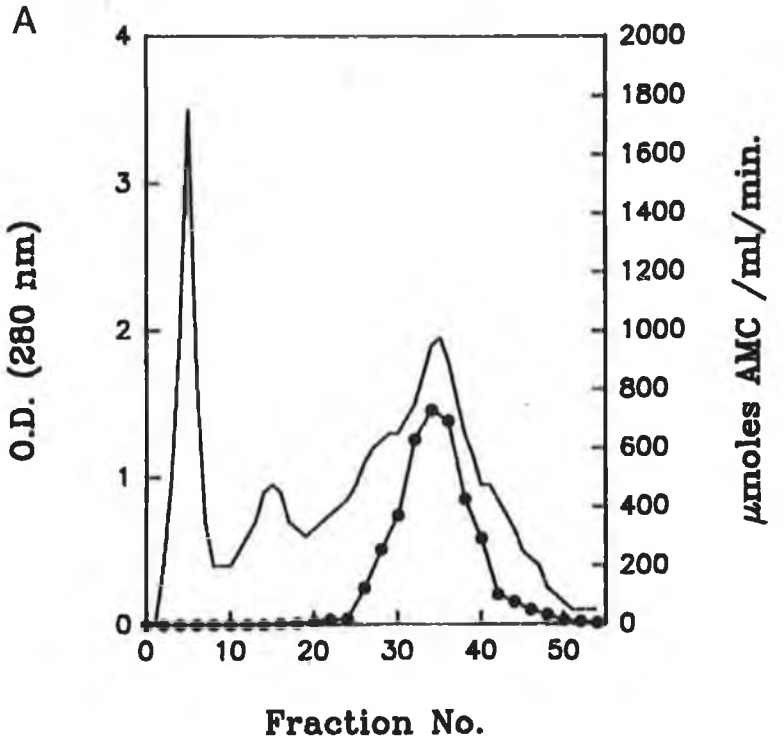


Figure 3.6

Purification of F. hepatica *cathepsin L-like proteinase. 25 mg of concentrated adult worm E/S products were separated on a 120 ml Sephacryl S-200 column. Five ml fractions were collected and monitored by absorbance at 280 nm for protein content. Each fraction was also assayed for cathepsin activity using the fluorogenic substrate Z-F-R-AMC. Fractions 25-42 were pooled and passed over a QAE-Sephadex ion exchange column (profile not shown) in order to separate any remaining Group 2 proteinases from the cathepsin L-like proteinase.*

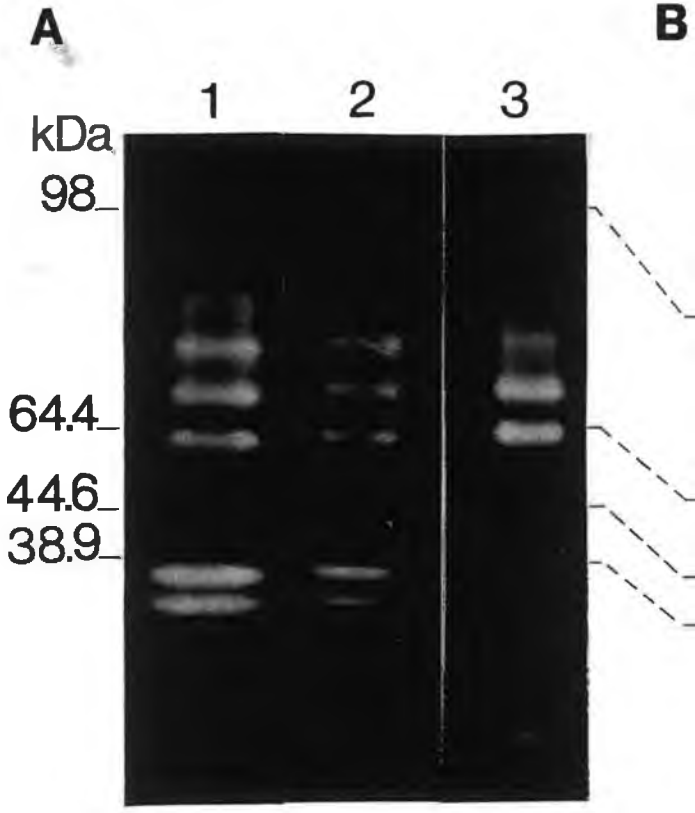


Figure 3.7

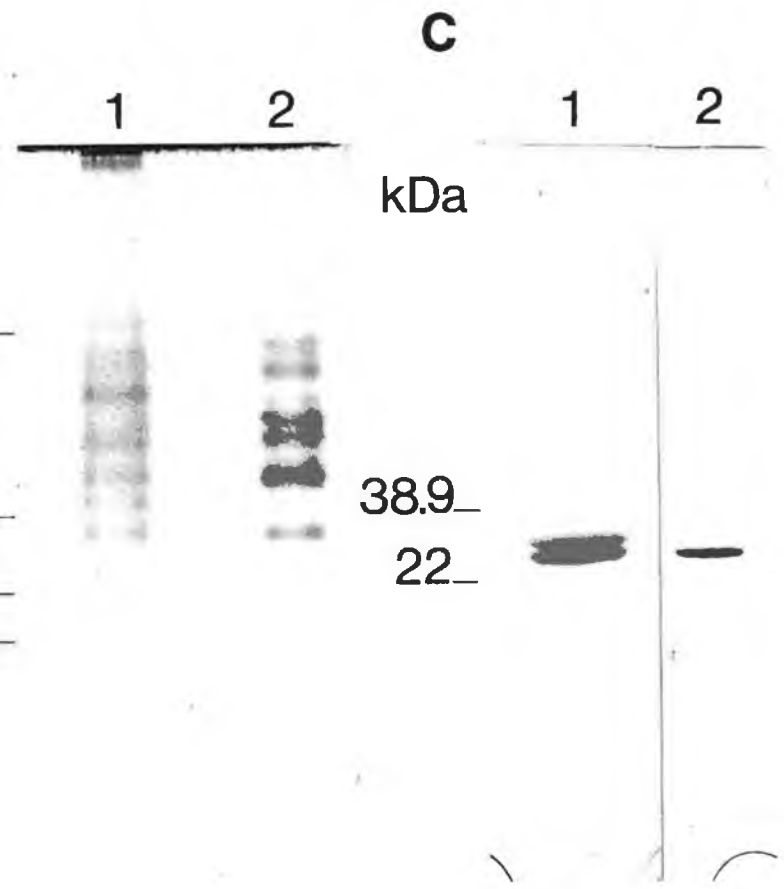

C
1
2
1
2
kDa
38.9
22

Figure 3.7

Electrophoretic analysis of purified cysteine proteinase.

***A**. GS-PAGE analysis of proteinases, in total adult fluke E/S products (lane 1), in the pooled fraction following gel filtration chromatography (lane 2) and the purified cathepsin L in the unbound fraction of the QAE Sephadex A-50 column (lane 3).*

***B**. Analysis of the adult fluke E/S products (lane 1) and QAE Sephadex unbound fraction (lane 2) by non-reducing SDS-PAGE demonstrating that under these conditions the cysteine proteinase migrates as several protein bands in the 60-90 kDa range.*

***C**. Analysis of adult fluke E/S products (lane 1) and QAE Sephadex unbound fraction (lane 2) by reducing SDS-PAGE reveals that the cysteine proteinase migrates as a single band of 27 kDa.*

Table 3.1

Purification of Fasciola hepatica *cathepsin L-like proteinase.*

	Protein (mg)	Enzyme activity Total (U)	Enzyme activity Specific (U mg^{-1})	Purification fold	Yield (%)
E/S products	460.0	400	0.87	1.0	100.0
Pooled gel filtration fractions	49.5	202	4.09	4.7	50.6
QAE run through	7.0	49	69.2	8.0	12.1

obtained, which was 20 residues long, was found to be most homologous with sequences in the N-terminal region of other known cathepsin Ls. In this region the liver fluke proteinase shows 63% identity with chicken liver cathepsin L (Wada & Tanabe, 1986), 58% with bovine (Turk *et al.,* 1985) and human (Mason *et al.,* 1986) liver cathepsin Ls, 53% identity with a cathepsin L from rat liver (Towatari & Katunuma, 1988) and a protozoan parasite *Trypanosoma cruzi* (Cazzulo *et al.,* 1989). A cathepsin B-like proteinase, Sm 31, has been identified in the related parasitic trematode, *Schistosoma mansoni* (Klinkert *et al.,* 1989). The liver fluke proteinase shows only 32% similarity to the Sm 31 molecule in this N-terminal region. One striking feature of the liver fluke cathepsin L-like proteinase sequence that is not found in the other sequences examined, is the additional alanine residue at the N-terminal end of the sequence.

3.5 Immunoblotting studies.

Immunoblot analysis was used to determine the specificity of rabbit antibodies raised against the purified cathepsin L-like proteinase from *F. hepatica* (Figure 3.9). Antibodies were raised in a white New Zealand rabbit. When this antisera was used to probe blots of adult fluke E/S products and purified enzyme separated under non-reducing conditions, the antibodies reacted with several polypeptides with a molecular size range between 60-90 kDa (Figure 3.9A, lanes 1 and 2). When identical samples were separated under reducing conditions and probed with the antisera, specific binding was observed with only one band of 27 kDa in both adult fluke E/S products and purified proteinase (Figure 3.9B, lanes 1 and 2). No reaction was observed with pre-immune sera (Figure 3.9A and B, lanes 3 and 4). These results

	-1	1	2	3	4	5	6	7	8	9	10			11	12	13	14			15	16	17	18	19
F. hepatica (C-L)	A	V	P	D	K	I	D	P	R	E	S			G	Y	V	T			G	V	K	D	Q
Chicken liver (C-L)	-	A	*	R	S	V	*	W	*	*	K			*	*	*	*			P	*	*	*	*
Bovine (C-L)	-	L	*	*	S	V	*	W	*	*	K			*	G	*	*			P	*	*	Y	*
Human liver (C-L)	-	A	*	R	S	V	*	W	*	*	K			*	*	*	*			P	*	*	N	*
Rat liver (C-L)	-	I	*	K	T	V	*	W	*	*	K			*	C	*	*			P	*	*	N	*
T. cruzi (c-p)	-	A	*	A	A	V	*	W	*	A	R			*	A	*	*			A	*	*	*	*
S. mansoni (Sm 31)	-	I	*	S	N	F	*	S	*	K	K	W	P	*	C	K	S	I	A	T	I	D	*	*

Figure 3.8

N-terminal amino-acid sequence of the purified cysteine proteinase of F. hepatica. *The determined sequence of 20 residues is shown compared to the N-terminal sequence of cathepsin Ls from chicken liver (Wada & Tanabe, 1986), bovine liver (Turk* et al., *1985), human liver (Gal & Gottesman, 1988), rat liver (Towatari & Katunuma, 1988),* Trypanosoma cruzi *cathepsin L like proteinase (Cazzulo* et al., *1989), and a cathepsin B purified from* Schistosoma mansoni *(Klinkert* et al., *1989). Identical residues are highlighted by the asterisks.*

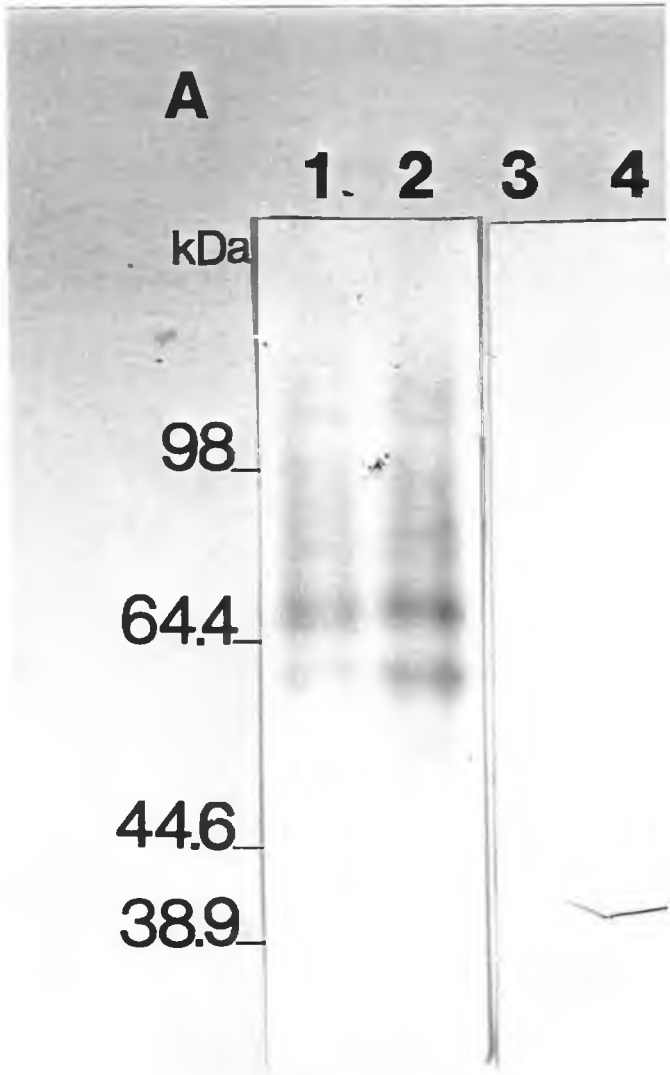


Figure 3.9

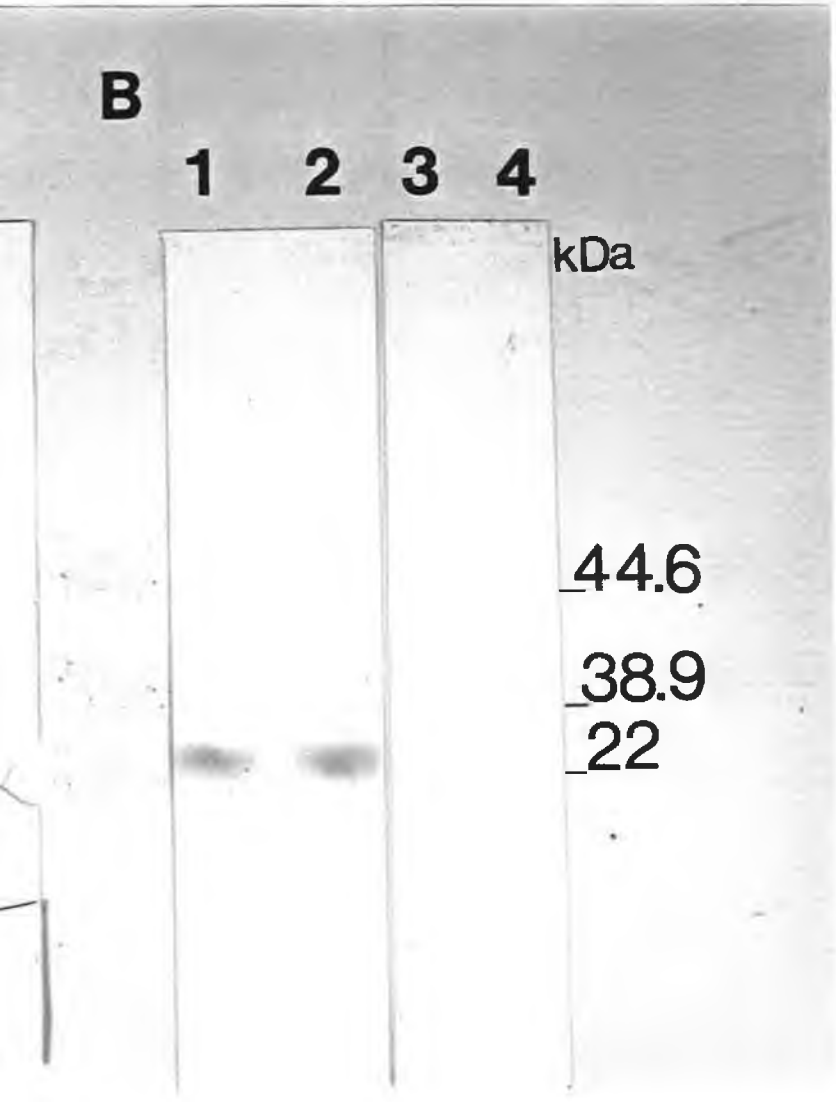

B
1
2
3
4
kDa
44.6
38.9
22

Figure 3.9

A. *Immunoblot analysis of adult fluke E/S products and purified cathepsin L-like proteinase under non-reducing SDS-PAGE conditions. Following transfer of electrophoretically separated adult fluke E/S products (lanes 1 and 3) and purified cathepsin L-like proteinase (lanes 2 and 4) to nitrocellulose, the filters were probed with anti-sera prepared against the purified enzyme (lanes 1 and 2) or control sera (lanes 3 and 4).*

B. *Immunoblot analysis of adult fluke E/S products (lanes 1 and 3) and purified cathepsin L-like proteinase (lanes 2 and 4) under reducing SDS-PAGE conditions. Nitrocellulose filters were probed with antiserum prepared against the purified cathepsin L-like proteinase (lanes 1 and 2) or with control sera (lanes 3 and 4).*

correlate with the data showing that the purified cathepsin L-like enzyme migrates as a single band on reducing SDS-PAGE (Figure 3.7C, lane 2) and as multiple bands between 60 and 90 kDa on non-reducing SDS-PAGE (Figure 3.7B, lane 2).

3.6 Light- and electron-microscope immunolocalisation studies.

Cathepsin L proteinases are located in the lysosomes of mammalian cells (Barrett & Kirschke, 1980). To determine the localisation of the *F. hepatica* cathepsin L-like proteinase prior to its excretion/secretion into the culture media immunolocalisation studies were carried out at the light- and electron-microscope levels.

Three μm sections of a resin-embedded liver from a *F. hepatica*-infected rat were prepared. Examination of methylene blue-stained sections reveals that migrating flukes within the liver cause extensive perforations in the tissue, with a consequential disruption of the liver architecture (Figure 3.10A). Anti-cathepsin L-like proteinase antibodies were used to probe sequential sections of these liver flukes. Antibody labelling was observed in the immune effector cells surrounding the migrating fluke, indicating that the cathepsin L is secreted and is taken up by these cells (Figure 3.10 B). Binding of these antibodies is confined to the epithelial cells of the fluke gut. Fluorescence is of a granular nature and appears to be restricted to vesicles (Figure 3.10C).

Immunolocalisation studies at the electron microscope level, using gold labelled antibodies, confirmed that the cathepsin L-like proteinase is packaged in vesicles within the gut epithelial cells (Figure 3.10D). No labelling of fluke tissues was observed, at the light and electron microscope level, when normal rabbit serum was used (data not shown).

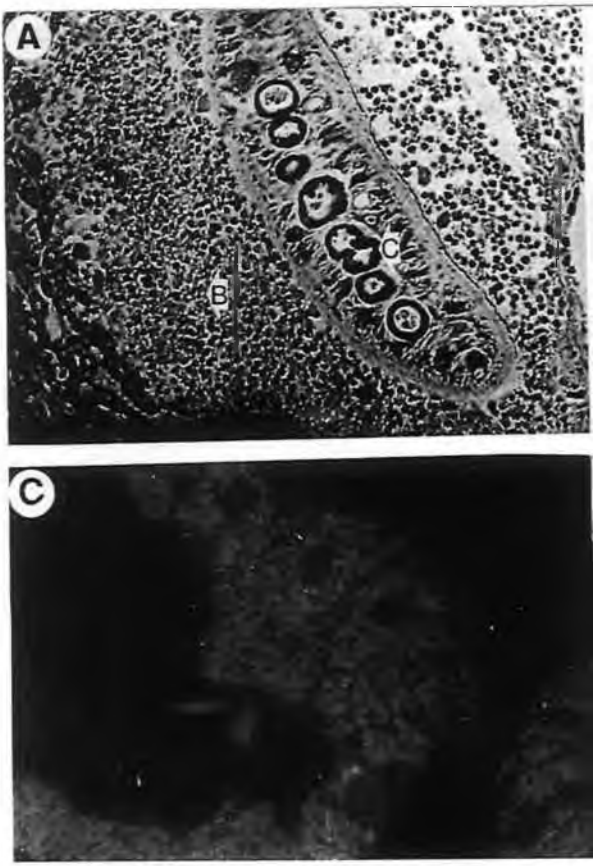
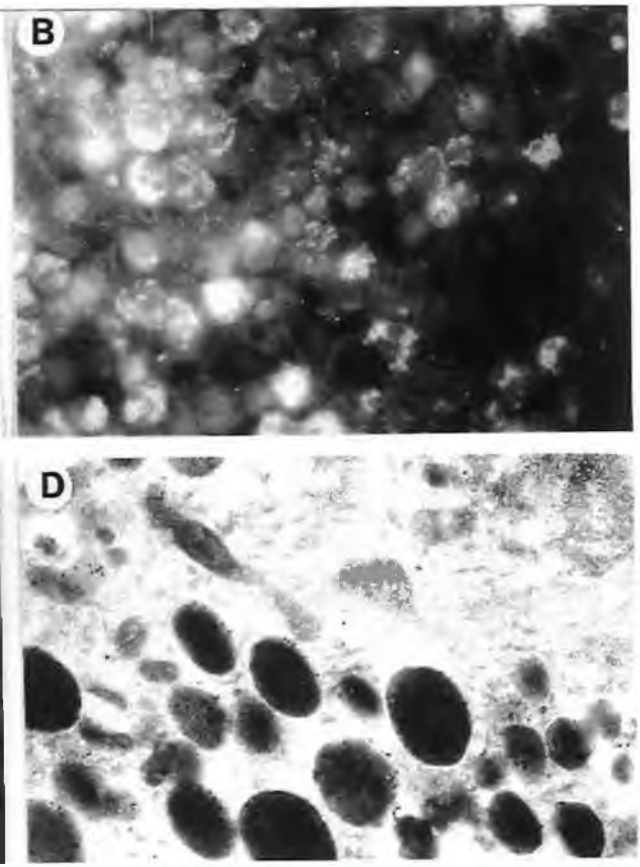


Figure 3.10


B

D

Figure 3.10

Immunolocalisation studies. Antiserum prepared against the purified cathepsin L-like proteinase was used to probe resin-embedded sections of liver fluke within the tissues of an infected rat host.

***A**. Methylene blue staining of sections of migrating fluke in liver mass.*

***B**. Immunostaining demonstrating that antibody labelling is located in immune effector cells surrounding the migrating fluke (indicated by B in panel A).*

***C**. Higher magnification of gut epithelial cells, immunostained with anti-cathepsin L-like proteinase serum, to demonstrate granular appearance of labelling.*

***D**. Immuno-gold labelling reveals that the cathepsin L-like enzyme is packaged in vesicles within the gut epithelial cells.*

3.7 Inhibition of proteinase activity with anti-cathepsin L-like proteinase antibodies.

The cathepsin L-like proteinase has been shown to be capable of cleaving antibody molecules within the hinge region (Figures 3.2B and 3.5A) and prevent antibody-mediated immune effector cell attachment to newly excysted juveniles *in vitro* (Carmona *et al.*, 1993). If the *in vivo* function of this cathepsin L-like proteinase is in the immune protection of the parasite while it migrates through the tissues of the host, blocking its activity should allow the host to overcome the infection. It would be of interest therefore, to demonstrate the inactivation of this cathepsin L-like proteinase by anti-cathepsin L antibodies.

3.7.1 Inhibition of GS-PAGE proteolytic activity.

Anti-cathepsin L IgG (2-20 μg) was mixed with 3 units of purified cathepsin L-like cysteine proteinase for 4 h at 4°C. The proteolytic activity of this mixture was then analysed by GS-PAGE. A decrease in the intensity of all of the bands from 60-90 kDa was observed in the presence of anti-cathepsin L antibodies. A reciprocal correlation was observed between the intensity of the proteinase bands and the quantity of IgG incubated with the enzyme (Figure 3.11A, lanes 1-8). No decrease in the intensity of any of the bands was observed when the cathepsin L proteinase was mixed with control IgG (Figure 3.11A, lane 9).

The observed decrease in proteinase activity indicates that the cathepsin L antibodies bind to and inhibit the activity of the proteinase in GS-PAGE gels in some manner. It is possible that the antibodies retard the proteinase within the polyacrylamide gels, a large protein band can be observed at the top of the gel lanes which may correspond to an antibody-proteinase complex. There is no breakdown of gelatin associated with this band, indicating that the proteinase

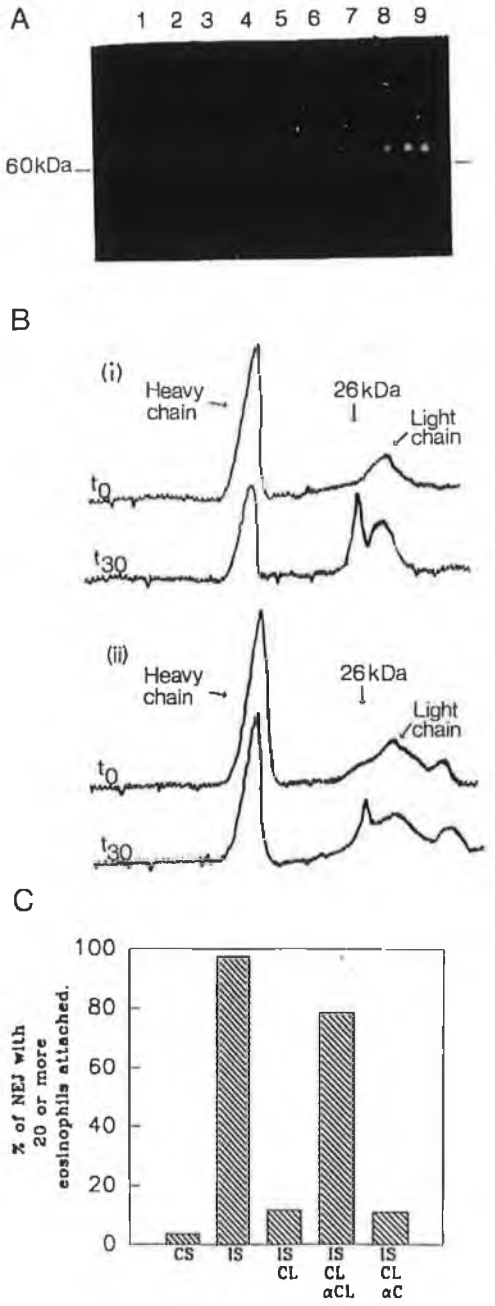

A
1 2 3 4 5 6 7 8 9
60kDa
B
(i)
Heavy chain
26kDa
Light chain
t0
t30
(ii)
Heavy chain
26kDa
Light chain
t0
t30
C
% of NEJ with 20 or more eosinophils attached.
100
80
60
40
20
0
CS
IS
IS CL
IS CL αCL
IS CL αC

Figure 3.11

Figure 3.11

***A**. GS-PAGE analysis of cathepsin L proteinase following incubation with anti-cathepsin L IgG. Three units of cathepsin L were incubated with varying amounts of anti-cathepsin L IgG (20-2 µg, lanes 1-8, respectively), or with 20 µg of non-immune rabbit IgG (lane 9) for 4h at 4°C. Samples were then analysed by GS-PAGE (Dalton and Heffernan, 1989).*

***B**. Neutralisation of cathepsin L activity by anti-cathepsin L IgG. Control rabbit IgG (i) and anti-cathepsin L IgG (ii) were incubated with 6 units of cathepsin L for 4 h at 4°C. The enzyme was then activated by the addition of 2-mercaptoethanol and EDTA and transferred to 37°C. Samples were removed at the beginning (t_0) and after 30 min (t_{30}) of the incubation and subjected to SDS-PAGE (Laemmli, 1970). The polyacrylamide gels were then analysed by densitometry.*

***C**. Antibody-mediated eosinophil attachment to juvenile flukes. Juvenile flukes were incubated in serum obtained from rats infected for five weeks with liver fluke (immune serum, IS) or control serum (CS) and 8 x 10^6 eosinophils at 37°C in wells of 24-well plates (Carmona* et al., *1993). To duplicate wells 6 units of purified cathepsin L (CL), with or without 55µg of anti-cathepsin L IgG (C-L) or control IgG (C) were added. Juvenile flukes were transferred to a slide after 2 h, examined at X40 and X100 magnifications, and the number of eosinophils attached to individual parasites counted. Those NEJ with more that 20 cells attached were scored as positive.*

is inactivated.

3.7.2 Inactivation of the IgG cleaving ability of the proteinase.

Six units of purified proteinase were incubated with 270 µg of either anti-cathepsin L IgG or control IgG at 4°C. After 4 h the enzyme was activated by the addition of reducing agents and transferred to 37°C for 30 min. The reaction was stopped by adding a cysteine proteinase inhibitor and the mixture was analysed by reducing SDS-PAGE. Densitometric scans of the gel demonstrate that when the purified cathepsin L-like proteinase is incubated with control IgG the heavy chain of the antibody is cleaved over time. This cleavage is observed by the gradual decrease in the height of the heavy chain peak and the corresponding gradual appearance of a peak at approximately 26 kDa representing the fragments produced by the cleavage of the heavy chain (Figure 3.11Bi). This result is consistent with our previous data showing that the cathepsin L-like proteinase cleaves immunoglobulin heavy chains within the hinge region (Figure 3.1). When purified enzyme is incubated with anti-cathepsin L IgG, comparatively little of the heavy chain is cleaved and only a minor peak at 26 kDa is observed (Figure 3.11Bii). These results were consistently repeated over a number of experiments.

3.7.3 Antibody-mediated eosinophil attachment to juvenile flukes.

Carmona *et al.*, (1993) demonstrated that eosinophils attach to the surface of NEJ in the presence of immune sera from an infected rat; however, the cathepsin L-like proteinase released *in vitro* by both mature and immature liver fluke can prevent this antibody-mediated attachment by cleaving the immunoglobulin molecule. In this study we investigate if antibodies specific to

the cathepsin L-like proteinase can inhibit the IgG cleaving action of the enzyme and thus allow the antibody-mediated attachment of effector cells to the surface of the NEJ.

Eosinophils are shown to bind to the surface of NEJ *in vitro* in the presence of serum from infected rats but not in the presence of normal rat sera (Figure 3.11C). Addition of purified cathepsin L-like proteinase to the assay prevents this antibody-mediated eosinophil attachment to the juvenile flukes (Figure 3.11C); however, purified cathepsin L-like proteinase which had been incubated with anti-cathepsin L IgG prior to addition to the assay, did not prevent eosinophil attachment to newly excysted juveniles. Purified cathepsin L-like proteinase incubated with control IgG remains active and hence eosinophil attachment does not occur (Figure 3.11C). These results were consistently repeated over several experiments.

3.8 Cloning and sequencing of PCR amplified cysteine proteinase gene fragments.

It has been shown in two previous studies that conserved structural motifs, identified by alignment of several members of both the serine and cysteine proteinase families, can be used to design generic molecular probes for amplification of serine and cysteine proteinase gene fragments using the polymerase chain reaction (Sakanari *et al.,* 1989; Eakin *et al.,* 1990). For the present study, we have used the same strategy to amplify fragments of the gene/genes encoding the cathepsin L-like proteinase of *F. hepatica.* Eakin *et al.,* (1990) designed two active site oligonucleotide primers based on the conserved sequences surrounding the cysteine and asparagine active site residues of cysteine proteinases. The gene fragment which is amplified

between these oligonucleotides is approximately 500 bp in length.

In this study we used the asparagine (antisense) primer as described by Eakin *et al.*, (1990), with a primer which we designed using the N-terminal sequence of the cathepsin L-like proteinase, in order to amplify the gene fragment encoding the cathepsin L-like enzyme (see Figure 2.1 in Section 2.2.19).

3.8.1 PCR amplification of cysteine proteinase gene fragments.

When the generic oligonucleotide primer for the cysteinyl active site sequence motif of cysteine proteinases (Eakin *et al.,* 1990), and the direct N-terminal sequence primer, from the cathepsin L-like proteinase of *F. hepatica,* were employed in PCRs using adult *F. hepatica* worm cDNA as template, a doublet at approximately 500-600 bp was observed, as judged by agarose gel electrophoresis (Figure 3.12, lane 2). Based on published cysteine proteinase sequences, this was the expected size of the cysteine proteinase gene fragments.

3.8.2 Subcloning and sequence analysis.

The PCR reaction was subcloned into the direct cloning vector Invitrogen pCR II, or Puc19. White colonies were picked and screened by PCR amplification with the original primers, and those containing inserts of the expected size were isolated. Two clones contained 500-600 bp inserts (Figure 3.12, lanes 3 and 4). Clone A was isolated from cells transformed with the ligation involving the Invitrogen direct cloning vector, and clone B was isolated from cells transformed with ligated Puc19. Both of these clones were characterised by DNA sequencing.

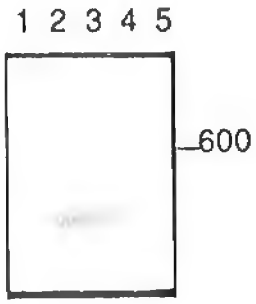


Figure 3.12

Analysis of gene fragments amplified by the polymerase chain reaction using generic cysteine proteinase primers with cDNA isolated from adult F. hepatica worms as template(lane 2). Twenty % (10 µl) of the total PCR reaction volume was analysed by 4% agarose-TAE gel electrophoresis using ØX174, digested with the restriction enzyme Hae III, as a marker (lanes 1 and 5). Subcloned cysteine proteinase gene fragments migrated as a band at approximately 500-600 bp (Clones A and B in lanes 3 and 4 respectively).

Figure 3.13 shows the alignment of the predicted amino acid sequence of Clones A and B with each other and with a spectrum of cysteine proteinase amino acid sequences; Sm 31 a cathepsin B-like proteinase from the related parasitic trematode *Schistosoma mansoni* (Klinkert *et al.,* 1989), papain from papaya (Drenth *et al.,* 1971), chicken liver cathepsin L (Dufour *et al.,* 1987), human liver cathepsin L (Gal & Gottesman, 1988), cruzain from *Trypanosoma cruzi* (Eakin *et al.,* 1992) and a cathepsin L-like cysteine proteinase isolated from *Fasciola* spp. (Yamasaki & Aoki, 1993).

For both the gene fragments obtained, regions of identity with other cysteine proteinases could be observed around the amino acids representing the active site residues as well as other structural motifs common to cysteine proteinases. Most notably the histidine residue at position 159, which is required for ion pair formation (Lewis *et al.,* 1981) with the active site cysteine, is present in both sequences. Also the glycine at position 66, which is involved in substrate binding in papain (Eakin *et al.,* 1990), as well as the buried acidic residues Glu^{35} and Glu^{50} are all conserved in the gene fragments isolated in this study. These glutamic acid residues are notably absent from the Sm 31 molecule which is consistent with other cathepsin B-like proteinases (Dufour, 1988). All the cysteine residues are present in the gene fragments, one being the active site cysteinyl residue (Cys^{25}) while the others are probably involved in disulphide bridges as in papain (Kamphuis *et al.,* 1984). Notably absent are potential N-linked glycosylation sites (NXS/T).

Analysis of sequence identity (Table 3.2) showed that the two *F. hepatica* cathepsin L-like cysteine proteinase gene fragments are 78% identical to each other.Both clones showed greater similarity to chicken liver cathepsin L (52% identity) than to any other protein sequence, and were only 26% identical to

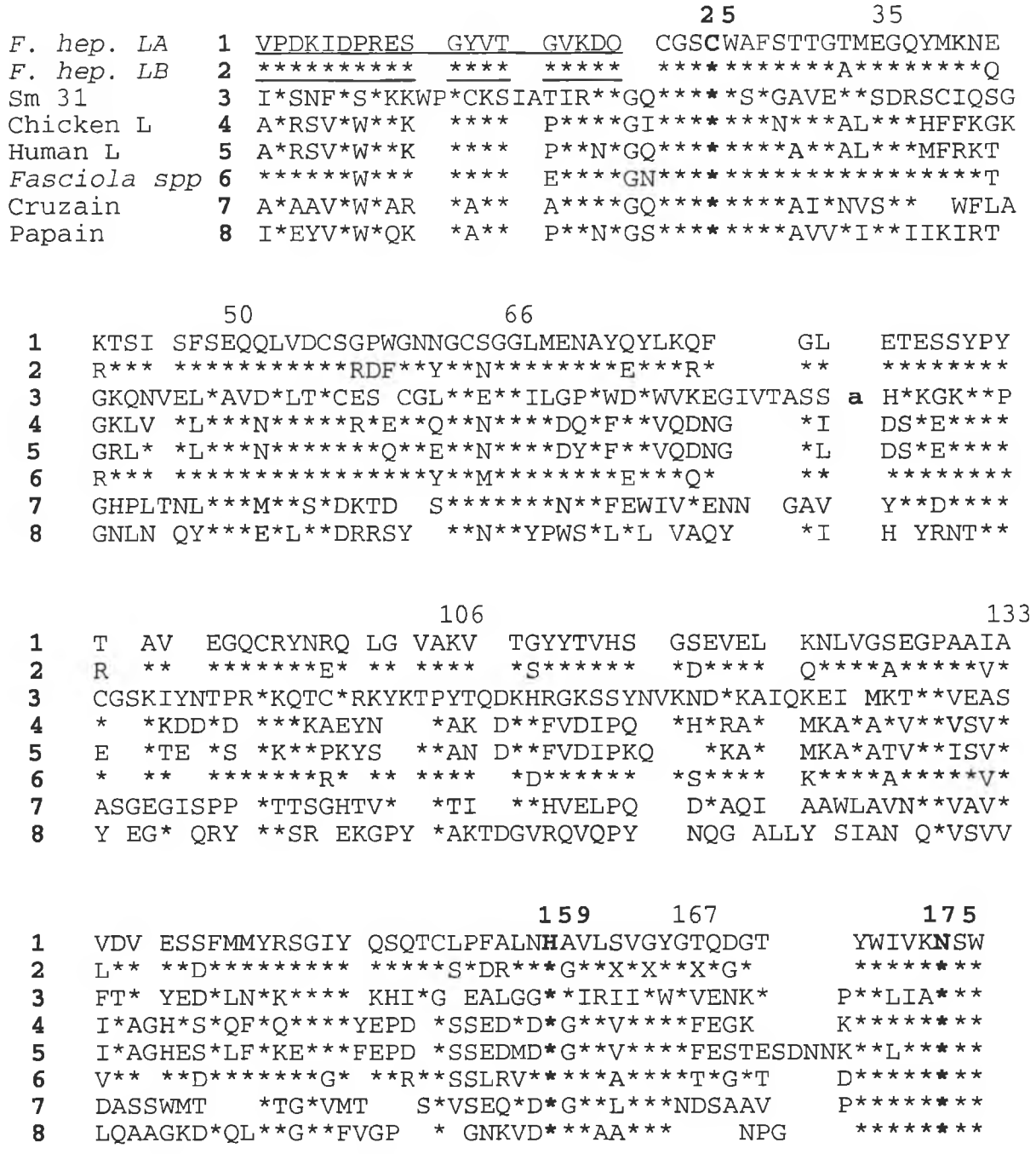

25 35
F. hep. LA 1 VPDKIDPRES GYVT GVKDQ CGSCWAFSTTGTMEGQYMKNE
F. hep. LB 2 ********** **** ***** ***********A********Q
Sm 31 3 I*SNF*S*KKWP*CKSIATIR**GQ*****S*GAVE**SDRSCIQSG
Chicken L 4 A*RSV*W**K **** P****GI********N***AL***HFFKGK
Human L 5 A*RSV*W**K **** P**N*GQ*********A**AL***MFRKT
Fasciola spp 6 ******W*** **** E****GN********************T
Cruzain 7 A*AAV*W*AR *A** A****GQ*********AI*NVS** WFLA
Papain 8 I*EYV*W*QK *A** P**N*GS*********AVV*I**IIKIRT
50 66
1 KTSI SFSEQQLVDCSGPWGNNGCSGGLMENAYQYLKQF GL ETESSYPY
2 R*** **********RDF**Y**N********E***R* ** ********
3 GKQNVEL*AVD*LT*CES CGL**E**ILGP*WD*WVKEGIVTASS a H*KGK**P
4 GKLV *L***N*****R*E**Q**N****DQ*F**VQDNG *I DS*E****
5 GRL* *L***N*******Q**E**N****DY*F**VQDNG *L DS*E****
6 R*** ***************Y**M********E***Q* ** ********
7 GHPLTNL***M**S*DKTD S*******N**FEWIV*ENN GAV Y**D****
8 GNLN QY***E*L**DRRSY **N**YPWS*L*L VAQY *I H YRNT**
106 133
1 T AV EGQCRYNRQ LG VAKV TGYYTVHS GSEVEL KNLVGSEGPAAIA
2 R ** *******E* ** **** *S****** *D**** Q****A*****V*
3 CGSKIYNTPR*KQTC*RKYKTPYTQDKHRGKSSYNVKND*KAIQKEI MKT**VEAS
4 * *KDD*D ***KAEYN *AK D**FVDIPQ *H*RA* MKA*A*V**VSV*
5 E *TE *S *K**PKYS **AN D**FVDIPKQ *KA* MKA*ATV**ISV*
6 * ** *******R* ** **** *D****** *S**** K****A*****V*
7 ASGEGISPP *TTSGHTV* *TI **HVELPQ D*AQI AAWLAVN**VAV*
8 Y EG* QRY **SR EKGPY *AKTDGVRQVQPY NQG ALLY SIAN Q*VSVV
159 167 175
1 VDV ESSFMMYRSGIY QSQTCLPFALNHAVLSVGYGTQDGT YWIVKNSW
2 L** **D********* *****S*DR***G**X*X**X*G* ********
3 FT* YED*LN*K**** KHI*G EALGG**IRII*W*VENK* P**LIA***
4 I*AGH*S*QF*Q****YEPD *SSED*D*G**V****FEGK K********
5 I*AGHES*LF*KE***FEPD *SSEDMD*G**V****FESTESDNNK**L*****
6 V** **D*******G* **R**SSLRV*****A****T*G*T D********
7 DASSWMT *TG*VMT S*VSEQ*D*G**L***NDSAAV P********
8 LQAAGKD*QL**G**FVGP * GNKVD***AA*** NPG ********

Figure 3.13

Figure 3.13

Alignment of the predicted amino acid sequences of the cloned PCR-amplified gene fragments, encoding F. hepatica *cysteine proteinases* (F. hep LA *and* F. hep LB), *with the amino acid sequences of a cathepsin B-like proteinase isolated (Sm 31) isolated from* S. mansoni *(Klinkert* et al., *1990), chicken liver cathepsin L (Dufour* et al., *1987), human cathepsin L (Gal & Gottesman, 1988), a cysteine proteinase isolated from* Fasciola spp. *(Yamasaki & Aoki, 1993), Cruzain isolated from* T. cruzi *(Eakin* et al., *1992), and papain from papaya (Drenth* et al., *1971). Asterisks denote the amino acid residues which are identical to their corresponding residue in the deduced amino acid sequence of the gene fragment* F. hep LA, *X denotes an undetermined residue, and gaps are introduced for best fit. The underlined sequence of* F. hepatica *clones A and B corresponds to the N-terminal sequence of secreted* F. hepatica *cathepsin L-like proteinase, purified from adult worm E/S products. The active-site residues based on those in the enzyme papain, cys^{25}, his^{159} and asn^{175}, are shown by* ***C***, ***H*** *and* ***N*** *respectively. The unconserved loop present in the Sm 31 molecule was omitted in order to conform to the papain numbering system (Drenth et al., 1971), so that a=KENHTGCEPYPFPKCEH.*

Table 3.2

Percent identity of predicted amino acid sequences of F. hepatica *cathepsin L-like cysteine proteinase gene fragments A and B with the corresponding sequences of other known cysteine proteinases (Sm 31, Klinkert* et al., *1990; chicken cathepsin L, Dufour* et al., *1987; human cathepsin L, Gal & Gottesman, 1988;* Fasciola *sp. proteinase, Yamasaki & Aoki, 1993; Cruzain, Eakin* et al., *1992; Papain from papaya, Drenth* et al., *1971).*

	F. hepatica (Clone A)	*F. hepatica* (Clone B)
F. hepatica A	-----	78%
F. hepatica B	78%	-----
Sm 31	26%	26%
Chicken L	52%	52%
Human L	49%	49%
Fasciola spp.	88%	85%
Cruzain	37%	37%
Papain	33%	33%

the cathepsin B-like molecule isolated from *S. mansoni*. The percent identity values were calculated as the number of identical amino acids per total number of amino acid residues, allowing gaps in the sequence to achieve the best alignments. This data clearly demonstrates that we have cloned cathepsin L-like gene fragments from *F. hepatica*.

CHAPTER FOUR
DISCUSSION

4.0 DISCUSSION.

Howell (1966), demonstrated that immature *Fasciola hepatica* release enzymes *in vitro* that are capable of cleaving collagen and postulated that *in vivo* these enzymes are involved in the penetration of the liver tissue.
In a study by Dalton & Heffernan, (1989), it was shown that when immature and mature *F. hepatica* were maintained in culture for 16 hours they released proteolytic enzymes, and they speculated that these enzymes were important in the feeding and migration of the parasite. Eleven proteinases were observed as distinct bands in gelatin-substrate SDS-PAGE. All of the proteinases observed in the GS-PAGE gels were classified as thiol or cysteine proteinases due to their inactivation by thiol proteinase inhibitors and their enhanced activity in the presence of reducing agents. The proteinases were divided into two groups based on the pH range in which they were most active (Group 1, comprising the proteinases in the molecular size range of 60-90 kDa, and being most active at pH 3.0-4.5, and Group 2, with those proteinases in the 27.5-50 kDa molecular size range and having a pH optimum in the range 4.5-8.0) (Dalton & Heffernan, 1989).

Chapman & Mitchell, (1982) showed that the *in vitro*-released products of *F. hepatica* contain a proteinase(s) that cleave immunoglobulin G into Fab and Fc fragments in a cathepsin B or papain like manner. Their characterisation of this activity was based on examining the effects of various cysteine proteinase enhancers and inhibitors, on the ability of total immature fluke E/S products to digest immunoglobulin and other substrates such as hemoglobin and casein. They suggested that these enzymes may prevent antibody activating effector functions, such as complement fixation, in the vicinity of the migrating fluke,

affording them some protection from immune attack. The IgG cleaving activity in the *in vitro* released products was characterised as a thiol or cysteine proteinase with a pH optima of 3.5-4.5. It is likely that this immunoglobulin cleaving proteinase corresponds to one or possibly all of the thiol proteinase activities identified in the Group 1 cysteine proteinases as described by Dalton & Heffernan, (1989).

In this study we first focused our attention on the proteinases involved in the cleaving of immunoglobulin because of their obvious implied role in protecting the parasite against host immune attack. This study is confined to adult *F. hepatica* E/S products although this enzyme is also present in immature fluke E/S products (Dalton & Heffernan, 1989; Carmona *et al.,* 1993).
When a purified monoclonal antibody IgG2a was incubated with total adult fluke E/S products a very specific cleavage of the antibody molecule occurs close to the papain cleavage site within the antibody hinge region. Our analysis by SDS-PAGE (Figure 3.1) revealed that incubation of the proteinase papain with the immunoglobulin molecule resulted in the cleavage of the 50 kDa heavy chain into 4 fragments in the region 22-28 kDa. This is the expected result since papain has two cleavage sites within the hinge region of the heavy chain of IgG molecules, that are very close to one another (Smyth & Utsumi, 1967). SDS-PAGE analysis of the IgG molecule cleaved by E/S products showed only two distinct bands, at 22 kDa and 28 kDa (Figure 3.1, lanes 2, 4 and 6), indicating that the proteinase in the E/S products responsible for the IgG cleavage has only one specific hydrolysing site within the hinge region of the heavy chain of the immunoglobulin molecule.

We suspected that the actual number of proteinases released *in vitro* by *F. hepatica* might have been less complicated than that indicated by GS-PAGE (Dalton & Heffernan, 1989), because it was surprising that all of the proteinases observed in these gels were cysteine proteinases.

We decided to subject the E/S products, harvested from adult worms, that had been isolated from the bile ducts of infected cattle, to molecular size separation by HPLC. Three major protein peaks were eluted from this column. Peak I protein(s), showing a molecular mass >150 kDa, did not contain proteolytic activity when analysed on a (0.1%)-gelatin substrate (10%)-polyacrylamide gel. When this peak was analysed for IgG cleaving activity, none was observed. This major protein peak may contain an as yet unidentified proteolytic activity. This may be because the protein was unstable in the presence of SDS or it could also prove to be a proteinase which can digest gelatin, but not to components small enough to diffuse from the gel. Gelatin substrate SDS-PAGE analysis of protein Peak II (at 45 kDa approximately) revealed several proteolytic bands in the molecular size range of 25-50 kDa that corresponded to the Group 2 enzymes as identified by Dalton & Heffernan, (1989) that had a pH optima in the range 4.5-8.0. Fractions of this peak were also analysed for IgG cleaving activity but none was detected (Figure 3.2B).

When Peak III was subjected to GS-PAGE several proteolytic bands between 60 and 90 kDa were observed (Figure 3.3A). These proteinases correlated with those described by Dalton & Heffernan, (1989) as Group 1 proteinases which showed optimal activity in the pH range of 3.0-4.5. Since all of the bands that are observed on the GS-PAGE gels by Dalton & Heffernan, (1989) appear to be represented by only Peaks II and III after HPLC

separation, this lead us to conclude that maybe there were only two proteinases (one of 45 kDa and the other of 30 kDa approximately) that were responsible for the breakdown of gelatin by E/S products in gelatin substrate polyacrylamide gels.

The reason why the proteinases migrate as multiple bands in GS-PAGE is unclear. A possible explanation may be that these enzymes undergo proteolytic breakdown (including autoproteolysis) in the presence of SDS giving rise to several smaller, but still active, fragments. Alternatively electrophoresis, in non-reducing conditions, of the proteinases may cause them to act uncharacteristically, particularly in the presence of SDS. This unusual phenomenon may be due to aggregation of the proteinases. It may also be possible that the proteinases bind to the gelatin while travelling down the gel resulting in retardation and the formation of a ladder-like pattern. However, this is unlikely since the same pattern of proteinase activity is observed repeatedly with a number of different E/S preparations.

Detection of thiol proteinases is not biased by GS-PAGE; in other studies using this method, serine proteinases of *Plasmodium falciparum* (Rosenthal *et al.,* 1987) and metalloproteinases of *Strongyloides stercoralis* (McKerrow *et al.,* 1990) have been identified. However this method is selective since it allows the detection only of those proteinases that are stable in the presence of SDS. Hence, it is also possible that *F. hepatica* releases other proteinases that are not detected by this particular method.

When fractions of protein Peak III from the HPLC separation were incubated with a monoclonal IgG2a molecule in the presence of mercaptoethanol and EDTA, and analysed by SDS-PAGE we observed digestion of the IgG heavy chains into two fragments of 28 and 22 kDa (Figure 3.2B). Hence the IgG

cleaving activity in the adult *F. hepatica* E/S products is associated with HPLC Peak III.

We performed several experiments to demonstrate that the immunoglobulin cleaving enzyme in E/S products is a cathepsin-like cysteine proteinase. We used Z-F-R-AMC to detect cathepsin-like activity. Fractions from the HPLC analysis were incubated with the synthetic fluorogenic substrate Z-F-R-AMC. Both cathepsin Ls and cathepsin Bs can cleave this substrate. The proteinases hydrolyse the peptide bond between the arginine residue and the amino methyl coumarin leaving group; the free AMC can than be detected using a fluorometric spectrophotometer (Barrett & Kirschke, 1980). Cathepsin-like activity was associated with the Peak III protein only (Figure 3.2A). To classify the proteolytic activity associated with Peak III, as either cathepsin L- or B-like in its properties, we employed the technique of overlaying GS-PAGE gels, following electrophoresis, with different fluorogenic substrates (Robertson *et al.*, 1990). Three synthetic peptide substrates were used: Z-R-AMC, which is cleaved only by cathepsin H-like proteinases; Z-R-R-AMC, which is hydrolysed by cathepsin B-like proteinases only and Z-F-R-AMC which is cleaved by both cathepsin L-like and cathepsin B-like proteinases but is preferentially hydrolysed by cathepsin L-like proteinases (Barrett & Kirschke, 1980). Analysis of the Peak III group of proteinases by this direct visualisation technique confirmed that all the enzymes in the 60-90 kDa size range were capable of cleaving Z-F-R-AMC, but did not hydrolyse either the Z-R-AMC or Z-R-R-AMC substrates (Figure 3.3B). This preference for the hydrophobic amino acid phenylalanine in the P_2 position is typical of cathepsin L-like proteinases (Barrett & Kirschke, 1980).

Inhibition studies were carried out to confirm the cathepsin-like nature of the

Peak III proteinase. Cathepsin-like proteinases (like all cysteine proteinases of the papain superfamily) contain an essential histidine residue in their active sites (Barrett & Kirschke, 1980). DPC is a an active site modifying agent which acts as a specific irreversible inhibitor of enzymes having histidines involved in their catalytic site (Dickenson & Dickinson, 1975). The DPC molecule forms an acyl-enzyme intermediate in which the acyl group is covalently bound to the histidine residue in the catalytic site. Modified active sites are unable to form the normal stable enzyme-substrate complex and hence the enzyme is inactivated. When a pooled sample of the Peak III fractions from the HPLC column was incubated with DPC, inhibition of the proteinase activity in the test-tube assay with Z-F-R-AMC (Figure 3.4B), IgG2a cleaving ability as analysed by SDS-PAGE (Figure 3.5A) and GS-PAGE activity (Figure 3.5B) was observed.

In the next series of experiments the diazomethyl ketone Z-F-A-CHN_2 was used. This reagent is a potent irreversible inhibitor of both cathepsin L-like and cathepsin B-like proteinases, but not other classes of proteinases or other subclasses of cysteine proteinases (Rosenthal *et al.,* 1989); however Z-F-A-CHN_2 inhibits cathepsin Ls more potently than cathepsin Bs (Barrett *et al.,* 1982). Peptidyl diazomethyl ketones are specific inactivators of thiol or cysteine proteinases being unreactive toward other classes of proteinases. They are unreactive with free cysteine, but react irreversibly with cysteine proteinases. A high degree of specificity for individual cysteine proteinases has been obtained by the use of appropriate peptide sequences (Green & Shaw, 1981; Barrett *et al.,* 1982). Diazomethyl ketones form a stable covalent bond with the sulphur atom of the active site cysteine-25 residue (numbering according to papain see Drenth *et al.,* 1971), this orients the inhibitor such that

alkylation of the cysteine residue may take place. Alkylation converts the free sulphydryl groups of the cysteine residues to stable S-carboxymethylcysteine residues and thus inactivates the enzyme (Drenth *et al.,* 1976). Z-F-A-CHN_2 inhibited all the proteolytic bands from 60-90 kDa in Peak III as observed on GS-PAGE (Figures 3.5B and 3.5C).

In summary, all the enzymes in Group 1 (60-90 kDa, Dalton & Heffernan, 1989) elute as a single peak on HPLC chromatography. The enzyme activity in this peak is capable of cleaving immunoglobulin molecules, has a pH optima of 4.5 and hydrolyses the synthetic fluorogenic substrate Z-F-R-AMC. They are all inhibited by DPC and Z-F-A-CHN_2 and therefore are cathepsin L-like in their cysteine proteinase activity.

The proteolytic enzymes identified by Dalton & Heffernan, (1989), and shown here to be responsible for IgG cleavage represent good target candidates for immuno- or chemotherapy of fascioliasis since inhibition of their activity may interfere with parasite feeding and migration. If as suggested by Chapman & Mitchell, (1982), these proteinases do play a role in immune evasion by inactivating host antibody in the vicinity of the migrating fluke, then a successful vaccine may be one that would induce high titres of anti-proteinase antibodies. The high levels of protection against infection consistently observed in rats immunised with immature fluke *in vitro* -released products (Rajasekariah *et al.,* 1979) may be mediated by antibody inactivation of parasite proteinases. Knox & Kennedy, (1988) showed that antibodies prepared against proteinases released by *Ascaris suum* could bind to and inactivate these enzymes.

Purification of the proteinase(s) responsible for the IgG cleavage in an

active form would allow characterisation of the enzyme(s), as well as the investigation of the potential of such a molecule(s) as a vaccine candidate. Purification by HPLC was inadequate due to the small quantity of proteinase that was obtained from a single run. The starting material i.e. adult fluke E/S products, was readily available; hence a purification protocol that required a larger volume of E/S products and would yield a greater amount of purified cathepsin L-like proteinase was required.

The cathepsin L-like cysteine proteinase was purified to homogeneity, from the E/S products of adult *F. hepatica* isolated from the bile ducts of infected cattle, by a scheme employing gel filtration and ion exchange columns (Figure 3.6 and Table 3.1). A rapid and simple purification of the enzyme was achieved by (a) using the same mobile phase for both gel filtration and ion exchange columns, thereby avoiding concentration steps and buffer changes between columns; and (b) performing the ion exchange chromatography at pH 7.0 at which the cathepsin L-like proteinase does not bind to the matrix. Other cysteine proteinases adhere to the column and are thus separated from the cathepsin L-like enzyme. These other proteinases have been purified and characterised in our laboratory by Dowd *et al.,* (1994a).

The *F. hepatica* cathepsin L-like proteinase appears to be very labile throughout the purification process resulting in a low yield (12.1%), this is despite the fact that all of the chromatography and concentration procedures were carried out at 4°C. Mature cathepsin L has been reported as not been stable for very long at neutral pH (Mason *et al.,* 1985; Dufour *et al.,* 1987). This low yield is comparable with that achieved for the cysteine proteinase of *Trypanosoma cruzi* (8%, Cazzulo *et al.,* 1989), and is considerably better than that obtained for both the human liver (5.1%, Mason *et al.,* 1985) and rat kidney

(2.7%, Bando *et al.,* 1986). The purification-fold of the cathepsin L-like proteinase of this study is low (7.95) in comparison with those of both human liver (3565, Mason *et al.,* 1985) and rat kidney (655, Bando *et al.,* 1986) but again is comparable with that of the cysteine proteinase isolated from *T. cruzi* (16.2, Cazzulo *et al.,* 1989). As can be seen from reducing SDS-PAGE analysis of the E/S products of adult liver fluke worms (Figure 3.7C), the cathepsin L-like cysteine proteinase is one of only two major protein bands in the culture medium. The former proteinases (human liver and rat kidney cathepsin Ls) were isolated from whole tissue homogenates, a source in which you would expect to find a lot more contaminating molecules than are present in the E/S products of *F. hepatica,* and hence such enzymes would require a longer and more intensive purification procedure than that necessary for the cathepsin L-like proteinase of *F. hepatica.*

The apparent molecular size of the fluke cathepsin L-like enzyme is 27 kDa, as determined by SDS-PAGE under reducing conditions (Figure 3.7C), which is consistent with the size of other cathepsin Ls (Wada & Tanabe, 1986; Turk *et al.,* 1985; Mason *et al.,* 1986); however, the enzyme migrates as several bands ranging from 60-90 kDa when analysed by non-reducing SDS-PAGE, and each of these bands is capable of digesting gelatin (Figures 3.7A and 3.7B). Antibodies prepared against the purified enzyme bind to the 27 kDa reduced form of the enzyme and to each of the molecules in the 60-90 kDa region that represent the non-reduced form (Figure 3.9).

These bands in the 60-90 kDa region have been previously described as a single group of cysteine proteinases (Group 1, Dalton & Heffernan, 1989), because all are inhibited by leupeptin, E-64 and iodoacetamide and have a similar pH optimum. Therefore, we have shown that these Group 1 enzymes

(a) can be resolved as a single protein band on reducing SDS-PAGE (Figure 3.7C); (b) can be purified to homogeneity using gel filtration and ion exchange columns; (c) hydrolyse the synthetic peptide substrate Z-F-R-AMC (Figure 3.2A); (d) are capable of cleaving IgG molecules in a manner similar to papain (Figures 3.1 and 3.2B); (e) have a pH optimum for activity, at 4.5 (Figure 3.4A); and (f) are all inhibited by DPC and Z-F-A-CHN_2 (Figures 3.5B and 3.5C).

It is now clear that these multiple bands are representative of a single proteinase activity that is a cathepsin L-like cysteine proteinase. It is not known whether some form of aggregation takes place in the presence of detergent (although the aggregates are not multimers of 27 kDa) or if the native protein behaves aberrantly during electrophoresis under non-reducing conditions therefore giving rise to the multiple banding pattern which is consistent with every preparation of E/S products and purified proteinase of *F. hepatica*.

In order to confirm the identification of this proteinase as a cathepsin L-like enzyme, the N-terminal sequence of the protein was determined, and then aligned with sequences of other known cysteine proteinases. When comparing the amino acid sequences of cysteine proteinases, the sequences can be arbitrarily divided into three regions (Baker, 1980): an amino terminal (or active-site cysteinyl) region, a central one and a carboxyl-terminal (or active-site histidyl region). All the sequences in the N-terminal region contain the cysteine-rich site, where Cys^{25} (numbering according to papain, Drenth *et al.*, 1971) is the active site cysteine residue. In this region (N-terminal) homologies between different cysteine proteinases are the highest (Dufour, 1988). Hence, by knowing the N-terminal sequence of the cathepsin L-like proteinase released *in vitro* by *F. hepatica* we would be able to confirm its classification by

comparing the amino acid sequence with sequences of other known cathepsin Ls.

Comparison of the first 20 N-terminal amino acids obtained for our purified enzyme with that of other cysteine proteinases, identified the *F. hepatica* enzyme as having greater similarity to other cathepsin L proteinases than to any other protein sequences in the database (Genbank). Thus the cysteine proteinase released *in vitro* by *F. hepatica* is likely to be the fluke analogue or at the very least a related proteinase of cathepsin L.

This is the first cathepsin L-like enzyme to be identified in parasitic trematodes. A cathepsin B-like proteinase (Sm 31) has been identified in the related parasitic trematode, *Schistosoma mansoni* (Klinkert *et al.,* 1989), however the proteinase described here shows only 32% homology to the Sm 31 molecule in the N-terminal region. Presumably the homology between the two sequences would drop considerably when comparing the whole amino acid sequences as then the central region of the predicted amino-acid sequence would have to be considered. This is the region which is known to display the lowest homology when sequences of cysteine proteinases are compared (Dufour, 1988).

The N-terminal sequence which we obtained for the cathepsin L-like proteinase of *F. hepatica* falls short of the cysteine-rich motif (C-G-S-C-W), that surrounds the active site cysteine-25 residue and is seen in all cysteine proteinases (Dufour, 1988), by just two amino acids. However, the glutamine residue at position 19 (Figure 3.8) which is conserved in all the cysteine proteinase sequences that are known, plays a significant role in catalysis. It is believed to stabilise the intermediate complex by forming a hydrogen-bond between its NH_2 group and the carbonyl oxygen of the P_1 residue of the

substrate (Drenth *et al.*, 1976). This residue (Gln^{19}) is also conserved in the N-terminal sequence of the cathepsin L-like proteinase of *F. hepatica*.

Cathepsin Ls are synthesised as preproenzymes which are processed to the proenzyme (Smith & Gottesman, 1989). Further processing gives rise to a mature form which can consist of a single chain polypeptide or an active-site heavy chain and light chain linked by a disulphide bond (Barrett & Kirschke, 1980). It is not clear whether the two chained form results from limited proteolysis of cathepsin L during the purification procedure or whether cathepsin L does exist as a double-chain form within lysosomes (Dufour *et al.*, 1987). The autocatalytic cleavage specificity of cathepsin L at low pH and possible differences in the cleavage site specificities of the cellular proteinases *in vivo* may cause differences in the processing products formed *in vivo* and *in vitro* (Smith & Gottesman, 1989).

If the *F. hepatica* proteinase existed as a two chain form, separation of the two chains would have been necessary to make N-terminal sequence determination possible, as was the case when the N-terminal amino acid sequences of the heavy and light chains of human cathepsin L (Mason *et al.*, 1986) and rat liver cathepsin L (Towatari and Katunuma, 1988) were determined. Since a single N-terminal sequence was obtained for the *F. hepatica* proteinase we assume that this enzyme exists in the single chain form of the proteinase *in vitro*.

The sequence Ala-Xaa-Ala has been reported as the most frequent sequence preceding the signal peptidase cleavage site of cysteine proteinases (Perlman & Halvorson, 1983). According to the signal hypothesis, a signal sequence, once having initiated export of a growing protein chain across the rough endoplasmic reticulum, is cleaved from the completed protein

(prepro-form) at a specific site to yield the pro-form of the protein which can then be compartmentalised and/or further processed depending on the protein in question.

The N-terminal sequence of the *F. hepatica* cathepsin L-like enzyme begins one amino acid earlier than that of other cathepsin L sequences presented in Figure 3.7 and may suggest (since the extra amino acid is alanine) that a peptidase cleavage site may also be responsible for the processing of the pro-form of the cathepsin L-like enzyme into the mature proteinase. It may also indicate a different transportation procedure or localisation, or indeed it may be part of the secretion mechanism of the *F. hepatica* cathepsin L-like proteinase.

The precise function of the lysosomal cysteine proteinases which are predominantly cathepsins is unclear, but they are generally thought to play a role in the intracellular protein degradation (Barrett & Kirschke, 1980). Immunological studies demonstrated that the cathepsin L-like proteinase of *F. hepatica* is packaged in vesicles within the epithelial cells that line the gut wall of the parasite (Figure 3.10). These cells are known to go through phases of secretion when these vesicles exude their contents into the gut (Robinson & Threadgold, 1975), and therefore the proteinase can reach the exterior of the parasite as a result of regurgitation.

A cysteine proteinase of *T. cruzi* showing 65% homology to chicken liver cathepsin L in the N-terminal region (Cazzulo *et al.,* 1989) has also been demonstrated as having its activity localised to the lysosomal organelles in the epimastigote stage of *T. cruzi* (Bontempi *et al.,* 1989). The proteinase was suggested to participate in host tissue damage directly, by secretion from the parasites, which may facilitate rupture of host cells, or incidentally, by leakage

of the proteinase upon parasite death and lysis (Murta *et al.*, 1990). Cathepsin L-like proteinase activity has also been observed in *Plasmodium falciparum* trophozoite food vacuoles (Rosenthal *et al.*, 1988). This enzyme is responsible for intraerythrocytic hemoglobin digestion (Rosenthal *et al.*, 1993). Yamasaki *et al.*, (1989) have purified a 27 kDa cysteine proteinase from the *Fasciola* spp. Immunocytochemical studies (Yamasaki *et al.*, 1992) have since shown that this enzyme is localised in the secretory granules of the intestinal epithelial cells. In mammalian cells, the localisation of cathepsin L to the lysosomes provides it with an acidic environment, for activity and stability, while restricting its action to only those proteins that can enter this compartment (Bond & Butler, 1987). There are no reports of cathepsin L being actively secreted from such cells, indeed it is only found extracellularly in pathological situations (Bond & Butler, 1987). Troen *et al.*, (1987) have reported that the major excreted protein of malignantly transformed mouse fibroblasts is a secreted thiol proteinase, which is identical with mouse cathepsin L (Portnoy *et al.*, 1986) but it contains a different polyadenylation site. Mason *et al.*, (1987) have since described this major excreted protein of malignant mouse fibroblasts as a catalytically active form of cathepsin L with an N-terminal extension peptide. It was thought likely that these extension peptides were regions of the proteins which were sensitive to hydrolysis within the lysosome, and hence were not seen on the isolated stable forms of the mature enzymes. The proteinase does not require cleavage of this extension peptide to become active (Mason *et al.*, 1987). The presence of the additional N-terminal peptide stabilised the secreted form of the enzyme at neutral pH and would seem to suggest a different processing or packaging mechanism for the excreted form of mouse cathepsin L that is associated with tumour cells (Mason *et al.*, 1987).

Cysteine proteinases such as the cathepsins are not only localised in lysosomes of various types of mammalian cells but also in secretory granules in endocrine cells (Docherty *et al.,* 1984; Taugner *et al.,* 1985; Im *et al.,* 1989; Watanabe *et al.,* 1989), where they or related enzymes are indicated in prohormone cleavage (Marx, 1987), and in gastro-duodenal mucosa (Furuhashi *et al.,* 1991). Since the cathepsin L-like proteinase is located in vesicles in the intestinal epithelial cells of adult *F. hepatica*, which may well be the secretory granules (Figure 3.10), it is possible that whilst the flukes migrate through the host liver the cathepsin L-like enzyme may be secreted to the exterior of the parasite. Once secreted from the fluke it could play a role in immunoevasion by cleaving host immunoglobulin and thus preventing antibody-mediated immune-effector cell attachment to the parasite.

Immune evasion mechanisms exploited by helminth parasites in infected hosts, can be categorised into avoiding initial induction of immune responses, compromising selected arms of the immune system and disabling the short range offensive mounted by various effector mechanisms (Maizels *et al.,* 1993). Of these comprising selected arms of the immune surveillance by cleaving immunoglobulins has been known to exert effects in some helminthic infections. Auriault *et al.,* (1981) described how both bound and free IgG undergo proteolytic cleavage by proteinases released *in vitro* by schistosomula of *Schistosoma mansoni*, which produces peptidic fragments that are liberated into the culture medium. Thus, the Fab fragment and hence the antibody activity is lost. Fragments resulting from the proteolytic cleavage of IgG have been shown to reduce the phagocytic activity of macrophages (Auriault *et al.,* 1980). Proteolytic cleavage of immunoglobulins into 8-10 kDa fragments by an extract of *Dirofilaria immitis* microfilarial proteinase has also

been reported (Tamashiro *et al.*, 1987).

The cathepsin L-like proteinase released by the protozoan parasite *T. cruzi*, "cruzipain", is believed to play a role in the defence mechanism of the parasite against the immune response of the host (Bontempi & Cazzulo, 1990). It (cruzipain) is believed to be responsible for the intracellular proteolysis of endocytosed IgG molecules. It has also been shown to cleave IgG molecules within the hinge region. The Fab fragment was only slightly degraded, but the Fc fragment was extensively hydrolysed to small peptides. Hence it is believed, that that as well as hydrolysing endocytosed IgG, the proteinase also plays a role in immune evasion through a "fabulation mechanism", by destroying the Fc moiety of the antibody molecule and leaving an intact Fab fragment able to bind to the antigen but not to activate the complement cascade (Bontempi & Cazzulo, 1990).

Cathepsin Ls have also been implicated in the reduced immunocompetence of tumor-bearing animals. MEP, a precursor of mouse cathepsin L, secreted by transformed mouse fibroblasts, interferes with antigen processing in the antigen presenting cells, by cleaving the antigenic determinant. It is thought that due to the high levels of secreted cathepsins by some tumors that they (the cathepsins) could contribute to the depressed immunocompetency exhibited by tumor-bearing animals (McCoy *et al.*, 1988).

In view of the fact that the cathepsin L-like proteinase of this study cleaves immunoglobulin, and is secreted by all stages of liver fluke that exist in the mammalian host, including the early invasive NEJ, this enzyme may play a role in immune protection, even as the parasite is crossing the gut wall of the host (Carmona *et al.*, 1993). To investigate further the implications of the immunoglobulin cleaving ability of this proteinase, an eosinophil attachment

assay was developed, and it was found that addition of the purified cathepsin L-like proteinase, NEJ or adult fluke E/S products to the antibody-mediated eosinophil attachment assay resulted in > 70% reduction in the number of parasites with eosinophils attached (Carmona *et al.*, 1993). Therefore, liver fluke cathepsin L can prevent *in vitro* the antibody mediated attachment of eosinophils to parasites. It is suggested that the enzyme may perform a similar role *in vivo*. Thus, the cathepsin L-like proteinase is a potential target for either vaccine design and/or drug development due to its implied role in immune evasion and its presence in all stages of the fluke life cycle.

The success of cathepsin L as a vaccine would depend on, if antibodies induced in vaccinated animals were capable of neutralising the effect of the cathepsin L-like proteinase. In this study, we investigated the ability of antibodies raised in rabbits to purified enzyme to inactivate the proteinase. Purified anti-cathepsin L IgG2a molecules incubated with cathepsin L-like enzyme inhibited the proteolytic action of the proteinase on GS-PAGE and immunoglobulins (Figure 11A and 11B). Furthermore, purified proteinase that had been preincubated with anti-cathepsin L antibodies did not prevent the antibody mediated attachment of eosinophils to NEJ (Figure 11C). The inhibition data presented in this work demonstrates that antibodies that bind to and neutralise the enzyme activity can indeed be produced. Knox & Kennedy, (1988) also reported the inhibition of proteinases released by the parasitic larval stages of *Ascaris suum* by serum antibody from infected hosts. Neutralising antibodies may act by conformationally modifying the active site of the enzyme or by physically hindering the ability of the enzyme to gain access to its cleavage site.

Liver fluke may have a two-pronged mechanism of immunoevasion. Secretion of the cathepsin L-like proteinase may reduce the number of immune effector cells that attach to the parasite, and continual glycocalyx turnover may prevent any eosinophils that do manage to attach to the parasite to remain sufficiently long enough to allow degranulation and destruction of the parasite (Hanna, 1980). Vaccination of animals with cathepsin L could induce antibodies that can paralyse the fluke's immune protection mechanism, and result in the elimination of the parasite. Cathepsin L is present in the very early stages of the parasite cycle in the mammalian host (Carmona *et al.*, 1993). Neutralisation of the enzyme at this stage would eliminate worms before they reached the developmental stage, which causes the liver pathology associated with fascioliasis.

To search for particular characteristics unique to the primary structure of the parasite proteinase which might be exploited for use in drug and/or vaccine design, and in order to further our characterisation of the cathepsin L-like proteinase released by *F. hepatica*, we employed the technique of PCR to amplify a gene fragment encoding the cysteine proteinase in question. This procedure was used successfully to determine the primary structure of cysteine proteinases of nematode (Sakanari *et al.*, 1989) and protozoan (Eakin *et al.*, 1990) parasites, and could be extended to develop a system for producing easily purifiable recombinant proteinase for use in vaccine trials, as well as for analysing mutant forms of the enzyme.

Genomic DNA although more readily attainable is not ideal for use in this procedure, it has been found that when genomic DNA is used, many spurious amplification products are obtained. To avoid this problem and also to obtain

uninterrupted sequence we used cDNA, prepared from mRNA isolated from adult *F. hepatica* worms. Using molecular oligonucleotide probes based upon the consensus amino acid sequence of eukaryotic cysteine proteinases around the asparagine active site residue (Eakin *et al.,* 1990), and the N-terminal sequence determined for the cathepsin L-like proteinase purified in this study, we expected to amplify a gene fragment of approximately 550 bp.

In fact two gene fragments of approximately 550 bp were amplified from the cDNA (Figure 3.12). Subcloning of the entire PCR reaction using two different methods, yielded two clones with different nucleotide sequences. Both sequences show significant sequence identity with the sequences of other known cathepsin Ls (Table 3.2). The predicted amino acid sequence of the cysteine proteinases of these two gene fragments show that they are not identical; they are however quite similar to one another (78% identity). They are most closely related (88% identity) to a cysteine proteinase encoding gene isolated recently from *Fasciola* spp. (Yamasaki & Aoki, 1993). Aside from the similarity to the *Fasciola* spp. proteinase both gene fragments show considerable similarity to chicken cathepsin L (52% identity; Dufour *et al.,* 1987) and human cathepsin L (49% identitiy; Gal & Gottesman, 1988). This observation may indicate that *F. hepatica* is more closely related, on an evolutionary scale, to avian and mammalian species than it is to protozoans.

Three residues make up the catalytic triad of all cysteine proteinases: the cysteine at position 25 (Cys^{25}), the histidine residue at position 159 (His^{159}) and the asparagine at position 179 (Asn^{175}) (Dufour, 1988) [numbering according to papain (Drenth *et al.,* 1971)]. See Figure 4.1 for a linear representation of the important residues in the amino acid sequence of the proteinase. The Asn^{175} residue orients the His^{159} imidazole ring by forming a

Figure 4.1

A schematic representation of the cathepsin L-like gene fragments, indicating residues which are important for the catalytic activity of cysteine proteinases belonging to the papain superfamily, and more specifically for cathepsin L-like P_2 subsite specificity.

*The glutamine residue at position 19 and the tryptophan at position 179, responsible for hydrogen bonding in the P_1 subsite position, along with the catalytic triad (C^{25}, H^{159} and N^{175}) are represented in bold type (**Q, C, H, N,** and **W**). The glycine residues at position 65 and 66 and the other residues involved in the formation of the P_2 subsite pocket are represented in italics The glutamic acid residues which are found in positions 35 and 50 in cathepsin L-like cysteine proteinases, but are absent from cathepsin B-like proteinases, are underlined (E).*

hydrogen bond with it; Trp^{177}, (another important residue) shields this hydrogen bond from solvent attack (Kamphuis *et al.*, 1985). The imidazole ring of the His^{159} residue is then situated next to the Cys^{25} residue with which it forms an imidazole-thiolate ion-pair. One possible sequence of events in the catalytic mechanism is: the substrate binds to the enzyme by bonding with residues such as Gln^{19} which hydrogen bonds to the residue in the P_1 substrate position, and Gly^{65} and Gly^{66} which bind to the P_2 position; it (the substrate) then undergoes attack by the ion-pair i.e the sulphydryl group of Cys^{25} attacks the carbonyl carbon atom of the substrate, forming the acyl-enzyme intermediate, the imidazolium group donates its extra proton to the nitrogen of the peptide bond which facilitates the expulsion of the leaving group of the substrate (Drenth *et al.*, 1976; Lewis *et al.*, 1981).

The antisense primer was designed to the conserved sequence surrounding the active site Asn^{175} residue. No conclusions can be drawn about the presence or absence of this residue in the DNA fragment for the cathepsin L-like proteinase as it is present in the amplifying primer. However we may conclude that the sequence of the *F. hepatica* cathepsin L-like proteinase DNA gene fragment is sufficiently similar to the primer, over this sequence motif, to allow primer annealing to occur. The presence of the Cys^{25} and the third active site residue, His^{159}, is important in confirming the classification of this enzyme. The amino acids surrounding the three catalytic residues are highly conserved in both the cathepsin L-like gene fragment sequences. This indicates that these proteinases probably have similar catalytic mechanisms to other cysteine proteinases eg. papain. Indeed the hydrophobic nature of the cysteine and asparagine domains (segments located after Cys^{25} and His^{159}), and the hydrophillic character of the central region (residues 80-120) is maintained

within the sequences of both the cathepsin L-like proteinase gene fragments emphasising that their mode of action is probably similar to related cysteine proteinases.

The glycine residues at positions 65 and 66 form part of the active site wall (Kamphuis *et al.,* 1985), and are responsible for binding to the substrate at the P_2 position (Drenth *et al.,* 1976). Extensive similarity again exists in the amino acids surrounding these residues (Gly^{65} and Gly^{66}). We have observed the specificity of the cathepsin L-like proteinase of this study for a hydrophobic residue in the P_2 position in our earlier fluorogenic peptide substrate analysis. Table 4.1 details the other residues which are important in forming the P_2 subsite pocket of papain, human cathepsins B and L, and bovine cathepsin S (Bromme *et al.*, 1994). The corresponding residues for the two gene fragments of the cathepsin L-like proteinase isolated in this study are also included.

Differences in the substrate specificity of papain-like proteinases is determined by differences in the S_2-P_2 site interactions (Bromme *et al.*, 1994). Changes in the P_2 residues can therefore bring about changes in the specificity of the cysteine proteinase in question. Bromme *et al.*, (1994), showed that using site directed mutagenesis the substrate specificity of the binding site of human cathepsin S could be altered to a cathepsin L- or a cathepsin B-like specificity.

Both the gene fragments isolated in this study have identical residues at all the crucial positions for P_2 site specificity, except at position 160. A single substitution at this position has not been noted as being responsible for a dramatic change in specificity (i.e cathepsin L to B) (Bromme *et al.,* 1994), however, the substitution of alanine for glycine at this position may be responsible for the subtle differences that have been detected between the

cathepsin L1 and cathepsin L2 proteinases in our laboratory (Dowd *et al.,* 1994a).

Whether or not Clones A and B represent two genes, one encoding for cathepsin L1 and the other encoding for cathepsin L2, or two variants encoding for either cathepsin is undeterminable at this stage. However, a third gene fragment has also been isolated and sequenced in our laboratory using this technique (Katherine Kilbane, personal communication) which is 98 % identical to clone A, and hence would seem to represent a variant of the Clone A gene fragment. Given the substantial differences between Clone A and B gene fragments (22 % dissimilarity in amino acid sequence), and their striking overall similarity to chicken and human cathepsins L, it is not unreasonable to suggest that Clones A and B represent two gene fragments, one encoding for cathepsin L1 and the other encoding for cathepsin L2 .

The buried residues Glu^{35} and Glu^{50} are also conserved in the sequences isolated in this study. These glutamic acid residues are notably absent from the Sm 31 molecule (Klinkert *et al.,* 1989), which is consistent with other cathepsin B-like proteinases (Dufour *et al.,* 1988).

All the cysteine residues found in other cathepsin L-like proteinases are present in the gene fragments, one being the active site cysteinyl residue while the others are probably involved in disulphide bridges as in papain (Kamphuis *et al.,* 1984).

The absence of potential N-linked glycosylation sites (NXS/T), would support the theory of a different mechanism by which the *F. hepatica* proteinase is processed (Smith *et al.,* 1993a; Yamasaki & Aoki, 1993), and transported into the secretory vesicles (Smith *et al.,* 1993a) rather than the traditional lysosomal location of cathepsins (Barrett & Kirschke, 1980). Glycosylation has

Table 4.1

Amino acid residues involved in the P_2 subsite pocket of papain (pap), human cathepsins B, L (H [CB]and H [CL] respectively) and bovine cathepsin S (B [CS]) (Bromme et al., *1994), and the corresponding amino acid residues of* F. hepatica *gene fragments A and B (FhA [CL] and FhB [CL]) isolated in this study.*

Residue	Pap	(B) CS	(H) CL	(H) CB	(FhA CL	FhB) CL
67	Tyr	Phe	Leu	Tyr	Leu	Leu
68	Pro	Met	Met	Pro	Met	Met
133	Val	Gly	Ala	Ala	Ala	Ala
157	Val	Val	Leu	Gly	Leu	Leu
160	Ala	Gly	Gly	Ala	Ala	Gly
205	Ser	Phe	Ala	Glu	---	---

been shown not to be essential for enzymatic function (Smith & Gottesman, 1989), but N-linked glycosylation sites do occur in the lysosomal cathepsin Ls (residue 29, chicken cathepsin L, Dufour *et al.,* 1987; residue 106, human cathepsin L, Gal & Gottesman, 1988) and in the cathepsin L-like proteinase of the protozoan parasite *T. cruzi* (residues 47 and 167, cruzain, Eakin *et al.,* 1992). Yamasaki & Aoki (1993), did not report the presence of any N-linked glycosylation sites in the cysteine proteinase which they isolated from *Fasciola* spp.

Lysosomal enzymes are sequestered within the lumen of the endoplasmic reticulum and undergo a variety of post-translational modifications before being sorted to their appropriate cellular destination. Soon after the assembly of the polypeptide chain, the asparagine linked mannose oligosaccharides on lysosomal enzymes are covalently modified by the addition of N-acetylglucosamine 1-phosphate. In the Golgi body apparatus these residues are removed to generate monoesters, which bind to mannose-6-phosphate receptors. The receptor-ligand complexes are transported in Golgi-derived vesicles to prelysosomes where the vesicles discharge their contents. There the lysosomal enzymes are released from the mannose phosphate receptors which are recycled back to the Golgi apparatus (McIntyre & Erickson, 1991). Secretory proteins are not processed in this manner and hence the absence of any N-linked glycosylation sites would suggest a different procedure for the processing of the cathepsin L-like proteinase released by *F. hepatica*.

The highest degree of similarity between all the sequences is seen in the active site domains. Most deletion, insertions and substitutions occur in the middle region which is far removed from the active site and hence does not interfere with major catalytic characteristics. Cathepsin B has an insertion of 17

amino acids in this region (Figure 3.13) which seems to have little effect on the organisation of the active site residues, but is probably somewhat responsible for the difference in the substrate preference of this enzyme (Dufour, 1988).

It has been reported that the gene encoding "cruzain", a cysteine proteinase also related to cathepsin L enzymes isolated from *T. cruzi*, is organised in the genome as an array of at least six tandemly repeated copies (Eakin *et al.*, 1992). Yamasaki & Aoki, (1993) have reported the isolation of another cysteine proteinase clone which is 76% identical to the gene from *Fasciola* spp. which they published recently. We have described here two gene fragments from *F. hepatica* which are 78% homologous to each other. It is possible that these fragments are representative of a tandemly repeated gene as is present in *T. cruzi* (Eakin *et al.*, 1992). The amino acid substitutions may be inconsequential or may result in functional differences among the expressed proteinases.

Recently Heussler & Dobbelaere, (1994) have reported the cloning of a proteinase gene family of *F. hepatica* by reverse transcription PCR. Using degenerate oligonucleotide primers derived from conserved cysteine proteinase sequences, 7 different cysteine proteinase cDNA clones were amplified from RNA isolated from adult *F. hepatica* worms. Five of these clones were related to cathepsin L-type proteinases, while the remaining two were related to cathepsins B. Heussler & Dobbelaere's data suggest that some members of this proteinase gene family are present in multiple copies on the genome, and they have revealed differences in the levels of steady state mRNA expression for some of these proteinases, as well as a stage-specific gene expression for one of the cathepsin L-like clones (Heussler & Dobbelaere, 1994).

The cathepsin L of this study is one of the two major proteins present in the

E/S products of adult *F. hepatica*. The second protein which binds to the QAE column during the purification procedure has also been characterised. Although it differs subtly from the proteinase of this study, this second major protein is also cathepsin L-like in its characteristics, and to this end has been termed cathepsin L2 (CL2) (Dowd *et al.*, 1994a), where cathepsin L1 (CL1) is the proteinase of interest in this study. Thus the two gene fragments described here are perhaps representative of the two different cathepsin Ls present in the *in vitro* released products of adult *F. hepatica* worms.

In conclusion, we have purified and characterised a cathepsin L-like proteinase from the *in vitro* released products of *F. hepatica*. This is the first cathepsin L-like proteinase to be described for a parasitic trematode. It is responsible for the cleavage of immunoglobulin molecules *in vitro* and has other implied roles in feeding and motility. Inclusion of the purified proteinase in an eosinophil adherence assay prevents antibody-mediated immune-effector cell attachment to NEJ and hence protects the NEJ from destruction by the immune-effector cells. Antibodies raised in a rabbit to the purified proteinase, inhibit the proteolytic activity of the cathepsin L-like proteinase. When these antibodies are added to the eosinophil assay, they inhibit the action of the proteinase, hence the immune-effector cells are able to attach to and destroy the NEJ. Since this proteinase has an important biological role and has been shown to be present in all stages of *F. hepatica*, it is an ideal candidate molecule for vaccine and/or drug design. The partial gene fragment described in this study will enable future workers to obtain the full gene(s) for this proteinase. Expression of this enzyme in a eukaryotic system would allow analysis of the structure/function relationship, give information as to the

processing mechanisms of the proteinase, and allow analysis of mutant forms of the proteinase, therefore acquiring valuable information for the design of antiparasitic drugs which specifically block parasitic proteolytic action.

CHAPTER FIVE
REFERENCES

5.0 REFERENCES

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CHAPTER SIX
APPENDIX

Revised version
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ADULT *SCHISTOSOMA MANSONI* EXPRESS CATHEPSIN L PROTEINASE ACTIVITY

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Running title: Schistosome cathepsin L

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Keys words: *Schistosoma mansoni*; cysteine proteinases; cathepsins L and B

Abbreviations: GS-PAGE, gelatin-substrate polyacrylamide gel electrophoresis; Z-, N-benzyloxcarbonyl-; Bz-, N-benzoyl-; AMC, 7-amido-4-methylcoumarin-HCl; PCR, polymerase chain reaction; PBS, phosphate buffered saline; EDTA, ethylene diaminetetraacetic acid; SDS, soium dodecyl sulfate.

Sequences herein have been assigned the GenBank accession number U07345.

ABSTRACT This report presents the deduced amino acid sequence of a novel cathepsin L proteinase from *Schistosoma mansoni*, and describes cathepsin L-like activity in extracts of adult schistosomes. Using consensus primers specific for cysteine proteinases, gene fragments were amplified from adult *S. mansoni* cDNA by PCR and cloned. One of these fragments showed marked identity to Sm31, the cathepsin B cysteine proteinase of adult *S. mansoni*, whereas another differed from Sm31 and was employed as a probe to isolate two cDNAs from an adult *S. mansoni* gene library. Together these cDNAs encoded a novel preprocathepsin L of 319 amino acids; this zymogen is predicted to be processed *in vivo* into a mature, active cathepsin L proteinase of 215 amino acids. Closest homologies were with cathepsins L from rat, mouse, and chicken (46-47% identity). Southern hybridization analysis suggested that only one or a few copies of the gene was present per genome, demonstrated that its locus was distinct from that of Sm31, and that a homologous sequence was present in *S. japonicum*. Because these results indicated that schistosomes expressed a cathepsin L proteinase, extracts of adult *S. mansoni* were examined for acidic, cysteine proteinasè activity. Based on rates of cleavage of peptidyl substrates employed to discriminate between classes of cysteine proteinases, namely cathepsin L (Z-phe-arg-AMC), cathepsin B (Z-arg-arg-AMC) and cathepsin H (Bz-arg-AMC), the extracts were found to contain vigorous cathepsin L-like activity. In contrast, complete inhibition of this activity was observed when the cathepsin L inhibitor Z-phe-ala-CHN_2 was included, which together demonstrated that the conspicuous, acidic cysteine proteinase activity in extracts of adult *S. mansoni* was cathepsin L-like. The cathepsin L may be crucial for schistosome metabolism of host hemoglobin.

INTRODUCTION

Blood flukes of the genus *Schistosoma* are the cause of the chronic and debilitating disease schistosomiasis which afflicts more than 250 million people in tropical regions. Infection follows penetration of the skin by cercariae, the aquatic larvae. During human infection, cercariae transform into schistosomula which migrate to the lungs, and subsequently to the liver before taking up residence in the vasculature of the intestines or bladder. Adult females release numerous eggs each day for many years. In *S. mansoni* infections, the eggs move through the intestinal wall and are liberated into the lumen of the bowel. Eggs are carried also with the circulation and are deposited in various body organs, particularly the liver. The host inflammatory response to the eggs is responsible for the tissue pathology associated with schistosomiasis [1].

Proteinases are known to be secreted from *S. mansoni* parasites at several stages during migration in the mammalian host. These proteinases are thought to be involved in a variety of functions including skin penetration by cercariae, nutrition, and movement of eggs through tissue [see 2 for review]. Cysteine-type proteinase activity has been identified in adult *S. mansoni* [3-5] and was classified as a cathepsin B based on its hydrolysis of the synthetic peptide substrate Cbz-arg-arg-AMC [5] and because the sequence of a transcript encoding the enzyme shows homology with mammalian cathepsin B genes [6]. This cysteine proteinase, termed Sm31, is immunogenic in infected humans, is considered an important diagnostic protein, and may function as a hemoglobinase [5-9]. Since the enzyme must play some important biological role in the metabolism of the schistosome, it is considered a potential vaccine and/or chemotherapeutic target [2].

Here we report that, using generic oligonucleotide primers for amplifying cysteine proteinases gene fragments from cDNA by the polymerase chain reaction (PCR), we have isolated DNA fragments that encode *S. mansoni* cysteine proteinases. One of these encoded Sm31 [6] whereas another encoded a different cysteine proteinase. Using the latter fragment as a probe, we have isolated several cDNAs from an adult *S. mansoni* cDNA library that encode a novel preprocathepsin L. In addition, we show that the conspicuous, acidic cysteine proteinase activity in extracts of adult *S. mansoni* tissues is cathepsin L-like.

MATERIALS and METHODS

Schistosomes, genomic DNA, soluble extracts of adult worms Mixed sex, adult *S. mansoni* (Puerto Rican strain) and *S. japonicum* (Philippine strain) were perfused from BALB/c mice 10 weeks after infection with 20 to 100 cercariae, and stored at -70°C for up to 6 months. Genomic DNAs from pools of adult worms were isolated as described [10]. Lysates of schistosomes were prepared in phosphate buffered saline (PBS) by two freeze-thaw cycles followed by sonication (25 x 10 s bursts at duty cycle 10% and output control 2, Branson Sonifier 250) (Branson Ultrasonics, Danbury, CT) at 4°C. The lysate was centrifuged for 30 min at 14,000 x *g* at 4°C and the supernatant (adult worm extract) retained. Protein concentration of the extracts was measured by a modified Lowry method (DC Protein Assay, Bio-Rad, Richmond, CA).

PCR amplification, cloning, sequencing mRNA was isolated directly from

tissues of ~50 adult *S. mansoni* by chromatography on oligo dT-cellulose (Micro Fast Track™, Invitrogen, San Diego, CA). Double stranded cDNA was synthesized from the mRNA using AMV reverse transcriptase, and RNaseH and DNA polymerase 1 (Riboclone™ Synthesis Kit, Promega, Madison, WI). Generic oligonucleotide primers for eukaryotic cysteine proteinases, described by Eakin *et al.* [11] (5' Cys-specific primer and 3' Asn-specific primer, of 4096- and 1024-fold degeneracy, respectively), a modified 5' Cys-specific primer (8-fold degenerate), based on the sequences of cysteine proteinases of nematodes [12-15] (Harrop, unpublished), and a sense strand primer (4-fold degenerate) based on the N-terminus of a cathepsin L of *Fasciola hepatica* [16] were employed to amplify gene fragments from adult *S. mansoni* cDNA. The sequences of the four oligomers are shown in Fig. 1. PCRs were carried out for 40 cycles using 50 ng cDNA as template, with denaturation at 94°C for 45 sec, primer annealing at 40°C for 1 min, and extension at 72°C for 2 min, with an initial step at 94°C for 2 min and a final extension for 10 min at 72°C.

PCR products were purified by phenol/chloroform extraction and ethanol precipitation, ligated into the plasmid vector pGEM-T™ (Promega), and the ligation products used to transform *Escherichia coli* strain DH5α by electroporation. Maxipreps of recombinant pGEM-T and pBluescript (below) plasmids were prepared from bacterial cultures using Qiagen-500 columns (Qiagen Inc, Chatsworth, CA). The nucleotide sequence of plasmid inserts was determined using universal forward and reverse primers, and gene-specific primers, the Taq DyeDeoxy™ Terminator Cycle Sequencing System (Applied Biosystems Inc. [ABI], Foster City, CA) and an automated DNA sequencer (ABI, model 373A). Oligomers

were synthesized using the β-cyanoethyl phosphoramidite procedure on a ABI model 308B DNA Synthesizer, and purified using Nensorb Prep cartridges (NEN-Dupont, Wilmington, DE). Both strands of plasmid inserts were sequenced. Analyses of nucleotide and deduced amino acid sequences were assisted by the GCG Package software, version 7 (Genetics Computer Group, Madison, WI), using the GenBank, PIR-Protein, and SwissProt databases.

Southern hybridization analysis Genomic DNAs (3 μg) from pools of adult *S. mansoni* or *S. japonicum* were digested with *Eco* RI, *Hind* III, or *Bam* HI (Biolabs, Beverley, MA), separated by electrophoresis through 0.8% agarose/Tris acetate EDTA, and Southern transferred to nylon membranes (Zeta-Probe, Bio-Rad). Inserts were excised from recombinant plasmids p466 and pB2 (see below) by digestion with *Bam* HI, separated from vector sequences by agarose gel electrophoresis, and purified by "glass milk" chromatography (GENECLEAN™, BIO 101, San Diego, CA). The inserts (50 ng) were radiolabeled with α^{32}P.dCTP (NEN-DuPont) by random oligomer priming and Klenow polymerase (Oligolabelling Kit, AMRAD-Pharmacia, North Ryde, NSW, Australia). Labeled insert DNA of p466 was hybridized to Southern blots at 65° C overnight in 1 mM EDTA, 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), and washed at 65° C for 2 h in 1 mM EDTA, 40 mM $NaHPO_4$, 5% SDS, then 2 h in 1 mM EDTA, 40 mM $NaHPO_4$, 1% SDS, as described [17]. Autoradiography was performed at -70° C using Kodak X-AR film and intensifying screens. The p466 probe was stripped from membranes by incubation in 500 ml 15 mM NaCl, 1.5 mM sodium citrate for 30 min at 95° C, after which the membranes were reprobed with the ^{32}P.pB2 insert.

Screening of a *S. mansonI* cDNA phage library A λZapI cDNA library constructed from adult *S. mansoni* mRNA [18] (library kindly provided by Dr. Graham F. Mitchell) was screened by nucleic acid hybridization using the ^{32}P-labeled insert of p466 as the probe, using Hybond-N (Amersham, Castle Hill, NSW, Australia) nylon membranes with hybridization and washing conditions as above. The pBluescript phagemid in positive λ clones was excised using P408 helper phage (Promega) and *E. coli* strain BB4 according to the λZapI manufacturer's instructions (Stratagene, San Diego, CA).

Analysis of proteinase activity Samples of extract (200 μg protein) were subjected to non-denaturing, non-SDS, gelatin-substrate-(0.1%)-polyacrylamide (10%) gel electrophoresis (GS-PAGE), as described [19]. (This procedure was employed, rather than regular SDS-PAGE, because SDS and other denaturing reagents inhibited hydrolysis of enzyme substrates [Dalton, unpublished].) Further, replicate gel lanes of the electrophoresed extract were incubated in 10 μM fluorogenic peptidyl substrate (below) in 100 mM sodium citrate, 10 mM cysteine, pH 4.5, for 15 min, after which they were transilluminated at 302 nm and photographed (Polaroid 667 film). In addition, samples of extract (2 μg to 25 μg protein) were assayed for cysteine proteinase activity in 100 mM sodium citrate, pH 4.5, containing 10 mM cysteine and the fluorogenic peptide substrates benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amido-4-methyl-coumarin (Z-phe-arg-AMC), benzyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin (Z-arg-arg-AMC), benzoyl-L-arginine-7-amido-4-methylcoumarin (Bz-arg-AMC) (Bachem, Bubendorf, Switzerland) at 10 μM. These three substrates were

employed in order to discriminate between classes of cathepsin-like endopeptidases, namely cathepsin L (Z-phe-arg-AMC), cathepsin B (Z-arg-arg-AMC) and cathepsin H (Bz-arg-AMC) [20]. The release of the fluorogenic moiety 7-amino-4-methyl coumarin (AMC) from hydrolyzed substrates was measured in a fluorescence spectrometer (Kontron, model SFM 25, Milan, Italy) with excitation at 370 nm and emission at 440 nm. Amount of enzymatically-generated AMC was calculated from the standard fluorescence intensity of authentic AMC (Sigma Chemical Co., St. Louis, MO). Inhibition assays were carried out using the peptidyl diazomethylketones, benzyloxycarbonyl-L-phenylalanyl-L-alanine-diazomethylketone (Z-phe-ala-CHN_2), and N-benzyloxcarbonyl-L-phenylalanine-L-phenylalanine-diazomethylketone (Z-phe-phe-CHN_2) (Bachem). These specific inhibitors of cathepsin L-like, cysteine proteinase activity [21, 22] were added to the assay at concentrations of 10 μM - 0.05 μM prior to the addition of the substrate.

RESULTS

PCR amplification of *S. mansoni* cysteine proteinase gene fragments When generic oligonucleotide primers were employed in the PCR with *S. mansoni* cDNA as the template, DNA fragments ranging in size from approximately 400 to 600 nucleotides were amplified using all three combinations of the three sense and one antisense primers (see Fig. 1) (data not shown). After the amplified cDNA sequences were cloned into pGEM-T and the nucleotide sequence of the inserts determined, it was apparent that several PCR products obtained using a pairing of the modified 5' Cys-specific primer and the 3' Asn-specific primer contained

sequences characteristic of cysteine proteinases, i.e. they exhibited the catalytic triad of Cys, His, and Asn residues [23, 24]. Although some of these clones (e.g. pB2, insert size 480 bp) contained inserts homologous to Sm31, another (p466, insert size 483 bp) contained a novel cysteine proteinase-like gene sequence distinct from Sm31 [6] (data not shown). By contrast, fragments amplified using other combinations of primers (i.e. other than the modified 5' Cys-specific primer with the 3' Asn-specific primer) did not ostensibly encode proteinases (not shown).

A transcript from adult *S. mansoni* encodes a cathepsin L-like proteinase

Using the radiolabeled insert of p466 as a probe, two positive clones (pSmCL1 and pSmCL6) were isolated from the *S. mansoni* λZapI cDNA library after screening about 1 x 10^5 phage plaques. Clones pSmCL1 and pSMCL6 contained recombinant inserts of ~1.1 kb and ~1.8 kb, respectively, overlapped over ~480 bp at the 3'-end of pSmCL6 and the 5'-end of pSmCL1, and together included an open reading frame of 319 amino acids including the catalytic triad residues Cys, His, and Asn. pSmCL6 contained a start codon and pSmCL1 contained a termination codon TAG, a potential polyadenylation signal (AAUA) and a poly(A) stretch, the latter probably representing part of the poly(A) tail and therefore the 3'-terminus of the mRNA (not shown) (GenBank accession # U07345). The 319 amino acids apparently comprise the sequence of a novel zymogen, of predicted MW of 35,444. Based on homology with other cysteine proteinases [25-28], the cleavage site of the pro-region peptide from the mature, processed enzyme (215 amino acids, ~23,888 MW) probably lies between residues 104 (asparagine) and 105 (isoleucine).

Homology comparisons showed closest identity to mouse cathepsin L (GenBank accession # P06797) at 46.6% over a 223 amino acid (aa) overlap, to chicken cathepsin L (P09648) at 46.4% over a 220 aa overlap, and to rat cathepsin L (P07154) at 46.2% over 223 residues. By contrast, the schistosome cathepsin L was only 26% homologous to the *S. mansoni* cathepsin B proteinase Sm31 (M21309; J03984). Figure 2 presents an alignment of sequences of this novel schistosome cathepsin L, cathepsins L from mouse and chicken, and sequences deduced from two recently isolated cDNAs (L1-*Fh* and L2-*Fh*) from another trematode parasite, *Fasciola hepatica*. (The *F. hepatica* sequences were from cloned PCR products of 460 bp and 480 bp obtained using adult *F. hepatica* cDNA as template and the 5' N-terminal *F. hepatica* cathepsin L primer in combination with the 3' Asn-specific primer [11] shown in Fig. 1 [Dalton, unpublished]. L1-*Fh* and L2-*Fh* may encode cathepsins L reported recently to be secreted by adult *F. hepatica* [16].) Conservation in sequence, particularly around the active site Cys, His, and Asn catalytic triad residues, and in length, with the cathepsins L of the other species was obvious which, together with an expected MW of 23,888, strongly supports our proposal that the polypeptide encoded by amino acid residues 105 to 319 represents the entire, mature form of the novel *S. mansoni* cathepsin L proteinase [20, 29].

Gene copy number for schistosome cathepsins L and B ^{32}P-inserts of p466 and pB2 hybridized to Southern blots of *S. mansoni* genomic DNA, but with dissimilar patterns. p466 hybridized to *Eco* RI fragments of 4.5 kb and 2.8 kb, to a 2.8 kb *Hin*d III fragment, and to a 4.5 kb *Bam* HI fragment. In addition, p466

hybridized to a 4.5 kb *Eco* RI fragment of *S. japonicum* genomic DNA. By contrast, pB2 hybridized to a single *Eco* RI fragment of ~2 kb, *Hin*d III fragments of 6 kb, and 4.5 kb, and to *Bam* H1 fragments of about 7 kb and 5.5 kb. pB2 did not hybridize to the *Eco* RI-digested genome of *S. japonicum* (Fig. 3). The simple patterns obtained with both probes are characteristic of single (or several only) copy genes, a finding consistent with a previous report for Sm31 [6].

Cysteine proteinase activity in schistosome extracts Aqueous, acidic *S. mansoni* extracts were analyzed for cysteine proteinase activity, in particular for activity ascribable to cathepsin L-like proteinases. A single proteinase activity capable of digesting gelatin was apparent after GS-PAGE (Fig. 4a). Replicate gel lanes showed marked differences in substrate preference after incubation with three fluorogenic peptidyl substrates. Potent Z-phe-arg-AMC hydrolyzing activity was observed migrating with a similar mobility to the gelatinolytic activity as a smear at the top of the gels (Fig. 4b, lane 2). By contrast, minimal activity was observed in gels incubated with Z-arg-arg-AMC, and little or no activity was detected against Bz-arg-AMC (Fig. 4b, lanes 1 and 3, respectively). When the release of enzymatically cleaved AMC (as visualized in the GS-PAGE gels) was subsequently quantified in test tube assays, the specific activities (nmol AMC released mg protein^{-1} min^{-1}) in the extracts for the fluorogenic substrates were Z-phe-arg-AMC, 58.3, Z-arg-arg-AMC, 0.97, and Bz-arg-AMC, 0.24. Complete inhibition of schistosome Z-phe-arg-AMC hydrolyzing activity was observed if Z-phe-ala-CHN_2 was included in the assay at 1.0 μM final concentration, and 72% inhibition observed at 0.1 μM inhibitor concentration. In contrast, at 1.0 μM and 0.1

μM concentrations of Z-phe-ala-CHN_2, Z-arg-arg-AMC hydrolyzing activity was inhibited only 70% and 45%, respectively. Similar inhibition was observed with Z-phe-phe-CHN_2 (not shown).

DISCUSSION

Using oligonucleotide probes based upon consensus sequences of eukaryotic cysteine proteinases, we amplified and cloned gene fragments from adult *S. mansoni* cDNA. Some were homologous to the previously characterized cathepsin B (Sm31) [6], but another (p466) showed significant sequence identity to cathepsin L-like proteinases. Subsequently, using p466 as a probe, we isolated, cloned, and sequenced two cDNAs from an adult *S. mansoni* λZapI library, that encode a novel cathepsin L cysteine proteinase. Sequence identity comparison and Southern hybridization analysis clearly showed that the gene encoding the cathepsin L was distinct from that encoding Sm31.

We employed three pairs of consensus primers in order to maximize the likelihood of amplification of proteinase gene sequences from cDNA. Only the pairing of the modified 5' Cys-specific and the 3' Asn-specific primers amplified authentic *S. mansoni* cysteine proteinase gene fragments. Since the same antisense oligomer was employed in the PCRs, it appears that inclusion of the modified 5' Cys-specific primer was crucial for amplification of the target sequences and may be related to the its reduced degeneracy (8-fold) in comparison to the 5' Cys-specific primer of Eakin *et al.* [11] which is 4096-fold degenerate. Indeed, when the sequences of the modified 5' Cys-specific and the 3' Asn-specific primers were compared with the cDNA sequence, the former matched at 16 of 17 residues,

and the latter at 22 consecutive residues (not shown) - sufficiently homologous, evidently, to hybridize to the cDNA template under the modest stringency of the PCR (40° C annealing temperature) and to result in the amplification of a novel cysteine proteinase gene.

The sequence of the cathepsin L transcript included an open reading frame of 319 amino acids, which apparently encodes the entire preprocathepsin L from *S. mansoni*. It can be expected that cleavage of the pro-region from the zymogen would release the mature, active cysteine proteinase [28]. Based on comparisons of amino acid sequences of other cysteine proteinases, which often include a prepro- sequence upstream of the mature enzyme sequence [25-29], the processed cathepsin L is expected to be comprised of 215 amino acids with an estimated MW of 23,888. This is of comparable size to other cathepsins L [22, 27].

Since the presence of the novel transcript demonstrated that adult *S. masnoni* express a cathepsin L proteinase, we examined soluble extracts from *S. mansoni* for novel cysteine proteinase activity, particularly for activity distinct from that ascribable to Sm31. Based on the rate of cleavage of three synthetic peptidyl substrates, the results indicated that the conspicuous cysteine proteinase activity in soluble, acidic *S. mansoni* extracts was cathepsin L-like. The rate of cleavage of Z-phe-arg-AMC by cathepsin L is up to 100-fold greater than that of Z-arg-arg-AMC; by contrast, cathepsin B can cleave both Z-phe-arg-AMC and Z-arg-arg-AMC substrates, but the rate of cleavage of the former substrate is only two to three times that of the latter [22]. Since sixty-fold more of the substrate Z-phe-arg-AMC (which has the hydrophobic amino acid phenylalanine in the P_2 position) was hydrolyzed by the schistosome extracts compared to Z-arg-arg-AMC, the soluble

extracts of *S. mansoni* clearly contained cathepsin L-like proteinase activity. GS-PAGE, fluorogenic substrate-PAGE analysis, and inhibition studies using the cathepsin L-specific inhibitors Z-phe-ala-CHN_2 and Z-phe-phe-CHN_2 [21-22] provided further evidence of cathepsin L-like activity in the extracts.

A novel cysteine proteinase activity (specifically hydrolysis of carbobenzoxy-phe-arg-7-amino-4-trifluoromethyl coumarin) has been reported from miracidia and sporocysts of *S. mansoni* [30]. Although the activity was not ascribed to a cathepsin L, and although the sizes of the partially purified enzymes (19 kDa and 36 kDa) do not conform with that predicted for the processed cathepsin L of adult *S. mansoni* (~24 kDa), it is not unlikely that larval schistosomes may also employ cathepsins L. Yoshino *et al.* [30] suggested a role for miracidial cysteine proteinase(s) in penetration of the intermediate snail host of the schistosome. Two recently reported cathepsins L from the related parasite *Fasciola hepatica* appear to be associated with tissue digestion by immature liver flukes and with immunologic evasion mechanisms including cleavage of immunoglobulins and inhibition of eosinophil attachment [16, 31, 32].

In addition to sequence differences and to substrate preferences between the cathepsin L and Sm31 proteinases, the differential patterns of hybridization of p466 and pB2 to the Southern blots of *S. mansoni* genomic DNA clearly demonstrated the dissimilarity in genomic organization and sequence of the cathepsins L and B genes. The patterns suggested that only one or a few copies of each of the cathepsin L and Sm31 genes were present in the *S. mansoni* genome, and the hybridization of p466 to *S. japonicum* DNA indicated the presence of a homologous cathepsin L in this species. Since we have also observed

cathepsin L-like activity (hydrolysis of Z-phe-arg-AMC) in extracts of adult *S. japonicum* with activity and inhibition profiles similar those of *S. mansoni* (Smith, unpublished), cathepsin L activity, hitherto not reported in adult *S. mansoni*, may be common in schistosomes. Indeed, in view of the recent reports of cathepsins L in *Fasciola* species as well [16, 31-33] (Fig. 2), this category of cysteine proteinase may be ubiquitous in parasitic flatworms.

Cysteine proteinases are reputed to be involved in the degradation of host hemoglobin by schistosomes [2-4]. To date, only the cathepsin B Sm31 has been characterized in detail and has by default been designated the schistosome hemoglobinase [2, 5, 6]. Since the specific activity of the cathepsin L proteinase in our schistosome extracts was sixty-fold greater than that of cathepsin B, it is feasible that the cathepsin L rather than the cathepsin B may play the leading role in the catabolism of hemoglobin to readily absorbable peptides [2, 34].

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FIGURE LEGENDS

Fig. 1 Nucleotide sequences of oligonucleotide primers employed to amplify cysteine proteinase gene fragments from adult *Schistosoma mansoni* cDNA. I = inosine, K = G/T, R = G/A, W = A/T, and Y = C/T.

Fig. 2 Alignment of amino acid sequences of cathepsins L from other species with the schistosome sequence deduced from pSmCL1 and pSmCL6. L-*Sm*, *S. mansoni*, L-mo, mouse (GenBank accession # P06797), L-ch, chicken (P09648), L1-*Fh* and L2-*Fh*, *Fasciola hepatica* [16] (Dalton, unpublished). Asterisks denote homology with L-*Sm*; gaps (-) have been introduced to maximize alignment; X denotes an undetermined residue; and . indicates that it is not known whether or not a residue exists at the position. The cysteine proteinase catalytic triad of residues (C, H, and N) are shown in boldface type, and numbering of amino acids is based on the papain system of Drenth *et al.* [24].

Fig. 3 Southern hybridization of ^{32}P-insert from p466 (cathepsin L-like gene probe) (panel A) and ^{32}P-labeled insert of pB2 (Sm31-like cathepsin B gene probe [6]) (panel B) to genomic DNAs from *Schistosoma mansoni* after *Eco* RI (lane 1), *Hin*d III (lane 2) and *Bam* HI (lane 3) digestion, and from *S. japonicum* after *Eco* RI digestion (lane 4).

Fig. 4 Identification of cathepsin L-like activity in soluble extracts of adult *Schistosoma mansoni*. Panel a: Samples of schistosome extract (200μg) were separated by gelatin-substrate (0.1%) polyacrylamide (10%) gel electrophoresis, subsequently incubated in 100 mM sodium citrate, pH 4.5, 10 mM cysteine, and finally stained with Coomassie Blue. Panel b: Replicate gel lanes were excised and incubated in the fluorogenic substrates Z-arg-arg-AMC (RR, lane 1), Z-phe-arg-AMC (FR, lane2), and Bz-arg-AMC (R, lane 3) at 10 μM in 100 mM sodium citrate, pH 4.5, 10 mM cysteine, and photographed while transilluminated with ultraviolet light at 302 nm.

Fig. 1 Smith et al revised MS # 108

5′ Cys-specific primer

```
5′ ACA GAA TTC CAR GGI CAR TGY GGI TCI TGY TGG 3′
     Eco RI   Q   G   Q   C   G   S   C   W
```

5′ modified Cys-specific primer

```
5′ GC TCA TGY TGG GCW KTC 3′
   G   S   C   W   A  F/V
```

5′ N-terminal *F. hepatica* cathepsin L primer

```
5′ ACA GAA TTC GGY TAT GTG ACT GGY GTG AAG G 3′
     Eco RI   G   Y   V   T   G   V   K
```

3′ Asn-specific primer

```
                  IGA
5′ TTA AAG CTT CCA   RTT YTT IAC RAT CCA RTA 3′
                  RCT
    Hind III W    S   N   K   V   I   W   Y
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Fig. 3 2 Smith et al.

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                10        20        30        40        50        60
L-Sm    IPKNFDWREKGAVTEVKNQGMCGSCWAFSTTGNVESQWFRKTGKLLSLSEQQLVDCDG--
L-mo    ***SV******C**P*****Q********AS*CL*G*M*L**********N****SHAQ
L-ch    A*RSV******Y**P**D**Q**********AL*G*H**TK***V*****N****SRPE
L1-Fh   ......................****X*****AM*Q*TMKNQRTSI*X********SRDF
L2-Fh   ......................**********TM*-XYMKNEKTSX*X********SGPW

                70        80        90        100       110       120
L-Sm    LDDGCNGGLPSNAYESIIKMGGLMLEDNYPYDAKN-EKCHLKTD-GVAVYINSSVNLTQ-
L-mo    GNQ******MDF*FQY*KEN***DS*ES***E**DG-S*KYRAE-FAVANDTGF*DIP*-
L-ch    GNQ******MDQ*FQYVQDN**IDS*ES***T**DD*D*RY*AE-YN*ANDTGF*DIP*G
L1-Fh   CNY******ME****YLKRF-**ET*SS***R*VEGQ-*RYNEQL***KV-T*YYTVHSG
L2-Fh   GNN**S***ME***QYLKQF-**ET*SS***T*VEGQ-*R-NRQL***KV-TGYYTVHSG

                130       140       150       160       170       180
L-Sm    DETELAAWLYHNSTISVGMNALL--LQFYQHGISHPWWIFCSKYLLDHAVLLVGYGVSE-
L-mo    Q*KA*MKAVATVGP***A*D*SHPS****SS**YYE--PN**SKN***G*******YEGT
L-ch    H*RA*MKAVASVGPV**AID*GHSSF****S**YYE--PD**SED***G**V****FEGG
L1-Fh   **V**QNLVGAEGPAA*ALD-VESDFMM*RS*-YQS--QT**PDR*N*G**X*X**XQGG
L2-Fh   S*AGLKNLVGSEGPAA*AVD-VESDFMM*RS*-YQX--QT*LPFA*N****X*D**TQGG

                190       200       210       220   226
L-Sm    --KNEPFWIVKNSWGVEWGENGYFRMYRG-DGSCGINTVATSAMIY
L-mo    DSNKNKY*L******S***ME**IKIAKDR*NH**LA*A*SYPVVN
L-ch    ----KKY********EK**DK**IYMAKDRKNH***A*A*SYPLV-
L1-Fh   ------Y*******.............................
L2-Fh   ------Y*******.............................
```

Fig 3

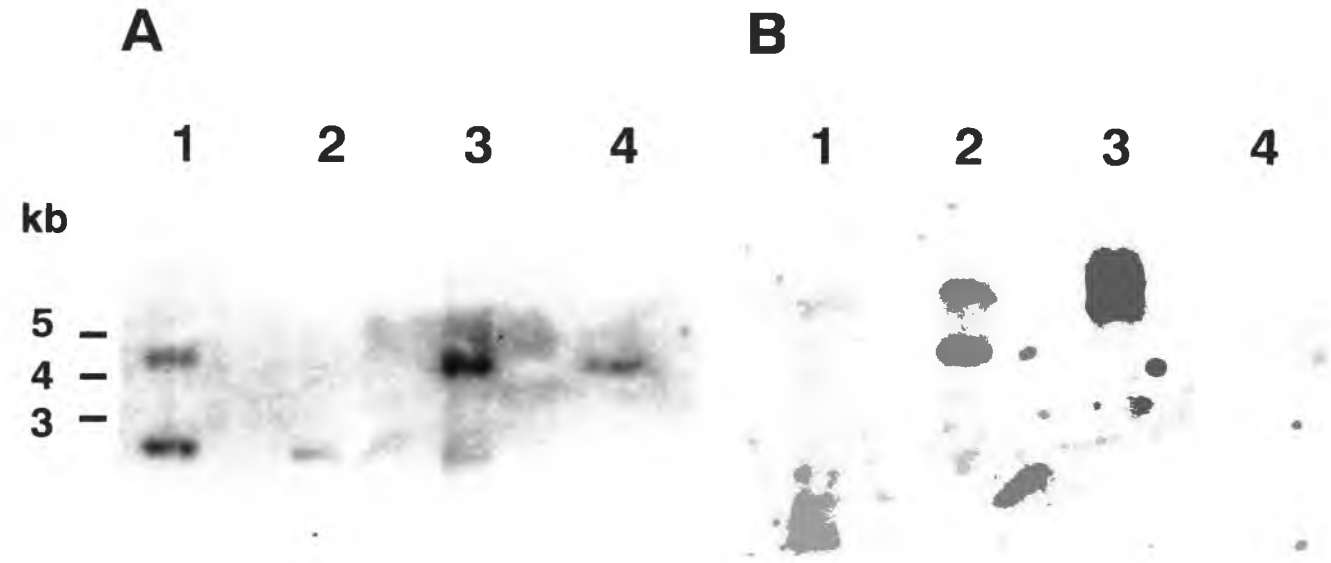
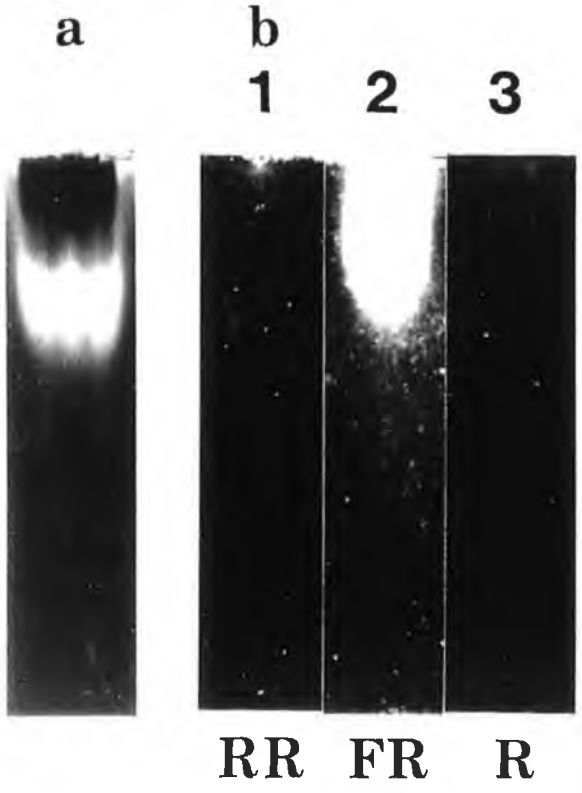


Fig 4



CURRICULUM VITAE

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Education

Secondary School (1980-1986)	Ursuline College Sligo, Finisklin Road, Sligo.
University (1986-1994)	Dublin City University, Dublin 9, Ireland.
B.Sc. Degree (1990)	Grade 1,Class 2 Hons in Biotechnology. Option courses: Genetics, Immunology, and Biochemical Engineering
Post-Graduate (1990-present)	Undertaking a Ph.D. through research, in Dr. J. P. Dalton's laboratory in Dublin City University investigating proteinases released *in vitro* by the parasite *Fasciola hepatica*. This included a six month visit to Dr. Paul J. Brindley's laboratory in The Queensland Institute of Medical Research, Brisbane, Australia, where proteinases of the related parasitic trematode *Schistosoma mansoni* were studied.

Research and Experience

Purification and characterization of a cathepsin L proteinase released *in vitro* by *Fasciola hepatica* . Techniques used included electrophoresis, HPLC analysis, protein chemistry, enzyme assays, polyclonal antibody production, immunofluorescence, PCR and cloning.

Investigation of the proteinase activity present in extracts of adult *Schistosoma mansoni* extracts involved the use of molecular biological techniques such as Polymerase Chain Reaction methods, primer design, DNA cloning and sequencing.

Outside Interests

Aerobics, basketball, reading, knitting and travelling.

Publications

Smith, A. M., Dowd, A. J., Heffernan, M., Robertson, C. D. and Dalton, J. P. (1993). *Fasciola hepatica*: A secreted cathepsin L-like proteinase cleaves host Immunoglobulin. *International Journal for Parasitology. Vol. 23, No. 8: 977-983.*

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Meetings/Abstracts

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Dowd, A. J., **Smith, A. M.**, Carmona, C. and Dalton, J. P. Purification and characterisation of a cysteine proteinase released by *Fasciola hepatica*. Irish Biochemical Society, Trinity meeting 1992.

Carmona, C., **Smith, A. M.**, Dowd, A. J. and Dalton, J. P. A *Fasciola hepatica* cathepsin L like proteinase prevents the adherence of eosinophils to newly excysted juveniles. Irish Biochemical Society, Trinity meeting 1992.

Smith, A. M., Dowd, A. J., McGonigle, S. and Dalton J. P. Purification of two cathepsin L-like proteinases secreted by adult *Fasciola hepatica*. British Society for Parasitology, Spring Meeting, April 1993.

Smith, A. M., Dalton, J. P., Clough, K. A., Harrop, S. A., Kilbane, C. L. and Brindley, P. J. Cathepsin L proteinase of *Schistosoma mansoni*. British Society for Parasitology, Spring Meeting, April 1994.

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