IDENTIFICATION AND CHARACTERIZATION OF THIOL PROTEASES RELEASED *IN VITRO* BY *FASCIOLA HEPATICA.*

THESIS SUBMITTED FOR THE DEGREE OF

MASTER OF SCIENCE

BY

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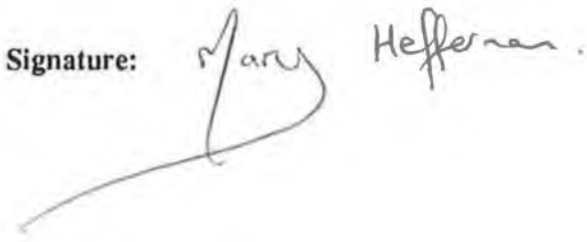
I declare that the work presented in this thesis was performed by me alone (unless otherwise stated) and has not been submitted to any other institution for an academic award.

Signature: 

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1.0 ABSTRACT

Fasciola hepatica excretory/secretory (E/S) products were obtained by culturing liver flukes *in vitro* for 16 hours. When these E/S products were analyzed by gelatin substrate sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) two groups of proteases were revealed. Group 2 proteases were active in the pH range 4.5-8.0, while Group 1 proteases were most active in the pH range 3.0-4.5. Activity of all proteases was enhanced by the reducing agents cysteine and DTT. Based on these studies and on inhibitor studies both groups of proteases were identified as thiol proteases. Also consistent with thiol proteases all of the enzymes bound to an organomercurial column. Antiserum against these thiol proteases eluted from this column was prepared. Using this antiserum in immunolocalization studies we showed that these enzymes are secreted by the gut epithelial cells. The antiserum was also used to screen an adult fluke cDNA expression library; however, no recombinant clones were identified. Rabbit IgG was incubated with the E/S products or papain. Analysis by SDS-PAGE revealed that both preparations resulted in the cleavage of IgG heavy chain into two fragments of 28 and 22 kDa. HPLC analysis of the E/S products yielded three major peaks. The IgG cleaving enzyme was found to be associated with peak III of approximately 15 kDa. Using the substrate Z-Phe-Arg-AMC we showed that this peak also contained cathepsin B activity. Consistent with cathepsin B enzymes the enzyme in peak III was found to be a thiol protease with optimal activity at pH 4.5. Gelatin substrate PAGE analysis showed that peak III correlated with the acidic Group 1 proteases. DPC, which inhibits enzymes containing histidine in their active site, totally inactivated the enzyme in peak III and inhibited the cleavage of IgG by this enzyme. This result is consistent with this enzyme being categorized as a cathepsin B since these enzymes have histidine residues involved in catalytic activity.

2.0 INTRODUCTION

Fascioliasis is caused by infection with the parasitic trematode *Fasciola hepatica* and has been estimated to cost the Irish Agricultural Industry £20 million annually due to sheep death and the condemnation of infected sheep and cattle liver. Included in this estimation are losses due to a reduction in milk yield, growth rate and sheep wool condition. The distribution of liver fluke disease is worldwide and the parasite is known to have a wide range of mammalian hosts. Although its importance lies in the damage it causes to sheep and cattle, cases of human fascioliasis have been found.

The life cycle of this Digenean trematode begins when the adult worm in the bile ducts releases fertilised eggs. The eggs, which are operculated, are carried to the intestine, and leave the body in the faeces. After embryonation (14-17 days), the opercula fly open and ciliated miracidia are liberated. The miracidia soon find the intermediate host, *Limnaea truncatula*, a snail commonly found in fresh water or damp pastures. It sucks onto its host and by the enzymatic action of its penetration gland, digests the host epidermis. The larva loses its own epidermis so becoming a sporocyst at which stage it enters the snail, the whole process taking only about 30 minutes. Inside the snail host the young sporocyst gives rise to rediae, daughter rediae and finally cercariae. A single miracidium can ultimately give rise to about 600 cercariae. Cercariae leave the snail to encyst on grass as metacercariae. Following ingestion by sheep and other hosts, the parasite excysts in the intestine and migrates through the gut wall and body cavity to the liver where it devours liver parenchyma on its way to take up residence in the bile ducts. The immature fluke becomes sexually mature and starts to lay eggs; hence the cycle starts again (Grove and Newall, 1979). These migrating fluke stages cause intestinal perforation along with necrosis and serious haemorrhage of the liver.

Migrating and developing *F. hepatica* elicit in their definitive host both humoral (Hanna 1980a, Chapman and Mitchell, 1982a) and cellular immune responses (Bennet, Hughes and Harness, 1980, Davies and Goose, 1981). The titre of specific antibody in the bloodstream of sheep and cattle reaches a maximum during the first six weeks of infection but declines once the flukes reach the bile ducts (Movsesijan and Jovanovic, 1975, Hanna and Jura, 1977). However, the production of antibodies has little protective significance to the host, at least during primary infection. Sheep seem to be unable to resist secondary and subsequent infections with *F. hepatica* (Reviews by Dawes and Hughes, 1964, 1970, Smithers, 1976) but infected cattle (Kendall, 1967, Ross, 1967) and rats (Hayes et al., 1972) can partially destroy a challenge infection. Multiple mechanisms to account for the parasite's ability to evade immune responses have been proposed, including the rapid turnover of the surface glycocalyx (Bennet, 1978, Hanna, 1980b, Duffus and Franks, 1980, 1981), the release of lymphotoxic substances (Goose, 1978), and the elaboration of proteases capable of cleaving host immunoglobulin (Chapman and Mitchell, 1982a).

During its development in the final host, a series of morphologically different granules appear in the tegument of *F. hepatica* (Bennet and Threadgold, 1975). These granules have been designated T0, T1 and T2. T0 granules are associated with metacercariae and newly excysted juveniles (NEJ), T1 with liver migratory stages and T2 with pre-bile and bile duct stages. Tegumental antigens appear to be expressed at different stages of development and this expression coincides with the presence in the tegument of the different granules (Bennet, 1978, Hanna, 1980a, 1980c, Dalton and Joyce, 1987). The earliest and strongest antibody response in infected cattle and sheep is against the T1 antigen. This is perhaps because the tegumental surface of the invading juvenile flukes is continually exposed to the host. However, after 6-7 weeks just before the maturing flukes reach the "immunological safety" of the bile ducts, the anti-T1 antibodies begin to level off before declining and anti-T2 antibodies appear. The latter eventually decline 5-6 weeks later. Using an indirect fluorescent antibody labelling

technique, Hanna (1980b) found that a section of mature fluke containing mainly T2 granules would show little fluorescence against antisera taken early in infection. In contrast, antisera taken late in infection i.e. 21 weeks post infection (PI) showed marked fluorescence to mature fluke tegument. The reverse was true for immature fluke containing T1 granules showing strong fluorescence with early antisera but not with antisera obtained later in infection. Hence the molecular composition of the surface glycocalyx changes which possibly affords the migrating flukes protection against mounting antibody responses and also may prepare them for the changing environment i.e. gut to liver to bile duct. The continual turnover of the glycocalyx estimated by Hanna (1980a) to take place every 3 hours may also protect the migrating flukes from immunological attack by simply shedding off any bound antibody.

Considerable variation exists between different hosts in their ability to develop host-protective immunity. Rats and cattle become resistant to reinfection while sheep and mice do not. Hayes et al. (1972) and Goose et al. (1973) reported a marked resistance in rats to reinfection with *F. hepatica* after one immunizing infection. This resistance is apparent within 2 weeks of initial infection (Goose's phD thesis, 1977, Hughes et al., 1977) but diminishes in aging infections (Hughes et al., 1977). Resistance does not depend on the presence of the primary infection at the time of challenge (Goose et al., 1973), and operates against intraperitoneally implanted metacercariae, juvenile or adult flukes as well as against orally administered metacercariae. Subcutaneous or intraperitoneal implantation with adult flukes does not stimulate marked resistance to challenge (Goose thesis, 1977, Anderson et al., 1977), and these implanted flukes may persist in the host for a long time. Haroun et al. (1980) however found that adult flukes in diffusion chambers implanted intraperitoneally elicited significant resistance to challenge. Some other workers have failed, however, to stimulate host protective immunity in rats (Davies et al., 1979, Lehner and Sewell, 1979) ; these contradictions may be due to different host strains and immunization protocols used.

Cattle, like rats, are able to resist reinfection and to eliminate primary infection. Corba et al. (1971) were able to transfer resistance from an infected calf to its monozygotic twin using lymphoid cells. However, Oldham and Williams (1985) found antibody-mediated mechanisms to be the most important in immunological resistance. Hillyer at al. (1987) reported a 55% reduction of fluke burdens in cattle using a mature fluke extract cross reactive with *Schistosoma mansoni* as sensitizing antigen. Hall and Lang (1978) successfully immunized cattle against infection using E/S antigens.

Attempts to immunize mice against infection have generally been unsuccessful (Lehner and Sewell, 1979, Rajasekariah et al., 1979, Burden et al., 1982) except for experiments reported by Lang (1976) and Lang and Hall (1977). Chapman and Mitchell (1982b) were also unable to induce resistance in mice using antigen extracts from 14-and 16-day-old flukes. The same extracts were used to immunize rats where resistance to challenge infection was observed.

Sheep are susceptible hosts and neither expel a primary infection nor become resistant on reinfection (Haroun and Hillyer, 1986). Attempts to induce an acquired resistance to challenge in sheep either by sensitization with a metacercarial infection (Sinclair, 1962), a mature fluke infection (Sandeman and Howell, 1981), with an immature antigen/sheep antibody complex (Sandeman et al., 1980) or homogenates of lymph nodes and spleen from infected sheep (Sinclair, 1971) have not been successful.

Wikerhauser (1961) showed that the development of *F. hepatica* in rabbits could be attenuated by irradiation of the metacercarial stage. He used irradiation doses of between 3 krad (kr) and 20 kr and he showed that very few of these flukes reached maturity, even though the treated metacercariae were still capable of hatching and infecting rabbits. Rabbits infected with these irradiated metacercariae did produce an antibody response. Hughes (1963) made various attempts to immunize mice, rabbits and sheep using *F. hepatica* metacercariae X-irradiated at 4 kr but all were without success. Boray (1969), using metacercariae irradiated

at 20 kr, failed to produce a resistance to oral *F. hepatica* challenge in either sheep or calves. Rats do not become resistant to a normal fluke challenge when sensitized with an oral infection of 4 kr γ-irradiated metacercariae (Hughes, Doy, Burden and Oldham, 1981). However, rats do become resistant when sensitized with an oral infection of 3 kr γ-irradiated metacercariae (Hughes et al., 1982). At irradiation levels of 4 kr or greater flukes do not develop beyond the T0 stage, hence the host does not receive the immunological stimulus from T1 or T2 granules. Therefore, Burden et al. (1983) suggested that for immunity to develop in rats, the sensitizing fluke infection must have developed to the stage where T1 and/or T2 granules are produced.

Hanna and Trudgett (1983) produced 6 monoclonal antibodies against a surface antigen of *F. hepatica* which was shown by ultrastructural localization to be present within the T1 secretory granules in the tegument of immature flukes (Threadgold, 1967). The T1 antigen was found to occur not only in the tegument but also in the gut and excretory system of juvenile and adult flukes. They also demonstrated that T0 and T1 granules share common epitope, as was suggested by Hanna (1980a). When isolated by immunoadsorption and separated electrophoretically under reducing conditions, T1-type antigen was found to consist of a polypeptide of molecular weight 50 kDa, possibly linked to polypeptides of molecular weights 25-40 kDa (Hanna and Trudgett, 1983). Although all the monoclonals appeared to bind the same T1 epitope, this was not the determinant reactive with antibodies in the serum of infected sheep and cattle. Vaccination trials using a purified T1 antigen did not produce significant levels of resistance in rats and mice (Hanna and Trudgett and Anderson, 1988).

The E/S products of *F. hepatica* have been recognized as important in the development of antibody production in fascioliasis (Lang et al., 1977, Howell et al., 1979, Sandeman et al., 1981). Rajasekariah et al. (1979) found that *in vitro* E/S products from immature flukes produced significant resistance to challenge in rats but not in mice. E/S products from 13- to 14-day-old flukes were found to provide a slight degree of protection to a challenge

infection (Burden et al., 1982b). Haroun et al. (1982) showed that the protective capacity of serum from infected cattle was eliminated by absorption of the serum with the culture fluid from adult flukes before serum transfer to the rat.

Cross reactions occur between *F. hepatica* and other helminth parasites. Probably the most important is the cross reactivity between *S. mansoni* and *F. hepatica* which has been extensively studied (Hillyer, 1976, 1982). Schistosomiasis is responsible for 800,000 human deaths throughout the world each year (Capron et al., 1987). Capron et al. (1968) first discovered that Schistosomes and *F. hepatica* shared common and /or cross reactive antigens. Infection with *F. hepatica* has offered some protection against challenge with *S. mansoni* organisms. Hillyer (1976) demonstrated that mice infected with *F. hepatica* for 9 weeks developed 56% less *S. mansoni* worms than controls. Christensen et al. (1980) found that mice infected with *F. hepatica* from around 3.5 weeks before, to 3 days post-challenge developed resistance to challenge with *S. mansoni.* The reverse is also the case : mice infected with *S.mansoni* developed antibodies reactive with *F. hepatica* worm antigens by 7 weeks of infection (Hillyer and Sagramosa de Ateca, 1980). Cross protection therefore between *F. hepatica* and *S. mansoni* occurs in both directions. More recently, monoclonal antibodies have been produced to demonstrate this cross reactivity between *F. hepatica* and *S. mansoni* (and other trematodes) and immunofluorescent studies with this monoclonal antibody has shown different anatomic localizations of these antigens by the trematodes e.g. these antigens were confined to the surface of adult *S. mansoni*, while they were localized mainly in the parenchymal tissue of immature and mature *F. hepatica* (Aronstein et al., 1985a, 1985b, 1986).

Vaccination offers an attractive adjunct to existing helminth control measures based on chemotherapy. Unfortunately, however, the early optimism of workers in this field has in few instances been justified. *F. hepatica* larvae and adults have evolved very efficient mechanisms of "immune evasion". As already mentioned, a mechanism reported by Goose (1978) to account for the parasite's ability to evade the immune response could be the release of

substances in the E/S products which are toxic to lymphocytes. When juvenile *F. hepatica* were incubated *in vitro* with serum and peritoneal cells from normal rats, 4.9% of the flukes were coated with cells. However, when incubated in serum and peritoneal cells from *F. hepatica*-resistant rats, 75.8% of the flukes were coated with cells. When he included E/S products from *F. hepatica* in the immune serum the number of flukes with cells attached was reduced to 10.9%. Cellular attachment is likely to be an early step in the immune destruction of the fluke by the host. The results obtained by Goose (1978) clearly shows that the *F. hepatica* E/S products do prevent this attachment *in vitro*. Hanna (1980b) suggests that this active principle in the E/S products toxic to immunocompetent cells may be a proteolytic enzyme. Chapman and Mitchell (1982a) found that *F. hepatica* E/S products contain thiol proteases capable of cleaving immunoglobulin (Ig) of mouse, rat, rabbit and sheep. Because the Ig is cut into Fab and Fc fragments (Fig 1), 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 specific cleavage of the IgG at its hinge region is similar to the action of papain on the immunoglobulin molecule (Fig 2). They showed that the proteolytic activity of the secreted enzymes is optimal at pH 3.5-4.5, but activity is also present at pH 7.0. Proteolysis was shown to be enhanced in the presence of dithioreitol (DTT) and cysteine. Based on these studies and on studies using protease inhibitors, the *F. hepatica* enzyme was described as a cathepsin B-like protease.

The thiol or cysteine proteases are a member of the general group of proteases (Ward, 1985) (Fig 3). These thiol, or SH-dependent proteases have been identified in plants (Glazer et al., 1971), animals (Barrett et al., 1980), viruses (Bazan et al., 1988), bacteria (Morihara et al., 1974), and eukaryotic microorganisms (North, 1982). They are activated by reducing agents e.g. cysteine and are susceptible to sulphydryl reagents. All are endopeptidases with a critical cysteine residue in the active site. Cys-25 has been identified in the active site of the thiol protease papain by irreversible inhibition with [^{14}C]-iodoacetate and with the active site

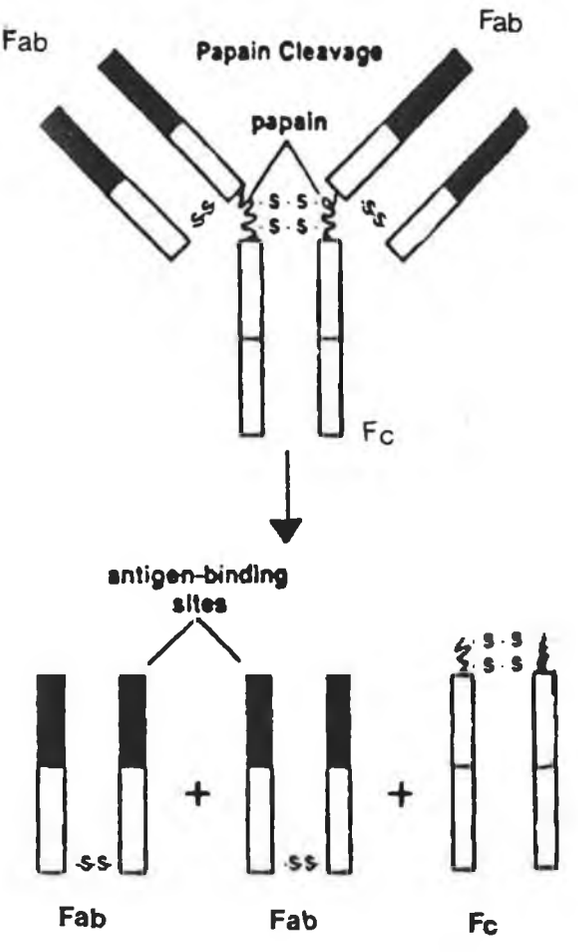

Fig. 1. Cleavage of IgG by papain to produce Fab and Fc fragments.

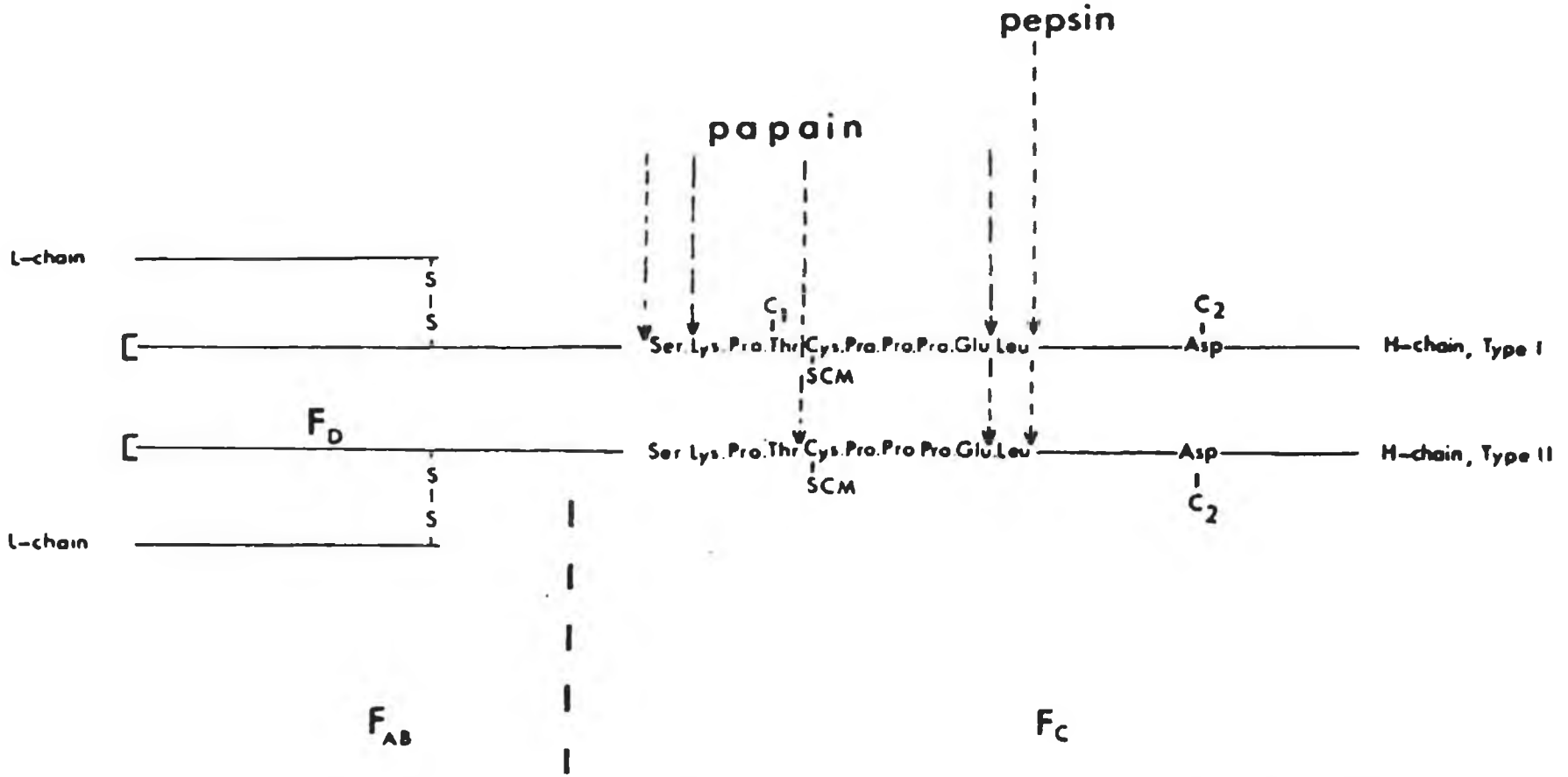

Fig. 2 **Chain structure of rabbit IgG, showing detailed structure at the hinge region. The arrows indicate alternative sites of papain cleavage. Molecules lacking the carbohydrate C_1 undergo additional cleavage at the NH_2 group of the adjacent cystine residue.**

(Smith et al., 1967)

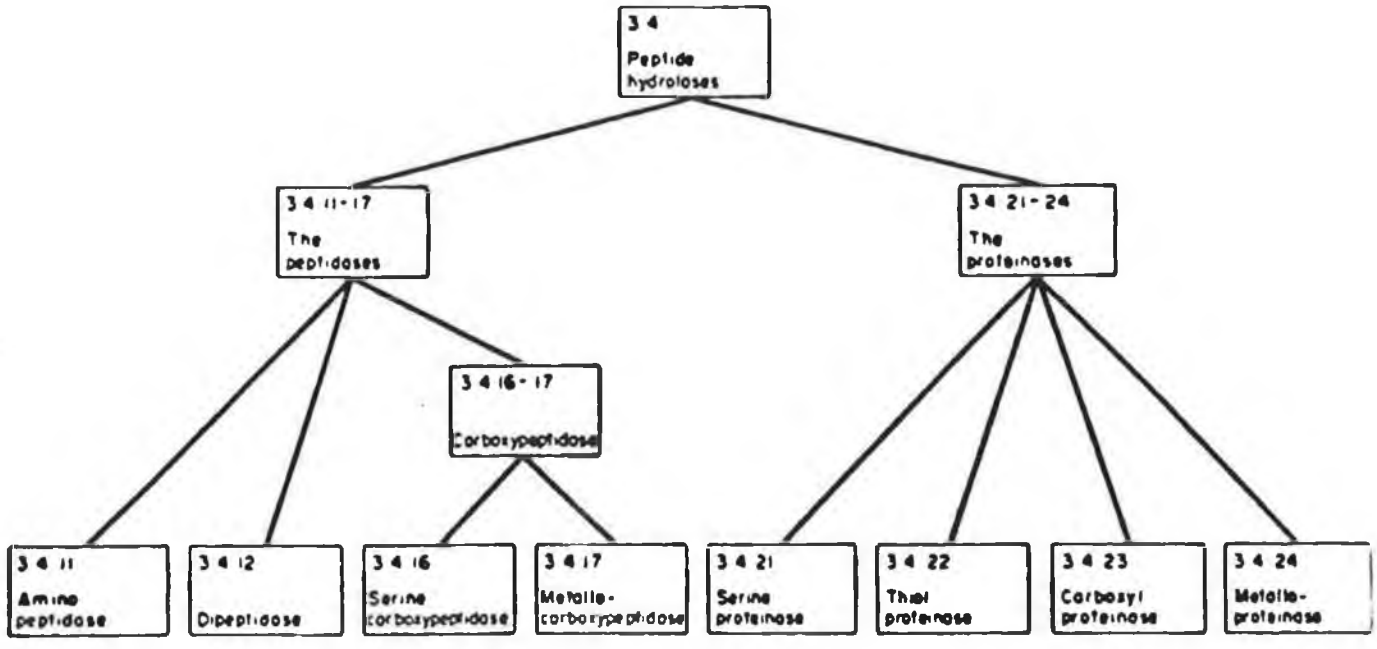

Fig. 3. Classification scheme for the peptide hydrolases. (Ward, 1985).

directed irreversible inhibitor chloroketone (Lowe et al., 1966). Reagents more specific for the active site cysteine residue have been described ; these include 2, 2'-dipyridyl disulphide used at pH 4 (Brocklehurst & Little, 1973), and more recently the peptidyldiazomethanes (Green & Shaw, 1981). The diazomethanes are unreactive with free cysteine, but react irreversibly with cysteine proteases, and not with some serine, aspartic and metalloproteases. L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) rapidly inactivates papain and other cysteine proteases (Barrett et al., 1982). Also in the active site of papain (and other thiol proteases) a histidine residue has been found (Drenth et al., 1971). Of the lysosomal thiol proteases cathepsins B, H and L are among the most abundant ; cathepsins T, N, P and S appear to be minor constituents (Barrett, 1977).

Cathepsin B is an acidic thiol protease and has been identified in various stages and species of parasitic helminths (Chapman & Mitchell, 1982, Dresden et al., 1979). Sequence analysis has demonstrated that cathepsins B and H are related to each other and to the plant thiol proteases papain and actinidin (Takio et al., 1983). The term cathepsin, meaning to digest, was proposed for acidic protease activity found in aqueous extracts of a variety of animal tissues (Colowick and Kaplan, 1970) . The different cathepsins are differentiated on the basis of substrate specificity and sensitivity to various inhibitors, properties which have led to their identification and characterization in a variety of normal mammalian tissues (Kirschke et al, 1980, Barrett, 1977) and in human (Mort et al., 1980) and mouse mammary carcinomas (Recklies et al., 1982). One of the more specific substrates for cathepsin B which distinguishes it from other cathepsins is its preference for hydrolyzing substrates which have a pair of arginine residues such as Z-Arg-Arg-AFC (Barrett and Kirschke, 1981). Inhibitors of Cathepsin B include iodoacetate, chloroquine, leupeptin and antipain. The pH range for optimal catalytic activity is 3.0-4.0 (Colowick and Kaplan, 1970). Cathepsin B is unstable above pH 7.0. Many published reports agree in attributing a molecular mass of 24-27 kDa to cathepsin B (Barrett, 1977).

Several organisms produce enzymes capable of cleaving immunoglobulins. IgA cleaving proteases are found in *Neisseria gonorrhoeae*, *N. meningitidis*, *Streptococcus pneumoniae* (the three leading agents of bacterial meningitis), *Haemophilus influenzae*, *S. pneumoniae*, *S. sanguis* and other species that infect the oral cavity (O'Reilly et al., 1986) (**Fig.4**). Only the pathogenic Neisseriae produce IgA1 protease (Mulks and Plaut, 1978, Plaut et al., 1975); non-pathogenic or opportunistic species do not produce this enzyme. The organisms often colonize mucosal surfaces, where the predominant isotype of antibody is IgA. Cleavage of secretory IgA would presumably enhance the ability of an organism to survive on mucosal surfaces. These IgA proteases are specific for IgA, usually the IgA1 isotype and they cleave the IgA1 heavy chain into two fragments, Fab and Fc (Plaut, 1983). The peptide sequences which these enzymes hydrolyze vary between organisms, but they occur within the hinge region of the heavy chain (this region is missing in human IgA2 , and thus this molecule is not cleaved). In IgA1 the hinge region contains an unusual, repeated polypeptide that is notably rich in proline. The IgA1 protease genes from *N. gonorrhoeae* (Koomey et al., 1982, Pohlner et al., 1987) and *H. influenzae* (Bricker et al., 1983) have been cloned and expressed in *E.coli.*

Eisen and Tallon (1977) reported that the protozoan, *T. pyriformis*, was capable of excreting "fabulating" enzymes capable of cleaving both bound and free Ig to produce univalent fragments which bind or remain bound to the ciliar antigens, and protect the animals from further antibody treatment. This protects the parasite, as cell bound Fab would not activate complement or macrophages.

Cysteine proteases secreted by malaria parasites have been found. It is not yet known, however, if these proteases cleave Ig. The lack of information about these proteases may be related to the short time-lapse of the merozoite-erythrocyte invasion step and the loss of activity due to external release of the proteases into the plasma. However, some research has been carried out on these proteases. Parasite proteases appear to have important roles at several different stages of the erythrocytic cycle of malaria parasites: (a) trophozoites degrade

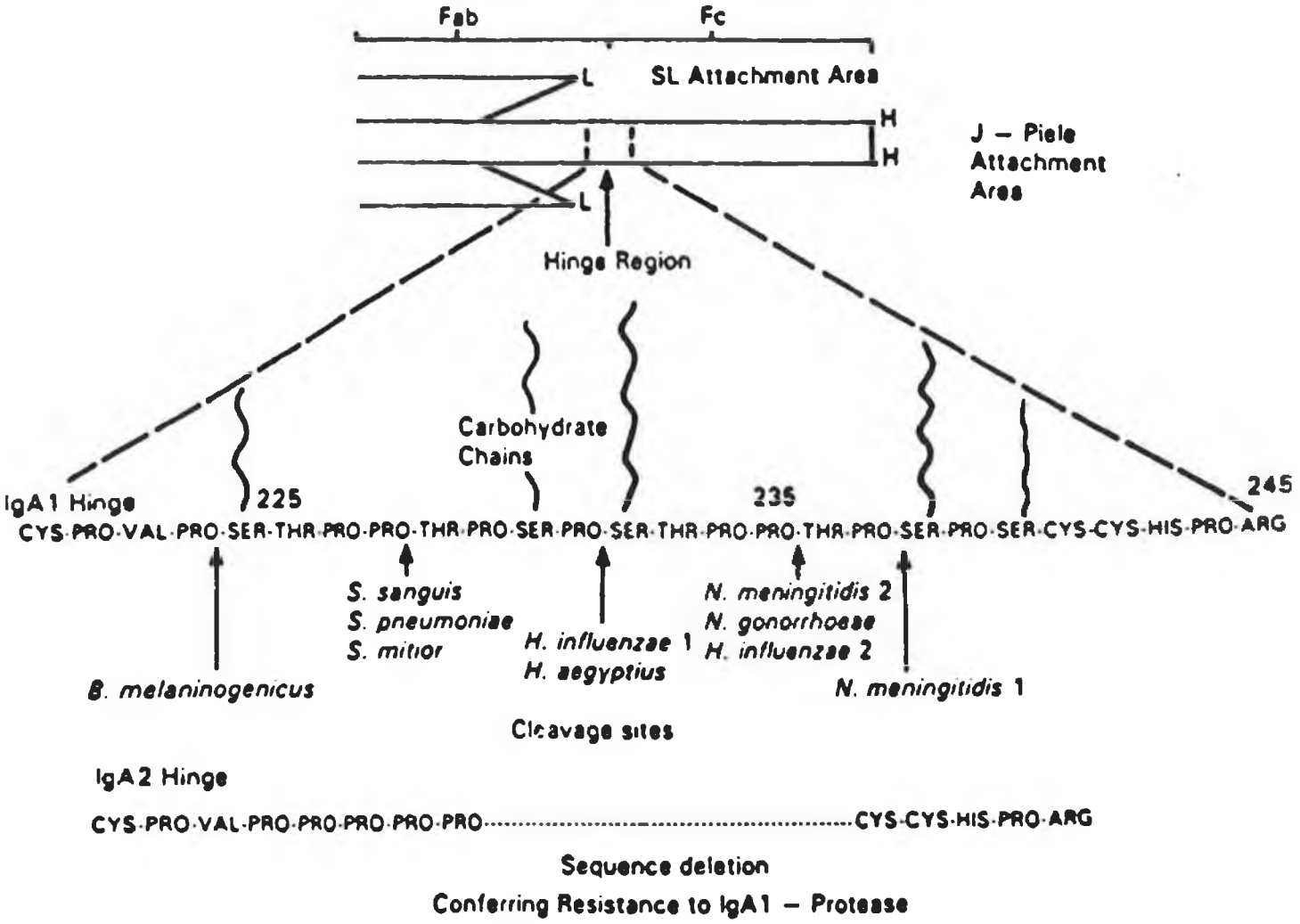

Fig. 4. Comparison of the cleavage sites of IgA1-ptotease from various bacterial sources. (O'Reilly et al., 1986).

host erythrocyte hemoglobin to provide amino acids for protein synthesis (Sherman, 1979); (b) schizont and merozoite proteins are proteolytically processed during the final stages of schizogony (Holder and Freeman, 1982, David et al., 1984) ; (c) the proteinase inhibitors chymostatin and leupeptin prevent the rupture of erythrocytes by mature schizonts (Hadley et al., 1983, Lyon et al., 1986); and (d) chymostatin inhibits the invasion of erythrocytes by merozoites (Hadley et al., 1983). Rosenthal et al. (1987) discovered three proteinases secreted by *Plasmodium falciparum* and each was active at a different stage of the life cycle. Proteinase activity was detected at 28 kDa with trophozoites, at 35-40 kDa with schizonts, and at 75 kDa with merozoites. They have since found that the 28 kDa trophozoite proteinase is efficiently inhibited by highly specific diazomethylketone and fluoromethylketone, inhibitors of cathepsin B and L. (Rosenthal et al., 1989). They suggested that specific inhibitors of the proteinases might provide new means for antimalarial chemotherapy.

Proteases were detected in aqueous extracts of the canine heartworm, *Dirofilaria immitis*. Enzymes were found capable of hydrolysing Azocoll, a general proteinase substrate, at pH's 7, 8 and 9 (Tamashiro et al., 1987). Sensitivities to a variety of protease inhibitors indicated that these enzymes were of the serine class of proteins. IgG was hydrolyzed into low M.Wt. fragments by both the microfilarial extract and viable microfilariae.

Acidic proteinases secreted from *S. mansoni* were first described by Timms and Bueding, (1959). It has been reported that these proteases have an acidic pH optimum (Timms et al., 1959, Deelder et al., 1977, Dresden et al., 1981), and molecular masses of approximately 27 kDa (Grant et al., 1971) to 32 kDa (Deelder et al., 1977). Zussman and Bauman (1971) reported that proteolytic activity from *S. mansoni* was inhibited by a variety of compounds including dithiocarbamates, cyanates, iodoacetic acid and TLCK. By the use of specific inhibitors it was established that these adult worm proteinases belong to the class of thiol proteinases (Deelder et al., 1977, Dresden et al., 1981). Asch & Dresden (1979) found that extracts of the eggs of *S. mansoni* exhibited proteolytic activity. They found these enzymes

to behave in a similar mode to cathepsin B. Dresden et al. (1979) reported the release of a thiol protease from adult *S. mansoni.* This enzyme had a marked specificity for hemoglobin and its properties also resembled those of a cathepsin B. Auriault et al., (1981) demonstrated that 4h-20h old *S. mansoni* secrete proteolytic enzymes. At least two types of proteinase activities have been found. One is an endoprotease activity with trypsin-like activity, with an optimum pH of 7.0 and an optimum temperature of 45°C. The other is a metalloaminopeptidase with an optimum pH of 7.0 and a temperature of 37°C. They showed that bound and free IgG both undergo proteolytic cleavage producing peptidic fragments in the culture medium. Auriault et al. (1981) suggested that the IgG molecules are initially cleaved into large fragments by the endoproteases and then these fragments are rapidly hydrolysed into small peptides by both the aminopeptidases and endoproteases. They ascribe this activity to possible escape mechanisms at the host-parasite interface.

Therefore, it appears that many parasitic helminths that penetrate or feed on host tissues secrete proteolytic enzymes (Hotez et al., 1985, Mc kerrow et al., 1985, Petralanda et al., 1986, Sakanari et al., 1988). Such enzymes play an important role in the migration of *F. hepatica* in its host. The parasite enters its host by penetrating the gut wall and then feeding on and burrowing through the liver mass before migrating into the bile duct. Howell (1966) demonstrated that immature fluke release enzymes *in vitro* capable of cleaving Azo-collagen. He suggested that *in vivo* these enzymes are involved in the penetration of the liver tissue. As already mentioned, Chapman & Mitchell (1982a) showed that *F. hepatica* secrete protease(s) capable of cleaving IgG and showing activity similar to that of cathepsin B or papain. They suggested that this action on immunoglobulin molecules may afford the parasite protection from host immune attack. We suggest that these IgG-cleaving enzymes may be those that resulted in the detachment of peritoneal cells including eosinophils from NEJs in the presence of immune serum and E/S products as observed by Goose (1978).

Various enzyme activities have been identified in the E/S products from *F. hepatica* including

collagenase, hemoglobinase (Locatelli and Paoletti, 1969) and IgG-cutting activity. However, a complete protease analysis of these *in vitro*-released products has not been reported. The aim of this study is first to investigate how many proteases are released by the fluke ; these proteases are then purified and biochemical characteristics such as pH optimum, apparent M.Wt., inhibitor studies and substrate specifity of the purified enzymes determined. Particular attention is paid to the IgG-cutting enzyme which was identified as a cathepsin B.

3.0 MATERIALS

AGAR AIDS

Gelatin Beem Capsules

BACHEM AG BUBENDORF SCHWEIZ

Z-Phe-Arg-Amino-Methyl-Coumarin (AMC)

BDH CHEMICALS Ltd., POOLE, ENGLAND

Ammonium Persulphate, analar grade

N,N'-Methylenebisacrylamide (Bisacrylamide), electrophoresis grade

L-cysteine

D-glucose

Glycine

Sodium Dodecyl Sulphate (SDS)

Tetramethylenediamine (TEMED)

Tris (hydroxymethyl) methylamine (2-amino- (hydroxymethyl) propane-1,3-diol) (TRIS)

Triton X-100, scintillation grade

BIO-RAD

Affi-Gel 501 Organomercurial Agarose

FLOW LABORATORIES

Gentamycin Reagent Solution

Microslides

GIBCO LABORATORIES

RPMI Medium

Bacto-tryptone

Bacto-yeast extract

ICN BIOLOGICALS Ltd.

Anti-rabbit IgG-Alkaline Phosphatase Conjugate

Anti-rat IgG (H+L)-Alkaline Phosphatase Conjugate

Anti-rabbit IgG-Fluoroscein (FITC) Conjugate

Anti-rat IgG-FITC Conjugate

KOCH LIGHT Ltd., SUFFOLK, ENGLAND

Dithioreitol (DTT)

Nitric Acid

Sodium Bicarbonate

Sodium Carbonate

PLASTIPAK

Sterile Syringes

POLYSCIENCES INC., WARRINGTON, PA

JB4 Plastic Resin

RIEDEL DE HAEN

Acetic Acid

Bromophenol Blue

Calcium Carbonate

Citric Acid

Formaldehyde

Glycerol

Isopropanol

Magnesium Chloride-6-Hydrate

Mercaptoethanol

Methanol

Potassium Chloride

Potassium Dihydrogen Phosphate

Sodium Acetate

tri-Sodium Citrate-2-Hydrate

Sodium Chloride

di-Sodium EDTA

Sodium Dihydrogen Phosphate

di-Sodium Hydrogen Phosphate

Sodium Hydroxide

Tween 20

Zinc Chloride

SCHLEICHER & SCHUELL

Nitrocellulose paper

SIGMA CHEMICAL COMPANY Ltd., DORSET, ENGLAND

Agarose, molecular biology grade

Ampicillin

5-Bromo-5-Chloro-3-Indolyl Phosphate (BCIP)

Coomassie Blue Dye

Diethyl Pyrocarbonate (DPC)

Dimethylformamide (DMF)

trans-Epoxysuccinyl-L-Leucyl Amido (4-Guanidino) Butane E-64

Freund's Complete and Incomplete Adjuvant

Gelatin

HEPES

Iodoacetimide

Isopropyl -D-thiogalactopyranoside (IPTG)

Leupeptin

MW-SDS 70L MWt Marker kit

Pre-stained Molecular wt Markers

Nitro Blue Tetrazolium (NBT)

p-Nitrophenyl phosphate (Sigma 104 Phosphatase)

Papain

Phenyl Methyl Sulphonyl Fluoride (PMSF)

Potassium Dichromate

Protein A-Agarose

Silver Nitrate

4.0 METHODS

4.1 PREPARATION OF EXCRETORY/SECRETORY (E/S) PRODUCTS

4.1.1 Isolation of flukes from rat livers

Immature *F.hepatica* were teased from the livers of female white Wistar rats five weeks after oral administration of 30 metacercariae obtained from *Limnaea truncatula* (Compton Paddock, U. K.). Mature flukes were removed from the bile ducts of rats twelve weeks after infection. Flukes were washed six times in phosphate-buffered saline (PBS), pH 7.3 at 37°C and then cultured overnight. Some flukes were also removed and homogenized by hand in cold PBS in a glass-glass homogenizer. The homogenate was centrifuged at 10,000 x g for 30 min and the supernatant stored at -20°C.

4.1.2 Isolation of flukes from bovine liver

Mature *F.hepatica* were removed from the bile ducts of naturally infected cattle (Anglo-Irish Meat Processor's abbatoir, Ballymun, Dublin). The flukes were washed six times in PBS, pH 7.3 at 37°C.

4.1.3 *In vitro* maintenance of flukes

The PBS-washed flukes from rat or bovine livers were cultured (six immature flukes or one mature fluke per ml) in RPMI-1640, pH 7.3, containing 2% glucose, 1% rat serum, 30mM Hepes, 25 mg/l gentamycin and 7.5% sodium bicarbonate buffer. For purification and High Pressure Liquid Chromatography (HPLC) analysis of proteases, flukes from bovine livers were cultured in the same medium except that the rat serum was omitted. After 16h incubation, in 4% CO_2 atmosphere and at 37°C, the culture medium (containing *in vitro*

released products) was removed and centrifuged at 10,000 x g for 30 min. The supernatant was divided into aliquots and stored at -20°C. This preparation is referred to as fluke excretory/secretory (E/S) products.

4.2 PURIFICATION OF ENZYME

4.2.1 Affinity Chromatography

Affinity Chromatography with Affi-Gel 501 Agarose was used to purify thiol proteases from the adult fluke E/S products. The organomercurial agarose readily binds any free sulfhydryls (Barrett, 1973). Before applying sample to the column, the fluke E/S products were dialysed against column buffer (0.05M Na acetate pH 4.6, 0.2M NaCl, 1mM EDTA). The entire procedure was carried out at 4°C. A ml column was prepared in a 2 ml syringe. The column was washed with 5 bed volumes of distilled water followed by 2 bed volumes of column buffer. E/S products (40mls) were then passed through the column at a flowrate of 1ml/minute. When the entire sample had passed through the column, the bed was washed with 10 bed volumes of column buffer. Bound molecules were eluted by the addition of 10mM cysteine, the competing sulfhydryl, to the column buffer.

4.2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were analyzed by one dimensional SDS-PAGE according to the method of Laemmli and Favre (1973) on gels containing 10% or 15% (W/V) polyacrylamide, 0.27% (W/V) bisacrylamide, 0.373M Tris/HCl, pH 8.8, 0.1% (W/V) SDS, 0.03% (W/V) ammonium persulphate and 0.008% (W/V) TEMED. The stacking gel contained 3% (W/V) polyacrylamide, 0.08% (W/V) bisacrylamide, 0.125M 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 (0.12M Tris/HCl, pH 6.8, 5% (W/V) SDS, 10% (W/V) glycerol and 0.01% (W/V) Bromophenol Blue R) or reducing sample buffer (same as non-reducing buffer except 5% mercaptoethanol is included). The sample and sample buffer

were combined in a 1:1 ratio.

Gels were run in a vertical slab gel apparatus in electrode buffer containing 0.024M Tris/HCl, 0.186M glycine and 0.1% (W/V) SDS pH 8.3 at 35mA for 4-5 hours at room temperature. The proteins were stained 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 hour at room temperature. Destaining was carried out in 20% (V/V) methanol, 10% (V/V) acetic acid.

4.2.3 Gelatin-substrate SDS-PAGE

Gelatin-substrate SDS-PAGE was adapted from the method of Heussen and Dowdle (1980). Liver fluke E/S products (25µl) or PBS- extracted flukes (10µl) were mixed with an equal volume of non-reducing sample buffer. The samples were applied to a 10% SDS-polyacrylamide gel as already described with the exception that both stacking and separating gel contained 0.1% gelatin. After electrophoresis the gels were washed for 1 hour in 'incubation buffer' containing 2.5% Triton X-100 with one change. The gels were then immersed in incubation buffer alone for 12 or 24 hours at 37°C. Four different incubation buffers were tested : 0.1M glycine, pH 8.0, pH 7.0 and 0.1M sodium citrate, pH 4.5, pH 3.0. Gels were then stained in Coomassie Brilliant Blue R solution as already described.

4.3 PREPARATION OF ANTISERA

4.3.1 Antiserum against flukes

The five week-infected rats from section 4.1.1 were each bled by cardiac puncture on the same day as the flukes were removed. Uninfected rats, serving as controls, were bled simultaneously. Antiserum from ten weeks infected rats was a gift from Dr. Teresa Sloane and Dr. Patrick Joyce (U.C.D., Belfield, Dublin). The collected sera were stored at -20°C.

4.3.2 Antiserum against fluke E/S products

Antisera were produced against the following antigens:

A - crude fluke E/S products

B - thiol proteases purified by organomercurial affinity chromatography from E/S products

C - a protease of molecular weight (M.Wt.) 76 kDa excised and eluted from PAGE

D - a protease of M.Wt. 60 kDa excised and eluted from PAGE

E - a combination of proteases of M.Wts 28 kDa and 27.5 kDa excised and eluted from PAGE

4.3.3 Immunization with E/S products and purified thiol proteases

Inoculations of rats with total E/S products were carried out intraperitoneally ; in the first inoculation, antigen was emulsified in an equal volume of Freund's Complete Adjuvant and in all subsequent boosts antigen was emulsified in an equal volume of Freund's Incomplete Adjuvant. In each case the amount of protein introduced into the animal was the same whether it be the first inoculation or any subsequent boost. The rats were boosted 3-4 times. Anti-thiol protease antibodies were prepared in rabbits. The animals required 5 boosts subcutaneously to produce a good antibody response. Ten days after each boost a blood sample was taken and the antibody response monitored by Immunofluorescence and ELISA. Ten days after the final boost the animal was bled. (See table 1 for immunization regime).

4.3.4 Immunization with proteins eluted from polyacrylamide gels

Fluke E/S products were run on a 10% SDS-PAGE and stained with Coomassie Brilliant Blue R. The protein band pattern was similar to the protease pattern produced when the same sample was run on a gelatin substrate SDS-PAGE. The bands of molecular weights 76, 60, 28/27.5 kDa corresponding to proteases of the same molecular weights were cut out. The

Table 1
The following table indicates the species of animal immunised and the quantity of protein administered to the animal.

ANTIGEN	SPECIES OF ANIMAL IMMUNIZED	AMOUNT OF PROTEIN ADMINISTERED AT EACH INOCULATION
A (total E/S product	BALB/C RAT	Approx. 200μg
B (purified thiol protease)	RABBIT	Approx. 300μg
C (76 kDa)	BALB/C RAT	Approx. 10μg
D (60 kDa)	BALB/C RAT	Approx. 10μg
E (28/27.5 kDa)	BALB/C RAT	Approx. 20μg

bands 28/27.5 kDa were cut out together. The gel slices were individually homogenized by hand in PBS in a glass-glass homogenizer. The gel emulsion was then combined with an equal volume of adjuvant before immunization into the animal. (See table 1 for immunization regime).

4.4 ASSAYS TO MONITOR ANTIBODY PRODUCTION

4.4.1 Enzyme-linked-immunoabsorbent assay (ELISA)

The presence of antibodies against the antigen was tested by an enzyme linked immunoabsorbent assay (ELISA). 96-well polyvinyl plates were coated with 50µl of fluke E/S products (200µg/ml) per well and incubated at 37°C overnight. The excess protein binding sites on the wells were blocked by incubating in 200µl of Buffer A Solution (0.1% Tween and 2% Foetal Calf Serum in PBS) for 1 hour at 37°C. After one hour the wells were emptied and washed 3 times with Buffer A Solution. The infected serum from two host species was diluted appropriately in Buffer A as was non-infected serum and 100µl of the dilutions added to each well. After 1 hour incubation at 37°C, the wells were again emptied and washed 3 times with Buffer A. To detect the antibody which binds antigen a second antibody which is conjugated to an enzyme was dispensed into the wells (200µl/well). A 1 in 2000 dilution of Alkaline Phosphatase labelled rabbit anti-rat IgG (H+L) and a 1 in 1500 dilution of Alkaline Phosphatase labelled Anti-rabbit IgG (Fc) were used as the conjugated antibodies to detect bound rat and rabbit antibodies, respectively. The second antibody was diluted in the Buffer A solution. After incubation for 1 hour at 37°C the wells were then washed 3 times in Buffer A solution. 100µl of the substrate solution (1mg/ml of p-nitrophenyl phosphate in 0.1M glycine Buffer, pH 10.4, 0.001M $MgCl_2$ and 0.001M $ZnCl_2$) was added to each well. 5 minutes after substrate addition, the Optical Densities of each well were read in an EL-307 ELISA reader (BIO-TEK Ltd.) using a 405nm filter. The titre of antibody in each serum sample was taken to be that dilution of serum which gave a discernible reading above controls of similar dilutions on the ELISA reader.

4.4.2 Immunofluorescence

Antigenic localization was determined by immunofluorescence. Mature flukes from naturally infected cattle were washed in PBS and fixed with 10% Formaldehyde + $CaCO_3$ for 30 minutes (while shaking). The flukes were then washed in several changes of PBS and dehydrated quickly in a graded alcohol series to absolute ethanol. After 24 hours infiltration in JB4 Plastic Resin (monomer + catalyst) the fluke were placed in dry gelatin BEEM capsules which were half filled with the JB4 embedding mixture (monomer + catalyst + accelerator). The resin was left to polymerise at room temperature for 30-45 minutes. The capsules were then filled, the fluke orientated upright and the capsule sealed to exclude the air. The resin was left to polymerise at room temperature for 30 minutes. Sections, 6μm, were cut using an LKB Pyramatome ; sections were cut using a dry glass knife, collected with forceps and transferred to PBS drops on microslides. The slides were left to dry.

The sections of fluke were then exposed to the infected and noninfected serum serially diluted in 0.1% Tween 20 in PBS. Diluted sera (10μl) was applied to the sections and the slide left in a humid container for 30 minutes at room temperature. The serum was then rinsed off 5 times with 0.1% Tween and PBS and the sections were labelled for 30 minutes in a humid container at room temperature. A 1 in 80 dilution of fluorescein (FITC) labelled sheep anti-rat and a 1 in 80 dilution of FITC-labelled sheep anti-rabbit were used as the conjugated antibodies to test the rat and rabbit sera, respectively. The labelled antibodies were diluted in 0.1% Tween 20 and PBS. The preparations were examined under a fluorescence microscope.

4.4.3 Western Blotting

Western blotting was performed with gels following electrophoresis (Laemmli and Favre, 1973). The gel and a sheet of nitrocellulose paper (NCP) were soaked in a dish of transfer buffer (20mM Tris/NaOH, pH 8.3, 100mM Glycine) for 20 minutes. After 20 minutes, a sheet of Whatman (wetted in transfer buffer) was placed on a BIO-RAD Scotch Brite pad and the

gel placed on the filter paper. All air bubbles were eliminated. The NCP was then placed on the gel again ensuring absence of air bubbles. Another sheet of Whatman (wetted in transfer buffer) was placed on the NCP and the sandwich closed and inserted into a BIO-RAD trans-blot vertical cell pre-filled with transfer buffer. The sandwich was inserted so that the NCP was nearest the anode. The cell was run at 150mA for 8 hours, or overnight.

When electroblotting was complete, the NCP was removed for immunodetection. The excess binding sites were blocked by incubating the NCP in buffer A solution (0.1% Tween 20 + 2% FCS in PBS) for 1.5 hours at room temperature (RT). The NCP was then removed and placed in PBS + 0.1% Tween 20 + an enzyme-labelled antibody (1µl/ml) for 1 hour at RT. The NCP was then transferred to the substrate solution (66µl Nitro Blue Tetrazolium (NBT)[1] solution and 50µl 5-Bromo-5-Chloro-3-Indolyl phosphate (BCIP)[2] solution per 15 ml of substrate buffer[3]).

1 75mg/ml NBT in 70% dimethylformamide, stored at 4°C n a dark bottle

2 50mg/ml BCIP in dimethylformamide, stored at 4°C n a dark bottle

3 0.1M Tris, 0.1M NaCl, 50mM $MgCl_2$, pH 9.5.

4.5 CHARACTERIZATION OF CRUDE E/S PRODUCTS

4.5.1 Inhibitor studies : total E/S products

Inhibitors were added to immature fluke E/S products or PBS extracts 10 minutes prior to mixing with sample buffer and to all gel incubation buffers at final concentrations of PMSF, 10 mM ; iodoacetimide, 10mM ; leupeptin, 5µg/ml ; E-64, 5µg/ml ; EDTA, 10mM ; DTT, 5mM ; and cysteine, 10mM. Stock solutions of 100mM PMSF were prepared in isopropanol.

4.5.2 Digestion of IgG by papain and by mature E/S products

IgG was recovered from normal rabbit serum by Affinity Chromatography using Protein A-Agarose as described by the manufacturer. To test for the presence of an IgG-cleaving

papain-like enzyme in the fluke E/S products four reaction mixtures were prepared :-

1) rabbit IgG only (isolated from rabbit serum by affinity chromatography)

2) 100µl rabbit IgG, 1mM EDTA, 25mM mercaptoethanol ; make up to 200µl in PBS

3) 100µl rabbit IgG, 100µl crude E/S products, 1mM EDTA, 25mM mercaptoethanol

4) 100µl rabbit IgG, 1µl of 1mg per ml papain, 1mM EDTA, 25mM mercaptoethanol

The samples were then incubated at 37°C for 1 hour.

After incubation, 30µl was taken from the tubes and an equal volume of 2X reducing buffer added to each. The samples were boiled for 2 minutes and then run on a 15% SDS-PAGE. Molecular weight standards were also applied to the gel α_2-macroglobulin (180 kDa), ß-galactosidase (116 kDa), fructose-6-P-kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa) and triosephosphate isomerase (26.6 kDa). The proteins were then electroblotted onto Nitrocellulose Paper by Western Blotting and the IgG and IgG digestion products detected using an anti-rabbit IgG-alkaline phosphatase conjugate.

4.6 CHARACTERIZATION OF E/S PRODUCTS FROM HPLC

4.6.1 Protease activity of fractions from HPLC

Crude E/S products was concentrated 10-fold by freeze-drying and dialysed overnight against 0.1M potassium phosphate buffer pH 7.0. The sample (50µl) was filtered through a 0.45µm membrane filter (Gelman Sciences) and then subjected to molecular sieve HPLC at room temperature. The latter was performed with a Waters Model 510 HPLC pump and a Waters lambda-max Model 481 liquid chromatography spectrophotometer.

The gel filtration column used was a TSK 3000 SW column. The mobile phase was 0.1M potassium phosphate buffer pH 7.0. A flowrate of 0.3 mls/min., an absorbance of 280 nm and a sensitivity range of 0.01-0.05 were used. Once the sample was applied to the column 0.3 ml fractions were collected every minute. The M.Wts of the proteins were determined after calibration of the column with proteins of known M.Wts :- IgG2a (150 kDa), bovine serum albumin (66 kDa), Horse radish peroxidase (45 kDa), and lysozyme (14.3 kDa). The fractions (20µl) were assayed for activity using gelatin substrate SDS-PAGE.

Fractions were also analyzed for cathepsin B activity using Z-Phe-Arg-AMC as a substrate. This procedure was adapted from Rege et al (1989) by Curtin (unpublished). The assay mixture (1ml total volume) consisted of 2-20µl fraction, 5mM DTT and 26.8 mol of substrate in 0.1M sodium citrate buffer pH 4.5. After incubation for one hour at 37°C, the release of the fluorescent leaving group AMC was monitored in a Perkin-Elmer 204 Fluorescence Spectrophotometer (exciter wavelength : 370 nm, analyzer wavelength : 440 nm). Enzymatic activity was expressed as µmolAMC/hr/ml.

Total E/S products and fractions from the HPLC analysis were analyzed for enzyme activity. 10µl of each fraction was used in the reaction mixture and 2µl of the total E/S products was

used as control.

4.6.2 Inhibition by Diethyl pyrocarbonate (DPC)

Different concentrations of Diethyl pyrocarbonate (DPC) were incubated with an equal volume of total E/S products for one hour at room temperature. An equal volume of 2X non-reducing sample buffer was added to each sample and these were run on a 10% SDS-PAG. The final concentrations of DPC in the E/S products were 0, 5 and 50mM DPC.

Inhibition by DPC (50mM-0.025mM) was investigated using a pool of the fractions which showed cathepsin B activity with the substrate. 2µl of this pooled sample in 48µl of 0.1 M sodium citrate buffer pH 4.5, was incubated with 50µl of the inhibitor for one hour at room temperature. This mixture was then added to the cathepsin B assay.

4.6.3. Enzymatic activity at different pHs

The pooled cathepsin B fractions were investigated at different pHs for enzymatic activity using the following buffers :- 0.1 M sodium citrate pH 3.0-5.0, 0.1 M sodium phosphate pH 6.0, and 0.1 M glycine pH 7.0 and 8.0. The substrate was also prepared in these buffers.

Inhibition of enzyme activity by DPC at different pHs was investigated. The final concentration of DPC was 50 mM. An equal volume of the inhibitor was added to the sample 5 minutes before adding to the assay mixture.

4.6.4 Digestion of monoclonal antibody IgG2a

IgG2a purified by Protein A was a gift from Dr. Edward Pearce, NIAID, National Institutes of Health, Bethesda, Maryland. The IgG2a digestion by the HPLC fractions was analyzed using 40µl of the fraction in question, 40µl of PBS, 1µl of mercaptoethanol and 1µl of 100 mM EDTA. This was incubated at 37°C for one hour. 20µl of 5X reducing sample buffer was

added and the samples were boiled for 2 minutes and loaded on a 10% SDS polyacrylamide gel.

Inhibition of IgG digestion by DPC was investigated using a pool of cathepsin B fractions. The final concentration of inhibitor was 200 mM. The pooled fractions were incubated in equal volumes (20µl) with the inhibitor for 30 mins at room temperature, and was then added to the reaction mixture (30µl IgG2a, 30µl PBS, 1µl mercaptoethanol, 1µl 100 mM EDTA) and incubated for one hour at 37°C. Samples were loaded onto a 10% SDS polyacrylamide.

4.7 IMMUNOSCREENING OF AN ADULT FLUKE cDNA LIBRARY

An adult *F. hepatica* cDNA library constructed in λgt11 was very kindly supplied by Dr. Alan Trudgett and Dr. Alan McNair, Queen's University, Belfast. The λgt11 was plated on *E. coli* host strain Y1090. Approximately 1 X 10^3 pfu per 1µl of library were obtained as determined by growth on LB plates (bottom agar : 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, 2% agarose ; top agar : same as bottom agar except only 0.7% agarose included) supplemented with 0.2% maltose and 100µg/ml ampicillin. After 8 hours of growth at 42°C, the plates were overlaid with nitrocellulose filters impregnated with 10 mM isopropyl-D-thiogalactopyranoside (IPTG), an inducer of the lac Z gene expression. After incubation for 2 hours at 37°C, the exact position of the filters on the agar was marked using a sterile needle. The filters were then removed and washed 5 times in PBS/0.1% Tween 20. The filters were then blocked in PBS/0.1% Tween 20/2% FCS for 2 hours at RT. They were washed in PBS/0.2% Tween 20 and then incubated in a 1:100 dilution of rabbit anti-thiol proteases for 1 hour at RT. The filters were then washed again in PBS/0.2% Tween 20 and incubated in an anti-rabbit-alkaline phosphatase. After 1 hour incubation the filters were washed and the substrate (NBT,BCIP) added and allowed to develop for 5-10 minutes. Once a positive recombinant clone was identified on the filter, the corresponding plaque was removed using a sterile glass Pasteur pipette. The agar plug obtained in this manner was suspended in SM

buffer (10 mM Tris/HCl pH 8.0, 2mM $MgSO_4$) and 3µl of fresh chloroform and left at 4°C overnight. The suspension was vortexed and a sample was reinfected as before into *E. coli* and plated on *E. coli* Y1090.The phages were rescreened as already described.

4.7.1 Characterization of recombinant fusion protein

E. coli Y1090 was infected with 1) parental phage and 2) recombinant phage. The infected bacteria were plated as before. After 8 hours incubation at 42°C plates were overlaid with SM buffer and the proteins were eluted by incubation overnight with gentle agitation. The overlay SM buffer was centrifuged at 8,000 x g for 10 mins at 4°C to remove bacterial debris. Aliquots of both lysates were electrophoresed and transferred to NCP. Filters were blocked and incubated in 1) 1:1000 of rabbit anti-ß-galactosidase antibody, 2) 1:100 of rabbit anti-thiol proteases and 3) 1:100 normal rabbit serum. Antibody bound to both filters was detected by incubation with a second antibody, anti-rabbit alkaline phosphatase.

5.0 RESULTS

5.1 IDENTIFICATION OF *F. hepatica* E/S PRODUCTS

Culture medium in which immature *F. hepatica* were maintained for 16 h was analyzed by gelatin-substrate SDS-PAGE. Following electrophoresis of four samples on a slab gel, the gel was sliced, and individual slices incubated at pH 8.0, 7.0, 4.5 or 3.0. A total of 11 clear bands representing proteolytic activity were visualized following staining of the gel (**Fig. 1a**). These proteases could be divided into two groups based on the pH range in which they were active; Group 1 consisted of five proteases of M.Wts 88, 83, 80, 76 and 60 kDa showing optimal activity in the pH range 3.0-4.5 and Group 2 consisted of six proteases of M.Wts 46, 44, 42, 40, 28 and 27.5 kDa which were most active in the pH range 4.5-8.0 (Fig. 1a). To confirm that these proteases were of fluke origin both rat serum and PBS extracts of rat liver were analyzed by gelatin-substrate SDS-PAGE. No proteolytic activity was detected in these samples (data not shown).

Gelatin substrate SDS-PAGE analysis showed that many proteases ranging in molecular sizes from >100 kDa to <10 kDa were present in the PBS extracts of immature flukes (**Fig. 1b**). In comparison, relatively few proteases are released *in vitro* by these parasites and many did not co-migrate with major proteases in the immature fluke PBS extract (Fig. 1b). This suggests that the proteases identified in the *in vitro*-released products were selectively released by immature flukes, and were not the result of damaged or disintegrating parasites. Gels shown in Fig. 1b were incubated at pH 4.5, since at this pH all proteases could be visualizsd (see Fig 1a).

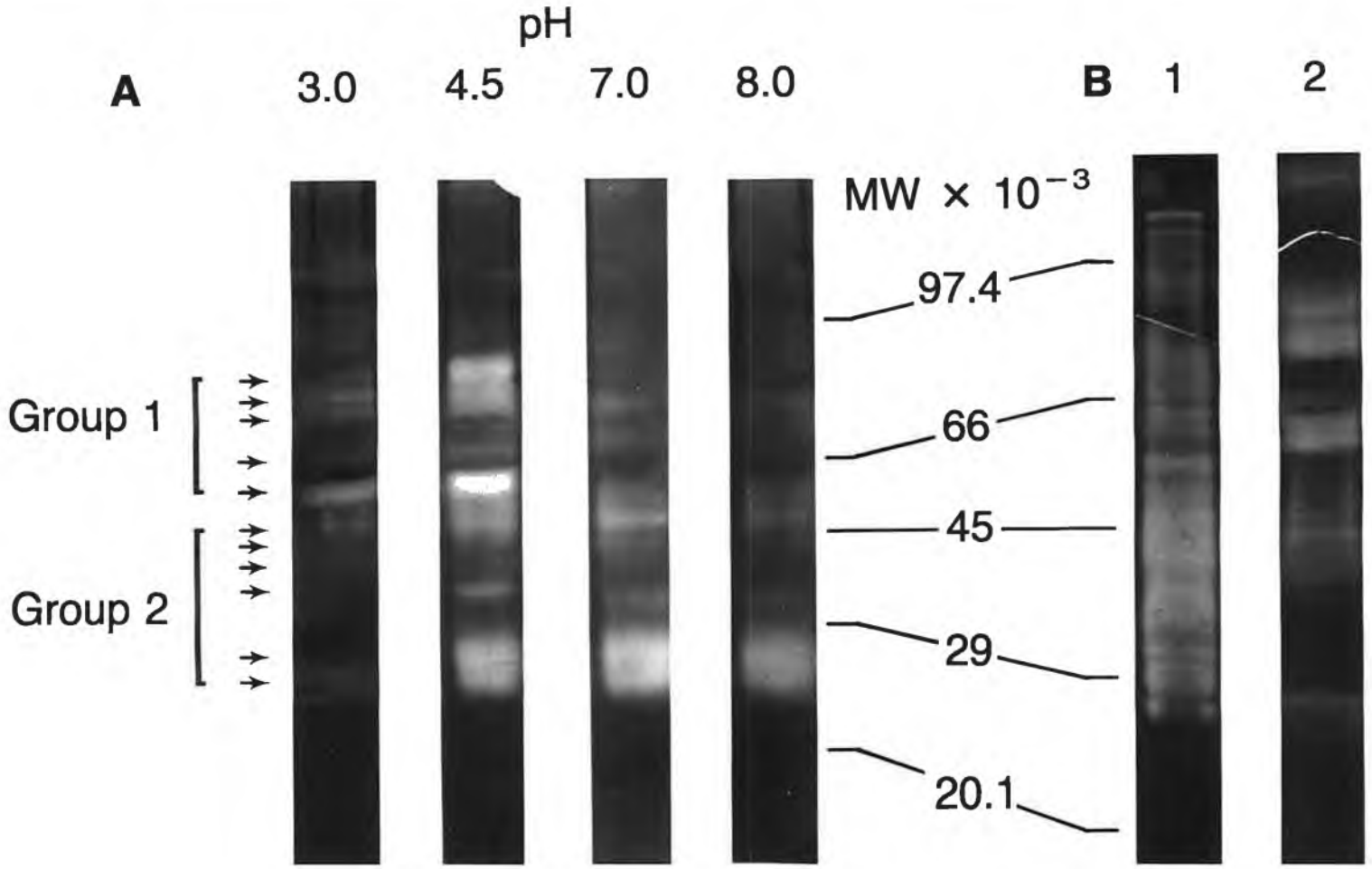

Fig 1. A. Identification of immature *F. hepatica in vitro*-released proteases in 10% gelatin substrate SDS-PAGE. Following electrophoresis, gels were incubated in 0.1 M sodium citrate, pH 3.0, 0.1 M sodium citrate, pH 4.5, 0.1 M glycine, pH 7.0 or 0.1 M glycine pH 8.0 for 24 h. Proteases are divided into two groups based on the pH range in which they are active. B. Comparison of proteases present in PBS extracts of immature flukes (lane 1) with those released *in vitro* (lane 2). Gels were incubated in 0.1 M sodium citrate, pH 4.5 for 12 h. Molecular weight markers were phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa) and soybean trypsin inhibitor (20.1 kDa).

5.2 COMPARISON OF IMMATURE AND MATURE FLUKE PROTEASES

The proteases released *in vitro* by immature and mature flukes were compared. The proteolytic profiles were similar with the exception that the immature fluke 40 kDa protease was a very minor protease released by mature flukes (**Fig. 2**, arrowed). The comparison shown in Fig. 2 was carried out at pH 4.5; comparisons carried out at other pHs did not reveal any additional differences (data not shown).

5.3 INHIBITION OF PROTEOLYTIC ACTIVITY

Protease inhibitors were used to determine the class of each protease in the immature fluke E/S products products. These inhibitors were added to the sample before application to the gel and to all gel washing and incubation buffers. The metal chelator, EDTA, had no effect on any protease activity; however, the thiol protease inhibitors leupeptin and E-64 inhibited all proteases (**Fig. 3a**).

Since these experiments suggested that all the proteases detected in the immature fluke E/S products products were thiol proteases, I tested additional thiol protease inhibitors, PMSF and iodoacetimide (Waxman, L , 1981), and also the reducing agents cysteine and DTT , which can enhance or stabilize thiol proteases. PMSF and iodoacetimide inhibited all enzymes, but at the concentrations used, they were less effective than leupeptin and E-64, since some activity of the 88-, 83-, 80- and 60 kDa proteases remained (**Fig. 3b**, lanes 3 and 4). All enzymes showed increased activity when cysteine or DTT was added to the samples (Fig. 3b, lanes 2 and 5). In other experiments DTT was added to samples 10 min after the addition of PMSF; and was included in all washing and incubation buffers with PMSF; all proteases retained activity or showed slightly enhanced activity demonstrating that DTT could reverse the inhibitory effect of PMSF (data not shown). These studies indicate that all proteases detected by gelatin-substrate SDS-PAGE were thiol proteases.

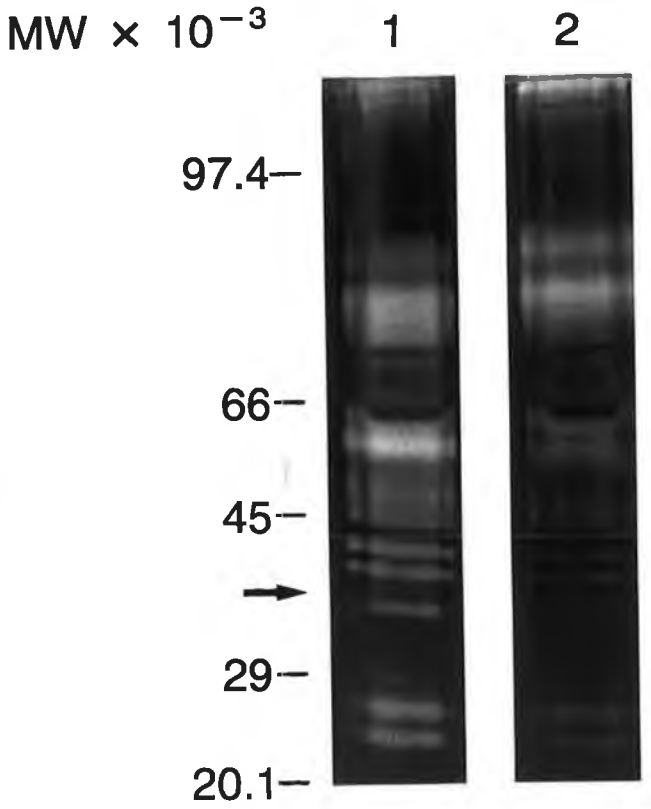

Fig 2. Comparison of proteases released *in vitro* by immature flukes (lane 1) and mature flukes (lane 2). The 40 kDa protease released by immature flukes is arrowed.

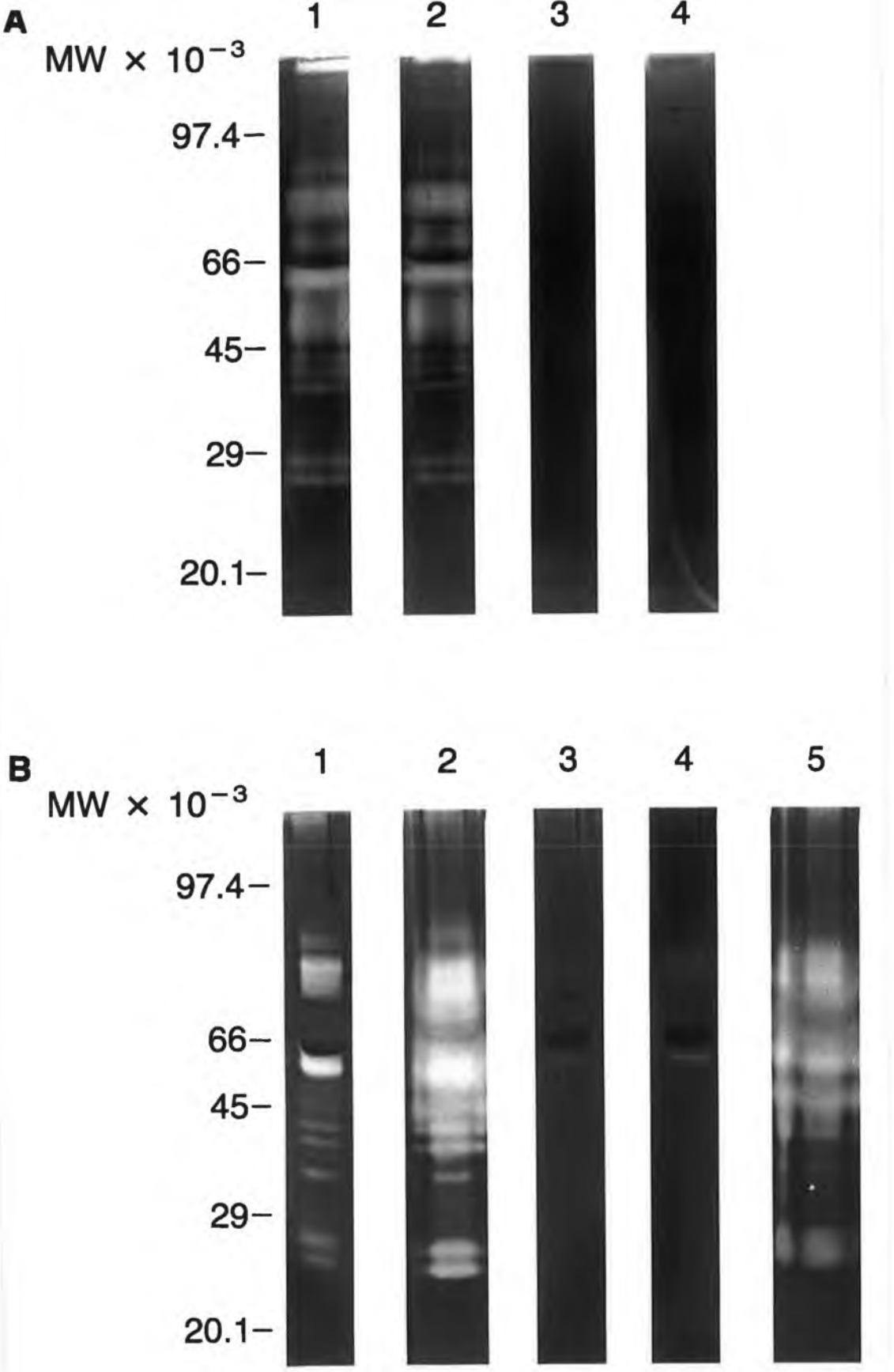

Fig 3. Effect of protease inhibitors on proteases in the E/S products of immature *F. hepatica*. Protease inhibitors were added to samples 10 min before mixing with sample buffer and loading onto gels (see text for concentrations) and also into all washing and incubation buffers. Panel (A) shows results of E/S products with no inhibitor (lane 1) EDTA (lane 2) leupeptin (lane 3) and E-64 (lane 5) added. Panel (B) shows E/S products with no inhibitor (lane 1) DTT (lane 2) PMSF (lane 3) iodoacetimide (lane 4) and cysteine (lane 5) added. Gels were incubated in 0.1 M sodium citrate, pH 4.5.

5.4 AFFINITY CHROMATOGRAPHY

The use of Affinity Chromatography to purify thiol proteinases using an organomercurial linked to Sepharose, and elution with -SH-containing compounds was reported by Barrett (1973). Since the *F. hepatica* proteinases are also thiol proteinases this technique was employed to confirm their thiol nature and also to purify the enzymes. When mature fluke E/S products were applied to the column, the results on a gelatin substrate SDS-PAGE showed that all of the 11 proteinases bound to the column and could be eluted by using 10 mM cysteine (Fig 4).

In this study we developed a cathepsin B assay (see later) so only purification data for this enzyme could be estimated. The calculation of the yield of purified cathepsin B and the purification factor can be seen in Table 2. Approximately 19.3% of cathepsin B activity and 7.4 % of the total protein applied to the column was recovered in the purified fraction, resulting in a 2.6-fold increase in specific activity of the cathepsin B.

5.5 IMMUNIZATION WITH THE PURIFIED THIOL PROTEASES

Antibodies to the thiol proteases were produced for the purpose of localizing the proteases within the fluke tissues and immuno-screening of cDNA libraries. The purified thiol proteases were injected into a rabbit and every 3 weeks the animal was boosted. The rabbit received 6 injections in total. 10 days after the 3rd, 4th and 5th and 6th booster injections, the serum was assayed for antibody production by ELISA. The antibody response is indicated in the graph shown in Fig 5a.

The final titre of antibody in the rabbit serum after the 6th booster injection was >1600. This sera was used for immunoscreening of cDNA library. 3 rats were each inoculated with individual proteases eluted from the gel (28/27.5 kDa, 60 kDa, 76 kDa). The animals were then boosted 5 times at regular intervals. An ELISA was then carried out at the final bleed taken 10 days after the final booster. The antibody response is shown in Fig 5b. The results

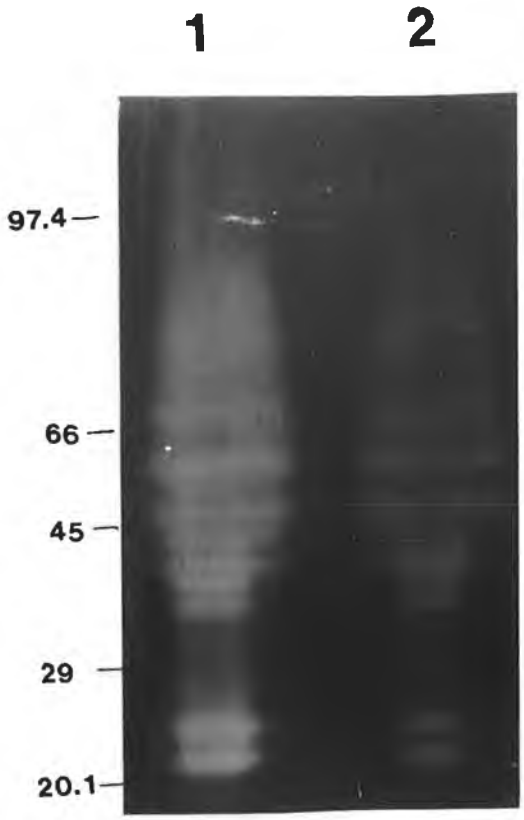

Fig. 4. Gelatin substrate SDS-PAGE analysis of E/S products from *F. hepatica* before (lane 1) and after (lane 2) purification on Affi-gel 501 column.

TABLE 2. Purification of thiol proteases from *F. hepatica* adult worms.

Fraction	Volume (mls)	Protein		Activity		Specific Activity units/mg protein	Yield	Purification factor
		mg/ml	Total mg	Units/ml	Total units			
E/S before column purification	40	0.071	2.84	76.8	3072	1082	100%	1
Purified protease (cathepsin B)	10	0.021	0.21	59.2	592	2819	19.3%	2.6

Protein was assayed by the method of Bradford (1976). Z-Phe-Arg-AMC was the substrate used for cathepsin B activity.

Fig 5a

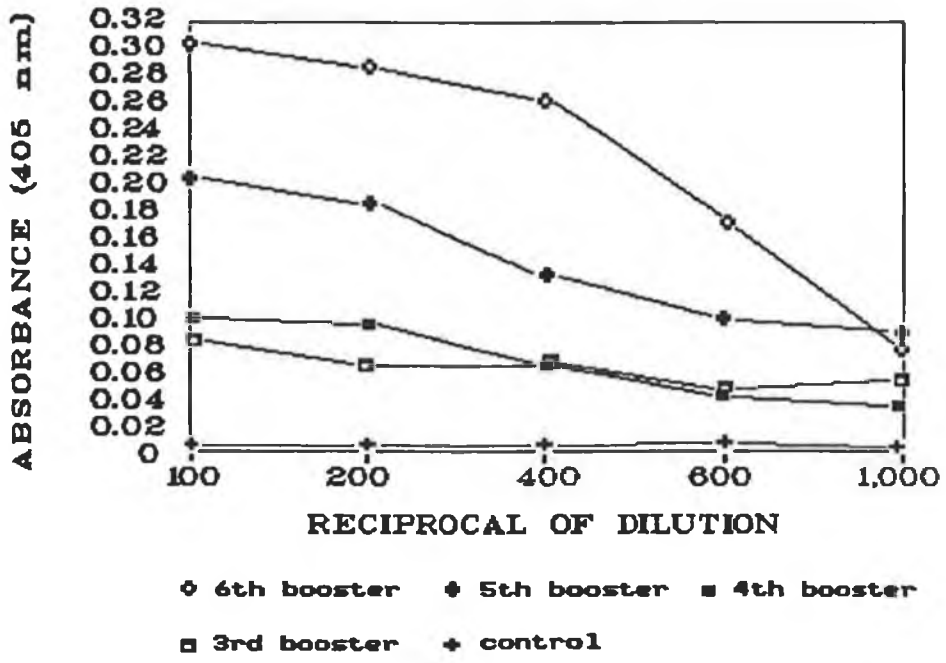

Fig 5b

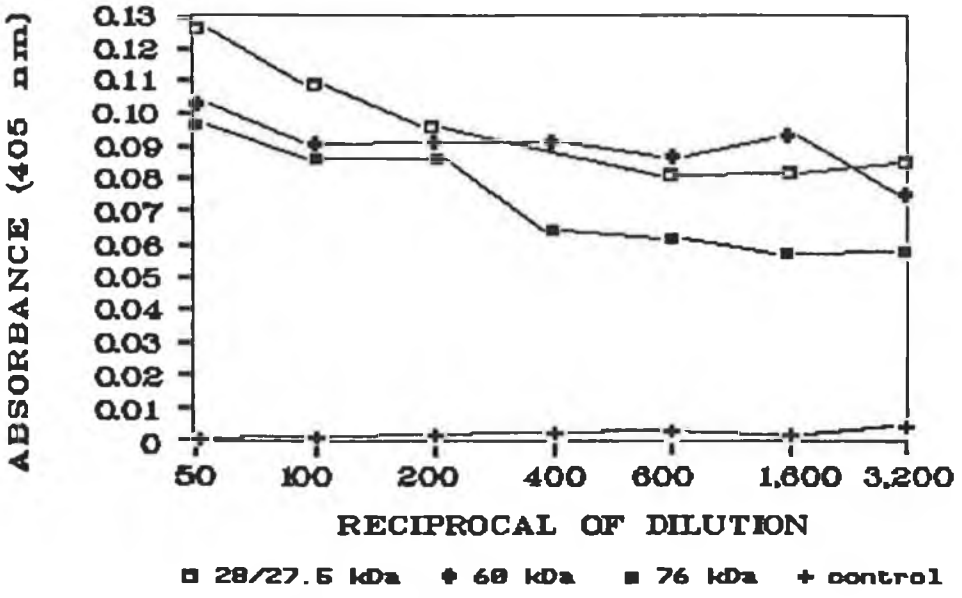

Fig 5a. ELISA results of antibody response of rabbit injected with *F. hepatica* – affinity purified thiol proteases.

Fig 5b. ELISA results of antibody response of 3 rats each injected with a protease from *F. hepatica* of M.Wt. 28/27.5 kDa, 60 kDa or 76 kDa.

show that the antibody titres in all three rats was very low; anti-28/27.5 kDa was >100, anti-60 kDa and anti-76 kDa were <100.

5.6 ANALYSIS BY IMMUNOFLUORESCENCE MICROSCOPY

The anatomic localization of the antigenic target recognized by a number of antisera was studied by indirect immunofluorescence microscopy. The sections used were plastic embedded mature flukes. The antiserum applied to the section, its localization in the fluke and the intensity of fluorescence are outlined in Table 3. Normal rabbit and normal rat serum did not show any fluorescence.

With the 5 weeks PI rat serum, the tegumental surface showed only very slight fluorescence. The cells lining the gut cecum, in contrast were very brightly labelled. The vitelline glands and the parenchyma were also fluorescently labelled. In comparison, the 10 weeks PI rat serum showed a very strong fluorescent labelling evenly distributed in the tegument. The cells lining the gut exhibited bright labelling also. Fluorescence was bright in the vitelline glands and throughout the parenchyma.

The tegumental surface was very strongly labelled with the rat anti-E/S products (mature fluke), as were the cells lining the gut cecum. Bright fluorescence was distributed throughout the parenchyma and also in the vitelline glands.

With the rabbit anti-thiol protease serum, there was no labelling in the tegument. However, very bright fluorescence was observed in the cells lining the gut. The vitelline glands showed no fluorescence and labelling in the parenchyma was just discernible.

For the rat anti-protease gel bands, labelling was only present in the cells lining the gut. Rat anti-28/27.5 kDa showed weak but obvious labelling in the gut. However, the fluorescence exhibited by anti-60 kDa and anti-76 kDa was just discernible but was also located in the gut.

TABLE 3. Indirect fluorescent antibody labelling of JB4-embedded mature *F. hepatica*.

ANTISERUM (1:50)	LOCALIZATION IN THE LIVER TISSUE			
	TEGUMENT	GUT	VITELLINE GLANDS	PARENCHYMA
5 Weeks PI rat serum	+	++++	+++	+++
10 weeks PI rat serum	++++	+++	+++	+++
Rat anti-adult E/S products	++++	++++	+++	+++
Rabbit anti-protease	-	++++	-	+
Rat anti-28/27.5 kDa	-	++	-	-
Rat anti-60 kDa	-	+	-	-
Rat anti-76 kDa	-	+	-	-

++++, very bright labelling; +++, bright labelling;

++, labelling clearly discernible; +, labelling just

discernible, -, unlabelled.

This lack of fluorescent labelling correlated with the antibody titre of these three antisera which was very low (Fig 5b).

5.7 DIGESTION OF IgG BY PAPAIN AND BY CRUDE E/S PRODUCTS

In this study we made a direct comparison of antibody-cleavage by papain and mature fluke E/S products. Purified rabbit IgG was incubated with papain or E/S products and the cleavage products detected by immunoblotting using alkaline phosphatase-conjugated-goat-anti-rabbit IgG which was specific for rabbit IgG heavy chain. *F. hepatica* E/S products resulted in cleavage of rabbit IgG heavy chain into a specific fragment with an apparent M.Wt. of approximately 22 kDa (Fig 6). This major fragment is also produced when rabbit IgG is incubated with papain. Therefore the IgG-cleaving enzyme in *F. hepatica* E/S products cuts this IgG at or close to the papain cleaving site.

We have shown that *F. hepatica* release 11 distinct proteases which can be divided into two groups based on their optimum pH range ; the higher M.Wt. group is active in the pH range 3.0-4.5 ; the lower M.Wt. group are most active in the pH range 4.5-8.0. All of the enzymes are thiol proteases and can be purified by Organomercurial Affinity Chromatography. To determine which of these enzymes was capable of cleaving IgG, we subjected the *F. hepatica* proteases to molecular size separation on a HPLC TSK 3000 SW column.

5.8 HPLC ANALYSIS

Molecular sieve HPLC yielded three major peaks and several minor peaks (Fig 7). A sample from each HPLC fraction was incubated with a mouse IgG2a monoclonal antibody to determine which peak represented the IgG-cleaving enzyme. As can be seen from Fig 8 only fractions 19-27 cleaved the IgG molecules ; this activity corresponded to the major peak III. Each fraction was also tested for cathepsin B activity using Z-Phe-Arg AMC as a substrate. This activity again correlated with the major peak III (Fig 7). To determine the molecular size of the proteases in peaks I, II and III we subjected a sample of pooled fractions from

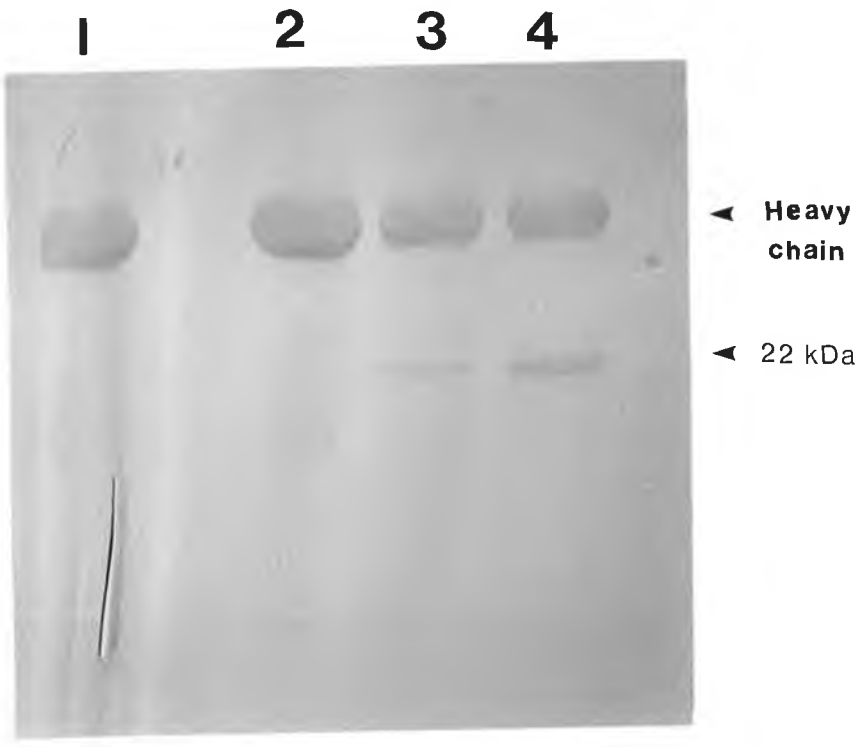

Fig 6. Electrophoretic transfer blot analysis of digestion of IgG by both mature fluke E/S products and papain to produce a specific fragment of 22 kDa. Lane 1 : Rabbit IgG incubated at 37°C for 1 hour at pH 7.0 (Reaction mixture A), lane 2 : reaction mixture A, with mercaptoethanol and EDTA (Reaction mixture B), lane 3 : reaction mixture B, with mature fluke E/S products, lane 4 : reaction mixture B, with papain. The gel was 15 % polyacrylamide and was run under reducing conditions. The IgG and digestion products were detected using an anti rabbit-alkaline phosphatase conjugate which binds one end of heavy chains only.

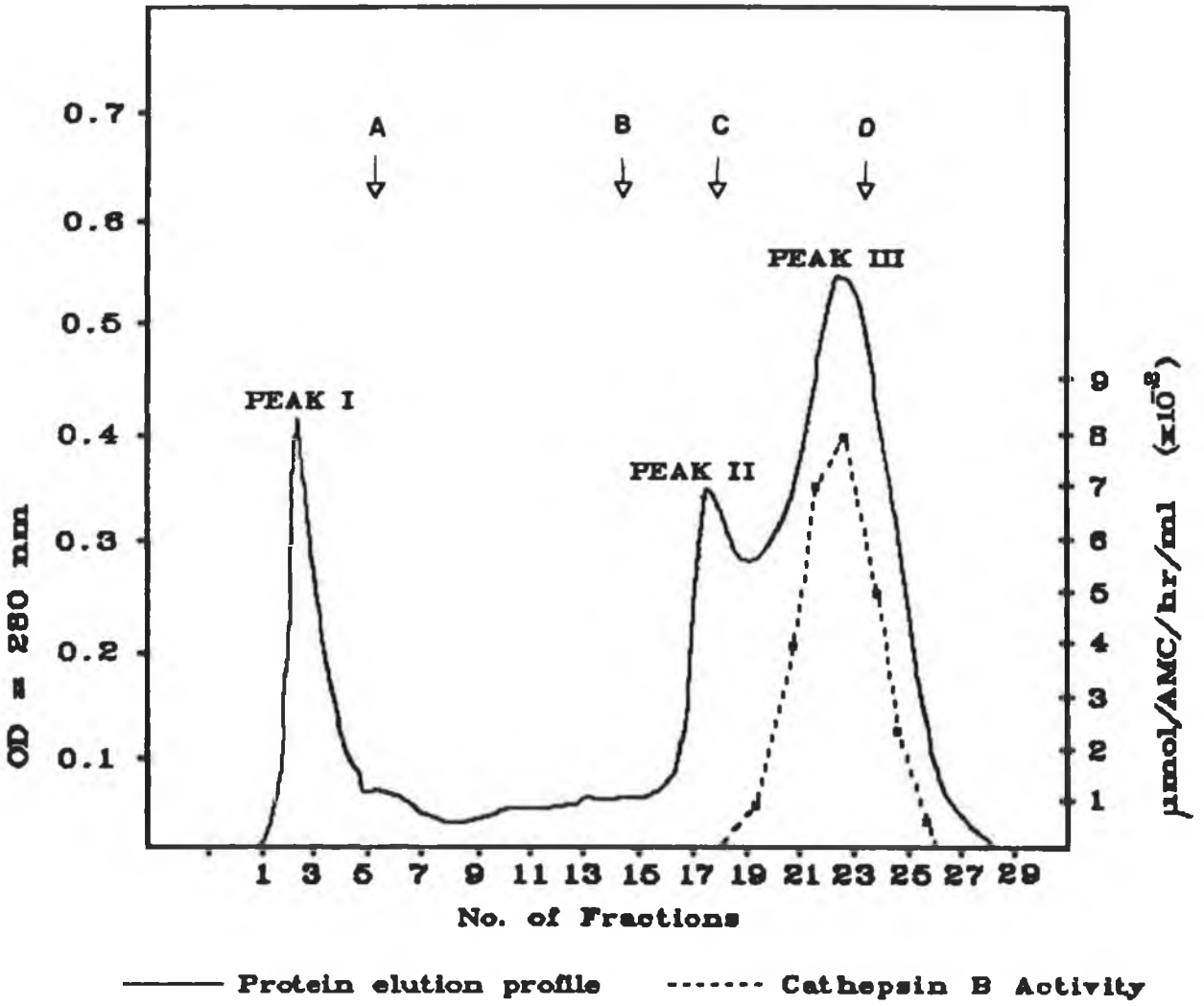

Fig. 7 Elution profile of the *in vitro*-released products of *F. hepatica*

The column was equilibrated with the following standards:

A	-	Immunolgobulin G	150,000
B	-	Bovine serum albumin	66,000
C	-	Horse Radish Peroxidase	45,000
D	-	lysozyme	14,300

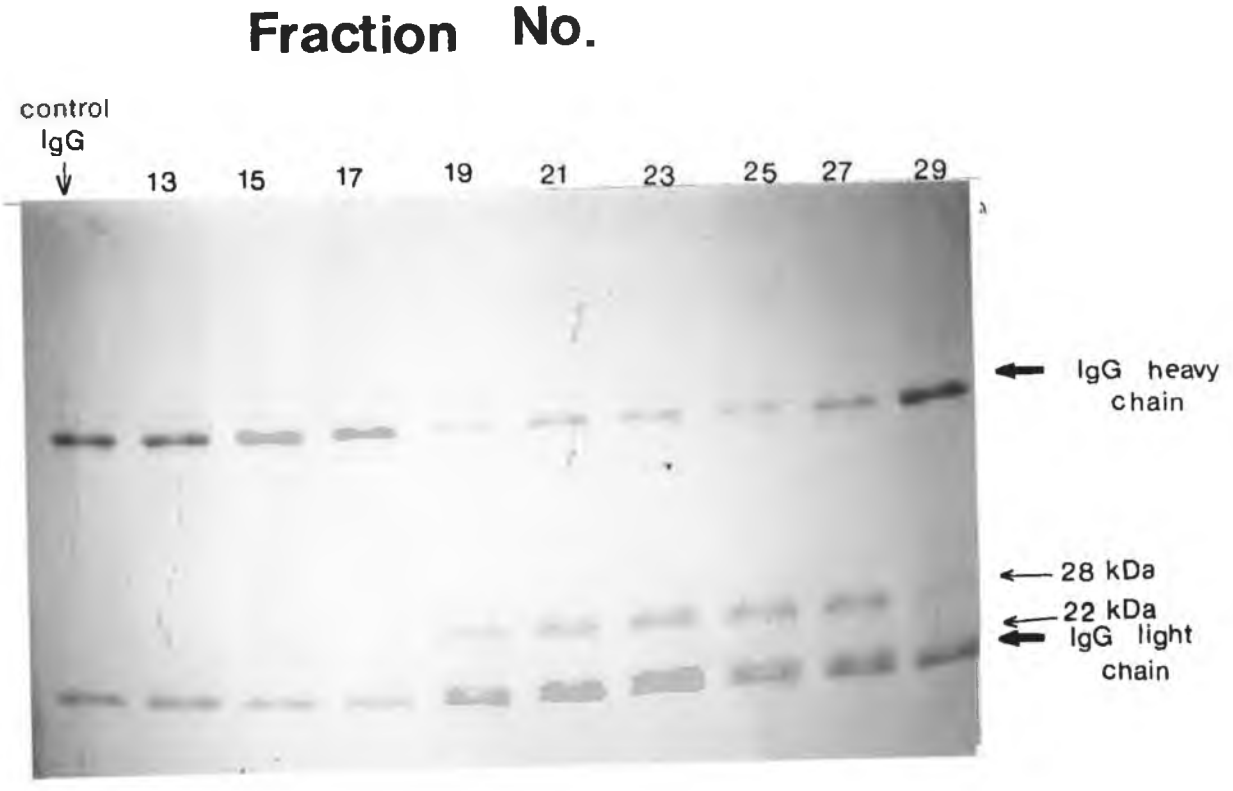

Fig 8. Reducing SDS-PAGE demonstrating the IgG-cleaving ability of fractions collected from the HPLC. Only fractions 19-27 cleaved IgG heavy chain into two specific fragments of 22 kDa and 28 kDa.

these regions to gelatin substrate SDS-PAGE. Peak I showed no activity on these gels. Peak II showed multiple bands from 46-25 kDa, and peak III which represented the IgG-cleaving enzyme exhibited multiple bands in the molecular size range 90-60 kDa (Fig 9). Peak II ban correlated with the proteases described as Group 2 above i. e. those with a pH optimum between 4.5-8.0. Peak III bands correlated with those described as Group 1 above i.e. those with a pH optimum between pH 3.0-4.5.

5.9 INHIBITION STUDIES WITH DIETHYL PYROCARBONATE (DPC)

DPC is an active site specific modifying reagent which binds to histidine amino acid residues that are involved in catalytic activity. We carried out inhibition studies with this reagent a) to determine whether the cathepsin B enzyme present in fluke E/S products contained histidine in its active site and b) to clearly demonstrate that the multiple bands of Group 1 were derived from a single enzyme of peak 3.

5.9.1 Inhibition of Cathepsin B.

Samples of cathepsin B (peak III) were incubated with various concentrations of DPC for 10 mins and then assayed for activity on Z-Phe-Arg-AMC. Cathepsin B activity decreased with increasing DPC concentration ; 98% inactivation of activity was achieved at 25mM DPC (Fig 10).

The pH optimum for cathepsin B activity using the Z-Phe-Arg-AMC substrate is shown in Fig 11a. This enzyme shows optimum activity at pH 4.5. Inhibition studies with DPC (50 mM final concentration) were carried out over a range of pHs. DPC was most effective in the pH range 3.0-4.5. DPC had little or no effect on activity at pH 8.0 (Fig 11b). At pH 4.5 complete inhibition could be achieved in less than one minute (data not shown).

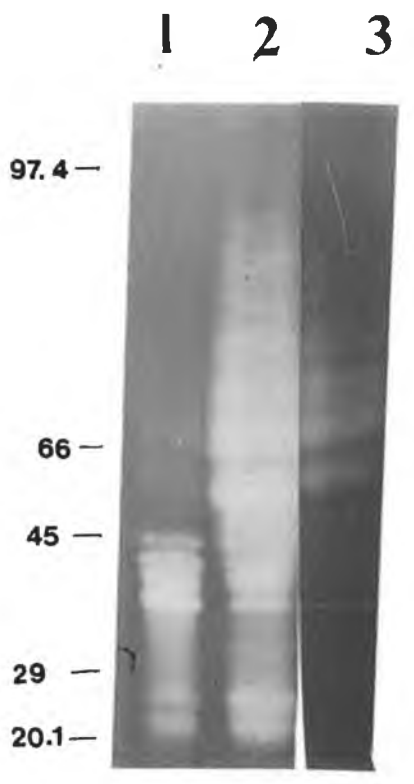

Fig 9. Gelatin substrate SDS-PAGE analysis of Peak II (lane 1), total E/S before HPLC (lane 2) and Peak III (lane 3).

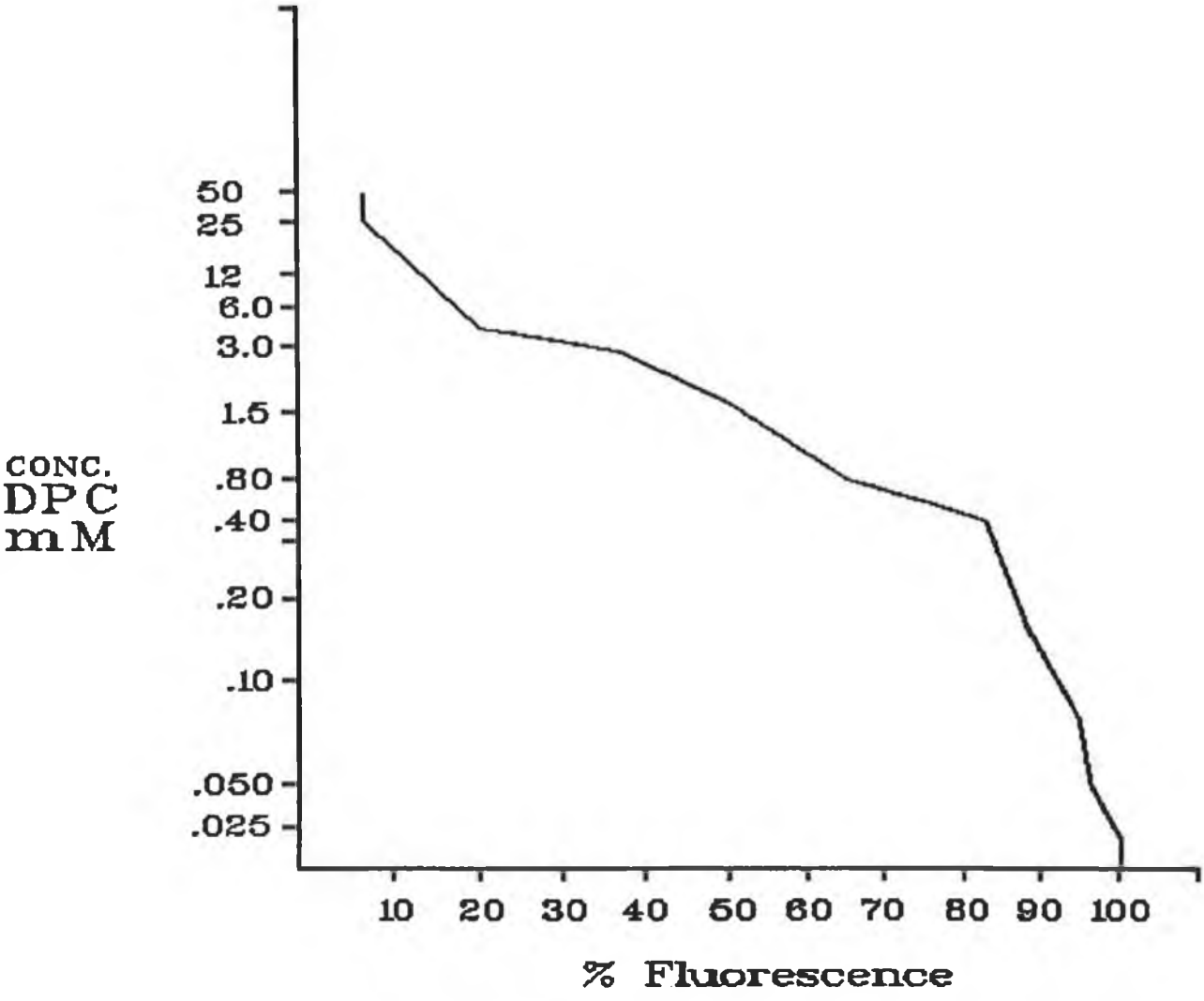

Fig 10 Graph depicting the percent inhibition of protease activity against DCP concentration.

Fig 11a

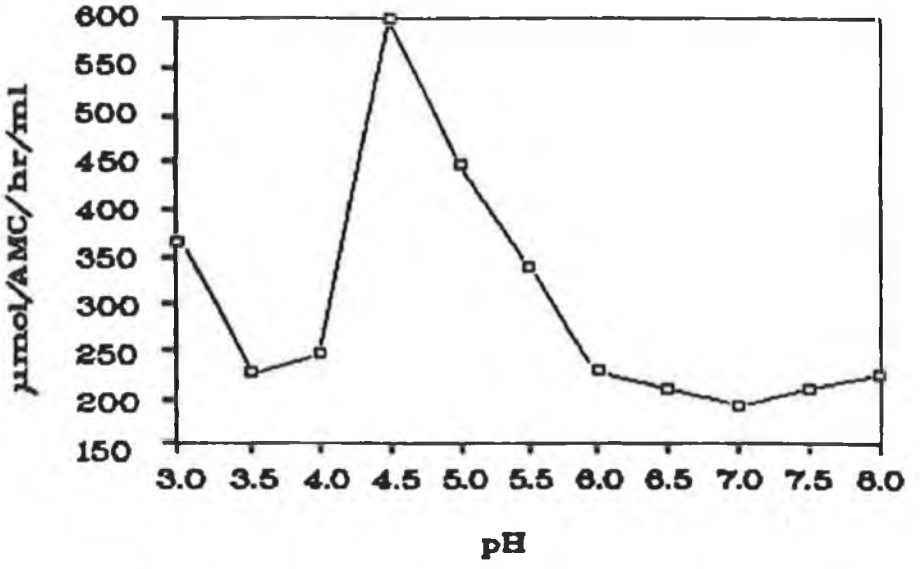

Fig 11b

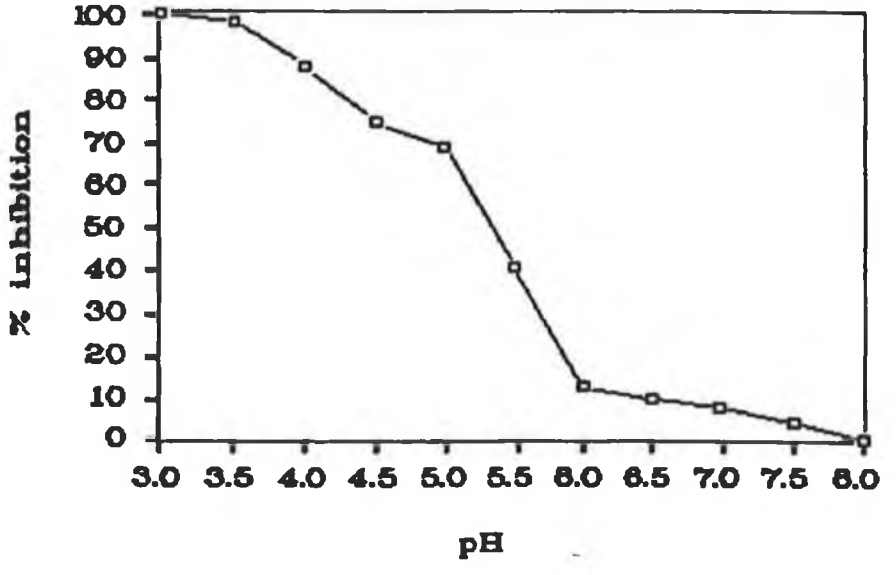

Fig IIa pH optimum of protease activity on Z-Phe-Arg-AMC.

Fig IIb The inactivation of enzyme activity on Z-Phe-Arg-AMC under different pH conditions.

5.9.2 Inhibition of cathepsin B enzymes in gelatin substrate SDS-PAGE

DPC was added to samples of total E/S products at a final concentration of 0, 5 and 50 mM before application to gelatin substrate SDS-PAGE. All the Group 1 proteases in the molecular size range 90-60 kDa were inactivated at 50 mM (Fig 12, lane 2). Little or no reduction in activity of these bands is seen at 5 mM (Fig 12, lane 3) compared to control (Fig 12, lane 1).

5.9.3 Inhibition of IgG cleavage by cathepsin B

DPC was mixed with a sample of cathepsin B (peak III) for 10 mins before adding IgG to the mixture. Since this reaction was carried out in PBS at pH 7.3 it was necessary to add DPC at a final concentration of 200 mM. At this concentration it can be seen that IgG cleavage by cathepsin B is almost completely blocked (Fig 13, lane 3) compared to controls without DPC (Fig 13, lanes 1 & 2).

5.10 IMMUNOSCREENING OF AN ADULT WORM cDNA LIBRARY

The anti-thiol proteases antiserum prepared in the rabbit was used to screen an adult worm cDNA expression library constructed in λgt11. Screening of 3 plates each with approximately 1.3 X 10^5 plaques yielded one positive recombinant clone. This clone was plated out again as before and rescreened. All clones were found to be positive. Extracts from infecting *E. coli* Y1090 infected with 1) parental phage and 2) recombinant phage were obtained. These were analyzed by immunoblotting using anti-ß-galactosidase and anti-thiol proteases antiserum (Fig 14). The anti-ß-galactosidase antiserum reacted with ß-galactosidase at 116 kDa in extracts of *E. coli* infected with wild-type or recombinant phage i.e. there was no difference in the M.Wt. of the ß-galactosidase present in these two extracts. Furthermore, the rabbit anti-thiol proteases did not detect any fusion protein. A protein band of 170 kDa was detected with the anti-thiol protease antibody but was also observed when control serum was used. These observations indicated that the isolated clone contained no insert and hence our original positive clones were false positives.

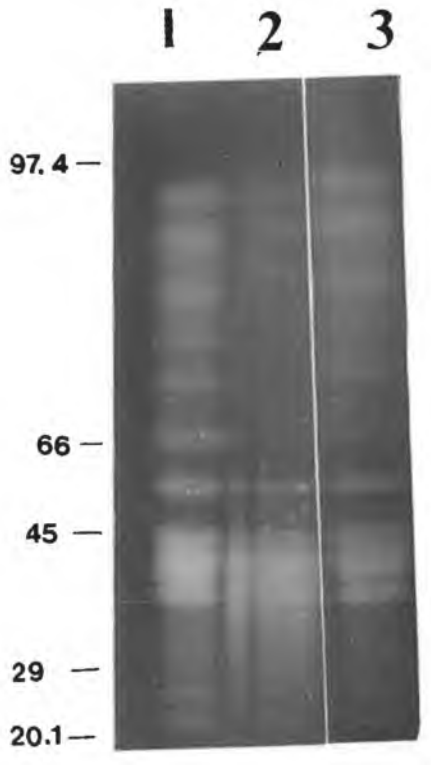

Fig 12. Gelatin substrate SDS-PAGE analysis of the inactivation of the Group 1 proteases only from total E/S products of *F. hepatica* using 0mM (lane 1), 50mM (lane 2) and 5mM (lane 3) DPC.

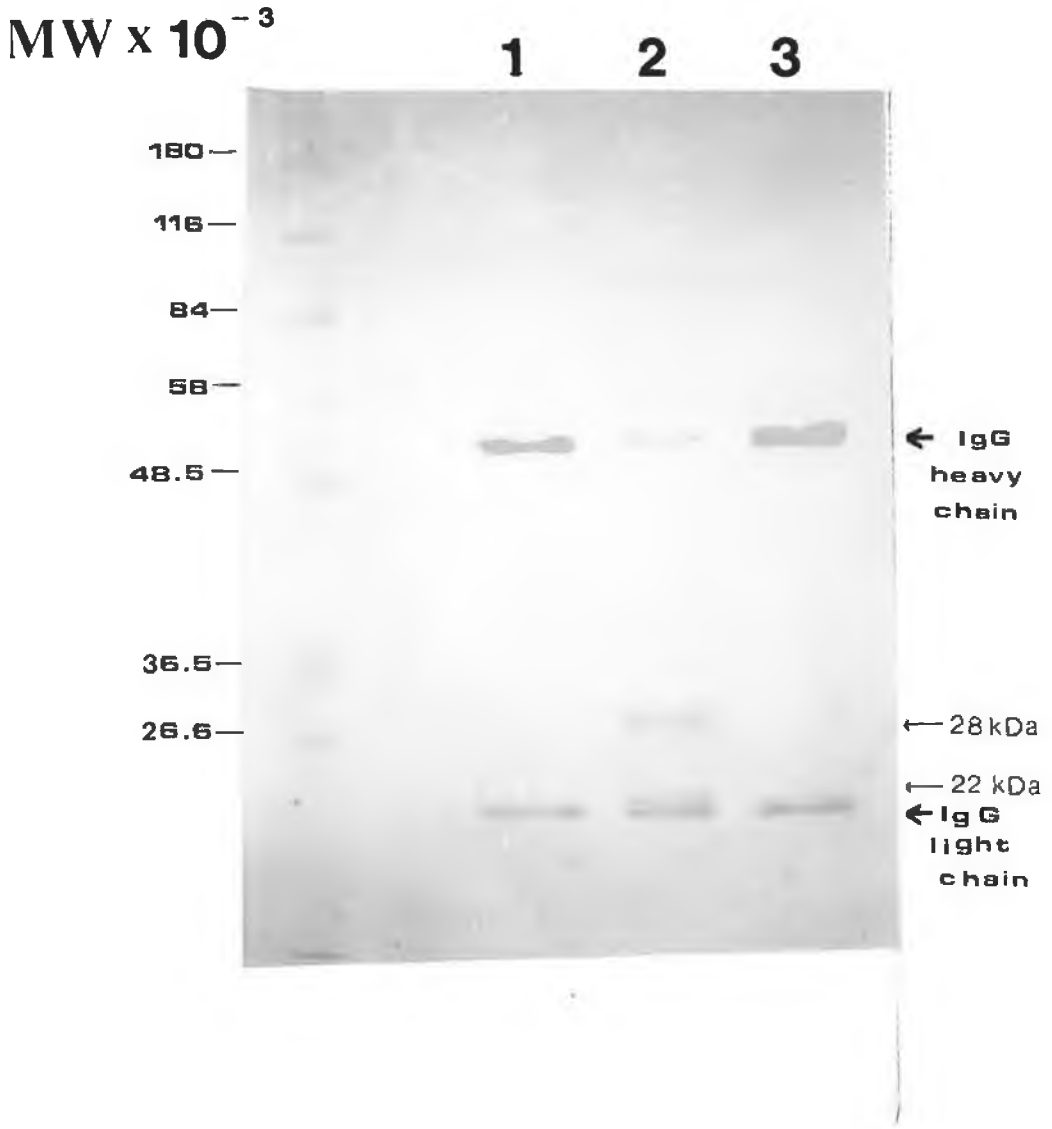

Fig 13. Inhibition of IgG-cleaving enzyme from *F. hepatica* with DPC. Lane 1:IgG only, lane 2: IgG and sample from pooled peak III fractions and lane 3: IgG, peak III and DPC (final conc. 200mM).

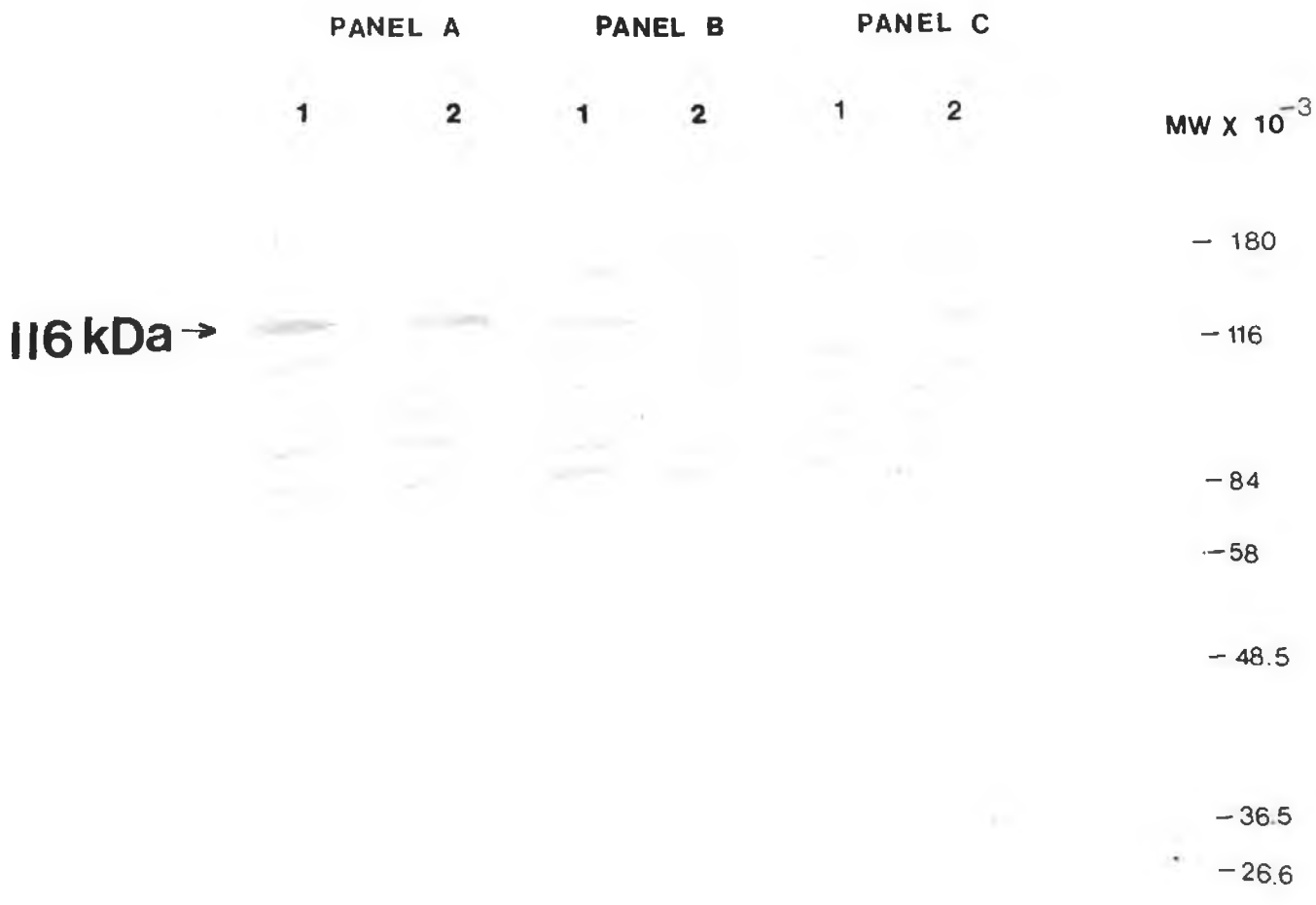

Fig 14. Western Blot analysis of recombinant fusion protein. Lysates of *E.coli* Y1090 infected with parental (lanes 1) or recombinant phage (lanes 2) were subjected to 7.5% SDS-PAGE before electroblotting to nitrocellulose. Filters were probed with rabbit anti-ß-galactosidase (panel A), rabbit anti-thiol proteases (panel B) or normal rabbit serum (panel C). The bound antibody was detected using anti-rabbit-conjugated alkaline phosphatase.

6.0 DISCUSSION

By incorporating the appropriate substrate in an SDS-polyacrylamide gel the identification of several classes of enzymes can be demonstrated while simultaneously providing values of their apparent M.Wts. In the present report we used gelatin substrate SDS-PAGE to detect proteolytic activity. Polyacrylamide was copolymerized with gelatin and the enzyme sample was applied to the gel. After electrophoresis, the SDS was removed from the gel to prevent destabilization of the enzymes. To allow digestion of the gelatin by the proteases, the gel was incubated in buffer at an appropriate pH and temperature. Proteases then degraded the gelatin to components small enough to diffuse from the gel. Therefore, only nondegraded gelatin remaining in the gel stained with Coomassie Blue ; clear bands were visible where the proteases had migrated and digested the gelatin. To prevent inactivation of the enzymes we combined the enzyme sample with nonreducing sample buffer (i.e. without mercaptoethanol and no boiling) and applied the sample to the gel without boiling it. However, since the conditions were nonreducing the observed M.Wts of the proteases are not completely accurate.

By this method, we showed that when immature and mature *F. hepatica* were maintained in culture for 16 hr they released proteolytic enzymes. Whether proteolytic enzymes are also released *in vivo* is unknown ; however we speculate that these enzymes are important in the immune evasion, feeding and migration of the parasite. These functions may not be separate since degradation of the host proteins and tissue during migration would provide the parasite with substrates for further digestion and adsorption. The Group 2 proteases i.e. 46, 44, 42, 40, 28 and 27.5 kDa were most active between pH 4.5 and 8.0. We speculate that most digestion is carried out by the Group 1 proteases i.e. 88, 83, 80, 76 and 60 kDa which show optimal activity in an acidic environment between pH 3.0 and 4.5. We also propose that this

group of enzymes may contain the acidic protease cathepsin B responsible for the digestion of host IgG as described by Chapman and Mitchell (1982a).

Mature flukes do not penetrate host tissue, but rather reside in the lumen of the bile ducts. Damage to the bile duct wall caused by the tegumental spines allows adult flukes access to nutrients and cells in the blood ; this is evidenced by the presence of hemoglobin in the digestive tract. Since adult worms have access to nutrients similar to that of immature flukes, it is not surprising that they also elaborate many of the same proteases. Of particular interest, however is the 40 kDa protease released by immature flukes, which was very minor in the mature flukes *in vitro*-released products. This stage-specific release may indicate an important role for this enzyme in the degradation of substrates encountered by the flukes predominantly in the liver mass. Such a substrate may be the liver cell matrix protein collagen.

All of the proteases were classified as thiol proteases due to their inactivation by thiol protease inhibitors and their enhanced activity in the presence of reducing agents. Evidence that all of these proteases bound to the Affi-gel 501 column and were subsequently eluted with 10 mM cysteine also confirms their thiol nature. This column was therefore used to purify the thiol proteases from our original crude E/S products. Cathepsin B activity was detected in the E/S products using the synthetic substrate Z-Phe-Arg-AMC which is specific for this particular enzyme. The specific activity of the cathepsin B enzymes in the mature fluke E/S was 1082. Rege et al. (1989) purified a cathepsin B-like enzyme from adult worm extracts using conventional methods. The specific activity obtained by these authors (also using Z-Phe-Arg-AMC as substrate) in total fluke extracts was only 24. Following purification the specific activity was 1143. It would appear therefore that the cathepsin B enzyme is a major component in the mature fluke E/S since the specific activity in this preparation is close to that obtained by Rege et al. (1989) for highly purified cathepsin B. HPLC analysis of mature fluke E/S (see below) confirmed that the cathepsin B enzyme is associated with

a major protein peak. Affinity chromatography resulted in a 2.6-fold increase in specific activity for cathepsin B.

Preparing antisera against the purified thiol proteases enabled us to determine their localization in adult fluke tissues by immunofluorescence. The antibody response was monitored by ELISA every 3 weeks. The final titre of antibody in the rabbit serum was sufficient to produce very strong fluorescent labelling. Antibody responses obtained against individual proteases eluted from the gel however were very low. The reason for this very low antibody titre was attributed to the very small quantities of protein administered to the rats or to the low antigenicity of these proteins in rats.

Antisera taken from infected rats at 5 and 10 weeks after infection were used in immunofluorescent studies on mature fluke sections. Labelling occurred within the tegument, parenchyma, vitelline glands and gut ceca with both antisera. However, the tegumental labelling with sera taken 5 weeks after infection was much weaker than that obtained with the 10 weeks post infected sera. This result confirms the observation by Hanna (1980a, b, c) that the mature fluke surface displays T2 antigens to which antibodies are present in 10 weeks PI rat serum but not in 5 weeks PI rat serum. There was also a strong fluorescent labelling by both 5 week and 10 week immune sera along the lining of the gut. Antibody titres against these gut antigens were higher in the 5 week PI rat serum than in the 10 week PI rat serum. Hanna (1980) and Hanna and Trudgett (1983) showed that T1 antigens share common epitopes in common with antigens present in the gut epithelium.

Immunofluorescent studies carried out on mature fluke sections using antiserum prepared in th rat against total E/S products localized E/S antigens within the tegument, parenchyma, vitelline glands and gut ceca. However, immunofluorescence using rabbit anti-thiol proteases showed specific labelling in the gut region of the fluke. The antigenic cross reactivity between tegumental T1 antigens and antigens within the gut lining (Hanna, 1980b, Hanna and

Trudgett, 1983) obviously does not involve the fluke proteases as all antibodies prepared against these showed no tegumental binding. This specific labelling to the gut when compared with the widespread labelling throughout the tegument, parenchyma, vitelline gland and gut when the rat anti-total E/S was used suggests that the Affi-gel column successfully purified the thiol enzymes away from other proteins present in the E/S.

Fluorescence using antibodies prepared in rats specifically against the 28/27.5, 60 and 76 kDa proteases confirmed the specific secretion of proteases (in both Group I and Group II) from the fluke gut epithelial cells. Obviously, the large surface area of the gut provides the fluke with ample enzyme to run a highly efficient digestive system ((3-4 hr gut-emptying-time reported by Geroc (1975)). Our result confirms previous work which has shown that the ceca are the major source of proteolytic activity in the fluke homogenate (Howell, 1973). Locatelli and Beretta (1969) demonstrated that the flukes can disrupt gelatin sheets *in vitro* but are prevented from doing this when the pharynx is ligated ; they concluded from this that the fluke enzymes are not secreted through the worm cuticle or excreted through the excretor pilum. This indicates that these enzymes are therefore associated with the gut. It would appear that the cecal epithelium synthesizes the enzymes, these are then discharged into the lumen for extracellular digestion and can reach the exterior of the parasite as a result of regurgitation. Within the cecal epithelial cells enzymes may be involved in intracellular digestion which is considered to take place (Halton, 1967).

Chapman and Mitchell (1982) found that thiol proteases from *F. hepatica* exhibited papain-like activity. Although they did demonstrate the cleavage of IgG by total E/S products on SDS-PAGE, they did not compare it directly with papain-cleavage of IgG. Our analysis by SDS-PAGE and Western blotting revealed that both the fluke E/S and papain resulted in cleavage of IgG heavy chain into a specific fragment of approximately 22 kDa. The light chain was not detected because our enzyme-conjugated second antibody was specific only for heavy chain. Furthermore, as we detected only one fragment of the split heavy chain it would

appear that this second antibody only reacts with one end of the heavy chain. We conclude however that F. hepatica releases an enzyme capable of cleaving at, or close to, the papain cleavage site.

We suspected that the actual number of proteases released *in vitro* by *F. hepatica* may be less complicated than that indicated by gelatin substrate SDS-PAGE. For example, a protease that aggregates may have been represented as multiple bands. Secondly, auto-proteolysis may have given rise to several active fragments. We were also surprised that all of the proteolytic enzymes identified by this method were cysteine or thiol proteases. Detection of thiol proteases is not biased by gelatin substrate SDS-PAGE since in other studies using this method, serine proteases of *Plasmodium falciparum* (Rosenthal et al., 1987) and metalloproteases of *Strongyloides stercoralis* (McKerrow et al., 1990) have been identified. However, this method is selective since it allows the detection only of those proteases that are stable in the presence of SDS. Hence, it was also possible that *F. hepatica* releases other proteases not detected by this particular method.

We therefore decided to subject the E/S products to molecular size separation by HPLC. As we suspected, the number of proteases released by the fluke appeared much less complicated upon HPLC analysis. Only two of the three major protein peaks yielded by HPLC demonstrated protease activity by gelatin substrate SDS-PAGE. Peak I protein(s), showing a molecular mass >150 kDa, did not digest gelatin when run on a 10% polyacrylamide gel. This may be because the protein was too large to diffuse into the gel ; a lower percentage polyacrylamide may detect any proteolytic activity by this protein. Alternatively, it may be a protease which is unstable in the presence of SDS or it could also prove to be an protease which can digest gelatin, but not to components small enough to diffuse from the gel. Peak I was also analyzed for IgG-cleavage ; however no cleavage was observed. Since we believe enzymes play such an important role in the survival of the parasite we speculate that this major peak I exhibits an as yet unidentified proteolytic activity.

Gelatin substrate SDS-PAGE analysis of peak II revealed several proteolytic bands between 25 and 50 kDa. These proteases correlated with the original Group 2 enzymes which showed optimal activity in the pH range 4.5-8.0. We suspect that since all of these protease bands are represented by one peak on HPLC analysis, there may be only one enzyme with a molecular mass of 45 kDa, which runs abberantly in the gel. The reason why the protease(s) migrate in this manner is unclear. A possible explanation may be that this enzyme undergoes 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 proteases may cause them to act uncharacteristically, particularly in the presence of SDS. Since this enzyme (from Group 2) is most active at physiological pH, we speculate that it is involved in extracorporeal digestion of host tissue e.g. liver, serum proteins or bile duct wall. Activity of this enzyme on IgG was investigated, however the IgG remained intact.

When peak III was subjected to gelatin substrate SDS-PAGE several proteolytic bands between 60 and 90 kDa were observed. These proteases correlated with those described earlier as Group 1 and which showed optimal activity in the pH range 3.0-4.5. This group of enzymes would also appear to originate from one enzyme which runs aberrantly in the gel. Why this is so is again unclear but it may be due to an aggregation of this protease. It may also be possible that the protease binds to the gelatin on its way down the gel resulting in retardation and the formation of a ladder-like pattern. However this is unlikely since the same pattern of protease activity is observed repeatedly with a number of different E/S preparations.

Whatever their behaviour under gelatin substrate SDS-PAGE, we achieved to separate the two groups of enzymes, each group with a different pH optimum, into two enzymes on the basis of molecular size. Peak III was found to possess a molecular mass of 15 kDa. Using the substrate Z-Phe-Arg-AMC, we showed that the fractions representing peak III contained

cathepsin B activity. Consistent with cathepsin B enzymes this enzyme in peak III was a thiol protease with optimal activity at pH 4.5. Therefore, the Group 1 enzymes (now responsible for cathepsin B activity) may be the IgG-cleaving enzymes reported by Chapman and Mitchell (1982) ; they found these IgG-cutting enzymes to have cathepsin B-like activity. We therefore incubated our peak III fractions with IgG and by SDS-PAGE analysis we observed digestion of IgG heavy chains into two fragments of 28 and 22 kDa. The 22 kDa fragment probably corresponds to the heavy chain fragment found earlier after IgG-digestion by total E/S. Therefore peak III contains a cathepsin B-like enzyme which cleaves IgG at or close to the papain cleavage site. Further work needs to be carried out to investigate activity of this cathepsin B on other substrates. Simpkin et al. (1980) described an acidic protease from *F. hepatica* with similar characteristics to the one described in this study. Their enzyme was maximally active at pH 3.9-4.0, and had an apparent molecular mass of approximately 12 kDa. Hemoglobinase activity was observed at this pH, but no report was made of its activity on immunoglobulin. This enzyme was not assigned to any particular class. Rege et al. (1989) reported a cysteine protease from *F. hepatica* which was optimally active at pH 6.0 and had an apparent molecular mass of 14.5 kDa. They had preliminary data that this enzyme was capable of digesting hemoglobin, collagen and IgG.

DPC is an active site modifying reagent which irreversibly inhibits enzymes containing histidine in their active site. This reagent totally inactivated the enzyme from peak III at a concentration of 25 mM at pH 4.5. This result is consistent with the enzyme being classified as a cathepsin B since these enzymes have histidine residues involved in catalytic activity. O'Connor et al. (1987) reported successful modification of an amino peptidase by DPC at pH 8.0. We found however that DPC had no effect on the cathepsin B enzyme at pH 8.0. A pH profile demonstrates that the effect of DPC on the enzyme decreased with increasing pH, being most effective at the lower pH's, and with little or no effect at pH's 7.0 and 8.0. However it is generally accepted that if a functionally active residue has been modified by DPC or any other active site modifying reagent then the pH dependence for activity loss

follows the pH optimum activity curve. This trend would be expected since the pH activity profile of enzymes reflect the pH at which important proton-donating or proton-accepting groups in the enzyme catalytic site are in their required state of ionization. It follows therefore that this same state of ionization is required for modification of the residue by DPC (histidine in this case) and thereby producing the same activity loss profile. We observed such behaviour by the DPC modification of cathepsin B demonstrating the success of our modification procedure.

DPC also inhibited IgG-cleavage by the total E/S products and pooled peak III fractions showing that IgG-cleavage can be attributed to the cathepsin B enzyme. Addition of DPC to a sample of total E/S products prior to electrophoresis specifically inactivated all of the Group 1 enzymes (molecular size 60-90 kDa). This confirms that all of the Group 1 proteolytic bands from gelatin substrate SDS-PAGE contain a histidine in their active site and do originate from one enzyme, which by HPLC analysis is 15 kDa and which we classified as a cathepsin B. Selective inhibition of Group 1 indicates that Group 2 is different to Group 1 with respect to its active-site conformation i.e. there is no histidine residue present in the active-site of Group 2 i.e. peak II.

We have identified the cathepsin B enzyme which cleaves IgG in the hinge region at or close to the papain cleavage site. The biological significance *in vivo* of an IgG-cleaving enzyme of this type is unknown. Obviously, cleavage of host IgG would result in the release of the Fc portion. Although the Fab fragment can still bind to the parasite's surface ; without the Fc portion , the Fab cannot attract macrophages which would normally carry out the destruction of the parasite. Furthermore, Auriault et al. (1980b) reported the inactivation of rat macrophages by peptides which are produced when schistosome larvae proteases cleave IgG. The inactivation, therefore of the cathepsin B enzyme *in vivo* would make it more difficult for the parasite to evade the host immune system. Host IgG would remain intact and carry out its function of parasite destruction.

Goose (1978) reported that *F. hepatica* protects itself from host immune defenses by producing substances which are toxic to its host's lymphocytes and other immunocompetent cells. He found that peritoneal cells from *F. hepatica*-infected serum attached to the NEJ surface but were detached on addition of E/S products. IgG most likely constitute the junctions between the cell NEJ surface and these attached cells. We speculate, therefore, that the cathepsin B-like enzyme reported in this study is responsible for cleaving the IgG and detaching the cells from the parasite's surface. Further work on immune adhesion of cells (particularly those responsible for immune destruction) to *F. hepatica* and subsequent addition of the cathepsin B isolated in this report, would be very interesting. Hanna (1980) did postulate that the lymphotoxic substances referred to by Goose (1978) were proteolytic enzymes. Goose (1978) suggested that since these E/S products (containing lymphotoxins) must be in greater abundance in the actively feeding immature stage of the fluke than in the very early or very late adult stage of the fluke then it would be important to time the passive transfer of serum ; transfer at the time of challenge before the flukes were producing E/S products would be effective, but transfer later, when E/S products are in abundance would not confer protection.

Immunological attack directed against parasitic enzymes by immunization with purified enzymes has been proposed in a number of host-parasite systems (Sinclair, 1970, Clegg and Smith, 1978). Reich and Zorzopulus (1980) proposed that the observed acquired resistance of cattle against infestation with the tick *Boophilus microplus* may have been attributed to anti-enzyme antibodies induced in the cattle. Knox et al. (1988) reported the release of proteases by the parasitic larval stages of *Ascaris suum*. They found that these proteases were inhibited by serum antibody from infected animals. They proposed that these proteases may be responsible for cleavage of surface bound IgG and hence their inactivation by antibody could contribute to impairment of survival of the parasite. Both proteolytic activity from *Ancylostoma caninum* (Thorsen et al., 1956) and lipolytic activity from *Nippostrongylus muris*

(Thorsen et al., 1953) have been shown to be inhibited by immune sera. Antibodies to an enzyme produced by *Babesia bovis* in infected erythrocytes can induce host protection (Commins et al., 1985). However in contrast Rothwell et al. (1973) reported that the binding of antibody to antigenic acetylcholinesterase which is secreted by many parasitic nematodes, did not inhibit enzyme activity. To our knowledge, the ability of proteases from *F. hepatica* to elicit a protective response has not been reported. However, when E/S products from immature *F. hepatica* were used to immunize rats against an oral challenge infection, partial protection was achieved (Rajasekariah et al., 1979). Burden et al. (1982b) found E/S from 13- to 14-day old flukes to provide a slight degree of protection to a challenge infection. These E/S products presumably contained proteases including IgG-cutting cathepsin B.

It has been shown that *F. hepatica*-infected rat serum can confer partial protection on a naive recipient (Hayes et al., 1974, Rajasekariah and Howell, 1979). However, it has not been proven that this protection is attributed to antibodies induced by *F. hepatica*. Chapman and Mitchell (1982) suggested that liver damage may increase the level of α_2-macroglobulin in the serum of *F. hepatica* infected animals and proposed that factors other than IgG could be responsible for the transfer of protective activity. Perhaps, therefore, the observed protection conferred by the *F. hepatica*-infected rat serum is due to the presence in the serum of increased levels of protease inhibitors rather than anti-parasite antibodies. Lewert et al. (1959) demonstrated the inhibition of collagenase-like enzymes from *S. mansoni* by *S. mansoni*-infected human sera. They attributed this inhibition to non-specific inhibitors, and not antibodies which they found were elevated in an active infection.

Obviously, if cathepsin B from *F. hepatica* are responsible for the fluke's evasion of the host immune response then neither the concentration of protease inhibitor nor the titre of anti-proteinase antibodies in the serum of an infected animal prove sufficient to neutralise the enzyme activity. The reasons for this are unclear. In this study, a reasonably high antibody

titre of anti-thiol proteases was obtained in the rabbit. However, we do not know if antibodies produced were capable of inactivation of cathepsin B activity or whether the anti-cathepsin B titre was very high. Since cathepsin B is naturally present in mammals the host immune system may not recognise the complete cathepsin B from the parasite as foreign and it is therefore immunologically tolerated and little antibody response is induced. Further analysis of the cathepsin B released from *F. hepatica* would be required to compare its homology to mammalian cathepsin B. A significant similarity has been shown between mammalian cathepsin B and a cysteine proteinase from *Plasmodium falciparum*. It is the active site histidine region of the cathepsin B which showed greatest similarity to the Plasmodium sequence. However, apart from this region, the similarity between the proteins was weak overall. If this was also the case between mammalian and *F. hepatica* cathepsin B's then we would expect that any antibodies produced would be directed against non-homologous regions and hence not against homologous regions close to the active site. Therefore the antibodies produced may not interfere with the enzyme activity even following binding. Another reason why these parasites might avoid neutralization may be that the antibodies or serum protease inhibitors are unable to cope with such large quantities of cathepsin B which are continually released by the fluke.

The anti-thiol proteases antiserum prepared in the rabbit was used to screen an adult worm cDNA expression library constructed in λgt11. Our objective for carrying out this experiment was to be able to isolate cDNA's encoding the proteases, to determine their sequence for characterization and homology searches and to produce recombinant fusion proteins for vaccination trials. However, we did not succeed in detecting any recombinant clone using this antisera. At this stage we can only suggest several reasons for this failure including 1) the titre of antibody may not have been sufficiently high enough to detect any recombinant clones, 2) the antibodies may be specific only for the glycosylated epitopes (if they exist) on the protease secreted by the fluke and 3) the protease expressed in *E. coli* may assume a conformation not recognized by the antibodies. The antibodies produced in rabbits may be

directed predominantly to conformational epitopes rather than to linear sequences and hence may not bind to *E. coli*-produced fusion proteins which would lack the native conformation. Hybridization with a suitable oligonucleotide probe would probably prove a more successful method in our case to screen for a positive recombinant clone. Our identification of a false-positive recombinant clone may have been due to some anti-*E. coli* antibodies present in the antiserum despite our efforts to adsorb them out.

In order to obtain sufficient quantities of protein from these parasites for characterization and vaccination, recombinant DNA technology has generally been used. Recently, Sakanari et al. (1989) showed that conserved structural motifs could be used to make generic molecular probes for amplification of serine protease gene fragments from the DNA of parasitic organisms using the polymerase chain reaction (PCR). PCR can offer a more convenient method of amplifying a specific DNA segment. 25 cycles of PCR amplification increase the amount of the target sequence by around a million fold with high specificity. Eakin et al. (1990) used the same strategy ; they amplified cysteine protease gene fragments from 3 protozoan parasites *Trypanosoma cruzi*, *T. brucei*, and *Entamoeba histolytica* by the PCR from genomic DNA using degenerate oligonucleotide primers. These primers were designed based upon amino acid sequences flanking the active site cysteine and asparagine residues that are conserved in the eukaryotic cysteine proteases analyzed to date. The amplified DNA fragments were subcloned and sequenced. This PCR method may prove to be a successful approach to amplify a gene fragment from the DNA of thiol proteases released from *F. hepatica*. The gene fragments isolated by this method provide probes to obtain the full length cDNA's and genes. The laborious and time-consuming efforts of enzyme purification and antibody production can be circumvented. Characterization of these gene products will provide biochemical information on the function of these enzymes.

By studying the hinge region of IgG after its digestion by cathepsin B we can locate the exact target where the enzyme cleaves. Coupled with experiments using synthetic peptides one could

learn the specificity of the enzyme for peptide bonds. Investigations on the mode of action of inhibitory agents may lead to a rational chemotherapy of this parasitic infection. To study the location of IgG digestion by the cathepsin B I would suggest purification of the Fc fragment by passing the digested IgG fragments through a *Staphylococcus aureus* protein A column. The Fc portion binds to the protein A and can be eluted, leaving the cleaved hinge portion accessible for N-terminal sequencing. O'Reilly et al. (1986) has sequenced IgG fragments resulting from IgG-cleavage by proteases from some bacteria including *N. gonorrhoeae*, *N. meningitidis* and *S. pneumoniae*. He found these proteases to cut in the hinge region of the heavy chain. It would be interesting to compare the peptide sequences which these proteases hydrolyze with that which the cathepsin B from *F. hepatica* hydrolyzes.

In conclusion, we have identified two groups of proteases released from *F. hepatica*. Group I is a cathepsin B-like enzyme showing a molecular mass of 15 kDa and which cleaves IgG at or close to the papain cleavage site. I speculate that this group may also be responsible for hemoglobinase activity. The function of the Group II proteases have yet to be characterized. Further work should be carried out on the 40 kDa protease from Group II which is released by immature fluke but was very minor in the mature fluke E/S. This enzyme may exhibit collagenase activity since at this stage the immature fluke is actively burrowing through the liver tissue. One must be cautious however when analyzing these proteases on gelatin substrate SDS polyacrylamide gels. We proved from using HPLC that the proteases ran aberrantly in these gels, which were run under non-reducing conditions, and the results were therefore misleading. Also not all proteases of interest were detected by this method. Finally, we believe the cathepsin B to be of great significance in host immune evasion by *F. hepatica*. We have isolated this enzyme and demonstrated its cleavage of IgG *in vitro*. Further studies should involve PCR to amplify the gene fragment for cathepsin B, then sequencing the gene and finally the expression of this gene. If this could be accomplished, followed by further study of this enzyme and its inhibition then the prospect of either chemotherapy or vaccine development would look very promising.

7.0 BIBLIOGRAPHY

Anderson, J.C., Hughes, D.L. and Harness, E. 1975. The immune response of rats to subcutaneous implantation with *Fasciola hepatica*. Br. Vet. J. 131, 509-517.

Aronstein, W.S., Dalton, J.P. and Strand, M., 1985a. A *Schistosoma mansoni* surface glycoprotein cross-reactive with a T1 antigen of *Fasciola hepatica*. American Journal of Tropical Medicine and Hygiene 34, 889-897.

Aronstein, W.S., Dalton, J.P., Weiss, J.B. and Strand, M., 1985b. Identification and characterization of a major *S. mansoni* glycoprotein antigen cross-reactive with *Fasciola hepatica*. American Journal of Tropical Medicine and Hygiene 34, 879-888.

Aronstein, W.S., Lewis, S.A., Norden, A.P., Dalton, J.P. and Strand, M, 1986. Molecular identity of a major antigen of *Schistosoma mansoni* which cross-reacts with *Trichinella spiralis* and *Fasciola hepatica*. Parasitology. 92, 133-151.

Asch, H.L. and Dresden, M.H. 1979. Acidic thiol proteinase activity of *Schistosoma mansoni* egg extracts. J. Parasitol. 65, 543-549.

Auriault, C., Joseph, M., Dessaint, J.P. and Capron, A. 1980. Inactivation of rat macrophages by peptides resulting from cleavage of IgG by Schistosoma larvae proteases. Immunol. Lett. 2, 135-139.

Auriault, C., Ouaissi, M.A., Torpier, G., Eisen, H. and Capron, A. 1981. Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomula. Parasite Immunol. 3, 33-44.

Barrett, A.J., 1973. Human cathepsin B1. Purification and some properties of the enzyme. Biochemical Journal 131, 809-822.

Barrett, A.J., 1977. Proteinases in mammalian cells and tissue. In : J.T. Dingle (ed.), Research monographs in cell and tissue physiology. Vol. 2. North-Holland, New York, pp. 1-55, 181-

208.

Barrett, A.J. and McDonald, J.K. 1980. Mammalian Proteinases:a Glossary and Bibliography, Vol. 1: Endopeptidases. Academic Press, London.

Barrett, A.J. and Kirschke, H. 1981. "Cathepsin B, Cathepsin H, Cathepsin L". In: Methods Enzymol. Proteolytic Enzymes. Part C. 80, 535-561.

Barrett, A.J., Kembhavi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M. and Hanada, K., 1982. L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane(E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. Biochem. J. 201, 189-198.

Bazan, J.F. and Fletterick, R.J., 1988. Viral cysteine proteases are homologous to the trypsin-like family of serine proteases : structural and functional implications. Proc. Natl. Acad. Sci. USA 85, 7872-7876.

Bennett, C.E. and Threadgold, L.T., 1975. *Fasciola hepatica* : Development of tegument during migration in mouse. Experimental Parasitology 38, 38-55.

Bennett, C.E., 1978. The identification of soluble adult
antigen on the tegumental surface of juvenile *Fasciola hepatica*. Parasitology 77, 325-332.

Bennett, C.E., Hughes, D.L. and Harness, E., 1980. *Fasciola hepatica* : Changes in tegument during killing of adult flukes surgically transferred to sensitized rats. Parasite Immunology 2, 39-55.

Boray, J.C. 1969. "Experimental fascioliasis in Australia". In "Advances in Parasitology". B. Dawes(editor). Academic Press. New York. 7, 95-210.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 72, 248-258.

Bricker, J., Mulks, M.H., Plaut, A.G., Moxon, E.R., Wright, A. 1983. IgA1 proteases of *Haemophilus influenzae*:cloning and characterization in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA. 80, 2681-2685.

Brocklehurst, K. and Little, G. 1973. Reactions of Papain and of low-Molecular-Wt thiols with some aromatic Disulphides. Biochemical Journal. 133, 67-80.

Burden, D.J., Hughes, D.L. and Hammet, W.C., 1982a. ***Fasciola hepatica*****:Antibody coating of juvenile flukes in the intestinal lumen of resistant rats. Res. Vet. Sci. 32, 44-47.**

Burden D.J., Harness, E. and Hammet, N.E. 1982b. ***Fasciola hepatica*****:Attempts to immunise rats with metabolic and somatic antigens derived from juvenile flukes. Vet. Parasitol. 9, 261-266.**

Burden, D.J., Bland, D.L., Hughes and Hammet N.C. 1983. ***Fasciola hepatica*****:development of the tegument of normal and γ-irradiated flukes in infection in rats and mice. Parasitology, 86, 137-145.**

Capron, A., Biguet, J., Vernes, A. and Afchain, D., 1968. Structure antigenique des helminthes. Aspects immunologiques des relations hote-parasite. Pathologie Biologie 16, 121-138.

Capron, A., Dessaint, J.P., Capron, M., Ouma, J.H. and Butterworth, A.E. 1987. Immunity to schistosomes:Progress toward vaccine. Sci. 238, 1065-1072.

Chapman, C.B. and Mitchell, G.F., 1982a. Proteolytic cleavage of immunoglobulin by enzymes released by *Fasciola hepatica*. Veterinary Parasitology 11, 165-178.

Chapman, C.B. and Mitchell, G.F. 1982b. ***Fasciola hepatica*****:Comparative studies on fascioliasis in rats and mice. Int. J. for Parasitol. 12, (1), 81-91.**

Christensen, N.O., Monrad, J., Nansen, P. and Frandsen, F., 1980. ***Schistosoma mansoni*** **and** ***Fasciola hepatica*** **: Cross-resistance in mice with single-sex schistosoma infection. Exp. Parasitol. 49, 116-121.**

Clegg, J.A. and Smith, M.A. 1978. Prospects for the development of dead vaccines against helminths. Adv. Parasitol. 16, 165-218.

Colowick, S.P. and Kaplan, N.O. 1970. Cathepsins. In : Methods in Enzymology. Vol. XIX (Proteolytic enzymes) (Ed. by Perlmann and Lorand).

Commins, M.A., Goodger, B.V. and Wright, I.G. 1985. Proteinases in the lysate of bovine erythrocytes infected with ***Babesia bovis*****:initial vaccination studies. Int. J. Parasitol. 5, 491-495.**

Corba, J., Armour, J., Roberts, R.J. and Urquhart, G.M. 1971. Transfer of immunity to

Fasciola hepatica infection by lymphoid cells. Res. Vet. Sci. 12, 292-295.

Dalton, J.P. and Joyce P. 1987. Characterization of surface glycoproteins and proteins of different developmental stages of *Fasciola hepatica* by surface radiolabelling. J. of Parasitology. 73(6), 1281-1284.

David, P.H., Hadley, T.J., Aikawa, M. and Miller, L.H. 1984. Processing of a major parasite surface glycoprotein during the ultimate stages of differentiation in *Plasmodium knowlesi.* Mol. Biochem. Parasitol. 11, 267-282.

Davies, C. and Goose, J. 1979. Preliminary investigation into the killing of newly excysted juveniles of *Fasciola hepatica* in immune rats. Parasitology 79, xlviii.

Davies, C. and Goose, J., 1981. Killing of newly excysted juveniles of *Fasciola hepatica* in sensitized rats. Parasite Immunology 3, 81-86.

Dawes B. and Hughes D.L., 1964. Fascioliasis : The invasive stages of *Fasciola hepatica* in mammalian hosts. In "Advances in Parasitology" B. Dawes (editor). Academic Press, New York. 2, 97-168.

Dawes, B. and Hughes, D.L., 1970. Fascioliasis : The invasive stages in mammals. Advances in Parasitology 8, 259-274.

Deelder, A.M., Reinders, P.N. and Rotmans, J.P. 1977. Purification studies on an acidic protease from adult *Schistosoma mansoni.* Acta Leidensia. 45, 91-103.

Drenth, J., Jansonius, J.N., Koekoek, R. and Wolthers, B.G., 1971. The structure of papain. Adv. Protein Chem. 25, 79-115.

Dresden, M.H. and Deelder, A.M., 1979. *Schistosoma mansoni* : Thiol proteinase properties of adult worm "hemoglobinase". Exp. Parasitol. 48, 190-197.

Dresden, M.H., Rutledge, M.L. and Chappell, C.L. 1981. Properties of an acidic thiol proteinase from *Schistosoma mansoni* adults. Biochem. Molec. Parasitol. 4, 61-66.

Duffus W.P.H. and Franks D., 1980. *In vitro* effect of immune serum and bovine granulocytes on juvenile *Fasciola hepatica.* Clinical and Experimental Immunology 41, 430-440.

Duffus W.P.H. and Franks D., 1981. The interaction *in vitro* between bovine immunoglobulins and juvenile *Fasciola hepatica.* Parasitology 82, 1-10.

Eakin, A.E., Bouvier, J., Sakanari, J.A., Craik, C.S. and McKerrow, J.H. 1990. Amplification and sequencing of genomic DNA fragments encoding cysteine proteases from protozoan parasites. Molec. and Biochem. Parasitol. 39, 1-8.

Eisen, H. and Tallon, I. 1977. *Tetrahymena pyriformis* recovers from antibody immobilisation by producing univalent antibody fragments. Nature 270, 514-515.

Geroc, A. 1975. Repellent-like principle as a "mode of action" of antifasciolics (abstr.). In "Proceedings of the 7th International Conference of the World Association for the Advancement of Veterinary Parasitology, Thessaloniky, Greece July 14-16 1975".

Glazer, A.N and Smith, E.L., 1971. Papain and other plant sulphydryl proteolytic enzymes. In : The Enzymes, 3rd eds. (P.D. Boyer, ed.), Academic Press, New York.

Goose, J., and Macgregor, M. 1973. Naturally Acquired immunity to *Fasciola hepatica* in the rat. Br. Vet. J. 129, xlix-lii.

Goose, J. Thesis, Brunel University. 1977.

Goose, J., 1978. Possible role of excretory/secretory products in evasion of host defences by *Fasciola hepatica* Nature 275, 216-217.

Grant, C.T. and Senft, A.W. 1971. Schistosome proteolytic enzyme. Comp. Biochem. Physiol. 38B, 663-678.

Green, G.D.J. and Shaw, E., 1981. Peptidyl diazomethyl ketones are specific inactivators of thiol proteinases. J. Biol. Chem. 256, 1923-1928.

Grove and Newall. 1979. "Animal Biology" 9th ed. University Tutorial Press.

Hadley, T., Aikawa, M. and Miller L.H. 1983. *Plasmodium knowlesi*:Studies on invasion of rhesus erythrocytes by merozoites in the presence of protease inhibitors. Exp. Parasitol. 55, 306-311.

Hall, R.F. and Lang, B.Z. 1978. The development of an experimental vaccine against *Fasciola hepatica* in cattle. Proceedings of the U.S. Animal Health Association. 82, 55-60.

Halton, D.W. 1967. Observations on the nutrition of digenetic trematodes. Parasitol. 57, 639-660.

Hanna, R.E.B. and Jura, W., 1977. Antibody response of calves to a single infection of

Fasciola gigantica determined by an indirect fluorescent antibody technique. Research in Veterinary Science 22, 339-342.

Hanna, R.E.B., 1980a. ***Fasciola hepatica*** **: glycocalyx replacement in the juvenile as a possible mechanism for protection against host immunity. Experimental Parasitology 50, 103-114.**

Hanna, R.E.B., 1980b. ***Fasciola hepatica*** **: An immunofluorescent study of antigenic changes in the tegument during development in the rat and sheep. Experimental Parasitology 50, 155-170.**

Hanna, R.E.B., 1980c. ***Fasciola hepatica*** **: autoradiography of protein synthesis, transport and secretion by the tegument. Experimental Parasitology 50, 297-304.**

Hanna, R.E.B. and Trudgett, A.G., 1983. ***Fasciola hepatica*** **: development of monoclonal antibodies and their use to characterize a glycocalyx antigen in migrating flukes. Parasite Immunology 5, 409-425.**

Hanna, R.E.B., Anderson, A. and Trudgett, A.G., 1988. ***Fasciola hepatica*** **: studies on vaccination of rats and mice with a surface antigen prepared from fluke homogenate by means of a monoclonal antibody. Research in Veterinary Science 44, 237-241.**

Haroun, E.M., Hammond, J.E. and Sewell, M.M.H. 1980. Resistance to ***Fasciola hepatica*** **in rats and rabbits following implantation of adult flukes contained in diffusion chambers. Res. Vet. Sci. 29, 310-314.**

Haroun, E.M., Hammond, J.A. and Sewell, M.M.H. 1982. Absorption of protective components from serum of cattle infected with ***Fasciola hepatica.*** **Res. Vet. Sci. 33, 263-264.**

Haroun, E.M. and Hillyer, G.V. 1986. Resistance to fascioliasis-a review. Vet. Parasitol. 20, 63-93.

Hayes, T.J., Bailer, J. and Mitrovic, M. 1972. Immunity in rats to superinfection with ***Fasciola hepatica.*** **Journal of Parasitology 58, 1103-1105.**

Hayes, T.J., Bailer, J. and Mitrovic, M. 1974. Serum transfer of immunity to ***Fasciola hepatica*** **in rats. J. Parasitol. 60, 722-723.**

Heussen, C. and Dowdle, E.B. 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerised substrates. Anal.

Biochem. 102, 196-202.

Hillyer, G.V. 1974. Immunity to Schistosomes using heterologous trematode antigens-A review. Parasitol., 14, 263-283.

Hillyer, G.V., 1976. Can we vaccinate against schistosomes? Fed. Proc., 35, 2568-2571.

Hillyer, G.V. and Sagramoso de Ateca, L., 1980. Antibody responses in murine schistosomiasis and fascioliasis. Am. J. Trop. Med. Hyg., 29, 598-601.

Hillyer, G.V. and Serrano, A.E. 1982. Cross protection in infections due to *Schistosoma mansoni* using tegument antigens of *Fasciola hepatica*. J. Infect. Dis. 145, 728-732.

Hillyer, G.V., Haroun, E.M., Hernandez, A. and Soler de Galanes, M. 1987. Acquired resistance to *Fasciola hepatica* in cattle using a purified adult worm antigen. Am. J. Trop. Med. and Hyg. 37, 363-369.

Holder, A.H. and Freeman, R.R. 1982. Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and a monoclonal antibody. J. Exp. Med. 156, 1528-1538.

Hotez, P.J., Le Trang, N., McKerrow, J.H. and Cerami, A. 1985. Isolation and characterization of a proteolytic enzyme from the adult hookworm *Ancylostoma caninum*. J. Biol. Chem. 260(12), 7343-7348.

Howell, R.M. (1966). Collagenase activity of immature *Fasciola hepatica*. Nature 209, 713-714.

Howell, M.J. 1973. Localization of proteolytic activity in *Fasciola hepatica*. J. Parasitol. 59, 454-456.

Howell, M.J. and Sandeman, R., 1979. *Fasciola hepatica* : Some properties of a precipitate which forms when metacercariae are cultured in immune rat serum. Int. J. Parasitol. 9,41-45.

Hughes, D.L. 1963. Some studies on the host-parasite relations of *Fasciola hepatica*. PhD thesis, London University.

Hughes, D.L., Harness, E. and Doy, T.G., 1977. Loss of ability to kill *Fasciola hepatica* in sensitized rats. Nature (London) 267, 517-518.

Hughes, D.L., Harness, E. and Doy, T.G., 1981. The different stages of *Fasciola hepatica* capable of inducing immunity and the susceptibility of various stages to immunological attack in the sensitized attack. Res. Vet. Sci. 30,93-98.

Hughes, D.L., Hanna, R.E.B. and Doy, T.G. 1982. Antibody response in cattle, sheep and rats to infection with irradiated metacercariae of *Fasciola hepatica*. Res. Vet. Sci. 32, 354-358.

Kendall, S.B. 1967. Resistance of the host to *Fasciola hepatica*. Proceedings of the Royal Society of Medicine. Great Britain. 60, 168-169.

Kirschke, H., Langner, J., Riemann, S., Wiederanders, B. and Bohley, P. 1980. In Proteinases and Tumor Invasion (Strauli, P., Barrett, A.J. and Baici, A., eds) pp. 69-79, Raven Press, New York.

Knox, D.P. and Kennedy M.W. 1988. Proteinases released by the parasitic larval stages of *Ascaris suum*, and their inhibition by antibody. Molec. and Biochem. Parasitol. 28, 207-216.

Koomey, J.M., Gill, R.E. and Falkow, S. 1982. Genetic and biochemical analysis of gonococcal IgA protease:clonong in *Escherichia coli* and construction of mutants of gonococci that fail to produce the activity. Proc. natn. Acad. Sci. 79, 7881-7885.

Laemmli, U.K. and Favre, M. 1973. Maturation of the head of bacteriophage T4, I. DNA packaging events. J. Mol. Biol. 80, 575-599.

Lang B.Z., 1967. Host-Parasite relationships of *Fasciola hepatica* in the white mouse 11 studies on acquired immunity. J. Parasitol. 53, 21-30.

Lang, B.Z., 1976. Host-parasite relationships of *Fasciola hepatica* in the white mouse. VII. Effect of anti-worm incubate sera on transferred worms and successful vaccination with a crude incubate antigen J. Parasitol. 62, 232-236

Lang, B.Z. and Hall, R.F. 1977. Host-parasite relationships of *Fasciola hepatica* in the white mouse. VIII. Successful vaccination with culture incubate antigens and antigens from sonic disruption of immature worms. J. of Parasitol. 63, 1046-1049.

Lehner, R.P. and Sewell, M.M.H. 1979. Attempted immunization of laboratory animals with metabolic antigens of *Fasciola hepatica*. Vet. Sci. Communications. 2, 337-340.

Lewert, R.M., Lee, C.L., Mandlowitz, S. and Dusanic, K.G. 1959. Inhibition of the

collagenase-like enzymes of cercariae of *Schistosoma mansoni* by serum S and serum fractions. J. Infect. Dis. **105**, 180-187.

Locatelli, A. and Beretta, C. 1969. Detection of amino acid N in saline or serum media incubated with *Fasciola hepatica* and proteolytic activity exerted by liverfluke *in vitro*. Archivio Veterinario Italiano. **20**, 385-391.

Lowe, G. 1966. Structural Relationships between some Plant and Animal Proteases. Nature. **212**, 1263-1264.

Lyon, J.A. and Haynes, J.H. 1986. *Plasmodium falciparum* antigens synthesized by schizonts and stabilized at the merozoite surface when schizonts mature in the presence of protease inhibitors. J. Immunol. **136**, 2245-2251.

McKerrow, J.H., Jones, P., Sage, H. and Pino-Heiss, S. 1985. Proteinase from invasive larvae of the trematode parasite *Schistosoma mansoni* degrade connective tissue and basement-membrane macromolecules. Biochem. J. 231, 47-51.

Mckerrow, J.H., Bindley, P., Brown, M., Gam, A.A., Staunton, C. and Neva, F.A. 1990. *Strongyloides stercoralis* : Identification of a protease that facilitates penetration of skin by the infective larvae. Exp. Parasitol. **70**, 134-143.

Mitchell, G.G.B., Armour, J., Ross, J.G. and Halliday, W.G. 1981. Successful passive transfer of resistance to *Fasciola hepatica* infection in rats by immune serum and transfer factor. Res. Vet. Sci. **30**, 246-247.

Morihara, K., 1974. Comparative specificity of microbial proteinases. Adv. Enzymol. **41**, 179-243.

Mort, J.S., Recklies, A.D. and Poole, A.R. 1980. Characterization of a thiol proteinase secreted by malignant Human Breast Tumours. Biochim. Biophys. Acta **614**, 134-143.

Movsesijan, M. and Jovanovic, B. 1975. Immune response of sheep to *Fasciola hepatica* infection. Res. Vet. Sci. **18**, 171-174.

Mulks, M.H. and Plaut, A.G. 1978. IgA protease production as a characteristic distinguishing pathogenic from harmless Neisseriae. New Eng. J. Med. **299**, 973-976.

North, M.J., 1982. Comparative biochemistry of the proteinases of eukaryotic microorganisms.

Microbiol. Rev. 46, 308-340.

O'Connor, B. and O'Cuinn, G. 1987. Active Site Studies on a Narrow-Specificity Thyroglobulin-Hydrolysing Pyroglutamate Aminopeptidase Purified from Synaptosomal Membrane of Guinea-Pig Brain. J. Neurochem. 48(3), 676-680.

Oldham, G. and Williams, L. 1985. Cell mediated immunity to liver fluke antigens during experimental *Fasciola hepatica* infection of cattle. Parasite Immunol. 7, 503-516.

O'Reilly, T.M. and Bhatti A.R. 1986. Proteases of the pathogenic neisseria:possible role in infection. Microbios. 45, 113-129.

Petralanda, I., Yearzabal, L. and Pressons, W.F. 1986. Studies on a filarial antigen with collagenase activity. Mol. Biochem. Parasitol. 19, 51-59

Plaut, A.G., Gilbert, J.V., Artenstein, M.S. and Capra, J.D. 1975. *Neisseria gonorrhoeae* and *Neisseria meningitidis* : extracellular enzyme cleaves human immunoglobulin A. Science 190, 1103-1105.

Plaut, A.J. 1983. The IgA1 proteases of pathogenic bacteria. A. Rev. Microbiol. 37, 603-622.

Pohlner, J., Halter, R., Beyreuther, K. and Meyer, T. F. 1987. Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. Nature (London) 325, 458-462.

Rajasekariah, G.R., Mitchell, G.F., Chapman, G.B. and Montague, P.E., 1979. *Fasciola hepatica* : Attempts to induce protection against infections in rats and mice by injection of excretory/secretory products of immature worms. Parasitology 79, 393-400.

Recklies, A.D., Mort, J.S. and Poole, A.R. 1982. Secretion of a thiol proteinase from Mouse Mammary Carcinomas and its characterization. Cancer Research 42, 1026-1032.

Rege, A.A., Herrera, P.R., Lopez, M. and Dresden, M.H. 1989. Isolation and characterization of a cysteine proteinase from *Fasciola hepatica* adult worms. Molec. and Biochem. Parasitol. 35, 89-96.

Reich, C.I. and Zorzopulos, J. 1980. *Boophilus microplus* : cattle anti-enzymes to larval phosphomonoesterases. Exp. Parasitol. 50, 272-277.

Rosenthal, P.J., Kami,K., McKerrow, J.H. and Leech, J.H. 1987. Idetification of the three stage-specific proteinases of *Plasmodium falciparum*. J. Exp. Med. 166, 816-821.

Rosenthal, P.J., McKerrow, J.H., Rasnick, D. and Leech, J.H. 1989. ***Plasmodium falciparum*****:inhibitors of lysosomal cysteine proteinases inhibit a trophozoite proteinase and block parasite development. Molecular and Biochemical Parasitology. 35, 177-184.**

Ross, J.G. 1967. Studies of immunity to ***Fasciola hepatica*****:Acquired immunity in cattle, sheep and rabbits following natural infection and vaccine procedures. J. of Helminthology. 41, 393-399.**

Rothwell, T.L.W., Ogilvie, B.M. and Love, R.J. 1973. Acetylcholinesterase secretion by parasitic nematodes. II. ***Trichostrongylus spp.*** **Int. J. Parasitol. 3, 599-608.**

Sakanari, J. and McKerrow, J.H. 1988. Characterization of a protease excreted by the invasive stage of the "sushi worm" Anisakis simplex. J. Cell. Biochem. Suppl. 12B, 299.

Sakanari, J.A., Staunton, C.E., Eakin, A.E., Craik, C.S. and McKerrow, J.H. 1989. Serine proteases from nematode and protozoan parasites:Isolation of sequence homologues using generic molecular probes. Proc. Natl. Acad. Sci. USA. 86, 4863-4867.

Sandeman, R.M., and Howell, M.J. and Campbell, N.J., 1980. An attempt to vaccinate sheep against ***Fasciola hepatica*** **using a juvenile fluke antigen sheep antibody complex. Res. Vet. Sci. 29, 255-259.**

Sandeman, R.M. and Howell, M.J. 1981. Precipitating antibodies against excretory/secretory antigens of ***Fasciola hepatica*** **in sheep serum. Veterinary Parasitology 9, 35-46.**

Sherman, I.W. 1979. Biochemistry of Plasmodium (malaria parasites). Microbiol. Rev. 43, 453-495.

Simpkin, K.G., Chapman, C.R. and Coles, G.C. 1980. ***Fasciola hepatica*****:A Proteolytic Digestive Enzyme. Exp. Parasitol. 49, 281-287.**

Sinclair, K.B. 1962. Observations on the clinical pathology of bovine fascioliasis. Br. Vet. J. 118, 37-53.

Sinclair, L.J. 1970. The relationship between circulating antibodies and immunity to helminthic infections. Adv. Parasitol. 8, 97-138.

Sinclair, K.B. 1971. Resistance to ***Fasciola hepatica*** **in sheep: attempts to transfer resistance with lymph node and spleen homogenates. Br. Vet. J. 127, 125-136.**

Smithers, S.R., 1976. Immunity to trematode infections. In "Immunology of Parasitic Infection" (S.Cohen and E.Sadun, eds.), pp. 296-332. Blackwell,Oxford/London/Edinburgh/Melbourne.

Takio, k., Towatari, T., Katunuma, N., Teller, D.C. and Titani, K., 1983. Homology of amino acid sequences of rat liver cathepsin B and H with that of papain. Proc. Natl. Acad. Sci. USA 80, 3666-3670.

Tamashiro, W.K., Rao, M. and Scott, A.L. 1987. Proteolytic cleavage of IgG and other protein substrates by *Dirofilaria immitis* microfilarial enzymes. J. Parasitol. 73(1), 149-154.

Thorsen, R.E. 1953. Studies on the mechanism of immunity in the rat to the nematode *Nippostrongylus muris*. Am. J. Hyg. 58, 1-15.

Thorsen, R.E. 1956. Proteolytic activity in extracts of the oesophagus of adults of *Ancylostoma caninum* and the effect of immune serum on this activity. Int. J. Parasitol. 42, 21-25.

Threadgold, L.T., 1967. Electron microscope studies of *Fasciola hepatica*. 111. Further observation on the tegument and associated structures. Parasitology 57, 633-637.

Timms, A. and Bueding, E. 1959. Studies of a proteolytic enzyme from *Schistosoma mansoni*. Brit. J. Pharmacol. 14, 68-73.

Ward, O.P. 1985. "Proteolytic Enzymes". In:Commercial Biotechnology Vol. 3. Ed. by Murray Moo-Young.

Waxman, L., 1981. Calcium-activated proteases in mammalian tissues. Methods in Enzymology 80, 664-680.

Wikerhauser, T. 1961. Immunonobiologic diagnosis of fascioliasis . II. The in vitro action of immune serum on the young parasitic stage of *Fasciola hepatica*-a new precipitin test for fascioliasis Vet. Arch. 31, (314), 71-80.

Zussman, R.A. and Bauman, P.M. 1971. Schistosome hemoglobin protease;search for inhibitors. J. Parasitol. 57, 233-234.

8.0 APPENDIX : RESEARCH COMMUNICATIONS

Papers Published

Dalton, J.P. and Heffernan, M. (1989). Thiol proteases released *in vitro* by *Fasciola hepatica*. Molecular and Biochemical Parasitology. 35, 161-166.

Meetings

Heffernan, M. and Dalton, J.P. Analysis of *Fasciola hepatica* proteases using zymograms. Poster presented for the Biochemical Society. (1988).

Heffernan, M., Smith, A., Curtin, D., Mc Donnell, S., Ryan, J. and Dalton, J.P. Characterization of a cathepsin B proteinase released by *Fasciola hepatica*. Poster presented for the Biochemical Society. (1990).