Identifying “hidden” antigens in the liver fluke, *Fasciola hepatica*

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Abstract

Fasciola hepatica is responsible for substantial economic losses and animal welfare issues within the agricultural sector worldwide. The increasing incidence of fasciolosis, coupled with the emergence of flukicide resistance, makes vaccination an attractive alternative control strategy. Hidden antigens extracted from the gut of blood feeding parasites have proven to be excellent vaccine candidates against haematophagous parasites, most notably *Haemonchus contortus* and *Rhipicephalus (Boophilus) microplus*. Here, as a first step towards a prototype liverfluke vaccine an attempt to identify hidden gut antigens in *F. hepatica* was made. Proteomic analysis on extracts of adult *F. hepatica* was used to identify molecules exclusively found within the membrane-bound fraction including four proteases; cathepsin B2, legumain-2, a putative lysosomal pro-x-carboxypeptidase precursor and a saposin-like protein. Histological sections of adult *F. hepatica* were screened with a panel of 21 lectins to identify those with an affinity for glycoproteins on the parasite's gut and to inform subsequent lectin affinity chromatography. Seven lectins showed affinity for the gut region, with peanut (PNA) and jacalin (JAC) lectins binding to glycoproteins on either the gastrodermal cells or gut lamellae, respectively. PNA and JAC were then used to purify glycoproteins from the crude S3 extract by affinity chromatography. The resultant fractions were separated by SDS-PAGE and the protein profiles analysed by mass spectrometry. The enriched lectin-binding fractions shared a number of proteins but one of note that was exclusively identified in the PNA-binding fraction was a cathepsin D-like aspartyl protease, which had not previously been studied in *F. hepatica.* The proteolytic activities of three somatic extracts of adult *F. hepatica* were therefore investigated. The ability of the respective fractions to digest haemoglobin, a potential food source, was measured in the presence/absence of class-specific enzyme inhibitors. These analyses confirmed the presence of cathepsin D-like aspartyl protease activity capable of digesting haemoglobin optimally at pH 2 - 2.5. Further characterisation of the cathepsin D-like aspartyl (FhCatD) protease revealed it to be highly conserved within trematodes, to be localized to the gastrodermis of immature (10 day) and adult fluke, and to be more highly expressed, at the RNA level, in the Newly Excysted Juveniles (NEJ) than adult stages. Western blot analysis of native somatic extracts, enriched lectin-binding fractions and recombinant FhCatD using antisera from naturally infected sheep, showed some recognition of the recombinant FhCatD but did not provide clear evidence that the cathepsin D is strongly antigenic during natural infection.

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Author’s declaration

I declare that the work presented in this thesis is my own original work and has not been submitted for any other degree or qualification.

Heather McDougall

Date

Abbreviations

NEJ	Newly Excysted juveniles
bTB	Bovine tuberculosis
BCG	Bacillus Calmette-Guérin
SEPA	Scottish Environmental Protection Agency
NOAH	National Office of Animal Health
TCBZ	Triclabendazole
Th	T helper
B cells	B lymphocytes
T reg	T regulatory cells
TCR	T cell receptors
MHC	major histocompatibility complex
INF- γ	interferon-γ
IL	interleukin
Ig	immunoglobulins
ESGP	eosinophil secondary granule proteins
MBP	major basic protein
ADCC	antibody-dependent cellular cytotoxicity

GST	glutathione *S*-transferase
FABP	fatty acid binding protein
FheCL	*F. hepatica* Cathepsin L
LAP	Leucine aminopeptidases
NHmec	leucine-7-amino-4-methylcoumarin
FhLAP	*F. hepatica* Leucine aminopeptidase
Hb	Haemoglobin
r	recombinant
Ac-APR	*A. caninum* Cathepsin D
MS	Mass spectrometry
LC-ESI-MS/MS	Liquid chromatography – Electron spray ionisation - Tandem mass spectrometry
ToF	Time-of-flight
MOWSE	MOlecular Weight SEarch
EST	expressed sequence tags
2D	two dimensional
MALDI-ToF	matrix-assisted laser desorption/ionisation Time of flight
LCM	laser capture microscopy
PBS	phosphate buffered saline

HB	homogenising buffer
S1	PBS-soluble
S2	membrane-associated
S3	membrane-bound
BSA	Bovine Serum Albumin
BCA	bicinchoninic acid
KEGG	Kyoto Encyclopaedia of Genes and Genomes
SAP	saposin-like protein
OCT	Optimal Cutting Temperature
DEPC	diethylpyrocarbonate
E/S	excretory/secretory
LWB	lectin wash buffer
STWS	Scots tap water substitute
SDS PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
PNA	peanut agglutinin
JAC	jacalin agglutinin
HRP	Horse radish peroxidase
Na-APR-1	*N. americanus* Cathepsin D

DTT	dithiothreitol
TCA	trichloroacetic acid
CatDFS	7-methoxycourin-4-acetyl-gly-lys-pro-ile-leu-phe-phe-arg-leu-lys(DNP)-D-arg-amide
MSA	Multiple sequence alignment
PTM	Post translational modifications
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
PepA	Pepstatin A
FhCatD	*F. hepatica* cathepsin D

1 General Introduction

Liver fluke disease or fasciolosis, is an economically important disease of sheep and cattle worldwide. It is estimated to cost the global agricultural sector approximately US $2 billion (Bennett and Ipjelaar, 2005; McGonigle *et al*., 2008; Spithill *et al*., 1999) and within Scotland account for losses of around £50 million per annum (Dr G.B. Mitchell, Personal communication). These losses are attributable to mortality, reduction in milk and meat production, secondary bacterial infections, expensive anthelmintic treatment and condemnation of livers at slaughter, Table 1.1 (Garcia *et al*., 2008; McKenna *et al*., 2002; Schmidt and Roberts, 2005).

Table 1.1. Liver condemnation data for Great Britain in 2010 (Data courtesy of The organisation for the English Beef and Lamb Executive)

Liver rejection data (fluke) 2010		**Throughput**	**Liver condemnation**	**% Liver condemnation**	**Estimated loss (£) from liver rejection only***
England	Cattle	1,547,151	306,499	19.81	£1,225,996
	Sheep	8,625,486	700,982	8.13	£ 911,277
Wales	Cattle	135,563	38,126	28.12	£152,504
	Sheep	3,672,596	165,877	4.52	£215,640
Scotland	Cattle	518,461	143,271	27.63	£573,084
	Sheep	1,463,044	128,560	8.79	£167,128

*sheep liver = £1.30, cattle liver = £4.00

Fasciolosis is an example of an emerging/re-emerging human parasitic disease in the Andean countries (Bolivia, Peru, Chile, Ecuador), the Caribbean area (Cuba), northern Africa (Egypt), western Europe (Portugal, France and Spain) and the Caspian area (Iran and neighbouring countries) (Mas-Coma, 2004). It is spurred on by both environmental changes (warmer, wetter climate) and man-made modifications such as an increase in animal movements and intensification of livestock farming (Mas-Coma *et al*., 2005). Over the last decade there has been a substantial increase in the number of fasciolosis cases recorded in the UK, (Figure 1.1).

Previously the disease was restricted to the wetter western areas of the U.K. but today spreads to the Eastern and Northern areas which were previously considered too dry and cold to host fluke (Kenyon *et al.,* 2009; Baird, 2010). Climate change has caused a combination of wetter summers and milder winters which aids the survival of both the infective stages on pasture and the parasite's intermediate mud snail host (Kenyon *et al.,* 2009; van Dijk *et al.,* 2009; Fox *et al.,* 2011). Furthermore, an acute liver fluke warning was issued in January 2009 by the Scottish Agricultural College stating that this was the cause of numerous deaths amongst sheep in south-west Scotland, despite the routine treatment with flukicides in October (Mitchell and Rundle, 2009).

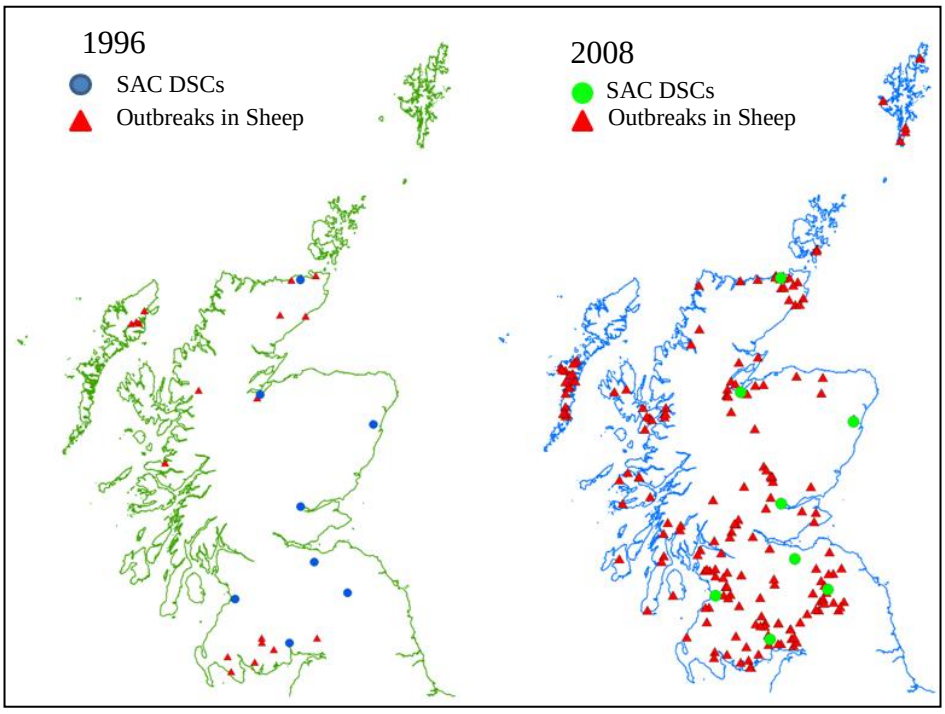

Figure 1.1: Recorded fluke outbreaks in Scottish sheep flocks. This illustrates the increase in occurrence of outbreaks (red triangle) from those in recorded 1996 (green map) to 2008 (blue map). The locations of Scottish Agricultural College (SAC) disease surveillance centres (DSCs) in 1996 (blue circle) and 2008 (green circle) are also shown. (Data kindly provided by SAC and reproduced by kind permission of Dr G.B. Mitchell)

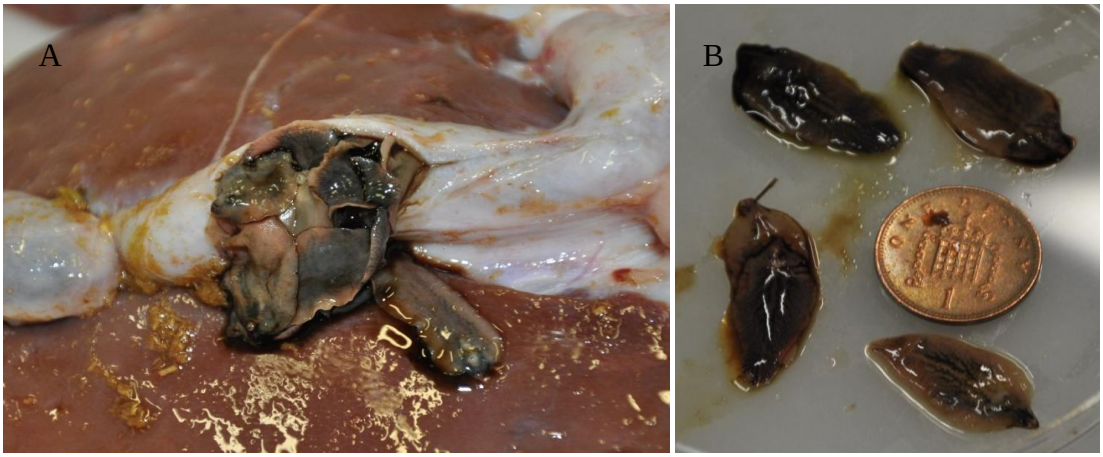

Figure 1.2: Adult *F. hepatica*, A) emerging from the bile duct of an 11 month old lamb and B) after removal from the liver

1.1 Fasciola hepatica

Fasciolosis is caused when the definitive host (herbivorous mammals or humans) ingest the infective metacercarial cysts of digenean trematodes from the genus *Fasciola.* Two species, *Fasciola hepatica* and *Fasciola gigantica* are involved. These are leaf-shaped parasitic flatworms which infect the liver and bile duct of their host and can be up to 30 mm in length (Schmidt and Roberts, 2005), see Figure 1.2. The geographic distribution varies between these two species, where *F. hepatica* is ubiquitous throughout all regions (Mas-Coma *et al*., 2005) and *F. gigantica* is restricted to equatorial regions (Torgerson and Claxton, 1999). Within the U.K., *F. hepatica* is the causative agent of fasciolosis.

1.2 Liver fluke Life-cycle

The life-cycle of *F. hepatica* is complex and comprises a number of developmental stages, as shown in Figure 1.3. Adult stages of the parasite reside in the liver and bile duct system of their definitive hosts, e.g. sheep and cattle. A large proportion of an adult fluke's body consists of reproductive organs (Hanna *et al.,* 2006) and each parasite has the potential to shed up to 25,000 eggs per day (Happich and Boray, 1969). These are deposited in the host's faeces onto pasture and undergo embryonation in 9 to 10 days given warm (above 10°C) and wet conditions (Schmidt and Roberts, 2005). The eggs hatch into ciliated, free-living larval stages known as miracidia when conditions are wet. Each miracidium actively seeks and penetrates an intermediate host, typically molluscs belonging to the genera *Lymnaea*, *Pseudosuccinea*,

Galba and *Stagnicola* (Rognlie *et al.*, 1994; Shubkin *et al.*, 1992). Within the UK the intermediate *F. hepatica* stages have to date only been detected in *Galba* (*Lymnaea) truncatula*. However, in Ireland intermediate stages of *F. hepatica* have been detected in molluscs of the *Succinea* sp. and *Radix peregra* (Relf *et al.*, 2008). The miracidia respond to light, which bring them near the water's surface increasing their chances of encountering a potential intermediate host (Kalbe *et al.*, 1997; Saladin, 1979). High temperatures dramatically reduce the miracidium life-span to as little as 6 hours at 25 °C (Smith and Grenfell, 1984). However, a miracidium will only successful infect a snail if it locates and penetrates the snail within 3 hours of hatching (Kalbe *et al.*, 1997). Once inside the snail, each miracidium migrates through the host tissue undergoing metamorphosis and transforming into the next larval stage, the sporocyst. This then migrates to the snail's digestive gland and grows further. Within the sporocyst, there are a number of germinal cells which replicate to give rise to the next larval stage, the rediae, which are freed when the sporocyst ruptures. The germinal balls within the redia also replicate and develop to give rise to the final larval stage, the cercaria. Cercarial shedding is initiated by the presence of fresh water (Kendall, 1951; Walton, 1918). Cercariae are tadpole-like with a discoidal body, long tail, oral sucker and ventral sucker in the centre of the body, similar to that seen in the adult fluke. The cercariae leave the snail by migrating through the digestive tissues to the salivary gland. They swim freely in the water and become encysted on vegetation then develop into metacercariae, the infective stage. The metacercariae are ingested along with vegetation by the definitive host (Acosta *et al*., 2008; Andrews, 1999). The parasite then undergoes excystation into the newly excysted juvenile (NEJ) stage in the definitive host's small intestine (Andrews, 1999). Following this they penetrate through the intestinal mucosa into the peritoneal cavity where they browse on the available tissue. They then migrate to the liver arriving there 4 to 6 days post-infection (Andrews, 1999). The immature fluke burrows through the liver tissue, feeding on available tissue and causing extensive haemorrhage and fibrosis before eventually reaching the bile ducts around 7 weeks post-infection. They then complete development to sexually mature adults, begin to produce eggs and the cycle repeats (Andrews, 1999; Schmidt and Roberts, 2005). The parasite's life-cycle is highly prolific and the asexual multiplication within the snail host can lead to rapid and extensive contamination of pasture with fluke cysts. (Schmidt and Roberts, 2005).

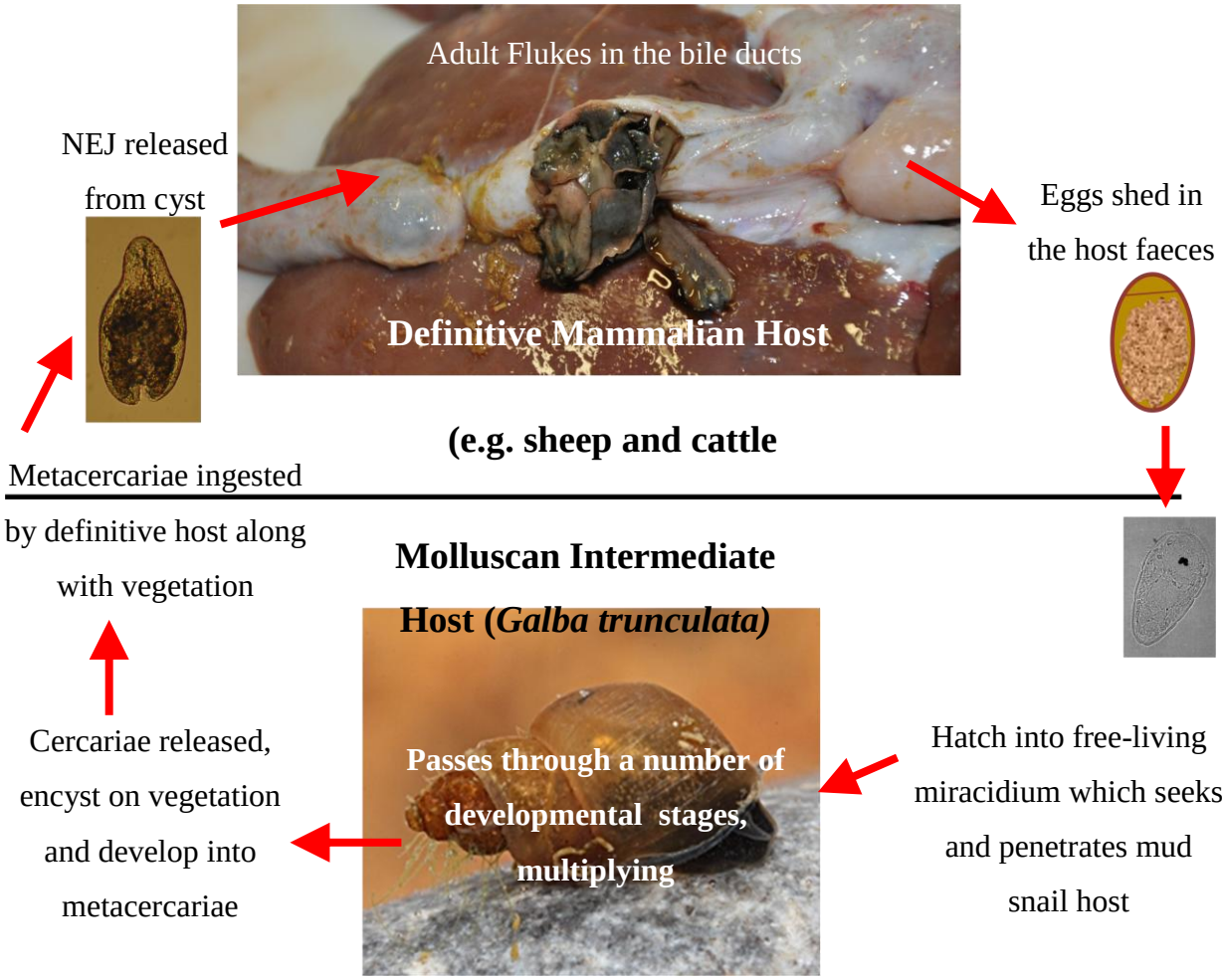

Figure 1.3: The life-cycle of the liver fluke, *Fasciola hepatica*. Adult flukes in the bile ducts of the definitive host (e.g. sheep, cattle) shed eggs in the faeces. Eggs hatch on pasture into miracidia. These actively seek and penetrate a suitable snail intermediate host. Once inside the snail, they undergo a number of developmental stages through sporocyst, rediae and cercariae. The free-swimming cercariae exit the snail host and encyst on vegetation as metacercariae, the infective stages. These are then ingested by the mammalian definitive host. The metacercariae then excyst into newly excysted juveniles, which make their way to the liver and to the bile duct system, developing into egg laying adults and the cycle continues.

1.3 Pathogenesis and Clinical Signs

Infection with *F. hepatica* comprises two phases: firstly the parenchymal phase, where immature flukes migrate through the liver parenchyma, and secondly, the biliary phase, which coincides with their maturation into adults residing in the bile ducts. The parenchymal phase begins when NEJ flukes penetrate the intestinal wall, following which they then migrate within the abdominal cavity and penetrate the liver or other organs (Behm and Sangster, 1999).

Clinical signs are closely associated with the severity of the disease which is determined by the level of infection, nutritional plane of the animal and also on the individual host species and breed (Behm and Sangster, 1999). Fasciolosis can be classified as acute, sub-acute or chronic (Osman *et al.*, 1998).

1.3.1 Acute Fasciolosis

Acute fasciolosis occurs when large numbers of the immature stages of fluke migrate simultaneously through the liver parenchyma of the definitive host. This normally occurs from October through to spring after animals become infected in the summer or from late spring until early summer if initially infected during winter, Table 1.2 (Ollerenshaw, 1959). However, recently within the U.K, acute fasciolosis has been reported throughout the year as opposed to the traditional spring and autumn infections (Kenyon *et al.*, 2009; Sargison, 2011). The extensive tissue damage caused by the migration of NEJ can result in a reduction of liver function and intraperitoneal haemorrhage (Behm and Sangster, 1999; Fetcher, 1983). This is often characterised by anaemia, weight loss, diarrhoea and an enlargement of the liver but infected animals often show no symptoms of an acute infection (Armour *et al*., 1970; Fletcher, 1983; Smith, 1978). Acute infections in dairy herds have been reported to result in significant production losses and extended periods between calving (Charlier et al 2007). Acute fasciolosis may cause sudden death in sheep and has been reported to cause losses of up to 25% of the animals within flocks (Behm and Sangster, 1999). Cattle rarely die from the acute form of the disease unless very young calves are subject to a large intake of metacercariae (Dalton *et al.*, 2003).

Table 1.2: The seasonality of fasciolosis within U.K sheep flocks and the developmental stages responsible for the type of disease. (Data adapted from Abbott *et al.*, 2009)

Disease Type	Peak Incidence months	Predominant stage of parasite present
Acute	July – December	Immature
Sub-acute	October – January	Adult and immature
Chronic	January - April	Adult

1.3.2 Sub-acute Fasciolosis

Subacute fasciolosis normally peaks between October and January after a large intake of metacercariae but over a more prolonged period than that causing the acute form (Table 1.2). Furthermore, both adult and immature flukes can be present simultaneously (Abbott *et al.*, 2009). Infected animals often develop haemorrhagic anaemia with this type of fasciolosis (Smith, 1978).

1.3.3 Chronic Fasciolosis

Chronic fasciolosis is associated with the establishment of adult stages in the bile duct and the shedding of eggs. This type of disease in sheep is often seen between January and April, Table 1.2 (Abbott *et al.,* 2009). It has a number of associated symptoms including weight loss, paleness, submandibular oedema (bottlejaw), and wool break in sheep (Behm and Sangster, 1999; Fletcher, 1983). The liver function of infected animals can be compromised leaving them more susceptible to secondary infections such as the bacterium *Clostridium novyi*, resulting in a condition known as Black's disease (Smith, 1978). Other clinical outcomes such as abortions and reduced milk production in lactating animals have also been associated with the condition (Fetcher, 1983). There are concerns about the effect of a chronic fluke infection on the reliability of the bovine tuberculosis (TB) test, which relies on cell-mediated immune responses. Experimental co-infections with *F. hepatica* and *Mycobacterium bovis* brought into

question the predictive capacity of TB tests (Flynn *et al.,* 2007). Infection with *F. hepatica,* as discussed later, skews the host's immune response, decreasing interferon γ (INF-γ) production (Flynn *et al.,* 2007; Flynn *et al.,* 2009). This compromises the reliability of the bovine TB test which relies on an INF-γ response (Flynn *et al.,* 2007). Furthermore, a study involving 3026 dairy herds found a negative relationship between diagnosis of bTB and exposure to *F. hepatica* (Claridge *et al.,* 2012). Chronic fasciolosis only results in death when animals are severely weakened by the infection (Fletcher, 1983; Smith, 1978).

1.4 Control and treatment of Fasciolosis

Fasciolosis is difficult to control for a number of reasons, some of which include; amplification of the intermediate stages of the parasite within the snail host which rapidly creates high levels of infective cysts on pasture, a lack of natural immunity to infection in the definitive host and the presence of wildlife reservoirs (Andrews *et al,* 1999). However, it can be controlled by using a combination of strategies in order to reduce heavy pasture contamination and to an extent prevent definitive host contact with the infective stages (Brunsdon, 1980).

1.4.1 Pasture management

The intermediate stages of *F. hepatica* develop within the mud snail *G. trunculata* as mentioned above. These snails inhabit wet and marshy environments (Kendell, 1949). These areas are likely to harbour high levels of the infective metacercariae after they exit the intermediate host. Wet areas of fields can be fenced off to prevent contact of the host with infective stages or drained to make them less habitable for the snails, thus reducing the number of infective metacercariae produced (Osborne, 1967). However, this is a costly method and it is not always feasible or practical (Wilson *et al.,* 1982).

1.4.2 Snail control

Molluscicides, aiming to kill the snail intermediate host, have provided successful short-term control of infective stages present on the pasture and have proven cost effective in the past (Crossland, 1976; Urquhart *et al.,* 1970). However, they have gained little support and are now considered to be environmentally/ecologically unacceptable (Wilson *et al.,* 1982). Despite being very effective against the snails they are non-specific and often kill other species

including fish and crabs (Roberts and Suhardono, 1996). In addition, there are now regulations (outlined by the Scottish Environmental Protection Agency, SEPA) which restrict the use of chemicals such as molluscicides on pastures. Furthermore, their effect is often short-lived as the snails, being hermaphrodite, have a huge potential for rapid repopulation once molluscicides clear from the environment and can therefore repopulate an area in a short time (Roberts and Suhardono, 1996).

There are a number of plants which possess natural mollscicidal activity. These include some plants from the *Eucalyptus spp.* (Hammond *et al.,* 1994; Zhou *et al.,* 1993) and the *Euphorbiales spp.* (Singh and Agarwal, 1988). The leaves of *Eucalyptus spp.* and the latex from *Euphorbiales* are toxic to snails *in situ* (Singh and Agarwal, 1988; Hammond *et al.*, 1994). To date, trials have not been carried out *in situ* and it is unclear how effective these would be at controlling the snails in the field (Torgerson and Claxton, 1999).

Snails are naturally predated by arthropods, amphibians, reptiles, birds and rodents (Torgerson and Claxton, 1999). Normally they exist in equilibrium with their predators, rapidly increasing when conditions are favourable (Torgerson and Claxton, 1999). The intensive farming of ducks and geese has been shown to eradicate snails from pastures (Levine, 1970) but this method is, again, not always feasible. The *Sciomyzid* fly larvae (Berg, 1953) and the snail-killing fly *Llione albiseta* (Germally, 1988) have also been suggested as potential predators of *G. trunculata* and are thus potential biological control species. Introducing other snail species which will compete for the same habitat but which do not host intermediate stages of *F. hepatica*, such as *Zonitoides nitdus,* has also been proposed as a control method to reduce pasture contamination of the infective metacercariae (Ximenes *et al.,* 1993).

1.4.3 Treating infected animals

Despite a number of available methods to manage fasciolosis, ultimately in domestic livestock liver fluke disease is controlled by treating infected animals with anthelmintics, specifically fasciolicides or flukicides (Roberts and Suhardono, 2008; Torgerson and Claxton, 1999). There is a range of such products available, which typically fall into one of 4 chemical classes; benzimidazoles, salicylanilides, nitrophenols and halogenated hydrocarbons. These anthelmintics differ in efficacy, price, safety and the stage of parasite which they target, as summarised in Table 1.3 (Torgerson and Claxton, 1999). These chemicals leave residues in

the meat and milk of treated animals and thus can have lengthy withdrawal periods (up to 65 days) preventing their sale for human consumption during this time (National Office of Animal Health, (NOAH), www.noahcompendium.co.uk/Compendium/Overview/-41030.html).

Table 1.3: Currently available flukicides for use in sheep, adapted from Fairweather and Boray (1999).

Chemical Class	Anthelmintic	Age of parasite targeted (Weeks)
Benzimidazole	Albendazole	>12
	Triclabendazole	1
Halogenated phenol	Bithionol	>12
	Hexachlorophene	12
	Niclofolan	12
	Nitroxynil	8
Salicylanilide	Brotianide	12
	Closantel	6 to 8
	Oxyclozanide	12
	Rafoxanide	6

The benzimidazole derivative, Triclabendazole (TCBZ), has been the predominant drug of choice for treating liver fluke infections for over 20 years (Brennan *et al*., 2007). This is largely because it is effective against early immature and adult stages of the parasite, whereas most of the other flukicides only show activity against the later stages as highlighted in Table 1.3 (Fairweather and Boray, 1999).

In the 1990s, the first evidence of TCBZ resistant fluke populations was reported in Australia, just over a decade after this drug class was introduced (Overend and Bowen, 1995). Resistance has since been reported in a number of countries including Ireland (Anon, 2005), U.K. (Mitchell *et al.,* 1998; Thomas *et al.,* 2000), The Netherlands (Gaasenbeek *et al.,* 2001) and Spain (Alvarez-Sanchez *et al.,* 2006). The true extent of resistance to TCBZ is currently unknown (Fairweather, 1995) and resistance to the salicylanide compound clostantel, another extremely important and useful flukicide, has also been reported in Australia (Fairweather and Boray, 1999).

1.5 Immunology

1.5.1 The host immune system

The host immune system protects it from invading pathogens using layers of defence mechanisms of increasing specificity. Initially, physical barriers, such as the epithelium, prevent the infectious agents from entering the host. If these are breached the hosts' innate, followed by the adaptive, immune system act to try to expel the pathogen. The innate immune system is fast acting and uses non-specific defence mechanisms to expel invading bodies. The specific acquired immune system is slower acting but highly adaptable (Mulcahy *et al.*, 1999).

CD4+ T helper cells (Th), B lymphocytes (B cells) and T regulatory (Treg) lymphocytes have critical roles in host defence and immunoregulation (Hirahara *et al.*, 2011). T cells are a major source of cytokines and bear receptors (TCR) which recognise antigen peptides (T cell epitopes) in association with major histocompatibility complex (MHC) presenting cells (Bhattacharya and Sinha, 2006). Th cells can be divided into subsets which each differ in phenotype and function, e.g. Th1, Th2 and Th17 cells (Hirahara *et al.*, 2011).

The Th1 cells produce interferon-γ (INF- γ), interleukin-2 (IL-2) and interleukin-3 (IL-3) which promote a type 1 immune response (O'Neill *et al.*, 2000). These cytokines promote the production of activated macrophages, antibodies, mediate delayed type hypersensitivity reactions and inflammatory responses (O'Neill *et al.*, 2000). This response is often elicited against invading intracellular organisms (viruses, bacteria and protozoa) but can also be effective against extracellular organisms. For example, induced immunity to schistosomiasis in mice is mediated by activated macrophages, which can kill larval *Schistosoma mansoni* in vitro (James *et al.*, 1982).

Th2 cells produce a number of cytokines including interleukin-4 (IL-4), interleukin-5 (IL5), interleukin-6 (IL-6) and interleukin-10 (IL10) which promote a type 2 immune response. This has been shown to be important in the control of helminth infections (Urban *et al.*, 1995; Else *et al.*, 1994; Mulcahy *et al.*, 1999). The cytokines promote B cell proliferation, the secretion of immunoglobulins (IgA, IgG1 and IgE) and mediate production / activation of mast cells and eosinophils. Eosinophils bind to antibodies on the surface of extracellular organisms, such as *F. hepatica* and release compounds, such as nitric oxide which are toxic to the invading pathogen (Anthony *et al.*, 2007; Mulcahy, 1999).

The cytokines released by the different T cell subsets regulate the type of immune response generated. For instance, the type 1 cytokine INF- γ suppresses type 2 responses where as IL-4, IL-10 and IL-13 inhibit the effects of INF- γ and thus the development of type 1 responses (Mulcahy, 1999).

1.5.2 The host immune response to infection with *F. hepatica*

To date, the definitive host's immune response to infection with *F. hepatica* is not fully understood. Studies show that infection with *F. hepatica* provokes a Th2 biased response, which has been shown to be important in the expulsion of some helminth parasites (Mulcahy *et al.,* 1999). The expulsion of helminth parasites by Th2 responses has been demonstrated in rodent models with an established infection of *Heligmosomoides polygyrus* (Urban *et al.*, 1995), *Nippostrongylus brasiliensis* (Urban *et al.*, 1998), *Trichuris muris* (Else *et al.,* 1994) and *Trichinella spiralis* (Ahmad *et al*., 1992). The importance of the Th2 response has been further demonstrated in murine models, where infection of IL-4 deficient mice with the gastrointestinal nematode, *Trichuris muris* results in a patent infection in an inbred mouse strain which would normally resist infection (Bancroft *et al.,* 1998). Although the definitive host's immune response toward *F. hepatica* is thought to be predominately Th2 biased, in the early stages of infection Th1 cells are thought to also play a role (Antony *et al.,* 2007; Herbert *et al.,* 2004; Moreau & Chauvin, 2010; Rodríguez-Sosa *et al.,* 2002), see Figure 1.4. During the migratory phase of the NEJ and immature fluke, Th1 cells produce INF- γ which induces classically activated macrophages. These macrophages bind to the parasite and produce nitric oxide (Noel *et al.,* 2004), which is toxic to the fluke (Antony *et al.,* 2007; Herbert *et al.,* 2004; Moreau & Chauvin, 2010; Rodríguez-Sosa *et al.,* 2002). When the infection proceeds to later (chronic) stages the immune response becomes predominately Th2 biased.

The cytokine IL-4 activates and promotes B cell proliferation to produce antibodies IgE and IgG1 (Antony *et al.,* 2007; Moreau & Chauvin, 2010; Urban *et al.,* 1992). IL-4 also stimulates IL-5 production which causes rapid proliferation of eosinophils which rapidly migrate to the site of infection under the direction of a number of cytokines including IL-4 and IL-3 (Antony *et al.,* 2007; Moreau & Chauvin, 2010; Urban *et al.,* 1992). Eosinophils release compounds, such as eosinophil secondary granule proteins (ESGPs), which in murine models are toxic to the invading *F. hepatica* and *Schistosoma* (Antony *et al.,* 2007; Sabin *et al.,* 1996; Behm &

Ovington, 2000; Klion & Nutman, 2004). Together, the increased production of IgE, IgG1 and IL-5 contribute to the production of antibody-dependent cellular cytotoxicity (ADCC). The binding of antibodies, IgE and IgG1, to the surface of the fluke is then recognised by receptors present on mast cells, as shown in a rat infection model (Van Milligen *et al.*, 1998). These then secrete vasoactive amines and other mediators of inflammation resulting in the degranulation of eosinophils which releases toxic chemicals onto the parasites surface, such as major basic protein (MBP) and protamine sulphate which can kill the invading NEJ (Duffus, 1980) and also amplify the Th2 response through the production of cytokines IL-5 (Antony *et al.*, 2001; Mulcahy *et al.*, 1999). ADCC is thought to be the principal mechanism in clearing helminth infections (Mulcahy *et al.*, 1999). For example, NEJs from immune rats were coated with IgG1 antibodies and surrounded by eosinophils which were not observed in naïve controls, suggesting that the NEJs are killed by an eosinophil-mediated cytotoxic response (Van Milligen *et al.*, 1998), which is also seen in murine models infected with Schistosome (Sabin *et al.*, 1996). Finally, the secretion of interleukin-13 stimulates the production of alternatively activated macrophages which assist in tissue repair and can result in fibrosis, as seen in the bile ducts of chronic fasciolosis in cattle (Moreau & Chauvin, 2010).

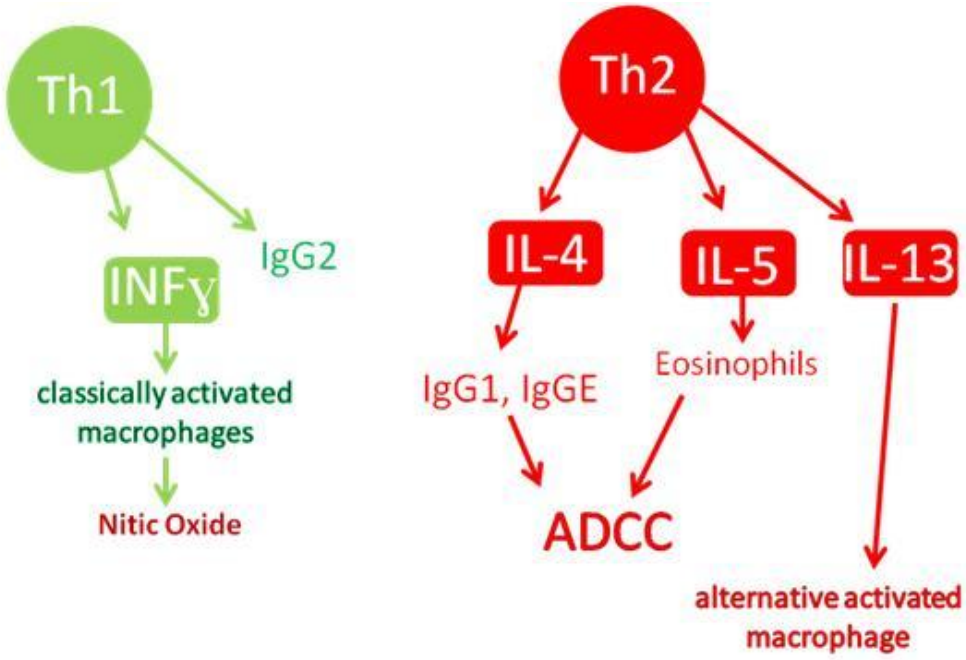

Figure 1.4: Definitive host immune mechanisms produced against *F. hepatica* infection. Two mechanisms have been proposed to occur against *F. hepatica*. (a) The initial phase of infection involves classically activated macrophages which bind to the parasite and induce nitric oxide production, which is harmful to the fluke. This is produced by Th1-type cytokines. (b) During the later (chronic) phase, Th2 cells produce the cytokines interlekin-4 (IL-4) and interlekin-5 (IL-5) which induce antibody and eosinophil production. This in turn evokes antibody-dependent cellular cytotoxicity (ADCC) which causes the degranulation of eosinophils and releases of toxic mediators such as major basic protein, eosinophil cationic protein, and reactive nitrogen intermediates toward the parasite. This phase inhibits the production and function of Th1 cytokines. Finally, alternative activated macrophages produce molecules that are toxic to the fluke and participate in fibrosis and tissue repair (adapted from Antony *et al.*, 2011).

1.5.3 Parasite evasion of immune attack

Flukes have evolved a number of mechanisms to evade the immune system and thus survive for long periods within their host (Fairweather and Boray, 1999). However, the ways in which the parasite evades or modulates the immune system are not fully understood. The final residence in the bile ducts is a relatively immunologically ‘safe’ environment from immune attack (Hanna, 1982) for the parasite but, to get there, it must evade the immune system as it

migrates through the intestinal wall and liver tissue (Haroun and Hillyer, 1986). Only 5-10% and 20-25% of the inocula in cattle or sheep, respectively, reach maturity in experimental infections, indicating that a large proportion of the NEJ either fail to enter the gut or are killed during the migrating phase (Haroun and Hillyer, 1986; Piacenza *et al.,* 1999).

The tegument of *F. hepatica* differs from that of related species such as the schistosomes. Instead of the schistosomes' two-lipid bilayer (thin layer composed of two layers of hydrophobic molecules) liver flukes have a single surface membrane covered with a tough glycocalyx (carbohydrate-based structure) (Threadgold, 1976). This tough tegument protects the liver fluke from the immune system in a number of ways. Using surface radio-labelling techniques, Dalton and Joyce (1987) showed that the glycoproteins on the surface of the NEJ, immature (liver) and the adult (bile duct) stage differed. So, as the parasite develops from a NEJ through to the adult stage, the surface composition alters, presenting the immune system with a changing target, therefore protecting the parasite from immune recognition by specific antibodies (Tkalcevic *et al.,* 1995, 1996). The glycocalyx on the surface is also shed and replaced approximately every 3 hours as the parasite migrates to the bile ducts (Hanna, 1980). This prevents the definitive host's immune defense mechanisms, such as the eosinophils, making sufficient contact with the fluke to cause damage to their surface (Hanna, 1980). In addition to this, the glycocalyx shed from the parasite binds any circulating antibodies, which then prevents further upregulation of the host immune response (Duffus and Franks, 1981).

The tracts made by flukes in the liver tissue are filled with immune effector cells such as T and B lymphocytes, macrophages and granulocytes (eosinophils and neutrophils) but these are not attached to any of the parasites present (Meeusen *et al.,* 1995). Flukes from a secondary infection (where a host which has previously been infected with *F. hepatica* but the infection was cleared and the host reinfected) are never found in cavities generated by flukes in the primary infection which indicates flukes may avoid areas where there are high levels of immune response mechanisms (Meeusen *et al.,* 1995). Parasites may also modulate short range immune responses, this may explain the presence of undamaged flukes in tissues filled with immune effector cells (Meuusen *et al.,* 1995). The way in which the parasites do this is not fully understood, but they may secrete enzymes such as glutathionine *S*-transferase to deactivate the effector cells (Brophy *et al.,* 1990; Creaney *et al.,* 1995). Carmona *et al.,* (1993) identified a cathepsin L protease in NEJ excretory secretory (E/S) material and showed that it

can prevent antibody-mediated attachment of eosinophils to this stage, thus evading immune detection.

Furthermore, parasites release immunosuppressive factors to modulate the definitive host's immune system (Sandeman and Howell, 1981; Zimmermann *et al.*, 1983). As an infection proceeds in an ovine host the proliferative capacity of the peripheral blood lymphocytes reduces after just 4 weeks of infection, indicating that modulatory effects occur as the parasite migrates to the liver tissue (Zimmermann *et al.*, 1993; Mulcahy *et al.*, 1999). In addition, the fluke's E/S products can block the differentiation and maturation of eosinophils by bone marrow cells in mice (Milboume & Howell, 1990; Milboume & Howell, 1993). A 28kDa protein isolated from the E/S fluid can mimic the action of IL-5 (Milboume & Howell, 1993) and, thus, direct the definitive host's immune system to a less effective Type 2 response, allowing the host to tolerate the parasite leading to chronic infection (Clergy *et al.*, 1996). Furthermore, the *F. hepatica* E/S proteases, cathepsin L1 and cathepsin L2, can also degrade all subclasses of human IgG, which assist the parasite in evading immune attack (Berasain *et al.*, 2000).

1.5.4 Immunity to infection with *F. hepatica*

Numerous attempts have been made to raise a natural immunity to infection with *F. hepatica* in sheep using primary homologous infections (where naïve animals are infected with low numbers of *F. hepatica* cysts), infections with metacercariae and inocculating with somatic or secreted antigens (Spithill *et al*., 1999; Spithill and Dalton, 1998). Despite evidence that there is a degree of variability between sheep breeds in their resistance to infection with *F. hepatica* (Boyce *et al*., 1987), there is little evidence to suggest that sheep sensitized to infection of *F. hepatica* (previously infected) develop any protective immunity to reinfection (Boyce *et al*., 1987; Sandeman and Howell, 1981; Sinclair, 1971). Sheep are capable of producing an antibody response towards experimental infection with *F. hepatica* which is predominately IgG1 and peaks 5 to 6 weeks after a primary infection (Movsesijan, 1974). This has been exploited in the development of diagnostic tests which detect circulating antibodies, however, this response does not appear to be protective (Mitchell *et al*., 1981).

Furthermore, when antibodies from sheep experimentally infected with *F. hepatica* are passively transferred to animals which were subsequently challenged, there was a 64%

reduction in the number of flukes recovered from the liver (Mitchell *et al*., 1981). This indicates that the lack of inducible protective immunity in sheep may be related to insufficient cellular responses (Mulcahy *et al*., 1999) or modulation by the parasite to evade immune recognition as discussed previously.

Cattle are more resilient than sheep to infection following previous exposure to the parasite suggesting a difference in immune response between the two species (Mulcahy *et al*., 1999). Unlike sheep, fasciolosis is rarely fatal in cattle which often self-cure between 9 and 26 months after infection (Mulcahy *et al*., 1999). Firstly, they have substantially larger livers than sheep and secondly the chronic infection causes a condition called "pipestem fibrosis", where biliary ducts appear white from fibrosis and inflammation. This is rarely seen in sheep (Mulcahy *et al.,* 1999). This condition creates a physical barrier between the host and the parasite making it difficult for parasites to navigate their way through the liver tissue, thus preventing feeding and ultimately killing the fluke (Jones *et al.,* 1997). A number of studies have found that cattle previously exposed to infection with *F. hepatica* were less susceptible than naïve animals to reinfection (Doyle, 1971; Ross, 1967). Studies indicate with increasing liver fibrosis there is a decrease in the establishment of mature adult parasites, indicating that cattle's protection occurs at the liver capsule (Anderson *et al*., 1978; Doy and Hughes, 1984). In addition, NEJs were still able to migrate through the peritoneal cavity wall (Anderson *et al*., 1978; Doy and Hughes, 1984; Mulcahy *et al*., 1999; Ross, 1967).

1.5.5 Vaccination

Vaccination would certainly be a desirable alternative control strategy to treating animals with flukicides, due to consumer concerns about chemical residues in food and (Science for Environment Policy, 2008) the presence of anthelmintic resistant parasite populations (Overend and Bowen, 1995; Anon, 2005; Mitchell *et al.,* 1998; Thomas *et al.,* 2000; Gaasenbeek *et al.,* 2001; Alvarez-Sanchez *et al.,* 2006). A commercial vaccine to prevent fasciolosis is currently not available (McManus and Dalton, 2006). However, immunoprophylactic control of fasciolosis has been attempted in both sheep and cattle using injection with either parasite extracts or defined functional parasite antigens which differ in protection level elicited (Spithill and Dalton, 1998). These antigens include glutathione *S*-transferase (GST), cathepsin L-like cysteine proteases, fatty acid binding protein (FABP),

leucine aminopeptidases (LAP) and fluke haemoglobin (Spithill and Dalton, 1998). These will each be discussed in turn.

1.5.5.1 Glutathione *S*-transferase

Purified GST from adult *F. hepatica* was the first defined antigen to induce high levels of protection in sheep (Sexton *et al*., 1990). Vaccination with native GSTs initially failed to reduce worm burdens in rats experimentally infected with *F. hepatica* (Howell *et al*., 1988), although a subsequent challenge trial with sheep receiving multiple injections elicited a significant 57% reduction in worm burden at *post mortem* (Sexton *et al*., 1990). Protection (as measured by percentage reduction in parasite burden to non vaccinated controls) has also been elicited in trials with cattle (Estuningsih *et al*., 1997; Morrison *et al*., 1996). The level of protection (measured by faecal egg count and worm burdens) was shown to be dependent on the adjuvant used, with GST in Quil A/Squalene Montanide 80® (Quil A/SM) resulting in a 69% reduction in worm burden (Morrison *et al*., 1996; Spithill and Dalton, 1998). GSTs belong to a family of isoenzymes which are involved in cellular detoxification, including the initial steps of detoxification of xenobiotics and endogenous toxic compounds such as anthelmintics (Spithill *et al*., 1999; Spithill and Dalton, 1998). They have been identified in all parasitic helminths (Brophy *et al*., 1990; Brophy and Pritchard, 1994) and proposed to have an important role in the helminth parasite's homeostasis and survival (Brophy *et al*., 1990; Brophy and Pritchard, 1994). They were chosen as candidate vaccine antigens because homologous native GST proteins from the closely-related *Schistosoma* spp., were shown to reduce parasite burden in mice (40-43%) and rats (50-72%) with two vaccinations of GST before subsequently being challenged with 15000 cercariae (Balloul *et al.*, 1987; Brophy and Pritchard, 1994). Five GST isoenzymes between 23-26.5kDa in size, have been purified from adult *F. hepatica* (Brophy *et al*., 1990; Howell *et al*., 1988; Wijffels *et al*., 1992). Immunolocalisation studies, using antisera raised in rabbits against a native *F. hepatica* GST cocktail, showed a widespread distribution of the GST throughout the tissues of the adult fluke e.g. within the intestine, the parenchymal cells, the tegument and the adjacent muscle cells (Howell *et al*., 1988; Wijffels *et al*., 1992). Furthermore, GST was localised to the intestine and associated with the lamellar surface (Wijffels *et al*., 1992). A similar analysis, using the NEJ, localised GST to the parenchymal cytoplasm, cytoplasmic extensions of the parenchymal cells in the subtegumental area and the excretory ducts and not the intestinal epithelium (Creaney *et al*., 1995). This altered distribution within the intestine of the adult compared to

the juvenile relates to different morphology and functions of the gut at different life-stages (Spithill *et al*., 1999). The juvenile gut contains few lamellae and gut cells have a secretory function, which is in contrast to the dual secretory/absorptive role in the adult gut (Bennett and Threadgold, 1973).

In vitro experiments in which anti-GST antibodies from trial sheep (those vaccinated with GST) were tested for their ability to inhibit GST activity, found no direct correlation between inhibition of GST activity *in vitro* and fluke burden, suggesting that inhibition of GST function by antibody binding is not responsible for the observed protection (Morrison *et al*., 1996).

1.5.5.2 Cathepsin L-like Proteases

Cysteine endopeptidases are found in many parasitic worms, with an important role in host/parasite interactions (McKerrow, 1989; Dalton and Heffernan, 1989; Dalton *et al*., 2003; Williamson *et al.,* 2003). Two gut-associated cysteine endopeptidases have been isolated from adult *F. hepatica.* These have been identified as cathepsin L-like cysteine proteases and termed cathepsin L1 (27 kDa, FheCL1) and cathepsin L2 (29.5 kDa, FheCL2), respectively (Mulcahy and Dalton, 2001). They have attracted considerable attention due to their predominance in the E/S products of adult and juvenile flukes (Dalton *et al*., 2003). These enzymes are homologous to mammalian lysosomal cathepsin L proteases, with the nucleotide sequences sharing 45% similarity with mammalian homologues (Tort *et al*., 1999). In liver flukes, cathepsin L-like proteases are secreted into the gut lumen following ingestion of host blood and liver tissue and participate in digestion of host tissues (Smith *et al*., 1993b; Yamasaki *et al*., 1992). Liver flukes have a blind-ending gut and constantly regurgitate their gut contents so these secreted enzymes have an additional role in tissue penetration and immune evasion (Spithill and Dalton, 1998). For example, i*n vitro* experiments have shown that FhCatL1 can cleave immunoglobulin in the hinge region and prevent antibody-mediated attachment of eosinophils to NEJ (Carmona *et al*., 1993; Goose, 1978; Smith *et al*., 1993a; Spithill and Dalton, 1998). FhCatL2 can cleave fibrinogen, in a similar manner to thrombin but at unique cleavage sites, to cause the formation of an insoluble blood clot, which can prevent the access of immune effector cells to the parasite's surface (Dowd *et al*., 1995; Spithill and Dalton, 1998). Both cathepsin Ls can cleave intracellular matrix proteins including collagen, laminin and fibronectin (Berasain *et al*., 1997; Howell *et al*., 1988).

Cathepsin B has been identified as a major protease released by juvenile flukes (Wilson *et al*., 1998). This protease shows similar properties to those exhibited by cathepsin L in the adult stages. For example, it has been localised to the gut epithelium of NEJs (Creaney *et al.* 1995), can aid immune evasion (Carmona *et al.,* 1995; Chapman and Mitchell, 1982; Smith *et al.*, 1993) and assist migration through host tissues (Wilson *et al.,* 1998).

The cathepsins have been considered as valuable candidates because of their involvement in crucial biological processes and their ability to digest a wide range of host substrates (Spithill and Dalton, 1998). A number of vaccine trials have identified their potential as vaccine candidates either as native or recombinant proteins (Dalton *et al*., 1996; Piacenza *et al*., 1999). An initial trial in sheep by Wijffels *et al* (1994) found that vaccination with a mixture of CatL1 and CatL2 did not significantly reduce fluke burdens in comparison to non-vaccinated controls. However, the parasites' egg output was reduced by 69.7 % and those eggs produced had an 80% reduction in viability (Wijffels *et al*., 1994). Trials in cattle have also provided promising results. When used individually in vaccination experiments, both the cathepsin L molecules significantly reduced worm burdens, egg output and egg fecundity (Dalton *et al.*, 1996). Cattle were vaccinated three times at 28 day intervals, then challenged with 500 metacercariae 28 days after final vaccination and killed 16 weeks post challenge. Results from vaccinated animals were compared with those from control animals (vaccinated with adjuvant only), where the native CatL1 reduced worm burdens by up to 69% (Dalton *et al*., 1996). Although the actual mechanisms which provide protective immunity have not been identified, there are two proposed explanations (Spithill *et al*., 1999; Wijffels *et al*., 1994). Firstly, the inhibition of cathepsin L activity may prevent the parasite digesting food sources and thus have secondary consequences including a reduction in egg production. This is supported by the observations of Dalton *et al* (1996), who found adults recovered from CatL1-vaccinated cattle were reduced in size. Secondly, it is proposed that a different tissue-located cathepsin L is required for egg production but is unable to act because vaccination induces cross-reacting neutralising antibodies, thus inhibiting egg production (Spithill *et al*., 1999). Adult flukes recovered from CatL1-vaccinated sheep were found to be normal in size but their fecundity was reduced by 67 % (Wijffels *et al*., 1994). Substrate specificity studies suggest that cathepsin L may play a role in processing the vitelline B precursor protein, a major eggshell protein of *F. hepatica* (Spithill et al., 1999). Furthermore, immunolocalisation studies have

also localised Cat L to the Mehlis' gland, an important organ in egg production (Wijffels *et al*., 1994).

1.5.5.3 Fatty acid binding proteins

The FABP antigen belongs to a family of proteins involved in binding and transporting a range of hydrophobic ligands across membranes (Spithill *et al*., 1999). FABP was the first defined and purified antigen fraction to be tested as a vaccine against fasciolosis (Hillyer *et al*., 1977; Hillyer, 1979). A set of proteins was purified from an extract of adult *F. hepatica* that cross-reacted with antisera raised in mice immunised with soluble proteins from the closely-related human blood fluke, *S. mansoni* (Hillyer *et al*., 1977). A subset of these *F. hepatica*–derived proteins was termed $Fh_{SMIII(M)}$. This subset was shown to reduce worm burdens of *F. hepatica* in immunised mice (by 69-78%) (Hillyer, 1985) and calves (55%) (Hillyer *et al*., 1987). The protective antigen in this fraction was identified as a 12kDa protein and termed Fh12 (Hillyer *et al*., 1988). The sequence of this protective antigen was identified when polyclonal rabbit antiserum against native Fh12 was used to screen a *F. hepatica* cDNA library (Rodriguez-Perez *et al.,* 1992). Immunoreactive clones were found to encode a homologue of the cytoplasmic FABP family known as Sm14, originally isolated from *S. mansoni* (Moser *et al*., 1991). The *Fasciola* cDNA encoded a protein of 14.7 kDa and was thus termed Fh15 (Spithill *et al*., 1999). There is still some debate as to whether Fh12 and Fh15 represent the same protein, as they are similar in size, however several FABP isoforms do exist in *F. hepatica* (Spithill *et al*., 1999). Fh15 is not only protective against fasciolosis but also cross protects against schistosomiasis (Hillyer *et al.,* 1977; Hillyer *et al.,* 1979; Hillyer *et al.,* 1985; Spithill *et al.,* 1999). Vaccination is thought to protect animals by interfering with the parasite's uptake of fatty acids (Spithill *et al*., 1999).

1.5.5.4 Leucine Aminopeptidase

Aminopeptidases catalyse the removal of amino acids from the N-termini of peptides and proteins. They are found in tissues and cells in both membrane-associated and soluble forms (Acosta *et al*., 2008; Piacenza *et al*., 1999). Leucine aminopeptidases (LAP) have not fully been characterised in helminths but there is evidence to support their participation in vital life-cycle processes. Within the human blood fluke, *S. mansoni,* the enzyme is localised to the gut and tegument. Furthermore, egg hatching is inhibited by bestatin, a LAP inhibitor (McCarthy

et al., 2004; Xu and Dresden, 1986). Amongst many functions, aminopeptidases participate in terminal degradation of proteins, protein maturation and regulatory processes of cellular metabolism (Bachmair *et al*., 1986). An enzyme capable of cleaving a leucine aminopeptidase- specific substrate, leucine-7-amino-4-methylcoumarin (NHMec), was identified from soluble *F. hepatica* extracts and termed FhLAP (Acosta *et al.*, 2008). FhLAP has been associated with the epithelial cells that line the parasite's digestive tract and proposed to function in the final stages of the catabolism of peptides generated by the degradation of host tissue by endoproteases such as the cathepsin L proteases (Acosta *et al*., 2008).

Vaccination of sheep with native FhLAP induces the highest levels of protection reported to date (Piacenza *et al*., 1999). Sheep immunised with FhLAP alone had 89% reduced fluke burdens and, when used in combination with the native cathepsin L1 and L2, fluke burdens were reduced by 76 % against challenge infection in sheep (Acosta *et al*., 2008; Piacenza *et al*., 1999). More recently, a recombinant form, rFhLAP, has protected rabbits from infection with *F. hepatica* (Acosta *et al.*, 2008). Vaccinated rabbits were immunised twice with rFhLAP in Freund's adjuvant (control rabbits with PBS and Freund's complete adjuvant) at 4 week intervals then challenged orally with 50 metacercariae two weeks after the final immunisation. Animals were slaughtered 20 weeks after the first immunisation and the number of flukes present in the liver and bile ducts counted. Worm burdens in rFhLAP vaccinated rabbits were reduced by 79 % in comparison to the non-vaccinated controls (Acosta *et al.*, 2008). However, despite the early promise of the native and recombinant LAP, a vaccine based on this antigen has still not reached commercial reality.

1.5.5.5 Fluke Haemoglobin

Haemoglobin (Hb) was isolated from *F. hepatica* E/S material (McGonigle and Dalton, 1995) and has been shown to provide protection when used as a vaccine antigen alone or in combination with the cathepsins (Dalton *et al*., 1996). Cattle which were vaccinated with Hb combined with CatL2 in Freund's complete adjuvant had a reduction in fluke burdens of 72 % in vaccinated compared to control animals (Dalton *et al.*, 1996). If these levels of protection could be reproduced, this could form the basis of a viable vaccine (Spithill *et al*., 1999). Furthermore, the reduction in fluke burden was accompanied by a reduction in egg viability of 30 – 75 %. Dalton *et al* (1996) suggested that the oxygen storage properties of Hb may be vital in areas of low oxygen tension, for example, in the bile duct. Thus, since egg production by *F.*

hepatica requires oxidative metabolism, its egg development may be compromised by immunisation with Hb (Spithill *et al*., 1999). Cattle immunised with Hb alone, Hb/Cat L1 and Hb/CatL2 had reduced worm burdens (43.8 %, 51.9 % and 72.4 %, respectively) and reduced egg viability (30-75%, 0-80%, 0.7%, respectively) in comparison to non-vaccinated controls (Dalton *et al.*, 1996). The anti-fecundity component of the vaccine is an attractive attribute and would help to reduce pasture combination (Taylor *et al*., 1994).

1.5.6 Vaccination against helminth parasites: Conventional and "Hidden" antigens

Although helminth infections can be controlled to an extent by the use of anthelmintics and pasture management, the occurrence of drug-resistant populations has spurred an interest into developing vaccines (Anon, 2005; Mitchell *et al.*, 1998; Thomas *et al.*, 2000; Gaasenbeek *et al.*, 2001; Alvarez-Sanchez *et al.*, 2006). The first commercial vaccine to be developed against a helminth parasite was "Dictol", an attenuated live vaccine against the bovine lungworm, *Dictyocaulus viviparus* (Jarrett *et al.*, 1955; Jarrett and Sharpe, 1963). Unfortunately, the success of using attenuated larvae to immunise against infection with *D. viviparus* has not extended to other helminths (Smith, 1999). Vaccination of sheep with a recombinant GST antigen (GST-45W) protected against infection with the cestode *Taenia ovis* (Johnson *et al.*, 1989). However, despite eliciting high level of protection (up to 98% reduction in cestode burdens) in small and large scale field trials the vaccine never reached commercial availability (reviewed in Rickard *et al.*, 1995). Sheep can develop a natural immunity to infection with the gastrointestinal nematode, *Teladorsagia circumcincta*, with a continued trickle infection of infective larvae (Smith *et al.*, 1984; Stear *et al.*, 1999). However, the mechanisms of naturally acquired immunity are not fully understood but appear to be complex, involving a combination of local hypersensitivity, cell mediated, antibody and inflammatory responses (Halliday *et al.*, 2007; Halliday *et al.*, 2009; Halliday *et al.*, 2010; Stear *et al.*, 1999; Smith *et al.*, 1984; Smith, 1999). The complicated development of natural immunity and the lack of available helminth vaccines highlight how difficult it is to develop an effective parasitic vaccine.

Targeting secreted antigens released by parasites has been a popular starting point for vaccine studies (Smith, 1999). This method has identified antigens which have elicited protective immunity against a number of parasites including *F. hepatica*, as previously discussed. These antigens are termed "natural" or "conventional" antigens as they are recognised by the host

during the course of a natural infection (Smith, 1999). An alternative approach is to target antigens which are not exposed to the host's immune system during a natural infection, i.e. " hidden" antigens. This approach proved successful with the cattle tick, *Rhipicephalus (Boophilus) microplus*. This vaccine, termed TickGARD™, was a antiparasite vaccine and reached commercial viability. The active component in the TickGARD vaccine was a recombinant form of the Bm86 antigen but, due to a reduction in demand, this product is no longer commercially available (Willadsen *et al*., 1995). A reduction in worm burdens and egg output has also been elicited against the blood-feeding gastrointestinal nematode, *Haemonchus contortus* by immunising with "hidden" antigens from the parasite's gut (Smith *et al*., 1994; Smith *et al*., 2000). Native intestinal antigens have been purified, characterised and shown repeatedly to reduce both egg counts and worm burdens (Knox and Smith, 2001; Knox *et al*., 2003; Smith *et al*., 1999; Smith *et al*., 1994; Smith *et al*., 2000). The most effective of these antigens identified by this approach was a proteolytic enzyme complex termed *Haemonchus* galactose-containing glycoprotein or H-gal-GP, which is involved in digesting the blood meal (Murray and Smith, 1994). Similarly, mice who were immunised three times with the hookworms aspartyl protease cathepsin D (Ac-APR-1), then challenged with *Ancylostoma duodenale,* had a reduction in the small intestine worm burden of 69 % (Ghosh & Hotez, 1999; Williamson *et al.,* 2002; Williamson *et al.,* 2004). The vaccinated animals were also protected from anaemia, with haemoglobin levels within a normal range (12.45 g/dl), compared with non-vaccinated dogs which exhibited anaemia (9.5 g/dl) indicating Ac-APR-1 disrupted the parasite's ability to feed (Loukas *et al.,* 2005). Immunoglobulins from the vaccinated animals also neutralised the catalytic activity of the Ac-APR-1, indicating the vaccination inhibited the parasites ability to feed (Loukas *et al.,* 2005).

This approach takes advantage of the fact that these parasite's are blood-feeders. Thus, for TickGARD™, the intestinal antigens from *H. contortus* and Ac-APR-1 success relies on parasites ingesting a blood meal in order to be exposed to the circulating antibodies for the vaccine to be effective.

Targeting "hidden" antigens could prove to be a novel and successful strategy for vaccinating against *F. hepatica*. The adult liver fluke lives in a highly vascularised environment and feeds on surrounding tissue. The adult flukes ingest haemoglobin and their branched gut is often found filled with partially digested blood, see Figure 1.5 (Smyth and Halton, 1983).

Furthermore, blood will most certainly be ingested, whether deliberately or not, by the juvenile fluke migrating through the liver (Oslen, 1986). Some animals infected with *F. hepatica* exhibit severe levels of anaemia. This may be related to blood loss from tissue damage caused as the parasite migrates but could also be the result of blood ingestion by the parasites (Sandeman and Howell, 1981; Vengust *et al*., 2003).

1.5.7 The gut of *Fasciola hepatica*

The fluke's gut changes as the parasite develops from a NEJ through to the adult stage (Dawes, 1962). The gut of the fluke can be separated into two distinct regions; the foregut (mouth, pharynx and oesophagus) and the paired intestinal caeca. The intestinal caeca are highly branched, blind-ending and embedded within the body tissues of the fluke making them difficult to dissect out, see Figure 1.5. This is in contrast to some nematodes e.g *H. contortus*, which possess a through gut lined with microvilli which form a brush border (Knox and Smith, 2001). The cells of the NEJ digestive system are different to those of the adults as they have no structural features associated with digestive function (Gallagher and Threadgold, 1967). They have few lamellae which are small and irregular where as the lamellae of the mature adults are numerous, long and regular in shape (Gallagher and Threadgold, 1967). The caeca elongate as the parasite develops and only become branched when the immature fluke enters the liver, around 8 to 10 days post infection (Dawes, 1962; Sukhdeo and Sukhdeo, 2002). The gut cells of the NEJ are all in a secretory phase, containing a large number of dense secretory vesicles. These contain hydrolytic enzymes which aid the digestion of tissue as the juvenile migrates through the gut wall and liver tissue (Bennett and Threadgold, 1973). The epithelial cells of the fully developed gut consist of a single continuous layer of cells of one basic type, varying in height (Robinson and Threadgold, 1975). Unlike the NEJ, the cells lining the gut differ from the adult fluke in their functional state, switching between an absorptive (Gress and Threadgold, 1959) and secretory phase (Müller, 1923; Dawes, 1962). The cells possess lamellae, which help amplify the gut surface area (Gress and Threadgold, 1959; Robinson and Threadgold, 1959; Fairweather *et al.,* 1999). These are analogous to the microvilli that line the nematode gut. Neighbouring gut cells are generally in different phases of the cycle so that absorption and digestion are occurring simultaneously and thus continuously (Fairweather *et al*., 1999).

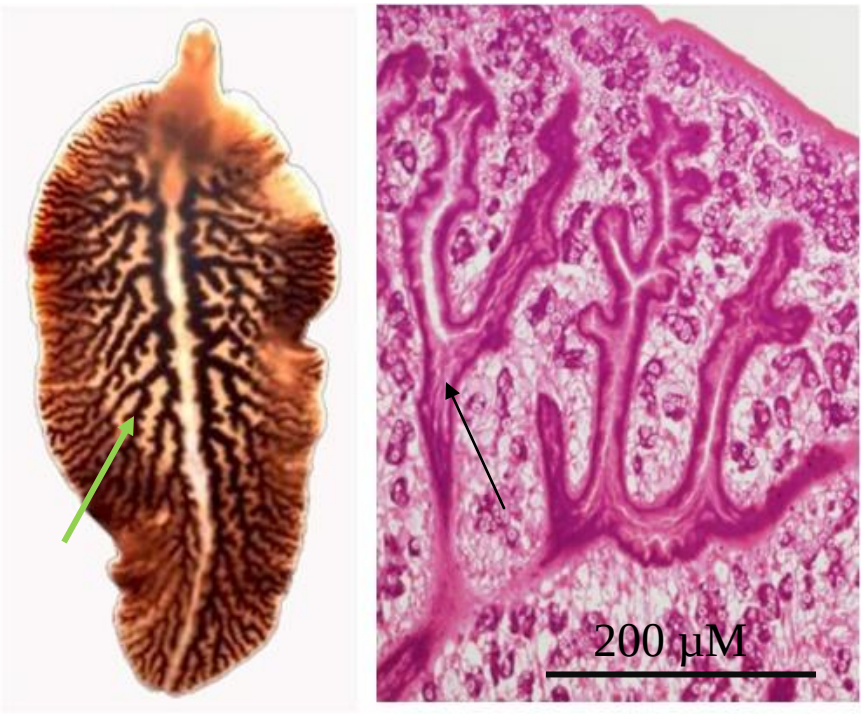

Figure 1.5: Adult *F. hepatica* showing A) the branched gut system spread throughout the parasite's entire body (green arrow), and B) a Haematoxylin and Eosin (H&E) stained cross-section of the gut region (black arrow).

Cells in the secretory phase possess dense secretory vesicles, abundant and active Golgi complexes and an extensive network of cytoplasmic organelles arranged in a random manner (Thorsell and Björkman, 1965) see Figure 1.6. The secretory activity of cells was studied using radioactively labelled amino acids over a time course (Hanna, 1975). This indicated that molecules enter the cell at the baso-lateral membrane followed by secretion at the apical membrane (Hanna, 1975). Cells in the absorptive phase have no secretory bodies but possess mitochondria and endoplasmic reticulum which are arranged in structured parallel rows, see Figure 1.6. Further, experiments by Thorsell and Björkman (1965) and Pantelouris and Gresson (1959) confirmed that epithelial cells were capable of absorption using radiolabelled amino acids and iron, respectively.

Initially, digestion is proposed to occur extracellularly in the lumen and is completed within the gut cells (Fairweather *et al.,* 1999). The fluke diet alters as the parasite develops, the immature flukes feeding on hepatic cells and also ingesting blood as they migrate through the liver tissue (Dalton *et al.*, 2004). Finally, as an adult, resident in the bile duct, the fluke's diet

consists largely of blood and in some cases bile duct epithelium (Dalton *et al.,* 1994; Smyth and Halton, 1983).

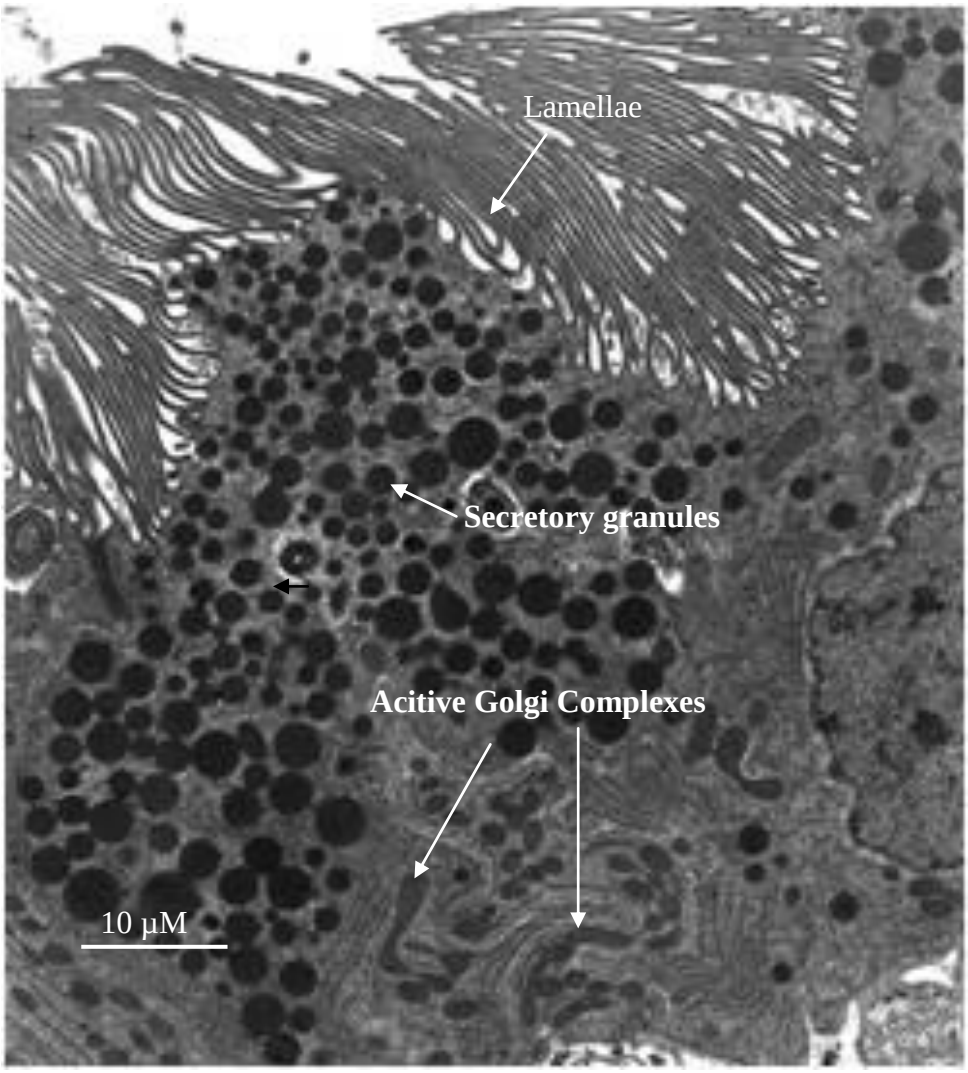

Figure 1.6: The secretory cell phases of *F. hepatica* gut cells, (Picture kindy provided by Professor Ian Fairweather).

The exact repertoire of enzymes possessed by each developmental stage and how they function is not yet completely understood. However, the enzymes used by each developmental stage do differ, with only a small number shared throughout development. For example, three cysteine proteases have been isolated from NEJ flukes (CL 3, CL 4, CL 6),of which only CL3 is present in the adult stages (Cancela *et al.,* 2010; Fairweather *et al.,* 1999; McGinty *et al.,* 1993). A summary of reported proteases is shown in Table 1.4. The different digestive enzymes allow the developing parasite to adjust to the different environments encountered as it progresses through the liver. For example, predominant cathepsin L3 in the NEJ has collagenase activity, which is essential for the invasion process (Corvo *et al.,* 2009), where as the adult cathepsin L1 is involved in haemoglobin degradation (Robinson *et al.,* 2008). The initial step of digestion is proposed to occur at the lining of the lamellae, with the digestion products then being endocytosed and further digested inside the cell (Fairweather *et al.,* 1999). Proteases from the E/S products of adults have been shown to be capable of digesting haemoglobin and host immunoglobulins (Carmona *et al.,* 1993; Smith *et al.,* 1993b; Yamasaki *et al.,* 1992). This indicates a dual role for the enzymes, in both nutrient acquisition and immune evasion as discussed earlier (Fairweather *et al.,* 1999).

Table 1.4: Digestive proteases identified in the newly excysted juveniles (NEJ), immature (3-5 weeks) and adult stages of *F. hepatica.*

Protease	Stage	Reference
cathepsin D	Adult, NEJ	Unpublished (ABJ97285.1)
cathepsin L1	Adult, Immature	Dalton and Heffernan, 1989, Smith *et al.,* 1993; Carmona *et al* 1993
cathepsin L2	Adult, Immature	Dalton and Heffernan, 1989Dowd *et al.,* 1994; Carmona *et al* 1993
cathepsin L3	NEJ, Adult	Cancela *et al.,* 2004
cathepsin L4	NEJ	Cancela *et al.,* 2004
cathepsin L6	NEJ	Cancela *et al.,* 2010
cathepsin B1	NEJ	Cancela *et al.,* 2004
cathepsin B2	NEJ	Cancela *et al.,* 2004
cathepsin B3	NEJ	Cancela *et al.,* 2004
legumain	Adult, NEJ	Unpublished (CAC85636.1)
leucine aminopeptidase	Adult	Acosta *et al.,* 2008; Piacenza *et al.,* 1999

1.5.8 Project Aims

Given the huge economic burden of fasciolosis on the world-wide agricultural industry (Bennett & Ijpelaar, 2005) and the occurrence of fluke populations resistant to available anthelmintics (Overend and Bowen, 1995; Anon, 2005; Mitchell *et al.*, 1998; Thomas *et al.*, 2000; Gaasenbeek *et al.*, 2001; Alvarez-Sanchez *et al.*, 2006) vaccination against *F. hepatica* would be an additional option in the control of fasciolosis. However, as mentioned above, animals show little or no protective immunity following infection and current vaccine candidates have yet to reach commercial availability. As a novel approach in *F. hepatica*, the project aims to focus on identifying novel hidden and/or gut-associated proteins in a similar approach to the work which led to the identification of protective gut antigens in *H. contortus* (Knox and Smith, 2001).

It is evident that the lead "hidden" antigen candidates currently identified from *H. contortus* are enzymatically active glycoprotein complexes obtained from detergent soluble extracts (Knox and Smith, 2001). These were initially identified, characterised and, ultimately, purified from Triton X-100 soluble parasite extracts (Smith *et al.*, 1994). Thus, the objectives of this project are as follows;

1. The protein components of three somatic extracts generated from adult *F. hepatica* (water-soluble, membrane-associated and membrane-bound, respectively) will be investigated to identify proteins which are enriched within the membrane-bound fraction. Furthermore, Laser Capture Micro-dissection (LCM) will be attempted to identify if transcripts of any of these proteins are within the parasite's gut.

2. A panel of lectins will be used to screen sections of adult fluke to identify those with an affinity for glycoproteins present on the parasite's gut. Gut-specific lectins will be subsequently used to enrich the membrane-bound extract for gut specific glycoproteins.

3. The membrane bound fraction will be investigated for evidence of proteolytic enzymes. The pH range of activity, ability to digest ovine haemoglobin and class of enzyme activity will be characterised.

4. Any candidates of interest will then be compared with homologous proteins in other parasite species, the expression characterised over the different life stages of *F. hepatica*, localised within the parasite and screened with definitive ovine host antibodies raised towards a natural fluke infection to assess whether they are “hidden” antigens.

2 Application of proteomics to identify proteins enriched within a membrane-bound fraction of adult *Fasciola hepatica*

2.1 Introduction

Identifying vaccine candidates for parasite control has historically involved vaccinating host animals with crude extracts or whole organisms before fractionating these to identify the protective components. This is a costly and lengthy method often taking decades to complete (Knox, 2000; Knox 2010).

Crude somatic extracts of parasites can be prepared in a number of ways. One approach is to homogenise parasites in a series of extraction buffers, starting with phosphate buffered saline (PBS) alone, then in PBS with Tween-20 and finally PBS with Triton X-100 (as described in Smith *et al* 1994). Proteins solubilised at each step can be loosely described as water-soluble, membrane-associated and membrane-bound, respectively (Smith *et al.*, 2000). Proteins within the membrane-bound fraction (Triton X-100 extract) have been a rich source of protective antigens against the blood feeding nematode, *H. contortus* (Smith *et al.*, 2000). The highly protective H-gal-GP complex was identified within this fraction (Smith *et al.*, 2000). Furthermore, proteases have, historically, made good vaccine candidates against helminth parasites. For example, the lead vaccine candidates, to date, against infection with *F. hepatica* are the proteases, cathepsin L1 and L2 (Dalton *et al.*, 1996; Mulcahy and Dalton, 2001) and leucine aminopeptidase (LAP) (Acosta *et al.*, 2008; Brophy and Pritchard, 1999). LAP has elicited the highest protection to date of a single antigen against *F. hepatica*, where immunising sheep reduces worm burden by up to 89 %, or when used in combination with the cathepsin L1 and L2, up to 76 % (Acosta *et al.*, 2008). Proteases have also proved to be successful vaccine candidates against the blood-feeding *Schistosoma spp., A. caninum*, *Necator americanus*, *H. contortus* and *B. microplus* (Dalton, 2001). Therefore, any protease which is exclusive to the membrane bound-fraction would be of particular interest as a potential vaccine antigen.

In this Chapter, a proteomic approach was used to identify proteins present in each of the three fractions and to identify those exclusive to the membrane-bound fraction. Proteomics was first used to describe the protein complement of a genome by Wasinger *et al.*, (1995). A combined approach of amino acid composition analysis and peptide mass fingerprinting was used to

identify novel functional proteins within *Mycoplasma genitalium* (Wasinger *et al.,* 1995). The proteome of organisms is dynamic and changes in response to the demands of both the external and internal environment (Barrett, 2009). Proteins are often the targets for therapeutic agents (Barret *et al.,* 2000). The amino acid sequence of proteins has been determined by Edman sequencing since 1950 (Edman *et al.,* 1950). However, the ability to separate proteins on two dimensional (2D) gels in combination with the development of mass spectrometry analysis allowed a rapid increase in proteomic opportunities during the 1990s (Graves and Haystead, 2002).

Mass spectrometry (MS) accurately determines the molecular mass of peptides and can be coupled to other instruments to allow sensitive and fast peptide sequencing (Ashton *et al.*, 2001). Liquid chromatography – Electron spray ionisation - Tandem mass spectrometry (LC-ESI-MS/MS) groups these three techniques to produce very sensitive analysis of peptides. Firstly, the sample is separated by high performance liquid chromatography (HPLC) at a very slow flow rate to produce small sample volumes for the ESI. The sample is then sprayed through a fine needle and a high voltage applied. This ESI step removes any solvents and produces small universally charged droplets of the sample in a gaseous phase which are then swept into a dual MS. The first MS fragments individual peptides with argon to release ions which are separated and detected in a Time-of-flight (ToF) MS. The ToF is determined by the ion's mass to charge ratio (mz), as ions are accelerated through an electric field and the time taken to travel a known distance to the detector is measured (ToF). As all the ions have the same charge, as an ion's size increases, so will the time to travel. The ToF can then be used to calculate the mass to charge ratio of the ion. This can then be used to determine the amino acid, and thus the sequence of the peptide (Roepstorff, 1997). Mascot is a search engine which can be used to identify the protein identity from the mass spectrometry data yielded from the peptides (Perkins *et al.,* 1999). Mascot uses a probability based scoring algorithm MOlecular Weight SEarch (MOWSE) which was first described by Pappin *et al.,* (1993). The significance of the MOWSE score depends on the size and complexity of the database searched.

Unlike a proteome, a genome is static containing all an organism's hereditary information (Barrett *et al.,* 2009). To date the only completed genome of a parasitic helminth is that of *S. japonicum* (Berriman *et. al.,* 2009) and the proteomes of many parasite helminths are still incomplete. To date, there is little known about the genome of *F. hepatica*. Currently, there are

only 3055 adult *F. hepatica* expressed sequence tags (EST) and 1677 newly excysted juvenile (NEJ) ESTs publically available (January 2012, NCBI, http://www.ncbi.nlm.nih.gov). Jefferies *et al* (2001) was the first to use basic proteomic techniques to identify major proteins in the E/S material from adult *F. hepatica*, despite an incomplete genome. Since then, a number of studies on *F. hepatica* have used proteomic analysis to help understand drug resistance to triclabendazole (Chemale *et al.*, 2010), to study stage-specific expression and for the classification of cathepsin L (Robinson *et al.*, 2008; Morphew *et al.*, 2011) and other vaccine candidates, including GST (Chemale *et al.*, 2006), to study proteins expressed by different developmental stages (Hernández-González *et al.*, 2010) or organ systems, such as the tegument (Ashton *et al.*, 2001; Wilson *et al.*, 2011) within *F. hepatica.*

In silico (computer-generated) bioinformatic analysis of an organism's genome can identify genes with various features such as function, motif signatures and cellular location. Proteomics can complement this genomics/bioinformatic approach because a viable vaccine candidate has to be expressed (Zagursky and Anderson, 2008). Gene expression cannot be determined by the genomic/bioinformatic approach, but proteomics can detect proteins which are actually expressed by the organism, for example, at different developmental stages or under different selection pressures (Grandi, 2001; Grandi 2002). One of the major proteomic techniques which have been used to assist the identification of potential candidates is two dimensional (2D) gel electrophoresis, which enables the construction of proteome maps and mass spectrometry (Grandi, 2001; Zagursky and Anderson, 2008; Barrett *et al.*, 2000). 2D gel electrophoresis allows comparison of proteins expressed by different developmental stages or in response to stimuli, for example probing with immune sera (Barrett *et al.*, 2000), or after exposure to drug treatment (Cooper *et al.*, 2004). The individual protein spots which are unique, for example to a developmental stage, can then be identified and analysed by matrix-assisted laser desorption/ionisation Time of flight (MALDI-ToF) analysis. In this study, using proteomic techniques, proteins exclusive to the membrane-bound fraction were sought and classified according to their biological function. The aim was to identify any proteases which are unique to the membrane-bound (S3) fraction. Homologies to exclusively membrane-bound proteases in other species will help indicate if they have a role in nutrient acquisition.

Although proteomic techniques can help identify peptides exclusive to the membrane-bound fraction, the tissue location of these can only be predicted by looking at homologues in other species. Using this in addition to other tools such as laser capture microscopy (LCM) will help identify if any of the identified proteins are represented by transcripts in the gut. LCM is a novel technique which allows the precise targeting and extraction of cells from tissues of interest (reviewed in Jones *et al.*, 2004). It is proposed to be a relatively simple technique where the biggest challenge is preparing the specimen in such a way as to preserve the cells for subsequent analysis and interpretation (Lee *et al.*, 2003; Scheidl *et al.*, 2002). In a parasitology context, LCM has the potential to analyse specific aspects of the host-parasite interaction; for example, it can be used to enable identification of parasites within host tissues by molecular means, analyse tissue-specific transcriptomes and allow living cells to be extracted for manipulation and subculturing (Jones *et al.*, 2004). These approaches are facilitated by the range of downstream analysis platforms which can be used on the captured cells, which include: DNA genotyping, RNA transcript profiling, cDNA library construction and proteomics (Fuller *et al.*, 2003). LCM has been used successfully to extract cells from the intestines of the hookworms, *A. caninum* and *N. americanus.* The mRNA from the extracted cells was used to generate tissue-specific cDNA libraries (Ranjit *et al.*, 2006). The hookworm gut is easily distinguished and separated from other tissues by the pseudocoelom, making it ideal for this technique (Jones *et al.*, 2007). In contrast, the gut of *F. hepatica* spreads extensively throughout the body of the parasite and is easily identified due to its branch-like structure thus makes it suitable for LCM, although to date it has not been attempted.

In conclusion, the work in this chapter aimed to identify proteins, ideally gut-derived, that were exclusive to the membrane-bound fraction of *F. hepatica* using a combination of proteomic analysis and LCM.

2.2 Materials and Methods

2.2.1 Analysis of somatic extracts by Liquid Chromatography - Electron Spray Ionisation -Tandem Mass Spectrometry (LC - ESI - MS/MS)

2.2.1.1 Parasite material

Live adult *F. hepatica* were retrieved from the bile ducts of naturally infected sheep from either ScotBeef Abattoir, Dunblane or Firth Mains farm, Penicuik. Parasites were washed twice with 1 x Phosphate buffered saline (PBS) (see Appendix 1) and frozen at -80°C until required.

2.2.1.2 Somatic extracts

The frozen flukes were thawed on ice and homogenised using an Ultra-turrax® T25 (IKA® – Labortechnik) with 5 ml of homogenizing buffer (HB) 1 (see Appendix 1) and centrifuged at 20,000 *g* for 20 min at 0°C. The supernatant (S1) was retained. The pellet was resuspended and hand homogenized in a Dounce homogeniser in HB 2 (see Appendix 1) then centrifuged at 16, 500 *g* for 20 min at 2°C. The supernatant (S2) was retained. The HB2 extraction was repeated, the supernatant discarded and the pellet was then resuspended and hand-homogenised using a Dounce homogeniser in buffer HB 3 (see Appendix 1) then mixed end-over-end for 30 min at 4°C. This extract was then centrifuged at 50,500 *g* for 30 min at 4°C and the supernatant (S3) retained. This resulted in the production of three crude somatic fractions representing water soluble proteins (S1), membrane-associated proteins (S2) and membrane-bound proteins (S3), respectively.

2.2.1.3 Protein determination

Protein concentrations of each extract were estimated using the bicinchoninic acid (BCA) ™ Protein Assay Kit (Pierce). Briefly, 11 Bovine Serum Albumin (BSA) standards were prepared by diluting a stock solution of 2000 µg/ml BSA in 1 x PBS (see Appendix 1) to concentrations ranging from 0-2 mg/ml. Extracts were diluted in 1 x PBS. Into a 96-well plate, 25 µl aliquots of each standard/extract were dispensed in triplicate into individual wells. A BCA mix was then prepared by mixing BCA reagent A with BCA reagent B (50:1 ratio). 200 µl of BCA mix was dispensed into each well, then plates incubated at 37ºC for 30 min. Absorbance was measured at 562 nm on a 96-well plate reader. A standard curve was plotted using the BSA standards and the protein concentration of the extracts determined from this.

2.2.1.4 Sample preparation

10 μg of each somatic extract (S1, S2 and S3) were loaded into sample wells of a Bis/Tris SDS-PAGE mini gel (4 -12 %, Invitrogen) and separated at 200V for 45 – 50 min. Resolved proteins were visualised using SimplyBlue Safe Stain ™ (Invitrogen). The MRI proteomics facility carried out the following procedures on the resolved gel. The stained gel lanes were excised and then sliced horizontally from top to bottom to yield 25 equal gel slices of 2.5mm depth. Each of the resulting 25 gel slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsinolysis procedures (Shevchenko *et al*., 1996).

2.2.1.5

Samples were transferred to HPLC sample vials and stored at +4^{o}C until required for LC-ESI-MS/MS analysis. Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon chromatography software (Dionex: http://www.dionex.com). A micro-pump flow rate of 246 μl/min^{-1} was used in combination with a cap-flow splitter cartridge, affording a $^{1}/_{82}$ flow split and a final flow rate of 3 μl/min^{-1} through a 5 cm x 200 μm ID monolithic reversed phase column (Dionex-LC Packings) maintained at 50°C. Samples of 4μl were applied to the column by direct injection. Peptides were eluted by the application of a 15 min linear gradient from 8-45% of solvent (80% acetonitrile, 0.1% ($^{v}/_{v}$) formic acid) and directed through a 3 nl UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (Esquire HCTplus^{TM}, Bruker Daltonics) via a low-volume (50μl/min^{-1} maximum) stainless steel nebuliser (Agilent, cat. no.G1946-20260) and ESI. Raw chromatography data were processed and Mascot compatible files created using DataAnalysisTM 3.2 software (Bruker Daltonics) with the following parameters: compounds (autoMS) threshold 1000, number of compounds 500, retention time windows were 2.0 min for C18 (30 min gradient) and 0.8 min for monolithic and C18 (9 min gradient) as described by Batycka *et al*., (2006).

2.2.1.6 Database Mining

The MASCOT compatible files generated in section 2.1.1.7 were submitted to two databases. First, data were submitted via the in-house MASCOT server and searched against an annotated *F. hepatica* expressed sequence tag (EST) database, kindly provided by Professor Peter Brophy (University of Wales, Aberystwyth) using the MASCOT search algorithm. Secondly, the MS/MS data were sent to Dr Neil Young (University of Melbourne, Australia) and searched against an annotated adult *F. hepatica* EST dataset. The presentation and interpretation of MS/MS data were performed in accordance with published guidelines (Taylor and Goodlett, 2005). To this end, fixed and variable modifications selected were carbamidomethyl (C) and oxidation (M), respectively, and mass tolerance values for MS and MS/MS were set at 1.5Da and 0.5 Da, respectively. Molecular weight search (MOWSE) scores obtained for individual protein identifications were inspected manually and considered significant only if; a) two unique peptides were matched for each protein and, b) each peptide contained an unbroken "*b*" or "*y*" ion series of a minimum of four amino acid residues.The trypsin digest breaks the protein into peptides. These are then fragmented under high energy dissociation conditions to create a series of fragments which are deemed ions. These are referred to as "b" (if the charge is retained on the N-terminus) or "y" ions (if the charge is maintained on the C-terminus).

Protein identifications were confirmed further as a MOWSE score of 29 or higher was statistically significant at the 95 % confidence level when searching against both datasets.

2.2.1.7 Analysis of data

Proteins which met the criteria outlined in section 2.2.1.6 were sorted to assess those which were; (i) shared between all the fluke extracts, (ii) shared between two of the extracts and (iii) unique to a single extract. S3-unique proteins were sorted into functional groups by a combination of information from Kyoto Encyclopedia of Genes and Genomes (KEGG) and critical review of the associated background literature.

2.2.2 Laser capture microscopy

2.2.2.1 Preparation of material

Adult parasites were collected from the bile ducts of sheep as described in Section 2.2.1.1, immediately placed onto a small disc of solid 10% gelatin (see Appendix 1) and snap frozen in liquid nitrogen-chilled isopentane. The frozen flukes were then wrapped in aluminium foil and stored at -80ºC until further required.

2.2.2.2 Transportation of material

The frozen blocks of parasites were transported to the Rowett Institute, Aberdeen in dry ice and placed immediately at -80ºC upon arrival.

2.2.2.3 Tissue preparation for Laser capture microdissection

The cryostat and tools for sectioning were cleaned to ensure that they were free of RNAses. The parasite material was warmed to -19ºC in the cryostat for around 10 min then fixed onto the chuck using Tissue-Tek OCT Compound (Agar scientific). 8 µm sections were cut and mounted on untreated slides (Thermo Shandon) which had been baked at 200ºC for a minimum of 2 hours. Slides were then kept on dry ice before warming to room temperature and immersion in a series of alcohols; 100% ethanol (1 min), 95% ethanol (30 sec), 75% ethanol (30 sec) and 50% ethanol (30 sec). 100 µl of Histogene ™ LCM frozen section stain (Arcturus) was pipetted onto the section on each slide and incubated for 1 min with gentle rocking. Slides were then immersed in 50% ethanol (30 sec) 75% ethanol (30 sec) 95% ethanol (30 sec), 100% ethanol (30 sec), and Xylene (1 min). Excess xylene was drained off and the slides dried in a desiccator for a minimum of 15 min.

2.2.2.4 Laser capture

The gut region of the parasite was identified visually and microdissected using the PixCell 11 Laser-capture Microdissection System (Arcturus) and captured on Capsure MacroLCM caps (Arcturus). Briefly, slides were placed, section up, on the microscope platform and kept in place under vacuum. The microscope was focussed on the 10 x objective and the gut brought into view. The LCM cap was then placed over the area of interest. The laser was focussed on the spot size of 7.5 µM. The 15 µM spot size was then selected and the power setting adjusted to 30 mW and the duration of pulse to 5 msec. Microdissection of gut cells was then

performed by pulsing with the laser under these conditions. This was repeated until all the gut in view had been captured. The polymer end of the cap was then inserted into a 500 µl Eppendorf tube containing 100 µl of lysis buffer (Absolutely RNA Microprep kit, Agilent). The RNA was then extracted using the Absolutely RNA Microprep kit (Agilent) according to the manufacturer's instructions. A 5 µl sample was taken for analysis of integrity and quantity and the remainder was frozen at -80ºC.

RNA quality and quantity were estimated using a Nanodrop™2000 (Thermo-Scientific). 1 µl of RNA was used to measure the A260 and A280 values and calculate the A260:A280 ratio for each sample, in triplicate. RNA quality was also assessed by running each sample on an Agilent 2100 Bioanalyser (Agilent) using an Agilent RNA 6000 Pico chip (Agilent) as described by the manufacturer.

2.2.2.5 RNA amplification

RNA captured from the LCM was amplified using the RIBO Amp HS plus High sensitivity kit (Arcturus), according to the manufacturer's instructions (www3.appliedbiosystems.com/cms/groups/mcb.../cms_085206.pdf). Two rounds of amplification were conducted and the process was stopped in the second round after the cDNA purification step, thus yielding double stranded cDNA. The Nanodrop™2000 (Thermo-Scientific) was used to assess if RNA amplification had been successful as described in section 2.2.1.7.

2.2.2.6 Transformation of competent cells with ligated cDNA

To enable ligation into the vector, an A-tail was added to the cDNA. Briefly, on ice the cDNA was mixed with dATP (0.2 mM), Taq DNA polymerase 10 x reaction buffer (Advantage ® cDNA PCR kit, Clontech) and Taq (5units, Advantage ® cDNA PCR kit, Clontech). This was incubated at 70ºC for 30 minutes then stored at -20ºC.

This was ligated into the pGEM®-T cloning vector (Promega) according to manufacturer's instructions. The ligated vector was then used to transform *Escherichia coli* JM 109 (Promega) competent cells, as per manufacturer's instructions. The transformed cells were spread on LB/IPTG/X-GAL/Ampicillin agar plates (see Appendix 1) and incubated overnight at 37 ºC. White colonies were picked and checked for inserts by colony PCR. Briefly, a sample of each colony was mixed with 5µl of dH_2O. On ice, this was mixed with 15 µl Biomix (Bioline), 5 µl of forward primer (T7 primer, 5'-TAATACGACTCACTATAGGG-3') and reverse primer

(SP6, 5'-ATTTAGGTGACACTATAG-3') at 5 µM. This was then amplified as follows in a thermal cycler (Applied Biosystems 2070): 1min at 95ºC (1 cycle); 30 sec at 95ºC, 30 sec at 55.4 ºC, 60 sec at 72ºC (25 cycles); 5 min at 72ºC. 5 µl of each reaction was mixed with 1 µl loading dye and separated on a 1% agarose gel with gel red (see Appendix 1) by electrophoresis, then viewed under a UV transilluminator.

2.2.2.7 Purification of plasmids for sequencing

Colonies with an insert were grown in LB broth containing ampicillin (See Appendix 1) overnight at 37ºC with shaking (250rpm). Plasmids were then purified using the Wizard ® Plus SV minipreps DNA purification system (Promega) according to manufacturer's instructions using the centrifugation protocol. The concentration of DNA was measured using the Nanodrop™2000 (Thermo-Scientific) and plasmids at an appropriate concentration were sent for automated sequencing (MWG).

2.2.2.8 Analysis of sequences

Sequencing results were assembled where possible into contigs using DNAstar® (http://www.dnastar.com/). Contigs (overlapping DNA fragments) or single nucleotide sequences were analysed by the BLASTx [Basic local alignment search tool, (Altschul *et al*, 1990)] programme on the National Centre for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). This programme compares the users nucleotide/protein sequences with those of known proteins or genes in the non-redundant database and calculates the statistical significance of the match to allow identities to be assigned to nucleotide/protein sequences submitted.

2.3 Results

2.3.1 Somatic extracts from adult *F. hepatica*

The protein profiles of the somatic extracts (S1, S2, and S3) were visualised by SDS-PAGE, see Figure 2.1. Protein concentrations were estimated at 3709 µg/ml, 1052 µg/ml and 708 µg/ml for the S1, S2 and S3 extracts, respectively. From this, the volume needed to load 10µg of protein per lane was calculated to ensure consistency between samples for subsequent analysis by LC-ESI-MS/MS.

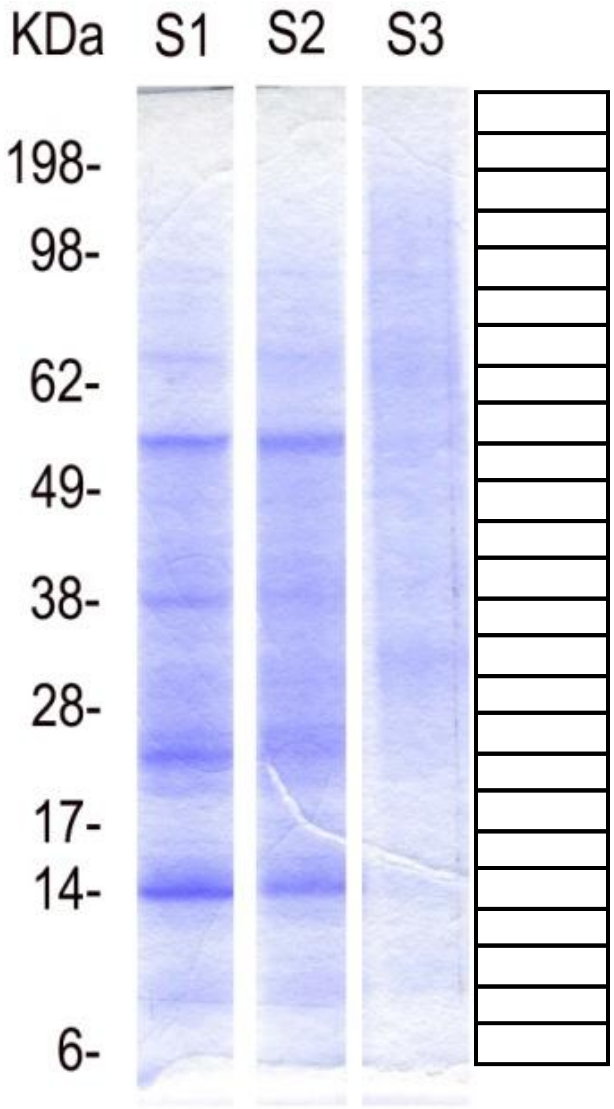

Figure 2.1 Somatic extracts from adult *Fasciola hepatica.* Soluble (S1), membrane associated (S2) and membrane-bound proteins (S3) shown in respective lanes. Grid to the right illustrates where the lanes were sliced for further analysis by LC-ESI-MS/MS

2.3.2 LC- ESI-MS/MS

Each lane as shown in Figure 2.1 was divided into 25 slices, as illustrated by the grid, for the LC-ESI-MS/MS analysis. The MS/MS results generated from searching against the two *F. hepatica* EST datasets were combined to provide the best coverage possible of data generated from the three somatic extracts. In total, 5153 peptides were matched against the datasets. In turn, these were matched against 1248 contigs, where each contig had two or more significant peptides with a consecutive sequence of 4 'b' or 'y' ions. However, 165 (13.2 %) of the contigs identified were classified as "of unknown function". The number of contigs unique to, or shared between, the three fractions is outlined in Figure 2.2. Although a large proportion of these contigs were shared between the extracts, there were considerable numbers of unique contigs within each fraction, namely S1 (254), S2 (240) and S3 (246).

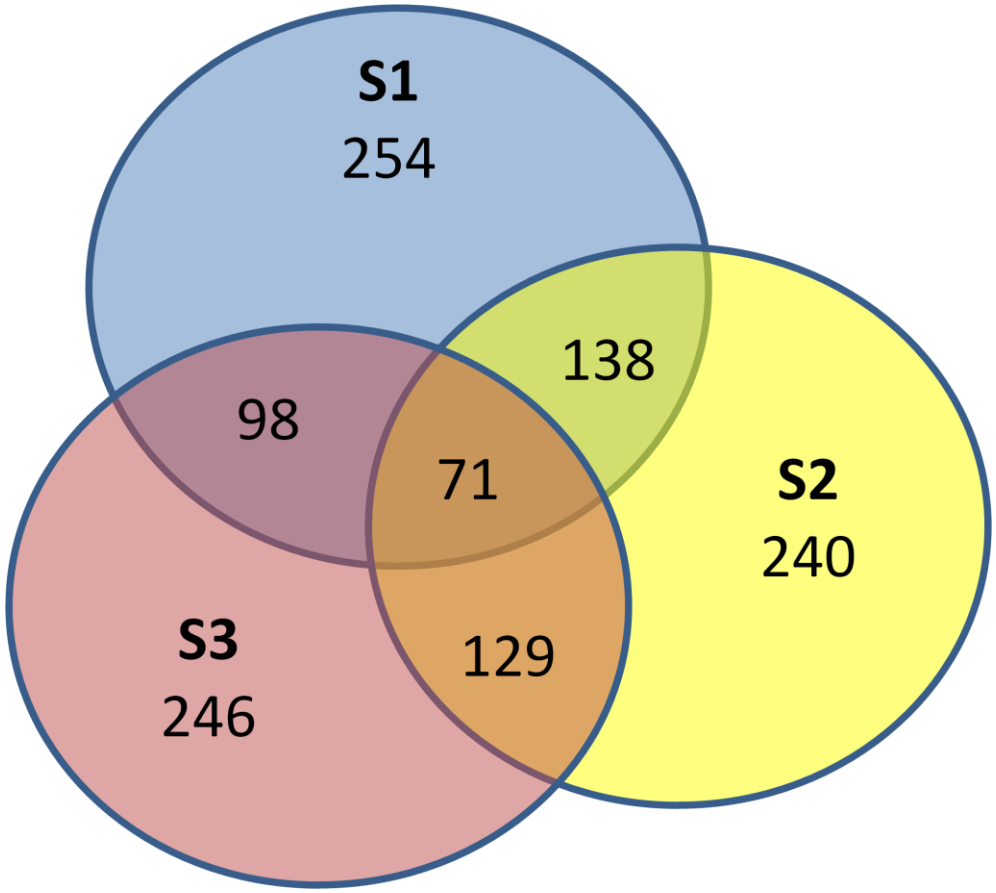

Figure 2.2: The significant contig hits in the three somatic extracts from adult *F. hepatica* from proteomic analysis. Overlapping areas indicate the number of shared contigs between fractions.

2.3.2.1 Unique S3 contigs and their functional classification

The annotated contigs unique to the S3 extracts were further characterised according to their function by KEGG analysis. Those contigs unique to this fraction are of particular interest as they are likely to be membrane-bound and thus would not be excreted or secreted by the parasite and may therefore represent true "hidden antigens". All the results are outlined in Appendix 2 and a subset of these appears in Table 2.1. The resultant hits were divided into a number of functional classes which includes; proteolytic, metabolic, transport, inhibition, cytoskeleton, transcription, gut-associated, signalling, gene transcription, heat shock and chaperone, ribosomal and any other function. The metabolic category had the largest proportion of hits, with 28% of the total divided into 7 different sub-classes. The databases searched were composed of contigs with assigned protein identities and often the same protein was represented by a number of different contigs. Although there were 246 contigs unique to the S3 fraction, some of the proteins that were identified were represented in other fractions. Therefore, the protein identities of each S3 contig (excluding hypothetical and unknown proteins) were searched against proteins present in the S1 and S2 fraction to confirm that the protein identity was truly unique to the S3 fraction. Ultimately, 111 contigs were identified that represented proteins exclusive to the S3 fraction. This encompassed 6 proteolytic, 36 metabolic, 20 transport, 2 inhibition, 5 cytoskeleton, 11 transcription, 0 gut-associated, 19 signalling, 1 gene transcription, 4 heat shock and chaperone and 1 ribosomal protein(s).

Out of the 111 contigs exclusive to the S3 fraction, and of particular interest, were the proteolytic enzymes specifically, cathepsin B2, Legumain-2 (both cysteine proteases), and a putative lysosomal pro-x-carboxypeptidase precursor (a serine/carboxypeptidase) as shown in Table 2.1. Proteases were of particular interest as they have historically made good vaccine candidates and also may have a vital role in nutrient acquisition by the parasite. In addition to these, secreted saposin-like protein, SAP-3, was also of interest as it is a gut-associated enzyme in the hookworm *A. caninum* (Don, 2007). Of these proteolytic enzymes, cathepsin B, cathepsin L and leucine aminopeptidase, were also represented in the other two fractions, S1 and S2. So, although the peptides were assigned to contigs which were unique to the S3 fraction, the protein identity of the contig was also represented in the S1 and S2 fractions and was, therefore, not exclusively S3.

The number of unique peptides for each contig is shown in Table 2.1, confirming that each contig met the minimum criteria of 2. The coverage of the full length protein sequence by the assigned unique peptides is shown under the percentage coverage. The protein (MOWSE) score is shown and all scores are above the threshold of 29, so scores are significant at the 95% confidence level.

Table 2.1: The proteolytic enzymes identified in the S3 fraction of *Fasciola hepatica* from searching two *F. hepatica* EST databases

Protein description	**S3 Unique?**	**Species**	**No. Peptides**	**% Coverage**	**Protein score**	**Protein mass (Da)**
***1.* Proteolytic enzymes**						
1.1 Cysteine proteases						
Cathepsin B2	Y	*T. szidati*	2	2.9	60	55648
Cathepsin B	N (1/2)	*S. japonicum*	4	7.1	111	49694
Calpain B	N (1/2)	*S. japonicum*	2	6.2	34	44322
Cathepsin B	N (1/2)	*F. gigantica*	4	7.1	55	46424
Cathepsin L	N (1/2)	*F. hepatica*	2	2.5	32	39516
Legumain-2	Y	*F. gigantica*	4	10.1	41	36523
Legumain-2	Y	*F.gigantica*	8	26.8	100	21886
Legumain-2	Y	*F. gigantica*	4	10.9	31	40793
1.2 Aminopeptidases						
Leucine aminopeptidase	N (1/2)	*F. hepatica*	2	15.4	49	15564
1.3 Serine / Carboxypeptidase						
Lysosomal Pro-X carboxypeptidase precursor	Y	*S. japonicum*	3	3.7	41	70862

Footnote: The full genus names are *Taenia szidati, Schistosoma japonicum, Fasciola gigantica, Fasciola hepatica, Clonorchis sinensis.* Although the proteases were identified from *F. hepatica* EST databases, some of the ESTs have been assigned identities by homology with proteins from other species. Although the peptides were assigned to a EST contig unique to the S3 fraction, the protein identity of the contig was sometimes represented in other fractions by different contigs. 'S3 unique' indicates whether these proteases are unique to the S3: Yes (Y) or No (N). If no, which other fraction it was also present in, S1(1) or S2(2), is also shown.

2.3.3 Transcripts located within the gut of *Fasciola hepatica*

2.3.3.1 Laser capture

Images were taken of the sections before and after laser capture and on the cap. These images are displayed in Figure 2.3. Gut tissue was excised successfully from the sections as illustrated by the before, after and cap shots shown in Figure 2.3.

2.3.3.2 RNA analysis

RNA extracted from the tissue captured from the gut could not be measured accurately with the Nanodrop (results not shown). Analysis of the laser captured gut tissue with the Bioanalyser also indicated that there was insufficient amounts of RNA or that the RNA extracted was degraded.

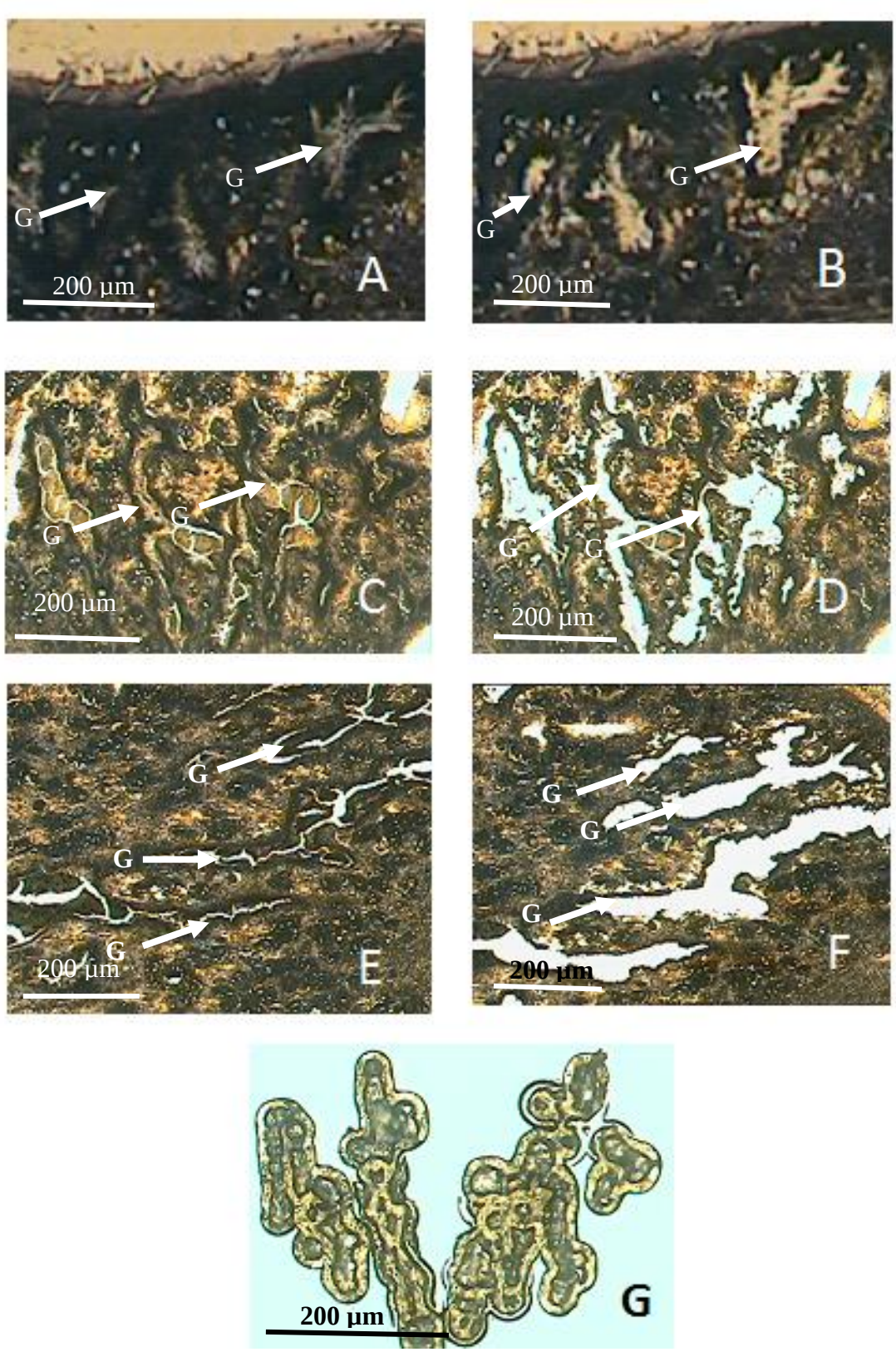

Figure 2.3: Images from laser capture microscopy. The gut (G) is illustrated by the white arrows before laser capture microscopy in panel A, C, E and after capture in B, D, F where the gut structure has been removed. Panel G illustrated the gut structure on the cap after it has been removed from the section.

2.3.3.3 cDNA synthesis and ligation into competent cells

Despite the low amount of RNA, an attempt was made to generate cDNA, which was then ligated into a vector for propagation in competent cells. Successful ligation was visualised initially by selecting white colonies, from blue/white screening. A colony PCR confirmed that these colonies did indeed have short insert sequences, see Figure 2.4. The pGEM®-T vector without any insert would be approximately 126bp in size. If the colony PCR product was larger it indicated that cDNA had been successfully ligated into the vector.

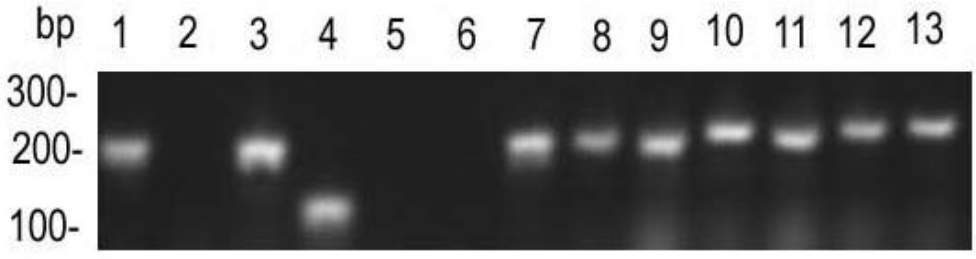

Figure 2.4: Colony Polymerase Chain Reaction (PCR) of cDNA generated from RNA extracted from laser captured *F. hepatica* gut cells after ligation into the pGEM®-T vector, lanes 1-13. The vector amplicon size without any inserts should be ~126bp in size, lane 4.

Colonies were sequenced (MWG, http://www.eurofinsdna.com) and searched against the NCBI non-redundant database using BLAST. The BLAST search found either no significant identities or identified the sequences as cloning vector fragments. Manually looking at the sequences indicated the fragments were 'junk' with repetitive sequences of bases, often with one base repeated in a row for 10-15 bases. So although the colonies appeared to contain cDNA inserts from RNA captured using LCM from *F. hepatica* gut, the cDNA could not be identified. This indicated RNA suitable for downstream analysis had not been captured using LCM.

2.4 Discussion

With advances in proteomics it is now possible to use this method to study aspects of host-parasite interaction, drug resistance to flukicides e.g. triclabendazole (Chemale *et al.,* 2010), protein expression by different developmental stages (Hernández-González *et al.,* 2010) or organ systems, such as the tegument (Ashton *et al.,* 2001; Wilson *et al.,* 2011) within *F. hepatica*. Here proteomics was employed to identify proteins, specifically proteases, which were exclusive to a membrane-bound fraction generated from adult *F. hepatica.* The membrane-bound fraction (S3) has been a rich source of protective antigens such as H11 and H-gal-GP against *H. contortus* and proteases have historically made good vaccine targets in a number of parasite species (McKerrow, 1989; Williamson *et al.,* 2003; Knox, 2010). For example, cathepsin D-like aspartyl proteases identified from the canine hookworm *A. caninum,* have elicited protection in immunised dogs, reducing worm burdens and worm fecundity (Loukas *et al.,* 2005; Williamson *et al.,* 2003). Vaccinating with cysteine proteases such as cathepsin L, which have a critical role in *F. hepatica* tissue invasion (Smith *et al.,* 1993b) and immune evasion (Smith *et al.,* 1993), reduces the worm burden of *F. hepatica* in immunised sheep and cattle (Mulcahy and Dalton, 2001). Here, proteolytic enzymes exclusive to the adult *F. hepatica* S3 fraction, and thus of interest included; cathepsin B2, legumain-2 and putative lysosomal pro-x-carboxypeptidase precursor. In addition to these, saposin-like protein SAP-3, was also of interest. Each of these will be discussed in turn.

Cathepsin B2, a cysteine protease, has been localised to the gut and tegument of *F. gigantica* and *S. mansoni* (Dalton *et al.,* 2006; Grevelding, 2006). Cathepsin B2 is expressed by the juvenile stages of *F. gigantica,* facilitating digestion of definitive host tissues and allowing migration through the liver parenchyma (Meemon *et al.,* 2004). The somatic extracts used here were prepared from adult stages of *F. hepatica*, indicating that cathepsin B2 is also expressed, at some level, in the adult stages of *F. hepatica*. However, cathepsin Bs have been identified in the E/S products of NEJ of *F. hepatica* (Wilson *et al.,* 2008). Here, cathepsin B was identified as exclusive to the membrane-bound fraction and thus is potentially "hidden". However, further analysis would be needed to confirm this.

Legumain is an cysteine protease originally identified from the leguminous plant, *Canavalia ensiformis*. Orthologues of this enzyme have also been identified in a number of species of helminth parasite, including *S. mansoni, S. japonicum, H. contortus* and *F. hepatica* (Sajid and

McKerrow 2002; Caffery *et al.,* 2000; Oliver *et al.,* 2006). It is proposed to have a role in the activation of cysteine proteases by cleaving the pro-enzyme form to activate the mature enzyme product (Knox *et al.,* 2003). Within *Schistosoma spp.,* legumain (SM32) has been shown to play a role in the digestion of haemoglobin by activating the endopeptidases involved in this process (Dalton and Brindley, 1996; Caffrey et al., 2000). Legumain has also shown potential as a diagnostic antigen against both *Schistosoma* (El-Sayed *et al.*, 1998) and the Chinese liver fluke, *Clonorchis sinensis* (Jung-Won *et al*., 2009). It was also identified in the E/S products of *C. sinensis* and contains a signal peptide (Jung-Won *et al*., 2009), which would indicate it is excreted by these species and thus not "hidden". As with cathepsin B2, although legumain was exclusive to the *F. hepatica* S3 (membrane-bound) fraction, further analysis would be required to confirm that it is genuinely membrane bound, as in other species it appears to be excreted and so not "hidden".

Pro-x-carboxypeptidase (prolylcarboxypeptidase) is a lysosomal protease which belongs to the S28 family of serine proteases (Tan *et al*., 1993). It exhibits optimal activity at acidic pH and has been identified in the helminths, *Caenorhabditis elegans* (Geier *et al*., 1999), *H. contortus* (Geldhof and Knox, 2008), *S. japonicum, C. sinensis* and in whole extracts of adult and immature stages of *F. hepatica* (Robinson *et al.*, 2009). Pro-x-carboxypeptidases have been implicated as anticoagulants in *H. contortus* (Geldhof and Knox, 2008) and *F. hepatica* (Robinson *et al*., 2009).

Saposin-like proteins, which include SAP-1, SAP-2 and SAP-3, have been shown to be expressed at different life stages in *F. gigantica* (Grams *et al.,* 2006). SAP-1 is expressed by the NEJ and is involved in the lysis of hepatic cells while SAP-2 and SAP-3 proteins, expressed by the adult stages, lyse erythrocytes and peripheral blood mononuclear cells (PMBC; Grams *et al.,* 2006). This has also been reported for clonorin, a saposin-like protein, isolated from the Chinese liver fluke, *C. sinensis* (Lee *et al.,* 2002). Erythrocyte lysis is likely to be required to initiate haemoglobin digestion and, hence, saposins may also be good vaccine candidates.

Proteomic screening for proteins exclusive to the S3 extract yielded some candidates of interest as outlined above. Although a large number of the proteins identified from the screen were assigned identities, there were a considerable number of peptides which were not. The *F. hepatica* genome remains incomplete and, to date, there are only 3055 adult *F. hepatica* ESTs

publically available (February 2012. NCBI, http://www.ncbi.nlm.nih.gov). This may explain why many peptides were not assigned a function. The genome of the closely related helminth *S. mansoni* encodes at least 11,809 genes (Berriman *et al.,* 2009). If the *F. hepatica* genome were completed and annotated a much larger number of the peptides in the current study would have been assigned an identity leading to the identification of more potential candidates.

The aim of this Chapter was to identify potential "hidden" antigens derived from the gut of *F. hepatica*. Obviously, the proteomic approach directed at the putatively membrane-bound S3 fraction cannot determine the precise tissue or sub-cellular compartment from which such proteins are extracted (or in which such proteins reside). In an attempt to identify which, if any, of these were present in the gut region, LCM was carried out. As discussed earlier LCM allows the precise targeting and extraction of cells from tissues of interest. The gut was visualised and extraction of gut tissue successful, as illustrated in Figure 2.3. However, subsequent steps in analysis were unsuccessful. The RNA appeared to be either of poor quality and/or too low a concentration to make cDNA for subsequent analysis. This was concluded as BLAST searches of inserts identified these as plasmid vector or with no significant hit.

Laser capture microscopy is a relatively novel method which is still being optimised for different requirements and applications. The method used to capture gut tissue here was first published by Nilaweera *et al*., (2009) and developed to isolate neurons from sections of hamster brain tissue. The LCM performed in this study was carried in collaboration with The Rowett Institute, Aberdeen, where the protocol by Nilaweera *et al.,* (2009) has been optimised for brain tissue. Here, this methodology failed to produce viable RNA and this could reflect subtle methodological differences. The protocol used here also differs from that used by Ranjit *et al.,* (2006) to explore the gut of the hookworms, *Necator americanus* and *Ancylostoma caninum.* Ranjit *et al.,* (2006) snap froze worms in Optimal Cutting Temperature (OCT, Tissue-tek), the slides were then washed with diethylpyrocarbonate (DEPC) water to remove OCT before being fixed and stained as described here. Furthermore, Ranjit *et al.,* (2006) dried slides in a fume hood for over 2 hours which was longer than the 15 min drying time here. The method used here also differed to that of Gobert *et al.,* (2009), who used LCM to study tissue-specific gene profiles in *S. japonicum*, who also washed slides with DEPC

treated water to remove OCT. The methods by Ranjit *et al.,* (2006) and Gobert *et al.,* (2009) may have been more suitable for use with *F. hepatica* and further studies with LCM could warrant using these approaches. However, the most likely explanation is that the RNA could have been degraded prior to LCM. The parasites were extracted around two hours after the liver was removed from the ovine host at post-mortem. The gut of *F. hepatica* is an enzyme-rich environment and may start to degrade almost immediately. Additionally, the parasite material was transported from MRI, Edinburgh to the Rowett Research Institute, Aberdeen on dry ice and, then the extracted RNA was transported back to Edinburgh in the same manner. Therefore, taking into consideration all the factors above, it is probable that poor starting material and transportation could be responsible for the poor yield of the RNA.

If this was to be repeated subsequently with *F. hepatica*, several factors would need to be addressed. Ideally, there would be a minimal distance between sample storage and processing for LCM/RNA extraction. Secondly, parasites for sectioning and LCM should be snap frozen alive to reduce any possibility of degradation. Unfortunately, MRI does not maintain the liver fluke life-cycle in house and samples were retrieved from naturally infected ovine hosts from abattoirs. It was often not possible to process livers on site and thus they had to be transported back to MRI. Working closely with groups who do have access to the parasite life-cycle and the necessary LCM and auxiliary facilities would thus be hopefully circumvent this problem.

The aims of this chapter were to identify exclusively membrane-bound proteases and to determine if transcripts of these were present in the parasite's gut. A subset of 4 *F. hepatica* proteases that reside within the membrane bound S3 fraction was successfully catalogued, which previously has not been carried out for *F. hepatica*. A large number of the proteins were shared between fractions, and these 4 proteases would warrant further investigation to identify where they are expressed and what role they have in haemoglobin digestion. Unfortunately, for technical/logistical reasons, the LCM approach could not be used to identify which, if any, of these, were expressed within the gut of *F. hepatica.* As membrane-bound gut antigens from *F. hepatica* could not be identified using the proteomic/LCM approach, in the next chapter, conjugated lectins will be used as an alternative method for enriching gut associated membrane-bound proteins.

3 Exploiting lectins to purify gut antigens from somatic extracts of *Fasciola hepatica*

3.1 Introduction

Fascioliosis in domestic livestock is traditionally controlled using fasciolicidal drugs. Triclabendazole has been the predominant drug of choice for treating liver fluke infections for over 20 years because of its efficacy against both juvenile and adult fluke (Brennan *et al*., 2007). However, the emergence of resistant parasites has spurred an interest in developing vaccines as an alternative means of control. Several promising *F. hepatica* vaccine candidates have been isolated to date, including glutathione-S-transferase (GST; Sexton *et al*., 1990), cathepsin L-like proteases (CatL; Dalton *et al*., 2003) and leucine aminopeptidase (LAP; Acosta *et al*., 2008). GST was selected because of parallels with the human blood flukes, where homologous native GST proteins from *Schistosoma* spp., were shown to reduce parasite burden in immunised mice (40-43 %) and rats (50-72 %) (Balloul *et al*., 1987; Brophy and Pritchard, 1994), CatLs due to their prominence in the excretory/secretory (E/S) products of adult and juvenile *F. hepatica* (Dalton *et al*., 2003) and LAP on the basis that it is associated with the epithelial cells which line *F. hepatica's* digestive tract (Acosta *et al*., 2008). The *F. hepatica* LAP study, the recent progress in vaccination against *Haemonchus* as well as positive outcomes in vaccination against the blood-feeding tropical cattle tick, *Rhipicephalus (Boophilus) microplus*, which led to the development of the first commercially available subunit vaccine, TickGARD™ (Willadsen *et al*., 1995), all indicate that targeting proteins expressed on the luminal surface of the intestine of haematophagous parasites could be a rich source of effective vaccine candidates. *F. hepatica* lives in a highly vascularised environment within the liver system, feeding on surrounding tissue and blood (Dalton *et al*., 2004; Oslen, 1986). Furthermore, blood is ingested, whether deliberately or not, by the juvenile fluke migrating through the liver parenchyma (Oslen, 1986). Thus *F. hepatica* may be vulnerable to the gut antigen approach to vaccination.

Note: Published, McAllister H.C., Nisbet, A.J., Skuce, P.J., Knox, D.P. (2011) Using lectins to identify hidden antigens in *Fasciola hepatica*. The Journal of Helminthology 85, 121-127

Most of the candidate "hidden" or gut antigens identified to date have been isolated from membrane-bound fractions and have glycosylated components, for example, H-gal-GP isolated from *H. contortus* (Smith *et al.,* 1994). Initial studies investigating *H. contortus* gut proteins involved physically dissecting out the parasite's gut then extracting the proteins. However, as discussed in Chapter 2, the *F. hepatica* gut is embedded in tissues and is very difficult to dissect out. Therefore, other means have to be used to extract and purify *F. hepatica* gut proteins. Lectins have proven to be valuable tools for separating glycosylated antigens from protein-rich extracts (Smith *et al*., 1994). Lectins are carbohydrate-binding proteins derived from plants, animals and micro-organisms, with specificity for terminal or sub-terminal carbohydrate residues (Leathem and Brooks, 1998). In *H. contortus,* the major vaccine candidates isolated from the intestine have all been localised to the microvillar surface of the intestinal cells (Knox and Smith, 2001), the equivalent structure to the gut lamellae in *F. hepatica.* This chapter aims to exploit the differential carbohydrate binding properties of lectins, in an attempt to identify those which bind specifically to the gut of adult *F. hepatica.* As described in Chapter 2, proteins in the membrane-bound extract are of particular interest as this has been a rich source of antigens in other helminth parasites (Smith *et al.,* 1994). By identifying lectins with specificity for the gut region, it may be possible to enrich the crude membrane-bound extract to subsequently isolate intestinal integral membrane proteins.

To identify the protein components of the enriched fractions, LC-ESI-MS/MS will be used, as described in Chapter 2. This will identify proteins which have been enriched by the selected lectins, which can then be classified by protein function. Again, proteases will be of particular interest as, historically, these have made very effective vaccine candidates (Dalton, 2001). For example, H-gal-GP identified from the membrane-bound fraction (Triton X-100 extract) of *H. contortus* reduces the egg counts and worm burdens of immunised sheep by 86 % - 93 % and 52 % - 75 %, respectively (Smith *et al.,* 2000). Furthermore, lead vaccine candidates to date against infection with *F. hepatica* are the proteases, cathepsin L1 and L2 (Dalton *et al.,* 1996; Mulcahy and Dalton, 2001) and LAP (Acosta *et al.,* 2008; Brophy and Pritchard, 1999).

3.2 Materials and methods

3.2.1 Parasites

3.2.1.1 Retrieval of parasites

Flukes from naturally infected ovine livers were obtained, at post mortem, from several sources; Professor Neil Sargison (RDSVS, Edinburgh), Professor Ian Fairweather (Queen's University, Belfast), Mr. S. Stevenson [Duncan Stevenson (Meats) Ltd, Dunblane], Scotbeef (Dunblane) and Dr Fiona Kenyon (Moredun). The parasites were recovered live from the gall bladder (if still intact) or from the bile ducts by slicing through the liver mass.

3.2.1.2 Storing parasites for somatic extract preparation

Parasites were washed in phosphate buffered saline (PBS) three times and frozen at -80^{o}C.

3.2.1.3 Processing parasites for paraffin wax sectioning

Parasites were washed three times in 1 x PBS (see Appendix 1) for 5 min, and fixed at room temperature in 4 % paraformaldehyde for 24-48 hours. They were then processed into paraffin wax blocks for histological sectioning using an automated processor. This took the fixed parasites through a series of alcohol washes as follows; 80% ethanol for 45 min; 95% ethanol for 75 min; 99% ethanol for 3×75 min; isopropanol for 2×90 min; isopropanol/xylene [1:1 v:v] for 90 min; xylene for 2×90 min; paraffin wax for 2×105 min. The dehydrated parasites were then placed in cassettes and embedded in wax. The blocks were stored at 4ºC until sectioned.

Wax sections were cut using a rotary microtome. 5 µm thick sections were then cut and floated in a 45ºC water bath. Sections were mounted on glass slides (Superfrost® Plus glass slides, Thermo Scientific). Slides were dried overnight at 45^{o}C and stored at 4^{o}C until required.

3.2.2 Lectin Screen on histological sections

3.2.2.1 Preparing wax sections for lectin staining

Mounted sections were dewaxed using an automated processor. This took slides through a series of washes as follows: xylene 3 min; xylene 1.5min; 2 x 74 OP 1.5 min; 74 OP/dH_2O

[1:1 v:v] 30 sec; dH_2O 1.5min. Slides were then kept immersed in dH_2O. Five minutes prior to staining, slides were immersed in lectin wash buffer (LWB), see Appendix 1.

3.2.2.2 Conjugated Lectins

Sixteen fluorescently labelled lectins of differing carbohydrate specificities (Vector Laboratories, UK) as listed in Table 3.1, were used to screen the mounted histological sections. Lectins were diluted to a working concentration of 5 μg/ml in LWB, see Appendix 1.

3.2.2.3 Haemotoxylin and Eosin (H/E) staining of sections

For reference, several mounted sections of adult flukes were stained with haemotoxylin and eosin by the following automated procedure: xylene, 180 sec; xylene, 90 sec; 74OP, 60 sec; 74OP, 90 sec; 74OP/water, 30 sec; water, 90 sec; haemotoxylin (nuclear stain), 360 sec; running water, 150 sec; acid alcohol [1% HCl in 70% ethanol], 1 sec; water, 60 sec; Scots Tap Water Substitute (S.T.W.S.) (see Appendix 1), 60 sec; water, 120 sec; eosin, 90 sec; water,150 sec; 70% alcohol, 60 sec; 95% alcohol, 45 sec; 90 sec; 3 x 74OP, 90 sec; 2 xylene, 120 sec; xylene.

3.2.2.4 Lectin probing of histological sections

Mounted sections of adult flukes were de-waxed by an automated procedure (Xylene, 90sec; xylene 165 sec; 74 OP 2x 90 sec; 74OP + dH_2O, 60 sec; dH_2O, 90 sec) and then probed with one of the panel of 16 lectins listed in Table 3.1. To do this, slides were placed horizontally in a dark moist chamber; 500μl of diluted lectin solution were pipetted directly onto the section and incubated for 2 hours at room temperature. Slides were then washed in darkness three times for 5 minutes with LWB. A negative control was prepared in the same way, replacing the lectin with an equal volume of LWB alone. Slides were mounted with a coverslip using Prolong® antifade with DAPI (Invitrogen) and stored at 4°C in the dark until viewed on an Axiovert 200 fluorescent microscope (Carl Zeiss Microimaging). The staining within the following organ systems was noted: gut lamellae, gastrodermal cells, tegumental syncytium, tegumental muscle layer, subtegumental cells, tegumental spines, egg shell material, eggs, vitelline follicles, Mehlis' gland, testis follicles, testis membrane, cirrus, ventral sucker, oral sucker and parenchyma.

Table 3.1 : Conjugated lectins used for screening histological sections of adult *F. hepatica* and/or Western blots of adult *F. hepatica* somatic extracts S1, S2 and S3

Lectin	**Used for Histological sections(H) and/or Western Blots (W)**	**Common name of source plant**	**Abbreviation**	**Glycan specificity**
Canavalia ensiformis	H and W	Jack bean	Con A	αMan> αGlc
Triticum vulgaris	H and W	Wheat Germ	WGA	β-GlcNAc
Lens culinaris agglutinin	H and W	Lentil	LCA	αMan> αGlc
Pisum sativum agglutinin	H and W	Pea	PSA	αMan
Arachis hypogaea	H and W	Peanut	PNA	Galβ1-3GalNAc
Artocarpus heterophyllus	H and W	Jackfruit (Jacalin)	JAC	αGal
Lycopersicon esculentum lectin	H and W	Tomato	LEL	βGlcNAc
Glycine max	H	Soybean	SBA	GalNAc or Gal
Vicia villosa lectin	H	Hairy Vetch	VVL	GalNAc
Solanum tuberosum lectin	H	Potato	STL	βGlcNAc
Erythrina cristagalli lectin	H	Coral tree	ECL	Galβ1-4GlcNAc
Ricinus communis agglutinin I	H	Castor bean	RCA I	βGal, βGalNAc
Dolichos biflorus agglutinin	H	Horse gram	DBA	Terminal αGalNAc
Sophora japonica agglutinin	H	Pagoda tree	SJA	βGal, βGlcNAc
Ulex europaeus agglutinin I	H	Gorse	UEA I	α-L-Fuc
Datura stramonium lectin	H	Thorn Apple	DSA	βGal, Galβ1-4GlcNAc, $(GlcNAc1\text{-}4)_n$

3.2.3 Preparation of somatic extracts from *F. hepatica*

Water-soluble (S1), membrane associated (S2) and membrane bound (S3) extracts were prepared exactly as described in Section 2.2.1.2.

3.2.3.1 Measurement of protein concentrations

Protein concentrations of each extract were measured using the BCA™ Protein Assay Kit (Pierce) as described 2.2.1.3.

3.2.4 Lectin probing of somatic extracts

3.2.4.1 SDS PAGE gel electrophoresis

Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS PAGE) was used to separate extracts, S1-S3, by molecular mass, as described in section 2.2.1.4. Precast NuPAGE® Novex Bis-Tris 4-12% gels (Invitrogen) were used and stained with Simplyblue® (Invitrogen).

3.2.4.2 Western blotting

Extracts S1-S3 were separated on 4-12% polyacrylamide gels at 200V for 50 minutes as outlined in section 3.2.4.1. Instead of staining, the gels were blotted on to nitrocellulose membrane using the X Cell II Invitrogen system (Invitrogen) according to the manufacturer's instructions. The nitrocellulose membrane was then divided into lanes and blocked in TNTT (see Appendix 1) overnight at 4ºC.

3.2.4.3 Probing Western blots with biotinylated lectins

Biotinylated lectins, as outlined in Table 3.1; ConA, WGA, LCA, PSA, PNA, JAC and LEL (Vector Laboratories), were prepared at a 1:1000 dilution (except ConA 1:2000) in TNTT. Electroblotted lanes of extracts were then incubated with one of the lectins for 1 hour at room temperature on a rotary shaker. After incubation, strips were washed 3 times in TNTT then incubated in Streptavidin-HRP (Thermoscientific, UK) at 1:1000 in TNTT for 1 hour and washed as before. Strips were developed with Sigmafast™ DAB with metal enhancer (Sigma-Aldrich, UK).

3.2.5 Lectin affinity purification

3.2.5.1 Peanut agglutinin and Jacalin agarose affinity column preparation

Affinity columns for both peanut agglutinin (PNA) and jacalin (JAC) lectins were prepared as follows. A 150 mm long x 10 mm diameter empty column apparatus was assembled according to the manufacturer's guidelines (Bio-rad) and washed thoroughly with dH_2O at room temperature to clean and check for any blockages or leaks. The column was then plugged with a stopper and 1 ml of 1 x LWB dispensed into the bottom of the column. 2 ml of the lectin-bound agarose beads were then dispensed into the column and the stopper removed. The column was packed by continually washing for 30 min with 1 x LWB, being careful not to allow the column to run dry. Once the column was packed, the stopper was replaced and the column filled very carefully with 1 x LWB, to create a positive meniscus. The top of the column was assembled ensuring no air bubbles were trapped. All the columns were then equilibrated and packed in LWB by washing overnight, at a flow rate of 6 mls per hour at 4ºC.

3.2.5.2 Lectin affinity chromatography of S3 Extract

1 ml of extract at 1 mg/ml was diluted four-fold in LWB. The diluted extracts were individually fractionated on either a JAC or PNA lectin-agarose column at 4ºC. First, the sample was loaded onto a fresh column at 6ml/hour ensuring any unbound protein was collected. The column was then washed with 5 x column volumes in LWB. Bound proteins were then eluted by washing the column with either 0.8M or 0.5M galactose in LWB for JAC and PNA, respectively, ensuring the eluates were collected. Bound and unbound proteins were buffer exchanged into 10mM Tris, pH7.4 by passing through a Sephadex G-25 column to remove detergents and sugars, which may interfere in later assays. Samples were then concentrated by ultracentrifiltration in Ultracel 10K concentrators (Amicon). The protein concentration for each sample was then estimated using a BCA™ Protein Assay Kit (Pierce) as described in section 3.2.3.1 and samples stored at -80ºC. To visualise purified fractions, samples were separated by SDS-PAGE (4-12%) and silver stained using Silverquest™ silver staining kit (Invitrogen).

3.2.5.3 Liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) of lectin-enriched fractions

The LC-ESI-MS/MS and database mining were carried out exactly as described in section 2.2.14 to section 2.2.1.6. Data were analysed as outline in section 2.2.1.7

3.3 Results

3.3.1 Lectin probing of histological sections

Seven of the lectins bound to the gut region of *F. hepatica,* as shown in Table **3.2**. However, none of these lectins bound exclusively to the gut. Many of the organ systems within tissue sections of the adult flukes were labelled by one or more of the panel of lectins screened. There were some examples of tissue–specific localisation as shown in Figure 3.1, panels A-D. The lectins STL, DBA and SJA bound exclusively to glycoproteins located on the testes membrane, the testes follicles and the subtegumental cells, respectively. These were the only lectins to show single tissue specificity but the intensity of staining in each of these cases was relatively low. Two lectins, UEA-1 and DSL, showed no affinity for any organ systems within the parasite. ConA bound intensely to all tissues throughout the parasite, with the exception of the tegumental spines. A similar pattern was seen with the lectins WGA, LCA and PSA but the staining intensity varied between the organ systems. JAC and LEL bound to molecules on the gut lamellae but not the gastrodermal cells whereas, conversely, PNA bound to carbohydrates on the gastrodermal cells and not the lamellae. This indicates tissue specificity to either the gut lamellae or gastrodermal cells as shown in Figure 3.2, panels A and B. Lectins of particular interest, i.e. those binding to the gut of *F. hepatica* identified from the screen of adult sections with fluorescently conjugated lectins were, therefore, ConA, WGA, LCA, PSA, PNA, JAC and LEL.

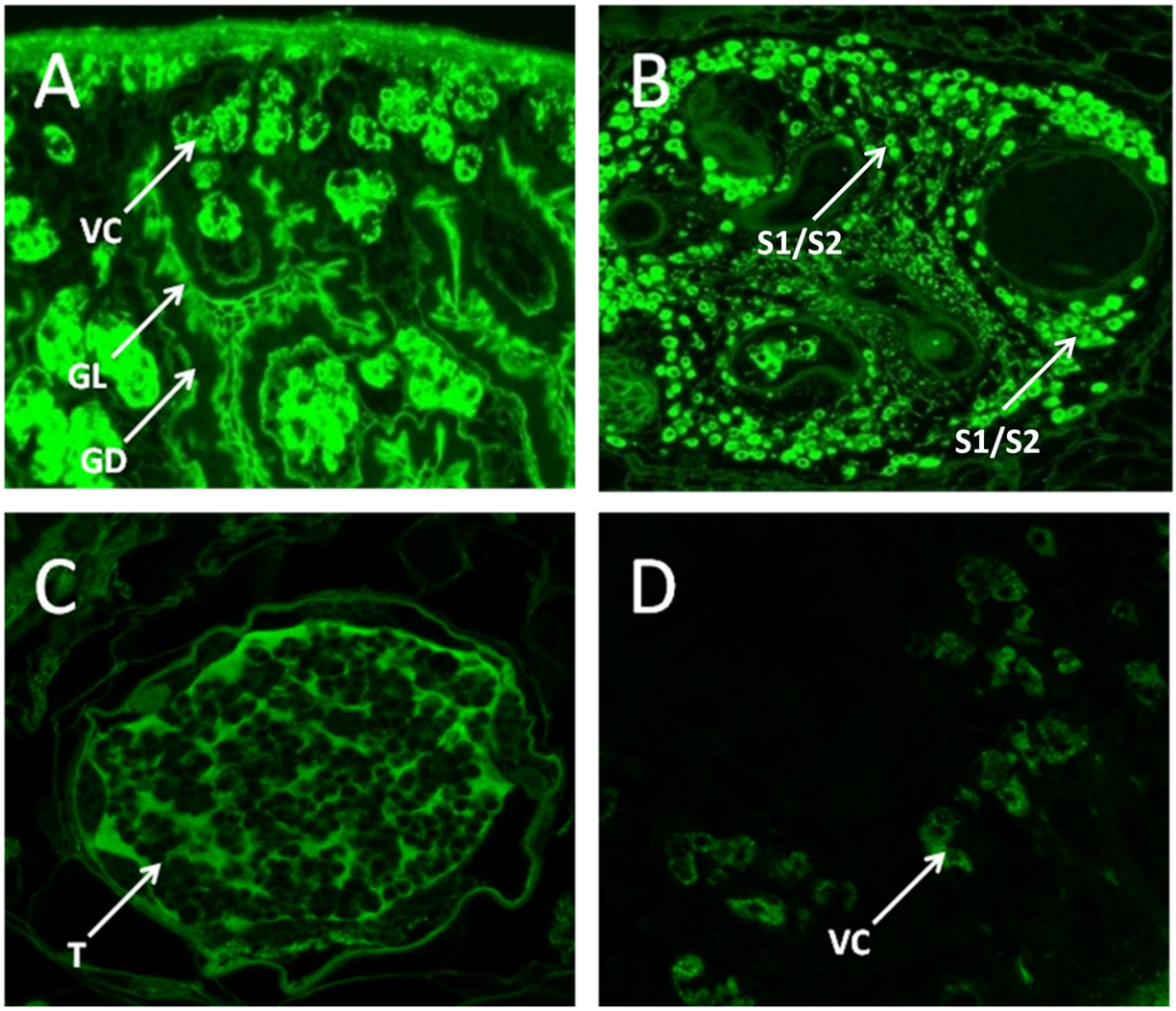

Figure 3.1: Lectin binding within the tissues of *F. hepatica.* Panel A shows the binding of concanavalin A to many tissues throughout the parasite, including the gut lamellae (GL), gastrodermis (GD) and vitelline cells (VC). Panel B shows the binding of *Lens culinaris* agglutinin to the presumed S1/S2 cells (arrows) of the Mehlis' gland. The selective binding of *Lycopersicon esculentum* lectin to the male tissues, for example, the testes (T) is shown in Panel C and of *Erythrina culinaris* agglutinin I to the female tissues, for example, the vitelline follicles (V) are shown in Panel D.

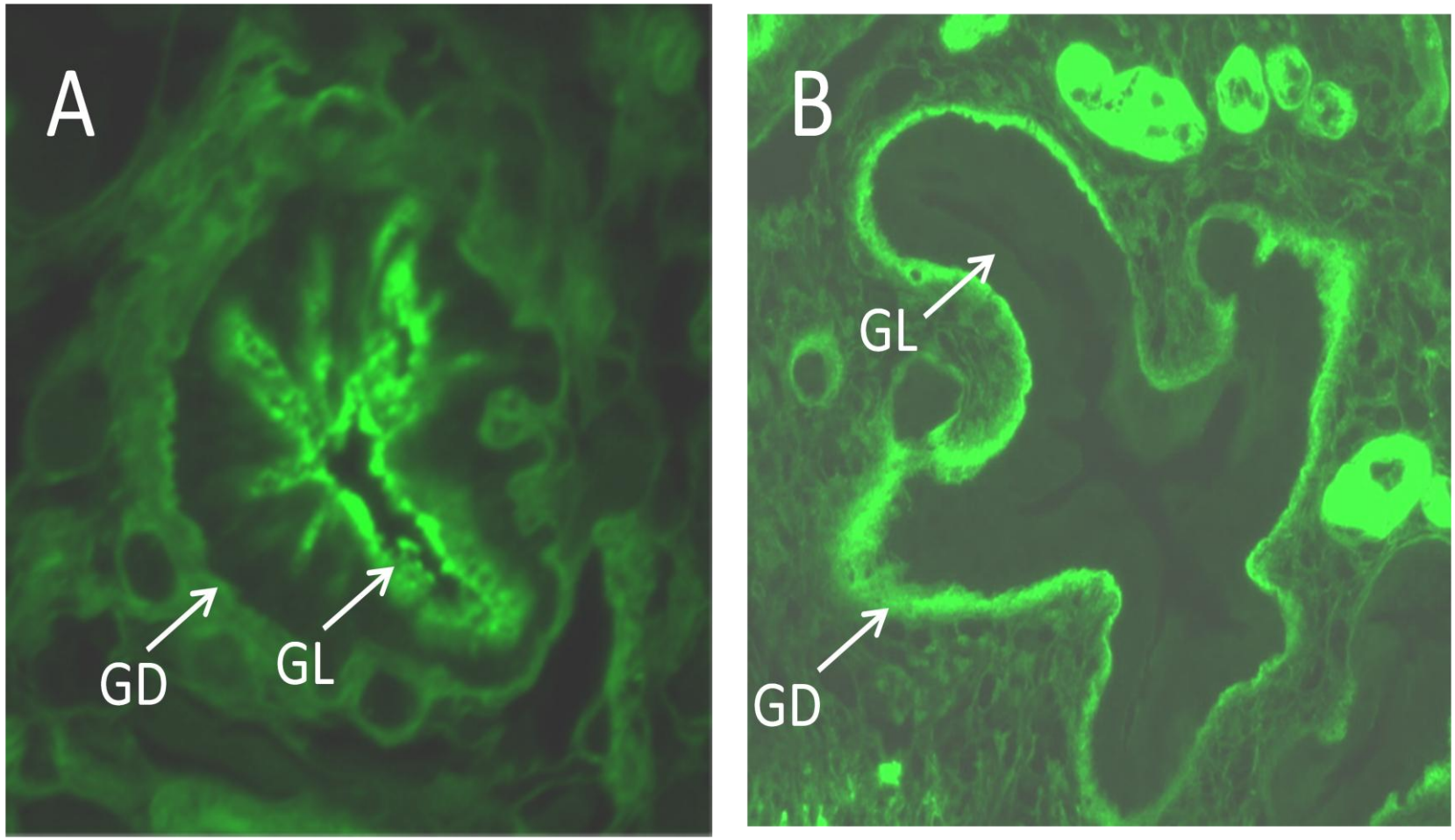

Figure 3.2 Selective lectin binding within the gut tissues of *F. hepatica*. Panel A shows the preferential binding of jacalin lectin to the gut lamellae (GL). Panel B shows the preferential binding of peanut agglutinin lectin to the gastrodermal cells (GD)

Table 3.2 Summary of lectin binding within the gut and other organ systems of adult *F. hepatica.* Other organ systems noted include; Syncytium (S), Muscle layer (M), Subtegumental cells (ST), Tegumental spines (T), Eggs (E),Vitelline follicles (VF), Mehlis' Gland (MG), Testes (T), Cirrus (C), Ventral sucker (VS), Oral sucker (OS), Parenchyma (P)

	Staining in the gut?		**Staining in other organ systems? (+/-)**												
Lectin	**Gut Lamellae**	**Gastrodermis**	**S**	**M**	**ST**	**TS**	**E**	**VF**	**MG**	**T**	**TM**	**C**	**VS**	**OS**	**P**
Con A	+++	+++	+	+	+	-	+	+	+	+	+	+	+	+	+
WGA	++	++	+	+	+	-	+	+	+	+	+	+	+	+	-
LCA	++	++	+	+	+	-	+	+	+	+	+	+	+	+	+
PSA	++	++	+	+	+	-	+	+	+	+	+	+	+	+	+
PNA	-	+	+	+	+	-	+	+	+	+	+	+	-	+	+
JAC	+++	-	+	+	+	-	+	+	+	+	+	+	+	+	+
LEL	++	-	+	+	+	-	-	-	-	-	+	+	+	+	+
SBA	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-
VVL	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-
STL	-	-	+	-	+	-	-	-	-	-	+	-	-	-	-
ECL	-	-	+	-	+	-	+	+	-	-	-	-	-	+	-
RCA I	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
DBA	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
SJA	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
UEA I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DSL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.3.2 Lectin probing of somatic extracts

Figure **3.3** shows the Western blots of the S3 extract incubated with the panel of lectins which reacted with the gut material on histological sections, as identified above; ConA, WGA, LCA, PSA, PNA, JAC and LEL. Each of the lectins tested reacted with proteins in the S3 extract and there was a difference in the staining patterns depending on the lectin used. The possibility that differential lectin binding could be used to enrich for different subsets of gut-specific membrane proteins was then tested by comparing the profiles of the glycoproteins which specifically bound JAC and PNA lectins.

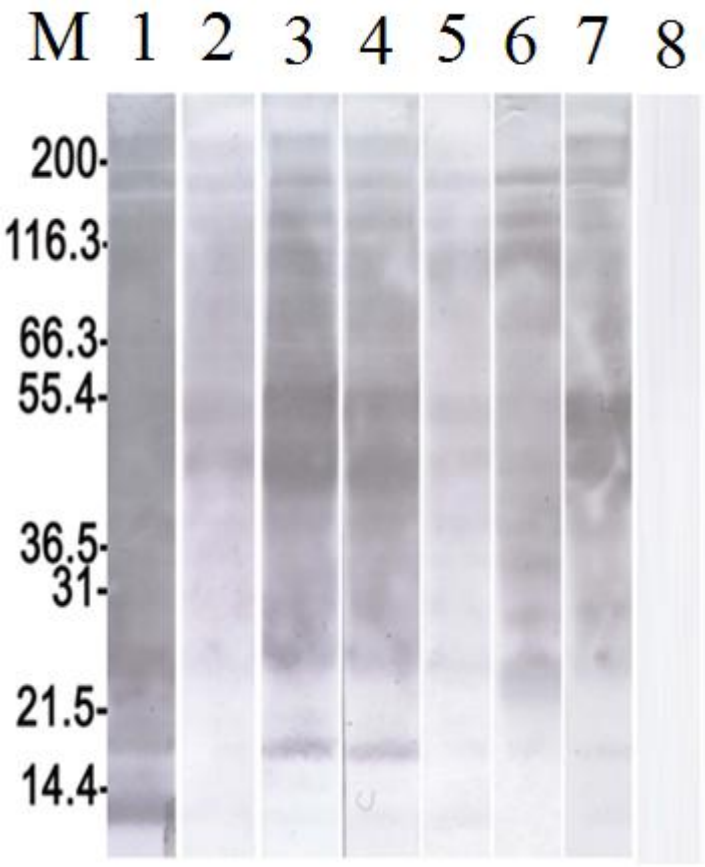

Figure 3.3. Lectin probing of *F. hepatica* membrane bound (S3) extract with the respective biotinylated lectins. Lane M, Markers. Lane 1, Con A. Lane 2, WGA. Lane 3, LCA. Lane 4, PSA. Lane 5, PNA. Lane 6, JAC. Lane 7, LEL. Lanes 1-7 were then incubated in strepdavidin-Horseradish peroxidise (HRP) and developed with Sigmafast™ DAB. Lane 8 is an example of a negative control, where the S3 was probed with Con A lectin then developed with DAB (no incubation in strepdavidin-HRP). Biotinylated lectins WGA, LCA, PSA, PNA, JAC and LEL and strepdavidin-HRP were prepared at a 1:1000 dilution in TNTT, ConA was diluted at 1:2000 in TNTT.

3.3.3 Lectin-affinity purification

The S3 extract was applied to JAC or PNA affinity columns and the respective unbound and bound fractions separated using SDS-PAGE and visualized by silver staining (Figure 3.4). Both lectins appeared to exhibit selectivity and bound relatively few proteins compared to the original extract. The eluate from the PNA affinity column contained a number of polypeptides over a broad size range but, nonetheless, clearly represented a subset of the original crude fraction. By contrast, JAC appeared to be more selective, the eluate containing only two faint bands around 62-65 kDa.

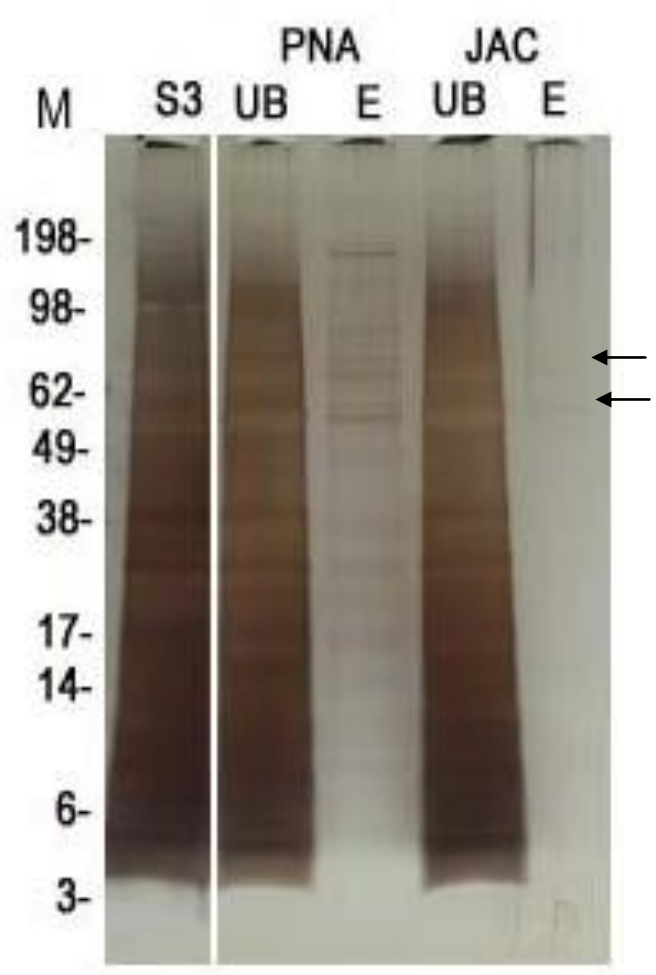

Figure 3.4: Enrichment of a crude detergent-soluble fraction (S3) by lectin affinity chromatography. The unbound fraction (UB) from the peanut agglutinin (PNA) and jacalin (JAC) columns are shown alongside the eluted (E) protein fraction from PNA and JAC. Arrows highlight the faint protein bands in the JAC eluate.

3.3.4 Proteomic analysis of JAC- and PNA- enriched fractions

The enriched fractions were analysed using LC-ESI-MS/MS to identify proteins which were present in the respective lectin-enriched fractions. Despite the fractions appearing relatively "simple", the sequences of the peptides identified from the S3 enriched JAC and PNA fraction identified 270 and 194 EST contigs in the Professor Peter Brophy EST database, respectively. From the identified ESTs, 160 and 113 represented proteins unique (not represented in the other lectin enriched fraction) to either JAC and PNA enriched fractions, respectively. A large proportion of the identified proteins from JAC and PNA were classed as hypothetical or unknown. This accounted for 42% and 30% of the identified EST contigs in the JAC and PNA enriched fractions. The protein identities were sorted based on functional class, as shown in Appendix 3 for the JAC fraction and Appendix 4 for the PNA enriched fractions. The proteases enriched from the S3 by JAC and PNA lectins are highlighted in Table **3**.**3**.

For the purposes of identifying a putative gut antigen(s), proteolytic enzymes identified within the lectin-enriched fractions were of particular interest. There were 3 proteolytic enzymes identified from the JAC-enriched fraction, namely; cathepsin L, legumain – 2 and leucine aminopeptidase. In addition to these, the gut protein "secreted saposin-like protein", SAP-3, was also of interest as it is a gut-associated protein in the hookworm *A.caninum* (Don, 2007). Within the PNA enriched fraction, four proteolytic enzymes were identified, namely; cathepsin L1, legumain-1, putative lysosomal Pro-X carboxypeptidase precursor and cathepsin D-like aspartyl protease. Additionally, as with the JAC-enriched fraction, SAP-3 was also identified and of particular interest.

Table 3.3: Proteases identified from the PNA and JAC enriched S3 fraction from adult *F. hepatica.*

Lectin Fraction	Database Accession Number	Protein Description	Mowse Score	No. of unique peptides
JAC	fhep00187|2	cathepsin L	283.4	5
	fhep00889|1	cathepsin L	94.6	2
	fhep00344|1	cathepsin L protein	361.2	8
	fhep03421|1	cathepsin L protein	311.2	7
	fhep00577|1	cathepsin L protein	262.4	6
	fhep00623|2	cathepsin L protein	163.2	3
	fhep00739|1	cathepsin L1D	131.5	4
	fhep04385|1	cathepsin L1D	90.8	2
	fhep02273|1	cathepsin L-like protein	282.1	7
	fhep03629|1	secreted cathepsin L 1	203.8	5
	fhep02735|1	cathepsin L-like protein	246.9	5
	fhep02461|1	cathepsin L-like protein	209.5	5
	fhep42601|1	secreted cathepsin L2	272.6	6
	fhep00458|1	legumain-2	47.2	2
	fhep00030|1	leucine aminopeptidase	560.5	8
PNA	fhep00344|1	cathepsin L1 protein	118.6	2
	fhep01124|1	legumain-1	47.2	2
	fhep00076|1	putative Lysosomal Pro-X carboxypeptidase precursor	38.6	2
	fhep43071|1	cathepsin D-like aspartyl protease	154.8	3
	fhep01837|1	cathepsin D-like aspartyl protease	317.4	7

3.4 Discussion

Here, lectin binding to tissue sections and extracts has been used in an attempt to facilitate the isolation of gut-specific glycoproteins from adult *F. hepatica*. Seven of the lectins tested, namely Con A, WGA, LCA, PSA, PNA, JAC, and LEL bound to the gut lamellae or to the gastrodermal cells. In addition, they also bound to elements within other tissues and organ systems but the intensity of staining differed depending on the tissue and lectin in question. For example, ConA lectin stained almost every anatomical region with equally bright intensity while others (LEL, ECL, JAC, PNA) bound selectively to male (LEL) and female (ECL) reproductive tissues. Similarly, JAC bound to the gut lamellae but not the gastrodermal cells while, conversely, PNA bound to the gastrodermal cells but not the lamellae (Figure 3.1; Panels A to F).When membrane-bound protein extracts were further fractionated using PNA or JAC affinity chromatography (Figure 3.2, panels A and B), it became clear that lectins did offer a means to isolate distinct, and simplified, protein sub-sets from detergent-soluble extracts of adult fluke.

Lectins have previously been used with considerable success for isolating candidate vaccine antigens by affinity chromatography as shown in the preparation of the gut-associated protective antigens H11 and H-gal-GP, from the parasitic nematode, *H. contortus,* (Knox and Smith, 2001; Smith *et al*., 1994; Smith *et al*., 2000a; Smith *et al*., 2000b). The gastrodermal cells of *F. hepatica* contain several known vaccine candidates for fluke, including cathepsin L1 and GST (Collins *et al.*, 2004; Creaney *et al.*, 1995). However, the gut cannot be dissected out to identify which proteins reside here. Thus, lectins were used to screen sections of adult *F. hepatica* which indicated that JAC had some specificity for the gut lamellae and PNA showed some specificity for the gastrodermis. While the Western blots described here confirmed that the selected lectins bound to glycosylated molecules within the S3 somatic fraction (Figure **3**.**3**), these blots did not indicate that binding was particularly selective with, for example, the PNA and JAC binding profiles being almost indistinguishable. When used as affinity ligands, these lectins selected smaller subsets of glycoproteins in comparison to those of the original S3 protein fraction. A number of proteins identified by PNA and JAC were shared. The eluate from PNA contained a series of bands undoubtedly less complex than the original sample and the JAC eluate contained two faint protein bands around 62-65kDa in

size. The brush border in *H. contortus* has proven to be a rich source of antigens and both JAC and PNA have been used to purify protective antigens from somatic fractions of *H. contortus* (Knox and Smith, 2001; and Smith, 2001; Knox *et al*., 2003; Smith *et al*., 2000).

The proteomic analyses of the lectin-enriched fractions revealed differences between the respective lectins' affinity for different glycoproteins. The JAC-enriched fraction contained three proteolytic enzymes (cathepsin L, Legumain – 2 and LAP) and a gut-associated protein (SAP-3). The PNA-enriched fraction contained four proteolytic enzymes and a gut-associated protein; cathepsin L1, legumain-1, putative lysosomal Pro-X carboxypeptidase precursor, cathepsin D-like aspartyl protease and the SAP-3 protein. Proteolytic enzymes or gut-associated proteins identified uniquely in the S3 dataset in the previous chapter included cathepsin B, Legumain-, a putative pro-X-Carboxypeptidase precursor and the SAP-3 protein. Additionally, cathepsin L1 was also identified in the S3 fraction but was not unique to this fraction as it was also identified in S1 and S2 fractions. CatL1 has been extensively studied and characterised in *F. hepatica*. It is secreted by all developmental stages within the definitive host and facilitates migration by digesting tissue, immune evasion and feeding (Mulcahy and Dalton, 2001). Native cathepsin L1 has been trialled as a vaccine candidate where it protected immunised animals from infection in both sheep (72 % reduction in parasite burden) and cattle (79 % reduction in parasite burden) when administered with Freund's adjuvant (Mulcahy and Dalton, 2001).

One protein which was not previously identified in the S3 fraction, but was enriched by the PNA lectin in the current study, is the cathepsin-D like aspartyl protease. Vaccination of dogs with recombinant cathepsin D-like aspartyl proteases (*Ac*-APR-1) has been shown to protect against infection, reducing egg output of *A. caninum* in comparison to non-vaccinated controls by 70 % (Loukas *et al.,* 2005). The cathepsin D aspartyl proteases identified from the canine hookworm, *A. caninum* (*Ac*-APR-1) and the human hookworm *N. americanus* (*Na*-APR-1), have been localised to the parasites' gut and shown to have a role in digesting host haemoglobin. This latter trait was also thought to be associated with host specificity. Although both forms of the cathepsin D were enzymatically active against canine and human haemoglobin but the rates of digestion were considerably higher against their own respective host blood samples (Williamson *et al.,* 2002). Further to this, hookworm cathepsin D has been

shown to be most active in acidic environments (Williamson *et al.,* 2002) and thus would be expected to function efficiently in the gut lumen of *F. hepatica* which is slightly acidic ~ pH 5.5 (Dalton *et al.,* 2006; Halton *et al.,* 1997). Blood-feeding parasites use a number of proteolytic enzymes which act in a cascade to facilitate haemoglobin digestion, whereby the action of one allows the next to act and so forth (Williamson *et al.,* 2003). The initial step of haemoglobin digestion is proposed to be performed by the aspartyl proteases, followed then by cysteine proteases, metalloproteases and finally the exopeptidases, in that order (Williamson *et al.,* 2003). The disruption of just one of these steps, especially an early and possibly rate-limiting step, could have a profound effect on the ability of the parasite to digest haemoglobin, thus causing it to starve. Cathepsin D has been identified in a number of blood-feeding parasites including Schistosomes (Brindley *et al.,* 2001), *A. caninum* and *N. americanus* (Brown *et al.,* 1995). The traits which have been associated with cathepsin D from studies in other parasites, include the ability to digest host haemoglobin (Williamson *et al.,* 2003), localisation to the gut and activity in acidic environments (Loukas *et al.,* 2005; Williamson *et al.,* 2003) make the cathepsin D identified in this study a very compelling gut antigen candidate in *F. hepatica* and worthy of further investigation. The next steps would be to characterise this protein and assess whether *F. hepatica* cathepsin D is localised to the gut and has the ability to digest host haemoglobin under acidic condition as proposed to occur within the *F. hepatica* gut lumen (Dalton *et al.,* 2006; Halton *et al.,* 1997).

This study also provides valuable information regarding the distribution of carbohydrates throughout the parasite's complex organ systems. It is evident that some carbohydrates are shared amongst tissues, whereas others have a more specific tissue distribution. The lectins ConA, WGA, LCA, PSA, PNA, JAC and LEL all bound to molecules in gastrodermal tissues but have specificity for differing sugars such as α-D-mannose, α –D-glucose, N-acetylglucosamine, N-acetylgalactosamine, glucose and galactose (Leathem and Brooks, 1998). The selective binding of JAC to molecules on the gut lamellae and PNA to those on the gastrodermal cells indicates that the sugars α-galactosidase and galactosyl (β-1, 3) N-acetylgalactosamine are present and may be specific to these tissues within the gut region. Previous studies have indicated that α-linked acetylgalactosamine sugars, derivatives of

galactose, are found on proteins within the gut of *F. hepatica* (Wuhrer et al., 2004), and this would be consistent with the present findings. In addition, the sugars N-acetylglucosamine and N-acetylgalactosamine have also been identified in the gut of the blood fluke, *S. japonicum* (Gobert *et al*., 1998). H-gal-GP, the highly protective antigen identified from the gut of the nematode, *H. contortus*, is a galactose-containing glycoprotein complex (Smith *et al*., 1999; Smith *et al*., 1994) and is purified using lectins with specificity for N-acetylgalactosamine (Smith *et al*., 1994). This lectin screen indicates that similar glycan moieties are also present on glycoproteins in the gut tissues of *F. hepatica.*

This lectin screen also highlights the diversity and complexity of glycoconjugates present throughout the organ systems of *F. hepatica*. The significance of the presence or absence of certain carbohydrates is not fully understood but the patterns seen here are consistent with previous studies in *F. hepatica* and other homologous trematode species. The aim here was to identify any lectins which could selectively isolate glycoproteins from tissues of specific interest, in this case the gut. Unfortunately, many carbohydrate molecules which are present on these tissues are also shared with other organ systems and no single lectin bound exclusively to the gut. Nonetheless, the selective binding of JAC to molecules on the gut lamellae was exploited in order to purify a relatively simple subset of proteins from a detergent extract of adult fluke. From this, cathepsin D was highlighted as a potential candidate for future work because it has many of the hallmarks/characteristics of previously successful vaccine antigens. Work in the subsequent chapters will focus on characterising this novel protease using enzymatic activity assays and immunohistochemistry.

4 Investigation of proteolytic activity in somatic fractions of *F. hepatica*

4.1 Introduction

The importance of proteolytic enzymes as protective vaccine antigens has been discussed in previous chapters. There are a number of proteins within *F. hepatica* which possess proteolytic properties and provide high levels of protection when used in vaccination experiments. These include CatL1, CatL2 (Mulcahy and Dalton, 2001) and LAP (Acosta *et al.*, 2008).

The presence of proteolytic activity in PBS-soluble homogenates of adult *F. hepatica* was first demonstrated by Howell (1966) using azocoll as a substrate. This proteolytic activity was later localised to the gut caeca of adult flukes by mounting fresh sections of adult flukes on Ilford photographic plates and incubating at 37 ºC, then examining plates for evidence of lysis (Howell, 1973). Proteases can be divided into a number of functional clans defined on the basis of amino acids in the active site that are essential for activity and include the aspartyl-, cysteinyl-, glutamyl-, metallo-, asparaginyl-, serine-, and threonine-dependent proteases (Rawlings *et al.*, 2010). A clan is divided into a number of families each comprising homologous proteolytic enzymes. Therefore, two proteases within a family share a significant proportion of their amino acid sequence, specifically with regard to the peptidase subunit region (Rawlings *et al.*, 2010). Four protease clans have been identified to date within *F. hepatica.* These include clan AA, CA, DA and MF and account for 29 putative proteases, not all of which have been characterised experimentally for their proteolytic properties (Rawlings *et al.*, 2010).

F. hepatica is known to ingest host blood during its migrating stages (Fairweather *et al.*, 1999) and as adults in the bile duct (Smyth and Halton, 1983; Dalton *et al.*, 2004). However, the mechanism of haemoglobin digestion remains unclear. Recently, it has been proposed that the acidic pH of the parasite's gut relaxes the haemoglobin structure, making it susceptible to proteolysis by cathepsin L1 (FheCL1), which is present in and secreted by adult and juvenile fluke (Lowther *et al*, 2009). These authors showed that FheCL1 could degrade haemoglobin to small peptides, predominantly of 4-14 residues, but could not release free amino acids. They

suggested that haemoglobin degradation was not completed in the gut lumen but that the resulting peptides are absorbed by the gut epithelial cells for further processing by intracellular di- and aminopeptidases to free amino acids that are distributed through the parasite tissue for protein anabolism. In other parasites, namely the malaria agent, *Plasmodium spp.*, the hookworms such as *N. americanus* and *A. caninum* and the trematodes *Schistosoma spp.*, aspartyl proteases are thought to initiate digestion by cleaving the globin backbone with subsequent unravelling of tertiary structures which exposes regions of the peptide backbone which can then be cleaved by other endopeptidases (Williamson *et al.*, 2003). A protease, active at pH 3, was detected in a PBS extract of *F. hepatica* and then isolated and partially purified (Rupova and Keilova, 1979). The protease cleaved denatured haemoglobin and proteolytic activity could be inhibited by Pepstatin A. The authors proposed that the protease probably played an important role in helminth nutrition. Despite its potential importance, this protease(s) has not been investigated since. Here the proteolytic properties of the PBS-soluble, membrane-associated and membrane-bound fractions of adult fluke were examined with a particular emphasis on haemoglobin digestion. These proteases may be pivotal in nutrient acquisition, thus making them potential vaccine candidates.

There are a number of assays available to characterise enzymatic activity but these often require purified or, at least, simplified extracts. The extracts (S1, S2 and S3) described previously, are complex mixtures of proteins as evidenced by the large number of proteins identified by the proteomics approach described in Chapter 2 and Appendix 2. Substrate gel analysis has been used successfully to characterise proteolytic activity in similar extracts from the sheep scab mite, *Psoroptes ovis* (Kenyon and Knox, 2002), the cattle nematode, *Ostertagia ostertagi* (Geldhof *et al*., 2000), the blood-feeding nematode, *H. contortus* (Knox *et al.*, 1993) and in *F. hepatica* E/S (Dalton and Heffernan, 1989). This technique involves separating the respective protein fractions by SDS-PAGE on gel matrices containing a substrate of interest, such as gelatin. The separated fractions can then be incubated in a range of buffers to ascertain the pH range of active proteases and an approximate molecular weight.

Substrate degradation can also be measured by reading a change in absorbance at 280 nm. This gives a readout for the extent of protein breakdown, as the higher the absorbance, the more short peptides and free amino acids (mainly tryptophan, tyrosine and phenylalanine) are

present. This technique has successfully been used to evaluate proteases in crude protein fractions from *P. ovis* (Nisbet and Billingsley, 2000). Furthermore, proteases cleave between specific residues depending on their functional class. Therefore, synthetic substrates containing a fluorescent tag, which is liberated when the substrate is cleaved between specific residues can be used to evaluate the presence of certain proteases within a crude fraction over a range of pH conditions. The amino acid composition of the substrate can vary, depending on the target enzymes which cleave at different sites.

In this chapter, the three fractions S1, S2 and S3 were investigated to help identify which active proteases are present. Subsequent analysis of their ability to digest haemoglobin in the presence of class-specific inhibitors was used to identify which classes of proteases are involved and highlight those of interest for further study.

4.2 Materials and methods

4.2.1 Somatic fractions

PBS-soluble (S1), membrane-associated (S2) and membrane-bound (S3) fractions were prepared from adult *F. hepatica* exactly as described in 2.2.1.2. The protein concentration of the S1, S2 and S3 was measured as described in 2.2.1.3.

4.2.2 Analysis of proteolytic activity with substrate gels

4.2.2.1 Preparation of SDS-PAGE slabs with protein substrates

Resolving gel solutions were prepared as follows;

4% Acrylamide: 2.91 ml of dH_2O, 1.2 ml of 1.5 M Tris-HCl resolving buffer with SDS at pH 8.8, 0.64 ml acrylamide (30 % solution).

12% Acrylamide: 1.63 ml of dH_2O, 1.2 ml of 1.5 M Tris-HCl resolving buffer with SDS at pH 8.8, 1.92 ml acrylamide (30 % solution).

These volumes of reagents were measured into 50 ml Falcon tubes (Greiner Bio-one CellStar) before adding 5 mg of protein substrate and allowing this to dissolve. Finally, 50 µl ammonium persulphate (10% w/v) and 5 µl TEMED (*N, N, N, N'*-tetramethylethylenediamine) were added to each. These were then dispensed between two glass plates with the aid of a gradient mixer and pump to ensure a continuous gradient of decreasing acrylamide concentration. The mixture was overlaid with 100 µl of isopropanol to disrupt any air bubbles and flatten the gel top. The gel was left to polymerise for 30 min at room temperature. The isopropanol was then removed using blotting paper and the stacking gel prepared as follows;

4% Stacking gel : 5.703 mls of dH_2O, 2.35 mls of 0.5 M Tris-HCl stacking buffer with SDS at pH 6.8, 1.253 ml acrylamide (30 % solution), 50 µl ammonium persulphate (10% w/v), 5 µl TEMED

As before, these reagents were measured into a Falcon tube, adding the ammonium persulphate and TEMED last. This was then carefully poured on top of the resolving gel to the

edge of the glass plates and the gel comb inserted gently to avoid the incorporation of air bubbles. Any spillages were carefully removed and the gels allowed to polymerise for 30 min at room temperature. Gels were used immediately or wrapped in damp tissue paper and stored at 4 °C for up to 1 week.

4.2.2.2 SDS-PAGE for substrate gel analysis

Protease activity in the three fractions (see Section 4.1.1) was determined by analysis on protein substrate gels. Gelatin is a non-specific substrate which can be broken down by all classes of proteolytic enzymes including serine, cysteine, aspartyl and metalloproteases. Therefore, gelatin was used to ascertain the presence of any proteolytic enzymes. Haemoglobin was subsequently used as a substrate as the presence of proteins which can digest haemoglobin are of particular interest in a putative blood-feeding parasite.

4µg of each of the three fractions S1, S2 and S3 were mixed with non-reducing sample buffer, see Appendix 1, and fractionated in 4 – 12 % SDS-PAGE gel slabs containing 0.1 % (w/v) substrate. The protein substrates used were gelatin and ovine haemoglobin (Sigma-Aldrich). Gels were electrophoresed at 200 V for 55 min in a Mini Protean® II Dual Slab Cell (BioRad, UK) in chilled SDS-PAGE substrate gel tank buffer, see Appendix 1. The buffer was chilled to ensure the proteases were not denatured by heat generated during electrophoresis.

Following electrophoresis, gels were washed 3 times for 20 min in 2.5% (w/v) Triton X-100 to remove the SDS from the gels. The gel slabs were then sub-divided and incubated in 0.1 M buffer at a range of pH values [pH 3–5, sodium acetate with and without the addition of 5 mM dithiothreitol (DTT); pH 7-9 Tris-HCl] for 16 h at 37 °C. DTT is known to activate cysteine proteases, which are generally active at acidic pH levels, so was not added to reactions at pH 7-9. After incubation, gels were stained with 0.25% Coomassie Blue and destained with Destainer, see Appendix 1, at room temperature. Clear/unstained zones of proteolysis were visualised using a transilluminator.

4.2.3 SDS-PAGE determination of the digestion of ovine haemoglobin

A stock solution of ovine haemoglobin substrate was prepared at a concentration of 2 mg/ml in distilled water. The substrate (10 µl) was mixed with 4 µg (3 µl) of either the S1, S2 or S3

extract or 3 µl PBS as a control. 2 µl of either 0.2 M sodium acetate buffer (pH 3-6, Appendix 1) or 0.2 M Tris-HCl (pH 7-9, Appendix 1) along with 5 mM DTT were then added and the samples incubated for 16 h at 37 °C.

An equal volume of reducing sample buffer (Invitrogen, UK) was then added to the reaction, which was heated to 70 °C for 10 min. Samples were then dispensed into wells of a precast 4-12 % SDS-PAGE gel (Invitrogen, UK) and electrophoresis carried out at 200 V for 45 min, as described in Section 2.2.1.4. After electrophoresis, gels were rinsed 3 times for 10 min in dH_2O, then stained with SimplyBlue ™ SafeStain (Invitrogen, UK) for 2 hr at room temperature, then destained with dH_2O. Proteolysis of haemoglobin was determined by the presence or absence of the haemoglobin doublet (14-15 kDa) in comparison to the control.

4.2.4 Measuring haemoglobin digestion at 280 nm

A stock solution of ovine haemoglobin substrate was prepared at a concentration of 2 mg/ml in distilled water. The following pH buffers were prepared, as outlined in Appendix 1; 0.2 M glycine-HCl (pH 2-3.5), 0.2 M citrate-buffer (pH 4-5), 0.2 M phosphate buffer (pH 6-7) and 0.2 M Tris-HCl (pH 8-9). The protein concentration of the *F. hepatica* extracts was adjusted to 1 mg/ml.

In a 1.5 ml Eppendorf tube, 175 µl of pH buffer and 50 µl of haemoglobin substrate were combined. Finally, 25 µl of extract were added (or dH_2O in the control), briefly vortexed and incubated at 37°C for 5 hours. Reactions were performed in triplicate. Reactions were terminated by the addition of 750 µl 15% trichloroacetic acid (TCA) at 4 °C and placed on ice for 30 min. These were then centrifuged at 18, 000 x *g* for 10 min and the absorbance of the supernatant at 280 nm was measured.

4.2.5 Digestion of cathepsin D-specific peptide by *F. hepatica* extracts

The aspartyl protease, cathepsin D, preferentially cleaves between hydrophobic amino acids such as phenylalanine (phe) residues. The fluorescently labelled peptide substrate, 7-methoxycourin-4-acetyl-gly-lys-pro-ile-leu-phe-phe-arg-leu-lys(DNP)-D-arg-amide (CatDFS), was used to investigate the presence of a cathepsin D-like protease in the three extracts. Cleavage between the two phe residues by a cathepsin D will cause release of the fluorescent

signal, which is detected using fluoremetry. This was used to calculate the turnover rate of the peptide at a variety of pH values as follows:

In a 10 mm black masking fluorometer cuvette, 2 µl of CatDFS in dH_2O (50 µM) were mixed with 97 µl of buffer (0.1 M Glycine-HCl, pH 2-3.5 and 0.1 M citrate-phosphate, pH 3–6.5). The background absorbance was used to blank the fluorometer and 1 µl of extract (1 mg/ml) was added to the mixture and a fluorescence reading initiated. Absorbance readings were taken every 5 sec for 150 sec, at excitation wavelength 330 nm and emission wavelength 390 nm. Each combination of extract/pH was performed in triplicate.

The fluorescence emission readings were plotted over time. From the linear period of the reaction, the turnover rate of the peptide substrate was calculated where:

$$\text{Turnover rate} = (X_1 - X_0) / (T1 - T_0)$$

where, X is the fluorescence reading at the start (0) and end (1) of a linear period, and T is the time (sec) at the start (0) and end (1) of the reaction.

The higher the turnover rate, the more efficiently the putative cathepsin D is working. This can be used to determine if a cathepsin D-like enzyme is present in the fractions and the optimum pH at which it functions.

4.2.6 Class-specific Proteolytic Inhibition

Class-indicative protease inhibitors were used to ascertain which enzyme classes were active in the fractions. These inhibitors were as follows; L- transepoxysuccinyl-leucylamido-[4-guanidino] butane (E64), a cysteine protease inhibitor (10 µM); Pepstatin A, an aspartyl protease inhibitor (10 µM). These were added to the respective S1, S2 and S3 protein extracts and incubated for a minimum of 1 hour at room temperature prior to incubation with substrate (gelatin substrate gel, ovine haemoglobin) and the appropriate pH buffer during reactions.

A two sample T-test was used on the quantitative readouts of these assays to establish if the class-specific inhibitors significantly reduced the activity of the extracts in comparison to the control.

4.3 Results

4.3.1 Substrate gel analysis of protease activity

Enzyme activity from each extract was characterised using gelatin substrate gel electrophoresis. Activity was evident in the S1 and S2 extracts at pH 3, 5, 7 and 9 and in the S3 at pH 3 and 5. The addition of 5mM DTT at pH3 and 5 served to enhance proteolysis to varying degrees in S1, S2 and S3, as shown in Figure 4.1.

Proteolytic activity from enzymes in the PBS-soluble (S1) and membrane-associated (S2) fractions were similar in respect to the areas of proteolysis visible across the pH range tested. Proteolysis was highest at pH 5 following the addition of DTT, where hydrolysis of substrate occurred in a zone covering 36 – 148 kDa size range. At pH 3, proteolysis of gelatin was evident at 36, 50-55 and 80-100 kDa. Gelatin proteolysis at pH 7 was evident at 36 and 50-64 kDa. Finally, at pH 9, gelatin proteolysis was evident at 36 and 50-64 kDa. Proteolysis by the membrane-bound (S3) fraction was more restricted than that of S1 and S2 being observed only at pH 3 and pH 5. As with the S1 and S2 extracts, most proteolysis in the S3 fraction was evident at pH 5 following the addition of 5 mM DTT, where two areas at 36 and 50 kDa were evident. At pH 3, proteolysis was evident at 50 and 105 kDa and was enhanced by the addition of DTT.

The addition of the class-specific protease inhibitors was used to ascertain which class(es) of proteases was present and, thus, responsible for proteolysis. Here, the addition of two inhibitors to block either cysteine (E64) or aspartyl (Pepstatin A) proteases was investigated. The addition of E64 reduced hydrolysis at pH 3 (Figure 4.2), pH 5, pH 7 and pH 9 (the latter summarised in Table 4.1) in all fractions with this inhibition being complete at all pHs and in the presence or absence of DTT. At pH 3, proteolysis was enhanced by the presence of Pepstatin A, both in the presence and absence of DTT, Figure 4.2 and Figure 4.3. This effect remained evident at higher pH values although the degree diminished markedly (data summarised in Table 4.1).

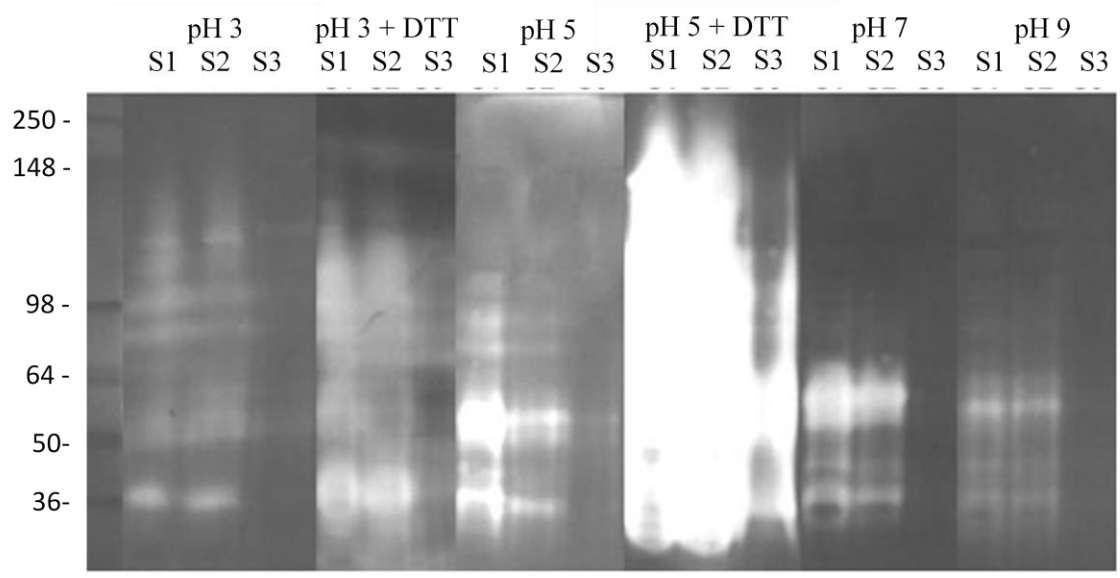

Figure 4.1: Protease activity of the *F. hepatica* PBS-soluble (S1), membrane-associated (S2) and membrane-bound (S3) extracts against the substrate gelatin at pH 3, pH 5, pH 7 and pH 9. Buffers used were 0.1 M sodium acetate (pH 3, pH 5) with and without the addition of 5 mM dithiothreitol (DTT) and 0.1 M Tris-HCl (pH 7, pH 9). Areas of white against the stained background indicate regions of proteolysis of the substrate, and thus the presence of active proteases.

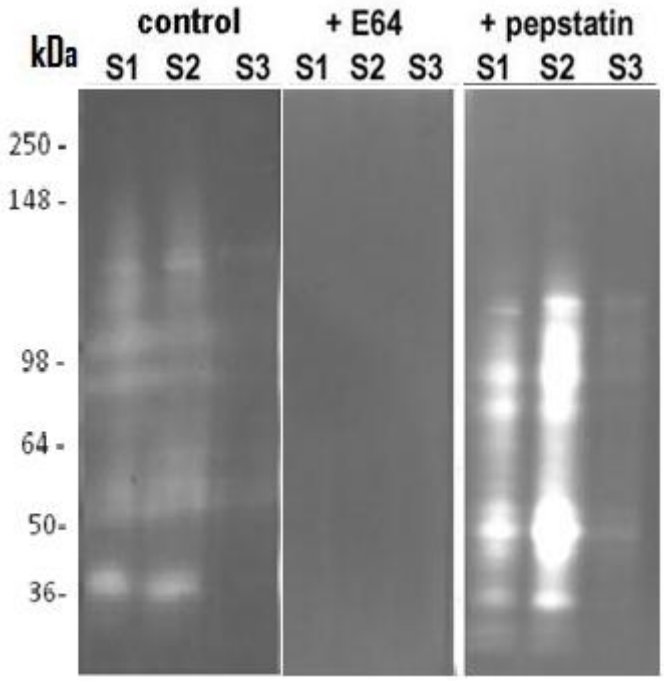

Figure 4.2: Protease activity of the *F. hepatica* extracts against gelatin at pH 3 with the addition of class-specific inhibitors. The control panel shows proteolysis in the respective fractions at pH 3 (0.1 M sodium acetate) in the absence of any class-specific inhibitors. The class specific inhibitors, E64 and Pepstatin A, were used at a concentration of 10 µM.

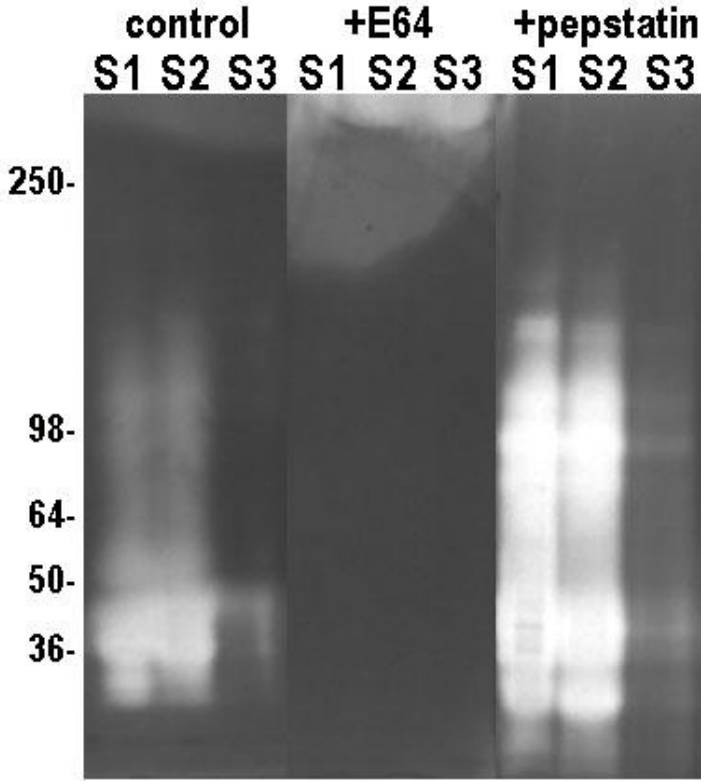

Figure 4.3: Protease activity of the *F. hepatica* extracts against gelatin at pH 3 in the presence of 5mM DTT. The control panel shows proteolysis at pH 3 with 5 mM DTT (0.1 M sodium acetate) in the absence of any class-specific inhibitors. The class specific inhibitors, E64 and Pepstatin A, were used at a concentration of 10 µM

Table 4.1: Summary of substrate gel analysis of gelatin proteolysis by soluble (S1), membrane-associated (S2) and membrane-bound (S3) extracts of *F. hepatica* at pH 3-9. 5 mM DTT was added at pH 3 and 5 (+) as it is known to enhance cysteine protease activity at acidic pHs. Low, moderate and high levels of proteolysis are indicated by +, ++, +++ respectively, and the areas where activity is evident noted in kDa. The class-specific inhibitors E64 (10 µM) and Pepstatin A (10 µM) were used to inhibit the action of cysteine and aspartyl proteases respectively.

	Size range of proteolysis (kDa)					
pH	3	3	5	5	7	9
DTT (+/-)	-	+	-	+	-	-
S1	++ (36, 50-55, 80-100)	++ (36, 50-55, 80-100)	+++ (36, 50, 70 – 105)	+++ (36–148)	++(36, 50-64)	+ (36, 50-64)
S2	++ (36, 50-55, 80-100)	++ (36, 50-55, 80-100)	++ (36, 50, 70 – 105)	+++ (36–148)	++ (36, 50-64)	+ (36, 50-64)
S3	+ (50, 105)	++ (36-50,64-105)	+ (50)	+++ (36, 50-64)	-	-
S1 + E64	-	-	-	++ (36-105, 110-140)	-	-
S2 + E64	-	-	-	++ (36-105, 110-140)	-	-
S3 +E64	-	-	-	+++ (36-105, 110-140)	-	-
S1 + PepA	+++(36, 50-55, 64-105)	+++ (36, 50-55, 80-100)	++ (36, 50, 70 – 105)	++ (36-105, 110-140)	+ (30-55)	+ (50-64)
S2+ PepA	+++(36, 50-55, 64-105)	+++ (36, 50-55, 80-100)	++ (3-105)	++ (36-105, 110-140)	-	+ (50-64)
S3+ PepA	+(50, 98)	++ (36-50,64-105)	+ (3-50)	+ (50-64)	-	-

4.3.2 Haemoglobin substrate gel analysis

Enzymatic activity against ovine haemoglobin in each extract was characterised initially by substrate gel electrophoresis. The S1 extract contained proteases which could digest haemoglobin at pH3 and pH5, as shown in Figure 4.4. There was also evidence of weak proteolysis from the S2 extract which was most prominent at pH 3 (Figure 4.8) with the addition of DTT. The S3 extract did not appear to contain any proteases which were capable of digesting haemoglobin in-gel, as shown in Figure 4.4.

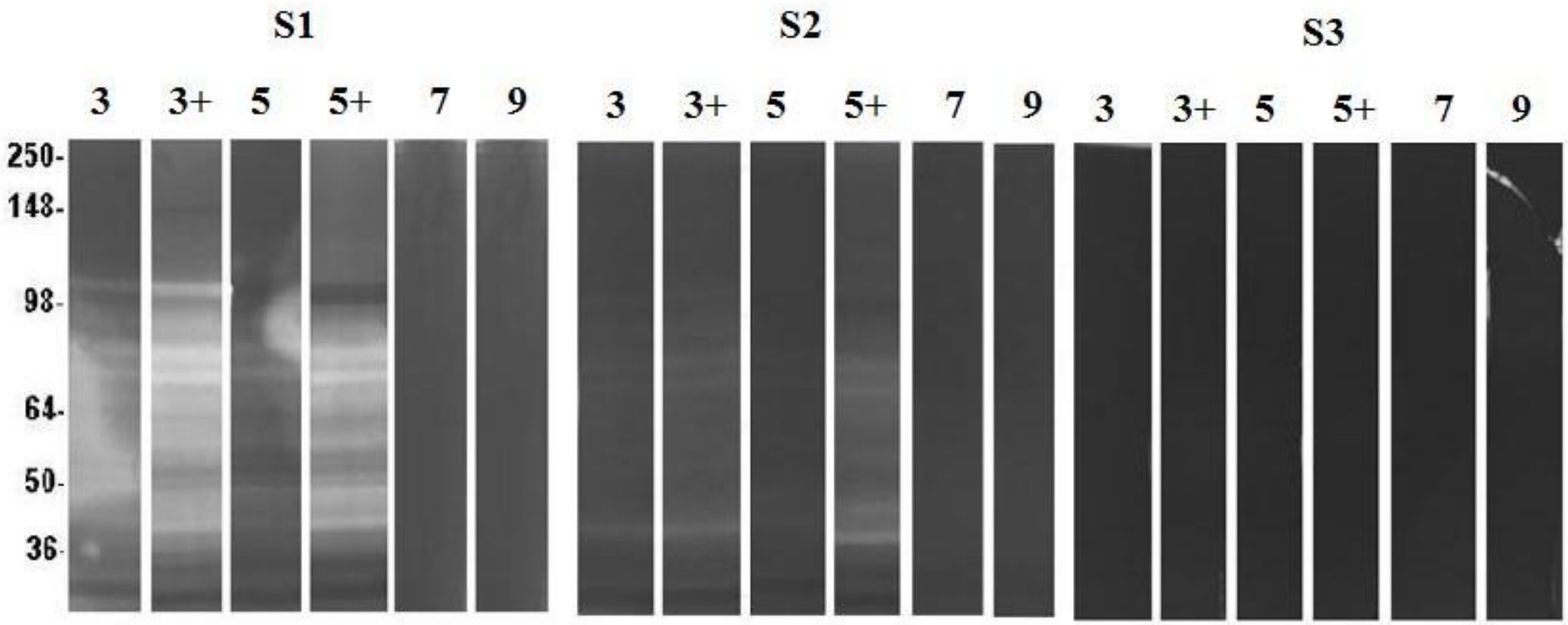

Figure 4.4: Substrate gel analysis to investigate the presence of proteases in the *F. hepatica* PBS-soluble (S1), membrane-associated (S2) and membrane-bound (S3) extracts capable of digesting ovine haemoglobin. Proteolysis was investigated at pH 3, pH 5, pH 7 and pH 9. The addition of 5 mM DTT (+) at pH 3 and pH 5 was also investigated.

4.3.3 SDS-PAGE analysis of haemoglobin digestion in solution

Substrate gels may not be the optimum method for investigating this process as the medium is static and, optimally, this process would occur in solution *in vivo*. Therefore, haemoglobin digestion was investigated using two further assays which would allow the enzymes present to interact with the substrate in solution. In addition, the active site region of aspartyl proteases is formed by two separate domains, each of which contributes a catalytic aspartate residue to the active site. This structure is disrupted by the SDS incorporated into a substrate gel and it is not always restored once the SDS is washed out (D.P. Knox, personal communication)

In order to investigate haemoglobin degradation under more favourable physiological conditions, it was decided to conduct the digestion in solution then visualise the breakdown of haemoglobin by SDS-PAGE. Haemoglobin digestion was determined by the breakdown of the haemoglobin doublet, which migrates at 14-16 kDa, digestion being apparent by the appearance of lower molecular weight peptides compared to the control (C). Results are shown in Figure 4.7 (S1), Figure 4.6 (S3).

Substrate digestion by the enzymes within the S1 (see Figure 4.5) and S2 (see Figure 4.6) fractions was apparent at pH 3, both with and without the addition of 5mM DTT, and also at pH 5 with the addition of 5mM DTT. There was no indication of degradation at pH7 or 9 or at pH 5 when DTT was absent. These results are consistent with those seen in the substrate gel analysis. Proteases within the S3 fraction also digested haemoglobin at pH3 and pH 5 in the presence of 5mM DTT. Although the addition of DTT enhanced proteolysis at pH 5, it did not appear to enhance it at pH 3, see Figure 4.6. In general terms, digestion due to the S3 fraction resulted in much more discrete breakdown products indicative of specific cleavage sites along the haemoglobin peptide backbone, particularly at pH 3.0 where there was an additional peptide present (see arrow, Figure 4.7)

The addition of inhibitors to the extracts produced equivocal results; these are displayed in Figure 4.7. The addition of E64 reduced proteolysis in all three extracts, however it did not completely inhibit degradation. The addition of Pepstatin A slightly reduced proteolysis, most notably at pH 5. It was difficult to determine the true extent of proteolysis and thus another assay was used to further investigate this.

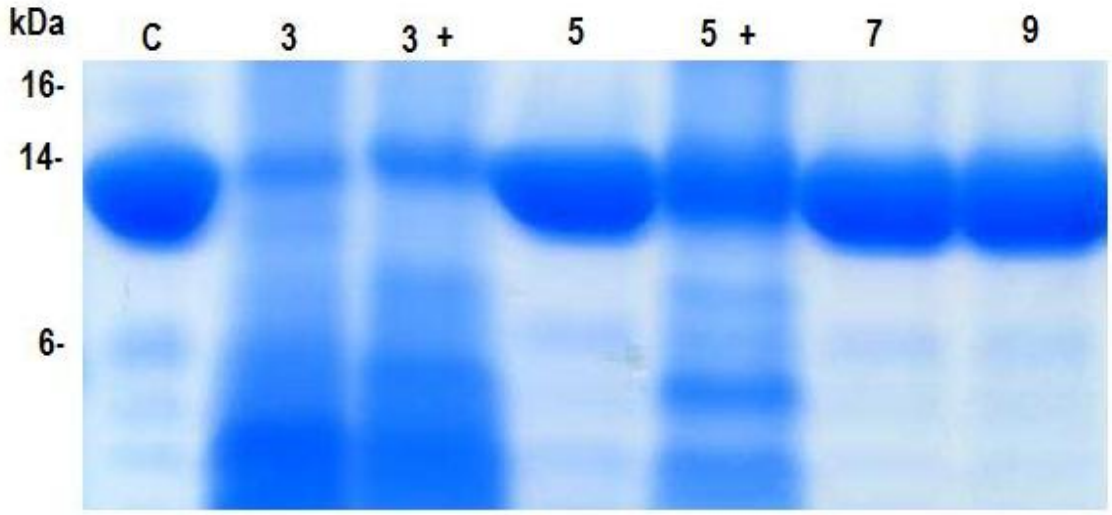

Figure 4.5: SDS-PAGE determination of ovine haemoglobin digestion by proteases within the PBS-soluble (S1) extract. This was investigated at pH 3, pH 5, pH 7 and pH 9. 5 mM DTT (+) was added at pH 3 and 5 as it is known to enhance cysteine protease activity at acidic pH. The haemoglobin doublet migrates at 14-15 kDa and is clearly visible, as a single broad band, in the control lane (C), where no S1 extract was present. Digestion was most prominent at pH3 with the haemoglobin doublet being clearly diminished. Almost identical results were obtained when the S2 extract was tested (data not shown)

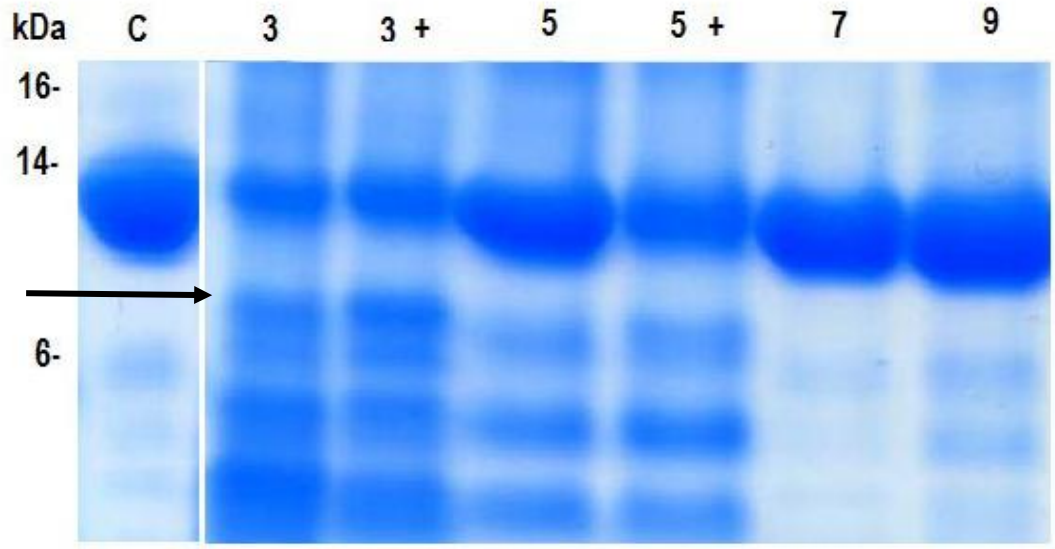

Figure 4.6: SDS-PAGE determination of ovine haemoglobin digestion by proteases within the membrane-bound (S3) extract. This was investigated at pH 3, pH 5, pH 7 and pH 9. 5 mM DTT (+) was added at pH 3 and 5 as it is known to enhance cysteine protease activity at acidic pH. The haemoglobin doublet migrates at 14-15 kDa and is clearly visible in the control lane (C), where no extract is present. Arrow indicates an additional peptide not present when haemoglobin was digested by the S1 and S2 extracts.

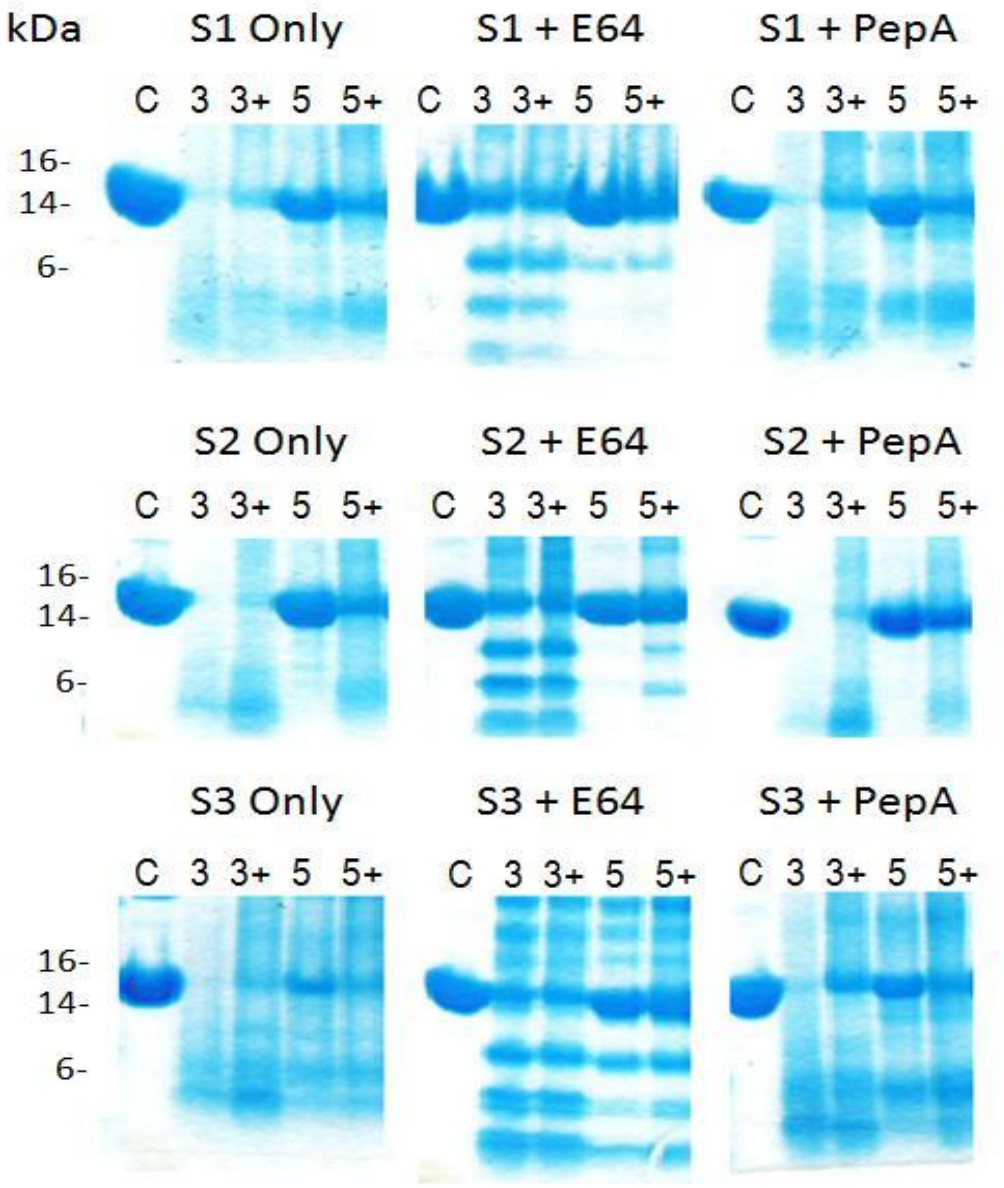

Figure 4.7: SDS-PAGE determination of ovine haemoglobin digestion by proteases within the respective *F. hepatica* extracts, PBS-soluble (S1), membrane-associated (S2) and membrane-bound (S3) at a range of pH, with/without the addition of class-specific inhibitors. Digestion was investigated at pH 3, pH 5, pH 7 and pH 9. 5 mM DTT (+) was added at pH 3 and pH 5, as it is known to enhance cysteine protease activity at acidic pHs. The haemoglobin doublet migrates at 14-15 kDa and is clearly visible in the control lane (C), where no *F. hepatica* extract was present. E64 specifically inhibits the function of cysteine proteases and Pepstatin A the function of aspartyl proteases, respectively.

4.3.4 Measurement of ovine haemoglobin digestion at 280 nm

To ascertain the degree of proteolysis within each fraction, the amount of solubilised protein was measured at 280 nm. This estimates the extent of protein breakdown, as the higher the absorbance, the more short peptides and free amino acids (mainly tryptophan, tyrosine and phenylalanine) are released. Haemoglobin digestion was only evident at acidic pH. The results are shown in Figure 4.8. The extracts follow a similar trend to each other in terms of digestion, showing two peaks of activity at pH 2.5 and pH 4, respectively. The highest degree of digestion was seen with the S1 and S2 extracts. Although digestion by S3 was less obvious, proteases capable of digesting ovine haemoglobin were clearly present.

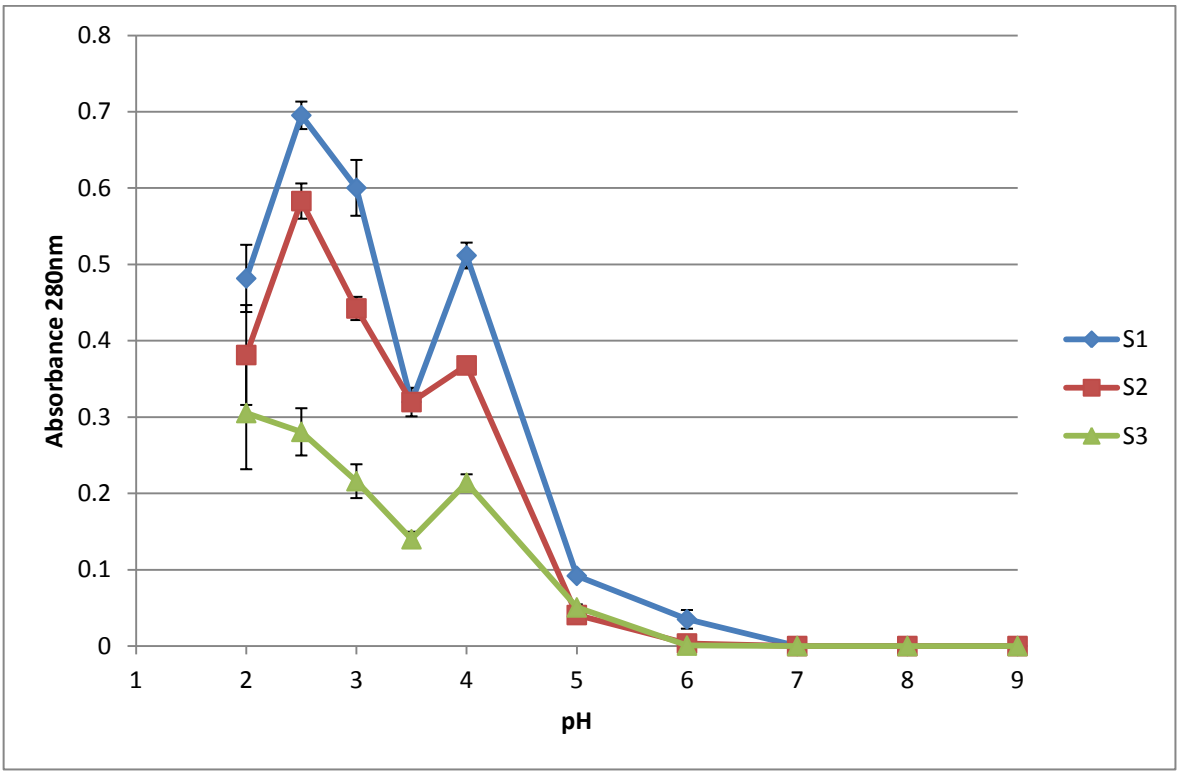

Figure 4.8: Digestion of ovine haemoglobin by the *F. hepatica* extracts, PBS-soluble (S1), membrane-associated (S2) and membrane-bound (S3), as determined by increased absorbance at 280nm over a range of pH. Buffers were used at the following concentrations; 0.2 M glycine-HCl (pH 2-3.5), 0.2 M citrate-buffer (pH 4-5), 0.2M phosphate buffer (pH 6-7) and 0.2 M tris-HCl (pH 8-9). The protein concentration of the extracts was 1 mg/ml. Protein hydrolysis (mean ± standard error) was measured at 280 nM using a negative control as the blank, which contained substrate and pH buffer but no *F. hepatica* extract (n =15).

4.3.5 Class-specific inhibition of proteases

To elucidate which classes of proteases were responsible for the digestion observed, the effects of two class-specific inhibitors were examined. The results for the extracts are shown in the following figures, Figure 4.8 (S1), Figure 4.9 (S2) and Figure 4.10 (S3).

The addition of E64 reduced hydrolysis by all extracts over pH 2-6, as summarised in Table 4.2. Focusing on pH 2-4, where haemoglobin hydrolysis was highest, the addition of E64 inhibited the hydrolysis of haemoglobin by all extracts from 82 - 100 %. Pepstatin A only had marked effects at pH 2 inhibiting proteolysis by all extracts, most notably by the S3.

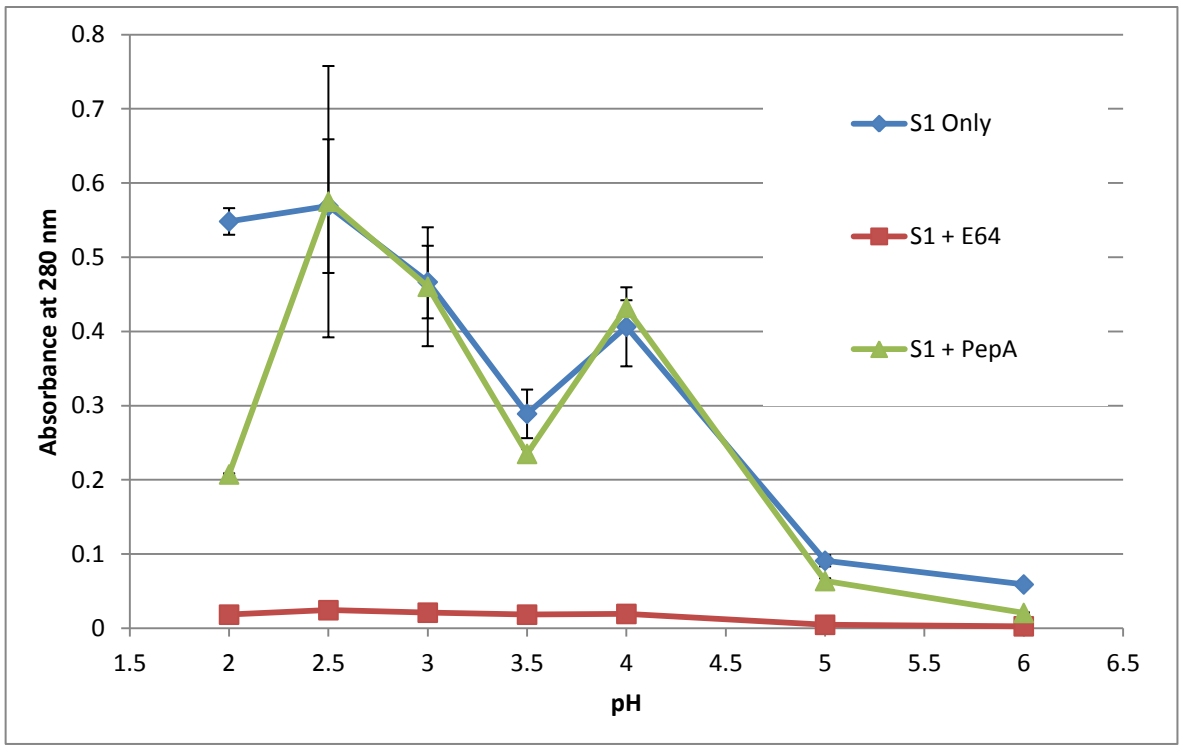

Figure 4.9: Digestion of ovine haemoglobin by the *F. hepatica* PBS-soluble (S1), extract as determined by absorbance at 280 nm over pH 2 - 6. The buffers, inhibitors and extracts used were as follows; 0.2 M glycine-HCl (pH 2-3.5), 0.2 M citrate-buffer (pH 4-5) and 0.2M phosphate buffer (pH 6). E64 (10 µM), pepstatin A (10µM), S1 (1 mg/ml). Protein hydrolysis (mean ± standard error) was measured at 280 nM using a negative control as the blank, which contained substrate and pH buffer but no extract.

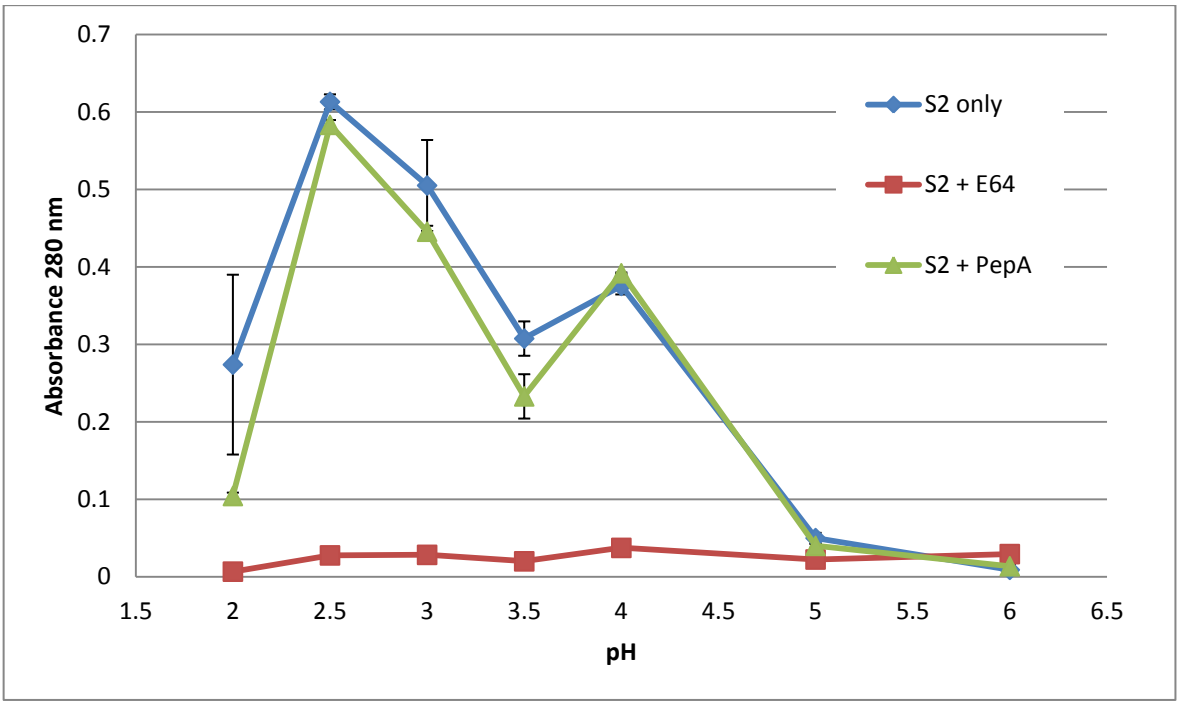

Figure 4.10: Digestion of ovine haemoglobin by the *F. hepatica* membrane-bound (S2), extract as determined by absorbance at 280 nm over pH 2 - 6. The buffers, inhibitors and extracts used were as follows; 0.2 M Glycine-HCl (pH 2-3.5), 0.2 M Citrate-buffer (pH 4-5) and 0.2M phosphate buffer (pH 6). E64 (10 µM), Pepstatin A (10µM), S2 (1 mg/ml). Protein hydrolysis (mean ± standard error) was measured at 280 nM using a negative control as the blank, which contained substrate and pH buffer but no extract.

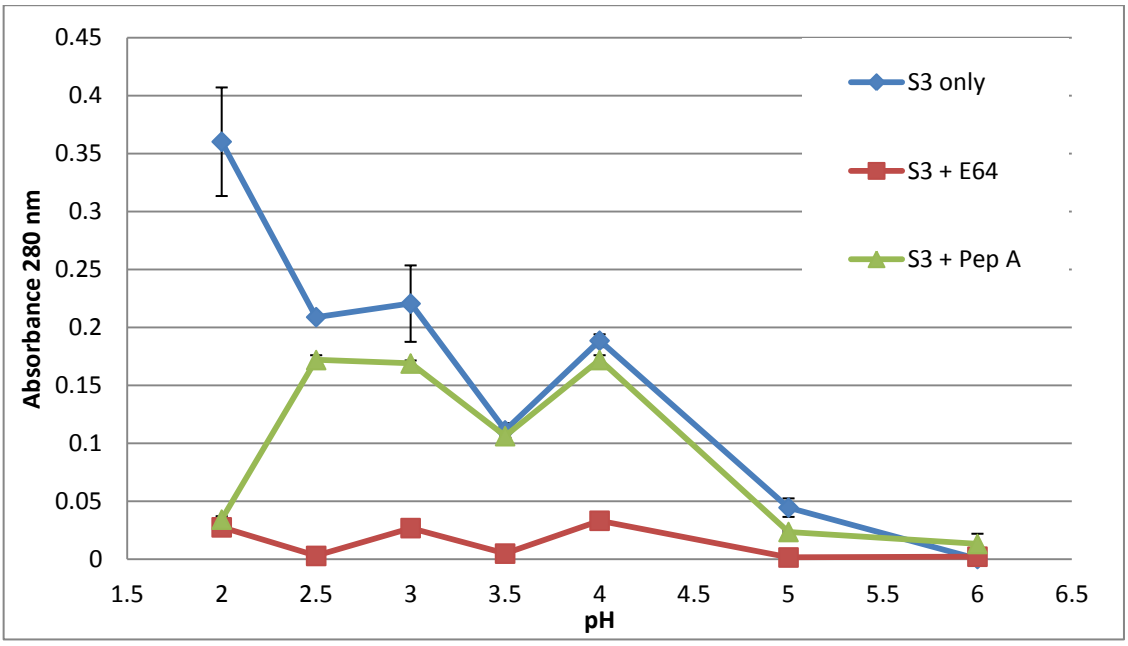

Figure 4.11: Digestion of ovine haemoglobin by the *F. hepatica* membrane-bound (S3), extract as determined by absorbance at 280 nm over pH 2 - 6. The buffers, inhibitors and extracts used were as follows; 0.2 M Glycine-HCl (pH 2-3.5), 0.2 M Citrate-buffer (pH 4-5) and 0.2M phosphate buffer (pH 6). E64 (10 µM), Pepstatin A (10µM), S3 (1 mg/ml). Protein hydrolysis (mean ± standard error) was measured at 280 nM using a control as the blank, which contained substrate and pH buffer but no extract.

Table 4.2: The percentage reduction in digestion of ovine haemoglobin with the addition of class-specific inhibitors by the *F. hepatica* S1, S2 and S3 extracts as determined by absorbance at 280 nm over pH 2 - 6. The buffers, inhibitors and extracts used were as follows; 0.2 M Glycine-HCl (pH 2-3.5), 0.2 M Citrate-buffer (pH 4-5) and 0.2M phosphate buffer (pH 6). E64 (10 µM), Pepstatin A (10µM) 1,10-phenathroline (1 mM), 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (1mM), S3 (1 mg/ml). Protein hydrolysis was measured at 280nM using a control reaction as the blank, which contained substrate and pH buffer but no extract.

	Percentage reduction of haemoglobin hydrolysis (estimated by change in absorbance at 280 nm)					
	S1		**S2**		**S3**	
pH	**E64**	**PepA**	**E64**	**PepA**	**E64**	**PepA**
2	96.66	62.19	97.57	61.92	92.32	90.47
2.5	95.72	0.00	95.49	4.84	98.56	17.70
3	95.50	1.36	94.39	11.81	87.76	23.41
3.5	93.66	18.69	93.50	24.27	95.51	4.49
4	95.24	0.00	90.06	0.00	82.33	8.83
5	94.87	30.04	55.33	20.00	96.27	47.01
6	95.48	64.97	100.00	0.00	100.00	0

4.3.6 Aspartyl protease-specific substrate

The presence of a cathepsin D-like enzyme was confirmed by the ability of all extracts to cleave CatDFS. The average peptide turnover rate for each extract at pH 2 – 6 is shown in Figure 4.12. Two buffers, which overlapped at pH 3, 3.5 (citrate and glycine-HCl) were used in the experiment to eliminate the possibility that either was having a direct role in the cleavage of the CatDFS peptide. As shown in Figure 4.21 the different buffers did not affect the cleavage of the substrate. The turnover rate of the peptide was greatest at pH 3 for S1/S2 and pH 2 for the S3 extract.

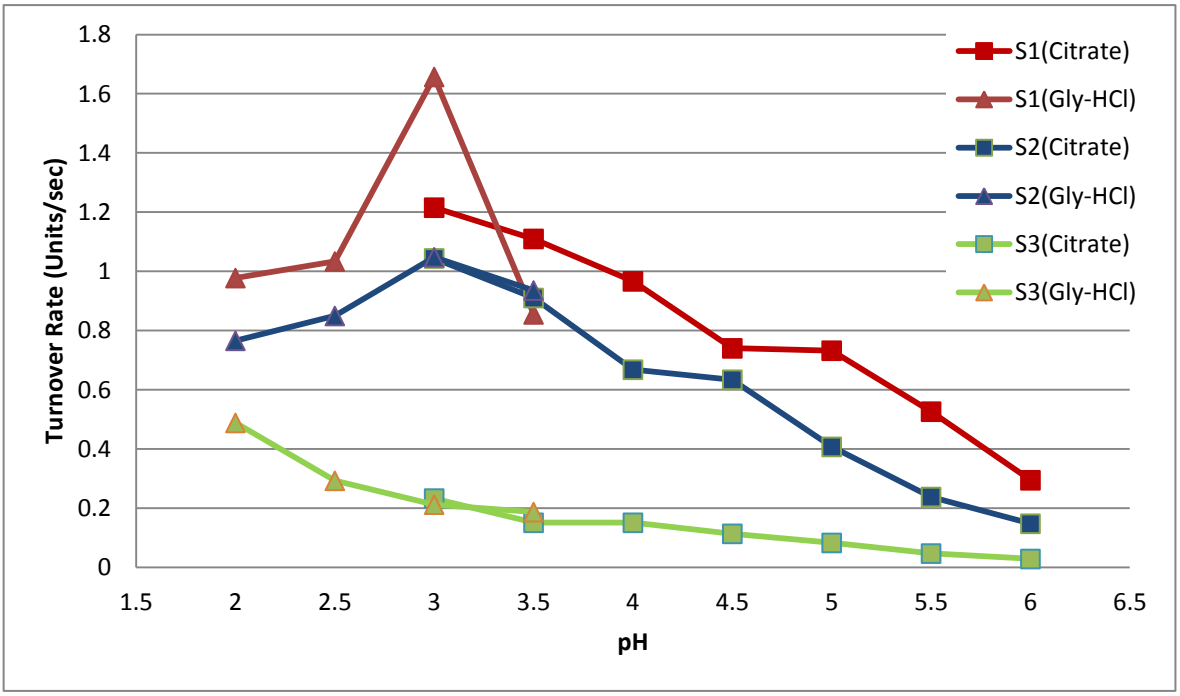

Figure 4.12: Average turnover rate of the cathepsin D-specific peptide 7-methoxycourin-4-acetyl-gly-lys-pro-Ile-leu-phe-arg-leu-lys(DNP)-D-arg-amide (CatDFS) by the *F. hepatica* PBS-soluble (S1), membrane-associated (S2) and membrane-bound (S3) extracts at a range of pH. Substrate, buffer and extracts were as follows; CatDFS (50 µM), S1 (1 mg/ml), S2 (1 mg/ml), S3 (1 mg/ml), Citrate buffer pH 3-6 (0.1 M) (shown by square markers), Glycine-HCl pH 2-3.5 (0.1 M) (Shown by triangle markers). Emission was measured every 5 sec for 150 sec, at excitation wavelength 330 nm and emission wavelength 390 nm to calculate the average turnover rate (± standard error).

4.4 Discussion

Protease activity was evident in all three *F. hepatica* fractions against the broad-spectrum substrate gelatin and also against ovine haemoglobin. Haemoglobin is likely to be part of the parasite's diet (Smyth and Halton, 1983). Class-specific inhibitors also indicated that cysteine and aspartyl proteases had some role in haemoglobin digestion within each of the three fractions.

Initially, the presence of active proteases was ascertained using gelatin substrate gel electrophoresis at a range of pH from pH 3 to 9. The addition of 5 mM DTT (a cysteine protease activator) enhanced hydrolysis indicating the presence of cysteine proteases, which is consistent with results from Chapter 2, where such enzymes were identified in the extracts by proteomics (see Appendix 2). Cysteine proteases are dominant components of the ES products of *F. hepatica* (Dowd *et al.,* 1994) and have also been shown to be capable of digesting host haemoglobin (Tort *et al.,* 1999). The addition of E64 and the apparent complete inhibition of substrate hydrolysis could indicate that cysteine proteases play a key role in digestion, possibly also initiating hydrolysis or activating other proteases. The addition of Pepstatin A did not cause such a profound reduction and, in some instances, actually increased hydrolysis of gelatin. A similar phenomenon was observed following the addition of EDTA and E64 to a Triton X-100 soluble mite extract from the ectoparasite, *Psoroptes cuniculi*, which resulted in increased hydrolysis of an aspartyl protease substrate (Nisbet & Billingsley, 1999). In the study presented here, aspartyl proteases present within liver fluke extracts may act to regulate other enzymes, thus their inhibition may serve to allow other proteases to act optimally. Furthermore, aspartyl proteases have been proposed to initiate haemoglobin hydrolysis in blood feeding parasites such as *H. contortus, N. americanus* and *A. caninum* (Williamson *et al.,* 2003).

The digestion of haemoglobin by liver fluke extracts was investigated using several different assays. Substrate gel analysis was carried out initially to ascertain the presence of enzymes capable of haemoglobin hydrolysis. Proteolysis within the S1 fraction was evident at pH 3 and pH 5 and the addition of 5 mM DTT enhanced hydrolysis in both cases. Proteolysis within the S2 fraction was evident at pH 3 and the addition of DTT enhanced proteolysis at pH 3 and pH 5. There was no evidence of haemoglobin digestion within the S3 fraction. Within *F. hepatica*, cysteine proteases have been shown to be capable of digesting host haemoglobin (Tort *et al.,* 1999). Here, cysteine proteases are present, as evidenced by the class-specific inhibition and the increase in hydrolysis

following the addition of DTT. Although there was evidence of in-gel digestion of haemoglobin substrate by S1 and S2, this was not observed in the S3. Therefore, the digestion of haemoglobin by the S3 fraction was further investigated by visualising the breakdown of the haemoglobin doublet using SDS-PAGE and further measuring hydrolysis by the change in absorbance at 280 nm over a range of pH, in an attempt to ascertain whether such proteases were active in the S3 fraction.

Haemoglobin hydrolysis in solution was investigated by visualising its breakdown using SDS-PAGE analysis. Hydrolysis was evident in S1 and S2 at acidic pH, which was consistent with the initial investigations using haemoglobin substrate gels. In addition, this analysis revealed that the S3 fraction also contained proteases capable of breaking down haemoglobin. At pH 3, in the S3 fraction there was an additional breakdown peptide which was not evident in S1 and S2 extracts. This peptide also disappeared following the addition of pepstatin A (see pH 3 Figure 4.6 and Figure 4.7 respectively), indicating that it could be the result of aspartyl protease activity. The addition of class-specific inhibitors, E64 and pepstatin A, indicated that cysteine and aspartyl proteases were involved, at least in part, in the digestion of haemoglobin. Inhibition with E64 reduced hydrolysis of haemoglobin by the S1, S2 and S3 fractions. The addition of pepstatin A increased hydrolysis by the S1 extract and decreased hydrolysis of the S3 fraction. As described before, a similar phenomenon was seen following the class-specific inhibition of a Triton X-100 extract from *P. cuniculi* (Nisbet & Billingsley, 1999). This highlights the complexity of the breakdown of haemoglobin and indicates that a number of enzyme classes may each have a role in hydrolysis and may also regulate the action of each other. Haemoglobin is hydrolysed by the action of a cascade of enzymes in *P. falciparum* (Francis *et al.,* 1994) and *N. americanus* (Williamson *et al.,* 2004). The initial step of haemoglobin degradation occurs by aspartyl proteases such as the plasmepsins I and II in *P. falciparum* (Francis *et al.,* 1994) and APR-1 and APR-2 in *N. americanus*, where haemoglobin is cleaved at the hinge region causing it to unravel and allow subsequent enzyme classes to act (Francis *et al.,* 1994). Haemoglobin is further broken down by enzymes belonging to the cysteine protease class, which then allow metalloproteases and endopeptidases to complete hydrolysis (Williamson *et al.,* 2003).

As the results obtained here were subjective, haemoglobin hydrolysis was investigated further by estimating the degree of hydrolysed protein by a change in absorbance of reaction supernatant at 280 nm. The higher the absorbance, the more short peptides and

free amino acids are present (mainly tryptophan, tyrosine and phenylalanine). A more extensive analysis of pH effect and inhibitor interaction with the extracts was also conducted. The S1, S2 and S3 fractions followed similar digestion profiles. All extracts had 2 peaks of activity at pH 2.5 and at pH 4. Hydrolysis was greatest in S1 followed by S2 and, finally, S3. There was no digestion evident at alkaline pH. This is consistent with previous results within this chapter and from other studies investigating haemoglobin digestion by blood-feeding parasites such as *P. cuniculi* (Nisbet & Billingsley, 2000), *R. microplus* (Mendiola *et al.,* 1996), *Ixodes ricinus* (Horn *et al.,* 2009), *Schistosoma spp*, *Ancylostoma spp., N. americanus* and *H. contortus* (Williamson *et al*., 2003). A number of protease classes have been proposed to function in the digestion of haemoglobin in blood-feeding parasites (Williamson *et al.,* 2003; Horn *et al.,* 2009). The addition of class-specific inhibitors in the work presented herein indicated that cysteine and aspartyl peptidases both had a role in the hydrolysis of haemoglobin by liver fluke extracts.

Proteolytic enzymes have been proposed to play a major role in the biology of *F. hepatica*, facilitating tissue invasion, migration (Newport *et al.,* 1988), immune evasion and nutrient acquisition (Brindley *et al.,* 1997; Brady *et al.,* 1999) while in the definitive host. The major proteins in the adult E/S products have also been identified as cysteine proteases (Dalton and Heffernan, 1989). Here, the presence of cysteine proteases in the S1, S2 and S3 extracts of adult flukes has also been demonstrated. A number of cysteine proteases have been identified from the parasite and listed on MEROPS (Peptidase database) (Rawlings *et al.,* 2012), including cathepsin L1, cathepsin L2 (Dowd *et al.,* 1994; Dowd *et al.,* 1997; Smith *et al.,* 1993), cathepsin L3 (Hamsen *et al.,* 2004) and cathepsin B (Wilson *et al.,* 1998). The zones of enzyme activity seen here by substrate gel analysis correspond in approximate molecular weight to previous findings (Smith *et al.,* 1993). Cathepsin L1 and L2 migrate at ~27 kDa, however, under non-reducing conditions, they migrate as multiple bands, each with enzymatic activity over 60 – 90 kDa and over a large pH range of 3-8 (Smith *et al.,* 1993), which was evident here. Furthermore, the presence of these enzymes was unequivocally demonstrated by peptides representing these proteases were identified by the proteomics approach described in Chapter 2 (also see Appendix 2).

A number of assays was employed here in an attempt to establish which classes of proteases were present within the fractions and capable of digesting haemoglobin. Substrate gel analysis allows separation of such crude mixtures and visualisation of hydrolysis by the extract. Furthermore, an approximate molecular weight can be assigned

to the proteases in question. However, the gel matrix medium is static and unlike that of the natural situation where the proteases substrate would be in solution. Although the results were convincing for gelatin digestion, the haemoglobin substrate proved to be difficult to visualise breakdown. Thus, a different method for visualising haemoglobin hydrolysis was used, which involved SDS-PAGE and UV absorption as a means of measuring lysis. This identified proteolytic activity within all fractions tested, S1, S2 and S3, against the haemoglobin substrate.

Aspartyl proteases have been proposed to act in the initial stages of haemoglobin digestion in haematophagous parasites (Williamson *et al.,* 2003). Cathepsin D, an aspartyl protease, was identified unequivocally in the PNA-enriched S3 fraction through LC-ESI-MS/MS analysis but was not identified in the crude extract; see Chapters 4 and 2, respectively. Following demonstration of a reduction of haemoglobin digestion with Pepstatin A (see Figure 4.11), the presence of a putative cathepsin D in the extracts (S1, S2 and S3) examined herein was confirmed by the ability of each of the extracts to hydrolyse a cathepsin D-specific substrate, CatDFS (see Figure 4.12) with greatest hydrolytic activity at pH 2-2.5. Cathepsin D has been identified in a number of blood-feeding parasites and when used as a vaccine elicited significant levels of protection in both native and recombinant forms. Mice immunised three times with recombinant aspartyl protease cathepsin D (Ac-APR-1) then subsequently challenged with *A. duodenale* had a 69 % reduction in the small intestine burden (Ghosh & Hotez, 1999; Williamson *et al.,* 2002; Williamson *et al.,* 2004). Also, vaccinated dogs were protected from developing anaemia, having haemoglobin levels within a normal range (12.45 grams/decilitre (g/dl)), compared with non-vaccinated dogs, which became clinically anaemic (9.5 g/dl), indicating immunisation disrupts the parasites ability to feed (Loukas *et al.,* 2005). Furthermore, vaccinated dogs has a 70 % reduction in worm burden in comparison to non-vaccinated controls (Loukas *et al.,* 2005). Mice immunised with the recombinant aspartyl protease, Ac-APR-1, also had a reduced intestinal burden of *A. caninum* (69 %) (Williamson *et al.,* 2002; Williamson *et al.,* 2004). Mice vaccinated with recombinant *S. japonicum* cathepsin D also have a reduction in worm burdens of 21-38 % but no notable reduction in fecundity despite a 20-40 % reduction in the number of female worms (Verity *et al.,* 2004).

The findings presented here highlight the complexity of the proteases contained within the three fractions generated from *F. hepatica* somatic extracts. Although proteases have been shown to digest haemoglobin, the precise ‘place’ of each enzyme within the cascade is

unknown. The extracts used here were crude mixtures of proteins, as shown in Appendix 2. Further fractionation would help to generate simpler fractions and thus establish optimal conditions for the functioning of the respective enzymes. However, within the gut there is likely to be a number of proteases being secreted concurrently, so there is also likely to be interaction between proteases in nature.

In conclusion, this chapter has extended studies of haemoglobin digestion is *F. hepatica* and indicates the classes of proteases capable of haemoglobin digestion which are present, at least in the adult stage of *F. hepatica*. The work presented here has identified the presence of a novel cathepsin D-like enzyme in the crude S3 extract which was not described in Chapter 2 but was enriched by the PNA lectin affinity chromatography approach described in Chapter 3. It is thought to function optimally below pH 2 and can digest ovine haemoglobin. Further investigation and characterisation of this protease will be carried out in the next chapter to determine its full-length amino acid sequence, to compare its sequence with those from related parasites and to localise its site of origin within the parasite. This should help establish the phylogenetic relationship of this *F. hepatica* cathepsin D to homologues from other species and help determine if it is expressed in or on the gut of the parasite. This latter investigation has implications for whether this cathepsin D is a hidden antigen or not and whether it could be vulnerable to the gut antigen approach to vaccination.

5 Characterisation of *F. hepatica* cathepsin D-like aspartyl protease

5.1 Introduction

Proteases play a vital role in processes crucial to the fluke's development and survival. These include nutrition (Brindley *et al.,* 1997; Brady *et al.,* 1999), invasion of host tissue (Newport *et al.,* 1988) and immunomodulation (Brophy *et al.,* 1990; Creaney *et al.,* 1995; Meuusen *et al.,* 1995). These proteases are, therefore potential vaccine candidates because they are proteins with a vital role in the parasite's survival (Smith and Smith, 1996; Wijffels *et al.,* 1994).

Haemoglobin is a potential food source for *F. hepatica* (Oslen, 1986) although how it is digested by the fluke is unclear. Haemoglobin digestion has been proposed to occur through the action of a cascade of enzymes within blood-feeding helminths, involving distinct classes of proteases in *Ancylostoma spp., N. americanus and H. contortus* (Williamson *et al*., 2003). Host haemoglobin is proposed to be cleaved initially by aspartyl proteases, then into smaller peptides by cysteine proteases and then further hydrolysed by metallopeptidases, acting in sequence. Finally, these peptides are broken down into amino acids by exopeptidases (Williamson *et al.,* 2003), Figure 5.1. Aspartyl proteases belong to the peptidase clan AA (Rawlings *et al.,* 2012, http://merops.sanger.ac.uk/index.htm). These proteases possess two catalytic aspartyl acid residues in their active sites which are highly conserved, where the sequence of nucleotides or amino acids has changed slightly or not at all during evolution (Williamson *et al.,* 2003). The A1 family of the clan AA contains the aspartyl protease, cathepsin D, which was enriched from the *F. hepatica* S3 extract by PNA lectin affinity chromatography, as described in Chapter 3. Cathepsin D is a lysosomal enzyme and has been identified in a number of protozoan and metazoan parasites, including *Plasmodium falciparum* (Banerjee *et al.,* 2002), schistosomes (Brindley *et al.,* 2001), *A. caninum* and *N. americanus* (Brown *et al.,* 1995). Furthermore, cathepsin D is involved in the digestion of host haemoglobin in *Plasmodium* (Francis *et al.,* 1997) and schistosomes (Brindley *et al.,* 2001). Optimal hydrolysis of haemoglobin by cathepsin D occurs in a host-specific manner as shown in the hookworms, where the cathepsin D from the canine hookworm can digest both canine and human haemoglobin but hydrolyses that of the canine host at a higher rate and vice-versa (Williamson *et al.,* 2002). The ability of recombinantly-derived versions of cathepsin D from the dog hookworm, and the human hookworm, to degrade host haemoglobin was studied by Williamson *et al* (2002), who

found these to be more efficient against their respective host haemoglobin substrate. Within the schistosomes (Brindley *et al.*, 2001), and the hookworms (Williamson *et al.*, 2001), cathepsin D is expressed by the gastrodermis of the adults. Localisation of the cathepsin D in histological sections of adult male and female schistosomes localised the expression to the gastrodermis of both sexes, but the expression was elevated in the females as indicated by more intense staining (Brindley *et al.*, 2001). Female schistosomes have a higher nutritional requirement than the males due to the demands of egg production. This demand, is proposed in part to be facilitated by the elevated expression of cathepsin D (Brindley *et al.*, 2001). In the hookworms, cathepsin D cleaves haemoglobin at the hinge region (between residue 33 (Phenylanlanine) and 34 (Leucine) on the alpha chain) which then allows the haemoglobin structure to 'unravel' and allow other proteases to act (Williamson *et al.*, 2002).

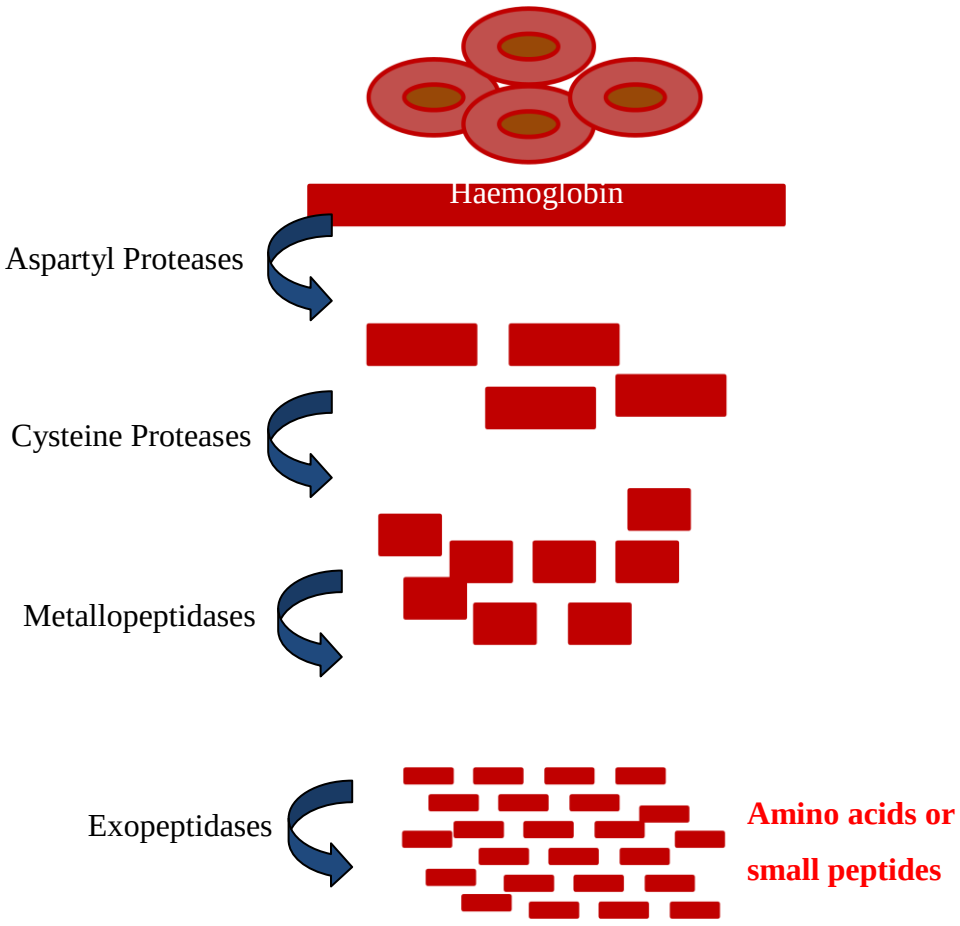

Figure 5.1: The proposed proteolytic cascade for haemoglobin digestion by blood-feeding nematodes (adapted from Williamson *et al.*, 2003)

One of the aims of this thesis attempted to identify proteins expressed on the surface of the gastrodermis which could be important for digestion but were not exposed to the host immune system during natural infection – equivalent to the "hidden" antigens of *H. contortus*. Work in the previous chapter provided evidence that a membrane protein extract from adult fluke had some aspartyl protease activity capable of contributing to haemoglobin digestion and unequivocal evidence of its presence by proteomic analysis of the PNA-enriched S3 fraction. In this chapter the *F. hepatica* cathepsin D, identified as a component of the peanut agglutinin lectin enriched S3 extract (Chapter 3), will be characterised in terms of its homology to published cathepsin D sequences by multiple sequence alignment (MSA) and phylogenetic analysis. Other characteristics of membrane proteins will be sought such as transmembrane anchors and glycosylation.

Glycosylation is a common post translational modification which occurs after the mRNA is translated into peptide sequence and the polypeptide has begun to fold (Hart, 1992). Glycosylation is important for protein folding and occurs as four types; N-linked, O-linked, C-mannosylation and glycophosphatidyl-inositol (GPI) anchor attachment (Hamby and Hirst, 2008). N-linked glycosylation is one of the most common and results from the addition of a glycan chain to an asparagine amino acid (Hamby and Hist, 2008). The sequence motifs required for N-glycosylation are N-x-S or N-x-T (where x is any amino acid except proline) (Blom *et al.*, 2004). In addition to this, identifying any possible transmembrane domains may help to elucidate whether this protein is stable within a membrane and could support the hypothesis that it is a membrane-bound protein as it was enriched from a membrane-bound *F. hepatica* (S3) extract using the PNA lectin (see Chapter 3). Antibodies raised against *F. hepatica* cathepsin D will be used to identify the site of expression within immature and adult stages of the parasite using immunolocalisation. The expression site is important in the context of a hidden antigen because it is vital that it is accessible to host antibodies, for example located in the gut region of the parasite. In addition, recognition, or lack of it, of the protein by sera from sheep which are naturally infected with *F. hepatica* will help to establish whether this enzyme is a genuine "hidden antigen" or is exposed to the host's immune response during the course of a natural infection. Finally, reverse transcription PCR will establish if transcripts encoding cathepsin D are present in the NEJ stages as well as adult stages of the parasite. This has implications for the range of stages susceptible if cathepsin D were to be used as a vaccine candidate. If cathepsin D is required by both NEJ and adult stages to

digest haemoglobin, antibodies raised towards cathepsin D may have a detrimental effect on the ability of both of these life stages to acquire nutrition.

5.2 Materials and Methods

5.2.1 Parasite material

Adult parasites were retrieved from the bile ducts of naturally infected sheep from ScotBeef Abattoir, Dunblane or Firth Mains, Penicuik. Parasites were washed two times with 1 x Phosphate buffered saline for 5 minutes and snap frozen in liquid N_2 in a cryovial and stored at -80°C until required.

5.2.2 Parasite extracts

Water-soluble (S1), membrane-associated (S2) and membrane-bound (S3) fractions were prepared from adult *F. hepatica* exactly as described in 2.2.1.2. The protein concentration of the S1, S2 and S3 was estimated as described in 2.2.1.3.

5.2.3 Trizol extraction of Total RNA from adult *F. hepatica*

Total RNA was extracted from flukes, stored as described in section 5.2.1, using Trizol reagent (Invitrogen), according to the manufacturer's recommendations. Briefly, parasite tissue was placed into a pre-chilled mortar and ground to a fine powder using a pestle, adding liquid nitrogen to keep it frozen. 2 ml Trizol was added per 100mg of parasite material and ground until a clear red colour. 1 ml aliquots were dispensed into sterile 1.5ml Eppendorf tubes and incubated at 15-30 °C for 5 minutes.

200µl chloroform were added to each aliquot then shaken vigorously for 15 seconds and incubated at room temperature for 10 minutes. These were then centrifuged at 12,000 x g for 15 minutes at 4°C. The mixture separates into an upper aqueous phase, an interphase and a lower red organic phase. The upper aqueous phase, which contains the RNA, was carefully removed and dispensed into a clean 1.5ml Eppendorf tube.

RNA was precipitated by the addition of 500 µl of isopropanol per tube which was inverted several times and incubated at room temperature for 10 minutes. Tubes were centrifuged at 12000 x g for 10 min at 4°C and the supernatant carefully discarded.

1ml of 75% ethanol was added per tube and vortexed and then centrifuged for 5 min at 7500 x g 4°C. The supernatant was discarded and the pellet briefly air dried. Each pellet was resuspended in 50 µl of RNase free dH_2O and heated to 60°C to dissolve, then pooled together. Two 3 µl aliquots were taken for gel electrophoresis and/or spectrophotometric analysis and the remainder frozen at -80°C.

5.2.4 RNA extraction form NEJ using RNAeasy®minikit

Approximately 150 metacercariae were excysted to NEJ by Ridgeway Research (kindly performed by Paula Martin) and preserved in 1 ml of RNAlater ® (Invitrogen) at room temperature for transportation, then stored at -80 ºC.

Total RNA was extracted using the RNAeasy ® minikit (Qiagen) protocol for animal tissues. Briefly, RNAlater ®-fixed tissue was removed from -80ºC storage and ground to a fine powder using a chilled mortar and pestle, adding liquid nitrogen to keep it frozen. 600 µl of buffer RLT were added and the mixture ground again. 600 µl aliquots were dispensed into clean 1.5 ml Eppendorf tubes and centrifuged at 18 000 x g for 3 min. An equal volume of 70% ethanol was added to the lysate and mixed. The mixture was then transferred to an RNAeasy spin column and centrifuged for 15 sec 8000 x *g*, which binds RNA to the spin column membrane. The flow-through was discarded and 500 µl of Buffer RPE added to the RNAeasy spin column and centrifuged 2 min 8000 x *g* to wash the RNA. The spin column was transferred to a new 1.5 ml Eppendorf tube and centrifuged at 18 000 x *g* for 1 min. The spin column was then placed into a 2 ml collection tube and 30 µl of RNAse free water pipetted onto the spin column membrane then centrifuged at 8000 x g for 1 min which elutes RNA from the membrane into collection tube.

5.2.5 RNA and PCR product analysis by gel electrophoresis and spectrophotometry

A 1% agarose gel was prepared by mixing 1g of agarose with 100 ml of 1 x TAE buffer (see Appendix 1) and heating until dissolved, then allowed to cool for 5 -10 min. 10 µl of 10,000 x Gel Red (Biotium) were added to the mixture and poured into an appropriate mould within a gel tank (Pharmacia Biotech GNA100). Once the gel was set, 1 x TAE buffer (see Appendix 1) was poured into the gel tank until the gel was submerged. 4 µl of RNA were added to 1 µl of 4 x DNA sample buffer (see Appendix 1) which were then loaded into one well of the 1% agarose gel alongside a molecular weight marker and separated by electrophoresis at 90V for ~30 min. The gel was removed from the gel tank and visualised under UV on a AlphaImager 2200 (Alpha Innotech). 4 µl of RNA were analysed using the Nanodrop®, as per manufacturer's instructions.

5.2.6 Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

The putative full-length coding sequence for *F. hepatica* cathepsin D was amplified by RT-PCR using the Superscript™One-step PCR kit (Invitrogen). First, forward and reverse

primers were designed for the 5' and 3' ends of the cathepsin D sequence deposited in the publically available GenBank database (accession number ABJ97285). The forward primer, 5'ATGAGACCGGTTCTCTTGATCT3' and reverse primer, 5'TTAAATCAAC AAATTCCAAAAGCTTA3' were produced by Eurofins (http://www.eurofins.co.uk/) and 10µM stock solutions made for each of these and stored at -20ºC.

On ice, a one-step RT-PCR reaction (Invitrogen) was set up by adding 25 µl of 2 x reaction buffer, 1 µl of *F. hepatica* RNA, 1 µl of forward primer at 10 µM, 1 µl of the reverse primer at 10 µM and 21 µl of dH_2O. This was briefly mixed and centrifuged. 1 µl of RT/Platinum *Taq* (Invitrogen) was then added to the tube and mixed. PCR was performed in a 2720 thermocycler (Applied Biosystems) with the following conditions;

a. cDNA synthesis: 1 cycle – 30 min at 50ºC, 2 min at 94ºC
b. PCR amplification: 40 cycles – 15 sec at 94ºC, 30 sec at 55ºC, 2 min at 70ºC
c. Final extension: 1 cycle – 7 min at 72ºC

3 µl of the reaction were added to 1 µl of DNA sample buffer and separated on a 1% agarose gel and visualised under UV as described above. The PCR product was then purified using the QIAQuick PCR purification kit (Qiagen), as per manufacturer's instructions.

5.2.7 Transformation of competent cells

The purified PCR product was ligated into the pGEM®-T cloning vector, as described by the manufacturer (Promega). The ligated vector was then used to transform JM 109 (Promega) competent cells, as per manufacturer's instructions. The transformed cells were spread on LB/IPTG/Ampicillin agar plates (see Appendix 1) and incubated overnight at 37 ºC. White colonies were picked and checked insert by colony PCR, as described in Chapter 2 section 2.2.2.6. Colonies with the insert were then cultured overnight in 10 ml of LB broth/Xgal/ampicillin (see Appendix 1). Plasmids were then purified with the Wizard ® Plus SV minipreps DNA purification system (Promega) according to manufacturer's instructions using the centrifugation protocol. The concentration of DNA was measured using the Nanodrop™2000 (Thermo-Scientific) and plasmids at an appropriate concentration were sent for automated sequencing (MWG, http://www.eurofins.co.uk/) using SP6 and T7 primers.

5.2.8 Multiple Sequence Alignment and identification of glycosylation sites and Transmembrane domains in *F. hepatica* cathepsin D

The inferred protein sequence of *F. hepatica* cathepsin D, derived from the RT-PCR amplification product (FhCatD) as described in section 5.2.7, was aligned with those from other species. Initially, the protein sequences of *A. duodenale* [FJ172357], *N. americanus* [AJ245459], *F. gigantica* [JF720347], *F. hepatica* [ABJ97285], *Clonorchis sinensis* [AAL14708], *S. japonicum* [CAX72343], *H. sapiens* [AAH16320], *H. contortus* [AJ577754] *H. contortus* [AF076608] *N. americanus* [J245458], *Opisthorchis viverrini* [AAZ39883], were aligned. This will allow comparison of residues in the active site clefts between the parasite species, which are described for cathepsin D in Merops (http://merops.sanger.ac.uk/). The sequences were then scanned for the presence of possible N-linked glycoslylation sites by identifying the amino acid series NxS or NxT (where x is any amino acid) (Blom *et al.*, 2004).

A second multiple sequence alignment of cathepsin D coding sequences including FhCatD, *Caenorhabditis elegans* [AAB06576], *C. sinensis* [AAL14708], *O. viverrini* [AAZ39883], *Ascaris suum* [ADY43078.1], *Anisakis simplex* [ACY38599], *Ancylostoma caninum* [AAB06575.1], *Ancylostoma ceylanicum* [AAO22152.1], *S. japonicum* [CAX79402.1], *F. hepatica* [AC104164.1] ADULT, *F. hepatica* [ABJ97285] NEJ, *F. gigantica* [ABJ97285], *A. duodenale* [FJ172357], *N. americanus* [AJ245459], *S. mansoni* [CCD78465], *H. contortus* [AJ577754], *H. contortus* [AF076608], *N. americanus* [J245458] *Homo sapiens* [AAA51922.1] was then conducted using the Clustal W2 programme (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The guide tree generated from the alignment was then used to generate a phylogenetic tree using Tree view (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Including more cathepsin D sequences will establish a better relationship of the FhCatD with that of other trematodes and nematodes.

The presence of a signal peptide was identified using Signal P 4.0 (Petersen *et al.*, 2011). Furthermore, any potential transmembrane domains (TM) were identified by submitting the FhCatD protein sequence to TMpred - Prediction of Transmembrane Regions and Orientation (http://www.ch.embnet.org/software/TMPRED_form.html) (Hofmann and Stoffel, 1993).

5.2.9 Purification of *F. hepatica* aspartyl protease by Pepstatin A affinity chromatography

A 150 mm long x 10 mm diameter empty column apparatus was assembled according to the manufacturer's guidelines (Bio-Rad) and washed thoroughly with dH_2O at room temperature. The column outlet was stoppered and 1 ml of Pepstatin A (PepA) binding buffer (see Appendix 1) dispensed into the column. 2 ml of Pepstatin A-bound agarose beads (Pierce) were then dispensed into the column and the stopper removed. The column was packed by continually washing with PepA binding buffer, being careful not to allow the column to run dry. Once the bead volume was adequately packed, the stopper was replaced and the column filled very carefully with PepA binding buffer, to create a positive meniscus. The rest of the column was assembled ensuring no air bubbles were trapped. The column was then equilibrated and packed in PepA binding buffer by washing overnight, 6 ml per hour at 4ºC.

2 ml of S3 was diluted to 7 ml with PepA binding buffer and loaded onto the column at 10 ml per hour. PepA binding buffer was washed through the column to remove any unbound material. The column was then washed with 20 ml of PepA elution buffer (see Appendix 1) and 1 ml fractions collected. The absorbance of fractions at 280 nm was measured. Fractions containing protein were pooled and concentrated with Amicon Ultra 10K (Millipore) as per manufacturer's instructions. A 10 µl sample were analysed by SDS-PAGE as described in section 3.2.4.1.

5.2.9.1 Matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) spectrometric analysis.

Where stated MALDI-TOF was used to identify the protein identity of protein bands of interest. This was performed by Kevin McLean of the Moredun Functional Genomics Unit. Briefly, protein bands of interest were excised, destained and reductively alkylated using DTT and iodoacetamide. The gel pieces were digested overnight with trypsin at 37 ^{o}C. Digests were then analysed on an Ultraflex II MALDI-ToF-ToF mass spectrometer (Bruker Daltonics), scanning the x to y Dalton region in reflectron mode producing monoisotopic resolution. The spectra generated were mass calibrated using known standards and the peaks deisotoped. Masses obtained were used for database searching with the MASCOT search engine using the in-house *F. hepatica* cathepsin D database with a 50 ppm mass tolerance window. Significant matches from the Peptide Mass Fingerprint

data were confirmed by MS/MS analysis using the search criteria detailed above and an MS/MS tolerance window of 0.5 Da.

5.2.10 Generation of a recombinant cathepsin D

5.2.11 Generation of recombinant cathepsin D using pET SUMO

5.2.11.1 Ligation of cathepsin D into pET SUMO vector

FhCatD PCR product from Section 5.2.6 was added in a 1:1 molar ratio of insert:vector by combining the following in an Eppendorf; 1µl cathepsin D PCR product, 1 µl 10 x ligation buffer, 2 µl pET SUMO vector (Invitrogen) (25 ng/ µl), 5 µl dH_2O, 1 µl T4 DNA ligase (4.0 Weiss Units). This was incubated for 24 hours at 15 ºC.

5.2.11.2 Transforming into competent cells

The construct was transformed into One Shot ® Mach1™ T1®competent cells (Invitrogen) as follows. One vial of cells was thawed on ice. 2 µl of ligation reaction were added and incubated on ice for 10 min. Cells were heat shocked at 42 ºC for 30 sec then immediately placed on ice. The transformation reaction was then incubated at 37 ºC for 1 hour, with shaking (200 rpm). After incubation, the transformation reaction was spread on LB plates with 50 µg/ml Kanamycin (See Appendix 1) and incubated overnight at 37 º C.

10 colonies were picked from plates and checked for inserts by colony PCR using SapphireAMP®Fast PCR mix (TaKaRa). A master mix was prepared by combining the following; 225 µl 2 x SapphireAMP®Fast PCR mix, 9 µl pET SUMO forward primer (5' AGATTCTTGTACGACGGTATTAG 3') (10 µM), 9 µl T7 reverse primer (5' TAGTTATTGCTCAGCGGTGG 3') (10 µM) and 207 µl dH_2O. 50 µl aliquots of this master mix were dispensed into 9 PCR tubes. Using a clean pipette tip, a swab was taken from each colony and mixed with master mix.

Reaction tubes were placed into a preheated 2720 thermocycler (Applied Biosystems) and processed through the following conditions;

a. Denaturation: 94 ºC for 1 Min (1 cycle)
b. PCR reaction: 98 ºC for 5 sec, 55ºC for 30 sec, 72ºC for 40 sec (30 cycles)
c. Extension: 72ºC for 1 minute, hold at 4ºC

Products were examined by agarose gel electrophoresis as described in section 5.2.5.

Another swab was taken from the transformants with the cathepsin D insert and added to 10 ml LB broth/Kanamycin (50 µg/ml). Cultures were incubated overnight at 37 ºC with shaking (200rpm). The plasmids were purified from the cultures using the Wizard Plus SV minipreps DNA purification system (Promega), using the centrifugation protocol. The concentration of the plasmid DNA was estimated using the Nanodrop then sent to Eurofins (http://www.eurofins.co.uk/) for sequencing.

Sequences were aligned using DNASTAR Lasergene 8 and translated into protein sequence using the sequence manipulation suite website (http://www.bioinformatics.org/sms2/) to check that the sequence was in-frame and capable of producing the correct translated product.

5.2.11.3 Expression of pET SUMO-cathepsin D fusion recombinant protein

Following transformation of BL21 (DE3) (Invitrogen, UK) competent cells with the pET SUMO-cathepsin D construct, the recombinant cathepsin D was expressed.

10 µg of plasmid from a colony containing the verified insert was added to a vial of competent cells BL21(DE3) One Shot (Invitrogen). This was incubated for 30 min on ice and heat shocked for 30 sec at 42 ºC and placed back on ice. The reaction was added to 250 µl of 42 ºC SOC and incubated at 37 ºC for 90 min with shaking. After incubation, the reaction was added to 10 ml of LB broth + ampicillin (See Appendix 1) and incubated overnight with shaking (200 rpm).

500 µl of overnight culture were added to two vials of 10 ml of LB broth and ampicillin (See Appendix 1). This was incubated for 2 hours at 37 ºC with shaking (200 rpm). When the OD_{280} was between 0.4 – 0.9 (ideally 0.6, exponential growth phase) IPTG (1mM) was used to induce one culture of transformed competent BL21(DE3) cells. Immediately after the addition of IPTG a 1 ml sample (labelled as time = 0) was taken from each culture, induced and uninduced. The samples were centrifuged at 3000 rpm for 10 min and the supernatant discarded. Pellets were stored at – 20 ºC. Cultures were incubated at 37 ºC for a further 5 hours taking a 1 ml sample from each culture every hour and processing as before.

Pellets were resuspended in 100 µl of binding buffer (see Appendix 1) and frozen at -80 ºC for 5 min, thawed and vortexed. This was repeated, then 5 µl of 20% Triton X-100 were

added and incubated on ice for 1 hour. Reactions were centrifuged at 13, 000 rpm for 10 min. The supernatant (soluble phase) was removed and dispensed into clean Eppendorf tubes. The pellet (insoluble) phase was resuspended in 100 µl binding buffer + 8M urea. A 10 µl sample from each time point and phase (soluble or insoluble) was taken and added to 5 µl Sample buffer and heated to 70 °C for 10 min. Samples were separated by electrophoresis on SDS-PAGE (4-12%) alongside molecular weight marker. When it was confirmed, by SDS-PAGE, that cathepsin D was successfully being expressed as described in section 5.2.5.10, the expression was carried out on a larger scale. A colony expressing cathepsin D was used to inoculate a 10 ml LB broth/Kanamycin (50 µg/ml) and grown overnight at 37 °C (200 rpm). 750 µl of the culture were used to inoculate 50 ml of LB broth which was then incubated at 37 °C for approximately 3 hours with shaking (200 rpm). When the OD_{600} was between 0.4 – 0.9 (ideally 0.6, exponential growth phase) a 1 ml sample was taken and centrifuged at 3000 rpm for 10 min and the supernatant was discarded. The remaining pellet was stored at – 20 °C. The culture was then induced to express the cathepsin D protein by the addition of IPTG (1mM) and incubating at 37 °C for 3 hours with shaking (200 rpm). After 3 hours the culture was transferred into a 50 ml Falcon tube and centrifuged for 20 min at 3000 x g. The supernatant was discarded and pellet stored at -80°C.

5.2.12 Purification of recombinant protein by nickel affinity chromatography

Nickel affinity chromatography was used to purify the recombinant cathepsin D. The cell pellet from section 5.2.11 was thawed and resuspended in 5 ml of binding buffer (see appendix 1). On ice, the cell pellet in binding buffer was sonicated for 2 x 20 sec, then refrozen at – 80 °C for 10 min. The pellet suspension was thawed and 500 µl of 20 % Triton X-100 in binding buffer added. This mixture was then incubated on ice for 30 min on a rotary shaker. 1.1 ml aliquots were centrifuged at 18 000 x g for 10 min, supernatant discarded and pellets stored at -80°C. As the recombinant cathepsin D was present in the insoluble fraction, 8 M Urea was added to the buffers to solubilise the protein during the subsequent purification process. One pellet was thawed and resuspended in 1 ml of binding buffer + 8 M Urea (see Appendix 1) by vortexing for 10 min. The suspension was then centrifuged at 14000 rpm for 10 min and the supernatant stored at 4 °C

Using a peristaltic pump, a 1 ml HisTrap™HP column (Amersham Biosciences) was washed with 5 ml dH_2O then 5 ml of binding buffer + 8 M Urea. 1ml of the urea

solubilised induced protein fraction was applied to the column at 1 ml per minute, retaining a subsample for later analysis (sample A). The column was then sealed and incubated for 1 hour at room temperature on a rotary shaker. After incubation, 5 ml of binding buffer with 8 M Urea was passed through the column and the first 1 ml eluted from the column collected for later analysis, labelled sample B. 1 ml of elution buffer 1 was applied onto the column and the eluate collected and labelled sample C. 1ml of elution buffer 2 was then applied onto the column and the eluate collected, sample D. 1 ml of elution buffer 3 was then applied onto the column and the eluate collected, sample E. Finally, 3 ml of elution buffer 4 was applied to the column and 1ml samples collected as sample F, G and H.

Samples A to H were mixed with sample buffer and separated on a 4- 12% Bis/Tris SDS PAGE by electrophoresis, at 200 V 50 min, to establish if purification was successful.

5.2.13 Purification of the recombinant cathepsin D by electro-elution

Although the protein had a C-terminal poly-histidine tag, it was not possible to purify the recombinant *F. hepatica* cathepsin D by nickel affinity chromatography. Therefore, the protein was purified by electro-elution. 1 ml aliquots of the recombinant protein mixture were separated by SDS-PAGE alongside SeeBlue®Plus2 Prestained standard (Invitrogen) by electrophoresis at 200 V for 45 min. The gel was stained with SimplyBlue™SafeStain (Invitrogen) for 1 hour at room temp, on a shaker, then destained with distilled water. After destaining, the band(s) representing the recombinant cathepsin D were excised from the gel using a clean scalpel blade and cut into small pieces.

The electro-elution tank (BioRad) was prepared as follows. The outer chambers of the electro-elution tank were filled with ½ x electrophoresis tank buffer (Tris glycine SDS), see Appendix 1. Dialysis membrane was soaked in water then stretched over the inside cups. The cup at the negative electrode was filled with ½ x electrophoresis tank buffer and the cup at the positive electrode was filled with ½ x electrophoresis tank buffer without SDS. The gel pieces were placed into the cup at the negative electrode. The cup at the positive electrode was filled with ½ x electroelution tank buffer until it covered the area between the cups, see Figure 5.2. The lid was attached and 50 V applied overnight. This causes the protein to migrate from the gel polyacrylamide pieces at the negative electrode to the positive electrode. Carefully, the buffer was removed and the blue solution (containing protein) at the positive electrode collected. A sample of this was separated by SDS-PAGE (as described in 3.2.4.1) alongside a molecular weight marker to confirm that

the purified protein was of the correct molecular mass. Multiple samples purified in this way were pooled and concentrated by centrifugation at 3500 x *g* using Amicon ultra10 K (Millipore) concentrators and then MALDI-TOF was used to confirm that the protein identity was cathepsin D as described in section 5.2.9.1. The recombinant cathepsin D generated here will be termed rFhCatD from now on.

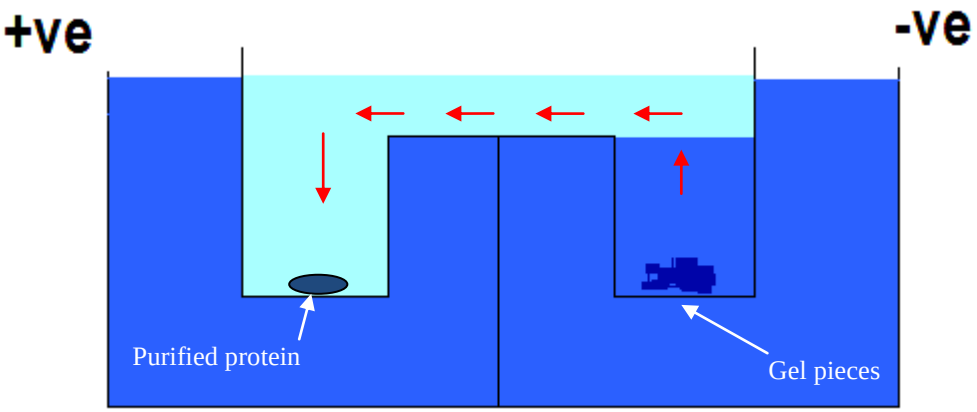

Figure 5.2 Electro-elution tank setup. The ½ x electrophoresis buffer with SDS is shown in blue and without SDS in light blue.

5.2.14 Western blot with immune sera

Approximately 10 µg of S1, S2, S3, RFhCatD, JAC-enriched S3, PNA-enriched S3, and cathepsin L1 (kindly provided by Professor Grace Mulcahy, University College Dublin, Ireland) were separated on 4-12% SDS-PAGE and electroblotted onto PVDF membrane as described in section 3.2.4.2. This was repeated to generate two replicate blots.

The PVDF was briefly stained with Ponceau S (see Appendix 1) to visualise lanes and then destained by two 5 min washes in 5 % acetic acid, then blocked for 1 hour with TNTT at room temp. One blot was incubated with serum taken from sheep 8 weeks after an experimental challenge with 500 *F. hepatica* metacercariae (kindly provided by Professor Grace Mulcahy, UCD). The other blot was probed with serum from naïve sheep. Sera were used at 1:200 dilution in TNTT and blots incubated for 1 hour at room temperature on a rotary shaker. Blots were washed 3 x 5 min in TNTT, then incubated in anti-sheep

immunoglobulin- (1:1000 in TNTT) for 1 hour at room temperature. The blot(s) were then developed with Sigma fast DAB (Sigma) for 5 min at room temperature.

5.2.15 Raising antibodies to rFhCatD in mice

Mice were used to raise antibodies against rFhCatD. The inoculation was prepared by combining 90 µl antigen (~125 µg/ml), 50 µl of 1 mg/ml Quil A adjuvant, 80 µl of 1 x PBS (see Appendix 1). Two BALB/c mice were inoculated with 100 µl of antigen/adjuvant suspension subcutaneously twice at 3 week intervals (by Louise Gibbard of the Moredun High Security Unit). Blood was taken prior to inoculation, 1 week after the second inoculation and the animals humanely exsanguinated. The serum was separated from other blood products by allowing the blood to clot at 4ºC overnight and pipetting the separated liquid from clotted red blood cells into a new tube. This was centrifuged at 1000 x *g* for 10 min and 50 µl aliquots of the supernatant stored at -20 ºC.

5.2.16 Immunoblotting of cathepsin D in *F. hepatica* extracts and protein fractions

To confirm the antisera generated in section 5.2.16 were capable of recognising rFhCatD, ten 10 µl aliquots of rFhCatD were separated on a 4-12 % SDS PAGE gel as described in section 3.2.4.1 and electroblotted onto NC as described in section 3.2.4.2. The NC was briefly stained with Ponceau S to visualise lanes, which were marked and cut then destained by two 5 min washes in 5 % acetic acid, then blocked for 1 hour with TNTT at room temperature. Lanes 1 to 5 were incubated for 1 hour at room temperature in decreasing concentrations of mouse anti-rFhCatD in TNTT (1: 500, 1000, 2000, 4000, 5000). Lanes 6 - 10 were incubated for 1 hour at room temp in decreasing concentrations of pre-immune mouse sera diluted in TNTT (1: 500, 1000, 2000, 4000, 5000). Lanes were washed three times for 5 min in TNTT. All lanes were then incubated in Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP (DAKO, P0260) at 1: 1000 in TNTT for 1 hour at room temperature. Lanes were washed three times for 5 min in TNTT. The blot was then developed with Sigma fast DAB (Sigma) for 5 min at room temp.

Once the working dilution of sera was established, 10 µl of S1, S2, S3, rFhCatD, JAC-enriched S3 and PNA-enriched S3 were separated on a 4-12 % SDS-PAGE gel as described in section 3.2.4.1 and the electroblotted onto NC as described in section 3.2.4.2. The blot was blocked in 1 x TNTT overnight at 4 ºC, then probed with the antisera (1: 5000) and rabbit anti-mouse HRP (1: 1000), as described above.

5.2.17 Immunolocalisation of FhCatD protein

The antisera generated in section 5.2.15 were used to immunolocalise proteins within tissue sections of juvenile and adult *F. hepatica.* Sections were prepared as described in section 3.2.2.1. Sections were dewaxed as described in section 3.2.2.4., then incubated for 20 min in 3 % hydrogen peroxide in methanol at room temperature with stirring. This inactivates any endogenous peroxidases from the sections to prevent non-specific staining. Sections were then rinsed with tap water and loaded into Shandon Sequenza slide racks (Thermo Scientific). Slides were then rinsed 3 times with immunolocalisation buffer (see Appendix 1). Sections were blocked with 100µl of 25% normal goat serum (NGS) in immunolocalisation buffer (see Appendix 1) for 30 min at room temperature. The histological sections were then incubated in either 100 µl of the pre-immune mouse sera (as a negative control) or 100 µl of serum raised against the rFhCatD, both diluted to 1:10,000 in 25% NGS/ immunolocalisation buffer (see Appendix 1). Slides were incubated at 4 ºC overnight. Sections were washed 3 x with immunolocalisation buffer then incubated with 100 µl HRP-conjugated goat anti-mouse IgG (Envision) 30 min at room temperature. Slides were washed 3 x with immunolocalisation buffer. 100 µl of DAB (1 ml substrate buffer with 20 µl DAB chromagen, Envision) were dispensed onto each slide and incubated for 8 min at room temperature, then washed with dH_2O. Sections were counterstained as follows; haemotoxylin 5 min, dH_2O 5 min, Scot's tap water substitute (STWS) (see Appendix 1) 5 min, dH_2O 5 min, 75% ethanol 5 min, 95% ethanol 5 min 100% ethanol 5 min, xylene 2 x 5 min. Slides were mounted with coverslips using Colsul mount (Thermo Scientific, UK) mounting medium and viewed on an Olympus BX50 microscope.

5.2.18 NEJ and adult stage expression of cathepsin D using RT-PCR

5.2.18.1 Primers

Primers were designed to amplify a fragment of *F. hepatica* cathepsin D (ABJ97285) (see Figure 5.3) and *F. hepatica* β-tubulin [GenBank accession: HM535959.1] (see Figure 5.4) using Primer3 (v. 0.4.0) (http://frodo.wi.mit.edu/primer3) (Rozen and Skaletsky, 2000).

The final amplified fragments for *F. hepatica* cathepsin D and β-tubulin are 211 bp and 174 bp in length respectively. The forward and reverse primers were produced by Eurofins (http://www.eurofins.co.uk/) and 10µM stock solutions made for each of these and stored t -20ºC.

GTGCGGGAAACAACTGGGCCAAGGGACATTACACCGAAGGTGCCGAGTTGGTTGACTCAGT
TTTGGATGTGGTTCGCAAAGAAGCTGAGTCATGCGACTGTCTCCAAGGCTTCCAGTTGACT
CATTCACTGGGTGGTGGCACCGGTTCTGGTATGGGTACCTTGCTCATCTCGAAGATCCGGG
AGGAGTATCCTGATCGGATCATGAACACTTTCTCCGTGGTCCCCTCACCCAAGGTATGTCA
AGCCAGGTCCAAATATGTTCATCAATATCTGTTTTATTTAGGTATCCGACACCGTTGTGGA
ACCATATAATGCTACCCTATCCGTACATCAGTTGGTCGAGAACACGGATGAG**ACGTATTGC**
ATCGACAACGAAGCTCTATATGATATCTGCTTCCGCACCTTGAAACTGACCACGCCAACCT
ACGGTGATTTAAACCATTTGGTGAGTGCCACCATGTCCGGTGTGACAACTTGTCTGCGTTT
CCCTGGTCAACTGAATGCTGATCTGCGAAAACTAGCCGTGAACATGGTTCCGTTCCCCCGT
TTGCACTTCTTCATGCCTGGTTTTGCTCCACTAACCAGTCGAGGTAGTCAACAATACCGTG
CTCTGACTGTACCTGAGTTGACTCAACAAATGTTTGATGCGAAGAACATGATGGCCGCTTG
TGATCCGCGTCACGGGCGCTACCTGACAGTTGCCGCCATGTTCCGTGGTCGCATGTCGATG
AAAGAGGTGGACGAGCAAATGTTGAATGTGCAGAACAAAATACAAT

Figure 5.3: The nucleotide sequence of *F. hepatica* cathepsin D (ABJ97285). Highlighted are the forward (red) and reverse primer binding sites (yellow). The final amplified fragment should be 211 bp in length.

ATGTTTTAAGGATTAAACTACGTCCATTTAAGACGACCCGCCAGGAGCTCAGCGAATATGG
GTCGCTCGACTGGGAATCATCACAACGGCTTTTTGGGAAATATGCAGGAAGAAACGGCTCT
ATTCCGGAGCAGCTAAATAACTATTTGGACGCTCAATATTATGGCGAAATCGGTATTGGAA
CGCCACCACAAACTTTCAAAGTCATTTTTGATACGGGGTCGTCAAACTTGTGGGTCCCGTC
AAAACGCTGCAGCTATCTCAGCTGGGCTTGTTGGCTACACAACAAATATAACTACGCTGCT
TCTTCAACTTATCAAGTTAATGGCACCGCTTTCAGTATTCAGTATGGAACCGGCAGTGTAT
CAGGTTTTATAAGCGTTGATTCATTTGAGGTTGGCGGTGTGGAGGTGAAAGGTCAACCATT
TGGGGAGGCTATCAAAGAACCTGGCATCGTTTTTGTGTTTGCTAAATTCGACGGTATCCTT
GGGATGGGATTTAGAAGCATATCTGTTGGTGGCCTGATTACCGTTTTTGAAAATATGATTG
CTCAAGGTCTAGTACCCGAACCTGTCTTCTCTTTTTACCTCAACAGAAATGCATCCGATCC
TGTGGGTGGCGAGCTTCTTCTCGGAGGGATCGATCCAAATTACTATACTGGTGACATTACC
TATGTGCCGGTCACTCATGAAGCATACTGGCAGTTCAAAGTTGATAAAATCGAGTTTCCTG
GTGTTTCAATTTG**CGCTGATGGTTGTCAAGCTA**TTGCTGATACAGGCACATCCCTCATTGC
CGGCCCGAAGAAGGAAGTTGACGCACTGAATGAGCAAATTGGAGGCACTTGGATGCCTGGA
GGTATCTACGTCGTGAATTGGGACAAGATTGATAATCTTTCTGCT**ATTACCTTTGTCGTGG**
CTGGGAGAAAAATGGTGTTTGAGGCTAAAGACTATC

Figure 5.4: The nucleotide sequence of *F. hepatica* β-tubulin [GenBank accession: HM535959.1). Highlighted are the forward (green) and reverse primer binding sites (yellow). The final amplified fragment should be 174 bp in length

5.2.18.2 RT-PCR

The RT-PCR reaction was set up on ice as outlined in **Table 5.1**.

Table 5.1: RT-PCR set up for investigating the expression of cathepsin D and β tubulin in NEJ and Adult stages of *F. hepatica.*

	2 x Reaction Buffer µl	Adult/NEJ RNA (µl)	F CD (10 µM) µl	RCD (10 µM) µl	F β T (10 µM) µl	R β T (10 µM) µl	dH_2O µl
1	25	NEJ (5)	-	-	1	1	17
2.	25	NEJ (5)	1	1	-	-	17
3	25	Adult (1)	-	-	1	1	21
4	25	Adult (1)	1	1	-	-	21
5	25	-	-	-	1	1	22
6	25	-	1	1	-	-	22

Reactions: 1. NEJ with β-Tubulin, 2. 1. NEJ with cathepsin D, 3. Adult with β-Tubulin, 4. Adult with cathepsin D, 5. β-Tubulin control, 6. cathepsin D control. Forward cathepsin D primer (F CD), Reverse cathepsin D primer (R CD), Forward β-Tubulin primer (F β T) Reverse β-Tubulin primer (Rβ T).

These were briefly mixed and centrifuged. Then, 1 µl of RT/Platinum *Taq* (1 Unit) (Invitrogen) was added to each tube, mixed. Reaction tubes were placed into a preheated 2720 thermocycler (Applied biosystems) and processed through the following conditions;

cDNA synthesis: 1 cycle – 30 min at 50°C, 2 min at 94°C

a. PCR amplification: 40 cycles – 15 sec at 94°C, 30 sec at 55°C, 2min at 68°C
b. Final extension: 1 cycle – 5 min at 68°C

5 µl samples were taken every 5 amplification cycles and stored at 4 °C. 3µl of the reaction were added to 1µl of DNA sample buffer and separated on a 1% agarose gel and visualised as described in section 5.2.5.

5.3 Results

5.3.1 Amplification of cathepsin D and alignment

The full-length *F. hepatica* cathepsin D (FhCatD) coding sequence was amplified, using primers designed to the respective 5' and 3' ends of the sequence, from total RNA extracted from adult flukes, by reverse transcription PCR (RT-PCR). RT-PCR yielded a product of the expected size (931 bp). This was ligated into a suitable plasmid vector and, in turn, transformed into competent cells in order to propagate the plasmid. Colony PCR confirmed the presence of the sequence in the vector and these plasmids were purified and sent for sequencing (MWG, Eurofins). The inferred amino acid sequence of the rFhCatD was aligned with the publically available *F. hepatica* cathepsin D sequence (ABJ97285) for comparison. The sequences shared 99% identity and glycosylation sites, transmembrane domains and signal peptides are highlighted in Figure 5.5.

5.3.2 Multiple sequence alignment and generation of a Phylogenetic tree

In order to ascertain the similarity of FhCatD to cathepsin D from other species, sequences were aligned using multiple sequence alignment to generate a phylogenetic tree. Amino acids in the active site clefts between all species were identical. The most closely related sequences were those of FhCatD, *F. gigantica* [JF720347] and *F. hepatica* [adult (ABJ97285) NEJ (AC104164)] sharing 99% identity. This high level of identity indicates that they are likely to represent the same cathepsin D gene. FhCatD shared the least similarity with *H. contortus* (AJ577754, AF076608) with only 24 % identity, see Table 5.2.

After the initial alignment, FhCatD was then aligned with a larger set of cathepsin D protein sequences to acsertain its relationship to cathepsin D of other species as before with pairwise alignment by Clustal W2. The subsequent guide tree was then used to generate a phylogenetic tree using Treeview (Page, 1996), this is shown in Figure 5.6. FhCatD was placed in a clade with the *F. hepatica* NEJ, *F. hepatica* adult and *F. gigantica* cathepsin D. Closely related were the enzymes of the other trematodes, the human blood flukes *S. japonicum* and *S. mansoni*. The nematode enzymes were also placed together within a single but different clade to the trematodes.

Scanning for transmembrane domains within FhCatD identified 4 significant transmembrane helicies which were located between residues; 1-19 (1800), 116- 144 (610), 177-195 (1309) and 348-374 (1112), see Figure 5.5. The scores for these were 1800,

610, 1309 and 1112 respectively where a score greater than 600 is significant. There was also a signal peptide identified at residues 1-16 with predicted cleavage between 16 and 17 (Petersen *et al.*, 2011).

```
F.hepatica[ABJ97285]      MRPVLLICLLFSAALCDVLRIKLRPFKTTRQELSEYGSLDWESSQRLFGKYAGRNGSIPE 60
FhCatD                    MRPVLLICLLFSAVLCDVLRIKLRPFKTTRQELSEYGSLDWESSQRLFGKYAGRNGSIPE 60
                          ************.********************************************

F.hepatica[ABJ97285]      QLNNYLDAQYYGEIGIGTPPQTFKVIFDTGSSNLWVPSKRCSYLSWACWLHNKYNYAASS 120
FhCatD                    QLNNYLDAQYYGEIGIGTPPQTFKVIFDTGSSNLWVPSKRCSYLSWACWLHNKYNYAASS 120
                          ************************************************************

F.hepatica[ABJ97285]      TYQVNGTAFSIQYGTGSVSGFISVDSFEVGGVEVKGQPFGEAIKEPGIVFVFAKFDGILG 180
FhCatD                    TYQVNGTAFSIQYGTGSVSGFISVDSFEVGGVEVKGQPFGEAIKEPGIVFVFAKFDGILG 180
                          ************************************************************

F.hepatica[ABJ97285]      MGFRSISVGGLITVFENMIAQGLVPEPVFSFYLNRNASDPVGGELLLGGIDPNYYTGDIT 240
FhCatD                    MGFRSISVGGLITVFENMIAQGLVPEPVFSFYLNRNASDPVGGELLLGGIDPNYYTGDIT 240
                          ************************************************************

F.hepatica[ABJ97285]      YVPVTHEAYWQFKVDKIEFPGVSICADGCQAIADTGTSLIAGPKKEVDALNEQIGGTWMP 300
FhCatD                    YVPVTHEAYWQFKVDKIEFPGVSICADGCQAIADTGTSLIAGPKKEVDALNEQIGGTWMP 300
                          ************************************************************

F.hepatica[ABJ97285]      GGIYVVNWDKIDNLSAITFVVAGRKMVFEAKDYIMKLSNMGRTVCVTSFIGIDVPVGPLW 360
FhCatD                    GGIYVVNCDKIDNLPAITFVVAGGKMVLEAKDYIMKLSNMGRTVCVTSFIGIDVPVGPLW 360
                          ******* ******.******** ***:**********************************

F.hepatica[ABJ97285]      ILGDVFIGSYYTVFDMGQKRIGFATTKRHSVSKPPLSVPMMGLKPAFRRQEEPRSAPPRN 420
FhCatD                    ILGDVFIGSYYTVFDMGQKRIGFATTKRHSVSKPPLSVPMMGLKPAFRRQEEPRSAPPRN 420
                          ************************************************************

F.hepatica[ABJ97285]      LLSFWNLLI 429
FhCatD                    LLSFWNLLI 429
                          *********
```

Figure 5.5: Multiple sequence alignment of FhCatD with adult *F. hepatica* cathepsin D (ABJ97285). Alignment was conducted using ClustalW2 by pairwise alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). * indicates where the amino acid is the same in both sequences and the active site clefts are highlighted yellow. Glycosylation motifs (NxS/T) are highlighted in green. Predicted transmembrane domains are highlighted in red and signal peptide underlined and in bold.

Table 5.2: The percentage DNA idenitity of FhCatD with cathepsin D from 18 other species.

	Accession number	Codon identity (%)
F. hepatica	ACI04164.1	98
F. gigantica	AEE69372.1	98
F. hepatica	ABJ97285.1	98
O. viverrini	AAZ39883	59
C. sinensis	AAL14708	56
S. mansoni	AAB63442	52
S. japonicum	CAX79402.1	51
H. sapiens	AAA51922.1	48
A. duodenale	FJ172357.1	45
A. suum	ADY43078.1	45
A. ceylanicum	AAO22152.1	44
A. simplex	ACY38599.1	42
A. caninum	AAB06575.1	40
C. elegans	AAB06576.1	28
N. americanus	AJ245459	27
N. americanus	J245458	27
H. contortus	AF076608	24
H. contortus	AJ577754	24

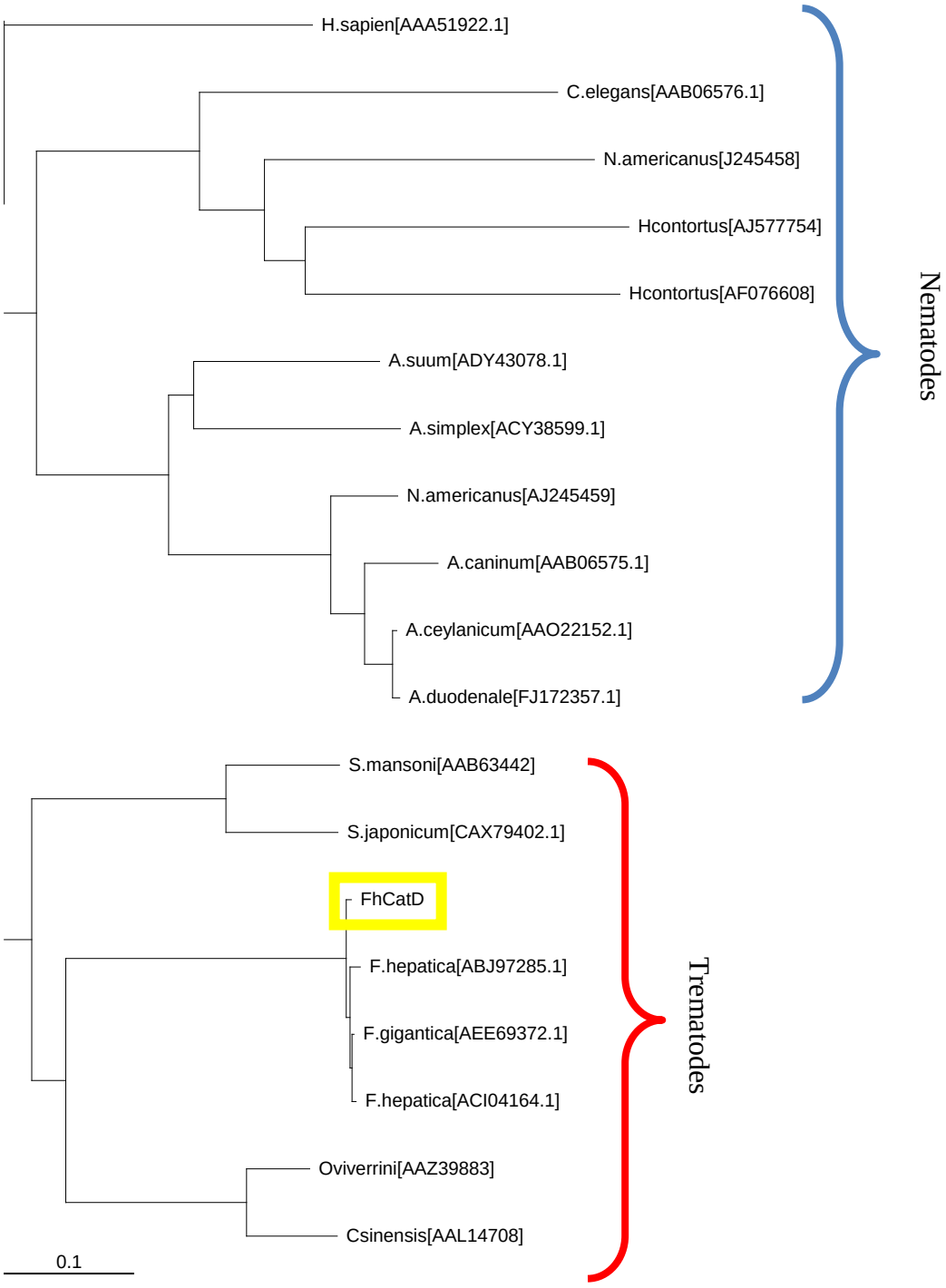

Figure 5.6: Relationship between selected cathepsin D molecules from eukaryotes, with emphasis on helminths. Clustal W2 used pairwise alignment to conduct multiple sequence alignment of the protein sequences. A phylogeny was created using the neighbour joining method and the subsequent guide tree used to generate a phylogenetic tree using Tree view (Page,1996).

5.3.3 Generating recombinant *F. hepatica* cathepsin D using pET SUMO

Cathepsin D was successfully ligated into the pET SUMO vector. The cathepsinD/pET SUMO (CatD-SUMO) construct was then used to transform JM 109 competent cells and colonies were checked for insert by colony PCR. Colonies which contained the insert were purified and sent for sequencing at MWG (http://www.eurofins.co.uk). A colony containing the insert with the desired sequence was then taken forward for protein expression. The CatD-SUMO was used to transform BL21(DE3) (invitrogen) competent cells for expression to obtain the recombinantly-derived protein. Samples were taken at 0, 1, 2 ,3, 4, and 5 hours after cells were induced with IPTG. The soluble and insoluble protein fractions were prepared from each bacterial pellet. A small sample of these was fractionated by SDS-PAGE, as shown in Figure 5.7

A prominent peptide of approximately 49 kDa, which is the predicted molecular weight for the protein encoded by the CatD-SUMO construct, was present in the induced insoluble fraction and absent from the non-induced cells. The band was excised from the gel and subjected to MALDI-TOF analysis. This confirmed its identity as *F. hepatica* cathepsin D, See Figure 5.8.

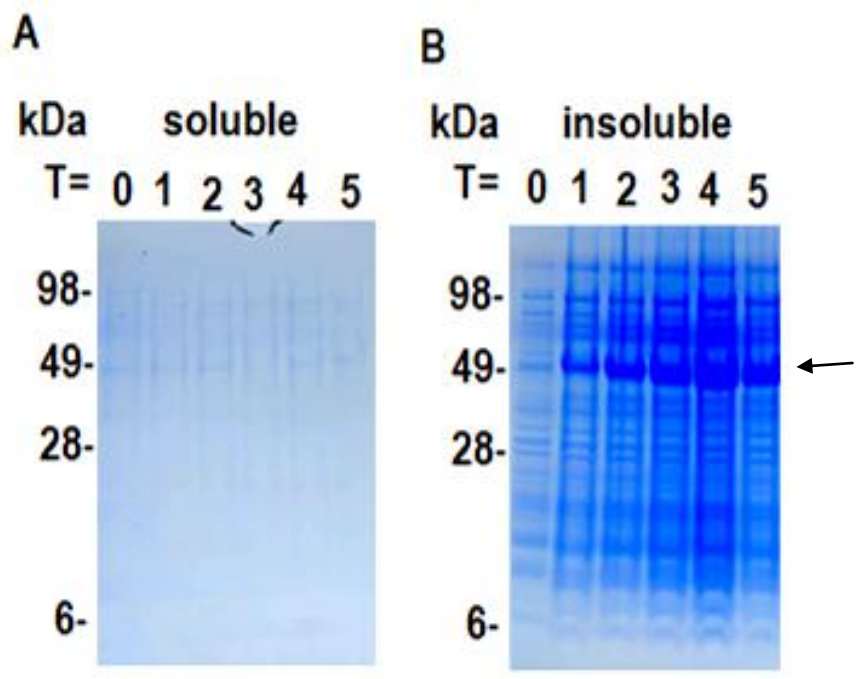

Figure 5.7: Induced expression of CatD-SUMO in BL21 cells. 1 ml samples were taken at times (T) 0, 1, 2, 3, 4, 5 hours post-induction with IPTG. Panel A shows the soluble fraction from each time point and panel B shows the insoluble fraction. A ~49 kDa band of increasing intensity over time is evident in the insoluble fraction as indicated by arrow.

Database searched: CathepsinD
MOWSE Score: 145.0
MW [kDa]: 48.1

Sequence Coverage [%]: 26.4
No. of unique Peptides: 14

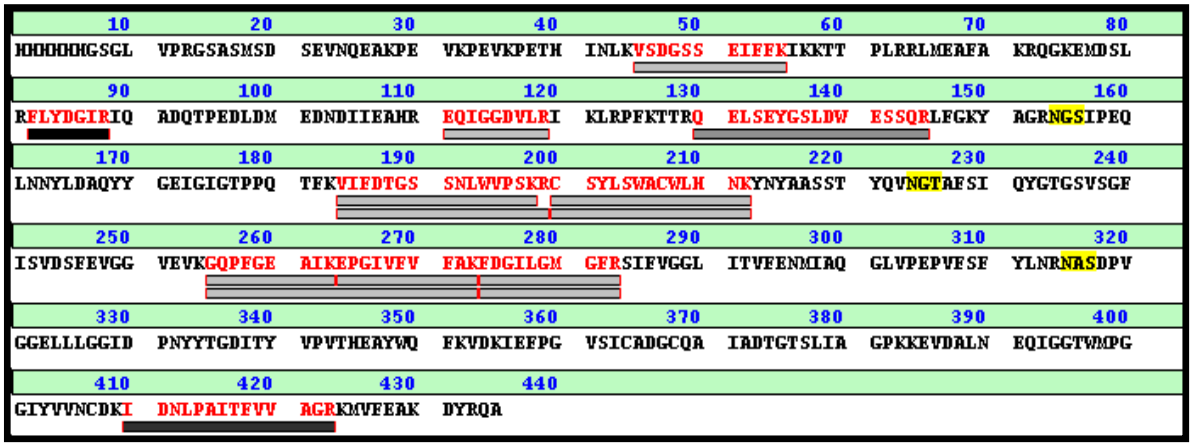

Figure 5.8: MALDI-TOF analysis of the 49 kDa fragment extracted from bacterial pellets.

5.3.4 Purification of recombinant FhCatD

Protein expression was scaled up by producing soluble and insoluble fractions as described in Section 5 2 11 3. Purification of the rFhCatD was then attempted by nickel affinity chromatography. Although the rFhCatD possesses a polyhistidine residue fusion peptide (verified by sequencing), it could not be purified by nickel affinity chromatography, result not shown. The recombinant cathepsin D was purified directly by electroelution. The purified recombinant cathepsin D is shown in Figure 5.9. Bands 1 and 2 were excised from the gel and subjected to MALDI-TOF analysis (performed by Kevin McLean of the Moredun Functional Genomics Unit) which confirmed these as *F. hepatica* cathepsin D. Band 2 was the correct size (49 kDa), the larger band 1 (Figure 5.9) was also confirmed as cathepsin D and is thought to represent a dimer.

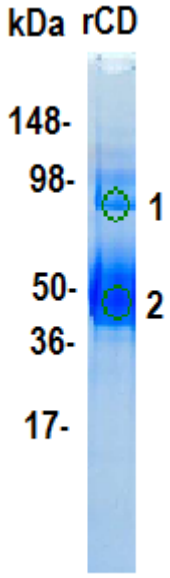

Figure 5.9: Purification of recombinant cathepsin D by electroelution. Band 1 and 2 were analysed by MALDI-TOF which confirmed both their identities as *F. hepatica* cathepsin D aspartyl protease. Band 2 is at the correct estimated size, where band 1 is thought to be a dimer which explains the higher molecular weight

5.3.5 Production of antisera to recombinant cathepsin D

Antiserum to the recombinant cathepsin D was raised in mice. The resultant sera were used to probe FhCatD at serial dilutions, along with pre-immune mouse sera. From this, a 1:500 dilution was used for future blots, see Panel A Figure 5.10. The sera were then used to probe the three *F. hepatica* extracts (S1, S2 and S3) and the lectin-enriched fractions. There was no evidence of cathepsin D in the S1, S2, S3 and JAC-enriched S3 fractions but its presence in the PNA enriched S3 was confirmed. This result is consistent with the outcome of the proteomic analysis from Chapter 2, but not with the enzymatic analyses in Chapter 4.

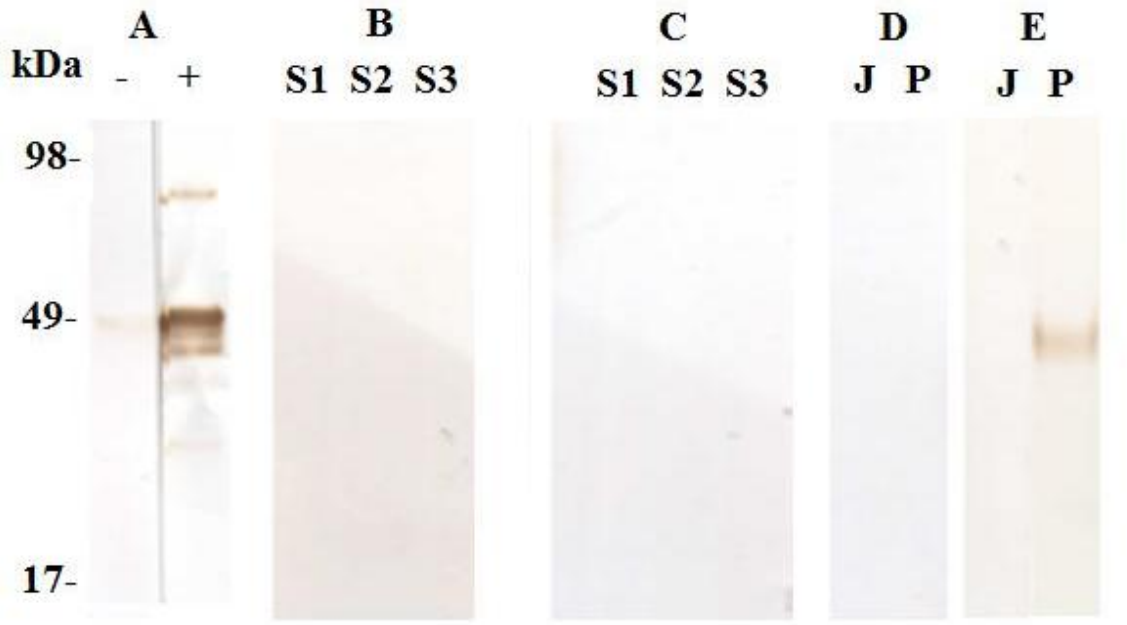

Figure 5.10 Reactivity of pooled mouse serum raised to rFhCatD against *F. hepatica* extracts. Extracts include PBS-soluble (S1), membrane-associated (S2) membrane-bound (S3), JAC-enriched S3(J) and PNA-enriched S3 (P). Panel A shows rFhCatD probed with sera raised to rFhCatD (+) and preimmune sera (-) at 1:500 dilution. Protein bands evident at 49 and 92 kDa represent cathepsin D, where 92 kDa is a cathepsin D dimer. Panels B and D are probed with pre-immune mouse sera at 1:500 dilution in TNTT. Panels C and E were probed with mouse anti rFhCatD at 1:500 dilution in TNTT.

5.3.6 Western blot of extracts with *F. hepatica* exposed serum

The availability of recombinant *F. hepatica* cathepsin D and PNA lectin-enriched native *F. hepatica* cathepsin D makes it possible to investigate if antibodies from sheep experimentally infected with *F. hepatica* bind both these forms. To address this, immunoblots were carried out using a pool of sera from sheep naturally infected with *F. hepatica* and also sera from naïve controls. From the results presented here, panel A Figure 5.11, it was clear that rFhCatD was bound by antiserum from naturally infected sheep and, thus, must be exposed to the host's immune system during the course of a natural infection. In addition, a number of proteins within the three extracts and PNA- and JAC- enriched S3 fractions were also bound.

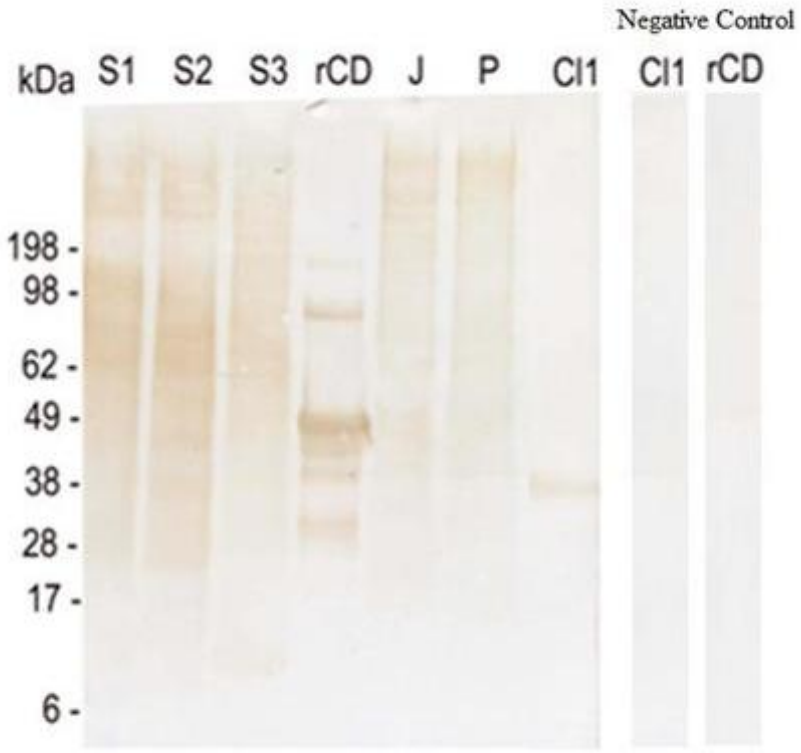

Figure 5.11: Reaction of pooled immune sera to the *F. hepatica* extracts (S1, S2 and S3), recombinant cathepsin D (rCD), JAC-enriched S3 (J), PNA-enriched S3 (P) and to cathepsin L1 (CL1), a positive control. Negative control lanes CL1 and rCD were probed with sera from sheep naïve to fluke infection (1:200 dilution) to confirm that recognition was specific.

5.3.7 Localisation of recombinant cathepsin D by Immunohistochemistry (IHC)

Cathepsin D was localised within sections of adult and immature *F. hepatica.* The anti-cathepsin D serum was tested at several dilutions and 1: 10,000 deemed the most suitable for immune-localisation. Within both stages, cathepsin D was localised to the gastrodermal region of the parasite, as shown in panel A Figure **5.12**. The negative control had low staining in comparison, Panel B Figure **5.12**. Within the immature (10 day) stages cathepsin D was again localised to the gut of *F. hepatica,* as shown in Figure 5.13.

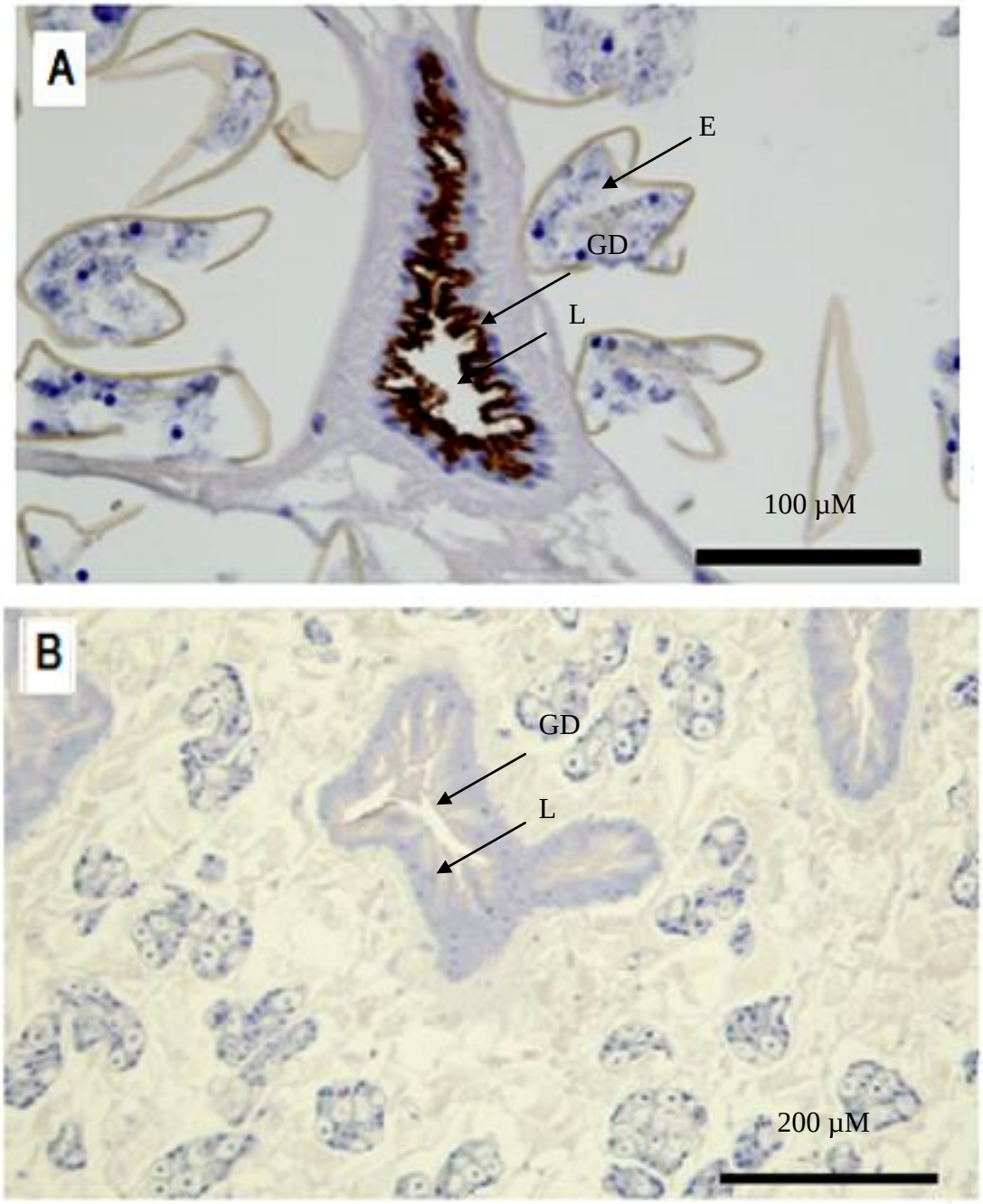

Figure 5.12: Localisation of cathepsin D in adult histological sections of *F. hepatica.* Panel A was probed with anti-cathepsin D sera, diluted 1: 10 000 in PBST. Panel B was probed with pre-immune mice sera as a negative control diluted 1: 10, 000 in PBST. The gastrodermal cells (GD), lamellae (L), eggs (E) are labelled accordingly.

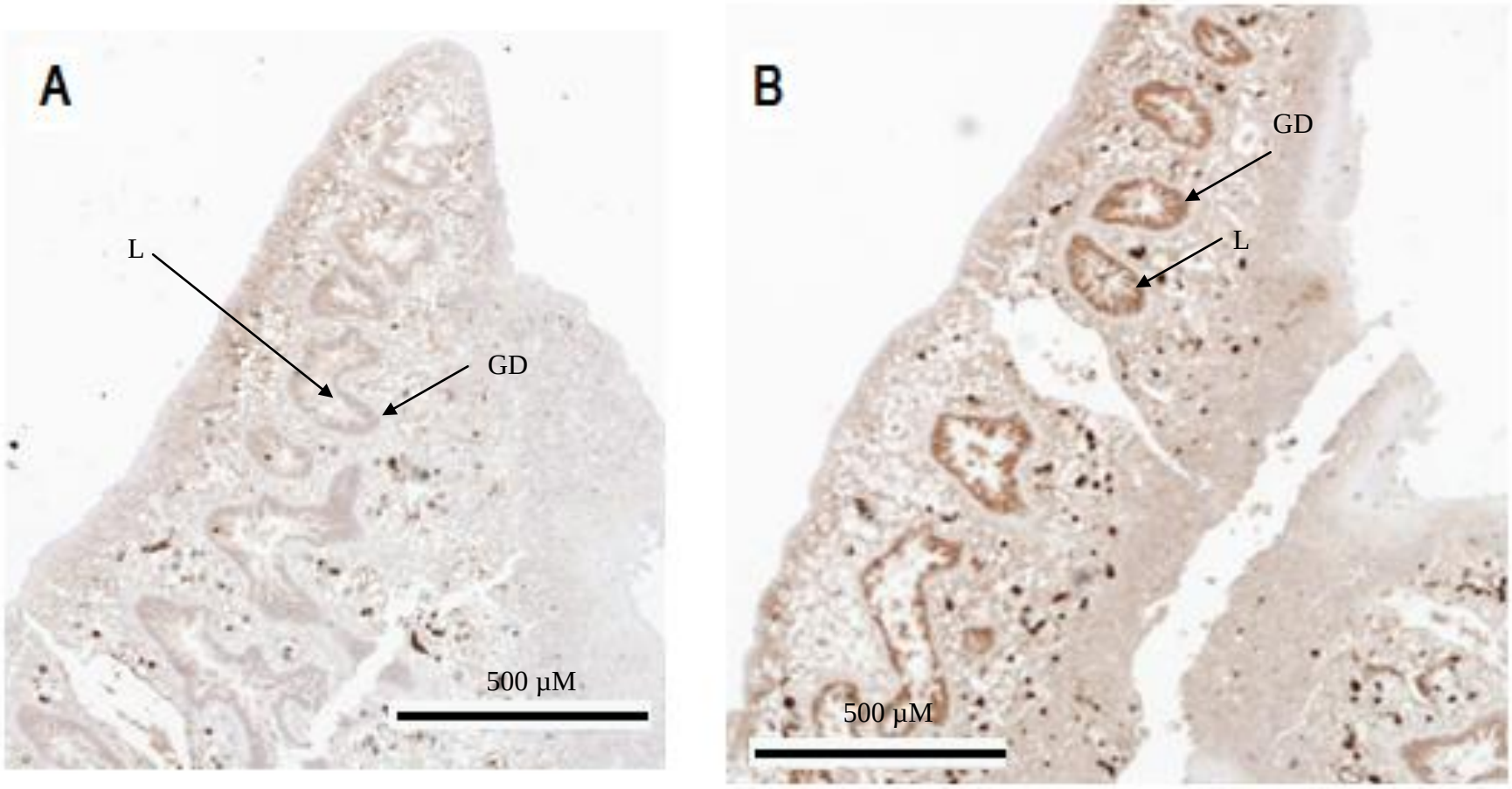

Figure 5.13 Localisation of cathepsin D in sections of the immature (10 days) stage of *F. hepatica.* Panel A was probed with pre-immune mouse sera as a negative control diluted 1: 10, 000 in PBST and Panel B was probed with anti- cathepsin D sera, diluted 1: 10, 000 in PBST. The gastrodermal cells (GD), lamellae (L) are highlighted.

5.3.8 Expression of cathepsin D in NEJ and adult stages of *F. hepatica*

It was evident that the adult stages of *F. hepatica* expressed cathepsin D as it was from a Triton X-100 soluble adult protein fraction (S3) that FhCatD was enriched using the PNA lectin, Chapter 2. However, it remains unclear whether the NEJ stages express cathepsin D. Primers were designed to amplify a fragment of β-tubulin, which was used as a housekeeping gene to allow comparison between the two stages. Internal primers were used to amplify a fragment of cathepsin D in both the NEJ and adult RNA.

Both life stages expressed the cathepsin D transcript, as shown in Figure 5.14. β-tubulin expression was evident from cycle 25 in the adults and cycle 30 in the NEJ stages. However, cathepsin D expression appeared at cycle 25 in the adults and cycle 25 in the NEJ stages. By inference, this indicates there is a slightly higher level of cathepsin D transcript present in the NEJ stages than that of the adults.

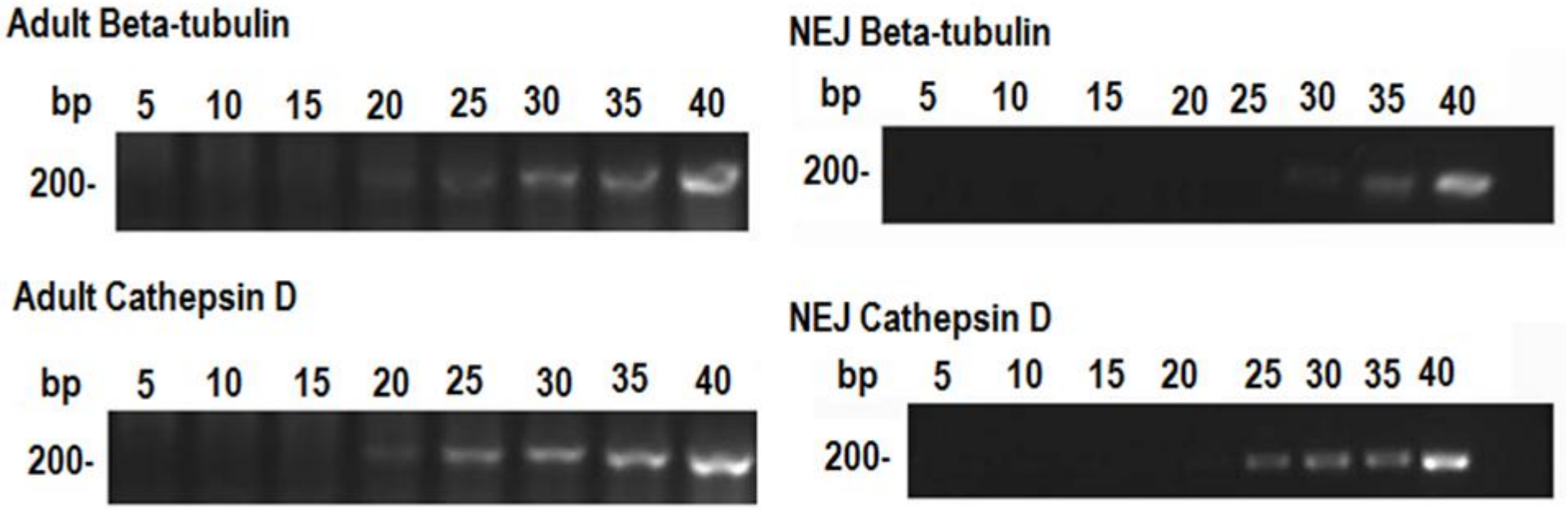

Figure 5.14: Expression of cathepsin D transcript by adult and NEJ stages of *F. hepatica.* The expression of cathepsin D was examined by performing RT-PCR with NEJ and adult RNA using internal cathepsin D primers. Expression of beta tubulin was included as an internal/housekeeping gene control to allow comparison between the life stages . A 5 µl sample were taken from each reaction at PCR cycle 5, 10, 15 20, 25, 30, 35 and 40. Base pair (bp) values from the 1kb plus DNA ladder (Invitrogen)

5.4 Discussion

Previous chapters have explored *F. hepatica* extracts using proteomics, lectins and enzymatic activity. The PNA lectin enriched a cathepsin D aspartyl protease which had previously not been identified in the crude fraction. There was also evidence of an aspartyl protease which has a possible role in the digestion of haemoglobin. This chapter has further characterised a *F. hepatica* cathepsin D-like aspartyl protease. There was a high level of identity between the protein sequence of FhCatD and cathepsin-D sequences from other parasites. Furthermore, the expression of FhCatD was localised to the gut of immature (10 day) and adult stages of the parasite. RT-PCR indicated that there may be a higher level of transcript in the NEJ compared to adult stages of the parasite. Probing native and recombinant versions of FhCatD with serum from sheep naturally exposed to *F. hepatica* did not provide clear evidence that the cathepsin D is strongly antigenic during natural infection.

PCR primers were designed to amplify the full length cathepsin D coding sequence from adult *F. hepatica* cDNA which was subsequently sequenced. This (FhCatD) showed 99% identity with an existing *F. hepatica* cathepsin D sequence (ABJ97285). The translated protein had 4 predicted transmembrane regions and carried a predicted signal peptide cleavage site. FhCatD was then aligned alongside 10 other cathepsin D enzymes from a range of parasites. There were high levels of homology between the cathepsin D proteases (see Table 5.2). This emphasises the importance of the cathepsin D structure to the function of the enzyme. It is proposed that aspartyl proteases have evolved from a common ancestor (Tang and Wong, 1987). In regards to the active site clefts, all sequences were identical between species. Establishing the relationship of FhCatD with other cathepsin D sequences placed FhCatD in a clade with the cathepsin D of *F. gigantica* and alignment found these shared 99% identity. The clade closest to these was composed of the cathepsin D from *S. mansoni* and *S. japonicum.* Like *F. hepatica* these are flukes, but they differ in morphology and habitual location (blood vessels as opposed to liver) (Schmidt and Roberts, 2005). These groupings were consistent with an alignment carried out by Suttiprapa *et al.*, (2009) to characterise Ov-APR-1, an aspartyl protease from the carcinogenic liver fluke *O. viverrini.* Aspartyl proteases have been proposed to function as digestive enzymes in some pathogens including *Plasmodium* (Goldberg *et al*., 1991), *A. caninum* (Harrop *et al*., 1996; Williamson *et al*., 2002, Williamson *et al*., 2003) and *S. japonicum* (Brindley et al., 2001). The aspartyl protease, cathepsin D is

thought to have a key role in haemoglobin proteolysis and host selection in hookworms (Williamson *et al.,* 2002). Furthermore, Na-APR-1 and Ac-APR-1 cleave at distinct sites within haemoglobin, with Ac-APR-1 preferentially cleaving at aromatic residues such as tryptophan and phenalanine and Na-APR-1 cleaving preferentially at hydrophobic, such as leucine, alanine and valine (Williamson *et al.,* 2002). Despite identical residues within the active site, subtle changes elsewhere in the protein have resulted in a different folding and ultimately different shape of the active site cleft (Williamson *et al.*, 2002). This has resulted in the distinct substrate cleavage positions of haemoglobin by cathepsin D from the different species and may also participate in substrate and host specificity (Williamson *et al.,* 2002).

Although it was possible to enrich cathepsin D using the PNA lectin in Chapter 2, the amount recovered was very low and a number of other proteins were present. A further attempt to purify large amounts of native cathepsin D was made using Pepstatin A affinity chromatography, however, this was unsuccessful. Previously, Pepstatin A chromatography was used in an attempt to enrich H-gal-GP from *H. contortus*. However, the complex bound so tightly to the Pepstatin A that it could not be eluted (Professor D. P. Knox, personal communication), which may explain why it was not possible to enrich FhCatD. Therefore, focus switched to generation of a recombinantly-derived version of FhCatD.

Insoluble rFhCatD was successfully generated using the pET SUMO system. The expressed rFhCatD could not, however, be purified by nickel affinity chromatography despite sequencing confirming the presence of the requisite poly histidine tag. Therefore, electroelution was used to purify the recombinant FhCatD directly from the bacterial proteins. The purified rFhCatD was then used to immunise mice and successfully raised cathepsin D-specific antibodies for immunolocalisation studies.

Immunohistochemistry localised rFhCatD to the gut of both immature (10 days) and adult stages of the parasite. Cathepsin D has also been localised to the gut of the hookworms *A. caninum and N. americanus* (Williamson *et al.,* 2002).

The main aim of this study was to assess the feasibility of identifying a ‘hidden antigen’ within *F. hepatica.* Therefore, to establish whether the FhCatD was genuinely a ‘hidden antigen’, Western blots were screened with sera from sheep naturally infected with *F.*

hepatica. Cathepsin L1, present in the E/S products of adult *F. hepatica* and widely used as a diagnostic antigen for infections of *F. hepatica* in the BIOX ELISA (Muino *et al.,* 2011), was used as a positive control. The outcome of these analyses was not clear. Relatively strong reactivity to FhCatD was noted, as shown in Figure **5.11** Nonetheless, this very equivocal reactivity combined with localisation to the gastrodermis (see below) and its partitioning in a PNA lectin-binding fraction of the membrane protein extract, is consistent with properties of known hidden antigens derived from *H. contortus* (Knox and Smith, 2001; Smith *et al.*,1999; Smith *et al.,* 1994; Smith *at al.*, 2003; Smith and Smith, 1996).

The immolocalisation data here show, clearly, that the cathepsin D is expressed in the gastrodermis of adult and juvenile *F. hepatica.* The parasite has no through-gut and regurgitates its gut contents every 2 -3 hours (Spithill and Dalton, 1998). If digestion of the haemoglobin took place extracellularly in the gut, these enzymes would be released from the parasite every few hours and, hence, may become exposed to the host immune system. The expression of *S. mansoni* cathepsin B, SmCB1 was located on the parasites gut by immunolocalisation studies (Sajid *et al.,* 2003), whereas Caffrey *et al.,* (2004) identified its presence in the gastrointestinal contents by a novel technique which measures the cleavage of a cathepsin B specific fluorgenic substrate. Adult *S. mansoni* were incubated in the fluorgenic substrate and stimulated to regurgitate their gut contents. By detecting fluorescence emission, digestion of the substrate could be detected, confirming the presence of SmCB1 in the gut contents (Caffrey *et al.,* 2004). This approach could help identify whether cathepsin D is present in the gut contents of *F. hepatica* and confirm if it is a secreted protein. Cathepsin D protease is expressed in a diverse range of mammalian cells and tissues but is located predominantly in lysosomes (Connor, 1998). It may then be excreted from the lysosomes into the gut lumen and then participate in lysis of ingested haemoglobin. Similarly, the aspartyl proteases of *A. caninum* (Ac-APR-1) and *N. americanus* (Na-APR-1) have also been localised to the parasite's gut but are soluble, secreted and function extracellularly (Williamson *et al.*, 2002), thus are not 'hidden' antigens. The two pepsin-like aspartyl proteases identified from the integral membrane complex H-gal-GP (Smith *et al..*, 2003) are considered true 'hidden' antigens (Smith *et al*, 1994). H-gal- GP has been localised to the brush border of *H. contortus* (Smith *et al.,* 1999). The FhCatD appears to be more like those of *A. caninum* and *N. americanus* where it is not an integral membrane and is secreted into the fluke's gut lumen which therefore suggests it is not a genuine "hidden antigen". However, this does not preclude

that FhCatD may potentially be a good vaccine candidate as previously proteins from the E/S material of *F. hepatica* have provided protection from infection with the parasite, for example the cathepsin Ls (Dalton *et al*., 1996; Piacenza *et al*., 1999). Vaccination with CatL1 and CatL2 can reduce the parasites' egg output by up to 69.7% and, further, those eggs produced have an 80% reduction in viability (Wijffels *et al*., 1994). When used individually in vaccination experiments, both the cathepsins significantly reduced worm burdens, egg output and egg fecundity (Dalton *et al.,* 1996). A vaccine trial with the FhCatD would identify if it confers protection from *F. hepatica.*

Expression of cathepsin D within the blood fluke *S. japonicum* has been demonstrated in the eggs, miracidia, and adults (male and female) (Verity *et al,* 1999). Within the adult stages, it is expressed on the parasite's gut and at higher levels in the females than males indicating that this enzyme plays a key role in the proteolysis of haemoglobin for nutrient acquisition to facilitate egg production (Brindley *et al.,* 2001). Unfortunately, the life stages of *F. hepatica* available for the project presented here were limited but reverse transcriptase PCR detected transcripts of cathepsin D in both the NEJ and the adults (those stages which are within the ovine host and of most interest). In contrast to adult *F. hepatica,* the NEJ do not contain any sexual organs (Bennett and Threadgold, 1973) but do have to migrate through the host to the liver, evading a number of immune effector mechanisms (Cancela *et al.,* 2010), as discussed in Chapter 1. Semi-quantitative RT-PCR indicated there may be a higher level of FhCatD transcript present in the NEJs than in adults and this may relate to the migration and evasion facets of the NEJ lifestyle. Cathepsin B is released by juvenile flukes (NEJs) and also similar to cathepsin L in the adult stages, facilitates migration and evasion of the host immune response (Wilson *et al,* 1998). Cathepsin B has been localised to the gut epithelium of NEJs (Creaney *et al.* 1995), and is thought to assist immune evasion (Carmona *et al.,* 1995; Chapman and Mitchell, 1982; Smith *et al.,* 1993) and migration through host tissues (Wilson *et al.,* 1998). Cathepsin D could also assist in these processes if it were excreted by this stage. The immature stages also shed and replace their glycocalyx every 3 hours while migrating to the bile duct to avoid immune recognition (Hanna, 1980). This will undoubtedly have a high metabolic demand and FhCatD may assist in nutrient acquisition to serve such a demand. Moreover, as both NEJ and adult stages contain the transcript it is plausible that they would both be affected if FhCatD were used as a vaccine. This is desirable, as the NEJ cause extensive damage when migrating through the host liver (Andrews *et al.,* 1999).

In conclusion, this chapter has characterised a cathepsin D-like aspartyl protease from adult *F. hepatica* which has previously not been published, to my knowledge. To date there is only one study which characterises a cathepsin D from a liver fluke, *O. viverrini* (Suttiprapa *et al.*, 2010). FhCatD had high levels of similarity with other nematode and trematode cathepsin D sequences. It is localised to the gut of both immature (10 day) and adult stages of the parasite, which is similar to the aspartyl proteases in *H contortus* (Smith *et al.*, 2003b) *A. caninum, N americanus* (Williamson *et al.*, 2002) *and S. japonicum* (Verity *et al.*, 1999). RT-PCR experiments suggested that there was a higher level of transcript in the NEJ compared to adult stages of the parasite. The NEJ grows rapidly and possibly has to process nutrients more rapidly than adults. No clear evidence of antigenicity during natural infection was found, consistent with the possibility that this cathepsin D may be a "hidden antigen" although, as discussed earlier, this is a very guarded conclusion. Vaccination studies with this protease would still be warranted given its localisation and possible function combined the with precedent from other blood-feeding parasites.

6 General discussion

Fasciolosis is caused by infection with the pathogenic flatworm *F. hepatica*. It is a serious production-limiting disease in sheep and cattle, responsible for huge losses within the livestock sector. Globally, these are estimated to be in excess of US $2 billion annually (Spithill *et al.*, 1999). These losses are attributable to mortality, reduction in milk and meat production, secondary bacterial infections, expensive anthelmintic treatment and condemnation of livers at slaughter (Garcia *et al.*, 2008; McKenna *et al.*, 2002; Schmidt and Roberts, 2005). Traditionally, fasciolosis was a problem in the wetter, milder west of the UK and Ireland (Baird, 2010; Kenyon *et al.*, 2009). However, over the last decade there has been an increase in the incidence of fasciolosis within the UK, with evidence of disease spreading from areas in the west to the east (See Figure 1.1). Control of *F. hepatica* is complicated by its complex life-cycle which involves both a definitive mammalian host and an intermediate mud snail host. The developmental stages within the intermediate mud snail host multiply asexually, therefore, a single egg shed from the definitive host has the potential to give rise to thousands of infective metacercariae (Schmidt and Roberts, 2005). Fasciolosis can be controlled to an extent by a combination of pasture management practices and methods to control the intermediate snail host populations, but often these approaches are impractical and only effective in the short term (Crossland, 1976; Urquhart *et al.*, 1970). Infection with *F. hepatica* is most commonly controlled by treating infected animals with anthelmintics (Roberts and Suhardono, 2008; Torgerson and Claxton, 1999), specifically, flukicides. Triclabendazole (TCBZ) has been the predominant compound for treating liver fluke infections since its introduction in the 1980s (Brennan *et al.*, 2007). This is because TCBZ is effective against early, immature and adult stages of the parasite, whereas most of the other flukicides only show activity against the adult stages (See Table 1.3) (Fairweather and Boray, 1999). The first evidence of TCBZ resistant fluke populations was reported in Australia (Overend and Bowen, 1995), and has since been reported in Ireland (Anon, 2005), United Kingdom (Mitchell *et al.*, 1998; Thomas *et al.*, 2000), The Netherlands (Gaasenbeek *et al.*, 2001) and Spain (Alvarez-Sanchez *et al.*,2006). Therefore, controlling fasciolosis by this method is unsustainable and alternative methods of control need to be investigated.

Vaccinating animals against *F. hepatica* is a desirable alternative control strategy to treating animals with flukicides due to consumer concerns about chemical residues in the food and environment (Science for Environment Policy, 2008) as well as the presence of anthelmintic resistant parasite populations. However, there has been limited success in developing vaccines against helminth parasites. "Dictol", an attenuated live vaccine against the bovine lungworm, *Dictyocaulus viviparus* was the first vaccine developed against a helminth parasite (Jarrett *et al.*, 1955; Jarrett and Sharpe, 1963) but its success has not extended to other helminths. Although sheep can develop natural immunity to some helminths, such as *T. circumcincta* (Smith *et al.*, 1984; Stear *et al.*, 1999), the mechanisms are not fully understood and there is little evidence to suggest that sheep sensitized to *F. hepatica* develop any protective immunity to reinfection (Boyce *et al*., 1987; Sandeman and Howell, 1981; Sinclair, 1971). The apparent lack of natural immunity and also lack of available helminth vaccines highlight how difficult it is to develop an effective fluke vaccine. However, through targeting secreted (natural) antigens released by parasites, protective immunity has been elicited against a number of parasites, including *F. hepatica* (Mulcahy and Dalton, 2001).

One of the biggest challenges in developing a vaccine against *F. hepatica* is the apparent lack of natural protective immunity in animals, particularly sheep, which have been exposed to the parasite (Spithill *et al*., 1999; Spithill and Dalton, 1998). However, there are a number of antigens which, when purified, have successfully elicited protection against infection with *F hepatica.* Sheep immunised three times with purified *F. hepatica* GST had a 57% reduction in worm burden at post mortem when compared to non- vaccinated controls (Sexton *et al*., 1990). Although immunising sheep with a mixture of the cathepsin L proteins (CatL1 and CatL2) did not reduce worm burdens, egg output was reduced by 69.7% and the eggs had an 80% reduction in viability (Wijffels *et al*., 1994). However, to date the highest level of protection elicited by a single antigen is by leucine aminopeptidase (LAP) (Piacenza *et al.*, 1999). Sheep were immunised with LAP in Freund's complete adjuvant twice at 4 week intervals then subsequently challenged with 500 metacercariae. Vaccinated animals had an 89% reduction in worm burden compared to non-vaccinated controls (Piacenza *et al*., 1999).

Some of these vaccine candidates were identified by investigating antigens which are essential for parasite survival including the cathepsin Ls, which have been shown to facilitate tissue and haemoglobin digestion (Smith *et al*., 1993b; Yamasaki *et al*., 1992) in addition to functioning

in immune evasion (Spithill and Dalton, 1998). Others were identified by investigating antigens homologous to protective vaccine candidates from other species such as the GSTs. These were chosen as candidate vaccine antigens (Sexton *et al.,* 1990) because homologous native GST proteins from *Schistosoma* spp. were shown to reduce parasite burdens in mice (40-43%) and rats (50-72%) (Balloul *et al.,* 1987; Brophy and Pritchard, 1994). Finally, other antigens were identified by investigating cross-protective antigens between fluke/parasite species; for example FABP (Fh12) was identified from a set of *F. hepatica* proteins which cross-reacted with antisera raised in mice to soluble proteins from the closely-related human blood fluke, *S. mansoni* (Hillyer *et al*., 1988; Hillyer *et al*., 1977). These antigens were shown to reduce *F. hepatica* worm burdens in immunised mice (by 69-78%) (Hillyer, 1985) and calves (by 55%) (Hillyer *et al.,* 1977).

An alternative approach, which has not yet been explored for the identification of vaccine candidates in *F. hepatica,* is the "hidden" antigen approach, which targets antigens not exposed to the host during the course of a natural infection (Munn, 1987). This approach has produced high levels of protection (up to 76%) against *R. microplus* tick infestation in cattle vaccinated with the gut-associated antigen BM86 (Willadsen *et al*., 1995). Also, native intestinal antigens from *H.contortus* have been purified and characterised and shown repeatedly to reduce both egg counts and worm burdens (Knox and Smith, 2001; Knox *et al*., 2003; Smith *et al*., 1999; Smith *et al*., 1994; Smith *et al*., 2000). Targeting "hidden" antigens could prove to be a novel and successful strategy for vaccinating against *F. hepatica*. Adult liver flukes live in a highly vascularised environment and their gut is filled with partially digested blood, see Chapter 1 Figure 1.5 (Smyth and Halton, 1983). Furthermore, as it migrates through the host the juvenile fluke ingests hepatic cells and haemoglobin (Oslen, 1986). Although it is not clear if any of the *F. hepatica* antigens investigated thus far are truly hidden, this project aimed to evaluate if it is possible to identify hidden antigens in *F. hepatica*. In attempting to do so, a number of techniques was employed including proteomics, exploiting the carbohydrate binding capacity of lectins and analysing the enzymatic activity of fractions of liver fluke.

Initially, a proteomics approach was employed to identify proteins within extracts of adult fluke which were exclusively localised to the S3 fraction of adult *F. hepatica.* Historically, an equivalent S3 fraction has been a rich source of gut-associated protective "hidden" antigens

from *H. contortus* e.g. H11 and H-gal-GP (McKerrow, 1989; Williamson *et al.,* 2003; Knox, 2010). The proteomic analysis identified four proteins of interest which were proteases exclusive to the adult *F. hepatica* S3 fraction; Cathepsin B2, Legumain-2, Lysosomal pro-x-carboxypeptidase precursor and Saposin-like protein (SAP-3). Cathepsin B2 is secreted by the juvenile stages of *F. gigantica* to facilitate digestion of host tissues and allow migration through the liver tissue (Meemon *et al.,* 2004). Legumain is a cysteine protease originally identified from the leguminous plant, *Canavalia ensiformis* and orthologues have been identified in *S. mansoni, S. japonicum, H. contortus* and *F. hepatica* (Sajid and McKerrow 2002; Carrey *et al.,* 2000; Oliver *et al.,* 2006) and it is also a potential diagnostic antigen of *Clonorchis sinensis* (Jung-won *et al.,*2009). Pro-x-carboxypeptidase is a lysosomal protease (Tan *et al.,* 1993) and is thought to function as an anticoagulant alongside saposin-like proteins to ensure effective lysis of ingested host blood in *H. contortus* (Geldhof and Knox, 2008; Oliver *et al.,* 2006). Saposin-like proteins identified from *F. gigantica* and the Chinese liver fluke, *C. sinensis* have a role in digesting host blood (Grams *et al.,* 2006; Lee *et al.,* 2002).

Despite the proteomic analysis being directed at the putatively membrane-bound S3 sub-fraction, this approach cannot determine the precise tissue or sub-cellular localisation of the protein(s) in question, so an alternative approach, Laser Capture Microscopy (LCM), was subsequently employed in an attempt to determine whether the transcripts encoding any of these molecules were located within the parasite's gut. This method permits isolation of specific tissues, in this case the liver fluke gut, from frozen or paraffin embedded tissue sections (reviewed in Jones *et al.,* 2004). Extracted RNA is then reverse-transcribed into cDNA and a representative sample cloned and sequenced to identify any tissue-specific transcripts. LCM is a relatively novel method which is still being optimised for different applications. While the method used in Chapter 2 was first published by Nilaweera *et al.,* (2009) unfortunately, it failed to produce viable RNA from liver fluke. This may be due to subtle methodological differences in the LCM method previously used to capture gut tissue from *N. americanus, A. caninum (Ranjit et al., 2006)* and *S. japonicum* (*Gobert et al., 2009).* However, the most likely explanation is that the RNA may have been degraded prior to LCM, as the parasites were extracted around two hours after the liver was removed from the ovine host at post-mortem.

As this thesis aimed to identify antigens derived from the gut of *F. hepatica*, an alternative approach, which sought to identify lectins with an affinity for glycoproteins on the gut of *F. hepatica,* was used. Lectins are carbohydrate-binding proteins derived from plants, animals and micro-organisms, with specificity for terminal or sub-terminal carbohydrate residues (Leathem and Brooks, 1998). Lectins have previously been used for isolating candidate vaccine antigens by affinity chromatography, as shown in the preparation of the gut-associated protective antigens H11 and H-gal-GP, from the parasitic nematode, *H. contortus,* (Knox and Smith, 2001; Smith *et al*., 1994; Smith *et al*., 2000a; Smith *et al*., 2000b). Chapter 3 explored the potential of using lectins to enrich glycoproteins from the S3 extract. To this end, an initial screen of histological sections of adult fluke with a panel of 21 different lectins, identified seven with an affinity for the gut of *F. hepatica.* Two of these lectins, PNA and JAC, preferentially bound to carbohydrates on the gastrodermal cells or lamellae, respectively. These were then chosen to enrich the membrane-bound S3 fraction, which was subsequently analysed by LC-ESI-MS/MS. This analysis revealed that the PNA- and JAC-enriched fractions shared a number of proteins. However, exclusive to the PNA lectin enriched S3 was a cathepsin D-like aspartyl protease, FhCatD, which had not previously been characterised in *F. hepatica*. This is a novel and potentially significant finding because immunisation of dogs with cathepsin D-like aspartyl proteases has been shown to protect against challenge infection by the hookworms, *A. caninum* and *N. americanus* (Loukas *et al.,* 2005). Furthermore, aspartyl proteases, HcPEP1 and HcPEP2, are key components in the H-gal-GP vaccine complex which gives protection against *H. contortus* (Smith *et al.,* 2003).

Proteases have, historically, made good vaccine candidates which emphasises the importance of further evaluation. The “hidden” antigen, H-gal-GP, comprises a complex of different enzyme components, including metallopeptidases and aspartyl proteases, and is involved in digesting the blood meal in *H. contortus* (Smith *et al.,* 2003; Ekoja and Smith, 2011). Thus, Chapter 4 aimed to identify which enzyme classes in the S1, S2 and S3 sub-fractions were participating in lysis of haemoglobin, a major protein component of host blood. Initially the broad substrate gelatin was used to establish the presence of active proteases. Gelatin-substrate gel analysis identified proteases which were active over a broad pH (3-9) and size range in all extracts. The addition of the reducing agent, DTT, enhanced proteolysis, confirming the presence of active cysteine proteases, some of which had previously been identified in the proteomic screen described in Chapter 2 (Appendix 2). A number of methods were used to

investigate haemoglobin digestion by the respective extracts. Haemoglobin hydrolysis was extensively investigated by measuring the amount of solubilised protein at 280 nm, which estimates the extent of protein breakdown. This identified two peaks of activity by all three extracts at pH 2-2.5 and at pH 4, with no evidence of digestion at alkaline pH. This finding was consistent with previous studies investigating haemoglobin digestion by blood-feeding parasites such as *P. cuniculi* (Nisbet & Billingsley, 2000), *R. microplus* (Mendiola *et al.*, 1996), *Ixodes ricinus* (Horn *et al.*, 2009), *Schistosoma spp, Ancylostoma spp., N. americanus* and *H. contortus* (Williamson *et al*., 2003). The addition of class-specific inhibitors to the haemoglobin lysis reactions (Section 4.1.11, Chapter 4) indicated that both cysteine and aspartyl peptidases had a role in the hydrolysis of haemoglobin by liver fluke extracts. Four protease classes have been proposed to function in a cascade in the digestion of haemoglobin in blood-feeding parasites (Williamson *et al.,* 2003; Horn *et al.,* 2009). Aspartyl proteases, such as cathepsin D, are thought to act in the initial stages of haemoglobin digestion (Williamson *et al.,* 2003). However, the cathepsin D identified from the PNA-enriched S3 fraction was not identified by proteomics in the crude S3 extract (see Chapters 3 and 2 respectively). This does not necessarily imply that cathepsin D was absent from these fractions, rather, it most likely reflects the resolution of the proteomic approach employed and that cathepsin D is present at a relatively low level. However, the addition of Pepstatin A inhibited haemoglobin hydrolysis at pH 2 in S1, S2 and S3, indicating the presence of an aspartyl protease in all three somatic extracts. To confirm that cathepsin D was present in these extracts, their ability to hydrolyse a cathepsin D-specific peptide 7-methoxycourin-4-acetyl-gly-lys-pro-ile-leu-phe-arg-leu-lys(DNP)-D-arg-amide (CatDFS) (see Figure 4.12) was examined. The substrate was hydrolysed by proteases from each extract with greatest hydrolytic activity at pH 2-2.5. Taken together, these experiments indicated the presence of an aspartyl protease active at pH2-2.5 which could hydrolyse a cathepsin D-specific substrate.

As the work described in Chapter 3 identified a cathepsin D in a PNA-enriched S3 extract and that described in Chapter 4 indicated its importance in haemoglobin digestion, Chapter 5 aimed to further characterise this novel *F. hepatica* cathepsin D. Cathepsin D is highly conserved among many eukaryotic species and is proposed to have evolved from a common ancestor (Tang and Wong, 1987). Sequence alignment and phylogenetic analyses demonstrated that the cathepsin D identified here shared a high degree of homology with previously deposited full coding sequences from *F. hepatica* NEJ [ABJ97285], *F. hepatica*

adult [AC104164.1] and *F. gigantica* [ABJ97285] cathepsin Ds, sharing approximately 99% identity with each. Using Pepstatin A affinity chromatography, it was not possible to enrich or purify the native FhCatD sufficiently for immunising mice, so a bacterially-derived recombinant version (rFhCatD) was generated using the Champion pET SUMO expression system. The recombinant protein was subsequently used to immunise BALB/c mice to generate antibodies for immunolocalisation studies. FhCatD was localised to the gut of both immature (10 day post infection) and adult stages of *F. hepatica* (see Figure 5.24 and Figure 5.26), a pattern consistent with the expression of cathepsin D in the hookworms *N. americanus* and *A. caninum* (Williamson *et al.*, 2002). Furthermore, semi-quantitative RT-PCR indicated that there was a higher level of FhCatD transcript in the NEJ stages than in the adult stages of *F. hepatica.* Probing immunoblots of rFhCatD and PNA-enriched S3 fractions with serum from sheep naturally exposed to *F. hepatica* demonstrated immunoreactivity to rFhCatD, indicating that it may not be a 'hidden' antigen, as antibodies from the serum bound both the recombinant and native antigen preparations. The initial steps of haemoglobin digestion by blood feeding parasites are proposed to involve aspartyl proteases (Williamson *et al.*, 2003), and, in *F. hepatica*, this is thought to occur at the lining of the lamellae, with the digestion products then being endocytosed and further digested inside the gut cell (Fairweather *et al.*, 1999). *F. hepatica* has a blind-ending gut and regurgitates its gut contents every 2 -3 hours (Spithill and Dalton, 1998). Therefore, if digestion of the haemoglobin took place extracellularly in the gut, these enzymes may be secreted by the parasite every few hours and thus be exposed to the host's immune response.

Ultimately, this thesis investigated whether there are 'hidden' antigens in *F. hepatica.* The project focused on a cathepsin D-like aspartyl protease and results from Chapter 5 indicate that this antigen maybe exposed to the host during the course of a natural infection. A number of hypotheses may possibly explain these findings. As mentioned above, *F. hepatica* regurgitate their gut contents every 2-3 hrs (Spithill and Dalton, 1998). However, to date the presence of cathepsin D in any of these extracts has not been documented (Cancela *et al.*, 2010). FhCatD appears similar to the aspartyl proteases of *A. caninum* (Ac-APR-1) and *N. americanus* (Na-APR-1) in amino acid sequence and has also been localised to the gut of *F. hepatica*, see Chapter 5 (Williamson *et al.*, 2002). The aspartyl proteases of the hookworms are soluble, actively secreted and function extracellularly (Williamson *et al.*, 2002). FhCatD contains a signal peptide and aspartyl protease activity was identified in the S1 and S2 fractions (PBS-

soluble and membrane-associated, respectively) which may suggest that FhCatD is also secreted. The two pepsin-like aspartyl proteases identified from the integral membrane complex H-gal-GP (Smith *et al..,* 2003) are considered true 'hidden' antigens (Smith *et al*, 1994). It may be that for an aspartyl protease to be a true 'hidden' antigen that it must form a complex with other insoluble membrane-bound components. Thus the aspartyl protease gut-associated antigens from *S. japonicum, N. americanus and A. caninum* all appear to be secreted, whereas those from the nematode *H. contortus* are resident and membrane bound. These differences could relate to variation in the architecture and/or function of the gut between the respective helminth species.

If a vaccine trial were to be conducted, larger quantities of the native FhCatD would be required. This could be facilitated by scaling-up the PNA lectin chromatography and/or by attempting different purification techniques. Large numbers of adult *F. hepatica* can be obtained from abattoirs and each adult parasite yields approx 3 mg of S3 extract (data not shown) so this could be feasible. Also, dose response trials could be conducted and it may be that the vaccine is very protective with very low protein concentrations required. Alternatively, vaccination studies with recombinant versions of cathepsin D have provided considerable levels of protection. For example, a study by Verity *et al.*, (2001) involved immunising mice four times with the bacterially-expressed form of *S. japonicum* cathepsin D (rSjASP-1) on days 0, 14, 56 and 105 then challenging 14 days after the last inoculation with 36 *S. japonicum* cercariae. Immunised mice had a reduced total worm burden (by 37.8 %) in comparison to non-vaccinated controls (Verity *et al.,* 2001). Mice immunised with the insect cell-expressed recombinant version, r*Sj*ASP-2 had a reduction in total worm burden of 20.9 % and in female worm burden by 29.9 % in comparison to non-vaccinated controls (Verity *et al.*, 2001). Therefore, it may be possible to use the recombinant form of FhCatD to conduct vaccination trials to assess the potential of this particular antigen.

Initial small scale trials in mice or sheep would be warranted to identify what effect vaccinating with FhCatD has on worm burden, egg fecundity and reducing liver damage. These trials could evaluate both native (if it proved possible to purify sufficient quantities for immunisation) and recombinant forms of FhCatD, which would indicate whether there is a difference in the protective capacity of these. Recently the first successful, reproducible protection against a helminth infection in sheep using a recombinant antigen cocktail was

described (Nisbet *et al.,* 2012). A cocktail of five recombinant immunogenic and three putatively immunosuppressive *T. circumcincta* molecules was used to immunise sheep which were then trickle challenged with *T. circumcincta* infective larvae. Vaccinated animals had an overall mean FEC reduction of 72% and 58% in two independent trials (Nisbet *et al.,* 2012). Furthermore, vaccinated sheep had 75% and 57% lower burdens of adult worms in the abomasal lumen, for trials 1 and 2 respectively, than those in the control groups (Nisbet *et al.*, 2012). The Human Hookworm Initiative aims to reduce the human suffering caused by hookworms through vaccination (Hotez *et al.,* 2003). Currently, the two lead vaccine candidates are GST-1 and *Na*-APR-1 which are used in combination (Hotez *et al.,* 2003). Combinations of native *F. hepatica* antigens (CatL1/L2 and LAP) have provided high levels of protection. For example, sheep immunised with a combined vaccine of LAP and CatL1/L2 had fluke burdens reduced by 76% in comparison to non-vaccinated controls (Acosta *et al*., 2008; Piacenza *et al*., 1999). It would be interesting to evaluate the effect of combining the FhCatD identified here with FhGST, FhCatL1/L2 or LAP had on the levels of protection. Moreover, identifying whether rFhCatD is enzymatically active, by investigating its ability to cleave host haemoglobin and the synthetic cathepsin D substrate (Chapter 5, CatDFS) would also be beneficial as it would provide more information on the nature of the recombinant form.

To assess the specific function of cathepsin D in *F. hepatica* further, RNA interference (RNAi) could be used. RNAi utilises double-stranded (ds) RNA to induce an intracellular cascade that ultimately results in the suppression of expression of homologous mRNA transcripts. To date, RNAi has been successfully applied in *S. mansoni* sporocysts (Boyle *et al*., 2003), *S. japonicum* larvae and schistosomulae (immature stages) (Skelly *et al*., 2003; Cheng *et al*., 2005) and *F. hepatica* NEJ (McGonigle *et al.,* 2008). The information yielded from suppression of FhCatD would help identify the possible roles of FhCatD in parasite development and survival. For example, suppressing *F. hepatica* cathepsin B and cathepsin L genes reduced the ability of NEJ to penetrate the gut during their migration to the liver, suggesting that these genes have an important role in tissue penetration (McGonigle *et al.*, 2008).

FhCatD could also be further characterised by investigating the expression levels throughout the different life-cycle stages of *F. hepatica* using quantitative real time polymerase chain (qPCR). qPCR has been previously used in *F. hepatica* to compare gene expression levels of

legumain 1 and legumain 3 between NEJ and adult stages (Cancela *et al.,* 2010). Here the expression levels of FhCatD in eggs, sporocysts, rediae, cercariae, metacercariae, NEJs, immature and adult flukes could be compared to identify which stages have the highest levels of expression. As with RNAi, this would help yield information regarding the possible roles of FhCatD depending on differences in expression levels throughout development. Furthermore, it may indicate which life-cycles stages a vaccine would be effective against. The gut of the NEJ has a largely secretory role with the dual absorptive/secretory function beginning to develop 10 days post-infection when the parasite reaches the liver (Bennett and Threadgold, 1073). The parasite has a fully functional dual absorptive and secretory gut once it has matured to an adult (Bennett and Threadgold, 1973; Wilson et al., 1998; Dalton et al., 2006) so it would be interesting to evaluate how the expression pattern of FhCatD changes as the gut develops.

Due to limitations on time and access to appropriate parasite material, only two attempts were made to use LCM to investigate the *F. hepatica* gut. Revisiting the LCM approach and optimising the protocol in line with the hookworm studies (as described in Ranjit *et al.,* 2006) and *S. japonicum* (Gobert *et al.,* 2009) may allow RNA of sufficient quantity and quality to be extracted from the parasite's gut. In the hookworm study, parasites were snap frozen in Optimal Cutting Temperature (OCT, Tissue-tek) and after sectioning were washed with diethylpyrocarbonate (DEPC) water to remove OCT (Ranjit *et al.,* 2006). The method used for *S. japonicum*, described by *Gobert et al., (2009),* also involved washing slides with DEPC treated water to remove OCT, but this was not done here. The availability of gut-specific RNA will help yield valuable information about the repertoire of proteins potentially expressed within or on the fluke's gastrodermal cells, which could then be further investigated for their potential as 'hidden' antigens. It is not altogether clear if cathepsin D is secreted by the fluke as, to date, it has not been reported in E/S material from adult *F. hepatica* (Morphew *et al.*, 2007). Antibodies from sheep naturally exposed to the parasite bind rFhCatD. As discussed in Chapter 5, it would be possible to evaluate whether a cathepsin D-like aspartyl protease (such as FhCatD) is present in the E/S material by a novel technique which has previously been used to measure the cleavage of a cathepsin B-specific fluorogenic substrate (Caffrey *et al.,* 2004). Parasites are incubated in the fluorogenic substrate (specific to that enzyme) and stimulated to regurgitate their gut contents. By detecting fluorescence emission, the digestion of the substrate could be measured, confirming the presence of that enzyme in the gut contents

(Caffrey *et al.*, 2004). This approach could help identify whether cathepsin D is present in the gut contents of *F. hepatica* and confirm if it is a secreted protein or not.

In conclusion, the work presented in this thesis has demonstrated unequivocally the presence of a cathepsin D-like aspartyl protease in a membrane-bound (S3) fraction of *F. hepatica*, which can be enriched by PNA lectin chromatography. Further characterisation of FhCatD demonstrated its involvement in the digestion of haemoglobin, which was optimal at pH 2-2.5 and confirmed by the cleavage of a cathepsin D-specific substrate. FhCatD was localised to the gut surface of both juvenile (10 days) and adult stages of the parasite. However, antibodies from sheep naturally exposed to *F. hepatica* bound rFhCatD indicating that it is unlikely to be a genuine "hidden" antigen. This thesis aimed to evaluate the "hidden" gut antigen approach to vaccination in *F. hepatica.* It may be that because of the secretory nature of its gut, that it is not feasible to identify such an antigen. Although FhCatD is potentially exposed, it does not preclude the possibility that this antigen could be highly protective. The higher transcript levels present in the NEJ compared to adult stages may also indicate that any resulting vaccine would be more effective against these stages. Ultimately the NEJ stages cause extensive damage when migrating through the host to the liver so targeting this stage is desirable. However, a vaccine trial would be required to determine if FhCatD is truly protective.

Appendix 1 : Standard Buffers and solutions

A.1 General Solutions and buffers

A.1.1 1 x Phosphate buffered saline (PBS)

A 10 x stock solution was prepared by dissolving 80 g sodium chloride, 2 g potassium chloride, 11.5 g di-sodium hydrogen orthophosphate and 2g potassium di-hydrogen orthophosphate in 1 litre of dH_2O. A working solution was prepared by diluting 1 in 10 in dH_2O

A.1.2 TNTT

In a beaker the following was combined; 50 ml 1 M Tris, 146.1 g NaCl, 2.5 ml Tween 20 and 0.5 g Thimerasol. This was made up to 5 litres with dH_2O and the pH adjusted to 7.4 using HCl.

A.1.3 Lectin wash buffer (LWB)

In a beaker the following was combined; 6.057g Tris, 8.7g NaCl, 0.203g $MgCl_2$, 0.111g $CaCl_2$ and made up to 500 ml with dH_2O. The pH was adjusted to 7.6 using concentrated HCl and the final volume made up to 1000 ml.

A.1.4 Scots tap water

3.5 g of Sodium bicarbonate and 20g of Magnesium sulphate were added to a beaker and made up to 1000 ml.

A.2 Protein extraction buffers

A.2.1 Homogenising buffer 1 (HB 1)

0.148g of EDTA were added to 400mls of 1 x PBS and pH adjusted to 7.4. HB stored at 4ºC until required. Immediately prior to using 1 ml of 100mM phenylmethylsulphonyl fluoride was added per 100mls of buffer.

A.2.2 Homogenising buffer 2 (HB 2)

0.148g of EDTA and 400ul of Tween 20 were added to 400mls of 1 x PBS and pH adjusted to 7.4. HB 2 stored at 4ºC until required. Immediately prior to using 1 ml of 100mM phenylmethylsulphonyl fluoride was added per 100mls of buffer.

A.2.3 Homogenising buffer 3 (HB 3)

4 ml of reduced triton x 100 were added to 200mls of 1 x PBS and pH adjusted to 7.4. HB 3 stored at 4ºC until required. Immediately prior to using 1 ml of 100mM phenylmethylsulphonyl fluoride was added per 100mls of buffer.

A.3 Laser Capture microscopy materials

A.3.1 10% Gelatin

10 g of gelatin were dissolved in 100mls of 1 x PBS by heating and stirring gently. This is prepared fresh as required.

A.3.2 Diethylpyrocarbonate (DEPC) treated water

1ml of 0.1% Diethylpyrocarbonate (DEPC) was dissolved in 1000ml of dH_2O. This was then autoclaved at 121ºC for 15mins and allowed to cool prior to use.

A.4 PCR and cloning

A.4.1 Tris-acetate-EDTA (TAE) buffer

A concentrated 50 x solution of TAE was made by combining 242 g Tris base, 57.1 ml Glacial acetic acid and 100 ml 0.5M EDTA (pH 8) then made up to 1 litre with dH_2O.

A.4.2 DNA sample buffer

A 6 x gel loading buffer was made by combining 0.25 % Bromophenol blue, 0.25 % Xylene cyanol FF, 40 % (w/v) Sucrose in water. This was stored at 4°C.

A.4.3 SOC media

2 g Bacto®-tryptone, 0.5 g Bacto®-yeast extract, 0.05 g NaCl, 1 ml 250 mM KCl was dissolved in 100ml of dH_2O. This was autoclaved at 121 ºC for 15 mins. Once cooled 0.5 ml filter-sterilised $MgCl_2$ (1.9 g $MgCl_2$ in 10 ml water) and 2 ml filter-sterilised glucose (1.8 g glucose in 10 ml water) were added. Aliquots were taken and stored at -20ºC.

A.4.4 Luria Bertani (LB) medium

LB medium was prepared by dissolving 10g Bacto-tryptone, 5 g Bacto-yeast and 5 g sodium chloride in 1 litre of dH_2O. This was autoclaved at 121ºC for 15mins.

A.4.5 LB agar

LB agar was prepared by dissolving 15g bacto-agar in 1 litre of LB medium and autoclaving at 121ºC for 15mins.

A.4.6 LB agar plates containing IPTG/X-GAL/Ampicillin

Agar plates were prepared by melting LB agar in a microwave, then cooling to 50ºC in a water bath. The following were added to 50ml of molten agar; 250 µl of 0.1 IPTG, 200 ul of ampicillin stock (25 µg/ml) and 80 µl of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). The agar was mixed and poured into petri dishes and allowed to set at room temperature. Petri dishes were stored at 4ºC until required.

A.4.7 LB plates with 50 µg/ml Kanamycin

Agar plates were prepared by melting LB agar in a microwave, then cooling to 50ºC in a water bath. The following were added to 50ml of molten agar; 50 µl of kanamycin (50 mg/ml) The agar was mixed and poured into petri dishes and allowed to set at room temperature. Petri dishes were stored at 4ºC until required.

A.4.8 10 ml of LB broth with ampicillin

Under a flame, 40 µl of ampicillin (25 µg/ml) were added to 10 ml of LB medium

A.4.9 10 ml of LB broth with kanamycin

Under a flame, 10 µl of kanamycin (50 mg/ml) were added to 10 ml of LB medium

A.5 Substrate gel electrophoresis materials

A.5.1 1.5 M Tris-HCl Resolving buffer with SDS, pH 8.8

90.8 g of tris and 2 g of SDS were added to a beaker and made up to 400 mls with dH_2O. This was allowed to dissolve with the addition of a low heat. Once dissolved the pH was adjusted to 8.8 with concentrated hydrochloric acid (HCl).

A.5.2 0.5 M Tris-HCl Stacking buffer with SDS, pH 6.8

30.285 g of Tris and 2 g of SDS were added to a beaker and made up to 400 mls with dH_2O. This was allowed to dissolve with the addition of a low heat. Once dissolved the Ph was adjusted to 6.8 with concentrated hydrochloric acid (HCl).

A.5.3 SDS PAGE non reducing sample buffer

A 2 x stock of non reducing sample buffer was prepared by dissolving 2 g sucrose, 8 mg bromophenol blue and 0.2 g SDS in 17.1 mls of 0.5 M Tris-HCl, pH 6.8.

A.5.4 2.5% (v/v) Triton X-100

2.5 ml of Triton X-100 were added to 97.5 ml dH_2O and dissolved

A.5.5 SDS PAGE Tank buffer for substrate gels

A 10 x stock solution was prepared by dissolving 144 g glycine, 70 g 2-Amino-2-hydroxymethyl-propane-1,3-diol (tris) and 10g sodium dodecyl sulphate (SDS) in 1 litre of distilled water. This solution was diluted to a 1 x solution in distilled water for use.

A.5.6 0.25% Coomassie Blue stain

In a beaker, 2.5 g of coomassie brilliant blue R-250 (BioRad) were dissolved in 450 ml Methanol by stirring. Once dissolved, 450 ml dH_2O and 100 ml glacial acetic acid were added. The mixture was then filtered through whatman paper

A.5.7 Gel destainer

In a beaker 450 ml of Methanol, 450 ml of dH2O and 100 ml of Acetic acid were combined.

A.6 Ph buffers

A.6.1 0.1 M Sodium acetate pH 3

4.1 g of sodium were added to 400 ml of dH_2O and the pH adjusted to 3 by the addition of concentrated acetic acid.

A.6.2 0.1 M Sodium acetate pH 5

4.1 g of sodium were added to 400 ml of dH_2O and the pH adjusted to 5 by the addition of concentrated acetic acid.

A.6.3 0.1M Tris-HCl pH 7

6.05g of Tris were added to 400 ml of dH_2O and the pH adjusted to pH 7 by the addition of concentrated HCl.

A.6.4 0.1 M Tris-HCl ph 9

6.05g of Tris were added to 400 ml of dH_2O and the pH adjusted to pH 9 by the addition of concentrated HCl.

A.6.5 0.2 M Sodium Acetate pH 3

8.2 g of sodium were added to 400 ml of dH_2O and the pH adjusted to 3 by the addition of concentrated acetic acid.

A.6.6 0.2 M Sodium Acetate pH 4

8.2 g of sodium were added to 400 ml of dH_2O and the pH adjusted to 4 by the addition of concentrated acetic acid.

A.6.7 0.2M Sodium Acetate pH 5

8.2 g of sodium was added to 400mls of dH_2O and the pH adjusted to 5 by the addition of concentrated acetic acid.

A.6.8 0.2 M Sodium acetate pH 6

8.2 g of sodium were added to 400 ml of dH_2O and the pH adjusted to 6 by the addition of concentrated acetic acid.

A.6.9 0.2 M Tris-HCl pH 7

12.1 g of Tris were added to 400 ml of dH_2O and the pH adjusted to pH 7 by the addition of concentrated HCl.

A.6.10 0.2 M Tris-HCl pH8

12.1 g of Tris were added to 400 ml of dH_2O and the pH adjusted to pH 8 by the addition of concentrated HCl.

A.6.11 0.2 M tris-HCl pH 9

12.1 g of Tris were added to 400 ml of dH_2O and the pH adjusted to pH 9 by the addition of concentrated HCl.

A.6.12 Stock 1 M DTT

1.5g of DTT were dissolved in 10 ml of dH_2O, stored at room temperature.

A.7 Protein expression and purification

A.7.1 IPTG solution

A 0.1 M solution of isopropyl β-D-1-thiogalactopyranoside (IPTG) was prepared by dissolving 238g in 10 ml dH_2O, filter sterilized and stored in 500 µl aliquots.

A.7.2 PepA binding buffer (0.1 M sodium citrate + 0.5 M NaCl, pH3)

29.412 g of sodium citrate and 2.92 g of sodium chloride were dissolved in 800 ml of dH_2O. The pH was adjusted to 3 with the addition of concentrated citric acid and the final volume made up to 1000 ml with dH_2O.

A.7.3 PepA Washing buffer (0.5 M NaCl)

2.92 g of sodium chloride were dissolved in 1000 ml of dH_2O.

A.7.4 PepA elution buffer (0.1 M Sodium carbonate + 0.5 M NaCl, pH 8.7)

8.4 g of Sodium bicarbonate and 2.92 g sodium chloride were dissolved in 800 ml of dH_2O. The pH was adjusted to 8.7 with concentrated HCl and the final volume made up to 1000 ml with dH_2O

A.7.5 Binding Buffer

20 ml 0.1 M Sodium Phosphate Buffer pH 7.4 were diluted with 80 ml dH2O. To this 2.92 g NaCl and 0.136 g Imidazole were added and allowed to dissolve.

A.7.6 Binding Buffer + 8M Urea

48 g of Urea were dissolved in 100 ml of Binding Buffer.

A.7.7 Elution Buffer 1

20 ml 0.1 M Sodium Phosphate Buffer pH 7.4 were diluted with 80 ml dH2O. To this 2.92 g NaCl and 0.34 g Imidazole were added and allowed to dissolve.

A.7.8 Elution Buffer 2

20 ml 0.1 M Sodium Phosphate Buffer pH 7.4 were diluted with 80 ml dH2O. To this 2.92 g NaCl and 1.36 g Imidazole were added and allowed to dissolve.

A.7.9 Elution Buffer 3

20 ml 0.1 M Sodium Phosphate Buffer pH 7.4 were diluted with 80 ml dH2O. To this 2.92 g NaCl and 2.38 g Imidazole were added and allowed to dissolve.

A.7.10 Elution Buffer 4

20 ml 0.1 M Sodium Phosphate Buffer pH 7.4 were diluted with 80 ml dH2O. To this 2.92 g NaCl and 3.4 g Imidazole were added and allowed to dissolve.

A.7.11 Elution Buffer Stock (with 8 M urea)

80 ml 0.1M Sodium Phosphate Buffer, 320 ml dH2O, 11.68 g NaCl and 192 g Urea were combined.

A.7.12 Elution Buffer 1 + 8M Urea

20 ml 0.1 M Sodium Phosphate Buffer pH 7.4 were diluted with 80 ml dH2O. To this 2.92 g NaCl and 0.34 g Imidazole were added and allowed to dissolve 100 ml Elution Buffer Stock

A.7.13 Elution Buffer 2 + 8M Urea

1.36 g Imidazole were added to 100 ml Elution Buffer Stock

A.7.14 Elution Buffer 3 + 8M Urea

2.38 g Imidazole were added to 100 ml Elution Buffer Stock

A.7.15 Elution Buffer 4 + 8M Urea

3.4 g Imidazole were added to 100 ml Elution Buffer Stock

A.7.16 1 x electrophoresis tank buffer with SDS

A 5 x Stock of electrophoresis was prepared by combining 30.8 g Tris, 144g Glycine, 10g SDS in a beaker then made up to 2 litres with dH_2O. A 1 x working stock was prepared by diluting 200 ml of stock with 800 ml dH2O .

A.7.17 ½ x electrophoresis tank buffer without SDS.

In a beaker 1.9 g Tris, 9 g Glycine were dissolved in 250 ml dH_2O.

A.7.18 Ponceau S stain

1g Ponceau S was added to 50ml acetic acid and made up to 1 litre with dH2O.

A.7.19 Immunolocalisation buffer

5 ml of Tween 80 were dissolved in 1000 ml of 1 x PBS. As required, 1 ml of normal goat serum was added to 10 ml of 1 x PBS + 0.5% Tween 80.

Appendix 2: Proteins identified as unique to the S3 fraction

Protein description	S3 Unique?	Species	No. Peptides	% Coverage	Protein length	Protein score	Protein mass	Contig
1. **Proteolytic enzymes**								
1.1 Cysteine proteases								
Cathepsin B2	Y	*T. szidati*	2	2.9	491	60	55648	10637
Cathepsin B	N (1/2)	*S. japonicum*	4	7.1	448	111	49694	11829
Calpain B	N (1/2)	*S. japonicum*	2	6.2	385	34	44322	12814
Cathepsin B	N (1/2)	*F. gigantica*	4	7.1	411	55	46424	14718
Cathepsin L	N (1/2)	*F. heptica*	2	2.5	363	32	39516	22509
Legumain-2	Y	*F. gigantica*	4	10.1	318	41	36523	11863
Legumain-2	Y	*F.gigantica*	8	26.8	190	100	21886	12100
Legumain-2	Y	*F. gigantica*	4	10.9	359	31	40793	18333
1.2 Aminopeptidases								

Leucyl aminopeptidase	N (1/2)	*F. hepatica*	2	15.4	143	49	15564	106739
1.3 Serine /Carboxypeptidase								
Putative Lysosomal Pro-X carboxypeptidase precursor	Y	*S. japonicum*	3	3.7	626	41	70862	11786
1.4 Metalloproteases								
Peptidase M16 precursor	Y	*C. sinensis*	3	4.1	581	66	65330	11902
2. Metabolism								
2.1 Amino Acid								
SJCHGC02362 protein	Y	*S.japonicum*	16	9.3	400	71	44046	12397
Pyruvate dehydrogenase	N (2)	*S.mansoni*	15	3	1058	51	116338	201
Threonine dehydratase	N (1)	*S.mansoni*	4	5.1	668	32	73723	886
Glutaminase, putative	Y	*S.mansoni*	6	6.3	574	29	64261	1676
Arginine n-methyltransferase	Y	*S. mansoni*	7	2.2	464	30	51468	2073

Glutamate--cysteine ligase catalytic subunit	Y	*S.japonicum*	6	7.4	609	27	67510	3181
SJCHGC01355 protein	Y	*S.japonicum*	15	8.6	476	103	53204	9995
Hadha protein	Y	*D. rerio*	30	12.1	281	53	31223	12059
Hadha Protein	Y	*X. tropicalis*	75	11.4	631	113	69060	11598
Hadha Protein	Y	*X. tropicalis*	10	7.9	367	132	39555	12191
2.2 ATP synthesis/Electron transport								
Ubiquinol-cytochrome c reductase complex 14 kDa protein	N (1/2)		2	8.4	178	28	20184	11878
NADH-ubiquinone oxidoreductase sgdh subunit, putative	Y	*S. mansoni*	3	14.1	270	112	31187	10902
NADH-ubiquinone oxidoreductase, putative	Y	*S. mansoni*	3	3.2	879	38	96342	12017
NADH:ubiquinone reductase 42kD subunit precurs	Y	*S. japonicum*	4	11.5	487	156	57153	12128
NADH dehydrogenase (ubiquinone) Fe-S protein 2	N (1/2)	*S. japonicum*	3	10.2	502	53	57229	55043

NADP transhydrogenase, putative	Y	*S. mansoni*	4	2.5	1265	68	133084	12159
Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor	N (1/2)	*S. japonicum*	3	10.8	418	59	46393	12806
Ubiquitin-protein ligase BRE1, putative	N (1/2)	*S. mansoni*	2	1.4	1526	25	174264	4515
ATP synthase, subunit d	N (1/2)	*S. japonicum*	3	20.1	219	106	25061	11921
SJCHGC06640 protein	Y	*S. japonicum*	3	6.6	500	91	55242	11401
AF303222_1 SNaK1	Y	*S. mansoni*	6	2	1826	40	201910	8926
SJCHGC07036 protein	Y	*S. japonicum*	2	6.9	216	53	23165	13280
tyrosine kinase	N(2)	*S. mansoni*	8	0.7	1054	31	114838	2814
calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type (calcium pump)	Y		3	3.4	1470	46	162738	288
peptidyl-prolyl cis-trans isomerase-like 4. ppil4	Y	*S. mansoni*	9	6.8	487	26	54253	551
cell division control protein, putative	N (1/2*)	*S. mansoni*	3	3	1029	47	109177	6292

SJCHGC05891	Y	*S. japonicum*	30	2.3	779	27	88749	3698
SMDR1	Y	*S. mansoni*	4	3.1	419	30	45119	8316
SMDR2	N(1)	*S. mansoni*	6	2.3	1331	54	148168	9153
smap1, putative	Y	*S. mansoni*	2	6.6	469	29	52745	916
ATP synthase gamma subunit, putative	N (1/2)	*S. mansoni*	3	11.2	330	99	36112	9531
F-type H+-transporting ATPase subunit f (SJCHGC06289 protein)	Y	*S. japonicum*	2	16	200	70	22442	54931
mitochondrial ATP synthase B subunit	N (2)	*C. sinensis*	3	25.5	157	119	17912	47829
plasma membrane calcium-transporting atpase, putative	N (1)	*S. mansoni*	4	2.7	1079	55	120394	7301
2.3 Carbohydrate								
succinate-Coenzyme A ligase, ADP-forming, beta subunit	N (1/2)	*S. japonicum*	4	2.1	479	30	53051	2268
glycogen phosphorylase, putative	Y	*S. mansoni*	6	2.7	1069	33	117962	2897

expressed protein [S. mansoni]	Y		10	3.7	753	29	79039	949
phosphatidylinositol 4-kinase	N (1/2)	*S. mansoni*	6	2.3	1446	28	163894	13262
alpha-glucosidase	N (1)	*S. japonicum*	3	5.6	665	101	74247	12094
2.4 Citric acid cycle								
succinate dehydrogenase complex, subunit B, iron sulfur	N (1/2)	*S. japonicum*	2	6.8	177	48	21099	13003
succinate dehydrogenase, putative	N (1/2)	*S. mansoni*	6	12.9	249	42	27562	12353
SJCHGC06566 protein	Y	*S. japonicum*	4	4.1	242	56	27256	7508
2. 5 Fatty Acid								
PREDICTED: similar to acyl-coa dehydrogenase	Y	*A. pisum*	2	5.2	690	48	76421	12285
2.6 Glycan								
SJCHGC00848 protein	Y	*S. japonicum*	6	12.5	634	202	73361	11988
oligosaccharyl transferase, putative	Y	*S. mansoni*	2	6.4	597	29	65055	8521

helicase, putative	N (1/2)	*S. mansoni*	6	3.3	1159	27	130461	4877
helicase, putative	N (1/2)	*S. mansoni*	6	3.3	1159	27	130461	4877
PREDICTED: similar to amylo-1,6-glucosidase, 4-alpha-glucanotransferase	Y	*G.gallus*	6	2.1	1886	45	207578	476
2.7 Other								
protein kinase	N (1/2)	*S. mansoni*	3	5.5	867	32	93958	3284
serine/threonine kinase	N (1/2)	*S. mansoni*	3	5.6	593	30	66537	14335
serine/threonine kinase	N (1/2)	*S. mansoni*	2	2.4	1227	40	132192	1725
serine/threonine kinase	N (1/2)	*S. mansoni*	4	4.1	591	27	66815	4039
serine/threonine kinase	N (1/2)	*S. mansoni*	4	4.1	591	27	66815	4039
serine/threonine kinase	N (1/2)	*S. mansoni*	2	4	505	33	55252	6869
serine/threonine protein kinase	N (1/2)	*S. mansoni*	3	4.9	628	25	68507	c2874
tyrosine kinase	N (1/2)	*S. mansoni*	4	6.2	838	63	92919	2448

tyrosine kinase	N (1/2)	*S. mansoni*	8	3.6	1420	38	156086	4503
Tyrosine-protein kinase CSK	N (1/2)	*S. japonicum*	6	1.8	885	26	97380	3900
Tyrosine-protein kinase CSK	N (1/2)	*S. japonicum*	6	1.8	885	26	97380	3900
cation-transporting atpase 13a1 (G-box binding protein), putative	N (1/2)	*S. mansoni*	3	2.3	1416	33	157321	5898
sphingomyelin phosphodiesterase	Y	*C. sinensis*	2	9.8	264	98	29512	12098
PREDICTED: similar to Dehydrogenase/reductase (SDR family) member 1	Y	*S. purpuratus*	16	22.6	106	39	11389	80532
predicted protein	Y	*N. vectensis*	5	3.1	416	49	44917	55504
SJCHGC06250 protein	Y	*S. japonicum*	2	2.6	811	97	92779	7569
histidine acid phosphatase, putative	Y	*S. mansoni*	2	19.4	129	37	13970	8437
Peptidyl--hydroxyglycine--amidating lyase	Y		2	6.5	463	29	52642	c11147
defender against cell death, putative	Y	*S. mansoni*	2	8.9	158	48	17841	13142

unknown [Glycine max]	Y		2	3	565	38	65800	4404
3. Transport								
PRA1 family protein 3		*S. japonicum*	2	8.4	262	77	29485	11639
phospholipid transport protein	N (2)	*S. mansoni*	2	5.5	326	32	37476	1078
sodium/dicarboxylate cotransporter-related	Y	*S. mansoni*	4	4.7	1339	37	150267	1019
integral membrane protein	Y	*S. mansoni*	5	14	429	153	48607	11756
putative Vesicle-associated membrane protein-associated protein A (SJCHGC09425 protein)	Y	*S. japonicum*	2	5.2	636	123	70940	11089
ABC transporter, putative	Y	*S. mansoni*	4	7.6	406	37	47051	20770
mitochondrial carrier protein, putative	N (2)	*S. mansoni*	3	7.5	548	77	60748	12171
signal recognition particle 54 kD protein, putative	Y	*S. mansoni*	4	6.5	672	29	74916	12550
SJCHGC09085 protein	Y	*S. japonicum*	3	5.7	714	47	79507	13548
mitochondrial phosphate carrier protein, putative	Y	*S. mansoni*	3	12.6	380	42	42459	20986

exocyst componenet sec8, putative	Y	*S. mansoni*	2	5.3	453	38	50312	2690
inositol transporter	Y	*S. mansoni*	2	1.3	844	32	93713	270
fer-1-related	N (1)	*S. mansoni*	3	9.6	552	57	60380	3000
Tubulin beta-2C chain	N (1/2)	*S. japonicum*	4	4	547	39	61257	3317
vacuolar membrane protein pep11, putative	Y	*S. mansoni*	2	3.4	504	26	56427	3405
multidrug resistance protein 1 (ATP-binding cassette C1), putative	Y	*S. mansoni*	6	11.4	517	29	58247	877
adapter-related protein complex 1 gamma subunit (gamma-adaptin)	Y	*S. mansoni*	2	2.1	1094	29	119304	1854
putative Golgi SNAP receptor complex member 2	Y	*S. japonicum*	6	11.4	403	37	45916	4383
exocyst complex component 1	Y	*S. mansoni*	2	6.6	482	27	53279	1425
mitochondrial 2-oxoglutarate/malate carrier protein	Y	*S. mansoni*	3	11.4	342	48	37597	354
translocon-associated protein, delta subunit	Y	*S. mansoni*	2	10.4	211	34	23970	12432

larval alpha-globin	Y	*H. retardatus*	2	12.2	123	31	13288	c76246
transmemberane protein, putative	Y	*S. mansoni*	2	4.8	567	69	64904	9563
clasp2 protein, putative	Y	*S. mansoni*	3	3.2	1204	35	134899	839
4. Inhibitors								
Phosphatase 2A inhibitor I2PP2A	Y	*S. japonicum*	2	8	387	26	42873	11757
rab GDP-dissociation inhibitor, putative	Y	*S. mansoni*	20	4.4	654	27	73002	12018
5. Cytoskeleton proteins								
hypothetical protein TcasGA2_TC013346	Y	*T.castaneum*	5	2.9	2446	41	271821	10
actin	N (1/2)	*T.similis*	6	15.7	451	145	50438	16962
hypothetical protein BRAFLDRAFT_118210	N (1/2)	*B. floridae*	3	3.2	1205	28	137556	21
PREDICTED: axonemal outer arm dynein intermediate chain 2	Y	*C. intestinalis*	2	3.5	837	26	91774	225
dynein heavy chain, putative	N (1/2)		2	6	548	34	62113	5394

nudix-type motif 6	Y	*X. laevis*	2	4.1	460	26	52201	8129
erythrocyte membrane protein, putative	Y	*S. mansoni*	3	4.2	993	29	108608	12243
troponin t, invertebrate, putative	Y	*S. mansoni*	3	7.4	568	35	64106	14984
actin-related protein 10, arp10	N (1/2)	*S. mansoni*	2	5.4	485	31	55327	4051
6. Transcription								
endonuclease-reverse transcriptase	N (1/2)	*S. japonicum*	3	5.3	472	27	51676	14338
chromatin assembly factor I P60 subunit, putative	Y	*S. mansoni*	3	2.2	773	33	87098	1656
RAB family	N (1/2)	*S. japonicum*	2	6.4	313	37	36440	17352
reverse transcriptase	N (1)	*Synthetic construct*	4	18.2	154	24	17458	19648
chromatin regulatory protein sir2, putative	Y	*S. mansoni*	3	6	664	27	74567	2295
trimeric G-protein alpha o subunit, putative	Y	*S. mansoni*	12	7.7	560	71	63993	408
mRNA-capping enzyme, putative	Y	*S. mansoni*	4	2.2	760	25	81250	70

splicing factor 3b, subunit 4 (fragment), putative	N (1/2)	*S. mansoni*	1	2.4	418	32	48941	7137
ccr4-not transcription complex, putative	N (1)	*S. mansoni*	3	4.1	1000	36	112992	7493
Homeobox protein ceh-18	Y	*S. japonicum*	2	7.6	368	28	41097	8007
flightless-I, putative	Y	*S. mansoni*	4	4.4	1071	31	120864	7861
pumilio, putative	Y	*S. mansoni*	2	4.2	780	30	87435	842
DNA polymerase zeta catalytic subunit	N (1/2)	*S. mansoni*	2	36	89	30	10273	c30321
heterotrimeric G-protein alpha subunit, GPA1-like protein	N (1/2)	*Laccaria bicolor S238N-H82*	16	17.2	128	27	13958	c88967
transcription initiation factor tfiid	Y	*S. mansoni*	2	22.9	105	33	12268	c89439
similar to cyclin K in Homo sapiens	N (1)	*S. japonicum*	2	6.5	154	39	17076	65443
reverse transcriptase [synthetic construct]	N (1)		2	5.6	372	26	42912	56639
serine-rich repeat protein , putative	Y	*S. mansoni*	2	3.7	490	27	54027	1268
PREDICTED: similar to endonuclease-reverse	N (1/2)	*S. purpuratus*	2	5.1	447	25	47727	7263

transcriptase								
sphingomyelin phosphodiesterase	Y	*C. sinensis*	2	4.2	625	107	70361	11752
sphingomyelin phosphodiesterase	Y	*C. sinensis*	2	9.8	264	98	29512	12098
PREDICTED: similar to transposase	N (2)	*S. purpuratus*	2	3.1	582	26	63797	881
7. Gut Proteins								
secreted saposin-like protein SAP-3	N (2)	*F. gigantica*	2	12.9	85	66	9803	14594
8. Signalling								
G-protein, beta subunit, putative	N (1/2)	*S. mansoni*	2	5.8	640	50	72427	11691
SJCHGC05537 protein	Y	*S. japonicum*	2	5.4	722	38	81328	10747
tubulin beta-2	N (1/2)	*F. hepatica*	4	4.8	495	34	55683	11855
glucose transport protein, putative	Y	*S. mansoni*	2	3.4	931	46	101651	11949
f-box and wd40 domain protein, putative	N(2)	*S. mansoni*	16	5.8	995	31	111060	12038
ormdl proteins, putative	Y	*S. mansoni*	3	6.1	788	56	87084	12144

pak-interacting exchange factor, beta-pix/cool-1, putative	Y	*S. mansoni*	2	2.3	1162	33	128582	1503
kalirin, putative	Y	*S. mansoni*	2	2.6	1260	27	139578	1647
expressed protein	Y	*S. mansoni*	4	2.4	1741	34	192870	2796
WD repeat protein 78	Y	*S. japonicum*	3	5.3	928	31	98790	4492
protein kinase	N (1/2)	*S. mansoni*	30	4.6	931	27	100920	4683
protein kinase	N (1/2)	*S. mansoni*	2	6.1	594	33	65359	4976
glycogen synthase kinase 3-related (gsk3) (cmgc group III)	Y	*S. mansoni*	60	3.7	1250	30	138851	5576
TGF-beta signal transducer Smad2, putative	Y	*S. mansoni*	24	5.5	1027	48	112863	617
PREDICTED: similar to germ cell associated 2 (haspin)	Y	*T. guttata*	3	4.2	984	26	111142	6684
inositol triphosphate 3-kinase C	Y	*S. mansoni*	6	4.6	411	36	47493	c10001
transient receptor potential cation channel subfamily m member	Y	*S. mansoni*	2	10.3	312	30	36580	c13353

SJCHGC09376 protein	Y		5	10.2	187	25	21906	c2580
histidine acid phosphatase	Y	*S. mansoni*	2	18.7	155	32	16306	c32624
long-chain-fatty-acid--CoA ligase	N (1)	*S. mansoni*	8	5.8	360	28	41495	c7934
signal peptidase 25 KDa chain	Y	*C. sinensis*	4	9.4	203	49	23072	57790
Receptor expression-enhancing protein 5	Y	*S. japonicum*	3	7.7	418	64	47993	11870
Gag-Pol polyprotein	N (1)	*S. japonicum*	3	6.1	734	27	82722	12662
TPA_exp: pol polyprotein	N (1)	*S. mansoni*	3	2.2	1291	34	144229	16444
TPA_exp: pol polyprotein	N (1)	*S. mansoni*	2	2.9	649	26	74051	1896
WD-repeat protein	Y	*S. mansoni*	2	4	450	43	48003	21859
coatomer protein complex, subunit alpha	N (1/2)	*Bos taurus*	3	1.9	1471	31	166006	4846
Ras guanine nucleotide exchange factor	Y	*S. mansoni*	4	2.9	799	26	87302	442
rgpr-related	Y	*S. mansoni*	3	8.4	581	30	65068	9347

9. Gene transcrition								
lung cancer metastasis-related (lcmr1) protein	Y	*S. mansoni*	2	7.5	371	31	41853	5073
10. Heat shock and chaperon proteins								
Heat shock protein 67B2	N (1/2)	*S. japonicum*	3	14.1	227	105	25182	10449
chaperonin containing t-complex protein 1, theta subunit, tcpq, putative	Y	*S. mansoni*	3	6.7	596	160	65611	11923
heat shock protein, putative	N (1/2)	*S. mansoni*	2	2.5	565	27	64422	12202
peptidyl-prolyl cis-trans isomerase, cyclophilin-type family protein	Y	*T. thermophila SB210*	3	27.6	163	141	18253	13035
cullin 3	Y	*S. japonicum*	6	5.5	854	33	94542	1172
SmIrV1 protein, putative	Y	*S. mansoni*	9	54.3	81	37	9207	9355
11. Ribosomal proteins								
Ribosomal protein L17 (SJCHGC09296)	N(1/2)	*S. japonicum*	4	8.3	229	29	25071	11754
60S ribosomal protein L13a, putative	N(1/2)	*S. mansoni*	4	3.5	231	28	27173	11831

pescadillo-related	Y	*S. mansoni*	12	4	831	33	93063	1404
small subunit ribosomal protein S5	N(1/2)	*S. japonicum*	2	5	558	44	60840	4989
12. Other Function								
Serologically defined colon cancer antigen 1	N (1)	*S. japonicum*	3	5.3	664	30	76898	5309
PREDICTED: similar to KIAA0174	Y	*H.magnipapillata*	2	10	201	28	23336	5991
Progesterone-induced-blocking factor	Y	*S. mansoni*	3	9.5	409	32	46126	c7596
SCAMP family	N (1/2)	*S. mansoni*	2	15.6	147	27	15746	c34708
putative F./S. cross-reactive protein	Y	*F. hepatica*	2	17.9	168	35	18312	c27650
AF303222_1 SNaK1	Y	*S. mansoni*	6	3.2	1825	163	204036	8926
tetraspanin		*S. japonicum*	1	1.5	741	42	83621	11957
IP15837p		*D.melanogaster*	3	3.4	861	29	94231	12230
Estrogen-regulated protein EP45 precursor		*S. japonicum*	5	10.8	574	266	65397	12332

sortingnexin-related		*S. mansoni*	3	8.9	483	34	55785	12387
tetraspanin-CD63 receptor , putative		*S. mansoni*	3	12.4	510	55	58080	12848
SJCHGC00563 protein		*S. japonicum*	2	3.6	392	56	44979	12967
regulator of chromosome condensation, putative		*S. mansoni*	3	3	910	27	102856	1378
metazoan probable membrane protein, putative		*S. mansoni*	4	6.2	761	42	84585	1412
cornichon, putative		*S. mansoni*	4	5.7	1310	40	139802	1579
tetraspanin, putative		*S. mansoni*	2	4.5	596	106	67336	3879
loss of heterozygosity 11 chromosomal region 2 gene a protein homolog (mast cell surface antigen 1) (masa-1), putative		*S. mansoni*	2	2.4	1094	50	122402	20995
SJCHGC09145 protein		*S. japonicum*	2	10.5	143	31	16132	261955
SJCHGC00713 protein		*S. japonicum*	2	4	347	28	38238	2509
PREDICTED: similar to IQ domain-containing protein D		E. caballus	3	3.9	719	37	77549	366

SJCHGC06304 protein		*S. japonicum*	3	3	1217	62	137090	5407
yip1-related	Y	*S. mansoni*	2	21.6	153	30	16599	109774
polyprotein		*S. japonicum*	3	15.9	151	30	17173	c3677
286 kDa polyprotein		*C. leprosis virus C*	2	2	1534	32	171152	9607
13. Hypothetical Proteins								
hypothetical conserved protein		*S. mansoni*	3	13.7	277	28	31722	13352
hypothetical protein		*S. japonicum*	3	4.8	888	38	102566	150
hypothetical protein		*S. mansoni*	2	2.8	740	30	83683	1614
conserved hypothetical protein		*Salinispora arenicola CNS-205*	2	8	387	30	43668	19968
hypothetical protein BRAFLDRAFT_118210		*B. floridae*	3	3.2	1205	28	137556	21
conserved hypothetical protein		*S. mansoni*	3	2.4	1689	33	192527	2577

hypothetical protein		*S. mansoni*	2	4	521	28	58700	2978
hypothetical protein		*S. mansoni*	2	4.3	350	48	39613	7914
hypothetical protein		*S. mansoni*	2	4.2	569	26	64948	8174
conserved hypothetical protein		*S. mansoni*	2	4.7	666	29	74581	1782
hypothetical protein		*S. mansoni*	3	5	899	33	96320	1020
hypothetical protein BRAFLDRAFT_74627		*B. floridae*	3	6.2	801	41	88402	10495
hypothetical protein		*S. mansoni*	3	9.3	496	44	53905	11814
hypothetical protein		*S. mansoni*	3	10.2	401	29	40006	11886
hypothetical protein		*S. japonicum*	3	6.5	415	38	46155	11910
hypothetical protein		*S. mansoni*	3	2	1280	33	143372	122
SJCHGC05740 protein		*S. japonicum*	2	12.6	206	28	22983	14838
hypothetical protein		*S. mansoni*	3	19	231	31	26344	c17628

hypotheticial protein		*S. japonicum*	1	8.3	192	41	22524	c27899
hypothetical protein		*S. mansoni*	2	11.1	144	27	15696	c67014
hypothetical protein		*S. mansoni*	2	12.2	123	50	13832	85108
hypothetical protein		*S. mansoni*	2	8.6	152	41	16820	62259
hypothetical protein		*Coprinopsis cinerea okayama*	2	23.8	122	43	13238	89766
hypothetical protein		*S. japonicum*	3	2.6	794	45	92812	4390
hypothetical protein		*S. mansoni*	2	2.4	757	25	85943	607
hypothetical protein		*S. mansoni*	3	8.1	445	27	49725	6601
12. Unknown								
expressed protein		*S. mansoni*	3	4.3	1189	41	131579	10076
CAZ318			18	7.3	717	44	80860	12911
Unknown			3	6.7	477	119	53467	11847

Unknown			2	8.4	405	39	44747	11319
expressed protein		*S. mansoni*	5	4.7	1077	58	121488	11056
CA			2	11.7	188	60	21194	12453
			4	4.3	1363	32	154400	1485
15153			2	9.6	374	29	42413	15153
16524			2	6.8	146	25	16244	16524
1738			2	14.3	189	38	21093	1738
expressed protein		*S. mansoni*	2	3.6	583	25	63592	1909
21880			2	9.7	319	33	36109	21880
expressed protein		*S. mansoni*	3	16.5	266	141	29309	21911
22198			2	6.7	267	39	29497	22198
expressed protein		*S. mansoni*	2	2.9	690	32	76475	2271

expressed protein		*S. mansoni*	2	8.2	291	26	32448	2299
232			2	5.7	598	27	67436	232
2325			2	3	880	38	93147	2325
2496			2	7.5	199	25	22328	2496
2710			3	4.4	996	48	109936	2710
SJCHGC04075 protein		*S. japonicum*	3	3.9	1084	41	118502	283
expressed protein		*S. mansoni*	2	6.9	320	40	35696	3009
308			2	1.8	896	33	99766	308
expressed protein		*S. mansoni*	3	3.2	1100	32	122615	3104
expressed protein		*S. mansoni*	2	5.4	350	27	39953	3289
3401			2	3	434	27	47856	3401
expressed protein		*S. mansoni*	2	2.3	731	39	81664	3575

3785			3	2.4	710	29	80577	3785
expressed protein		*S. mansoni*	2	2.4	916	31	102745	381
4030			2	6.7	375	30	40956	4030
427			2	6.4	423	29	46022	427
430			3	10.6	417	34	48036	430
SJCHGC09036 protein		*S. japonicum*	7	16.9	449	187	50350	404
462			3	8.1	421	31	45155	462
expressed protein			2	5.3	380	52	43596	4508
5195			2	3.8	524	27	57629	5195
expressed protein		*S. mansoni*	2	3.6	675	35	74533	5282
expressed protein		*S. mansoni*	4	2.7	1923	35	220165	548
5529			2	4.3	470	27	53193	5529

6013			2	12.6	253	27	29585	6013
expressed protein		*S. mansoni*	2	11.6	292	25	33119	7384
expressed protein		*S. mansoni*	1	1.1	796	27	89114	7663
expressed protein		*S. mansoni*	3	2.1	1274	35	141025	779
c13893			2	10.5	143	27	15824	c13893
c162320			2	11.9	135	28	15635	c162320
c169			2	2.8	714	30	77689	c169
c170809			1	7.8	128	27	14086	c170809
c17567			2	6.8	281	43	30992	c17567
8568			2	18.1	215	28	24275	8568
SJCHGC03581 protein		*S. japonicum*	2	9.3	226	35	25152	862
889			2	1.9	890	51	98428	889

9007			2	14.3	189	86	20985	9007
c105196			2	17.2	134	39	14147	c105196
c109127			2	22	123	26	13984	c109127
c11023			2	4.2	522	26	56164	c11023
c18606			2	17.1	146	32	16339	c18606
c20788			2	10.9	201	31	22219	c20788
c24695			2	35.2	71	31	8127	c24695
c25920			3	12.5	176	33	19799	c25920
c2836			3	11.7	412	25	44205	c2836
c35383			2	21.4	154	30	17223	c35383
c68013			2	18.1	144	34	15259	c68013
c69832			2	21.9	151	24	16931	c69832

c72475			2	14.6	144	30	15084	c72475
c80338			2	13.3	158	35	17410	c80338
c84817			2	11.3	239	27	26848	c84817
c8673			2	17.5	114	30	12087	c8673
c9468			3	16.9	189	31	22327	c9468
unknown		*S. japonicum*	2	3.9	690	59	76281	54913
predicted protein		*Laccaria bicolor S238N-H82*	2	17.8	118	89	12344	76963
polyprotein		*S. japonicum*	2	15.6	135	32	14994	76358
67810			2	26.4	110	25	12035	67810
64522			2	7.5	174	27	19564	64522
65198			2	9.3	150	26	16064	65198
SJCHGC08978 protein		*S. japonicum*	2	16.7	126	82	13928	69652

SJCHGC02303 protein		*S. japonicum*	2	7.1	524	31	58846	1132
SJCHGC09134 protein		*S. japonicum*	5	13	461	142	53149	11795

Appendix 3 : Proteins identified from JAC agglutinin affinity chromatography enriched

	Accession	Protein	MW (kDa)	Mowse Scores	No. of Peptides
1. Proteolytic enzymes					
1.1 Cysteine protease	fhep00187|2	Cathepsin L	194.1	283.4	5
	fhep00889|1	Cathepsin L	66.4	94.6	2
	fhep00344|1	cathepsin L protein	193.9	361.2	8
	fhep03421|1	cathepsin L protein	103.6	311.2	7
	fhep00577|1	cathepsin L protein	52.5	262.4	6
	fhep00623|2	cathepsin L protein	93.5	163.2	3
	fhep00739|1	cathepsin L1D	178.6	131.5	4
	fhep04385|1	cathepsin L1D	57.3	90.8	2
	fhep02273|1	Cathepsin L-like protein	100.1	282.1	7
	fhep03629|1	secreted cathepsin L 1	47.9	203.8	5

	fhep02735|1	RecName: Full=Cathepsin L-like protein	85.6	246.9	5
	fhep02461|1	RecName: Full=Cathepsin L-like protein	48.7	209.5	5
	fhep42601|1	secreted cathepsin L2	76.9	272.6	6
1.2 Aminopeptidase	fhep00458|1	legumain-2	119.7	47.2	2
	fhep00030|1	leucyl aminopeptidase	148.7	560.5	8
2. Metabolism					
2.1 Amino Acid	fhep07024|1	14-3-3 protein	61.2	127.1	4
	fhep01036|1	adenylate kinase	128.3	192.2	3
	fhep00922|1	Aldolase	71.0	90	2
	fhep00054|1	fructose-16-bisphosphatase-related	133.9	46.3	2
	fhep01092|1	fructose-bisphosphat	172.5	626.4	9
	fhep05385|1	fructose-bisphosphate aldolase	57.0	243.7	3

	fhep00365|1	hemoglobin F2	60.7	339.4	6
	fhep04489|1	hemoglobin F2	82.8	314.8	5
	fhep40916|1	hemoglobin F2	42.4	233.8	4
	fhep17472|1	Hydroxyacyl dehydrogenase	37.7	171.5	3
	fhep02757|1	hydroxyacyl-Coenzyme A dehydrogenase	65.7	42.6	2
	fhep11982|1	PREDICTED: Gag-Pol polyprotein	66.5	53.8	3
	fhep17681|1	PREDICTED: Gag-Pol polyprotein	29.1	35.2	2
	fhep28249|1	PREDICTED: similar to endonuclease	20.5	34.2	2
2.2 ATP synthesis/ Electron transport	fhep00563|1	acetyl-CoA carboxylase; methylc	218.8	1900	30
	fhep01948|1	ATP synthase alpha subunit mitosis	61.0	381.7	5
	fhep01951|1	ATP synthase, H+ transporting, mito	78.9	784.2	12
	fhep40820|1	ATP:ADP antiporter	84.6	353.8	7
	fhep09076|1	ATP:ADP antiporter	48.5	384.1	6

	fhep01654|1	branched chain ketoacid dehydrogenase	67.5	85.6	2
	fhep00533|1	ferritin-like protein	81.4	77.2	2
	fhep06472|1	glycine cleavage system H protein	30.0	161.9	3
	fhep23828|1	GTP:AMP Phosphotransferase	58.3	270.3	3
	fhep41156|1	mitochondrial ATP synthase B subunit	79.2	89.9	2
	fhep14046|1	NADP-dependent malic enzyme	33.7	304.8	7
	fhep00031|1	NADP-dependent malic enzyme	67.4	336.8	6
	fhep00755|1	NADP-dependent malic enzyme	49.4	115.5	3
	fhep00221|1	ornithine aminotransferase	154.7	923.8	16
	fhep00109|1	PREDICTED: Methylmalonyl-CoA	57.8	335.8	5
	fhep00640|1	prohibitin	106.6	59	2
	fhep40677|1	propionyl-CoA carboxylase beta	9.9	88.7	2
	fhep01734|1	Propionyl-CoA carboxylase beta	65.1	715.6	9

	fhep00451|1	propionyl-CoA carboxylase beta	73.1	38.9	2
	fhep07002|1	RecName: Full=Fatty acid-binding protein	39.9	217.6	5
	fhep01997|1	RecName: Full=Fatty acid-binding protein	97.8	622.4	10
	fhep39473|1	ubiquitin ligase E3 alpha-related	154.6	51.1	3
	fhep39850|1	UDP-glucose 4-epimerase	64.8	71.3	2
2.3 Carbohydrate	fhep00825|1	1-aminocyclopropane-1-carboxylate	140.3	191.4	3
	fhep00877|1	1-aminocyclopropane-1-carboxylate	131.9	120.6	2
2.4 Citric Acid Cycle	fhep00235|1	2-oxoglutarate dehydrogenase	120.5	75.1	2
	fhep20509|1	4a-hydroxytetrahydrobiopterin	28.6	155	2
	fhep01474|1	mitochondrial acetate:succinate CoA	78.9	257	4
	fhep02110|1	mitochondrial acetate:succinate CoA	66.6	208.8	2
	fhep04674|1	mitochondrial acetate:succinate CoA	60.5	588.8	10
	fhep39656|1	propionyl Coenzyme A carboxylase	27.2	299.5	4

2.6 Glycan	fhep02568|1	glucosamine-fructose-6-phosphat	105.8	31.7	2
	fhep02468|1	glutamate dehydrogenase	50.0	292	4
	fhep01294|1	glutamate dehydrogenase	101.1	1907.3	25
	fhep15603|1	glutamate dehydrogenase	85.5	1626.9	18
	fhep01196|1	glutamate dehydrogenase 1	134.3	1054.9	13
	fhep16197|1	glutamine-oxaloacetic transaminase	12.5	109.7	2
	fhep00463|1	glyceraldehyde 3-phosphate dehydrogenase	97.2	378.7	7
	fhep01437|1	glycerol kinase	66.1	32.8	2
	fhep21967|1	succinate dehydrogenase	31.7	309.5	7
	fhep00654|1	succinate dehydrogenase	51.4	360.2	6
	fhep39237|1	succinate dehydrogenase	52.4	247	5
	fhep04582|1	succinate dehydrogenase iron-sulfur	50.1	367.8	7
	fhep23326|1	succinate dehydrogenase iron-sulfur	18.8	216.8	3

	fhep14323|1	succinate-CoA ligase	65.1	264.2	6
	fhep26819|1	succinyl-CoA synthetase beta	30.8	162.4	4
	fhep00887|1	sugar ABC transport system	113.0	55.9	2
2.7 Other	fhep10435|1	dihydrolipoamide dehydrogenase	20.8	85.2	2
	fhep31296|1	dipeptidylpeptidase 3	12.9	45.1	2
	fhep00161|1	Fatty acid-binding protein	50.9	425.5	8
	fhep00817|1	Fatty acid-binding protein	50.6	402.1	7
	fhep02802|1	gelsolin	63.6	201.4	5
	fhep00225|1	Glutathione S-transferase	58.9	337.5	6
	fhep00210|1	Glutathione S-transferase	65.8	636.6	11
	fhep00725|1	glutathione transferase	71.7	731.6	12
	fhep01298|1	leucine-rich repeat family protein	110.4	34.1	2
	fhep00498|1	phospholipase d-related	176.7	181.2	4

	fhep03989|1	phosphorylase, putative	62.7	41.6	2
	fhep42010|1	protein disulfide-isomerase	108.2	97.9	2
	fhep00776|1	Protein disulphide isomerase	144.9	156.4	4
	fhep37375|1	putative Golgi SNAP receptor comple	19.2	41.2	2
	fhep01655|1	SmIrV1 protein	89.5	128.6	4
	fhep02579|1	SmIrV1 protein	120.9	61.9	2
3. Transport					
	fhep00078|1	cytochrome oxidase subunit 1	1220.3	178.4	2
	fhep00045|1	cytochrome oxidase subunit 1	1232.6	178	2
	fhep00305|1	dihydrolipoamide branched chain	52.1	57	2
	fhep00745|1	RecName: Full=Probable dynein	41.9	120.1	3
	fhep00051|1	tubulin alpha-5	76.4	38	2

5. Cytoskeleton Proteins					
	fhep01365|1	23 kDa integral membrane	73.3	350.7	4
	fhep01772|1	actin	125.3	999.9	19
	fhep01899|1	Actin 5C	86.5	736	13
	fhep02440|1	cyln2 (cytoplasmic linker protein	97.9	33.6	2
	fhep00648|1	transmemberane protein	94.7	151.3	3
6. Transcription					
	fhep09950|1	CDC5 cell division cycle 5-like	87.3	58.5	2
	fhep00767|1	DNA topoisomerase type I	66.6	35.7	2
	fhep31527|1	endonuclease-reverse transcriptase	17.0	51.9	2
	fhep19709|1	Reverse transcriptase	59.1	40.4	2

	fhep39086|1	glycyl-tRNA synthetase	65.1	33.8	2
	fhep14374|1	helicase	19.0	43.9	2
	fhep01907	KH domain-containing, RNA-binding	91.6	36.1	2
	fhep20867|1	PREDICTED: transcription factor	47.1	33.8	2
	fhep09770|1	TPA: endonuclease-reverse transcriptase	19.2	40.6	2
	fhep40455|1	TPA: endonuclease-reverse transcription	35.7	34	2
	fhep00484|1	trans-golgi protein gmx33-related	92.2	41.6	2
7. Gut Proteins					
	fhep04647|1	secreted saposin-like protein SAP-3	51.7	386.5	6
	fhep00672|1	secreted saposin-like protein SAP-3	57.9	316.9	6
	fhep04797|1	secreted saposin-like protein SAP-3	81.8	355.1	5
	fhep00865|2	secreted saposin-like protein SAP-3	125.9	314.6	5

	fhep04159|1	secreted saposin-like protein SAP-3	55.5	312.1	5
	fhep02977|1	secreted saposin-like protein SAP-3	57.9	241.8	5
	fhep05537|1	secreted saposin-like protein SAP-3	21.5	258	4
8. Signalling					
	fhep00933|1	beta-actin	60.0	509.8	8
	fhep06667|1	beta-actin	13.7	135.6	2
	fhep00178|1	Calcium-binding protein	83.8	164.3	4
	fhep38850|1	Gag-Pol polyprotein	44.3	30.4	2
	fhep00886|1	Lymphocyte cytosolic protein 1	68.4	218.2	4
	fhep00400|1	Lymphocyte cytosolic protein 1	70.8	156.3	3
	fhep00475|1	malate dehydrogenase	98.0	54.7	3
	fhep01631|1	mitochondrial malate dehydrogenase	64.4	567.1	9

	fhep12583|1	mitochondrial malate dehydrogenase	75.1	930	14
	fhep03618|1	mitochondrial phosphate carrier	48.7	210.8	4
	fhep40049|1	mitochondrial phosphate carrier	36.0	198.9	4
	fhep00377|1	monomeric Kunitz-type	28.6	267.4	4
	fhep00210|2	mu-glutathione transferase	61.8	548.5	8
	fhep21916|1	PREDICTED: similar to zinc finger	52.4	59.7	3
	fhep42252|1	PREDICTED: similar to zinc fingers	58.1	40.9	2
	fhep01048|1	zinc binding dehydrogenase	54.0	130.2	3
	fhep39190|1	zinc finger transcription factor	18.5	38.2	2
10. Heat shock and chaperone proteins					
	fhep20201|1	heat containing protein	40.2	40	2
	fhep05300|1	thioredoxin peroxidase	61.7	291.9	5

11. Ribosomal Proteins					
	fhep03071|1	39S ribosomal protein L15	99.9	125.9	2
12. Other					
	fhep02298|1	Amoebapore-like protein	153.7	190.1	5
	fhep38625|1	annexin	59.9	498.7	7
	fhep02287|1	annexin	78.0	215.1	4
	fhep01504|1	Annexin A13 (Annexin XIII)	59.3	445.2	8
	fhep01016|1	ARL2BP-like protein	78.5	37.7	2
	fhep05735|1	carboxypeptidase regulatory	54.9	35.1	2
	fhep00401|1	Chain A, Fasciola Hepatica Sigma Class	136.5	297.8	5
	fhep04938|1	Chain A, Fasciola Hepatica Sigma Class	60.7	110.3	2

	fhep00634|1	conserved hypothetical protein	64.7	32.4	2
	fhep41127|1	conserved hypothetical protein	20.6	32.1	2
	fhep01679|1	conserved Plasmodium protein	118.7	37.3	2
	fhep23090|1	ErfK/YbiS/YcfS/YnhG family protein	20.0	31	2
	fhep02067|1	Fh-KTM=6.751 kda monomeric Kunitz-type	27.4	230.6	3
	fhep00762|1	four and A half lim domains	138.9	85.6	2
	fhep04395|1	LOC613054 protein	75.8	764.3	13
	fhep00099|1	maturase K	99.9	37.8	2
	fhep27028|1	methylmalonic aciduri	60.2	78.9	2
	fhep42157|1	microrchidia 2a	76.8	57	3
	fhep00515|1	Negative cofactor 2 beta	56.6	34.9	2
	fhep00277|1	PREDICTED: similar to Annexin	33.0	166.6	3
	fhep01764|1	PREDICTED: similar to ENSANGP00	63.5	241.7	7

	fhep01062|1	PREDICTED: similar to Mal	71.3	252.7	4
	fhep01893|1	PREDICTED: similar to methylmal	140.5	125.3	4
	fhep25431|1	putative tetraspanin similar	37.0	73.3	2
	fhep22626|1	pyruvate carboxylase	30.2	230.3	5
	fhep00190|1	radixin	84.6	93.4	2
	fhep16370|1	sco1-related	76.4	73.8	2
	fhep00381|1	similar to Branched	90.6	512.7	9
	fhep05707|1	SJCHGC00820 protein	56.9	112.8	3
	fhep12956|1	SJCHGC01083 protein	50.9	46.3	2
	fhep16071|1	SJCHGC01281 protein	63.2	322.8	6
	fhep43835|1	SJCHGC01487 protein	59.9	46.8	2
	fhep40230|1	SJCHGC01836 protein	50.4	50.7	2
	fhep01243|1	SJCHGC01960 protein	54.1	331.5	6

	fhep01386|1	SJCHGC02792 protein	65.5	638.4	10
	fhep02839|1	SJCHGC03264 protein	56.6	56.3	2
	fhep00942|1	SJCHGC04324 protein	67.7	158.8	4
	fhep08325|1	SJCHGC06332 protein	60.6	64.6	2
	fhep01248|1	SJCHGC06566 protein	52.8	186.5	6
	fhep41433|1	SJCHGC06640 protein	54.3	184.4	3
	fhep00534|1	SJCHGC06703 protein	66.4	91.2	2
	fhep04589|1	SJCHGC09036 protein	52.8	132.7	4
	fhep11643|1	SJCHGC09036 protein	23.2	135.1	2
	fhep20324|1	SJCHGC09134 protein	29.2	143.4	2
	fhep06912|1	SJCHGC09380 protein	59.6	216.5	3
	fhep41308|1	SJCHGC09717 protein	29.0	50.4	2
	fhep07054|1	Tektin-3, putative	67.7	37.9	2

	fhep12673|1	uracil phosphoribosyltransferase 1	58.6	38.2	2
13. Unknown					
	fhep01032|1	hypothetical protein	93.9	397.4	6
	fhep02235|1	hypothetical protein	127.2	245.4	6
	fhep00340|1	hypothetical protein	143.8	197.2	5
	fhep24235|1	hypothetical protein	38.3	162.6	3
	fhep28022|1	hypothetical protein	17.6	105.6	3
	fhep01810|1	hypothetical protein	99.0	53.8	3
	fhep09189|1	hypothetical protein	70.0	52.3	3
	fhep00537|1	hypothetical protein	129.5	145.9	2
	fhep10701|1	hypothetical protein	80.9	56.5	2
	fhep00324|1	hypothetical protein	92.8	51.2	2

	fhep01077|1	hypothetical protein	38.1	51	2
	fhep18375|1	hypothetical protein	19.4	42	2
	fhep23497|1	hypothetical protein	46.0	36.5	2
	fhep00816|1	hypothetical protein	57.1	35.8	2
	fhep27421|1	hypothetical protein	18.4	35.5	2
	fhep01746|1	hypothetical protein	66.7	35.3	2
	fhep19990|1	hypothetical protein	51.7	34.6	2
	fhep11108|1	hypothetical protein	66.9	34.6	2
	fhep19687|1	hypothetical protein	64.0	33.3	2
	fhep07433|1	hypothetical protein	18.5	31.6	2
	fhep00920|1	hypothetical protein CUS_0203	101.5	33.6	2
	fhep03953|1	hypothetical protein ECEG_02466	51.3	220.8	5
	fhep04848|1	hypotheticial protein	64.5	62	2

	fhep44011|1	unknown protein	13.3	41.7	2
	fhep00759|1	No hits found	70.1	56.3	3
	fhep04999|1	No hits found	66.1	50.1	3
	fhep39094|1	No hits found	46.7	49.3	3
	fhep16349|1	No hits found	20.0	51.9	2
	fhep38387|1	No hits found	18.0	49	2
	fhep40890|1	No hits found	60.3	46.4	2
	fhep17533|1	No hits found	14.3	46	2
	fhep28204|1	No hits found	19.6	45.3	2
	fhep14579|1	No hits found	19.3	44.8	2
	fhep30344|1	No hits found	20.0	44.5	2
	fhep38260|1	No hits found	12.9	44.3	2
	fhep41263|1	No hits found	77.6	43	2

	fhep17611|1	No hits found	27.5	41.8	2
	fhep16517|1	No hits found	57.7	40.8	2
	fhep14838|1	No hits found	64.4	40.5	2
	fhep22783|1	No hits found	20.6	40.2	2
	fhep32990|1	No hits found	15.8	39.6	2
	fhep12534|1	No hits found	88.7	38.9	2
	fhep08634|1	No hits found	36.1	38.7	2
	fhep20969|1	No hits found	69.2	38.3	2
	fhep40378|1	No hits found	50.1	38.1	2
	fhep00212|1	No hits found	56.6	38	2
	fhep07471|1	No hits found	33.9	37.6	2
	fhep43756|1	No hits found	51.6	37.1	2
	fhep03509|1	No hits found	80.2	36.8	2

	fhep40833|1	No hits found	65.6	36.7	2
	fhep09319|1	No hits found	53.0	36	2
	fhep07240|1	No hits found	74.5	36	2
	fhep10397|1	No hits found	45.3	36	2
	fhep00968|1	No hits found	33.1	35.8	2
	fhep14145|1	No hits found	60.8	35.7	2
	fhep41397|1	No hits found	50.5	35.6	2
	fhep21767|1	No hits found	18.8	35.4	2
	fhep03570|1	No hits found	69.0	35.3	2
	fhep02292|1	No hits found	65.2	35.1	2
	fhep39677|1	No hits found	59.5	35	2
	fhep02563|1	No hits found	58.8	34.4	2
	fhep27784|1	No hits found	18.8	34.4	2

	fhep33503|1	No hits found	20.0	34.4	2
	fhep17865|1	No hits found	20.8	34.1	2
	fhep17046|1	No hits found	20.4	33.7	2
	fhep12698|1	No hits found	64.9	33.4	2
	fhep36682|1	No hits found	19.7	33.4	2
	fhep39591|1	No hits found	62.6	33.3	2
	fhep29531|1	No hits found	9.1	33.3	2
	fhep41698|1	No hits found	17.0	33.1	2
	fhep07714|1	No hits found	61.9	32.7	2
	fhep38361|1	No hits found	51.7	31.9	2
	fhep04461|1	No hits found	46.1	31.9	2
	fhep40422|1	No hits found	31.5	31.6	2
	fhep35742|1	No hits found	20.2	31.5	2

	fhep06454|1	No hits found	17.7	31.2	2
	fhep19223|1	No hits found	20.4	30.3	2
	fhep00829|1	PREDICTED	63.6	39.7	2
	fhep00068|1	predicted protein	52.6	102.7	4
	fhep05559|1	predicted protein	48.7	58.3	3
	fhep13884|1	PREDICTED: hypothetical protein	47.7	30.8	2
	fhep29778|1	Temporarily Assigned Gene name	21.6	108.9	2
	fhep01141|1	unknown	142.1	236.6	4
	fhep00259|1	unknown	61.5	44	2

Appendix 4 : Proteins identified from PNA agglutinin affinity chromatography enriched

	Accession	**Description**	Mowse Score	Scores	No. of Peptides
1. Proteolytic enzymes					
1.2 Cysteine protease	fhep00344|1	cathepsin L1 protein	193.9	118.6	2
	fhep01124|1	legumain-1	65.0	47.2	2
1.3 Serine /Carboxypeptidase	fhep00076|1	putative Lysosomal Pro-X carboxypeptidase precursor	137.2	38.6	2
1.1 Aspartyl proteases	fhep43071|1	cathepsin D-like aspartic protease	52.5	154.8	3
	fhep01837|1	cathepsin D-like aspartic protease	120.4	317.4	7
2. Metabolism					
2.1 Amino Acid	fhep01196|1	glutamate dehydrogenase 1	134.3	898	12
	fhep00045|1	cytochrome oxidase subunit 1	1232.6	85.8	2

	fhep01750|1	glutamine synthetase 1 2 (gluta...	65.5	40.6	2
	fhep01339|1	Triosephosphate	106.6	42.3	2
	fhep42010|1	protein disulfide-isomerase er-...	108.2	182.2	4
2.2 ATP synthesis/Electron transport	fhep02189|1	plasma memebrane H+-ATPase	32.1	38.3	2
	fhep39473|1	ubiquitin ligase E3 alpha-relat...	154.6	36.1	2
	fhep38669|1	Ubiquitin-protein ligase BRE1	56.6	32.8	2
	fhep40715|1	mitochondrial carrier protein	32.6	52.2	2
	fhep02352|1	PREDICTED: similar to sirtuin type...	68.4	33.5	2
	fhep20205|1	Tetraspanin-1 (Tspan-1)	16.5	94.3	2
	fhep01846|1	ATP-binding cassette protein	220.4	64.1	3
	fhep03618|1	mitochondrial phosphate carrier...	48.7	177	3
	fhep02715|1	tetraspanin 3	88.5	175.4	3
	fhep01948|1	ATP synthase alpha subunit mito...	61.0	128	4

	fhep04674|1	mitochondrial acetate:succinate CoA-.	60.5	196.4	4
	fhep40049|1	mitochondrial phosphate carrier...	36.0	198.8	4
	fhep40820|1	ATP:ADP antiporter	84.6	276.2	6
	fhep01951|1	ATP synthase, H+ transporting, mito..	78.9	463.2	8
2.3 Carbohydrate	fhep19796|1	alpha-glucosidase	23.3	76.4	2
	fhep00699|1	alpha-amylase	63.0	81.3	3
	fhep00563|1	acetyl-CoA carboxylase	218.8	232.1	7
2.4 Citric Acid Cycle	fhep23326|1	succinate dehydrogenase iron-sulfur p.	18.8	63.7	2
	fhep04582|1	succinate dehydrogenase iron-sulfur p.	50.1	191.7	3
	fhep00654|1	succinate dehydrogenase	51.4	240	5
	fhep21967|1	succinate dehydrogenase	31.7	244.8	7
2.6 Glycan	fhep00054|1	fructose-16-bisphosphatase-rela...	133.9	117.7	2
2.7 Other	fhep01349|1	cubilin (intrinsic factor-cobalamin .	96.3	41	2

	fhep01378|1	dolichyl-diphosphooligosacchari...	69.0	125.6	2
	fhep22574|1	podoplanin, isoform CRA_b	28.7	34.9	2
	fhep02298|1	amoebapore-like protein	153.7	117.2	3
	fhep39656|1	propionyl Coenzyme A carboxylase, b..	27.2	194.8	3
	fhep01734|1	propionyl-CoA carboxylase beta ...	65.1	240.8	4
	fhep22626|1	pyruvate carboxylase	30.2	286.4	6
3. Transport					
	fhep21916|1	PREDICTED: similar to zinc fing...	52.4	**49.3**	2
	fhep20177|1	PREDICTED: similar to zinc fing...	102.4	35.8	2
	fhep00599|1	protein SpAN precursor	70.2	100.1	2
	fhep39488|1	transmembrane transport protein...	73.1	121.2	2
	fhep40261|1	oxalate:formate antiporter	28.9	136.4	2

	fhep00304|1	cd63 antigen-like	78.7	129.7	3
	fhep38434|1	choline transporter-like protein 4 ...	34.5	136.9	3
	fhep02512|1	lysosome-associated membrane gl...	118.0	95	3
	fhep02802|1	gelsolin	63.6	207	4
	fhep32250|1	CDW92 antigen	24.0	295.2	5
	fhep00648|1	transmemberane protein	94.7	285.1	6
5. Cytoskeleton					
	fhep01772|1	actin	125.3	**470.5**	11
	fhep02671|1	copii-coated vesicle membrane p...	68.4	92.3	2
	fhep00063|1	tubulin alpha-2	185.5	73.6	2
6. Transcription					

	fhep28302|1	reverse transcriptase	19.6	**71**	2
	fhep08525|1	PREDICTED: similar to endonucle	28.0	32	2
	fhep06086|1	PREDICTED: similar to endonucle...	20.5	34.5	2
	fhep28249|1	PREDICTED: similar to endonucle...	20.5	36.5	2
	fhep01918|1	thioredoxin-glutathione reductase	189.4	40.1	2
	fhep00193|1	nep1	58.5	55.2	2
	fhep01221|1	nuclear autoantigenic sperm pro...	64.5	33.5	2
	fhep00660|1	peptidyl-prolyl cis-trans isome...	67.4	40.5	2
	fhep40917|1	prohibitin	90.3	199.6	5
	fhep42350|1	integrin beta subunit	37.6	316.2	6
	fhep00640|1	prohibitin	106.6	325.4	7
	fhep41948|1	Carbonic anhydrase 5B	125.6	422.9	8

7. Gut Proteins					
	fhep00865|2	secreted saposin-like protein SAP-3	125.9	**103.2**	3
8. Signalling					
	fhep05642|1	signal recognition particle 68 ...	88.6	**30.1**	2
	fhep15978|1	Gag-Pol polyprotein	40.8	46.7	2
	fhep30616|1	pol	64.6	33.2	2
	fhep08375|1	putative protein affecting Mg2+/Co2..	32.4	38.1	2
	fhep07174|1	TPA: TPA_exp: pol polyprotein	60.3	32.2	2
	fhep41142|1	fasciclin I-like protein	54.6	188	3
9. Gene Transcription					
	fhep42502|1	Ankyrin repeat domain-containing	92.6	**39.1**	2

10. Heat shock and Chaperon proteins					
	fhep00786|1	heat shock protein 70	163.3	**161.6**	3
11. Ribosomal Proteins					
	fhep00845|1	39S ribosomal protein L51	66.3	**50.2**	2
	fhep02068|1	40S ribosomal protein S3	78.4	40.6	2
	fhep19104|1	MGC84751 protein	46.9	43.6	2
12. Other					
	fhep00318|1	acidic fibroblast growth factor	66.5	**31.6**	2
	fhep39707|1	Breast cancer metastasis-suppre...	60.5	30.2	2

	fhep00330|1	Chain A, X-Ray Structure Of Fasciola	133.7	30.2	2
	fhep00634|1	conserved hypothetical protein	64.7	31.3	2
	fhep08659|1	conserved hypothetical protein ...	58.5	33.1	2
	fhep00867|1	SJCHGC01027 protein	102.8	31.8	2
	fhep01574|1	SJCHGC02721 protein	124.0	61	2
	fhep15534|1	SJCHGC02820 protein	34.3	33.2	2
	fhep24110|1	SJCHGC02821 protein	18.8	65.7	2
	fhep40771|1	SJCHGC03127 protein	83.8	42.7	2
	fhep15256|1	SJCHGC03776 protein	64.7	57.7	2
	fhep20531|1	SJCHGC06900 protein	31.6	34.2	2
	fhep11643|1	SJCHGC09036 protein	23.2	93.9	2
	fhep10785|1	SJCHGC09144 protein	68.6	32.8	2
	fhep15031|1	PREDICTED: polyprotein-like	22.2	31.3	2

	fhep17397|1	putative Fasciola/Schistosoma cross-r.	30.9	55.7	2
	fhep29633|1	GH11701	32.3	34.5	2
	fhep00207|1	PREDICTED: cytosolic phosphoeno	94.6	47.4	2
	fhep00230|1	PREDICTED: dentin matrix acidic...	92.5	41.3	2
	fhep26636|1	PREDICTED: similar to predicted...	14.8	65.9	2
	fhep20513|1	putative Cell division protein kina..	46.1	41.5	2
	fhep25431|1	putative tetraspanin similiar to ur..	37.0	171.2	2
	fhep16370|1	sco1-related	76.4	43.8	2
	fhep01040|1	Y box binding protein	87.2	43.5	2
	fhep01802|1	Minor tail protein H	92.0	41	2
	fhep38980|1	basigin related	73.6	138.1	3
	fhep20857|1	SJCHGC09595 protein	73.0	165.9	3
	fhep15871|1	DM9 domain-containing protein	48.4	117.3	3

	fhep42177|1	Immunoglobulin-like domain-containi..	28.1	154.9	3
	fhep20324|1	SJCHGC09134 protein	29.2	296.5	4
	fhep00942|1	SJCHGC04324 protein	67.7	220.6	5
	fhep04395|1	LOC613054 protein	75.8	433.8	8
	fhep01386|1	SJCHGC02792 protein	65.5	617.2	9
13. Unknown					
	fhep20342|1	hypothetical protein	97.3	**110.2**	2
	fhep20607|1	hypothetical protein	72.3	40.3	2
	fhep01668|1	hypothetical protein	80.9	48.3	2
	fhep24174|1	hypothetical protein	57.8	42.2	2
	fhep32773|1	hypothetical protein	17.3	64.5	2
	fhep01171|1	hypothetical protein	30.0	33.8	2

	fhep20462|1	hypothetical protein	25.9	119.7	2
	fhep17269|1	hypothetical protein	41.5	44.3	2
	fhep14703|1	hypothetical protein	101.6	51.4	2
	fhep37777|1	hypothetical protein	16.5	37.6	2
	fhep05671|1	hypothetical protein	58.3	36.2	2
	fhep02635|1	hypothetical protein	107.4	34.7	2
	fhep00450|1	hypothetical protein	70.6	33.8	2
	fhep15357|1	hypothetical protein	110.4	57.7	2
	fhep26109|1	hypothetical protein	34.1	53.8	2
	fhep00300|1	hypothetical protein	110.3	38.1	2
	fhep00779|1	hypothetical protein	64.6	39.1	2
	fhep42032|1	hypothetical protein	55.5	40.1	2
	fhep00416|1	hypothetical protein	93.4	35	2

	fhep13265|1	No hits found	113.7	93.1	2
	fhep29584|1	No hits found	16.6	81.4	2
	fhep23542|1	No hits found	15.1	55.1	2
	fhep17161|1	No hits found	22.9	54.7	2
	fhep02545|1	No hits found	46.7	50.6	2
	fhep10454|1	No hits found	18.8	50.5	2
	fhep18899|1	No hits found	20.2	50.2	2
	fhep23156|1	No hits found	14.0	49.2	2
	fhep43197|1	No hits found	14.5	47.9	2
	fhep25351|1	No hits found	32.6	47.8	2
	fhep26955|1	No hits found	14.3	45.8	2
	fhep26945|1	No hits found	30.1	45.6	2
	fhep29949|1	No hits found	19.8	45.5	2

	fhep17533|1	No hits found	14.3	44.5	2
	fhep19525|1	No hits found	22.5	44	2
	fhep14686|1	No hits found	52.6	43.6	2
	fhep17611|1	No hits found	27.5	43.2	2
	fhep42172|1	No hits found	38.1	43.2	2
	fhep20969|1	No hits found	69.2	43	2
	fhep13645|1	No hits found	18.3	42.7	2
	fhep18344|1	No hits found	71.3	42.6	2
	fhep38360|1	No hits found	72.8	42.4	2
	fhep19272|1	No hits found	15.3	42	2
	fhep07090|1	No hits found	9.5	40.9	2
	fhep18645|1	No hits found	18.0	40.6	2
	fhep04312|1	No hits found	53.4	40.3	2

	fhep26908|1	No hits found	19.3	39.5	2
	fhep02679|1	No hits found	20.5	39.5	2
	fhep20868|1	No hits found	15.9	39.1	2
	fhep00315|1	No hits found	103.8	38.6	2
	fhep22071|1	No hits found	19.8	38.2	2
	fhep21820|1	No hits found	17.6	37.4	2
	fhep15336|1	No hits found	17.7	37.1	2
	fhep34957|1	No hits found	15.0	35.8	2
	fhep29268|1	No hits found	34.9	35.5	2
	fhep00422|1	No hits found	79.6	35.2	2
	fhep18437|1	No hits found	20.3	34.7	2
	fhep03924	No hits found	56.7	34.6	2
	fhep06753|1	No hits found	52.3	34.2	2

	fhep06192|1	No hits found	19.9	34	2
	fhep30063|1	No hits found	38.7	33.8	2
	fhep31171|1	No hits found	8.5	33.7	2
	fhep11486|1	No hits found	19.5	33.3	2
	fhep37883|1	No hits found	10.2	33	2
	fhep06755|1	No hits found	65.6	32.7	2
	fhep00911|1	No hits found	7.3	32.5	2
	fhep08271|1	No hits found	19.8	31.8	2
	fhep41694|1	No hits found	28.2	30.8	2
	fhep41267|1	unnamed protein product	71.2	41.8	2
	fhep03647|1	PREDICTED: hypothetical protein XP_...	61.9	33.1	2
	fhep00340|1	PREDICTED: hypothetical protein...	143.8	67.3	2
	fhep42290|1	predicted protein	95.2	32.3	2

	fhep25409|1	hypothetical protein	48.4	89.1	3
	fhep08824|1	No hits found	29.3	56.7	3
	fhep07319|1	No hits found	36.0	54.9	3
	fhep02902|1	No hits found	43.0	47.5	3
	fhep02563|1	No hits found	58.8	47.4	3
	fhep00876|1	PREDICTED: hypothetical protein	74.2	73.7	3
	fhep01684|1	hypothetical protein	61.4	197	4
	fhep11734|1	No hits found	45.1	216.1	4
	fhep02234|1	No hits found	58.8	191.8	4
	fhep42569|1	unknown	25.0	203.1	4
	fhep00972|1	hypothetical protein	50.2	174.9	5
	fhep22091|1	No hits found	17.1	378.9	5
	fhep01475|1	hypothetical protein	73.9	419.4	8

Bibliography

Abbot, K.A., Taylor, M., and Stubbings, L.A (2009) Sustainable worm control for sheep 3rd Edition, A technical manual for veterinary surgeons and advisors. DEFRA (http://archive.defra.gov.uk/foodfarm/farmanimal/diseases/control/documents/scops-technical-manual-0903.pdf)

Acosta D., Cancela, M., Piacenza, L., Roche, L., Carmona, C., and Tort, J.F. (2008) Fasciola hepatica leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. *Moecular Biochemical Parasitology* 158, 52-64

Ahmad, A., Wang, C.H., Bell, R.G., (1992) A role for IgE in intestinal immunity. Expression of rapid expulsion of *Trichinella spiralis* in rats transfused with IgE and thoracic duct lymphocytes. *Journal of Immunology* 146, 3563-3570.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215, 403–410

Anderson P.H., Berrett, S., and Patterson, D.S. (1978) Resistance to Fasciola hepatica in cattle. II. Biochemical and morphological observations. *Journal of Comparative Pathology* 88, 245-251

Andrews, S.J. (1999) The life cycle of *Fasciola hepatica***.** Edited by: Dalton JP. Fasciolosis CABI Publishing, Oxin, UK

Bachmair, A., Finley, D., and Varshavsky, A. (1986) In vivo half-life of a protein is a function of its amino-terminal residue. *Science* 234, 179-186

Baird, G. (2010) Strategic liver fluke control in sheep. *Veterinary Times* 40, 12-14

Balloul, J.M., Grzych, J-W, Pierce, R.J. and Capron, A. (1987) A purified 28,000 dalton protein from *Schistosoma mansoni* adult worms protects rats and mice against experimental schistosomiasis. *Journal of Immunology* 138, *3448-3453.*

Batycka M., Inglis, N.F., Cook, K., Adam, A., Fraser-Pitt, D., Smith, D.G., Main, L., Lubben, A., and Kessler, B.M. (2006) Ultra-fast tandem mass spectrometry scanning combined with monolithic column liquid chromatography increases throughput in proteomic analysis. *Rapid Communication in Mass Spectrometry* 20, 2074-2080

Behm, C.A. Ovington, K.S. (2000). The Role of Eosinophils in Parasitic Helminth Infections: Insights from Genetically Modified Mice. *Parasitology Today 16, 203-210*

Behm, C. A. and Sangster, N. C. (1999) Chapter 6, Pathology, Pathophysiology and Clinical Aspects. *In* Dalton, J. P. *Fasciolosis* CABI international, Wallingford, *185-224*

Bennett, C. E., Threadgold, L. T. (1973) Electron microscope studies of *Fasciola hepatica*. XIII. Fine structure of newly excysted juvenile. Experimental Parasitology 34, 85-99.

Bennett, R. M. (2003). The 'Direct Costs' of Livestock Disease: the Development of a System of Models for the Analysis of 30 Endemic Diseases in Great Britain, *Journal of Agricultural Economics* **54**: 55-71

Bennet R. and lJpelaar, J. (2005) Updated estimates of the costs associated with 34 endemic livestock diseases in Great Britain. *Journal of Agricultural Economics* 56, 135-144

Bennett C.E. and Threadgold, L.T. (1973) Electron microscope studies of Fasciola hepatica. 13. Fine structure of newly excysted juvenile. *Exp.Parasitol* 34, 85-99

Berasain P., Goni, F., McGonigle, S., Dowd, A., Dalton, J.P., Frangione, B., and Carmona, C. (1997) Proteases secreted by Fasciola hepatica degrade extracellular matrix and basement membrane components. *J Parasitol* 83, 1-5

Berger, A. (2002). Th1 and Th2 responses: what are they? British medical Journal 12, 321-424

Berriman, M., Haas, B.J., LoVerde, P.T., Wilson, R.A., Dillon, G.P., Cerqueira, G.C., Mashiyama, S.T., Al-Lazikani, B., Andrade, L.F., Ashton, P.D., Aslett, M.A., Bartholomeu, D.C., Blandin, G., Caffrey, C.R., Coghlan, A., Coulson, R., Day, T.A., Delcher, A., DeMarco, R., Djikeng, A., Eyre, T., Gamble, J.A., Ghedin, E., Gu, Y., Hertz-Fowler, C., Hirai, H., Hirai, Y., Houston, R., Ivens, A., Johnston, D.A., Lacerda, D., Macedo, C.D., McVeigh, P., Ning, Z., Oliveira, G., Overington, J.P., Parkhill, J., Pertea, M., Pierce, R.J., Protasio, A.V., Quail, M.A., Rajandream, M.A., Rogers, J., Sajid, M., Salzberg, S.L., Stanke, M., Tivey, A.R., White, O., Williams, D.L., Wortman, J., Wu, W., Zamanian, M., Zerlotini, A., Fraser-Liggett, C.M., Barrell, B.G., El-Sayed, N.M. (2009). The genome of the blood fluke *Schistosoma mansoni*. *Nature* 460, 352-8

Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., Brunak, S. (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4**,** 1633-1649

Boyce, W.M., Courtney, C.H., and Thibideau, M. (1986) Heterologous resistance to Fasciola hepatica conferred upon rats by passive transfer of serum from different breeds of sheep. *Vet Parasitology* 22, 259-266

Boyce W.M., Courtney, C.H., and Loggins, P.E. (1987) Resistance to experimental infection with Fasciola hepatica in exotic and domestic breeds of sheep. *International Journal for Parasitology* 17, 1233-1237

Boyle, J.P., Wu, X.J., Shoemaker, C.B., Yoshino, T.P., 2003. Using RNA interference to manipulate endogenous gene expression in *Schistosoma mansoni* sporocysts. Molecular and Biochemical Parasitology 128, 205–215

Brady, C.P., Dowd, A.J., Brindley, P.J., Ryan, T., Day, S., Dalton, J.P. (1999). Recombinant expression and localization of *Schistosoma mansoni* cathepsin L1 support its role in the degradation of host haemoglobin. *Infection and Immunity* 67, 368-374.

Brennan G.P., Fairweather, I., Trudgett, A., Hoey, E., McCoy, McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G., and Brophy, P.M. (2007) Understanding triclabendazole resistance. *Experimental and Molecular Pathology* 82, 104-109

Brindley, P.J., Dalton, J.P., Day, S.R., Wong, J.Y.M., Smythe, M.L., McManus, D.P. (1997). Proteolytic degradation of host haemoglobin by schistosomes. *Molecular and Biochemical Parasitology* 89, 1-9.

Brindley PJ, Kalinna BH, Wong JY, Bogitsh BJ, King LT, Smyth DJ, Verity CK, Abbenante G, Brinkworth RI, Fairlie DP, Smythe ML, Milburn PJ, Bielefeldt-Ohmann H, Zheng Y, McManus DP.(2001) Proteolysis of human hemoglobin by schistosome cathepsin D. *Molecular Biochemical Parasitology* 15, 103-12.

Bruhn H. (2005) A short guided tour through functional and structural features of saposin-like proteins. *Biochemisty Journal* 389, 249–57.

Brophy P.M., Crowley, P., and Barrett, J. (1990) Detoxification reactions of Fasciola hepatica cytosolic glutathione transferases. *Molecular Biochemical Parasitology* 39, 155-161

Brophy P.M. and Pritchard, D.I. (1994) Parasitic helminth glutathione S-transferases: an update on their potential as targets for immuno- and chemotherapy. *Experimental Parasitology* 79, 89-96

Caffrey, C. R., Mathieu, M. A., Gaffney, A. M., Salter, J. P., Sajid, M., Lucas, K.D., Franklin, C., Bogyo, M. and McKerrow, J.H. (2000) Identification of a cDNA encoding an active asparaginyl endopeptidase of *Schistosoma mansoni* and its expression in *Pichia pastoris*. *FEBS Letters* 466, 244–248

Cancela, M., Acosta, D., Rinaldi, G., Silva, E., Durán, R., Roche, L., Zaha, A., Carmona, C., Tort, J.F. (2008) A distinctive repertoire of cathepsins is expressed by juvenile invasive *Fasciola hepatica. Biochimie* 90, 1461-1475

Cancela**,** M**.,** Ruétalo, N**.,** Dell'Oca, N**.,** da Silva**,** E**.,** Smircich**,** P**.,** Rinaldi**,** G**.,** Roche**,** L**.,** Carmona**,** C**.,** Alvarez-Valín**,** F**.,** Zaha, A., Tort, J.F. (2010) Survey of transcripts expressed by the invasive juvenile stage of the liver fluke *Fasciola hepatica. BMC Genomics* 11, 227

Carmona C., Dowd, A.J., Smith, A.M., and Dalton, J.P. (1993) Cathepsin L protease secreted by Fasciola hepatica in vitro prevents antibody-mediated eosinophil attachment to newly excysted juveniles. *Molecular Biochemical Parasitology* 62, 9-17

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

Chemale, G., Perally, S., LaCourse, E.J., Prescott, M.C., Jones, L.M., Ward, D., Meaney, M., Hoey, E., Brennan, G.P., Fairweather, I., Trudgett, A., Brophy, P.M. (2010) Comparative proteomic analysis of triclabendazole response in the liver fluke *Fasciola hepatica*. *Journal of Proteome Research* 9, 4940-51.

Chemale, Gustavo, Morphew, Russell, Moxon, Joseph V., Morassuti, Alessandra L., LaCourse, E.James, Barrett, John, Johnston, David A., and Brophy, Peter M. (2006) P*roteomic analysis of glutathione transferases from the liver fluke parasite, Fasciola hepatica.* Proteomics 6, 6263-6273.

Cheng, G.F., Lin, J.J., Shi, Y., Jin, Y.X., Fu, Z.Q., Jin, Y.M., Zhou, Y.C.,Cai, Y.M., (2005) Dose-dependent inhibition of gynecophoral canal protein gene expression in vitro in the schistosome (*Schistosoma japonicum*) by RNA interference. *Acta Biochim Biophys Sin. (Shanghi)* 37, 386–390.

Claridge, J., Diggle, P., McCann, C.M., Mulcahy, G., Flynn, R., McNair,J., Strain,S., Welsh, M., Baylis, M., Williams, D.J.L. (2012) *Fasciola hepatica* is associated with the failure to detect bovine tuberculosis in dairy cattle. *Nature Communications* 3, 853

Clery D, Torgerson P, Mulcahy G. (1996) Immune responses of chronically infected adult cattle to Fasciola hepatica. Veterinary Parasitology 62, 71-82.

Coffman RL, Ohara J, BondMW, Carty J, Zlotnik A, Paul WE. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* 136:4738–41

Cooper R.A.,Carucci D.J (2004) Proteomic Approaches to Studying Drug Targets and Resistance in Plasmodium. Current Drug Targets - Infectious Disorders 4, 41-51.

Corvo I, Cancela M, Cappetta M, Pi-Denis N, Tort JF, Roche L (2009) The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen. *Molecular Biochemical Parasitology* 167, 41-47

Creaney J., Wijffels, G.L., Sexton, J.L., Sandeman, R.M., Spithill, T.W., and Parsons, J.C. (1995) Fasciola hepatica: localisation of glutathione S-transferase isoenzymes in adult and juvenile liver fluke. *Experimental Parasitology* 81, 106-116

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

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

Dalton J.P., McGonigle, S., Rolph, T.P., and Andrews, S.J. (1996) Induction of protective immunity in cattle against infection with Fasciola hepatica by vaccination with cathepsin L proteases and with hemoglobin. *Infection and Immunity* 64, 5066-5074

Dalton J.P., Neill, S.O., Stack, C., Collins, P., Walshe, A., Sekiya, M., Doyle, S., Mulcahy, G., Hoyle, D., Khaznadji, E., Moirq, N., Brennan, G., Mousley, A., Kreshchenko, N., Maule, A.G., and Donnelly, S.M. (2003) Fasciola hepatica cathepsin L-like proteases: biology, function, and potential in the development of first generation liver fluke vaccines. *International Journal for Parasitology* 33, 1173-1181

Dalton, J.P., Skelly, P., Halton, D.W. (2004) Role of the tegument and gut in nutrient uptake by parasitic platyhelminths. *Canadian Journal of Zoology* 82, 2; Research Library

De Vries, E., Bakker, N., Krijgsveld, J., Knox, D.P., Heck, A.J.R., Yatsuda, A.P. (2009) An AC-5 cathepsin B-like protease purified from *Haemonchus contortus* excretory secretory products shows protective antigen potential for lambs. Veterinary Research 40, 41-52.

Don, Tegan Anita (2007). *Pore-Forming and Saposin-like Proteins from the Gut of Blood-Feeding Helminths* PhD Thesis, School of Molecular and Microbial Sciences , University of Queensland.

Dowd, A.J., Smith, A.M., McGonigle, S., Dalton, J.P (1994). Purification and characterisation of a second cathepsin L protease secreted by the parasitic trematode *Fasciola hepatica*, Eurpean Journal of Biochemistry 223, 91-98

Dowd A.J., McGonigle, S., and Dalton, J.P. (1995) Fasciola hepatica cathepsin L protease cleaves fibrinogen and produces a novel type of fibrin clot. *Eur.J Biochem.* 232, 241-246

Doy T.G. and Hughes, D.L. (1984) Fasciola hepatica: site of resistance to reinfection in cattle. *Exp.Parasitol* 57, 274-278

Doyle J.J. (1971) Acquired immunity to experimental infection with Fasciola hepatica in cattle. *Res.Vet.Sci.* 12, 527-534

Duffus,W.P., Franks, D. (1981) The interaction in vitro between bovine immunoglobulin and juvenile fasciola hepatica. *Parasitology* 82, 1-10

Duffus,W.P., Thorne, K., Oliver, R. (1980). Killing of juvenile *Fasciola hepatica* by purified bovine eosinophil proteins. *Clinical Experimental Immunology* 40, 336–344

Edman, P., Högfeldt, Erik; Sillén, Lars Gunnar; Kinell, Per-Olof (1950). Method for determination of the amino acid sequence in peptides. *Acta Chem. Scand.* **4**: 283–293

Eggleson, K.K. et al. (1999) Identification and characterization of falcilysin, a metallopeptidase involved in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. *Journal of Biological Chemistry* 274, 32411–32417

El-Sayed, L. H., Ghoneim, H., Demian, S. R., El-Sayed, M. H., Tawfik NM, Sakr I, Abou-Basha LM, Renganathan E, Klinkert MQ, Abou-Rawash N. (1998). Diagnostic significance of *Schistosoma mansoni* proteins Sm31 and Sm32 in human schistosomiasis in an endemic area in Egypt. *Tropical medicine & international Health* 3,721–727.

Else KJ, Finkelman FD, Maliszewski CR, Grencis RK (1994) Cytokinemediated regulation of chronic intestinal helminth infection. *Journal of Experimental Medicine* 179:347-351.

Ekoja, S.E., Smith, W.D. (2010). Antibodies from sheep immunized against *Haemonchus contortus* with H-gal-GP inhibit the haemoglobinase activity of this protease complex. *Parasite Immunology* 32, 731-738.

Estuningsih S.E., Smooker, P.M., Wiedosari, E., Widjajanti, S., Vaiano, S., Partoutomo, S., and Spithill, T.W. (1997) Evaluation of antigens of Fasciola gigantica as vaccines against tropical fasciolosis in cattle. *International Journal of Parasitology* 27, 1419-1428

Fairweather, I., Lawrence, T. E., Threadgold, L. T., and Hanna, B. (1999) Development of *F. hepatica* in the mammalian host. *In* Dalton, J. P.CABI Publishing, Wallington,

Flynn, R.J., Mannion, C., Golden, O., Hacariz, O., Mulcahy, G. (2007). Experimental Fasciola hepatica infection alters responses to tests used for diagnosis of bovine tuberculosis. *Infection and Immunity* 75, 1373-81

Fuller, A.P., Palmer-Toy, D., Erlander, M.G., Sgroi, D.C. (2003). Laser capture microdissection and advanced molecular analysis of human breast cancer. Mammary Gland Biology and Neoplsia 8, 335-345

Francis, S. E., Gluzman, I. Y., Oksman, A., Knickerbocker, A., Mueller, R., Bryant, M. L., Sherman, D. R., Russell, D. G., and Goldberg, D. E. (1994) Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. *EMBO J. 13, 306-317*

Gallagher. S. E., and L. T. Threadgold (1967) *Electron-microscope* studies of *Fasriola hepatica.* The interrelationship of the parenchyma with other organ systems. Parasitology *57*, 627-632.

Garcia L.G., Nicholson, K.L., Hoffman, T.W., Lawrence, T.E., Hale, D.S., Griffin, D.B., Savell, J.W., Vanoverbeke, D.L., Morgan, J.B., Belk, K.E., Field, T.G., Scanga, J.A., Tatum, J.D., and Smith, G.C. (2008) National Beef Quality Audit-2005: survey of targeted cattle and carcass characteristics related to quality, quantity, and value of fed steers and heifers. *J.Anim Sci.* 86, 3533-3543

Geldof, P., Clarebout, E., Knox, D.P., Agneessens, J., Vercruysse, J. (2000). Proteases released *in vitro* by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi*. Parasitology 121, 639-647

Geldhof, P., Knox, D. (2008) The intestinal contortin structure in *Haemonchus contortus*: an immobilised anticoagulant? *International Journal of Parasitolgy* **38,** 1579–1588

Geier, G., Banaj, H., Heid, H., Bini, L., Pallini, V., Zwilling, R. (1999). Aspartyl proteases in *Caenorhabditis elegans* - Isolation, identification and characterization by a combined use of affinity chromatography, two-dimensional gel electrophoresis, microsequencing and databank analysis. *European Journal of Biochemistry* 264, 872-879.

Ghosh, K., Hotez, P.J. (1999). Antibody-Dependent Reductions in Mouse Hookworm Burden after Vaccination with *Ancylostoma caninum* Secreted Protein 1. *The Journal of Infectious Diseases* 180, 1674-1681.

Gluzman, I.Y., Francis, S.E., Oksman, A., Smith, C.E., Duffin, K.L., Goldberg, D.E. (1994) Order and specificity of the Plasmodium falciparum hemoglobin degradation pathway. *Journal of Clinical Investigation* 93, 1602-8

Gresson , R.A.R., Threadgold, L.T. (1958) A Light and Electron Microscope Study of the Epithelial Cells of the Gut of *Fasciola hepatica. The Journal of Cell Biology* 6, 157-162.

Gobert G.N., Jones, M.K., and Stenzel, D.J. (1998) Ultrastructural analysis of the adult Schistosoma japonicum by lectin cytochemistry. *International Journal for Parasitology* 28, 1445-1452

Goldberg, D.E. Slater, A.F.G, Beavis, R., Chait, B., Cerami, A., Henderson, G.B. (1991). Hemoglobin Degradation in the Human Malaria Pathogen Plasmodium falciparum : A CatabolicPathway Initiated by a Specific Aspartic Protease. Journal of Experimental Medicine 173, 961-969

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J., Lopez, R. (2010). A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Research* 38, W695-9.

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

Grandi, G. (2001) Antibacterial vaccine design using genomics and proteomics. *Trends in Biotechnology* 19, 181-188

Graves, P.R., Haystead, T.A. (2002) Molecular biologist's guide to proteomics. Microbiology Molecular Biology Reviews 66, 39 –63

Halliday, A.M., McAllister, H.C. Smith, W.D. (2010). Kinetics of the local immune response in the gastric lymph of lambs after primary and challenge infection with Teladorsagia circumcincta. *Parasite Immunology* 32: 81–90.

Halliday, A.M., Morrison, W.I. Smith, W.D. (2009) Kinetics of the local cellular response in the gastric lymph of immune and susceptible sheep to infection with *Teladorsagia circumcincta. Parasite Immunology* 31, 402–411

Halliday, A.M., Routledge, C.M., Smith, S.K., Matthews, J.B. Smith, W.D. (2007) Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep. *Parasite Immunology* 29, 425–434.

Halton, D.W (1963). Some hydrolyticenzymes in the two dignetictrematodes. Proceedings of the 16thinternational congress of zoology. Washington D.C.1:29.

Hanna, R.E.B., (1975) *Fasciola hepatica* an electron microscope autoradiographic study of protein syntheisis and secretion by gut cells in tissue slices. *Experimental Parasitology* 38, 167-180

Hanna, R.E.B. (1982) *Fasciola hepatica:* antibody levels in sheep serum before and after treatment with anthelmintic. Research in veterinary Science33, 328-332.

Hanna, R.E.B., Cromie, L., Taylor, S.M., Couper, A., (2006) The effect of a parenteral ivermectin/closantel injection on the growth andreproductive development of early immature *Fasciola hepatica* in cattle. *Veterinary Parasitology* 142, 78–90.

Harmsen, M.M., Cornelissen, J.B.W.J.,. Buijs, H.E.C.M., Boersma, W.J.A., Jeurissen, S.H.M., Van Milligen. F.J. (2004) Identification of a novel *Fasciola hepatica* cathepsin L protease containing protective epitopes within the propeptide. *International Journal of Parasitology* 34 , 675–682

Harrop, S.A., Prociv, P., Brindley, P.J. (1996) Acasp, a gene encoding a cathepsin D-like aspartic protease from the hookworm Ancylostoma caninum, Biochem Biophys Res Commun. 227, 294-302.

Happich, F.A., Boray, J.C., (1969) Quantitative diagnosis of chronic fasciolosis. 2.The estimation of daily total egg production of *Fasciola hepatica*. *Australian Veterinary Journal* 45, 329–331.

Hammond J.A., Fielding, D., and Nuru, H. (1994) Eucalyptus: a sustainable self-delivery molluscicide? *Vet Research Communication* 18, 359-365

Haroun, E.T., Hilyer, G.V. (1986) Resistance to Facioliasis- a review. Veterinary Parasitology 20, 63-93

Herbert, D.R., Hölscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., Leeto, M., Kirsch, R., Hall, P., Mossmann, H., Claussen, B., Förster, I., Brombacher, F. (2004) Alternative macrophage activation is essential for survival during

schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity* 20, 623–635

Hernández-González, A., Valero, M.L., Sánchez del Pino, M., Oleagaa, A., Siles-Lucas, M. (2010) Proteomic analysis of in vitro newly excysted juveniles from Fasciola hepatica. *Molecular & Biochemical Parasitology* 172, 121–128

Hillyer G.V., del Llano de, D.A., and Reyes, C.N. (1977) Schistosoma mansoni: acquired immunity in mice and hamsters using antigens of Fasciola hepatica. *Experimental Parasitology* 42, 348-355

Hillyer G.V. (1979) Schistosoma mansoni: reduced worm burdens in mice immunized with isolated Fasciola hepatica antigens. *Experimental Parasitoogyl* 48, 287-295

Hillyer G.V. (1985) Induction of immunity in mice to Fasciola hepatica with a Fasciola/Schistosoma cross-reactive defined immunity antigen. *American Journal of Tropical Medicine and Hygeine* 34, 1127-1131

Hillyer G.V., Haroun, E.T., Hernandez, A., and de Galanes, M.S. (1987) Acquired resistance to Fasciola hepatica in cattle using a purified adult worm antigen. *American Journal of Tropical Medicine and Hygeine* 37, 363-369

Hillyer G.V., Garcia Rosa, M.I., Alicea, H., and Hernandez, A. (1988) Successful vaccination against murine Schistosoma mansoni infection with a purified 12 Kd Fasciola hepatica cross-reactive antigen. *American Journal of Tropical Medicine and Hygeine* 38, 103-110

Hillyer G.V. and Apt, W. (1997) Food-borne trematode infections in the Americas. Parasitology Today 61, 603-609

Hofmann, K., Stoffel, W., (1993) TMbase - A database of membrane spanning proteins segments. Biological Chemistry Hoppe-Seyler 374,166

Howell, M.J. (1973) Localization of Proteolytic Activity in Fasciola hepatica. *The Journal of Parasitology* 59, 454-456

Howell M.J., Board, P.G., and Boray, J.C. (1988) Glutathione S-transferases in Fasciola hepatica. *Journal of Parasitology* 74, 715-718

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

James SL, Sher A, Lazdins JK, Meltzer MS. (1982) Macrophages as effector cells of protective immunity in murine schistosomiasis. II. Killing of newly transformed schistosomula in vitro by macrophages activated as a consequence of Schistosoma mansoni infection. J*ournal of Immunology* 128,1535-40.

Jasmer, D. P., Lahmers, K. K. and Brown, W. C. (2007), *Haemonchus contortus* intestine: a prominent source of mucosal antigens. Parasite Immunology, 29: 139–151.

Jarrett, W.F.H., Sharp, N.C.C (1963). Vaccination against Parasitic Disease: Reactions in Vaccinated and Immune Hosts in *Dictyocaulus viviparus* Infection. *The Journal of Parasitology* 49, 177-189

Jarrett, W. F. H., Jennings, F.W., McIntyre, W.I.M., Mulligan, W., Urqhart, G.M., (1955) Immunological studies on *Dictyocaulus viviparus* infection. *Veterinary Record* 67, 291-296.

Jones, T.C., Hunt, R.D., King, N.W. (1997). Veterinary pathology (6th Edition). Williams & Wilkins USA, Page 659

Jones, B.F. and Hotez, P.J. (2002) Molecular cloning and characterization of Ac-mep-1, a developmentally regulated gut luminal metalloendopeptidase from adult *Ancylostoma caninum* hookworms. *Molecular Biochememical Parasitology* 119, 107–116

Jung-Won, J., Hyun-Na, J., Myoung-Ro, L., Shin-Hyeong, C., Hyeng-Il, C., Jung-Yeon, K., Young-Hee, L., Kwang-Jun, L., Woon-Mok, S., Dong-Min, K., Il-Chul, K., Byoung, C., and Tong-Soo, K. (2009). Identification of a serodiagnostic antigen, legumain, by immunoproteomic analysis of excretory-secretory products of *Clonorchis sinensis* adult worms. *Proteomics* 9, 3066–3078

Kalbe, M., Haberl, B., Haas, W., 1997, Miracidial host-finding in *Fasciola hepatica* and *Trichobilharzia ocellata* is stimulated by species-specific glycoconjugates released from the host snails. *Parasitology Research* 83, 806-812

Kendall, S.B., McCullough, F.S., (1951) The emergence of the cercariae of *Fasciola hepatica* from the snail *Lymnaea truncatula. Journal of Helminthology* 25, 77-92.

Kenyon F, Knox D (2002) The proteases of *Psoroptes ovis*, the sheep scab mite their diversity and substrate specificity. *Veterinary Parasitology* 105, 317-325

Kenyon F., Sargison, N.D., Skuce, P.J., and Jackson, F. (2009) Sheep helminth parasitic disease in south eastern Scotland arising as a possible consequence of climate change. *Veterinary Parasitology* 163, 293-297

Klion, A.D., Nutman, T.B. (2004). The role of eosinophils in host defense against helminth parasites. *The Journal of allergy and clinical immunology* 113, 30-37

Knox D.P. and Smith, W.D. (2001) Vaccination against gastrointestinal nematode parasites of ruminants using gut-expressed antigens. *Veterinary Parasitology* 100, 21-32

Knox D.P., Redmond, D.L., Newlands, G.F., Skuce, P.J., Pettit, D., and Smith, W.D. (2003) The nature and prospects for gut membrane proteins as vaccine candidates for Haemonchus contortus and other ruminant trichostrongyloids. *International Journal for Parasitology* 33, 1129-1137

Knox DP. (2000) Development of vaccines against gastrointestinal nematodes. Parasitology 120, S43-S61.

Knox, D.P. (2010) Parasite Vaccines: Recent Progress in, and Problems Associated with their Development. The *Open Infectious Diseases Journal* 4, 63-73.

Klinkert, M.Q., Bommert, K., Felleisen, R., Link, G., Doumbo, O., Beck, E., (1991). Immunological analysis of cloned *Schistosoma mansoni* antigens Sm31 and Sm32 with sera of schistosomiasis patients. *Tropical Medicine and Parasitology* 42, 319–324

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. and Higgins, D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947-2948.

Langley J.G. & Dunne D.W. (1992) Temporal variation in the carbohydrate and peptide surface epitopes in antibody-dependent, eosinophil mediated killing of *Schistosoma mansoni* schistosomula. *Parasite Immunology* 14, 185.

Leathem, A. J. and Brooks, S. A. (1998) Chapter 1, Light Microscopy. *In* Rhodes, J. M. and Milton, J. D. *Lectin Methods and Protocols* Humana Press Inc., New Jersey. *3-20*

Lee JY, Cho PY, Kim TY, Kang SY, Song KY, Hong SJ. (2002) Hemolytic activity and developmental expression of pore-forming peptide, clonorin. *Biochemical Biophysical Research Communication* 296, 1238–44.

Lehane, M.J., Billingsley, P.F.(1996) Biology of an insects midgut. Chapman and Hall, 2-6 boundary road, London,

Longbottom, D., Redmond, D. L., Russell, M.,Liddell, S., Smith, W. D. and Knox, D. P. (1997). Molecular cloning and characterisation of a putative aspartate protease associated with a gut membrane protein complex from adult Haemonchus contortus. *Molecular and Biochemical Parasitology* 88, 63–72.

Lowther J, Robinson MW, Donnelly SM, Xu W, Stack CM, et al. (2009) The Importance of pH in Regulating the Function of the *Fasciola hepatica* Cathepsin L1 Cysteine Protease. PLoS Negl Trop Dis 3(1): e369. doi:10.1371/journal.pntd.0000369

Loukas A, Bethony JM, Mendez S, Fujiwara RT, Goud GN, et al. (2005) Vaccination with Recombinant Aspartic Hemoglobinase Reduces Parasite Load and Blood Loss after Hookworm Infection in Dogs. *PLoS Med* 2, 295.

Mantovani A, Sica A, Locati M.(2005) Macrophage polarization comes of age. *Immunity* 23, 344–346.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A. (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437,** 376–380.

Mas-Coma, S., (2004). Chapter 19: Human fascioliasis. In: Cotruvo, J.A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D.O., Craun, G.F., Fayer, R., Gannon, V.P.J. (Eds.), World Health Organization (WHO), Waterborne Zoonoses: Identification, Causes and Control. IWA Publishing, London, pp. 305–322.

Mas-Coma S., Bargues, M.D., and Valero, M.A. (2005) Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology* 35, 1255-1278

McCarthy E., Stack, C., Donnelly, S.M., Doyle, S., Mann, V.H., Brindley, P.J., Stewart, M., Day, T.A., Maule, A.G., and Dalton, J.P. (2004) Leucine aminopeptidase of the human blood flukes, Schistosoma mansoni and Schistosoma japonicum. *International Journal for Parasitology* 34, 703-714

McGinty, A., Moore, M., Halton, D.W., Walker, B. (1993) Characteristics of the cysteine proteases of the common liver fluke *Fasciola hepatica* using novel, active-site directed affinity labels. *Parasitology* 106, 487-493.

McGonigle L., Mousley, A., Marks, N.J., Brennan, G.P., Dalton, J.P., Spithill, T.W., Day, T.A., and Maule, A.G. (2008) The silencing of cysteine proteases in Fasciola hepatica newly excysted juveniles using RNA interference reduces gut penetration. *International Journal for Parasitology* 38, 149-155

McGonigle S. and Dalton, J.P. (1995) Isolation of Fasciola hepatica haemoglobin. *Parasitology* 111, 209-215

McKenna D.R., Roebert, D.L., Bates, P.K., Schmidt, T.B., Hale, D.S., Griffin, D.B., Savell, J.W., Brooks, J.C., Morgan, J.B., Montgomery, T.H., Belk, K.E., and Smith, G.C. (2002) National Beef Quality Audit-2000: survey of targeted cattle and carcass characteristics related to quality, quantity, and value of fed steers and heifers. *Journal of Animal Science* 80, 1212-1222

McKerrow, J.H. (1989) Parasite proteases. *Experimental Parasitology* 68, 111-115.

McManus D.P. and Dalton, J.P. (2006) Vaccines against the zoonotic trematodes Schistosoma japonicum, Fasciola hepatica and Fasciola gigantica. *Parasitology* 133 Suppl, S43-S61

Mezo, M., Gonza´lez-Warleta, M., Carro, C., Ubeira, F.M. (2004) An ultrsensitive capture ELISA for detection of *Fasciola hepatica* corproantigens in sheep and cattle using a new monoclonal antibody (MM3). *The Journal of parasitology* 90, 845–852

Mendiola, J., Alonso, M., Marquetti, M.C., and Finlay, C. (1996). *Boophilus microplus*: multiple proteolytic activities in the midgut. *Experimental Parasitology* 82, 27–33

Meemon. K., Grams, R., Vichasri-Grams, S., Hofmann, A., Korge, G., Viyanant, V., Upatham, E.S., Habe, S., Sobhon, P. (2004). Molecular cloning and analysis of stage and tissue-specific expression of cathepsin B encoding genes from Fasciola gigantica. *Molecular Biochemal Parasitology* 136, 1-10.

Meeusen, E., Lee, C.S., Rickard, M.D., Brandon, M.R. (1995) Cellular responses during liver fluke infection in sheep and its evasion by the parasite.
ogy 17, 37-45.

Milbourne EA, Howell MJ. (1993) Eosinophil differentiation in response to Fasciola hepatica and its excretory/secretory antigens. International Journal of Parasitology 23, 1005-9.

Mitchell, G. B. B. (2008) Liver Fluke, in Diseases of Sheep, Fourth Edition (Aitken), Blackwell Publishing Ltd, Oxford, UK

Mitchell 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. *Research Veterinary Science* 30, 246-247

Mitchell G.B. (2002) Update on fasciolosis in cattle and sheep . *In Practice* 378-385

Mitchell G.F. and Rundle, K. (2009) Acute Liver fluke alert. *SAV Veterinary Investigation Centres*

Morphew, R.M., Wright, H.A., LaCourse, E.J., Woods, D.J., Brophy, P.M., (2007) Comparative proteomics of excretory-secretory proteins released by the liver fluke Fasciola hepatica in sheep host bile and during in vitro culture ex host. Molecular & Cellular Proteomics, 6:9, 63-972

Morrison C.A., Colin, T., Sexton, J.L., Bowen, F., Wicker, J., Friedel, T., and Spithill, T.W. (1996) Protection of cattle against Fasciola hepatica infection by vaccination with glutathione S-transferase. *Vaccine* 14, 1603-1612

Moser D., Tendler, M., Griffiths, G., and Klinkert, M.Q. (1991) A 14-kDa Schistosoma mansoni polypeptide is homologous to a gene family of fatty acid binding proteins. *Journal of Biogical Chemistry* 266, 8447-8454

Mosmann T.R., Cherwinski H., Bond M.W., Giedlin M.A. & Coffman R.L. (1986) Two types of murine helper T cell clone I. Definition according to profiles of lymphokine activities and secreted proteins. *The Journal of Immunology* **136,** 2348–2357

Mosmann T.R. & Coffman (1989) Heterogeneity of cytokine secretion patterns and function of helper T cells. *Advances in Immunology* **46,** 111–147

Movsesijan M, Jovanović. (1975). Immune response of sheep to Fasciola hepatica infection. *Research in Veterinary Science* 18, 171-4.

Muiño, L., Perteguer, M.J., Gárate, T., Martínez-Sernández, V., Beltrán, A., Romarís,F., Mezo, M., González-Warleta, M., Ubeira, F.M. (2011). Molecular and immunological characterization of *Fasciola* antigens recognized by the MM3 monoclonal antibody. *Molecular and Biochemical Parasitology* 179, 80–90

Mulcahy, G., Joyce, P., and Dalton, J. P. (1999) Chapter 10, Immunology of *Fasciola hepatica* infection. *In* Dalton, J. P. *Fasciolosis* CABI publishing, Wallington, *341-375*

Mulcahy G. and Dalton, J.P. (2001) Cathepsin L proteases as vaccines against infection with Fasciola hepatica (liver fluke) in ruminants. *Research in Veterinary Science* 70, 83-86

Müller, Die Nahrung von Fasciola hepatica und idre Nerdauung, Zoologischel Anzeiger (1923), pp. 273–281

Munn, E.A., Greenwood, C.A., Coadwell, W.J. (1987) Vaccination of young lambs by means of a protein fraction extracted from adult *Haemonchus contortus. Parasitology* 94, 385–397

Murray J. and Smith, W.D. (1994) Ingestion of host immunoglobulin by three non-blood-feeding nematode parasites of ruminants. *Research in Veterinary Science* 57, 387-389

Newlands GF, Skuce PJ, Nisbet AJ, Redmond DL, Smith SK, Pettit D, Smith WD (2006) Molecular characterization of a family of metalloendopeptidases from the intestinal brush border of *Haemonchus contortus*. *Parasitology* 133, 357-68.

Nisbet, A.J., McNeilly, T.N., Wildblood, L.A., Matthews, J.B. (2012) Control of a parasitic nematode in sheep by vaccination with a recombinant antigen cocktail. Proceedings of The British Society of Parasitology Spring Meeting, Strathclyde University, OR84, Page 66

Noel, W., Raes, G., Hassanzadeh Ghassabeh, G., De Baetselier, P., Beschin, A. (2004) Alternatively activated macrophages during parasite infections. *Trends in Parasitology* 20, 126–133

Nilaweera, K.N., Archer, Z.A., Campbell, G., Mayer, C.D., Balik, A., Ross, A.W., Mercer, J.G., Ebling, F.J., Morgan, P.J., Barrett, P. (2009). Photoperiod regulates genes encoding melanocortin 3 and serotonin receptors and secretogranins in the dorsomedial posterior arcuate of the Siberian hamster. *Journal of Neuroendocrinology* 21, 123–131

Newport, G.R., McKerrow, J.H., Hedstrom, R. (1988). Cloning of the protease that infection by schistosome parasites. *Journal of Biological Chemistry* 26, 13179 - 13184.

Oliver EM, Skuce PJ, McNair CM, Knox DP (2006). Identification and characterization of an asparaginyl protease (legumain) from the parasitic nematode, *Haemonchus contortus*. *Parasitology* 133, 237-44

Oslen O.W. (1986) Animal Parasites. Their Life Cycles and Ecology. 3,

Overend D.J. and Bowen, F.L. (1995) Resistance of Fasciola hepatica to triclabendazole. *Aust.Vet.J.* 72, 275-276

Page, R. D. M. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357-358

Pappin DJ, Hojrup P, Bleasby AJ (June 1993). "Rapid identification of proteins by peptide-mass fingerprinting". *Current Biology* **3** (6): 327–32

Petersen, T.N., Brunak, S., von Heijne, G.,Nielsen, H. (2011). SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nature Methods* 8,785-786.

Piacenza L., Acosta, D., Basmadjian, I., Dalton, J.P., and Carmona, C. (1999) Vaccination with cathepsin L proteases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. *Infection and Immunity* 67, 1954-1961

Poulin R. (2006) Global warming and temperature-mediated increases in cercarial emergence in trematode parasites. *Parasitology* 132, 143-151

Ranjit, N., Jones, M.K., Stenzel, D.J., Gasser, R.B., Loukas, A. (2006) A survey of the intestinal transcriptomes of the hookworms, *Necator americanus* and *Ancylostoma caninum*, using tissues isolated by laser microdissection microscopy. *International Journal for Parasitology* 36, 701–710

Rappuoli, R. (2001) Reverse vaccinology, a genome-based approach to vaccine development. *Vaccine* 19, 2688-2691.

Rawlings, N.D., Barrett, A.J., Baterman, A., (2010) MEROPS: The peptide database. *Nucleic Acid Research* 38, D227-233.

Redmond,D.L. et al. (1997)Molecular cloning and characterisation of a developmentally regulated putative metallopeptidase present in a host protective extract of Haemonchus contortus. *Molecular Biochemical Parasitology* 85, 77–87

Relf, V., Good, B., McCarthy, E. and de Waal, T. (2009). Evidence of *Fasciola hepatica*

infection in *Radix peregra* and a mollusc of the family Succineidae in Ireland. *Veterinary Parasitology*; 163: 152-155.

Richard Bennett and Jos IJpelaar. (2003) Economic Assessment of Livestock Diseases in Great Britain.

Rickard, M.D., Harrison, G.B., Heath, D.D., Lightowlers, M.W. (1995). Taenia ovis recombinant vaccine--'quo vadit'. *Parasitology* 110, S5-9

Roberts J.A. and Suhardono. (2008) Approaches to the control of fasciolosis in ruminants. *International Journal for Parasitology* 26, 971-981

Robinson MW, Tort JF, Lowther J, Donnelly SM, Wong E, et al. (2008) Proteomic and phylogenetic analysis of the cathepsin L protease family of the helminth pathogen, Fasciola hepatica: expansion of a repertoire of virulence associated factors. *Molecular and Cellular Proteomics* 7, 1111–1123.

Robinson, M.W., Tort, J.F., Lowther, J., Donnelly, S.M., Wong, E., Xu, W., Stack, C.M., Padula, M., Herbert, B., Dalton, J.P. (2008) Proteomics and phylogenetic analysis of the cathepsin L protease family of the helminth pathogen *Fasciola hepatica*: expansion of a repertoire of virulence-associated factors. *Molecular Cell Proteomics* 7, 1111-1123

Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P. and Ranganathan, S. (2009).An Integrated Transcriptomics and Proteomics Analysis of the Secretome of the Helminth Pathogen *Fasciola hepatica*. *Molecular &Cellular Proteomics* 8, 1891–1907.

Rodriguez-Perez J., Rodriguez-Medina J.R., Garcia-Blanco M.A., Hillyer G.V. (1992). *Fasciola hepatica*: molecular cloning, nucleotide sequence, and expression of a gene encoding a polypeptide homologous to a *Schistosoma mansoni* fatty acid-binding protein**.** *Experimental Parasitology* 74, 400-407

Rodríguez-Sosa, M., Satoskar, A.R., Calderón, R., Gomez-Garcia, L., Saavedra, R., Bojalil, R., Terrazas, L.I. (2002). Chronic helminth infection induces alternatively activated macrophages expressing high levels of CCR5 with low interleukin-12 production and Th2-biasing ability. *Infection and Immunity* 70, 3656-64.

Romagnani, S. (1992) Induction of T_H1 and T_H2 responses: a key role for the 'natural' response? *Immunology today* 13, 379-381

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

Roepstorff, P. (1997). Mass spectrometry in protein studies from genome to function. *Current Opinion in Biotechnology* **8**, 6-13.

Rozen, S., Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386

Sabin, E.A., Kopf, M.A., Pearce. E.J. (1996) *Schistosoma mansoni* Egg-induced Early IL-4 Production Is Dependent upon IL-5 and Eosinophils. *Journal of experimental medicine* 184, 1871-1878.

Sandeman R.M. and Howell, M.J. (1981) Response of sheep to challenge infection with Fasciola hepatica. *Research in Veterinary Science* 30, 294-297

Saladin, K.S., 1979, Behavioural parasitology and perspectives on miracidial host-finding. Z. Parasitenkd. 60, 197-210.

Sargison N.D. (2006) Investigation of poor scanning results. *UK Vet* 11, 52-56

Sargison, N.D. (2011) Diagnosis and economic consequences of triclabendazole resistance in *Fasciola hepatica* in a sheep flock in south-east Scotland. *Veterinary Record* **168,** 159

Sajid, M., McKerrow, J. H., (2002). Cysteine proteases of parasitic organisms. *Molecular Biochemical Parasitology 120*, 1–21

Science for Environment Policy (2008): European Commission DG Environment News Alert Service, edited by SCU, The University of the West of England, Bristol. News Alert Issue 95.

Schmidt G.D. and Roberts, L.S. (2005) Foundations of Parasitology 7. McGraw-Hill, 2006

Sexton J.L., Milner, A.R., Panaccio, M., Waddington, J., Wijffels, G., Chandler, D., Thompson, C., Wilson, L., Spithill, T.W., Mitchell, G.F., and . (1990) Glutathione S-transferase. Novel vaccine against *Fasciola hepatica* infection in sheep. *Journal of Immunology* 145, 3905-3910

Shevchenko A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analalytical Chemistry* 68, 850-858

Sinclair K.B. (1971) Acquired resistance to *Fasciola hepatica* in sheep. *British Veterinary Journal* 127, 125-136

Singleton DR, Stear MJ & Matthews L. (2010) A mechanistic model of developing immunity to Teladorsagia circumcincta infection in lambs. *Parasitology* 138: 1–11.

Sinha, J.K., Bhattacharya, S. (2006) A textbook of Immunology. B.K. Dhur of Academic publishers, 12/1A, Bankim Chatterjee Street, Kolkata-700 073.

Simpkin, K.G., Chapman, C.R.., Coles, G.C. (1980) *Fasciola hepatica:* A proteolytic digestive enzyme. *Experimental Parasitology* 49, 281–287

Skelly, P.J., Da'dara, A., Harn, D.A., (2003) Suppression of cathepsin B expression in *Schistosoma mansoni* by RNA interference. International Journal of Parasitology 33, 363–369

Skuce P.J., Sargison, N.D., Kenyon, F., Jackson, F., and Mitchell, G.B. (2008) New challenges for the control of helminth parasites of Scottish livestock in the face of climate change. *Livestock and Global Climate Change* 97-99

Smith, G., Grenfell, B.T., 1984, The influence of water temperature and pH on the survival of *Fasciola hepatica* miracidia. Parasitology 88, 97-104

Smith (1995) Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. Electrophoresis 19, 1090–1094

Smith A.M., Dowd, A.J., Heffernan, M., Robertson, C.D., and Dalton, J.P. (1993a) Fasciola hepatica: a secreted cathepsin L-like protease cleaves host immunoglobulin. *Int.J Parasitol* 23, 977-983

Smith A.M., Dowd, A.J., McGonigle, S., Keegan, P.S., Brennan, G., Trudgett, A., and Dalton, J.P. (1993b) Purification of a cathepsin L-like protease secreted by adult Fasciola hepatica. *Mol.Biochem.Parasitol* 62, 1-8

Smith, S.K., Smith, W.D. (1996). Immunization of sheep with an integral membrane glycoprotein complex of Haemonchus contortus and with its major polypeptide components. Research Veterinary Science 60, 1-6.

Smith S.K., Pettit, D., Newlands, G.F., Redmond, D.L., Skuce, P.J., Knox, D.P., and Smith, W.D. (1999) Further immunization and biochemical studies with a protective antigen complex from the microvillar membrane of the intestine of Haemonchus contortus. *Parasite Immunol.* 21, 187-199

Smith WD, Jackson F, Jackson E, Williams J & Miller HR. (1984). Manifestations of resistance to ovine ostertagiasis associated with immunological responses in the gastric lymph. Journal of Comparative Pathology 94, 591–601.

Smith W.D., Smith, S.K., and Murray, J.M. (1994) Protection studies with integral membrane fractions of Haemonchus contortus. *Parasite Immunol.* 16, 231-241

Smith W.D. (1999) Prospects for vaccines of helminth parasites of grazing ruminants. *International Journal for Parasitology* 29, 17-24

Smith W.D., Smith, S.K., and Pettit, D. (2000a) Evaluation of immunization with gut membrane glycoproteins of Ostertagia ostertagi against homologous challenge in calves and against Haemonchus contortus in sheep. *Parasite Immunol.* 22, 239-247

Smith W.D., Smith, S.K., Pettit, D., Newlands, G.F., and Skuce, P.J. (2000b) Relative protective properties of three membrane glycoprotein fractions from Haemonchus contortus. *Parasite Immunol.* 22, 63-71

Smith, W. D., Newlands, G. F., Smith, S. K., Pettit, D. and Skuce, P. J. (2003). Metalloendopeptidases from the intestinal brush border of Haemonchus contortus as protective antigens for sheep. Parasite Immunology 25, 313–323.

Smith, W. D., Skuce, P. J., Newlands, G. F., Smith, S. K. and Pettit, D. (2003). Aspartyl proteases from the intestinal brush border of Haemonchus contortus as protective antigens for sheep. Parasite Immunology 25, 521–530.

Spithill T.W. and Dalton, J.P. (1998) Progress in Development of Liver Fluke Vaccines. *Parasitology Today* 14, 224-228

Spithill, T. W., Smooker, Peter M., Sexton, J. L., Bozas, E., Morrison, C. A., Creaney, J., and Parsons, J. C. (1999) Development of vaccines against *Fasciola hepatica* . *In* Dalton, J. P. *Fasciolosis* CAB International, wallinford, UK, *377-401*

Stear MJ, Strain S & Bishop SC.(1999) How lambs control infection with Ostertagia circumcincta. Veterinary Immunology and Immunopathology 72, 213–218

Sukhdeo M.V.K. and Sukhdeo, S.C. (2002) Fixed behaviours and migration in parasitic flatworms. *International Journal for Parasitology* 32, 329-342

Suttiprapa S, Mulvenna J, Huong NT, Pearson MS, Brindley PJ, Laha T, Wongkham S, Kaewkes S, Sripa B, Loukas A (2009) Ov-APR-1, an aspartic protease from the carcinogenic liver fluke, *Opisthorchis viverrini*: functional expression, immunolocalization and subsite specificity. *International Journal of Biochemistry and Cell Biology* 41, 1148-56

Tang, J., Wong, R.N. (1987) Evolution in the structure and function of aspartic proteases Journal of Cell Biochemistry 33 , 53–63

Tan, F., Morris, P.W., Skidgel, R.A. & Erdos, E.G. (1993) Sequencing and cloning of human prolylcarboxypeptidase (angiotensinase C) Similarity to both serine carboxypeptidase and prolylendopeptidase families. Journal of Biological Chemistry 268, 16631-16638

Taylor G.K. and Goodlett, D.R. (2005) Rules governing protein identification by mass spectrometry. *Rapid Commun.Mass Spectrom.* 19, 3420-

Taylor S.M., Langridge, S.A., and Kenny, J. (1994) Anthelmintic suppression of Fasciola hepatica infections in sheep. *Vet Rec.* 135, 86-88

Torgerson, P. and Claxton, J. (1999) Chapter 4, Epidemiology and control. *In* Dalton, J. P. *Fascioloasis* CAB publishing, Wallington, *113-139*

Tort, Jose, Brindley, Paul J., Knox, Dave, Wolfe, Kenneth H., and Dalton, John P. (1999) Proteases and Associated Genes of Parasitic Helminths. *In* Baker, J. R. *Advances in Parasitology* Volume 43 edition. Academic Press, *161-266*

Tort, J., Brindley, P.J., Knox, D., Wolfe, K.H., Dalton, J.P.,(1999). Helminth proteases and their associated genes. Advancement Parasitol., 43 (1999), pp. 161–266

Thorsell, W., and Bjorkman, N. (1965) On the fine structure of the Mehlis' gland cells in the liver fluke, *Fasciola hepatica.* L. Zeitschrift fur parasitenkunde 26, 63-70.

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

Urban JF Jr, Maliszewski CR, Madden KB, Katona IM, Finkelman FD (1995) IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice. *J Immunol* 154:4675-4684.

Urban JF Jr, Noben-Trauth N, Donaldson DD, Madden KB, Morris SC, Collins M, Finkelman FD (1998) IL-13, IL-4R and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunity* 8, 255-264.

Van Milligen, F.J., Cornelissen, J.B., Hendriks, I.M., Gaasenbeek, C.P., Bokhout, B.A. (1998) Protection of Fasciola hepatica in the gut mucosa of immune rats is associated with infiltrates of eosinophils, IgG1 and IgG2a antibodies around the parasites. Parasite immunology 20,285-92

van D.J. and Morgan, E.R. (2008) The influence of temperature on the development, hatching and survival of Nematodirus battus larvae. *Parasitology* 135, 269-283

Vengust G., Klinkon, M., Bidovec, A., and Vengust, A. (2003) Fasciola hepatica: effects on blood constituents and liver minerals in fallow deer (Dama dama). *Vet Parasitol* 112, 51-61

Verity, C.K., McManus, D.P., Brindley, P.J. (1999) Developmental expression of cathepsin D aspartic protease in Schistosoma japonicum. International Journal for Parasitology 29, 1819-1824.

Verity, C. K., McManus, D. P. and Brindley, P. J. (2001), Vaccine efficacy of recombinant cathepsin D aspartic protease from *Schistosoma japonicum*. Parasite Immunology, 23: 153– 162

Veterinary Laboratories Agency. (2007) Veterinary Laboratories Agency Surveillance Reports 2007.

Veterinary Laboratories Agency. (2008) Veterinary Laboratories Agency Surveillance Reports 2008.

Walton, C.L., 1918, Liver rot of sheep, and bionomics of *Limnaea truncatula* in the Aberystwyth area. Parasitology 10, 232-266.

Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I. (1995). "Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium". *Electrophoresis* **7** (7): 1090–94.

WHO. (2007) Report of the WHO informal meeting on use of triclabendazole in fascioliasis control. WHO/CDS/NTD/PCT/2007,

Wijffels G.L., Sexton, J.L., Salvatore, L., Pettitt, J.M., Humphris, D.C., Panaccio, M., and Spithill, T.W. (1992) Primary sequence heterogeneity and tissue expression of glutathione S-transferases of Fasciola hepatica. *Exp.Parasitol* 74, 87-99

Wijffels G.L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J., Bowen, F., Friedel, T., and Spithill, T.W. (1994) Vaccination of Sheep with Purified Cysteine Proteases of Fasciola hepatica Decreases Worm Fecundity. *Experimental Parasitology* 78, 132-148

Willadsen, P., Riding, G.A., McKenna, R.V., Kemp, D.H., Tellam, R.L., Nielsen, J.N., Lahstein, J., Cobon, G.S. and Gough, J.M. 1989. Immunological control of a parasitic arthropod: identification of a protective antigen from *Boophilus microplus*. Journal of Immunology 143, 1346–1351.

Willadsen P., Bird, P., Cobon, G.S., and Hungerford, J. (1995) Commercialisation of a recombinant vaccine against Boophilus microplus. *Parasitology* 110 Supplement, S43-S50

Williams, S.A. *et al.* (1999) Helminth genome analysis: the current status of the filarial and schistosome genome projects. *Parasitology* 118, S19–S38

Williamson AL, Lecchi P, Turk BE, Choe Y, Hotez PJ (2004) A multienzyme cascade of hemoglobin proteolysis in the intestine of blood-feeding hookworms. Journal of Biological Chemistry 279, 35950–35957

Williamson, A.L., Brindley, P.L., Abbente, G., Prociv, P., Berry, C., Girdwood, K., Pritchard, D.I., Fairlie, D.P., Hotez, P.J., Dalton, J.P., Loukas, A., (2002) Cleavage of hemoglobin by hookworm cathepsin D aspartic proteases and its potential contribution to host specificity. The FASEB Journal 16,1458-1460

Williamson, A.L., Brindley, P.J., Knox, D.P., Hotez, P.J., Loukas, A. (2003) Digestive proteases of blood-feeding nematodes. Trends in Parasitology 19, 417-423.

Wilson, R.A., Wright, J.M., de Castro-Borges, W., Parker-Manuel, S.J., Dowle, A.A., Ashton, P.D., Young, N.D., Gasser, R.B., Spithill, T.W. (2011) Exploring the *Fasciola hepatica* tegument proteome. International Journal for Parasitology 41, 1347 1359

Wijffels, G.L., Salvatore, L., Dosen, M. (1994). Vaccination of sheep with purified cysteine proteases of Fasciola hepatica decreases worm fecundity. Experimental Parasitology 78, 132-148.

Wilson L.R., Good, R.T., Panaccio, M., Wijffels, G.L., Sandeman, R.M., and Spithill, T.W. (1998) Fasciola hepatica: characterization and cloning of the major cathepsin B protease secreted by newly excysted juvenile liver fluke. *Exp.Parasitol* 88, 85-94

Wilson, R. A., Smith, G., and Thomas, M. R. (1982) Fasciolosis. *The Population Dynamica of Infectious Disease: Theory and Applications.* Chapman & Hall, London, New York, *262-319*

Wuhrer M., Grimm, C., Dennis, R.D., Idris, M.A., and Geyer, R. (2004) The parasitic trematode Fasciola hepatica exhibits mammalian-type glycolipids as well as Gal(beta1-6)Gal-terminating glycolipids that account for cestode serological cross-reactivity. *Glycobiology* 14, 115-126

Xu Y.Z. and Dresden, M.H. (1986) Leucine aminopeptidase and hatching of Schistosoma mansoni eggs. *J Parasitol* 72, 507-511

Yamasaki H., Kominami, E., and Aoki, T. (1992) Immunocytochemical localization of a cysteine protease in adult worms of the liver fluke Fasciola sp. *Parasitol Res.* 78, 574-580

Zhai Y, Saier MHJ. (2000) The amoebapore superfamily. Biochim Biophys Acta 1469,87–99.

Zimmerman, G.L., Kerkvliet, N.I., Brauner, J.A., Cerro, J.E. (1983). Modulation of Host Immune Responses by Fasciola hepatica: Responses of Peripheral Lymphocytes to Mitogens during Liver Fluke Infections of Sheep. *The Journal of Parasitology* 69, 473-477