Expression of stage-specific *Fasciola* proteases and their evaluation in vaccination trials

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Declaration

I certify that except where due acknowledge has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and any editorial work, paid or unpaid, carried out by a third party is acknowledge.

Signature:

Ramamoorthi Jayaraj

Date:

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List of abbreviations

Ab	Antibody
ANGIS	Australian National Genomic Information Service
APC	Antigen presenting cell
bp	Base pairs
BSA	Bovine serum albumin
℃	Degrees centigrade
Cat	Cathepsin
CFU	Colony forming units
CIP	Calf intestinal phophatase
CMI	Cell-mediated immunity
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
Da	Dalton
DC	Dendritic cell
DIG	Digoxigenin
DNA	Deoxyribonuclease
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylenediamine tetra acetic acid, disodium salt
ELISA	Enzyme-linked immunosorbent assay
g	Relative centrifugal force
Gdn	Guanidine hydrochloride
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide

HCl	Hydrochloric acid
HRP	Horse radish peroxidase
Ig	Immunoglobulin
IFN-γ	Interferon gamma
IL 4	Interleukin 4
IPTG	Isopropyl-thiogalactoside
kb	Kilobase pairs
kDa	One thousand Daltons
LB	Luria Bertani broth
M	Molarity
MCS	Multiple cloning site
Mg	Milligram
Min	Minutes
mL	Millilitre
mM	Millimolar
MW	Molecular weight
NaCl	Sodium chloride
Na_2HPO_4	Di sodium hydrogen phosphate
NaH_2PO_4	Sodium di hydrogen phosphate
NaOH	Sodium hydroxide
NCS	New born calf serum
ng	Nanograms
Ni	Nickel
nM	Nanomolar
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Negative algorithm of hydrogen ion concentration

RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) amino methane
V	Voltage
X-gal	5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside
µg	Microgram
µL	Microlitre
µm	Micrometer

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Summary

The liver flukes *Fasciola hepatica* and *F. gigantica* cause infectious disease in ruminants and humans. The geographical range of these two parasite species (temperate and tropical respectively) ensures that infection can occur worldwide. Although anthelmintic treatment is effective against disease, emerging drug resistant strains leads to the development of a vaccine. However, despite several decades of research, there is no commercial vaccine available. Vaccine target antigens have been isolated and characterised from the fluke. The main challenge at present is to produce recombinant proteins in an immunologically active form using recombinant DNA technology. This is an essential step in *Fasciola* vaccine production.

Cysteine proteases are probably the most important facilitators of virulence in flukes and are produced by all stages of the fluke life-cycle. Two classes of cysteine protease are found in the excretory and secretory material of liver flukes- these are cathepsin L and cathepsin B. As such, the major aims of this thesis were to investigate the expression and purification of *Fasciola* recombinant cysteine proteins, and characterisation by SDS-PAGE and immunoblotting using monoclonal and polyclonal antibodies. These studies demonstrate the production of functionally active cathepsin L5, cathepsin L1g and cathepsin B in *S. cerevisiae* BJ3505 cells which will lead to vaccine candidate analysis and to explore host-parasite relationships.

The main goal of vaccine efficacy analysis and vaccine development is to observe immune responses in vaccinated animals. The second aim of this thesis was to determine the protective efficacy of stage specific target antigens against experimental infection. In addressing this issue, the protective efficacy of single and multivalent recombinant protein vaccinations of adult stage *F. hepatica* cathepsin L5, immature *F. gigantica* cathepsin L1g and juvenile *F. hepatica* cathepsin B were

analysed in Sprague Dawley rats against *F. hepatica* infection. In this study, the protective efficacy of anti-fluke vaccines was evaluated as parasitological parameters (recovered fluke burden, fluke body size and wet weight), pathological parameter (liver damage score), haematological parameter (leukocyte profile) and humoral responses (ELISA and western blotting) in vaccinated and control rats.

This study demonstrates that juvenile fluke target antigen-cathepsin B induces better immune protection than adult fluke antigen-cathepsin L5. The protective efficacy of cathepsin L1g was low compared to cathepsin B. The results of this study suggest that cathepsin B is a valuable target antigen for the development of an effective vaccine target against fluke infection. Cocktails of juvenile and adult stage fluke recombinant proteins (cathepsin B and L5) elicited the highest protective immunity against experimental infection and this combination showed not only reduction in fluke recovery and size of flukes, but also marked diminution in the intensity of liver lesions in vaccinated rats. The recombinant protein vaccines that hinder the transmission of infection *via* lowering the number of parasites in the host and block pathological symptoms in animals have been considered as potential components of disease control.

In order to assess the immunogenic property of an early infective stage fluke secreting cysteine protease as a vaccine candidate, DNA vaccination vectors encoding cathepsin B were analysed in BALB/c mice. In this study, the ability of four DNA vaccination strategies such as secretory, chemokine-activating, lymph node targeting vectors encoding cathepsin B were assessed by antibody titre, antibody avidity, western blotting and ELIPSOT assay. The elucidation of the relative vaccination potential in each of these constructs was revealed as enhanced antibody responses (on lymph node targeting), high antibody avidity (of chemo-attractant) or stronger cellular responses (of signal peptide carrying) in vaccinated animals. The results of this investigation demonstrate DNA vaccines have excellent

potential to induce both humoral and cellular immunity in vaccinated animals. The results have further validated the immunoprophylactic potential of a cathepsin B vaccine against *F. hepatica*.

In this study, we have expressed and attained high yields of *F. gigantica* cathepsin L1g from *E. coli* BL21, and compared this to a yeast-expressed system. This protease was over-expressed and formed insoluble inclusion bodies that were subsequently solubilised with urea or guanidine hydrochloride. In order to purify the urea-solubilised protein, step-wise urea gradient chromatography was used. Guanidine hydrochloride solubilised protein was not subjected to chromatography. For refolding of solubilised protein, a dilution and dialysis procedure was utilised. The expression and refolding yielded 2.5 mg of protease from one litre of bacterial culture compared to 300 µg/litre from a corresponding yeast culture. Proteolytic activity was confirmed by gelatin SDS-PAGE analysis.

Functionally active *Fasciola* proteins were purified from yeast. Protective immunity of the expressed proteins was observed in rats challenged with *Fasciola* infection. An enhanced immune response was observed when a juvenile fluke protein was used in targeting and non-targeting DNA vaccine forms. *Fasciola* protein was expressed in *E. coli* and was present in inclusion bodies in an inactive form. The active form of the protein was successfully obtained by refolding. In conclusion, the determination of the immune potential of recombinant stage specific antigens allows the development of effective vaccines against *Fasciola* infection.

Chapter 1

Literature review

1.1. Introduction

1.1.1. Fasciolosis

Fasciolosis is an infection mainly of ruminants and humans by digenetic trematodes, mainly *Fasciola hepatica* and *F. gigantica*. The former species has a world-wide distribution mainly in the temperate climates; but the latter species exists in tropical countries. Fasciolosis causes significant economic loss, as valued by animal productivity, estimated at approximately US$ 3.2 billion US dollars per annum to the global agricultural community with 600 million animals infected (Mas-Coma, 2005; McManus & Dalton, 2006; Spithill *et al*., 1997). *Fasciola* infects mainly sheep and cattle. Other animals that may be affected include goats, horses and pigs. Recently Mas coma (2005) reported that llmas in South America, camels in Africa and marsupials in Australia may be acting as reservoir hosts in these regions.

1.1.2. Distribution and prevalence of *Fasciola* infection

Fasciola infection occurs mainly in temperate regions and outbreaks have been reported throughout Europe. At present, fasciolosis is also highly prevalent in the US, Mexico, North Africa, New Zealand and Australia (Mas-Coma, 2005; Mas-Coma, 2007; McManus & Dalton, 2006). *F. hepatica* has become a major threat to humans in South America especially in Brazil, Argentina, Uruguay and also Andean countries such as Bolivia, Peru and Equador (Espino *et al*., 1998; Mas-Coma, 2005). *F. gigantica* infection has a more limited distribution because of the reduced survivability of snails (the intermediate host) in tropical regions. It is most prevalent in Africa (Egypt) and South East Asia (Mas-Coma, 2005; McManus & Dalton, 2006).

Both species have been reported in Japan, Korea, Taiwan and the Philippines (Agatsuma *et al*., 2000; Lim *et al*., 2007a).

The distribution and prevalence of the *Fasciola* infected cattle population in Queensland, Australia is depicted in Figure 1.1. Among the two species, *F. hepatica* is present in Australia and the livestock production loss has been estimated to be approximately US$ 80 million/year (Molloy & Anderson, 2006; Mulcahy & Dalton, 2001). In creeks within the Brisbane metropolitan area, a mixture of *F. hepatica* and *F. gigantica* were found about 25 years ago . As shown in Figure 1.1, the occurrence of *F. hepatica* infection is much more frequent in dairy herds than in beef herds. However, the dairy herds are usually in higher rainfall areas (southeast Queensland) with habitats suitable for the survival of aquatic snails .

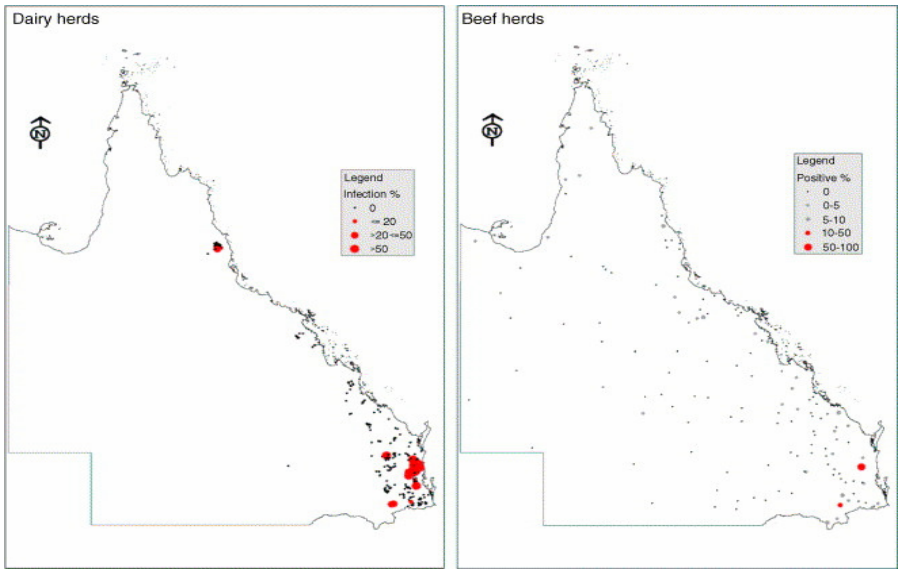


Figure 1.1`. The distribution and prevalence of *F. hepatica* infection in dairy and beef herds in Queensland, Australia (adopted from Molloy and Anderson, 2006). The percentage of infection and postive cases of *F. hepatica* are noted as red dots.

1.1.3. Control of liver fluke infection

Liver flukes are controlled by a combination of anthelmintic drugs and pasture management. A series of chemotherapeutic agents are available for the treatment of animal fasciolosis, including closantal, clorsulan, rafoxanide, nitroxynil and triclabendazole (Dalton *et al*., 2003b; Fairweather & Boray, 1999). The major anti-flukicide triclabendazole (Fasinex^{R}, Novartis Animal Health) is efficient against both juvenile flukes in the hepatic parenchyma and adult flukes in the bile ducts . However, triclabendazole resistant parasites have been reported in Europe and Australia . Most importantly, government and consumer concern about chemical and antibiotic residues in animal foods (milk and meat) and the environmental threat from chemical and pesticide (Molloscocide) use on pastures. Chemicals runoff into water supplies will make the chemical control of fasciolosis more difficult in the future (Dalton *et al*., 2003b).

1.1.4. Life cycle of *Fasciola* spp.

Two hosts (intermediate and definitive hosts) are involved in the liver fluke life cycle (Figure 1.2). The definitive host range is very broad which includes ruminants and humans (Boray *et al*., 1985; Mas-Coma *et al*., 1999). Intermediate hosts are fresh water snail species of the family Lymnacidae (Gastropoda: Basommatophora). Flukes attain maturity in the mammalian host bile duct and release eggs which are finally excreted with faeces. In damp and muddy areas the free swimming miracidium infect snails and this is followed by a series of developmental and multiplication stages such as sporocyst and redia.

After the development stages within the snail, the infective metacercariae emerges and get attached to the plant matter. The final host can be infected by eating contaminated aquatic plants such as water cress encysted with metacercariae. Following excystation in the gut of the host due to digestive juice activity, the newly excysted juvenile flukes penetrate the intestinal wall, enter the peritoneum and migrate into the hepatic parenchyma and the liver. Inside the liver, the immature flukes cause migratory tracts resulting in extensive haemorrhage and perforations (hepatic stage infection). This acute stage of infection can result in death in highly infected sheep, but rarely in cattle. At the final stage, flukes reach host bile duct. In chronic cases, infection causes oedema, bottle jaw, anaemia and thickening of bile duct (cirrhosis) (biliary stage infection) (Lim *et al.*, 2007a). The life span of adult fluke in man is between 9 and 13.5 years (Mas-Coma *et al*., 1999; Mas-Coma, 2005; Mas-Coma, 2007).

The infection usually evolves as a chronic disease, subacute and acute fasciolosis with high rates of mortality are not rare in small ruminants. In sheep and cattle, the juvenile flukes induce a granulomatous lesion in the hepatic parenchyma, with numerous macrophages, lymphocytes and eosinophils (Boray *et al.*, 1985; Mas-Coma *et al.*, 1999).

1.1.5. Human fasciolosis

Human liver fluke infections are classed as food borne zoonatic disease which is acquired by accidental ingestion of infective metacercariae. Human fasciolosis is an emerging health problem with an estimated 2.4-17 million people infected around the world with a further 180 million at risk of infection (Curtale *et al.*, 2005; Mas-Coma *et al*., 1999). The major clinical symptoms of human infection include fever, pain in the right hypochondrium, anorexia, persistent diarrhoea and vomiting .

Human infections are always associated with local endemic animal infections. Vegetable contamination with infective metacercariae (fresh water aquatic plants –

water cress) and free floating metacercariae in drinking water pipe lines play a major role in transmission of disease (Mas-Coma, 2007; Rokni *et al.*, 2003). In addition, parasites in humans produce eggs that play a major role in overall transmission of disease in third-world countries . Immuno-suppression due to *Fasciola* spp. leads to multi-parasitism (e.g. Giardiasis) and such super infection has been reported in Egypt and South America (Brady *et al.*, 1999; Mas-Coma, 2005).

1.1.5.1. Epidemiological reports of human liver fluke infection

Several epidemiological studies revealed that human infection is highly prevalent in the high altitude and rural poor regions of Bolivia, Peru and Equador (Curtale *et al.*, 2005; Esteban *et al.*, 1999; Mas-Coma *et al.*, 1999). Human infection is more prevalent among children of all ages in both sexes. This human pathogen can survive up to 13 years and lay up to 5000 eggs/gram faeces in infected Bolivian children who in turn act as disease carriers to humans and animals . The highest levels of human fasciolosis (due to *F. hepatica*) are noticed amongst the indigenous people of the Northern Bolivian Altiplano and prevalence is age-related with the highest infection rate in children aged 8-11 years . Human fasciolosis is also reported in the Guilan and Mazandaran provinces of Northern Iran with frequent outbreaks of up to 10,000 infections (Moghaddam *et al.*, 2004; Rokni *et al.*, 2002). Human infection in European countries occurs sporadically, but in Spain, France and Portugal there are approximately 50 to 100 cases per year (Mas-Coma *et al*, 2005).

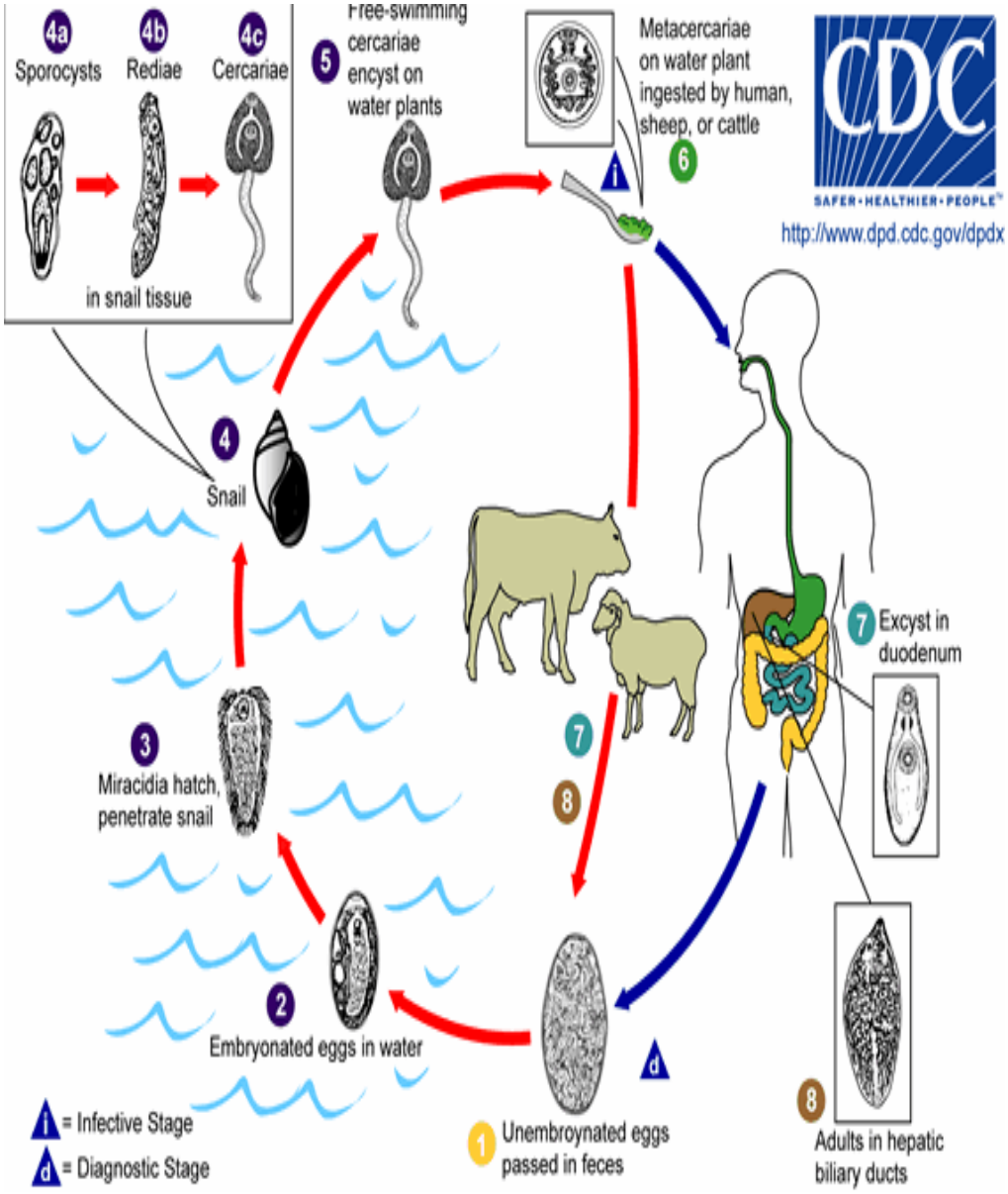


Figure 1.2. The life cycle of *F. hepatica*. Adopted from:

http://www.dpd.cdc.gov/dpdx/HTML/Frames/A-F/Fascioliasis/body_Fascioliasis_page1.htm).

Human and animal infections with the tropical liver fluke *F. gigantica* have been reported in Russia, Africa, Asia and South East Asia including Thailand and Vietnam . In Vietnam, starting from 1997 to 2001, approximately 500 new cases were identified using eosinophilia and ELISA titres as markers of infection with upper abdominal pain. However, in the same study, only 14 of 285 (4% postive) cases had *F. hepatica* eggs in stools .

1.1.6. Diagnosis of liver fluke infection

Traditional diagnosis of liver fluke infection has been made on the basis of detection of egg counts in faeces or flukes in the liver and bile ducts during post mortem. Faecal egg count detection is the conventional routine diagnostic method in livestock, but this procedure is cumbersome, labour intensive and shows low sensitivity (Molloy *et al.*, 2005; Molloy & Anderson, 2006; Reichel *et al.*, 2005). This method is unable to detect early infections as eggs appear at 10-11 weeks of post infection.

Commercial ELISA kits utilise sensitive and specific serological tests for diagnosing infection . The diagnosis of *F. hepatica* antibodies in milk and serum of infected cattle as well as serum from infected sheep was based on an indirect ELISA coated with the f2 antigen, and developed as a commercial kit by the Institut Pourier . Molloy *et al*. (2005) has evaluated this commercial kit (based on the ELISA) for detecting antibodies in milk and serum from sheep and cattle (*F. hepatica*) in Australia. The sensitivity and specificity of the ELISA was 98.2% and 98.3% using serum and 97.7% and 99.3% using milk in cattle. In sheep, the sensitivity and specificity of the ELISA was 96.9% and 99.4% using serum. The authors of this study conclude that this ELISA is considered as a potential routine diagnostic tool for *F. hepatica* infections in cattle and sheep.

1.1.6.1. Cathepsin protease based diagnosis

Carnevale *et al*. (2001) have used crude extracts or excretory-secretory products (ES) as antigen for the diagnosis of serum antibodies. However, the complex nature of ES products and its low specificity due to cross reactivity with other parasites prevented wide use of this method as a diagnostic tool for *Fasciola* infection. Cathepsin L protease is a major protease of *Fasciola* ES material, extensively used for vaccine development, and humoral responses are raised against the protein during infection. This resulted in the development of a reliable diagnostic test for *F. hepatica* infection in rats , sheep (Wijffels *et al*., 1994a), ruminants (Fagbemi *et al*., 1995; Fagbemi *et al*., 1997), and humans (Cordova *et al*., 1997; O'Neill *et al*., 1999).

Native cathepsin L protease extracted from ES products of adult flukes were used in serological screening which showed cross reactivity with other parasites in herbivores (Cornelissen *et al*., 1999b; Cornelissen *et al*., 2001a) and humans (O'Neill *et al*., 1998; O'Neill *et al*., 1999).

O'Neill *et al.* (1999) have assessed yeast produced recombinant cathepsin L1 enzyme for the diagnosis of human fasciolosis (Bolivian Altiplano regions) and its performance led to the development of the first standardized assay for the sensitive and specific diagnosis of human *Fasciola* infection. The cathepsin L based diagnosis (ELISA and western blotting) is a promising tool for the diagnosis of tropical fasciolosis in domestic animals and humans (Intapan *et al*., 2005; Raina *et al*., 2006; Rokni *et al*., 2002; Wongkham *et al*., 2005; Yadav *et al*., 2005). Humoral responses to cathepsin L appear at 2 to 4 weeks post infection, and increase steadily throughout the 10 week period. This is mainly due to their continuous expression throughout the life cycle of the parasite in sheep and cattle (Dixit *et al*., 2002; Dixit *et al*., 2004; Mulcahy *et al*., 1999). Recombinant pro-cathepsin B was

expressed in yeast and vaccine trials were performed in rats and sheep. After challenge infection with metacercariae, these recombinant proteins detected responses within 2 to 5 weeks of infection in sheep and within 5 weeks in rats. Thus *Fasciola* cathepsin B can play a pivotal role for diagnosis of the acute stage of fasciolosis (Law *et al.*, 2003).

1.1.7. Immune responses of infected animals against Fasciolosis

Natural fluke infection has been characterised as classical cellular stimulation mainly of macrophages and eosinophils along with IgE effector immune responses . In the early infection stage, dominant suppression of lymphocyte proliferation helps the pathogen survive during the immature invasive stage and hepatic migratory activity of juvenile flukes. Secretions during the invasive stage of flukes play a major role in the reduction of nitrous oxide/ free radical production by peritoneal macrophages which permits the flukes to bypass the deleterious effects of cell mediated defence mechanisms .

During the hepatic stage of infection with *Fasciola*, there appears to be a Th2 type immune response. Migratory juvenile and mature flukes ES material induce a Th2 response along with the down- regulation of Th1 responses. Cathepsin L plays a pivotal role in the immuno-modulatory effects in ruminants and suppressive activities against T cell proliferation (O'Neill *et al.*, 1999; O'Neill *et al.*, 2000; Prowse *et al.*, 2002). Migratory flukes finally reach the bile ducts of their host where they attain maturity and start secreting proteolytic enzymes by regurgitation for the purpose of digesting host haemoglobin. This digestive and secretory molecule may be considered as an immunologically important antigen due to the induction of a Th2 polarising activity during the chronic stage of infection (Dalton *et al.*, 2003a; McManus & Dalton, 2006; Spithill *et al.*, 1997).

Juvenile liver fluke is morphologically similar to adult stage and highly motile throughout its residence in the liver. After reaching the bile duct, the migratory activity reduces. Current vaccine candidates (Cathepsin L1, cathepsin L2, FABP, GST, aminopeptidase) mainly target the adult parasite in the bile ducts where a low concentration of antibodies and immune mediating effector cells are found. Thus, this site is considered to be an immunologically safe environment for adult flukes .

1.2. Evaluation of *Fasciola* protein antigens

The control of *Fasciola* infection is primarily by the use of anthelminthic drugs and pasture management. The appearance of drug resistance in the parasite and the aversion to the presence of chemical residues in food and the environment has increased the need for a vaccine as an alternative to chemotherapeutic drugs (Dalton, 2003 a). Spithill *et al*. (1997) observed in sheep and cattle that ruminants acquired resistance to both *F. hepatica* and *F. gigantica* infection after vaccination using irradiated metacercariae and crude parasite extracts.

The emergence of bioinformatics and biotechnology presents the possibility of discovering and synthesising potentially protective *Fasciola* antigen molecules capable of inducing immunological protection (Knox & Redmond, 2006; Smith & Zarlenga, 2006). Recent developments in molecular biology contribute to study the host-parasite interaction and vaccine development (Knox *et al*., 2001; Knox & Redmond, 2006). Genetic information related to the *Fasciola* organism such as EST databases, the mitochondrial genome sequence as well as phylogenetic analyses of the proteins of the ES has been generated, analysed and many of the biologically important molecules from *Fasciola* have been cloned (fatty acid binding proteins, glutathione S transferase, aminopeptidase, cathepsin L and cathepsin B) and expressed as recombinant proteins.

The production of vaccines by recombinant DNA technology or by naked DNA may eventually lead to commercialisation of *Fasciola* vaccines (Dalton & Mulcahy, 2001; Hillyer, 2005). Despite many attempts, no commercially viable vaccine against Fasciolosis has been developed. However, a number of *Fasciola* proteins have been evaluated as immunogens or vaccine candidates. A series of native and recombinant proteins from *Fasciola* spp. have shown to promise as vaccines. These include fatty acid binding proteins, glutathione S transferase, haemoglobin, leucine amino peptidases and cysteine proteases such as cathepsin L and cathepsin B (Dalton *et al*., 2003a; Hillyer, 2005; McManus & Dalton, 2006; Spithill *et al*., 1997).

1.2.1. Fatty acid binding protein (FABP)

This adult *F. hepatica* antigen is a major component of E/S material, and is involved in binding and transportation of hydrophobic ligands, especially long chain fatty acids and bile acids. The parasite is unable to synthesize their own fatty acids *de novo* and liver flukes may use the FABP to acquire fatty acids from the host serum . The FABP protein was termed Fh12 (12 kDa mass) (Hillyer *et al*., 1987; Hillyer & Soler de Galanes, 1988). Subsequent vaccination trials in Castillian sheep using Fh12 and rFh15 failed to induce high levels of protection, although anti-fecundity effects were observed . *F. gigantica* FABPs were used as vaccines in cattle, but only a 31% reduction in worm burden was observed (Estuningsih *et al*., 1997; Smooker *et al*., 1997). *F. hepatica* rFh15 in an adjuvant/immunomodulator (ADAD) system was tested in mice and sheep challenged with metacercariae. The ADAD system consist of an extract of *Polypodium leucotomos* (PAL) and/or a Quil A in a water/oil emulsion (30/70) with non-mineral oil (Montanide). The mice vaccinated with ADAD and Fh15 showed a survival rate of 40–50% and the sheep immunised with ADAD and Fh15 showed lower fluke recovery (43%) and less hepatic lesions than control animals . *E. coli* expressed *F. gigantica* FABP was used as a vaccine in

Freund's adjuvant to evaluate the protection in Indian buffalo (*Bubalus bubalis*) calves and vaccination showed a moderate level of protection in terms of reduced fluke burden (35.8%)(Nambi *et al*., 2005).

1.2.2. Glutathione S-transferase (GST)

GSTs constitute a large family of isoenzymes involved in cellular detoxification of xenobiotics and toxic compounds. *F. hepatica* GST was proposed as a vaccine candidate because *Schistoma* GSTs showed protection in laboratory animals against *F. hepatica* infection . The Spithill laboratory (1999b) has identified eight native and four recombinant isoenzymes of GST. Vaccination with GST showed 49 to 69% protection in Hereford and Swiss Red Holstein/Simmental cattle (McManus & Dalton, 2006; Spithill *et al*., 1997). Significant reductions in the faecal egg count were observed in all vaccine trials (up to 65%). Vaccination of cattle with *Schistosoma bovis* GSTs supplemented with different adjuvants such as with Quil A, aluminium hydroxide or Freunds adjuvant did not induce effective protection against challenge infection with *F. hepatica* metacercariae . These inconsistent protective efficacies elicited by this protein in both sheep and cattle vaccine trials lead largely to a discontinuation of its development as a vaccine candidate.

1.2.3. Leucine amino peptidase

Leucine amino peptidase is secreted from the gut of *F. hepatica* and may facilitate food digestion . A high level of protection (71%) was observed with a cocktail vaccine containing cathepsin L1 and amino peptidase. The recombinant version, expressed in *E. coli*, induced 81% protection in rabbits along with the least amount of damage to the liver of vaccinated animals. Using two dimensional gel electrophoresis and immunoblotting techniques, Marcilla *et al.* (2008) have found

that recombinant leucine aminopeptidase is recognised by sera from *Fasciola* infected humans in Peruvian Altiplano.

1.2.4. Haemoglobin

Haemoglobin is also proposed as an antigen in the ES material of adult *F. hepatica*. It was suggested that this protein may be essential for oxygen transport and/or storage in migrating flukes. A cocktail vaccine using *F. hepatica* cathepsin L2 /haemoglobin yielded up to 72% protection, but haemoprotein alone gave 43.8% protection and combination of the two cathepsin L1/haemoglobin induced 51.9% protection .

1.2.5. Cysteine proteases

Cysteine proteases play major roles in cellular, developmental and digestive processes, blood coagulation, inflammation and hormone processing of the parasite (Turk *et al.*, 2000; Williamson *et al.*, 2003). Mammalian lysosomal cysteine proteases (cathepsins B and cathepsin L) have been implicated in a variety of pathological processes such as cancer, tumour angiogenesis, and neuro-degeneration (Stahl *et al.*, 2007; Tort *et al.*, 1999; Turk *et al.*, 2000). *Fasciola* cysteine proteases present in ES material may facilitate biological functions such as parasite immune evasion, excystment/encystment, moulting and tissue invasion . *Fasciola* cysteine proteases are considered to be a potential target for the development of new immunotherapeutic and sero-diagnostic agents for the next generation of anti-parasite intervention.

1.2.5.1. Cathepsin L protease

The most abundant proteins in *Fasciola* ES materials are cathepsin L proteases which are encoded by an estimated seven genes . The majority of cathepsin L is secreted by epithelial cells lining the gut lumen (Collins *et al*., 2004) and facilitates parasite penetration of host tissues. Most of the cathepsin L cDNA's have been isolated from adult flukes (Wijffels *et al*., 1994a; Yamasaki & Aoki, 1993). Each stage in the fluke life-cycle contains the same genes, but only those genes needed for that stage are expressed (McManus & Dalton, 2006; Mulcahy & Dalton, 2001). A most striking feature is that cathepsin L and cathepsin B expression are developmentally regulated during maturation in the host, as juvenile parasites secrete cathepsin B (Grams *et al*., 2001; Meemon *et al*., 2004; Tkalcevic *et al*., 1995; Wilson *et al*., 1998), immature flukes secrete both cathepsin L and cathepsin B (Harmsen *et al*., 2004; Meemon *et al*., 2004), whereas a series of cathepsin L proteases are secreted by adult *Fasciola* (Dowd *et al*., 1995; Smith *et al*., 1993a; Smith *et al*., 1993b; Wilson *et al*., 1998). Jefferies *et al*. (2001) and Wijffels *et al*. (1994a) observed multiple cathepsin L's in adult fluke ES material using two dimensional gel electrophoresis and western blotting. Adult fluke cathepsin L and NEJ cathepsin B are the prominent proteolytic enzymes of their respective ES materials, although cocktails vaccines with these proteins have yet to be reported.

These proteolytic enzymes from the gastro-epidermal cells of *Fasciola* are secreted at approximately 0.5-1 µg/adult parasite/hour in an acidic environment (Collins *et al*., 2004). Most studies revealed that cathepsin L like proteases are the major enzymes which are homologues of the mammalian lysosomal cathepsin L (Dowd *et al*., 1994; Hawthorne *et al*., 2000; Heussler & Dobbelaere, 1994; Wijffels *et al*., 1994a; Yamasaki & Aoki, 1993). The involvement in biological roles that are essential for fluke survivability in the mammalian host makes this adult fluke protein (cathepsin L) an important vaccine target against infection.

Cathepsin L1 and cathepsin L2 have been studied extensively with different substrates (Dowd *et al*., 1994; Dowd *et al*., 1995; Dowd *et al*., 1997)**.** The cathepsin L's inhibit antibody-mediated eosinophil attachment to juvenile flukes and cleavage sites of cathepsin L1 and cathepsin L2 in the heavy chain of human IgG have been clearly demonstrated . *F. hepatica* cathepsin L suppress sheep T cell proliferation and down regulate surface CD4 expression on both human and ovine T cells (Prowse *et al*. 2002). In a comparative study, *F. hepatica* cathepsin L2 was able to cleave fibrinogen to produce a clot whereas *Fasciola* cathepsin L1 could not induce a clot. Both proteins comprise 326 amino acids, with 17 amino acids for the signal peptide, 90 amino acids for the pro region and 219 amino acids for the mature region (Dalton *et al*., 2003b). Smooker *et al*. (2000) described a cDNA encoding a seventh (at that time) cathepsin L, denoted as *F. hepatica* cathepsin L5 which encodes a proteinase showing 86% identity to cathepsin L1 and 80% identity to cathepsin L2. Both the cathepsin L1 and L2 cysteine proteases have shown proteolytic activity against a number of natural substrates such as gelatin (Dalton & Heffernan, 1989; Kesik *et al*., 2007), haemoglobin , collagen , immunoglobin , globin and albumin .

Cathepsin L is involved in protecting the parasite from the host immune system as it can effectively cleave immunoglobin in the hinge region, isolating Fab from the Fc region, preventing antibody mediated attachment of eosinophils to the parasite surface (Berasain *et al*., 1997; Carmona *et al*., 1993; Smith *et al*., 1993a). Cathepsin L may aid in suppression and/or immune modulation of Th1 responses and induction of nonprotective host Th2 responses (Dalton *et al*., 1996; O'Neill *et al*., 2000). Th1- specific responses to a bacterial pathogen, Bordetella pertussis, (IFN-γ indicative marker) were suppressed . Conversely, this enhanced non-protective Th2 response may favour the longevity of the parasite in the host . Cathepsin L is not highly immunogenic in the early stages of infection, and may be considered at that stage as a hidden antigen .

1.2.5.2. Cathepsin B protease

1.2.5.2.1. Cathepsin B as a potential vaccine candidate

Juvenile and immature stages of *F. hepatica* secrete cathepsin B-like proteases which are the major component of the ES material of newly excysted juvenile flukes (NEJ) (Creaney *et al.*, 1996; Tkalcevic *et al.*, 1995; Wilson *et al.*, 1998). It is released by early migratory flukes up to 5 weeks post infection, and may help in excystment and migration of young flukes into host tissues (Law *et al.*, 2003; Wilson *et al.*, 1998). A similar biological function (tissue invasion) has been reported for mammalian cathepsin B secreted by macrophages, osteoclasts and malignant tumour cells (Turk *et al.*, 2000; Williamson *et al.*, 2003). The high level of cathepsin B in the mammalian tissue invasion stage of *F. hepatica* is indicating that it may be required for successful parasitism. NEJ secreting cathepsin B show fluorogenic and gelatin substrate activity, and digest the heavy chain of immunoglobulin and bovine serum albumin . Therefore, *F. hepatica* cathepsin B of the NEJ and immature stages is regarded as a potential vaccine candidate and drug target against *Fasciola* infection (Creaney *et al.*, 1996; Law *et al.*, 2003; Wilson *et al.*, 1998)

1.2.5.3. Genomics of *Fasciola* cathepsin L and cathepsin B proteases

Many *Fasciola* gene and cDNA sequences are available in the GenBank database, although the entire genome sequence has yet to be solved. Many genes are only expressed at a specific stage in the life cycle. The gene sequence analysis of *F. hepatica* was initiated by Heussler and Dobbelaere. (1994) and *Fasciola* cathepsin L

database entries have been gradually increased since this time. At present 29 and 11 known cathepsin L and cathepsin B nucleotide sequences respectively are available (http://www.ncbi.nlm.nih.gov/sites/entrez). The three cDNAs encoding cathepsin B proteases from adult, NEJ, and metacercaria of *F. gigantica* were analysed for their stage- and tissue-specific expression by Northern hybridization, PCR techniques and RNA in situ hybridization .

1.2.5.4. RNAi studies with *F. hepatica* cathepsin L and cathepsin B

RNA interference (RNAi) is a recent genomic tool for exploiting the transcriptomic data (RNA-mediated interference) from parasites. Double stranded (ds) RNA stimulates an intracellular cascade which results in degradation or suppression of the expression of homologous mRNA transcripts . Double-stranded RNA is prepared from cDNA sequences and is then administered by microinjection into the germline syncytium . The ds RNA can spread across cell boundaries from the intestine and can be mediated by feeding worms on *E. coli* that are expressing parasite ds RNA or simply soaking worms in a diluted ds RNA solution. Currently, successful application of RNAi for the suppression of gynecophoral canal protein , cathepsin B , a facilitated diffusion glucose transporter (SGTP1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression has been studied in *Schistosoma* species.

McGonigle *et al*. (2008) showed inhibition of cathepsin B and cathepsin L in *F. hepatica* NEJ. RNAi silencing of cathepsin B and cathepsin L induced a marked reduction in transcript level as well as encoded proteins in the fluke gut . The *F. hepatica* cathepsin B and cathepsin L-RNAi on NEJs were shown to have specific effect on three factors: (a) mRNA transcript levels, (b) target protein expression, and (c) functionally, by altered NEJ gut penetration. Abrogation of expression of either

cathepsin L or cathepsin B induced abnormal locomotory phenotypic changes and subsequently reduced invasion of the rat intestinal wall. The results of this study concludes that both cathepsin B and cathepsin L proteases are essential to NEJ penetration of the gut, and that interference with the function of either cathepsin L or cathepsin B has a severe impact on worm virulence. The specific sensitivity of NEJs to RNAi yields broad ranging opportunities to further probe the biology of cysteine proteases genes in *F. hepatica*.

1.2.5.5. Structural analysis of *Fasciola* cathepsin L and B

Structural analysis is useful to identify regions of a target molecule which are likely to be immunogenic and therefore available for interaction with immune effectors. Molecular homology modelling of *Fasciola* cathepsin B using the MODELLER program was based on the human cathepsin B structure. Stereochemistry analysis of this protease paved the way to the discovery that the active site of this enzyme was partially buried by a unique structure called the occluding loop (Law *et al.*, 2003) (Figure 1.3). Molecular modeling studies were performed for cathpsin L5 where a single amino acid substitution in the mature protein may be responsible for the difference in specificity at the S2 subsite. Human cathepsin L was used as template for building these models . Using homology modelling, Irving *et al*. (2003) predicted the S_2 subsite specificity of cathepsin L5 and cathepsin L8, which have a leucine and a tryptophan at the crucial amino acid (#69), respectively. The structure of *F. hepatica* cathepsin L1 has been recently released .

1.2.5.6. Enzymatic activity of *Fasciola* proteases

Fasciola cysteine protease cathepsin L enzyme activity may be important in parasitic activities such as invasion of host tissues, digestion of host proteins,

helping the parasite to evade the host immune responses and mediation of molting of immature stages of flukes . Cathepsin B shows a unique feature in the active site of the enzyme known as the occluding loop (Figure 1.3). This structure partially occludes the active site of the enzyme, restricting access of extended substrates and inhibitors such as the cystatins. These proteases possess an essential cysteine residue that forms a covalent intermediate complex with substrate. Cathepsin B proteases are expressed in greater amounts in parasitic and non-parasitic nematodes such as *Haemonchus contortus*, *Ancyclostoma duodenale*, *Toxacara canis*, *Onchocerca volvulus* than in trematodes such as *Fasciola hepatica*, *Fasciola gigantica*, *Schistosoma mansoni*, *Schistosoma japanicum* (Brady *et al.*, 1999; Law *et al.*, 2003; Smith *et al.*, 1993a; Tort *et al.*, 1999). The functions of these various cathepsin B enzymes remain to be determined (Law *et al.*, 2003).

F. hepatica cathepsin proteases consist of a pre-region or signal peptide (12-20 amino acids), a prosegment (100 amino acids) and a mature enzyme (200 amino acids) . Adult cathepsins L's are expressed as an inactive protease and require proteolytic removal of their pro region in order to access active site residues. The substrate specificity of this proteolytic enzyme was assessed using synthetic fluorogenic peptides which were also used for characterisation of mammalian cathepsin L's, S's, K's (Z-Phe-Arg-NHmec) and cathepsin B (Z-Arg-Arg-NHmec) (Kirschke *et al.*, 1982; Smooker *et al.*, 2000). The two main forms of cathepsin L (L1 and L2) do not possess glycosylation sites in their mature form. Cathepsin L5, like L1 does not exhibit proteolytic activity against substrates containing proline, however the authors of this study have highlighted a single amino acid substitution that does affect the specificity of this protease.

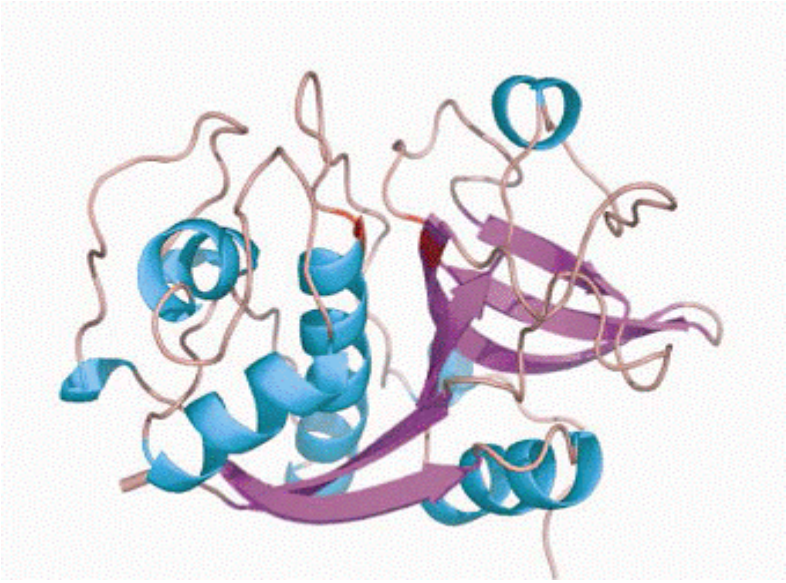
Recombinant cathepsin B yeast expression was initiated by Law *et al.* (2003), but they failed to demonstrate auto-processing. Cathepsin B was subsequently

expressed in the methylotrophic yeast *Pichia pastoris*. In order to obtain the active form of this enzyme, the expressed protease was processed by an asparaginyl endopeptidase via cleavage at an unusual position N-terminal to the normal cleavage site (Beckham *et al.*, 2006).

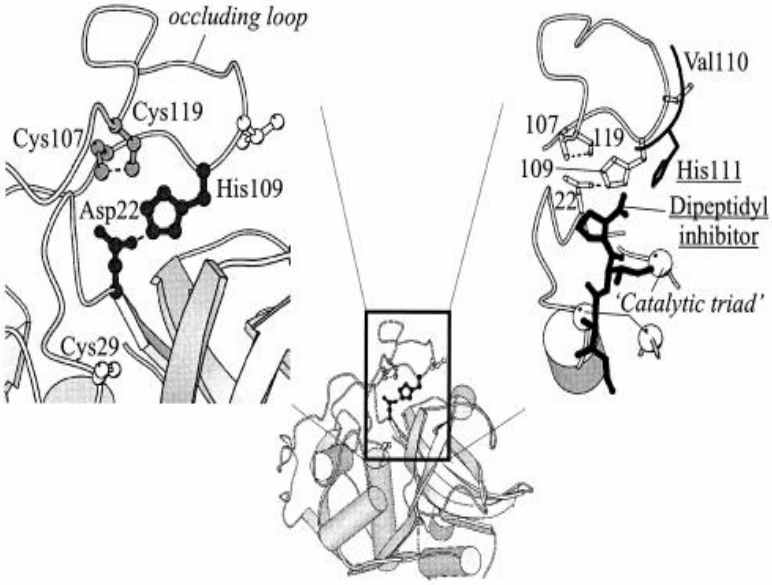
1.3. From *Fasciola* genes to protein vaccine candidate

Functional expression of cloned cathepsin genes has been used to determine the immunogenic potential of these proteases. Vaccine development processes are presented in Figure 1.4. The protective efficacy and success of these vaccine trails have demonstrated that each of these antigens delivered as proteins, can effectively elicit protection in terms of reduction in worm burdens, anti-fecundity, smaller fluke size, anti-pathology (less liver damage) and reduction in parasite egg output compared to non-vaccinated controls.

The native cathepsin L was isolated from *Fasciola* flukes and detailed biochemical and immunological assessment was performed independently by two research groups, Spithill (Australia) (Estuningsih *et al.*, 1997; Smooker *et al.*, 2000; Spithill *et al.*, 1997; Wijffels *et al.*, 1994a; Wijffels *et al.*, 1994b) and Dalton (previously in Ireland) (Dalton *et al.*, 1996; Dalton *et al.*, 2003b; Dowd *et al.*, 1994; Dowd *et al.*, 1997; Piacenza *et al.*, 1999; Roche *et al.*, 1997).



A



B

Figure 1.3. Molecular structure of *F. hepatica* (A) mature cathepsin L1 and (B) model of cathepsin B (Law *et al.*, 2003).

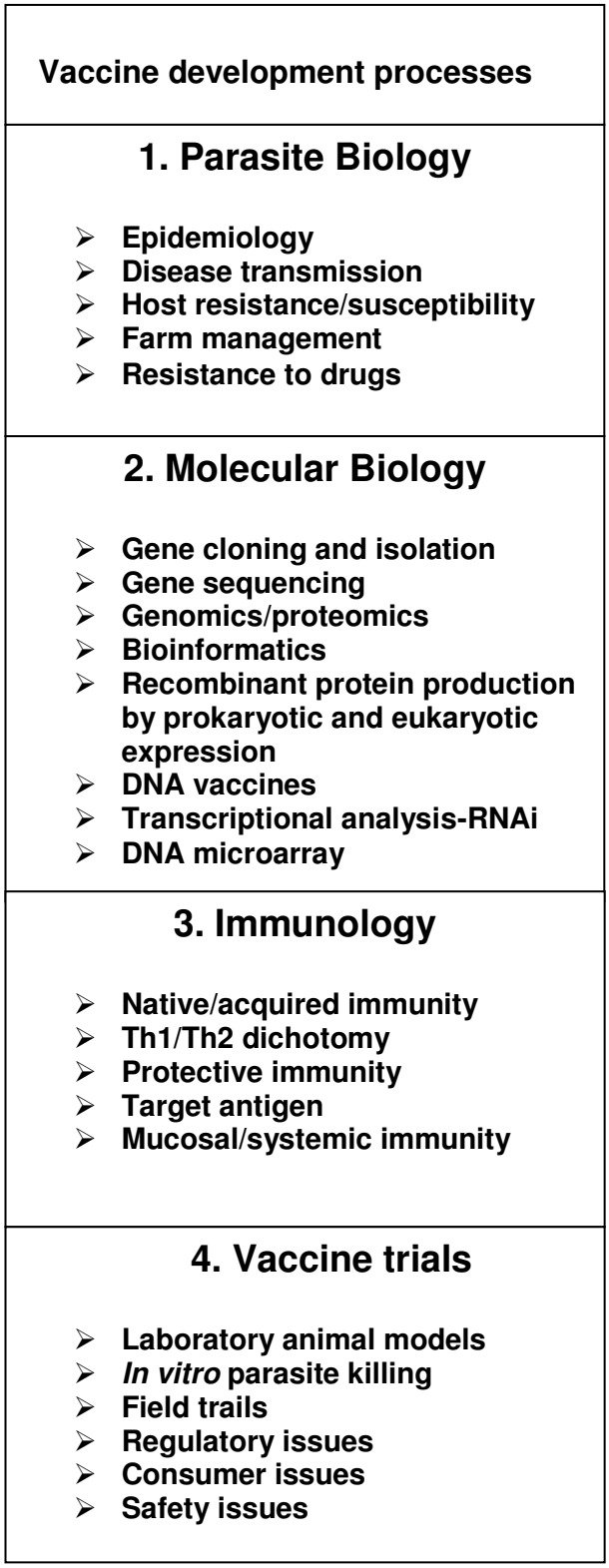


Figure 1.4. Involvement of four inter-related aspects in parasite vaccine development processes (adopted from).

1.3.1. Bacterial expression of cathepsin L and cathepsin B

Yamasaki *et al*. (2002) have expressed *Fasciola gigantica* cathepsin L1 proprotein and mature cathepsin L1 in *E. coli*, and inferred that the pro region of the protease is essential for correct folding of the protease. Kesik *et al*. (2007) expressed cathepsin W as inclusion bodies in *E. coli*, and this protein was used for enteral vaccination of rats against *Fasciola* infection. Though *E. coli* remains the preferred host for recombinant protein production, due to its ease of handling, cost effectiveness and high success rates, it often has shortcomimgs undesired effects such as the inability to perform post-translational modification and ends in inclusion body formation . The *E. coli* cytoplasm is a reducing environment that is unfavourable for correct disulphide bond formation . Proteins expressed in the *E. coli* system may lack biological function and antigenicity due to the lack of post translational modification, or misfolding. To obtain biologically active proteins, many renaturation methods have been implemented. These include dialysis, dilution and column chromatography .

1.3.2. Yeast expression of cathepsin

Saccharomyces cerevisiae has been developed as an alternative eukaryotic expression system and has been extensively studied as an expression system for *Fasciola* cathepsin L genes. Intracellularly expressed cathepsin L underwent proteolytic degradation during isolation. The recombinant protein can be secreted into the medium by co-expression with yeast expressed signal sequences such as α- secretion signal (Lipps *et al.*, 1996). Recombinant cathepsin L1 and cathepsin L2 were expressed and secreted by *S.cerevisiae* transformed with plasmids containg cDNA's (Dowd *et al*., 1997). These were correctly processed, secreted as mature enzymes and showed similar biochemical and substrate specificity to native

cathepsins (Brady *et al.*, 1999; Roche *et al.*, 1997). Then, these proteases were rapidly purified by gel and ultra filteration chromatography (Dalton *et al.*, 2003b). The cathepsin L1 and cathepsin L2 expression yields were low (100 µg/ L) (Dowd *et al.*, 1997; Roche *et al.*, 1997), but expression of *F. hepatica* cathepsin L5 in pFLAG has attained 2 mg/L of culture and can be purified by His_6 tag nickel chelate affinity chromatography and cation exchange chromatography .

Wild-type procathepsin L1 expressed in *Pichia pastoris* showed an intermolecular processing event resulting in an intermediate form. A high level of expression was observed, with no hyperglycosylation (Collins *et al.*, 2004; Dowd *et al.*, 2000). Excellent expression has been achieved (800 mg/L) and purification by His_6 tag affinity chromatography (Collins *et al.*, 2004). *Fasciola* cathepsin B was expressed in *P. pastoris,* yielded 40µg/ L and showed enzymatic activity (Beckham *et al.*, 2006).

1.3.3. Insect cells

Insect cell expression has been studied extensively in parasite recombinant protein production and has yielded recombinant proteins capable of inducing protective immunity against challenge infection . Baculovirus contains a gene encoding an abundant protein, polyhedron, which has a very strong promoter (polh). Polyhedrin is not important for viral replication and the gene can be replaced with heterologous genes which are then expressed in the system. Baculovirus expression allows correct folding with disulfide bond formation and post-translational modifications . *Fasciola* procathepsin L3 (Fhe CL3) was expressed in the baculovirus system and also *S.cerevisiae*, but only baculovirus expressed Fhe CL3 induced significant protection against liver flukes in challenged rats .

1.4. Vaccine trials with cathepsin L and cathepsin B

1.4.1. Vaccination with native cathepsin L

1.4.1.1. Ovine vaccine trials

Cathepsin L1 and cathepsin L2 vaccine potential have been extensively evaluated in sheep and cattle. Wijffels *et al*. (1994b) performed immunisation of native cathepsin L1 and L2 in sheep. This Freunds adjuvant vaccine formulation induced 70% reduction with faecal egg count and 67% reduction in worm fecundity, but did not show a signicant reduction in worm burden against challenge infection. In contrast, *F. hepatica* cathepsin L1 and cathepsin L2 vaccine trials were performed in Uruguayan sheep breeds which induced 34% and 33% fluke burden reduction and elicited 71% and 81% anti-fecundity respectively. A cocktail of the two enzymes showed 60% reduction in worm burden. Vaccination of Australian Merino sheep with two cathepsins induced 69% egg output reduction .

1.4.1.2. Bovine vaccine trials

Cattle vaccination against *Fasciola* infection is experimentally feasible, but more extensive vaccine research is needed before an effective vaccine is developed. The vaccine efficacy of both secreted cathepsin L1 and cathepsin L2 proteinases were tested alone or in combination with *F. hepatica* haemoglobin in cattle (Dalton *et al*., 1996; Mulcahy *et al*., 1999). These immunogens have given consistent levels of protection against infection in cattle which ranges from 38.2-68.5%. The combination of both antigens (L1 and L2) has never been tested in cattle, but when combined with haemoglobin, showed 51.9 % and 72.4% protection against infection. In bovine vaccine trials, reductions in egg production and egg viability ranging from 40 to 65% were observed, whereas cocktail vaccine with haemoglobin ranged from

80 to 98% reduction (Dalton *et al.*, 1996; Dalton & Mulcahy, 2001; Dalton *et al.*, 2003b). Cathepsin L's from adult *F. gigantica* have been tested in Brahman cross-bred cattle using DEAE dextran/sm as adjuvant. These vaccinations induced high antibody titres, but no reductions in faecal egg count . The above mentioned vaccine efficacy trials in sheep and cattle have been evaluated using native cathepsin L peptidases.

1.4.2. Vaccine trials with recombinant cathepsin L and B

Production and purification of recombinant cathepsin protease that mimic the levels of immunogenicity induced by native protease is needed. Expression of cathepsin B and L in yeast revealed that production of biologically active, native-like recombinant cathepsin protein is possible (Beckham *et al.*, 2006; Dowd *et al.*, 1997; Law *et al.*, 2003; Lipps *et al.*, 1996; Roche *et al.*, 1997). Recombinant cathepsin L expressed in *P. pastoris* was used as a vaccine in sheep and induced protection ranging from 35-45% and had an anti-fecundity effect of 50%. Recombinant procathepsin L3 was expressed by baculovirus and *S. cerevisiae*. Baculovirus expressed protease evoked 52% protection in rats, but yeast expressed protease did not induce a significant protective effect (Reszka *et al.*, 2005). Recombinant cathepsin W cysteine protease was expressed in the form of inclusion bodies, and used for intranasal immunisation in Holstein-Friesian calves and elicited 54% protection against infection, whereas 56.5% protection was achieved in Corriedale lambs . Native cathepsin B from NEJ or immature liver flukes is secreted in only small quantities, so performing vaccine trials with the native protein is difficult. Therefore recombinant FhCatB immunization was performed in rats. The yeast expressed protein was formulated in Quil A or Freunds adjuvant and induced IgG antibody titres of 10^6 and 10^5 respectively in vaccinated animals (Law *et al.*, 2003). There is no data on vaccine trials with recombinant cathepsin B protein and protection against challenge infection in animals.

1.4.4. Factors hindering *Fasciola* vaccine development

Although cellular infiltration and high anti-fluke antibody titres were observed in many immunisation studies, commercial success has not yet been attained. There are three factors responsible for slowing down the development of an effective vaccine:

1. The infective stage juvenile flukes after excysting from metacercariae invade three different locations of the host,

A. the wall of the small intestine

B. the peritoneal cavity

C. the hepatic parenchyma and liver surface, finally reaching the bile duct .

There are very few reports describing the influence of the site in the host (gut, peritoneum and liver) or stages of fluke life-cyle on the induction of innate and acquired immunity (McGonigle *et al*., 2008; Meeusen & Piedrafita, 2003; van Milligen *et al*., 1998).

2. Rapid migratory activity of immature flukes throughout hepatic tissues causes extensive haemorrhage, fibrosis and calcification which has been confirmed in goats and sheep . This activity makes it difficult for effective cellular defence mechanisms against flukes to operate.

3. Cathepsin L and cathepsin B cysteine proteases liberally secreted by all stages of *Fasciola* inhibit the lympho-proliferative responses by inducing (non-protective and immuno-pathological) Th2 responses via IL-4 and IgG1 (Brady *et al*., 1999; O'Neill *et al*., 2001).

Most vaccine candidates are extracted from adult parasites . No cocktail vaccine study has been conducted using various stage-specific *Fasciola* antigens for inducing protective immunity. However, our laboratory is interested in investigating the immune potency of three stage-specific cathepsins in providing a protective effect against experimental rat fasciolosis.

1.4.4.1. Immuno reactivity of rodent model against fluke infection

The immune response to *F. hepatica* infection has been evaluated in different mammalian hosts from rats to ruminants (Mulcahy *et al.*, 1998; Mulcahy *et al.*, 1999; Mulcahy & Dalton, 2001). Many mammalian hosts recognize potentially protective fluke antigens, but only rats exhibited the cellular effector mechanism for killing newly excysted juvenile flukes (Piedrafita *et al.*, 2001; Piedrafita *et al.*, 2007). Production of free radicals is 30 fold higher in the rat model than the mouse animal model when they were challenged with crude antigens.

Oxidative stress is a general mechanism whereby free radicals induce oxidative damages and reduce the antioxidant defences of the biological systems. Oxidative stress is a significant feature of chronic *F. hepatica* infection in grazing sheep (Saleh, 2008).

1.5. DNA vaccines

The use of plasmid DNA to induce the immune system to develop immunity against infectious disease provides a variety of practical benefits for large scale vaccine production which are not attainable with other forms of vaccines including recombinant protein or neoplastic cells . DNA immunisation against intracellular parasites has been demonstrated in *Leishmania* (Gurunathan *et al.*, 2000b), *Toxaplasma* and Malaria (Rainczuk *et al.*, 2004; Smooker *et al.*, 2004). Smooker *et al.* (2004) have reviewed the application of DNA vaccines against parasites.

1.5.1. Advantages of DNA vaccines

DNA vaccines offer a number of advantages than protein based vaccines. Plasmid DNA vaccines can encode multiple immunogenic epitopes and elicit both humoral and cell mediated immune responses . DNA vaccines are considered to be safe with no risk of reversion to a virulent form (Ulmer *et al.*, 1993). Plasmid vectors can be rapidly constructed, easily tested, produced and are cheaper to manufacture than protein vaccines .

DNA is thermostable and there is no need for a cold chain cycle (Ramsay *et al.*, 1997; Ramshaw & Ramsay, 2000), and therefore can be ideal for application in developing countries where the cost per dose of veterinary vaccines is the main concern (Watts & Kennedy, 1999; Watts *et al.*, 2000). The encoded protein can be presented to the immune system in the same manner as it occurs during native infection. Another major advantage is the capability of expressing immunogenic epitopes for a longer duration. DNA vaccines can be administered repeatedly without inducing anti-vector immunity. Hundreds of publications have now reported the efficacy of DNA vaccines in the lab animals than livestock models of infectious diseases, cancer and autoimmune diseases .

1.5.2. Properties of DNA vaccine vectors

The basic requirements for the backbone of a DNA plasmid vector are a eukaryotic promoter, a multiple cloning site, a polyadenylation sequence, a selectable marker and a bacterial origin of replication (Gurunathan *et al.*, 2000a; Smooker *et al.*, 2004), as depicted in Figure 1.5. A strong promoter is required for optimal expression in mammalian cells, and typically the strong promoter from cytomegalovirus (CMV) is used. A cloning site should be provided for insertion of the heterologous gene

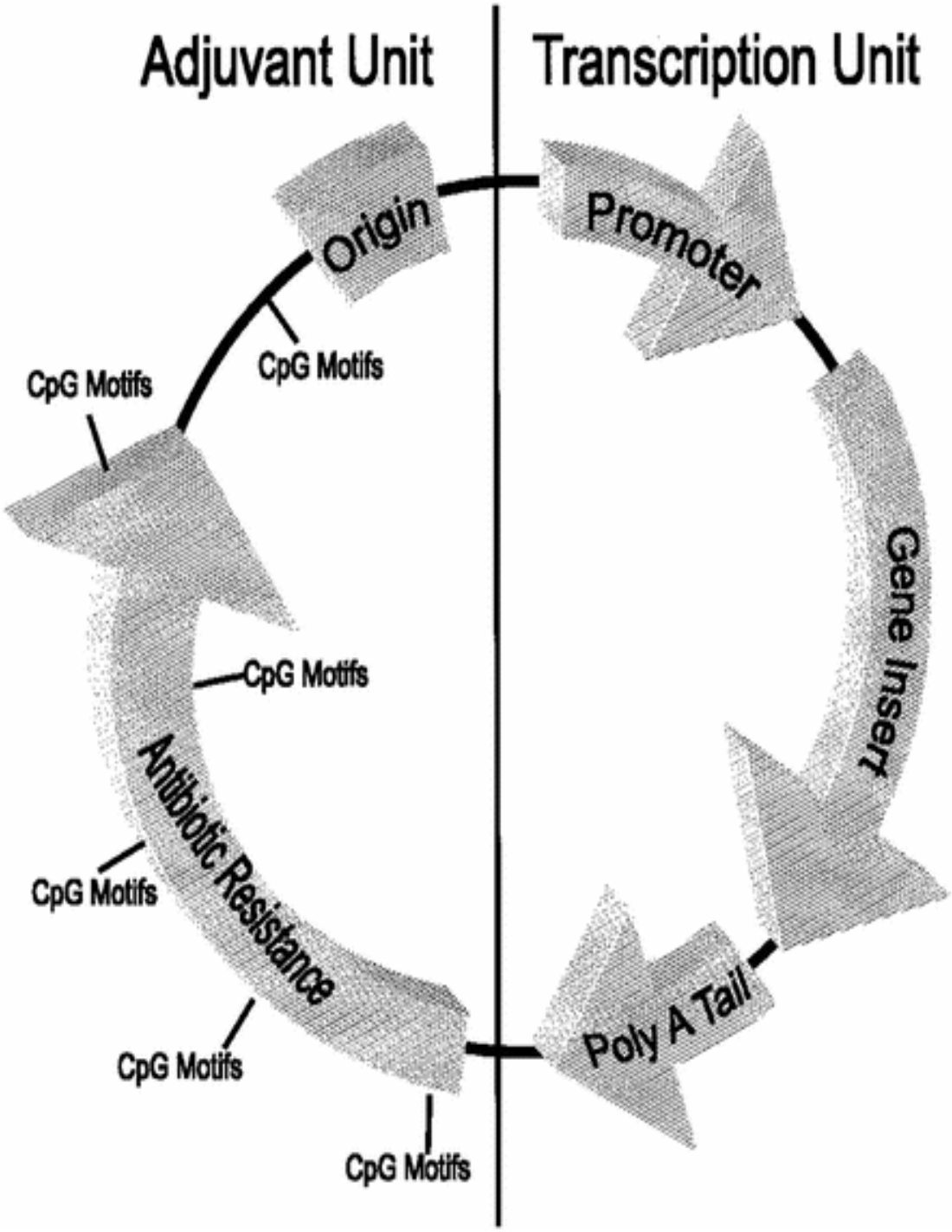


Figure 1.5. The back bone of DNA vaccine vector (adopted from)

and inclusion of a transcription termination sequence such as bovine growth (BGH) provides stabilisation of mRNA transcripts. The *E. coli* Col E1 origin of replication is used in DNA vaccines, due to its high copy number in bacteria enabling high yields of plasmid DNA on purification.

1.5.3. Immune responses after vaccination with DNA vaccines

1.5.3.1. Humoral immune responses

Plasmid DNA vaccination induces humoral immune responses against bacterial, parasitic and neoplastic proteins (Donnelly *et al.*, 2005; Gurunathan *et al.*, 2000a; Smooker *et al.*, 2004). After plasmid DNA encoded antigens are expressed *in vivo*, they can form a native structure with intact epitopes, therefore conformational epitopes can elicit neutralizing antibodies (Kumar & Sercarz, 1996; Ulmer *et al.*, 1993). Therefore DNA vaccination presents antigenically relevant epitopes to the immune system which may not be readily attainable by vaccination with other types of vaccines containing inactivated or irradiated organisms, or recombinant or purified native antigens which are delivered with adjuvant.

Longevity of humoral responses along with induction of antibody isotypes IgG, IgM and IgA has been seen in humans (Justewicz *et al.*, 1995; Justewicz & Webster, 1996). The induction of subclass serum antibodies IgG1, IgG2a and IgG2b were reported by number of authors (Kennedy *et al.*, 2006; Mitchell, 2002; Smooker *et al.*, 1999; Smooker *et al.*, 2001; Tachedjian *et al.*, 2003). Furthermore, IgE antibody production was stimulated by DNA vaccines encoding allergen, suggesting that this approach may be effective for attenuating allergic responses (Hsu *et al.*, 1999; Hsu *et al.*, 2001; Raz & Spiegelberg, 1999). Enhancement of immune responses against DNA encoded protein differs with dose and boosting, site and method of vaccination and co-injection with stimulatory molecules or cytokines (Boyle *et al.*, 1999; Donnelly

et al., 2005). Antibody titre is generally dependent on the nature of the antigen, dose and number of vaccinations (Boyle *et al.*, 1998; Deck *et al.*, 1997). After DNA vaccination, the antibody peak response occurs between 4-12 weeks post vaccination (Gurunathan *et al.*, 2000c).

1.5.3.2. Helper T cell responses

1.5.3.2.1. CD4+ helper T cell responses

In many animal models, it has been demonstrated that after vaccination with a DNA vaccine the encoded protein can induce CD4+ T cells and CTLs (Kumar *et al.*, 2001; Ulmer *et al.*, 1993; Ulmer *et al.*, 1994). Th1 type T-helper cells typically produce IFN-γ whereas Th2 cells typically secrete IL-4 and IL-5. IL-12 facilitates Th1 differentiation, whereas IL-4 facilitates Th2 differentiation . Bacterial DNA contains CpG motifs which can trigger the immune system to induce the IL-12 cytokine, which would generate Th1 responses, while protein, killed/inactivated vaccine typically induces Th 2 responses (Klinman *et al.*, 1997; Krieg *et al.*, 1998; Raz, 1997). Intramuscular DNA injection usually induces the IgG2a isotype and IFN-γ secretion which is consistent with a Th1 response (Boyle *et al.*, 1998; Donnelly *et al.*, 1995; Donnelly *et al.*, 1997; Gurunathan *et al.*, 2000b).

1.5.3.2.2. CD8+ helper T cells responses

Plasmid DNA encoded protein can be easily modified and the method of immunisation permits for optimisation of both quantitative and qualitative aspects of CTL responses . An effective method of inducing CD8+ T cells against dominant and sub-dominant epitopes is to generate the same breadth of responses as that induced by natural infection. Subdominant epitopes may be important in mediating an effector role in the absence of CTL responses to dominant epitopes. DNA

vaccines can elicit broad memory response to multiple epitopes (Fu *et al.*, 2004; Fu *et al.*, 1997).

1.5.3.3. Methods of vaccine delivery

Two main vaccine delivery methods for DNA vaccination include intra-muscular injection with saline and intra-dermal with saline or gold particles via a gene gun.

1.5.3.3.1. Intradermal delivery

Transfection of keratinocytes along with transfection of MHC class II expressing professional antigen presenting cells (APCs) is caused by biolistic intra-dermal DNA vaccination. The main advantage of gene gun immunisation is the amount of DNA required to achieve immune responses are only nanograms of DNA required, which is 100-1000 fold less than for intramuscular saline injection . Sixteen ng of plasmid has been found to induce an immune response using the gene gun, whereas 5000 times more DNA (both ID and IM route) was required to attain comparable results in mice via ID route (Pertmer *et al.*, 1995; Pertmer *et al.*, 2001). Gene gun delivery or subcutaneous (SC) injection of the DNA vaccine induced Th1-like response and presence of IgG2 antibodies in vaccinated animals gave rise to protective immunity against experimental challenge infection. Thus, DNA vaccine efficacy in sheep is strongly influenced by the route of vaccination (De Rose *et al.*, 2002).

1.5.3.3.2. Intramuscular immunisation

Intramuscular DNA injection elicits strong CD8+ responses, however muscle cells, which express MHC class I molecules, do not express B7.1 and B7.2 (CD 80 and CD 86), which are utilized for ligation of CD 28 on the T cell surface and provide the co-stimulatory signal (Gurunathan *et al.*, 2000a; Smooker *et al.*, 2004). After DNA

vaccination APCs have been shown to present the encoded antigen to T cells, rather than muscle cells (Doe *et al.*, 1996). There are several theories regarding antigen presentation after intramuscular DNA vaccination. It has been shown that the muscle cells cannot directly prime T cells, as they do not express appropriate co-stuimulatory molecules. Hence, the muscle cells act as a "factory" for protein production. In order for antigen to be presented to T cells, APC must take up the antigen. This can be resident APC's in the muscle bed, which either take up the plasmid, or protein secreted by muscle cells, or access protein via cross-presentation of antigen from muscle cells.

1.5.3.4. The importance of the dendritic cells (DC) in antigen presentation

Dendritic cells (DCs) play a major role in inducing immune responses after DNA immunisation. After vaccination, only a limited fraction of injected DNA molecules are available for DCs. The major DNA fractions are taken up by other cells (Keratinocytes or myocytes) which in turn produce encoded proteins, which can be recognised by B cells. These proteins can be efficiently captured and processed by DCs through receptor-mediated endocytosis and then presented via MHC class I and class II (Cross-priming) (Donnelly *et al.*, 2005; Smooker *et al.*, 2004; Steinman *et al.*, 1997; You *et al.*, 2001).

1.6. Improving the immunogenicity of encoded antigens of plasmid DNA

A rational approach to improve the efficacy of DNA vaccination would optimise 1. The vector backbone DNA sequence, 2. The co-expression of stimulatory sequences, 3. The delivery system used for the vector and targeting of vector for appropriate immune stimulation (Donnelly *et al.*, 1997; Gurunathan *et al.*, 2000a; Smooker *et al.*, 2004). The backbone of DNA could be further modified to enhance

immunogenicity via manipulation of DNA to include certain sequences. The CpG motifs in plasmid DNA induce a Th1 like pattern of cytokine production . It is possible to increase responses to DNA vaccine vectors by incorporating further CpG motifs into the DNA backbone of the plasmid .

There are various strategies designed to improve immune responses after DNA vaccination. Some of these are as follows:

1. Entrapment of DNA in liposomes or micro-particle encapsulation such as attenuated intracellular bacteria .

2. Cytokines and co-stimulatory DNA adjuvants enhance immune responses (IL-2; IL-4; GM-CSF) (Gurunathan *et al.*, 2000c).

3. Protein boosting: After priming DNA vaccination antibody responses are increased after a protein boost (Kennedy *et al.*, 2006; Rainczuk *et al.*, 2003).

4. Use of secretory signals encoded in vectors such as the VR1020 plasmid (Boyle *et al.*, 1999; Smooker *et al.*, 1999).

5. Targeting of APCs and lymph nodes (Boyle *et al.*, 1998; Drew *et al.*, 2001; Rainczuk *et al.*, 2003; Tachedjian *et al.*, 2003).

6. Electroporation of injected DNA vaccines .

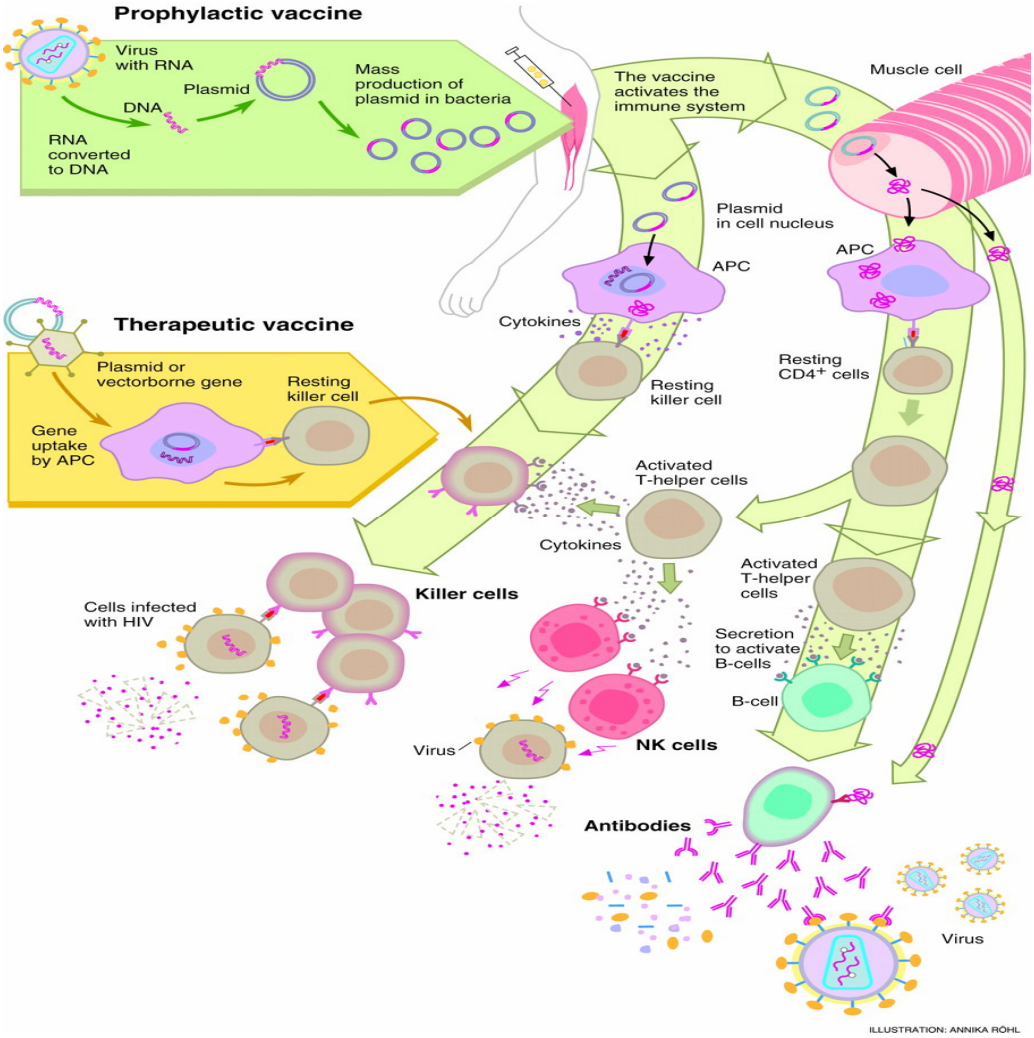


Figure 1.6. Immune mechanism of DNA vaccination (adopted from).

The gene of interest is inserted in a plasmid. Secreted proteins elicit cytokines, Th cells, and antibodies to act against infection via prophylactic and therapeutic vaccination.

1.6.1. Secretory and cytoplasmic sequences in plasmid DNA

Boyle *et al*. (1997) has demonstrated higher CTL responses and antibody titres after DNA immunisation with ovalbumin in a secreted form (Boyle *et al*., 1997). Rainczuk *et al.* (2003b) used pVR1020 to secrete encoded *Plasmodium falciparum* MSP4/5 protein against lethal malarial challenge. After three vaccinations, the IgG antibody titre attained a high level as well as significant protection upon protein boosting. *F. hepatica* glutathione S transferase targeting the encoded protein to the cytoplasm (VR1012) and extracellular compartment (VR1020) were evaluated *in vitro* (COS 7 cells) and *in vivo* (BALB/c mice). The secretory form of the DNA vaccine induced a mixed Th1/Th2 response after intramuscular vaccination . The active secretion of DNA encoded protein would allow optimal release of antigen from the transfected myocyte into the extracellular space where it could then either migrate to lymph nodes or be captured by migrating APCs. The expression of cytoplasmic antigen, would release either small doses of antigen trafficking to lymph node or make protein accessible after the death of transfected muscle cells (Boyle *et al*., 1997; Drew *et al*., 2000).

1.6.2. Chemo-attractant Monocyte chemotactic proteins (MCP3)

Chemokines are 8 to 12 k Da size peptides and two chemokine families are recognised based on the cysteine residues in their N-termini . Immature DCs express chemoreceptors from CCR1 to CCR6 . MCP3 has been shown to bind to chemokine receptor CCR1, CCR2 and CCR3 which are expressed on immune cells such as monocytes, T lymphocytes, eosinophils, basophils and dendritic cells (Gong *et al*., 1996; Xu LL *et al*., 1995). Poorly immunogenic encoded antigen can be efficiently rendered immunogenic by targeting them to APCs via differentially expressed chemokine receptors. In this way, many neoplastic and HIV antigens

fused with a pro-inflammatory chemo-attractant, induced potent and protective immunity in mice (Biragyn *et al.*, 2001). The chemokine fusion protein once internalised is degraded and can be presented using both the MHC class I and class II pathways . It has also been suggested that MCP3 fused antigens do not require adjuvants and are able to stimulate immune response *in vivo* and *in vitro* using relatively low doses (Biragyn *et al.*, 1999).

MCP3 is able to interact with at least three types of receptors,
1. MCP1 receptors on monocytes and basophils,
2. Selective Rantes receptor on basophils and eosinophils,
3. Selective M1P1α on basophils, eosinophils and neutrophils (Ben-Baruch *et al.*, 1995; Gong *et al.*, 1996).

1.6.3. Lymph node targeting cytotoxic lymphocyte antigen- 4

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed on activated T cells and shows strong binding affinity to both B7-1 (CD 80) and B7-2 (CD 86) molecules expressed on APCs (Huang *et al.*, 2000). It is a homologue of the T cell costimulatory molecule CD28 (Chambers *et al.*, 2001). Direct targeting of antigens to APCs via fusion to CTLA4, which in turn interacts with B7 enables antigens to be processed and presented to T cells with much higher efficiency, leading to a stronger and faster immune response (Boyle *et al.*, 1998; Huang *et al.*, 2000). Fusion with CTLA4 allows an encoded protein to be efficiently captured by APCs, processed in endosomes and presented to MHC class II by DCs to induce CD4+ Th cells and to cross- present internalised antigen to MHC class I to elicit CTL responses. This vaccination strategy can induce strong immunity and may provide a novel, generic DNA vaccine design for development of therapeutic and preventive DNA vaccination (Drew *et al.*, 2000; Drew *et al.*, 2001). Drew *et al.* (2001) used

CTLA4 and L-selectin targeted DNA vaccines in mice and sheep, and demonstrated that CTLA4-targeted immunogen generated antigen-specific antibody responses 30 fold higher than the non-targeted type. Kinetics of the antibody response after CTLA4 targeted DNA vaccinations were significantly faster than the non-targeted one. Rainczuk *et al*. (2003) found an enhanced antibody response at 20 weeks after CTLA4/MSP4/5 vaccination, irrespective of the route (intramuscular or intradermal). A study in sheep with CTLA4 fused to the *F. hepatica* cathepsin B showed enhanced speed and magnitude of the primary immune response .

1.7. *Fasciola* DNA vaccines

A DNA vaccine encoding a *Fasciola* antigen was first reported several years ago (Smooker *et al*., 1999; Smooker *et al*., 2001). The induction of humoral responses to the GST antigen was in a mode-specific manner. *Fasciola* GST cDNA subcloned into the cytoplasmic vector pVR1012 and secretory vector pVR1020 was injected intramuscularly into BALB/c mice. The cytoplasmic construct resulted in a Th1 type response, whereas the secretory construct yielded a mixed Th1/Th2 isotype response, as inferred by the titres of antibody isotypes. The humoral response and antibody isotype profile against *Fasciola* GST was influenced by the mode of vaccine delivery and targeting of the encoded antigen (Smooker *et al*., 2001).

Kofta *et al*. (2000) designed a pCDNA plasmid vaccine encoding a cysteine protease which was injected intramuscularly to male and female rats. All rats were challenged against fluke metacercariae, and male rats showed 100% protection, whereas female rats revealed 74% reduction in fluke burden.

The isotype profile induced after secretory construct VR1020/FABP vaccination showed an IgG1 dominant response with significant a IgE, IgG2a and 2b response

remarking a mixed Th1/Th2 response in mice (Smooker *et al.*, 2001). However VR1012/cathepsin L5 showed moderate IgG2a and no IgG2b response, but predominant IgG1, IgE responses indicating a predominantly Th2-like response (Smooker *et al.*, 2001). It should be noted that the VR1012/cathepsin L5 construct retained the native signal sequence for the protease, and hence it was secreted from host cells. The humoral and cellular response to DNA vaccination encoding the saposin like protein (Fh SAP-2) in secretory (FLAG-CMC/SAP) and cytoplasmic (FLAG/SAP-2) forms has been investigated both *in vivo* and *in vitro* (Espino *et al.*, 2005). They observed that vaccination with cytoplasmic construct induced a mixed Th1/Th2 response.

1.8. Scope and aims of the project

Fascioliasis is an economically important veterinary parasite disease. Although anti-helminthic drugs are effective against this organism, drug resistant strains have emerged . In addition, anti-flukicides have their own demerits against the environment and residues in animal food products. Vaccination offers an effective alternative to drug treatment. The complex lifecycle and immunomodulatory property of the parasite makes *Fasciola* vaccine development challenging. At each stage of fluke development, the parasite secretes different antigens. In addition, at different development stages the fluke resides at a different location in the definitive hosts.

The *Faciola* vaccine development process involves identification of stage specific antigens (juvenile, immature and mature stage), their subsequent isolation, recombinant protein expression, biochemical and immunological characterisation . The secreted cysteine protease family has been extensively studied, with the most promising antigens appearing to be cathepsin L (Dalton *et al.*, 2003b; Kesik *et al.*, 2007; Spithill *et al.*, 1997; Wijffels *et al.*, 1994b) and cathepsin B (Beckham *et al.*,

2006; Kennedy *et al*., 2006; Law *et al*., 2003; Meemon *et al*., 2004). Functional expression in yeast has proved to be more effective for cathepsin L (Dowd *et al*., 1994; Roche *et al*., 1997), cathepsin L5 and cathepsin B (Law *et al*., 2003) than other system.

Most *Fasciola* vaccine experiments have correlated protection against challenge infection with immunological parameters (IgG1, IgG2 and IgE or Th1/Th2) (Kesik *et al*., 2007; Mulcahy *et al*., 1998; Mulcahy & Dalton, 2001; Wedrychowicz *et al*., 2003; Wedrychowicz *et al*., 2007). Pathological (liver damage) and parasitological parameters (fluke recovery, size and wet weight of the flukes) are naked eye observations of the vaccine efficacy. Rats were challenged with natural infection of *Fasciola hepatica* showed a much higher immunoreactivity to cathepsin L proenzyme (Harmsen *et* al., 2004). Medium resistance group showed considerable macroscopic liver damage with calcification and dilatation of bile duct. Bovine, rodent and human beings belonged to this group. Rats are often used as a model to study immunity in cattle (Van Milligen *et al.,* 1998). Thus, rats are often used as a model to study immunity in cattle. Experimental oral challenge, liver score analysis, pathogenocity and immunity assessment in rodent model are evaluated easily for laboratory based vaccine analysis. However, our vaccine research team are interested in investigating the immune potency of cathepsin proteins using this classical infection rodent model of *Fasciola*.

Escherichia coli expression leads to production of inactive cathepsin L inclusion bodies which subsequently refold to active form of cathepsin L by using cumbersome renaturation methods (Kesik *et al*., 2007; Yamasaki *et al*., 2002). Cathepsin L contains cysteine residues and requires disulfide bond formation for proper folding of the protease, but expressed insoluble proteases consist of misformed disulfide bridges due to over expression. These misfolded proteases are

not active. In order to utilize these proteins fully, refolding into an active form was pursued.

In addition to identifying new antigens, it is advantageous to evaluate vaccine potential of novel vaccination strategies. DNA vaccines are a new proliferating vaccine research and have the capability of eliciting CD4+ and CD8+ T cell responses against infection. DNA vaccines are easily manufactured and transported to an endemic area without cold storage. *In vitro* and *in vivo* analysis of DNA vaccines *F. hepatica* cathepsin L5 was initiated by Smooker *et al*. (2001), ovine CTLA4 fused to cathepsin B was analysed by Kennedy *et al*. (2006) and the protective efficacy of cathepsin L was reported by Kofta *et al*. (2001). Each of these trials induced humoral responses against target antigen. The stunning sucessess in murine models demonstrate that DNA could be used to identify candidate antigens for more conventional vaccines and provide useful tools to understanding the immunology relavant to these models (Boyle *et al*., 1999). More over, these models may eventually unravel the mechanism of DNA uptake and immune induction. Low resistance group showed prolonged *Fasciola* infection with no calcification which includes sheep, mice and rabbit (Spithill *et al*., 1997). However, further immune induction studies are planned using the BALB/c mice to elucidate the relative vaccine effect of four different vaccine constructs encoding cathepsin B and before this approach can be applied to target species includes sheep, a more complete understanding of mechanism underlying antigen targeting and DNA vaccine delivery will be required. These mechanisms may then be manipulated for higher efficiency and thus efficacy in ruminants be augmented.

In summary, the aims of the work described in this thesis are:

1. To evaluate yeast expression, purification and immunological properties of adult *F. hepatica* cathepsin L5, immature stage *F. gigantica* cathepsin L1g and juvenile *F. hepatica* cathepsin B.

2. To determine the protective efficacy of these stage specific *Faciola* recombinant cathepsin proteins using the rat model of fasciolosis.

3. To characterise the immunological potency of various DNA vaccine constructs encoding cathepsin B using a mouse vaccination model.

4. To analyse *E. coli* expression and refolding of the of *Faciola gigantica* cathepsin L1g.

Chapter 2

Materials and Methods

2.1 General Procedures

Routine methods are presented in this section. Methods specific for studies described in individual chapters are presented there.All buffers, culture media solutions were prepared in Milli Q, using the Millipore water system unless otherwise specified. Liquid, agar media and glassware were sterilised by autoclaving at 121℃ for 20 mins. Glassware was washed in Pyroneg detergent by using a dishwasher (Galaxy scientific, Australia).

2. 2. Materials

2.2.1. Materials for specific application

Cell culture reagents

Concanavalin A	Sigma-Aldrich Pty. Ltd, USA
L- glutamine	Sigma-Aldrich Pty. Ltd, USA
HEPES Buffer	Cytosystem Pty Ltd
Incubator, for tissue culture	Forma Scientific
Newborn calf serum	Sigma-Aldrich Pty. Ltd, USA
Lipofectamine^{Tm} LTX reagent	Invitrogen
RPMI medium	Thermo Trace, USA
Tissue Culture Flask (25 cm^2, 50 cm^2, 75 cm^2)	Nunc, Denmark
Trypsin	Sigma-Aldrich Pty. Ltd, USA

ELISAs

Anti-rat IgG-Horse Radish peroxidise conjugate	Sigma-Aldrich Pty. Ltd, USA
Anti-mouse IgG-Horse Radish peroxidise conjugate	Sigma-Aldrich Pty. Ltd, USA
ELISA 96wellplate	Nunc, Denmark

Phosphate Buffered Saline (PBS)	Oxoid Australia Pty. Ltd
Skim milk powder	local supermarket
Sulphuric acid	BDH Chemicals, Australia
3, 3', 5, 5'-tetramethylbenzidine dihydrochloride	BD Pharmingen, USA
Tween^{R} 20	Sigma-Aldrich Pty. Ltd, USA

ELISPOT assay

IFN-γ capture antibody	BD Pharmingen, USA
IFN-γ biotin conjugated antibody	BD Pharmingen, USA
IL-4 biotin conjugated antibody	BD Pharmingen, USA
IL-4 capture antibody	BD Pharmingen, USA
Multi screen plates	Millipore, USA
Strepavidin Alkaline phosphatase	Sigma-Aldrich Pty.Ltd, USA

Enzymes

Restriction endonucleases such as, *BamH* I, *EcoR* I, *Kpn* I, *Pst* I, *Not* I, *Mlu* I were used in this study from Promega.

Calf intestinal alkaline phosphatise	Roche Diagnostics
DNase	Sigma-Aldrich Pty.Ltd, USA
RNase	Roche Diagnostics
T4 DNA ligase and buffer	Invitrogen, Australia

Molecular biology kits

ABI Prism BigDye Terminator Cycle	
Endo free plasmid Giga Kit	QIAGEN, Germany
EXPAND PCR System	Roche Diagnostics
QIA quick^{R} PCR purification kit	QIAGEN, Germany
QIAEX^{R}II Gel Extraction Kit	QIAGEN, Germany
Qiaprep Mini Spinkit	QIAGEN, Germany
Sequencing Ready Reaction Kit	Perkin-Elmer Corp, USA
TA cloning Kit	Invitrogen, Australia

PCR reagents

Gene specific primers were synthesised at Gene works, Melbourne, Australia.

dNTPS	Bioline, Australiai
Lambda DNA	Pharmacia LKB, Sweden
200µLPCRtubes	Axygen, USA

Plasmids, *E. coli* and yeast strain

pMCP3 was constructed by Dr. Peter Smooker

pCTLA-4 was a kind gift from Dr. Andrew Lew.

E. coli DH 5α	Stratagene, USA
E. coli BL21 DE3 pLysS	Novagen, USA
pVR1012 and VR1020	Vical Inc, USA
pCR2.1	Invitrogen, Australia
pRSET-A	Invitrogen, Australia
S. cerevisiae BJ3505	Kodak, USA

Protein purification

Membra-Cel^{Tm}15-kDa/cut-offDialysismembrane	Viskase, USA
Ni-nitrilotriacetic acid	Amersham-Biosciences, Australia
Chromatography column (5 mL and 2 mL)	QIAGEN, Germany

SDS-PAGE, SDS-gealtin-PAGE and western blotting

Anti-cathepsin B antibody was kindly supplied by Dr. David Piedrafita (Monash University, Melbourne), anti-cathepsin L monoclonal antibody was a kind from Dr. Rudi Grams (Thamasaat University, Thailand).

Anti-rat Alkaline phosphatase	Invitrogen
Anti-mouse Alkaline phosphatase	Invitrogen
Anti-His monoclonal antibody raised in mouse	Sigma-Aldrich Pty. Ltd, USA
Anti-rabbit Horse Radish Peroxidase conjugate	Sigma-Aldrich Pty. Ltd, USA
Ammonium persulphate (APS)	Sigma-Aldrich Pty. Ltd, USA
Gelatin from cold water fish skin	Sigma-Aldrich Pty. Ltd, USA
Mark12unstained protein marker	Invitrogen, Australia

4-Chloro-1-naphthol	Sigma-Aldrich Pty. Ltd, USA
L-Cysteine	BDH Chemicals, Australia
Dithiothreitol (DTT)	Bio-Rad Laboratories, USA
NBT/BCIP substrate solution	Roche Diagnostics, Australia
Nitrocellulose membrane	Bio-Rad Laboratories, USA
Sodium citrate	BDH Chemicals, Australia
Sodium dodecyl sulphate (SDS)	BDH Chemicals, Australia
SeeBlue^{R}Plus2 prestained standard	Invitrogen, Australia
TEMED (N, N, N', N'-tetramethylethylenediamine)	Bio-Rad Laboratories, USA
Triton-X 100	Sigma-Aldrich Pty. Ltd, USA
Trans-blot electrophoretic transfer cell	Bio-Rad Laboratories, USA
Whatmann blotting paper	Whatmann, USA

Two Dimensional gel analyses

IPTG strips (pH 3-11), rehydration buffer, equilibration buffer I and II, 4-20%,11 cm criterion precast gel, Protean IEF cell system, Electrophoretic unit were purchased from Bio-Rad Laboratories, USA.

Vaccine trials

Female BALB/c mice	Monash Animal Services, Australia
Male Sprague Dawley rats	Monash Animal Services, Australia
F. hepatica metacercariae	Macarthur Agricultural Institute, Australia
Syringe (1 mL, 5 mL, 10 mL, 20 mL, 50 mL)	Terumo pty Ltd, Australia
Quil A (*Quillaja saponaria* A extract)	Sigma-Aldrich pty. Ltd, USA

2.*2.2.* General chemicals and equipment

Acetic Acid, Glacial	BDH Chemicals, Australia
Acrylamide-bis-Acrylamide (40%)	BDH Chemicals, Australia
Acetone	BDH Chemicals, Australia

Agarose (DNA Grade)	Progen Industries, Australia
Albumin, Bovine serum	Sigma-Aldrich Pty. Ltd., USA
Ammonium acetate	Ajax Chemicals Ltd, Australia
Ammonium chloride	BDH Chemicals, Australia
Ammonium hydroxide	BDH Chemicals, Australia
Ammonium persulphate	Bio-Rad Laboratories, USA
Ampicillin	CSL, Melbourne
L- Arginine	Sigma-Aldrich Pty. Ltd., USA
Bacteriological agar	Oxoid Australia Pty. Ltd
Balance Analytical Balance	Sartorius GMBH, Germany
(ii) Balance (0.1-500g)	U-Lab, Australia
Bicine	Sigma-Aldrich pty Ltd, USA
Bromophenol blue	BDH Chemicals, Australia
Capillary tubes	Becton Dickenson & Co, USA
Cell strainer	BD Pharmingen, USA
Centrifuge	
(i) Microcentrifuge	Zentrifugen, Germany
(ii) Bench Top Centrifuge	Beckman, USA
(iii) High Speed Centrifuge	Beckman, USA
Centrifuge Tubes	
(i) 1.5 mL Eppendorf Tubes	Sarstedt, Germany
(ii) 10 mL Centrifuge Tubes	Greiner Labortechnik, Germany
(ii) 15 mL Centrifuge Tubes	Greiner Labortechnik, Germany
(iii) 50 mL Centrifuge Tubes	Greiner Labortechnik, Germany
4-Chloro-1-napthanol	Sigma-Aldritch Pty. Ltd., USA
Coomassie Brilliant Blue R-250	Bio-Rad Laboratories, USA
Coomassie Brilliant Blue G-250	Bio-Rad Laboratories, USA
Coverslips	Mediglass, Australia
Cryovials (1.8 mL)	Nalgene Company, USA

Electrophoresis Units	
(i) DNA	
a) Mini-gel	Pharmacia LKB, Sweden
b) Midi-gel	Bio-Rad Laboratories, USA
c) Maxi-gel	Pharmacia LKB, Sweden
(ii) Protein	
a) Mini Protean II gel system	Bio-Rad Laboratories, USA
b) Maxi Protean gel system	Bio-Rad Laboratories, USA
c) Protean IEF Cell	Bio-Rad Laboratories, USA
Ethylene glycol	Sigma-Aldrich Pty. Ltd, USA
Ethylenediamine tetra acetic acid (EDTA)	BDH Chemicals, Australia
Ethanol	BDH Chemicals, Australia
Ethidium Bromide	Roche Diagnostics, Germany
0.2µm, 0.45µm Filter (Acrodisc)	Gelman Sciences, USA
D-Glucose	BDH Chemicals, Australia
Glycerol	BDH Chemicals, Australia
Glycine	BDH Chemicals, Australia
Hydrochloric Acid (32%) v/v	Eqicell products Pty Ltd
Hydrogen peroxide (30%)	BDH Chemicals, Australia
Imidazole	Sigma-Aldrich Pty. Ltd., USA
Iodoacetamide	Bio-Rad Laboratories, USA
IPTG	Sigma-Aldrich Pty. Ltd., USA
Isoamyl alcohol	BDH Chemicals, Australia
Isopropanol	BDH Chemicals, Australia
Kanamycin	Sigma-Aldrich Pty. Ltd., USA
Lambda DNA	Pharmacia LKB, Australia
Lithium chloride	BDH Chemicals, Australia
Lysozyme	Roche Diagnostics, Germany
Magnesium chloride	Perkin Elmer, USA

B-mercaptoethanol	Bio-Rad Laboratories, USA
Methanol	BDH Chemicals, Australia
Microscopes	
(i) Light Microscope	Olympus Optical
(ii) Phase Contrast Microscope	Nikon Kogaku KK, Japan
PEG1000	Sigma-Aldrich Pty Ltd, USA
Penicillin/Streptomycin	Thermo Trace, USA
Petri Dish	Nunc, Denmark
pH meter	Radiometer, Denmark
Phenol	BDH Chemicals, Australia
Phenol/Chloroform/Isoamyl Alcohol	BDH Chemicals, Australia
Phosphate Buffered Saline (PBS)	Oxoid Australia Pty. Ltd
Polyethylene glycol 8000	Sigma-Aldrich Pty. Ltd., USA
Potassium acetate	BDH Chemicals, Australia
Potassium chloride	BDH Chemicals, Australia
Potassium hydrogen carbonate	BDH Chemicals, Australia
Silver nitrate	Sigma-Aldrich Pty. Ltd., USA
Sodium chloride	BDH Chemicals, Australia
Sodium hydroxide	BDH Chemicals, Australia
Sorbitol	Sigma-Aldrich Pty. Ltd, USA
Sulphuric Acid	BDH Chemicals, Australia
Transilluminator (UV)	Bio-Rad laboratories, USA
Tris-Base	Roche Diagnostics, Germany
Tris-HCl	Roche Diagnostics, Germany
Tryptone	Oxoid Australia Pty. Ltd
Yeast extract	Oxoid Australia Pty. Ltd
X-Gal	Sigma-Aldrich Pty. Ltd., USA

2.3. Bacterial and yeast culture methods

2.3.1. Solution and buffers

Ampicillin

Ampicillin was dissolved in 5 mL sterile Milli Q water, making a stock solution of 100 mg/mL and stored in 1 mL aliquots at –20℃. The ampicillin was added to media or broth at the final concentration of 100 µg/mL.

Isopropyl-β-D-thiogalactopyranoside (IPTG) 1 M stock

200 mg/mL IPTG filter sterilised through 0.2µm filter. Aliquots stored at -20°C.

TE buffer (10X)

Buffer contained 100 mM Tris and 10 mM EDTA, pH 8.0.

Kanamycin

Kanamycin was made up as a stock solution of 50 mg/mL in sterile Milli Q water and stored in 1 mL aliquots at -20℃. The working concentration of Kanamycin was 50 µg/mL.

2.3.2. Growth media

Luria-Bertani (LB) media

LB broth-0.5% yeast extract, 1% tryptone, 1% sodium chloride. When required, ampicillin was added to a final concentration of 100 µg/mL or 50 µg/mL of kanamycin. 2% bacteriological agar was added for preparing plates.

Minimal media +uracil+Lysine

0.67% (w/v) yeast nitrogen base, 2% (w/v) dextrose, 0.002% (w/v) uracil, 0.003% (w/v) lysine. Media was prepared as a 10X stock solution and filtered sterilised. For broth, stock was added to autoclaved Milli Q water and for preparing plate, 2% bacteriological agar was autoclaved and mixed with the stock solution.

YPD media

The components were 2% (w/v) D-glucose, 2% (w/v) peptone, 1% (w/v) yeast extract, 1.5% (w/v) agar.

YPHSM media

The components were 1% (w/v) yeast extract, 8% (w/v) peptone, 1% (w/v) D-glucose, 3% (w/v) glycerol, 20 mM calcium chloride. The components were dissolved with Milli Q water (1: 20 dilution), and then autoclaved prior to use.

2.3.3 Storage of bacterial and yeast strains

Bacterial and yeast liquid cultures were pelleted by centrifugation, resuspended gently in growth medium and glycerol to a final concentration of 50% glycerol and stored at -70°C.

2.3.4. Bacterial and yeast culture Conditions

Both *E. coli* strains (DH5α and BL21)were grown on LB broth at 37℃ shaking (120 rpm) for overnight.BJ 3505 strain was grown on minimal medium at 28℃ shaking (120 rpm) for 72 hours.

2.3.5 Bacterial and yeast strains

Table 2.1. Bacterial and yeast strains used in this study

Strain	Genotype	Source
E. coli DH5α	*supE ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Stratagene
E. coli BL 21(DE3) pLysS	F^- *ompT* *hsd*$S_B(r_B^- m_B^-)$ *gal dcm* (DE3) pLysS (Cam^R)	Novagen
S.cerevisiae BJ3505	*MATa ura3-52 trp1-Δ101lys2-208 gal2 can1Pep4::HIS3prbΔ1.6R*	Kodak

2.4. DNA manipulation methods

2.4.1. Buffers and reagents

Alkaline lysis solution I

The components were 50mMGlucose, 10mM EDTA, and 25mM Tris-Cl pH 8. The prepared solution stored at 4℃.

Alkaline lysis solution II

0.2 M NaOH, 1% SDS. The components were prepared using sterile MilliQ prior to use.

Alkaline lysis solution III

5 M potassium acetate. The solution prepared using MilliQ water and stored at 4℃.

X-Gal solution

X-Gal (100 mg) was dissolved in 5 mL of N, N'-dimethylformamide and stored at –20℃.

Isoproply-β-D thiogalactopyranoside (IPTG) (100 mM)

IPTG (1.2 g) was dissolved in 50 mL of sterile Milli Q water, filter sterilised and stored at 4℃.

NB buffer

0.15 M NaCl, 10 Mm bicine were prepared using 10 mL of Milli Q water

PEG-Bicine buffer

40% PEG 1000, 200 mM bicine were dissolved in 5 mL of Milli Q prior to use.

SBEG buffer

1M sorbitol, 10 M bicine, pH 8.4, 3% ethylene glycol dissolved in 5 mL of Milli Q water.

6x Loading buffer

40% sucrose, 0.25% (w/v) bromophenol blue and 0.25% xylene cyanol dissolved in sterile Milli Q water and stored at 4℃.

TAE buffer 50X

The buffer components were 24.2% (w/v) Tris-base, 5.17% glacial acetic acid and 1.86% EDTA (w/v) was dissolved in deionised water.

2.4.2. DNA purification methods

2.4.2.1. DNA vaccine preparation using Endotoxin free plasmid Giga kit (QIAGEN)

Plasmid DNA for vaccination was prepared from one litre of *E. coli* DH 5α using the Endotoxin free plasmid Giga kit according to the manufacturer's protocol. A single colony containing each DNA vaccine of interest was chosen after two rounds of selection on LB plates (supplemented with 100 µg/mL ampicillin or 50 µg/mL kanamycin). This was used to inoculate a starter culture of 10 mL kanamycin-LB broth (section 2.3.2). After overnight incubation, the grown culture was inoculated into 1 litre kanamycin-LB broth and this was incubated at 37°C for 16 hours with shaking (200 rpm). The grown culture was pelleted by centrifugation, resuspended in Tris-EDTA buffer supplemented with RNase A, lysed with NaOH/SDS and then neutralised with potassium acetate. The flocculent material was separated from DNA containing solution by vacuum-driven filtration through QIA filter. The filtered solution was then treated with "Endotoxin removal buffer" by incubation with on ice. The endotoxin free plasmid solution was then subjected to pre-equilibrated anion-exchange column purification, followed by a wash with Isopropanol and then eluted with elution buffer. The purified plasmid was precipitated using 0.7 volumes of isopropanaol and subjected to high speed centrifugation. The DNA pellet was washed with 70% ice cold ethanol, air dried and then resuspended in endotoxin free MilliQ water.

2.4.2.2. Miniprep alkaline lysis purification method

Ten millilitres of LB broth was inoculated with a single colony of bacteria and grown overnight with shaking at 37℃. The grown culture was pelleted by centrifugation at 4,000 x g for 3 minutes. The pellet was resuspended in 100 µL of ice-cold alkaline lysis solution I and incubated at room temperature for 5 min, followed by lysis with 200 µL of alkaline lysis solution II and then neutralised with 150 µL of ice-cold alkaline lysis solution III. The mixture was centrifuged for 10 min at 12,400 g and the resulted supernatant was then treated with an equal volume of phenol/chloroform (24 chloroform: 1 isoamyl alcohol) by inverting five times. The supernatant was collected after the centrifugation at 12,400 g for 5 min. The DNA was mixed with an equal volume of isopropanol and followed by centrifugation for 10 min at 12, 400 g. The DNA pellet was washed with 1 mL of ice-cold 70% ethanol and then air-dried for 15 min. The DNA pellet was finally resuspended in 40 µL of sterile Milli Q water.

2.4.2.3. Determination of DNA concentration

DNA concentration and purification were determined by measuring the absorbance of the DNA sample, diluted 1/200, in matching quartz cuvettes at 260 nm using a Schimadzu UV-160 spectrophotometer. An OD_{260} of one is equal to a DNA concentration of 50 µg/mL. An OD_{260}/OD_{280} ratio was used to measure the quality of the DNA. Ratios of 1.8-2.0 indicated good quality DNA.

2.4.3. Recombinant DNA techniques

2.4.3.1. Restriction endonuclease screening and phosphatase treatment of plasmid DNA

Restriction digestion of plasmid DNA (for example, pRSET-A vector) was scaled according to the quantity of plasmid to be prepared. The reaction mixture contains the following components and was incubated at 37℃ for 1 hour.

Plasmid DNA	50 to 100 ng
10X buffer	4 µL
Enzyme	1 Unit
Sterile MilliQ water	makes up to 40 µL

Phosphatase treatment of plasmids was performed by incubating 10 µg restriction digested plasmid DNA with 0.5 µL calf intestinal Alkaline phosphatase (CIP) in 10 µL 10X buffer for 45 minutes. After incubating for 45 minutes, the reaction was stopped by heat inactivation of the enzyme at 65℃ for 15 minutes.

2.4.3.2. DNA ligation

The sizes and concentrations of gel purified products (inserts) were estimated. Ligation was performed at ratio of 1 plasmid DNA molecule to 3 molecules of insert DNA with 1 unit of T4 DNA ligase in 1 µL T4 DNA ligase buffer. The reaction volume made up to a total of 10 µL with sterile Milli Q water and incubated in 16℃ for 16 hours. The mixture was then used for transformation in competent *E. coli* BL21 or DH 5 α cell through electroporation. The total amount of insert DNA was calculated using the following formula.

ng of insert = ng of vector X size of insert (kb) X 3/ size of vector (kb)

2.4.3.3. Transformation of competent *E. coli* with plasmid DNA

2.4.3.3.1. Preparation of competent cells

E. coli BL21 (DE3) pLysS or DH5α were grown in 10 mL of LB broth on a shaker at 37℃ for overnight. Two millilitres of this culture was used to inoculate 200 mL of pre-warmed LB broth in a flask and shaken vigorously at 37℃ until approximately mid-log phase, an absorbance at 600 nm of 0.4-0.5, was reached. The cells were chilled on ice for 30 min and collected by centrifugation at 4000 g for 10 min at 4℃. The supernatant was completely drained off before the cells were washed in 200 mL of ice-cold sterile Milli Q water and collected by centrifugation at 4000 g for 10 min. This step was repeated with 100 mL of ice-cold sterile Milli Q. The cell pellet was resuspended in 10 mL of ice-cold 10 % sterile glycerol and centrifuged as before. The cells were resuspended in a final volume of 0.7 mL of ice-cold 10 % sterile glycerol and divided into aliquots of 40μL each and stored at -70℃.

2.4.3.3.2. Electroporation of competent cells and blue - white colony screening

An aliquot of frozen electro-competent cells was thawed on ice for 15 min and mixed with DNA (approximately 40 ng) and transferred to an ice-cold electrocuvette with a 0.2 cm gap. The pulse settings used to deliver DNA into the cells were 2.5kV, 25mF and 200Ω. After the pulse, 1 mL of SOC medium was immediately added to the cells and they were incubated at 37℃ for 1 hour. One hundred microliters of transformed culture was plated out onto LB agar containing the appropriate antibiotic and/ or a final concentration of 100 μg/Ml, 40 μL each of 100 mM IPTG and 2% X-Gal plates. IPTG induced the promoter of the plasmid's *lac Z* gene. If *lac Z* had been disrupted

by the insertion of a foreign insert into multiple cloning sites, then no β-galactosidase was produced and colonies remained white. If however the plasmid was non-recombinant, active β-galactosidase acted on X- Gal to form a product that coloured colonies blue. Thus, cells transformed with recombinant and non-recombinant plasmids were discriminated based on colony color.

2.4.3.3.3. DNA sequence analysis and purification of PCR products for sequence analysis

All DNA sequencing was carried out using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions:

Terminator ready reaction mix	2.0 µL
Buffer	3.0µL
Template DNA	
Double stranded DNA	X µL (200-500 ng)
or PCR product	X µL (30-90 ng)
Primer (100 ng/mL)	0.5 µL
Milli-Q water	x µL
Total volume	25.0 µL

The PCR programme used for the sequencing of a DNA segment was as follows:

Number of cycles	Temperature	Duration
25	94℃	10 seconds
25	50℃	5 seconds
25	60℃	4 minutes

Two µL of sodium acetate (pH 4.6) and 50 µL of 95% ethanol were added to 25 µL of sequence reaction, mixed and placed on ice-bath for 10 minutes and then centrifuged for 30 minutes at 5000 g. The supernatant was discarded and the pellet was rinsed in 250 µL of 70% ethanol, vortexed briefly and then centrifuged again for 10 minutes at 5000 g. The pellet was air-dried and then analysed on ABI prism 377 DNA sequencer at Monash University, Clayton, Victoria, Australia.

2.4.4. Recombinant protein analysis

2.4.4.1. Buffer and stock solution

Bradford Coomassie reagent

0.05% Coomassie blue R-250 was dissolved in 25 mL 95% ethanol and 50 mL 88% phosphoric acid then made up to 500 mL using sterile Milli Q. To ensure thorough mixing of the components, the components were incubated at room temperature with shaking for 1 hour and then filtered through 0.45 µM filter and stored at 4℃.

Coomassie Brilliant Blue staining solution

0.05% Coomassie Brilliant Blue R-250 was dissolved in 250 mL methanol and 50 mL acetic acid. After the Coomassie Brilliant Blue had dissolved, the solution was diluted to 500 mL with sterile Milli Q.

Coomassie destaining solution

The solution components were 10% Ethanol and 10% acetic acid.

10x SDS-PAGE buffer

3% Tris Base, 14.4% Glycine and 1% SDS was dissolved in Milli Q pH 8.3. 1X dilution of the buffer was used for running SDS-PAGE.

SDS-PAGE sample buffer

The components were 20 mM Tris- HCl, pH 8, 50 % glycerol, 2.5% SDS, 5% mercaptoethonol, 0.02% bromophenol blue. For preparing non-reducing SDS-PAGE sample buffer, 5% mercaptoethonol was excluded from SDS-PAGE sample buffer.

Silver staining fixative solution

50% methanol, 10% acetic acid mixed in 100 mL of Milli Q

Silver staining solution

0.36% sodium hydroxide, 14.8 M ammonium hydroxide and 0.15% silver nitrate were used to prepare 100 mL of staining solution

Silver staining development solution

1% citric acid and 0.05% of formaldehyde were dissolved in 100 mL of Milli Q.

Triss buffered saline (TBS)

10 mM Tris base and 500 mM NaCl was dissolved in sterile Milli Q pH 7.4.

Western blot transfer buffer

25 mM Tris, 192 mM glycine, 20% methanol was dissolved in sterile Milli Q water.

Western blot blocking solution

5% skim milk powder was dissolved in TBS.

Western blot developing solution

The substrate, 30 mg 4-chloro-1-naphthol, was dissolved in 10 mL of Methanol. This solution was added to 50 mL of 1X TBS buffer, and 30 µL of hydrogen peroxide pH 7.4. The substrate NBT/BCIP, 200 µL of stock solution was added to 10 mL of 1XTBS, pH 7.4.

2.4.4.2. Bradford assay

A standard curve was set up from a 1 mg/mL BSA stock at 30, 60, 90, 120, 150, 180 and 210 µg/mL in 0.15 M NaCl. The purified protein samples were diluted 1/10 with 0.15 M NaCl in a total volume of 100 µL. Bradford reagent (1mL) was added to each standard and test solution and incubated at room temperature for 2 min. 200 µL of mixtures were loaded in duplicate into 96 well plates, and absorbance's read at 600nm.

2.4.4.3. Protein purification by dialysis method

Dialysis tubing of dialysis membrane

Dialysis tubing buffer I

2% sodium hydrogen carbonate and 1 M EDTA were dissolved into 800 mL of Milli Q and pH was adjusted to 8.0.

Dialysis tubing buffer II

1 M EDTA was dissolved into 800 mL of Milli Q and pH was adjusted to 8.0.

Dialysis of yeast supernatants

Prior to column purification, yeast supernatants were subjected to dialysis. After each use, the membrane was washed with Milli Q water several times and then immersed in boiling dialysis buffer I (by using gas burner) for 30 minutes. This was followed by washing the boiled membrane twice with Milli Q water and then immersion in boiling dialysis buffer II for 30 minutes. The membrane was stored in buffer II and or sterile Milli Q at 4℃ until use. After pouring the yeast supernatant, the ends of dialysis membrane were tied by plastic clips. The assembly was placed into 5 litres of Milli Q water containing 250 mM sodium chloride (dialysis buffer) and the dialysis was performed for 1 litre of yeast culture for 4 buffer changes and at least 4 hours per change.

2.4.4.4. Refolding of *E. coli* expressed proteins

Buffers and solutions

Inclusion body phosphate buffer

The buffer contained 50 mM Na_2HPO_4 and 300 mM NaCl

Bacterial cell lysis buffer

50 mM Na_2HPO_4, 0.5 M NaCl, 20 mM lysozyme, pH 8.0. The components were dissolved in Milli Q water prior to use.

Solubilisation buffer

50 mM Na_2HPO_4 pH 8.0, 10 mM EDTA and either 10 M urea and 10 mM β-mercaptoethonol or 6 M guanidine hydrochloride and 10 mM DTT (Gdn HCl). The components were dissolved in 10 mL of Milli Q water.

Urea gradient buffer

Urea from 10 M to 0 M in 50 mM Na_2HPO_4, 0.5 M NaCl, 10 mM β- mercaptoethonol and 20% glycerol, pH 8.0. The buffer was prepared using sterilised Milli Q.

Renatuation buffer

50 mM Na_2HPO_4, 50 mM NaCl, 5 mM EDTA, 4 mM GSH,0.4 mM GSSG, 0.7 M L-arginine mono hydrochloride, pH 10.5.

2.4.4.5. Ni-NTA affinity chromatography purification

Starter buffer or mixing buffer

Buffer components were 250 mM sodium dihydrogen phosphate and 2.5 M sodium chloride and the pH was adjusted to 7.6.

Running buffer

The buffer components were 350 mM sodium chloride, 25 mM sodium dihydrogen phosphate and 1 mM imidazole (50 mL in sterile Milli Q) and the pH was adjusted to 7.6.

Washing buffer

350 mM sodium chloride, 25 mM sodium dihydrogen phosphate and 10 mM imidazole was made up to 50 mL in sterile Milli Q and the pH was adjusted to 7.6.

Mono-S-buffer

25 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 5.0.

Chromatography column purification of proteins

The NTA resin containing polypropylene column (5 mL) was charged with 1 mL of 0.2 M Nickel sulphate and then 50 mL of sterile Milli Q passed through the Ni-NTA. This was followed by washing with 50 mL of the running buffer and dialysed yeast supernatants were loaded into the column. After washing with wash buffer, the protein was eluted using 100 to 400 mM imidazole (appropriate imidazole concentration optimised according to the protein visualisation in Coomassie stained SDS-PAGE). The eluted fractions were collected in eppendorf tubes and further analyses (by Bradford assay and SDS-PAGE) were performed.

2.4.4.6. SDS-PAGE, gelatin substrate PAGE, Coomassie and silver staining

2.5 mL of 1.5 M Tris pH 8.8, 100 µL of 10 % SDS, and 3.1 mL of 40% bis/acrylamide were added to 4.3 mL of Milli Q water. 50 µL 10% ammonium persulphate and 10 µL TEMED was added and mixture was mixed thoroughly prior to use. For preparing a SDS-gelatin separating gel, 0.5% gelatin was added in addition to above mentioned mixture.

SDS-gelatin PAGE buffer I

0.1M sodium citrate, pH 4.5 and 2.5% Triton X-100 was made up to 100 mL in Milli Q prior to use.

SDS-gelatin PAGE buffer II

0.1 M sodium citrate, pH 4.5 and 2 mM DTT or L- cysteine was made up to 100 mL in Milli Q prior to use.

Stacking gel (4%) for both SDS-PAGE and SDS-gelatin PAGE

1 mL of Tris pH 6.8, 40 µL of 10% SDS, 2.6 mL of Milli Q water, 20 µL 10% of ammonium per sulphate and 5 µL of TEMED was added and mixed thoroughly before use.

SDS-PAGE, SDS-gelatin PAGE, Coomassie and silver staining

The purified proteins from the Ni-NTA column was mixed with SDS-PAGE sample buffer (5:1) followed by vortexing for 2 minutes and then heated at 100℃ for 10 minutes in heating column. SDS-PAGE stacking gel wells were loaded with 10 to 20 µL of heated sample and electrophoresis was performed at 80 volts for 30 minutes and then 180 volts for 50 minutes. After electrophoresis, gels were stained with Coommassie Brilliant blue R 250, and destained with Coommassie destaining solution. If previously stained with Coomassie Blue, gels were rinsed with 50% methanol overnight with at least two changes. Gels were then fixed in silver staining fixative solution (2.4.4.1) for one hour with at least 3 changes. Following rinsing with Milli Q water the gels were immersed in silver staining solution (2.4.4.1) for 30 minutes with at least 3 changes. After rinsing with Milli Q water, the gels were incubated in developer (2.4.4.1) and development was stopped by adding 1% acetic acid.

For SDS-gelatin PAGE, protein samples were incubated with non-reducing SDS-PAGE sample buffer for 30 minutes at 37^0C. This was followed by electrophoresis at 200 volts for 1 hour at 4^0C, and the gels were incubated twice in buffer I for 30 minutes. The gels were then immersed in buffer II for 4 to 12 hours at 37^0C. After incubating with gentle shaking, gels were stained with Coommassie Brilliant blue R 250, destained and visualised under a light box.

2.4.4.7. Western blot

2.4.4.7.1. Electro-transfer of proteins to blotting membrane

Following electrophoresis, the gel, a piece of nitrocellulose membrane, scotch-brite pads and 4 pieces of Whatmann paper were immersed in western blot transfer buffer. To assemble the western blot, a pad was placed on the apparatus, followed by the gel, the membrane, the Whatmann paper, and another pad. The air bubbles

were removed by using a glass rod. The apparatus was assembled according to the manufacturer's instructions, and the western blot was performed at 75 volts for 1 hour.

2.4.4.7.2. Western blotting

The blotting membrane or strip was blocked for 1 hour in TBS/Skim milk (5% v/v) at room temperature and then the membrane was rinsed 3 times in 1X TBS. The primary antibody or vaccinated animal sera was diluted in 1X TBS/skim milk (1 % v/v), added to the membrane and incubated overnight at 4℃. After incubation, the membrane was washed thrice for 10 min in 1 X TBS (1% v/v). The secondary antibody, either HRP or Alkaline phosphatase conjugate (dilution according to manufacturer's instructions) was diluted in 1X TBS/skim Milk (1% v/v), added to the membrane and incubated for 1 hour shaking at room temperature. The membrane was then washed twice in 1X TBS (1% v/v). The membrane was developed by the addition of western blot substrate solution (2.4.4.1) (either 4-chloro-1-naphthol or NBT/BCIP) and incubated until the colour developed.

2.5. Vaccination

The DNA vaccination in BALB/c and recombinant protein immunisation in Sprague Dawley rats were performed with the approval of the RMIT and Monash University Animal Ethics committees respectively.

2.5.1 Buffers and reagents

ACK lysing buffer

NH_4Cl (0.15 M), $KHCO_3$ (10 mM) and Na_2EDTA (0.1 mM) dissolved in sterile Milli Q and pH adjusted to 7.4 with 1 M HCl and filter sterilised through a 0.2 µM filter.

ELISA coating buffer I

$NaHCO_3$ (8.4 g) was added to 100 mL of sterile Milli Q.

ELISA coating buffer II

Na_2CO_3 (10.6 g) was added to 100 mL of sterile Milli Q.

ELISA Substrate Solution

Equal volumes of reagent A and reagent B of TMB substrate were mixed prior to use.

ELISPOT blocking buffer

Newborn Calf Serum (5% v/v) was filter sterilised into sterile PBS.

ELISPOT coating buffer

Carbonate buffer 1 (4.53 mL) was added to Carbonate buffer 2 (1.82 mL) and diluted to 100 mL with sterile Milli Q. The pH was adjusted to 9.6 with either Carbonate buffer 1 or 2.

SDS-gelatin PAGE buffer I

0.1M sodium citrate, pH 4.5 and 2.5% Triton X-100 were diluted to 100 mL of Milli Q and prepared prior to use.

SDS-gelatin PAGE buffer II

0.1 M sodium citrate, pH 4.5 and 2 mM DTT or L- cysteine were diluted to 100 mL of Milli Q and prepared prior to use.

2.5.2. ELISA

The column purified protein was diluted to 5 µg/mL in carbonate coating buffer and 100 µL was added to 96 well plates and allowed to incubate overnight at 4°C. Plates were washed in PBS/Tween 20 three times and then blocked for 1 hour in PBS/skim milk (5% v/v). Vaccinated mice or rat serum was diluted in PBS/Tween 20 and skim milk (1% v/v) at the appropriate concentration and 100 µL was added to each well in duplicate. Serum was incubated for 2 hours at 37°C. Plates were washed 3 times in PBS/T, and any remaining liquid was removed by tapping the plates on paper

towels. Secondary antibody (either anti-mouse or anti-rat HRP conjugate) was then diluted in optimal concentration in PBS/Tween 20 and 100 µL was added to each well and incubated for 1 hour at 37℃. After washing plates 4 times in PBS/T and twice in PBS, 100 µL of substrate was added to each well and allowed to develop for 30 min. The reaction was then stopped by the addition 100 µL of 0.2 M sulphuric acid. Absorbances were then measured on ELISA plate reader at an absorbance of 450 nm.

2.6. Bioinformatics and statistics analysis

All the bioinformatics programs used in this thesis were found at the Australian National Genomic Information Service (ANGIS) (www.angis.org.au) or clone manager by Scientific and Educational Software. Statistical analyses were performed using SPSS (window version of 1.5) and Graphpad Prism (3.02 software).

Chapter 3

Recombinant yeast expression of secreted *Fasciola* cathepsin proteases

3.1. Introduction

Protein-based therapeutics are emerging as the fastest growing class of chemical compounds within the drug industry. Vaccine target antigens have been isolated and characterised from many endoparasites of veterinary and human importance. The main challenge at present is to produce these antigens in an immunologically active form using recombinant DNA technology. Recombinant DNA technology allows a vaccine candidate to be synthesised cheaply, safely and in bulk. The expressed antigens can then be evaluated as vaccines under laboratory and field conditions.

At present, there are two commercialised anti-parasite vaccines -TickGard and Gavac, that have been produced in *E. coli* and *Pichia pastoris* respectively. Many *E. coli* expressed nematode antigens have proven to be ineffective as vaccine molecules because they lacked the necessary post-translational modifications (Emery *et al*., 1993). The yeast expression system has been successfully used for nearly two decades in the biotechnology industry for the production of heterologous proteins of human, animal, plant or viral origin. *S. cerevisiae* is the most popular yeast for the expression of proteins as it is genetically well characterised and it has ability to perform many post-translational modifications such as O- or N- linked glycosylation, phosphorylation, disulphide bridge formation, proteolytic processing and folding (Dowd *et al*., 1997).

The liver fluke has a blind end gut and median pore at the posterior of the body. The contents of the parasite gut are regurgitated via the mouth and excreted through a median pore. *F. hepatica* ES products are proposed to have roles in crucial biological functions such as immune evasion, tissue penetration and nutrition. Two dimensional gel electrophoresis (2 DE) has been very useful in identifying target antigen analysis of ES material using polyclonal antisera raised against fluke proteins. Dalton and Heffernan (1989) analysed cysteine proteases that are secreted into the culture medium when flukes are incubated. N-terminal sequencing of the enzymes and biochemical analysis of these proteases showed that they consisted of two cathepsins, L1 and L2, which are proteins with distinct physiochemical properties and substrate specificities. Cathepsin L1 was purified from the ES products of *F. hepatica*, but the production of sufficient quantities of pure cathepsin L1 is a time consuming, complicated and expensive process. To solve this, recombinant cathepsin L1 and cathepsin L2 was expressed in yeast (Dowd *et al.*, 1997; Roche *et al.*, 1997). They observed that yeast expressed *F. hepatica* cathepsin L1 and cathepsin L2 exhibited biochemical properties in common with the *F. hepatica* native enzymes.

The cDNA sequences encoding juvenile *F. hepatica* cathepsin B as well as cDNAs from metacercariae, NEJ and adult *F. gigantica* were isolated (Heffernan *et al.*, 1991; Meemon *et al.*, 2004; Tkalcevic *et al.*, 1995; Wilson *et al.*, 1998). Yeast expressed cathepsin B was antigenic in rats, however the purified protein was unable to undergo autoprocessing (Law *et al.*, 2003). To improve autoprocessing a recombinant cathepsin B was expressed in *P. pastoris* and the purified enzyme was activated by low pH and the presence of dextran sulphate (Beckham *et al.*, 2006).

Cathepsin L and cathepsin B enzyme expression and purification from yeast was observed for clones isolated from other parasites such as *Schistosoma* (Brady CP *et al.*, 1999; Lipps *et al.*, 1996) and *Clonorchis sinensis* (Park *et al.*, 2001).

In the present investigation, *Fasciola* recombinant protein was expressed and purified from yeast, and characterised by SDS-PAGE and immunoblotting using monoclonal and polyclonal antibodies.

3.2. Materials and methods

3.2.1. Plasmid clones encoding cathepsins B, cathepsin L1 and cathepsin L5

Clones expressing recombinant cathepsin B cDNA (mutagenesis of pFLAG/CatB was performed to remove potential N-linked glycosylation sites, Asn-X-Ser/Thr, from the predicted protein prior to expression in *S. cerevisiae* to avoid hyper-glycosylation) and cathepsin L5 were previously constructed in the pFLAG expression vector by Dr. Peter Smooker (Law *et al.*, 2003). Proteins expressed in this system contain a poly-His tag to facilitate affinity chromatography. A similarly cloned cathepsin L1g construct was provided by Luke Norbury. Plasmid DNA was propagated in *E. coli* DH5α cells and extracted by the alkaline lysis miniprep method (see section 2.4.2.2).

3.2.2. PEG-Bicine transformation of *S. cerevisiae*

BJ3505 cells were cultured for 24 hours in 10 mL of yeast peptone dextrose (YPD) broth (see section 2.4.1) at 28^0C and then inoculated into 100 mL of YPD. The mixture was grown for four hours until the OD_{600} reach 0.6. 10 mL of aliquots were removed and centrifuged for 5 minutes at 4000 g and the cell pellet was resuspended in 5 mL of SBEG buffer (section 2.4.1). Following centrifugation for 3 minutes at 4000 g, the pellet was resuspended in 200 µL SBEG buffer and incubated at 28^0C for 5 minutes with shaking (120 rpm). Plasmid DNA (1 µg) was added, mixed and incubated at 28^0C for 10 minutes without shaking. The mixture was placed at -80^0C for 30 minutes. The suspension was thawed in a 37^0C water bath, 1.5 mL of PEG-Bicine buffer (section 2.4.1) added and incubated at 28^0C for 1 hour without shaking. This was followed by the addition of 2 mL NB buffer (section 2.4.1), and the mixture was then centrifuged for 3 minutes at 4000 g. The pellet was

then resuspended in 500 µL NB buffer and dispensed in 100 µL aliquots onto minimal media plates.

3.2.3. Cathepsin B, cathepsin L5 and cathepsin L1 protein expression

Expression and purification of cathepsin B and cathepsin L5 from *S. cerevisiae* BJ 3505 cells proceeded according to Law *et al.* (2003) and Smooker *et al*. (2000). pFLAG cathepsin B, pFLAG cathepsin L5 and pFLAG cathepsin L1g transformants were grown at 28^0C using shaking (120 rpm) in 10 mL minimal medium (section 2.3.1). After 72 hours growth, the cells were centrifuged and the cell pellet was put into YPHSM medium (one litre) and incubated with shaking at 120 rpm for 72 hours (see section 2.3.1).

3.2.4. Dialysis and Ni-NTA chromatography purification of recombinant proteins

After incubation and centrifugation to remove yeast cells, the culture supernatant was dialysed as described in section 2.4.4.3. The dialysed supernatant was mixed with mixing buffer (section 2.4.4.5) and the mixture inverted several times to ensure uniform mixing of solution. The whole mixture was centrifuged for 10 minutes at 9000 g (Beckman high speed centrifuge).

For purification of proteins from the yeast supernatant, Ni-nitrilotriacetic acid (NTA) resin was used to capture the hexahistidine tagged protein in a chromatography column. Five mL polypropylene columns were packed with 2 mL of NTA resin and charged with 0.2 M nickel sulphate. Purification proceeded as described in section 2.4.4.5. The protein was finally eluted with 250-400 mM imidazole (optimal imidazole concentration for cathepsin L5 and cathepsin L1g elution is 250 mM and cathepsin B is 400 mM). The eluted fractions were collected in 1.5 mL eppendorf tubes.

3.2.5. Protein concentration assay

Cathepsin protein concentrations were estimated using the Bradford method (refer to 2.4.4.2), with Coommassie Blue G 250 used as the dye reagent to measure the absorbance at 600 nm. Bovine serum albumin (BSA) was used to create a standard curve.

3.2.6. SDS-PAGE analysis

An aliquot (20 µL) of each eluted fraction from the column was boiled with SDS sample buffer (section 2.4.4.1) and separated by 12.5% SDS-PAGE. Protein was visualised by Coomassie Brilliant Blue staining (see section 2.4.4.6).

3.2.7. Autoactivation of *F. hepatica* cathepsin L5

Eluted fractions containing protease were pooled and dialysed in a mono-S-buffer (section 2.4.4.5). The dialysed procathepsin L5 proteins were mixed with 10 mM cysteine hydrochloride and incubated for 2 hours in a water bath (37^0C). After incubation, the mixture was applied to a Mono-S column and eluted using a buffer containing 250 mM NaCl. The eluted fractions were dispensed in eppendorf tubes. Aliquots of 20 µL of each fraction were run in SDS-PAGE and stained with silver nitrate.

3.2.8. Western blot analysis

F. hepatica ES material, *F. gigantica* ES material, *F. hepatica* native cathepsin L proteins and rat anti-cathepsin B sera were previously purified by Dr. David Piedrafita (Monash University, Melbourne), and yeast expressed *F. hepatica* cathepsin L2 was previously purified by Luke Norbury (RMIT University, Melbourne).

Native cathepsin L, ES material and recombinant cathepsin protease was separated on a 12.5% SDS-PAGE gel and transferred to a nitrocellulose membrane (see section 2.4.4.7.2). The membrane was incubated with either anti-cathepsin L specific monoclonal antibody (from Dr David Piedrafita, 1:3000 dilution) or anti cathepsin B rat sera (see Chapter 4, 1:2000 dilution) overnight at 4^0C, followed by incubation with anti-mouse alkaline phosphatase (1:5000 dilution) or anti-rat HRP (1:3000 dilution) conjugate. The membrane was developed by the addition of western blot substrate solution (either 4-chloro-1-naphthol or NBT/BCIP) (see section 2.4.4.1) and incubated until colour development.

3.2.9. Proteomic analysis of *F. hepatica* excretory and secretary products

3.2.9.1. First dimensional gel analysis of ES material

3.2.9.1.1. Passive rehydration of ES material

Two dimensional gel electrophoresis of ES material was performed essentially according to the manufacturer's instructions (BioRad). *F. hepatica* ES material (30 µg) with rehydration buffer (185 µL) was loaded into a rehydration tray. After removing the cover sheet from an IPG strip (pH 3-11) this was placed gently on the protein, and 2 to 3 mL of mineral oil was added to prevent evaporation, followed by covering the tray with a plastic lid and incubation at room temperature.

3.2.9.1.2. Isoelectric focusing of ES material

Isoelectric focusing (IEF) was performed on a Bio-Rad Protean IEF cell at 20°C using the following program:

a. 300 V for 1 h (step and hold);

b. 4,000 V for 2 h (linear voltage ramping until reaching 4,000 V); and

c. 4,000 V for 6.5 h (step and hold).

In the isoelectric focussing (IEF) tray, the two paper wicks were placed on each end of electrodes. After draining the mineral oil from the IPG strips, they were transferred to the IEF tray. The IPG strip was covered with 2 mL of mineral oil and placed in an IEF cell.

3.2.9.1.3. IPG strip equilibration and two dimensional gel electrophoresis

The mineral oil from the strip was decanted and 4 mL of equilibration buffer I was added and incubated for 10 minutes. After decanting, 4 mL equilibration buffer II was added onto the strips. Following 10 minutes of incubation at room temperature, this buffer was decanted. The equilibrated IPG strips were inserted into a 4-20 %, 11 cm criterion precast gel (Bio-Rad) assembly using a plastic comb. Agrose (Bio-Rad) was used to seal the gap between the IPG strip and the precast gel and placed in the electrophoresis cell. Electrophoresis was performed for 65 minutes at 200 volts. The gel was removed from the casting and stained with silver nitrate.

3.2.10. Silver staining of SDS-PAGE and two dimensional gels

The gels were carefully removed from the gel assembly and placed into a clean glass container (see section 2.4.4.6). The gel was fixed in a fixative (section 2.4.4.1). The gels were immersed in a silver staining solution (section 2.4.4.1). Finally the gel was developer (section 2.4.4.1). Once spots were visually obvious, the reaction was stopped by the addition of 1% acetic acid.

3.3. Results

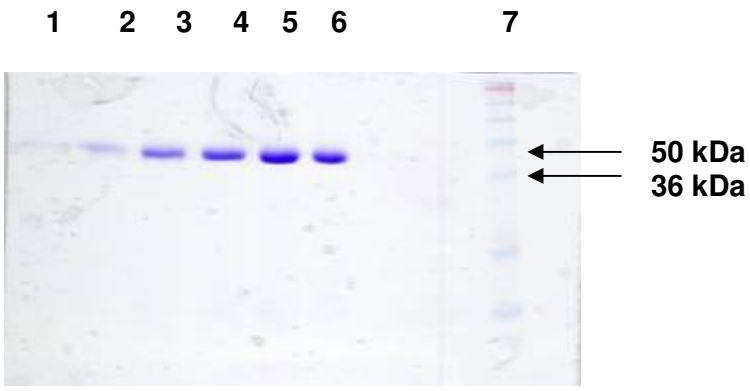
3.3.1. Expression and purification of recombinant *Fasciola* cathepsin proteins

Yeast expression of cathepsin B was performed using pFLAG cathepsin B transformed into BJ3505. Cathepsin L5 and cathepsin L1g expression used a pre-existing glycerol stock of *S. cerevisiae* containing the plasmids. After column chromatography, the cathepsin L's were eluted using a buffer supplemented with 250 mM imidazole, cathepsin B 400 mM imidazole (data not shown).

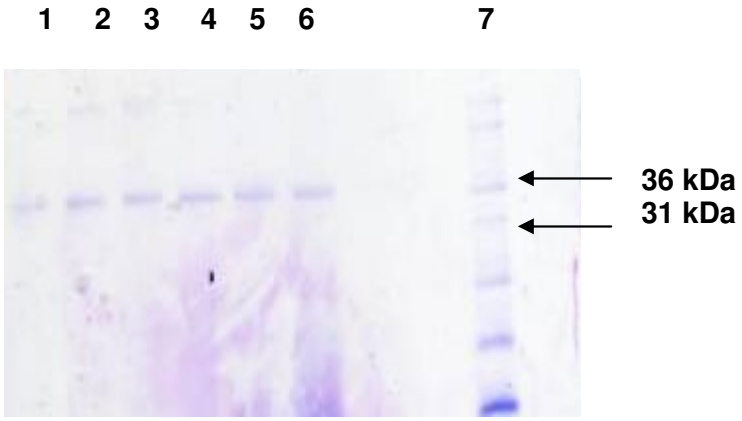
Analysis of 20 µL of each SDS-PAGE eluted fraction stained by Coomassie staining demonstrates the expression of each enzyme (Figures 3.1). The three purified cathepsins were observed at the predicted molecular masses, between 36.5 and 50 kDa in SDS-PAGE. From the gel (Figure 3.1), the fractions from 3 to 6 revealed procathepsin B. The mature cathepsin L5 was observed to autoprocess during purification, as shown by SDS-PAGE analysis (Figure 3.2). The purity of cathepsin L5 revealed a doublet form (Figure 3.1.C) where as cathepsin L1g (Figure 3.1.B) and cathepsin B (Figure 3.1.A) showed a single band in the stained gels.

3.3.2. Protein concentration of purified proteins

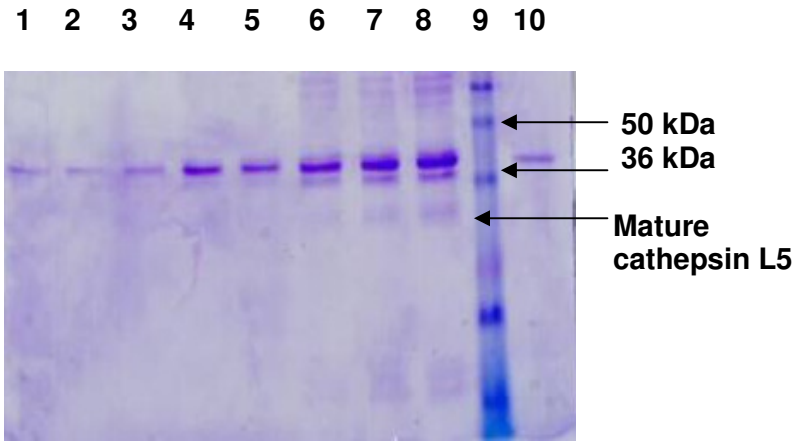
Ni-NTA column purified protein fractions were pooled and the concentration was assessed by the Bradford assay. The yield from one litre of yeast culture is presented in a Table 3.1.



A. Cathepsin B



B. Cathepsin L1g



C. Cathepsin L5

Figure 3.1. Eluted fractions of Ni-NTA column purified proteins resolved by 12.5% SDS-PAGE.

Eluted fractions of **(A). Cathepsin B.** Lanes 1-6, fractions after addition of elution buffer; lane 7, pre-stained protein marker. **(B). Cathepsin L1**. Lanes 1-6, fractions after addition of elution buffer; lane 7, Mark 12 unstained protein marker. **(C).Cathepsin L5.** Lanes 1-8, fractions after addition of elution buffer; lane 9, pre-stained protein marker; lane 10, ninth fraction.

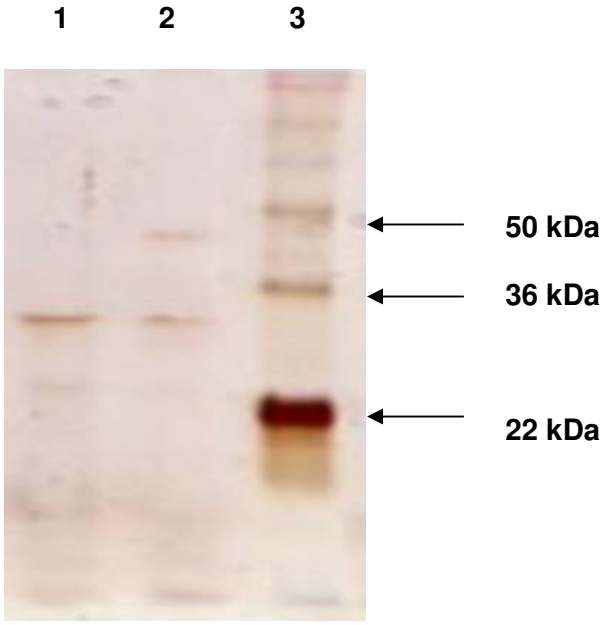


Figure 3.2. The autocatalytic processing of *F. hepatica* pro-cathepsin L5 observed by SDS-PAGE analysis.

Lane 1, activated fraction from the mono-S-column purification; lane 2, an eluted fraction from Ni-NTA column purification.

Table 3.1. The yield of recombinant proteins from one litre of yeast culture

Yeast expressed protein	Average yield of protein / litre of culture (µg / L)
Cathepsin B	500
Cathepsin L5	1300
Cathepsin L1g	300

3.3.3. SDS-PAGE analysis and Two dimensional gel electrophoresis (2 DE) of *F. hepatica* ES material

The ES material showed a major band at a molecular mass of 28 kDa in Coomassie stained gel, but was negatively stained using silver nitrate (Figure 3.3). The ES material showed a major band at a molecular mass of 28 kDa in Coomassie stained gel, but was negatively stained using silver nitrate (Figure 3.3)." Negative staining in silver gels is a well known phenomenon (although it is not really negative- it just means that there is an overabundance of protein at that point). This overloading was not evident in the 2D. With the aim of characterising proteins secreted by *F. hepatica* that could be involved in host-parasite interactions, the ability of these proteins to bind anti-cathepsin L and anti-ES antibodies was investigated. *F. hepatica* ES material was subjected to 2 DE and stained with silver nitrate. The isoelectric focussing pattern of adult *F. hepatica* ES material revealed up to 15 bands in pH range from 3 to 11. Several bands were located between pH 3-7 in the focussed and silver stained IPG strips as shown (Figure 3.4). In Figure 3.4, the double brackets indicate the presence of protein spots of similar molecular mass but differing isoelectiric points (Figure 3.4). The spots may be cathepsin L or possibly glutathione S transferase, which have approximately the same molecular mass. Some of the constituents of ES have been previously identified in several reports (Jefferies *et al.*, 2001; Robinson *et al.*, 2008; Wijffels *et al.*, 1994a) using peptide mass fingerprinting (PMF). A similar pattern was observed in 2 DE of *F. hepatica* ES material by Robinson *et al*. (2008). To attempt to confirm the identity of cathepsin L's within the ES material, western blotting of two dimensional gels was performed. However, this was unsuccessful, therefore dot blot and western blot analysis of ES material probed with cathepsin L (monoclonal) and ES antibodies (monoclonal and polyclonal) were performed.

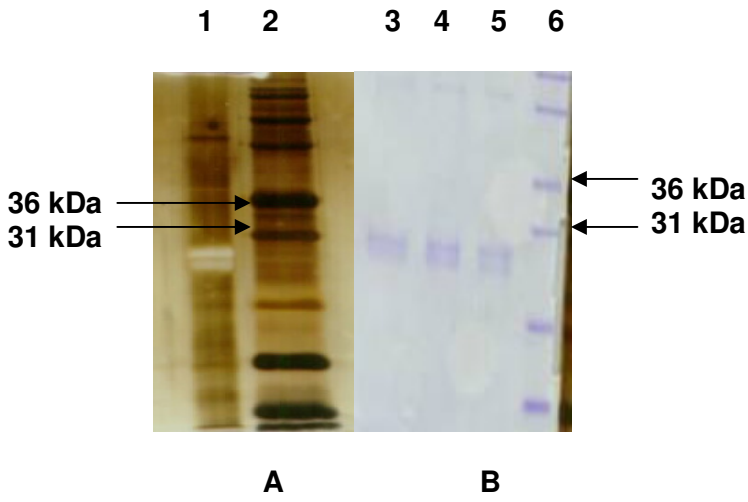


Figure 3.3. SDS-PAGE analysis of *F. hepatica* ES material (Composite image) **(A)**, *F. hepatica* ES material (0.5 µg) was run on SDS-PAGE and stained with silver nitrate; lane 1, ES material, lane 2, Mark 12 unstained protein marker; **(B)** lanes 3 to 5, ES material stained with Coomassie blue; lane 6, protein marker. In 2 DE of this same protein, ES material was fractionated into various spots according to their iso-electric points and molecular weights. So the region or spots in that gel stained by the silver staining did not make negative staining due to their small amount.

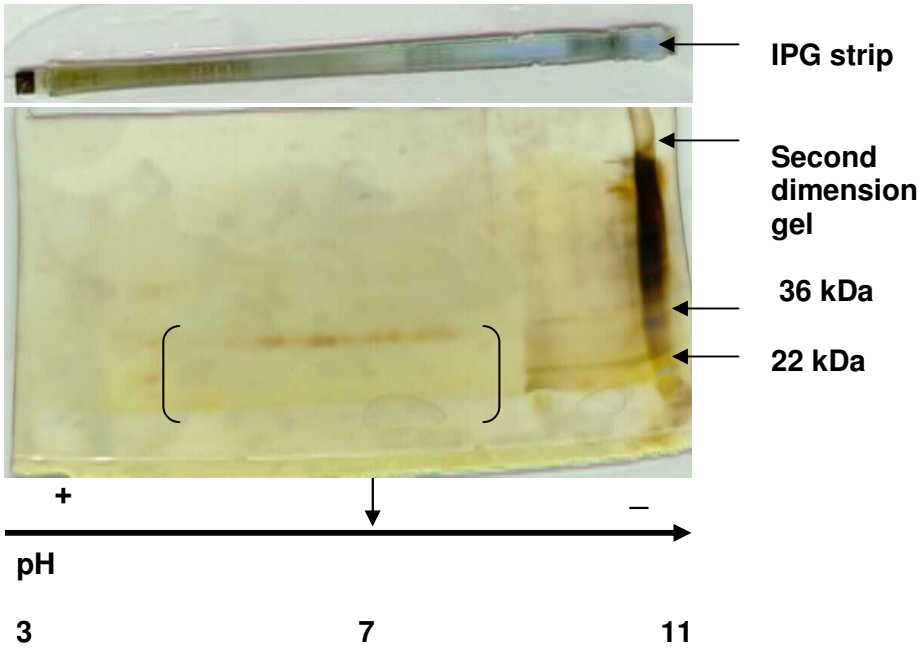


Figure 3.4. Isoelectric focussing and two dimensional gel electrophoresis of *F. hepatica* ES products

Adult ES products were run on a 11 cm ready IPG strip in the first dimension and then a criterion precast gel (Bio-Rad) in the second dimension and visualised with silver staining. The double brackets were indicated here for the identification of protein spots which may be cathepsin L, glutathione S transferase and FABP that have been previously identified in several reports (Jefferies *et al.*, 2001; Wijffels *et al.*, 1994) using peptide mass fingerprinting.

3.3.4. Immune reactivity of *Fasciola* proteins against a cathepsin L specific antibody and anti-cathepsin B sera

The reactivity of *F. hepatica* ES proteins and yeast-derived recombinant proteins with various antibodies was tested. The following antisera were used:

1. Monoclonal antibody raised against *F. gigantica* cathepsin L (from Dr Rudi Grams)
2. Rat anti-cathepsin B sera

A 29k Da protein in the ES material of both *Fasciola* species was recognised by the monoclonal antibody (Figure 3.5). Native cathepsin L and cathepsin L5 showed a strong reactivity to this antibody, whereas cathepsin L1g showed moderate reactivity and cathepsin L2 was only faintly recognised. The antibody recognised the mature cathepsin L5 (lower band after autoprocessing). As expected, the cathepsin L monoclonal antibody did not recognise cathepsin B. Cathepsin B specific rat sera reacts strongly with the yeast-expressed cathepsin B (Figure 3.6).

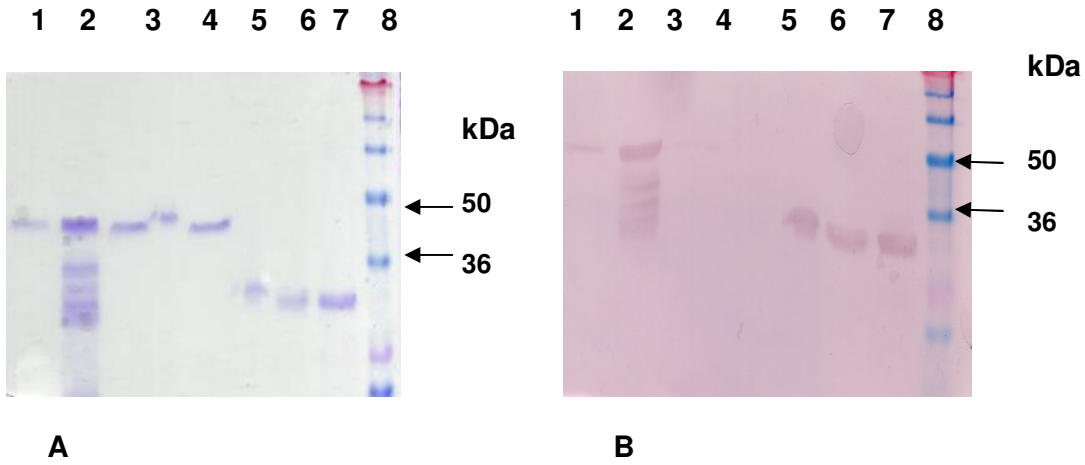


Figure 3.5. Coomassie-stained SDS–PAGE (A) and Western blot analysis using anti-cathepsin L antibody (B) of *Fasciola* proteins.

Lane 1, yeast expressed cathepsin L1g; lane 2, recombinant cathepsin L5; lane 3, yeast expressed cathepsin L2; lane 4, yeast expressed cathepsin B; lane 5, *F. hepatica* native cathepsin L; lane 6, purified *F. hepatica* ES material; lane 7, purified *F. gigantica* ES material; lane 8, pre-stained protein marker.

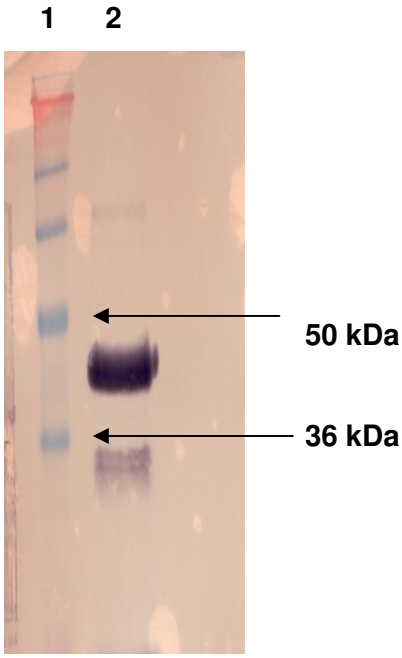


Figure 3.6. *F. hepatica* cathepsin B specific antibody raised in rats reacts with cathepsin B in a western blot.

Lane 1, pre stained protein marker; lane 2, recombinant cathepsin B. The cathepsin B specific rat sera showed strong reactivity to cathepsin B.

3.4. Discussion

3.4.1. Cathepsin B, cathepsin L5 and cathepsin L1g expression and purification

In order to progress to the vaccine trials discussed later in this thesis, it was necessary to optimise the expression and purification of the three cathepsin proteases, L5, L1g and B. These three recombinant proteins were expressed from cDNA generated from flukes at different life stages. Cathepsin L5 cDNA was isolated from adult fluke, while cathepsin B and L1g were isolated from NEJ and metacercariae, respectively. It is known that cathepsin B is expressed up to the age of 5 weeks, however it is not known if L5 and L1g are restricted to adult and juvenile expression, respectively.

The yeast expressed cathepsin B, cathepsin L5 and cathepsin L1g protein yield per litre of culture was higher than previous yeast expressed *F. hepatica* cathepsin L1 and cathepsin L2 by Dowd *et al* (1997) and Roche *et al* (1997) (100 µg/ L of yeast culture). In other studies, a high level of cathepsin L1 was expressed in *P. pastoris,* which carried a hexahistidine tag allowing purification by one step affinity chromatography procedure using Ni-NTA (800 mg/litre). The protease was expressed as a pro-mature enzyme which could be activated at low pH to fully active mature enzyme (Collins *et al.*, 2004). The recombinant cathepsin L5 that was used in these experiments was previously expressed in yeast and yielded 2 mg per litre of yeast culture .

Both *F. hepatica* cathepsin L1 and cathepsin L2 were previously purified using ultra-filtration and gel filtration chromatography on sephacryl S 200 HR columns (Dalton *et al.*, 2003b; Dowd *et al.*, 1997; Roche *et al.*, 1997). In the current experiments,

simple affinity chromatography has been used. Ni-NTA affinity column chromatography is an attractive tool for large scale purification of yeast expressed proteins. NTA resin is reusable and cost effective compared to gel filtration chromatography .

3.4.2. Biochemical analysis of *Fasciola* proteins

3.4.2.1. SDS-PAGE analysis

Using SDS-PAGE, the expressed proteins were separated on gels and the molecular weight was estimated between 36 and 50 kDa. Cathepsin L was found to be major component of ES. The mature, active cathepsin L5 migration pattern (approximate molecular weight is 28 kDa) behaved in the same way as *F. hepatica* ES (approximate molecular weight of the major band 29 kDa) in SDS-PAGE. The pattern of negative staining in the SDS-PAGE is mainly happened especially in the bands containing large amount of the proteins.

3.4.2.2. Two dimensional analysis of *F hepatica* ES material

There are growing numbers of reports involving *F. hepatica* secreted proteins, with a proteomic approach used to identify specific proteins (Allam AF *et al*., 2002; Jefferies *et al*., 2001) to target secreted proteins for analysis in humans and ruminants (Wijffels *et al*., 1994a). The isoelectric focussing of ES products demonstrates 17 bands from *F. hepatica* and 22 bands from *F. gigantica* between pH 3.5 to 10 . The results of this study suggest that isoelectric focussing of adult fluke ES material showed 15 bands in IPG strips. It was not possible to identify individual proteins due to the lack of resolution of these gels.

3.4.3. Immune reactivity of *Fasciola* proteins with monoclonal and polyclonal antigen specific antibodies

The expressed recombinant proteins and native proteins of *Fasciola* species showed immunoreactivity towards a cat L-specific monoclonal antibody and anti-cathepsin B rat sera in western blotting. The monoclonal *F. gigantica* cathepsin L detects recombinant cathepsin L5, recombinant cathepsin L1g and native cathepsin L, *F. hepatica* ES and *F. gigantica* ES. This result suggests that a common immunoreactive epitope exists in all these protein antigens. The results also suggest that there is common epitope expressed between *F. hepatica* and *F. gigantica.* The cathepsin L monoclonal antibody identifies both immature and adult stage cathepsins (cathepsin L1g and cathepsin L5). Cathepsin L5 showed strong reactivity to the monoclonal antibody.

3.5. Conclusion

The production of functionally active cathepsin L5, cathepsin L1 and cathepsin B in *S. cerevisiae* BJ3505 cells will allow for the evaluation of these proteins in a vaccine trial. Further experiments are required to analyse the protective immune responses of these recombinant proteins against experimental rat fasciolosis (chapter 4).

Chapter 4

Protective efficacy of *Fasciola* developmental stage specific recombinant antigens

4.1. Introduction

Liver flukes cause infectious disease in humans and ruminants world-wide. The causative pathogens are *F. hepatica* and *F. gigantica.* This emerging human pathogen is highly prevalent in South American countries and also in Northern Iran and Egypt (Esteban *et al.*, 2003). Sporadic cases has been observed in European countries and Australia . Even though anthelmintic treatment is effective against disease, drug resistant strains have been reported .

Due to the complexity of the life cycle and the immune suppressive activity of flukes, the vaccine development process has been slow. Many studies have concentrated on secreting antigens of the adult stage of the parasite. Adult flukes are found at the bile duct of the host where they are immunologically safe and not attacked by cell mediated defence mechanisms (Dalton *et al.*, 2003a; Hillyer, 2005; McManus & Dalton, 2006; Spithill *et al.*, 1997). Van Milligen *et al.* (1999) has highlighted the protective efficacy of NEJ fluke secreted proteins in rats.

Cysteine proteases are produced by all stages of the fluke and may facilitate biological functions such as immune evasion, excystment and tissue invasion . Among the secreted cysteine proteases, *F. hepatica* cathepsin L has been extensively studied (see for example (Dalton *et al.*, 2003b; Kesik *et al.*, 2007; Mulcahy *et al.*, 1999; Smooker *et al.*, 2000; Wedrychowicz *et al.*, 2007; Wijffels *et al.*, 1994b)).

F. hepatica cathepsin L5 (Irving *et al.*, 2003; Smooker *et al.*, 2000), cathepsin B (Beckham *et al.*, 2006; Kennedy *et al.*, 2006; Law *et al.*, 2003; Meemon *et al.*, 2004) and *F. gigantica* cathepsin L1 (Grams *et al.*, 2001; Tantrawatpan *et al.*, 2005; Wongkham *et al.*, 2005) appear to be most promising target antigens against *Fasciola* infection. In order to assess cysteine protein vaccine potential, recombinant protein expression is highly recommended. The yeast expression system has been proven to be fruitful for the functional expression of cathepsin L (Dowd *et al.*, 1994; Roche *et al.*, 1997), cathepsin L5 and cathepsin B (Beckham *et al.*, 2006; Law *et al.*, 2003). *F. hepatica* cathepsin L1 and cathepsin L2 have been expressed in *S. cerevisiae* (almost a decade ago) and rapidly purified from yeast culture, however no vaccine trials using these proteins have been reported to date (Dowd *et al.*, 1997; Roche *et al.*, 1997).

In experiments described in this chapter, the immune potential and vaccine efficacy of adult stage *F. hepatica* cathepsin L5, immature *F. gigantica* cathepsin L1g and juvenile *F. hepatica* cathepsin B recombinant proteins were analysed in Sprague Dawley rats against *F. hepatica* infection.

4.2. Materials and methods

4.2.1. Recombinant protein vaccine purification

The expression and purification of cathepsin B, cathepsin L5 and cathepsin L1g from *S. cerevisiae* BJ 3505 cells proceeded according to Law *et al.* (2003) and Smooker *et al*. (2000). The expressed proteins were purified by Ni-NTA affinity chromatography and analysed as described in Chapter 3.

4.2.2. Experimental animals

Sprague Dawley male rats were used in the vaccination experiment for testing yeast expressed protein vaccines for their protective efficacy against experimental rat *Fasciola* infection. The rats were six weeks old at the time of vaccination and ten weeks old at the time of challenge. Seven to nine rats were used per treatment group and rats were provided with food and water *ad libitum*. Animal experiments were performed under the supervision of the Monash University animal ethics committee, Australia.

4.2.3. Parasite

F. hepatica metacercariae were obtained from laboratory *Lymnae tomentosa* snail cultures at the Macarthur Agricultural Institute, Menangle, NSW, Australia and used for challenge infection of rats. Parasites were stored at 4^{o}C prior to infection.

4.2.4. Vaccination protocol and time line

Eight vaccine preparations were analysed and are listed in Table 4.1. On the first day of the experiment, the recombinant proteins and Quil A adjuvant were mixed with 0.9% saline. The rats were bled prior to protein vaccination. One primary dose along with two boosting doses of the vaccines was delivered to rats at fortnightly intervals (Figure 4.1). Two weeks after the third vaccination, rats were bled from the tail vein using a 25 gauge needle. All rats were infected with 25 metacercariae a day later.

4.2.5. *F. hepatica* challenge infection

Rats were challenged with 25 *F. hepatica* metacercariae/rat orally. Prior to challenge, metacercariae were observed under the microscope to ensure viability. Viable cysts were selected depending upon their morphology of parasite within the cyst. A stock solution was made consisting of 2000 viable metacercariae cysts mixed with 70 mL of 0.4% carboxy methyl cellulose (Sigma). The solution (1 mL/ rat) was mixed and administered orally to rats using a gavage needle. After each delivery, the gavage needle was flushed with Milli Q prior to the next oral dose.

4.2.6. Necropsy

Eight weeks after the oral infection, rats were humanely sacrificed and their livers were removed. Pathological lesions of livers were observed by the naked eye. Live adult flukes inside the lumen of bile duct were dissected out by using forceps and the whole liver was cut into small pieces (Figure 4.2), submerged in saline at 37^0 C for 1 hour and filtered through a 200 µM mesh sieve for detection and counting of immature and adult flukes.

4.2.7. Pathological and parasitological parameters

4.2.7.1. Anti-fluke effect analysis

The gold standard for anti- fluke vaccine development is the reduction in fluke numbers . A reduction in immature flukes in the liver tissue and adult fluke establishment in the bile duct determines the vaccine efficacy and protective immunity of experimental vaccines in rats (Valero *et al*., 1998; Valero *et al*., 1999; Valero *et al*., 2006). The total numbers of adult and immature flukes recovered from the each rat was counted. These worm burdens were compared with control groups and the reduction in fluke burden per group was calculated as described by Muro *et al*. (1997). The percentage of protection of vaccinated animals was calculated as follows:

Percentage of protection = 1-(Mean number of flukes found in immunised rats/ mean number of fluke recovery in challenged control rats) X 100

Percentage of maximal individual protection = 1-(Lowest mean number of fluke counts in individual vaccinated rats/ mean number of fluke burden in challenged control rats) X 100

The percentage of maximal individual protection in each vaccinated rat group was estimated as the least or no fluke recovered from an individual rat liver. The percentage of maximal individual protection in each vaccinated rat group will be 100 % if no flukes are observed in the vaccinated rats .

Table 4.1. The experimental design of the *Fasciola* recombinant protease vaccination trial. A total of 20 µg of protein (single or cocktail) in 200µl of 1mg/mL Quil A was administered to rats three times at two-weekly intervals.

Vaccination preparation	Rats/group	Antigen dose for each vaccination (µg)
1.Quil A adjuvant as control	8	PBS + 200 µg of Quil A
2. cathepsin B	8	20µg + 200 µg of Quil A
3. cathepsin L5	8	20µg + 200 µg of Quil A
4. cathepsin L1g	9	20µg + 200 µg of Quil A
Cocktail vaccinations		
5. cathepsin B/L5	8	10 µg of cathepsin B 10 µg of cathepsin L5 + 200 µg of Quil A
6. cathepsin B/L1g	7	10 µg of cathepsin B 10 µg of cathepsin L1g + 200 µg of Quil A
7. cathepsin L5/L1g	8	10 µg of cathepsin L5 10 µg of cathepsin L1g + 200 µg of Quil A
8. cathepsin B/L5/ L1g	7	6.6 µg of cathepsin B 6.6 µg of cathepsin L5 6.6 µg of cathepsin L1g + 200 µg of Quil A

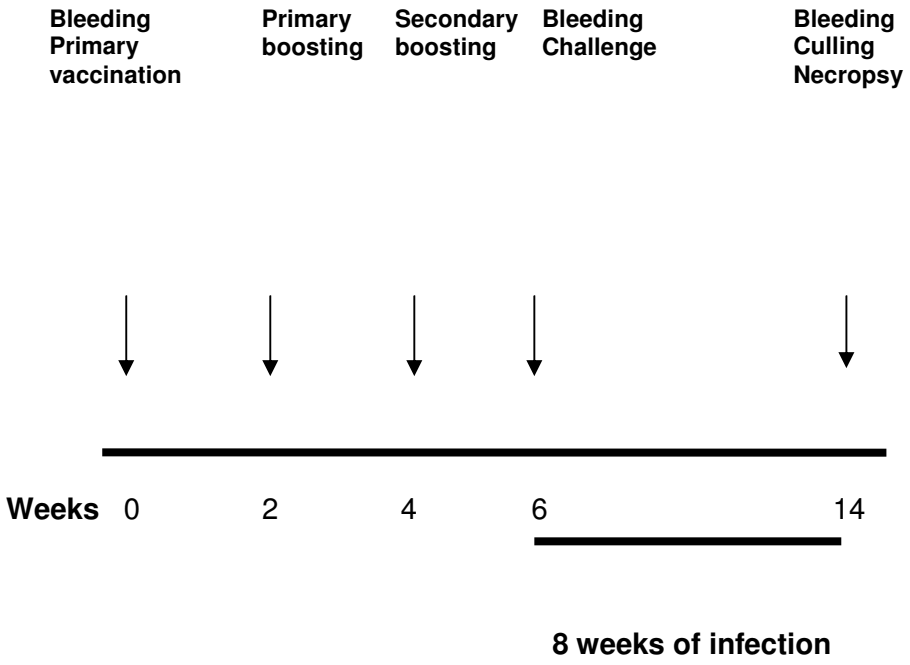


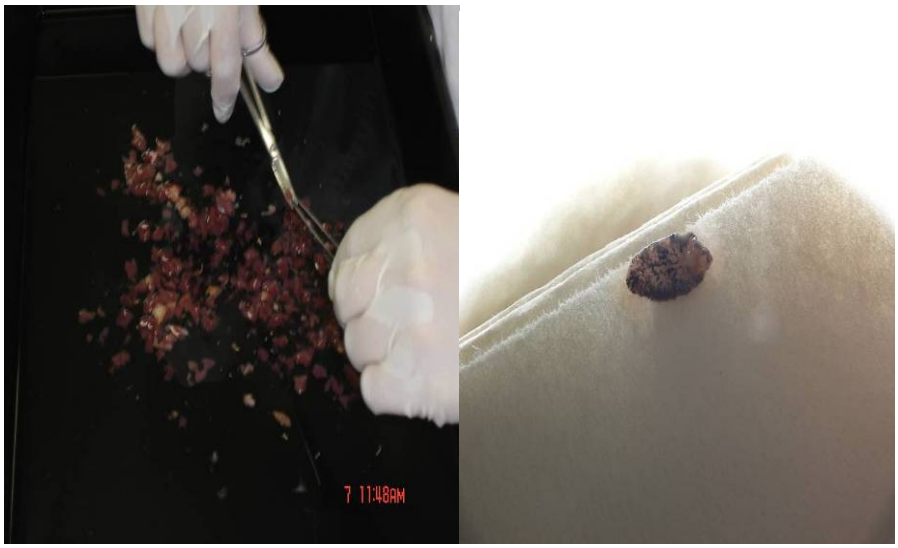
Figure 4.1. The experimental time line for vaccination, blood sampling and experimental infection in rats

4.2.7.2. Evaluation of liver damage

The following macroscopic changes in the liver were observed during the dissection of rats:

1. The change of colour in the hepatic parenchyma to greyish white.
2. Dilatation of the bile ducts and hyperplasia of ductile walls
3. Fibrosis of hepatic lobes, calcification and granuloma formation, mucopurulent discharge and outer surface scar lesions .

The scoring of liver damage was carried out blindly by Dr. David Piedrafita (Monash University, Melbourne). The quantitative degree of damage was scored 0 to 5 depending upon the severity and the intensity of lesions (Raadsma *et al*., 2007). The scoring scheme for liver damage is presented in Table 4.2.



A **B**

Figure 4.2. Sectioning of the liver was performed within the 60 minutes of culling of the rat.

Photograph **A**. The liver was cut into small pieces to enable identification of flukes; photograph **B**. A fluke collected from bile duct showing staining with bile pigments and blood. This fluke is again presented after washing with Milli Q water in Figure 4.6, photograph **(A)**.

Table 4.2. Liver damage score chart for *F. hepatica* metacercariae challenged animals

Liver index score	Macroscopic liver lesions
Score 5	Heavily damaged with extensive necrosis on more than 50% of the liver surface
Score 4	Heavy damage of up to 50% of the liver surface
Score 3	Moderate damage of up to 30% of the liver surface
Score 2	Light lesions of up to 15% of the liver surface
Score 1	Minor damage confined to not less than 5% of liver surface
Score 0	No signs of damage observed on the liver surface or hepatic lobes

4.2.8. *F. hepatica* fluke morphometry analysis

4.2.8.1. Body size and wet weight of recovered flukes

The influence of recombinant vaccines on the fluke body, size and wet weight are important factors of protection (Nambi *et al*., 2005; Ramajo *et al*., 2001; Valero *et al*., 2006). The body size is considered to be a good indicator of fluke development and it is estimated that in rats *Fasciola* measures approximately 6 mm before attaining maturity and entering the bile duct lumen (Dalton *et al*., 1996). The body lengths from head to tail and body width of each worm from all vaccinated and control rat groups were measured under a dissection microscope with a millimetre scale (Valero *et al*., 2002; Valero *et al*., 2005).

4.2.9. Haematological parameters

Peripheral blood smears were prepared from vaccinated and control rats and were stained using KWIK^{Tm} DIFF STAIN KIT (Thermo Electron Corporation). The number of neutrophils, eosinophils and lymphocytes were enumerated for pre-and post-challenge infection.

4.2.10. Immunological parameters

4.2.10.1. ELISA analysis

For ELISA, 96 well plates were coated with cathepsin B, cathepsin L5 or cathepsin L1g protein at 5 µg/ mL in carbonate binding buffer pH 9.6 and incubated overnight at 4^0C (see section 2.5.2). Sera from individual rats were serially diluted from a starting dilution of 1:1000 and bound antibodies were detected using anti-rat HRP conjugate IgG (1:5000 dilutions). The sera from the cocktail or individual vaccine groups were analysed against single cathepsins (for example, sera from rats vaccinated with cathepsin B, the cocktail of cathepsin B and L5, cathepsin B and L1g or cathepsin B, cathepsin L5 and cathepsin L1g were assessed against cathepsin B protein in the ELISA (plates coated with cathepsin B).A similar pattern of analysis was performed for each other protein, and therefore each group that was vaccinated with a particular protein was tested for titre against that protein. The reciprocal titre was calculated using an OD_{450} absorbance with cut-off 0.2 value, and each antibody titre was estimated in duplicate.

4.2.10.2. Immunoblotting of vaccinated sera against *Fasciola* antigens

For assessing immune reactivity of the vaccinated rat sera, cathepsin B, cathepsin L5 or cathepsin L1g were separated on 12.5 % SDS-PAGE and transferred to nitrocellulose membrane (see section 2.4.4.7.2). The membrane was probed with anti-cathepsin B, cathepsin L5 or cathepsin L1g anti-sera (1:3000 dilution) (pooled rat sera) from all vaccinated groups overnight at 4^0 C and then probed with anti-rat Ig - alkaline phosphatase (1: 3000) for 1 hour at room temperature and then finally developed with BCIP/NCT.

4.2.11. Statistical analysis

For each rat from all vaccinated and control groups, the protective efficiency determining parameters such as liver damage score, liver fluke count, wet weight, size of flukes and blood parameters were statistically analysed. For all the groups the mean and standard deviation and correlation was calculated using SPSS software. Statistical differences between vaccinated rat groups were analysed using one way ANOVA followed by Turkey's multiple comparison test (GraphPad (3.02 software), San Diego, USA).

4.3. Results

4.3.1. Effect of protease vaccination on worm recovery

The mean number of flukes recovered from from each rat is presented in Table 4.3, and the summary is given in Table 4.4. A diagrammatic representation of the percentage of protection (calculated as described in 4.2.7.1) is shown in Figure 4.3.

All groups of rats vaccinated with recombinant protein showed a statistically significant lowering of the numbers of recovered flukes. An 83% reduction in the fluke burden was observed in rats vaccinated with the cocktail vaccine containing cathepsin B and cathepsin L5. Fluke numbers in this group were also significantly lower than two other vaccinated groups, as shown in Table 4.3. The lowest percentage of reduction in fluke burden was observed in rats immunised with cathepsin L1g (49%). The cocktail vaccine groups showed a reduction in flukes ranging from 60 to 83%.

The percentage of maximal protection with individual rats was 100% in all cocktail and cathepsin B vaccinated rats (in other words, these groups each contained rats with no flukes). Importantly, vaccines containing cathepsin B and L5 protein showed 100% maximal individual protection in two rats.

Table 4. 3. The fluke recovery from the individual rats

Vaccinated rat group	Fluke counts in individual rats
Quil A	4,5,5,3,4,5,5,4
Cat B	1,2,2,3,3,2,0,1
Cat L5	3,2,2,2,2,2,2,2
Cat L1g	3,2,1,3,1,1,5,2,2
Cat B/L5	0,1,1,1,1,1,0,1
Cat B/L1g	3,0,2,2,2,1,2
Cat L1g/L5	3,1,3,2,1,0,1,3
Cat B/L1g/L5	2,3,4,1,1,2,0

Table 4. 4. Fluke recovery from the bile ducts and hepatic parenchyma. Mean fluke counts from the vaccinated and control rat groups.

Vaccine groups	Fluke recovery from bile duct	Fluke recovery from hepatic parenchyma	Mean fluke count with standard deviation	Statistical significance *P* <0.01	Statistical significance *P* <0.05
Control	18	17	4.37±0.74	-	-
Cat B	2	12	1.75±1.03	-	Vs control
Cat L5	6	11	2.12±0.35	-	Vs control
Cat L1g	10	10	2.22±1.30	-	Vs control
Cat B/L5	1	5	0.75±0.46	Vs control	Vs control, Cat L5, Cat L1g
Cat B/L1g	5	7	1.71±0.95	-	Vs control
Cat L1g/L5	6	8	1.75±1.16	-	Vs control
Cat B/L1g/L5	4	9	1.86±1.34	-	Vs control

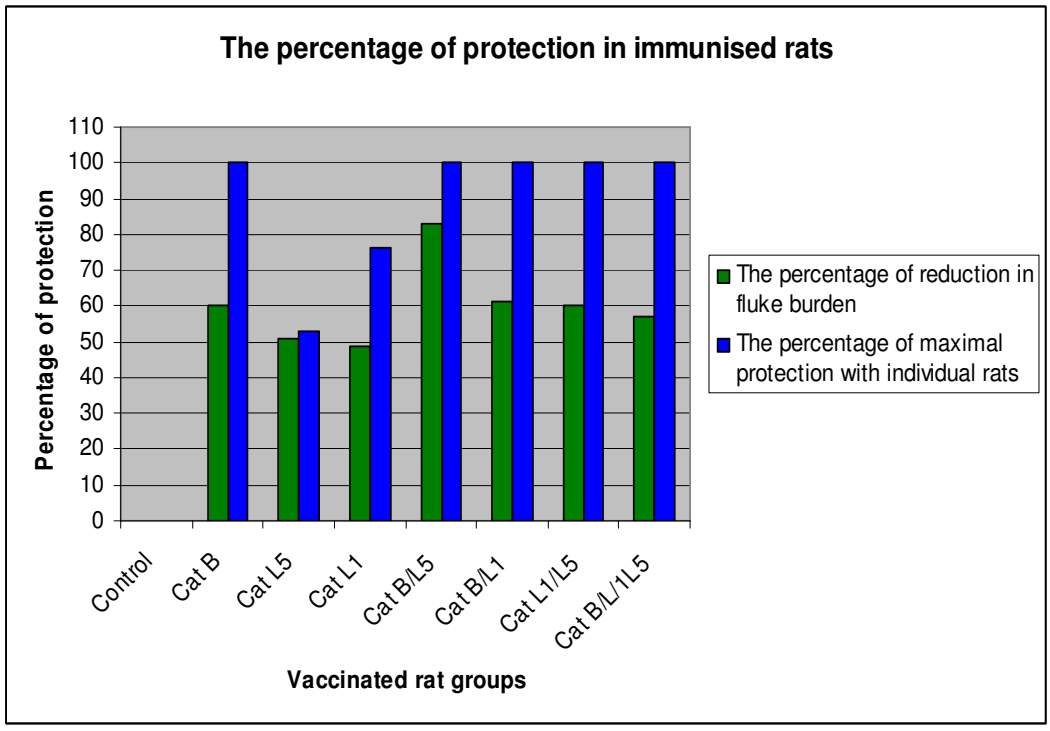


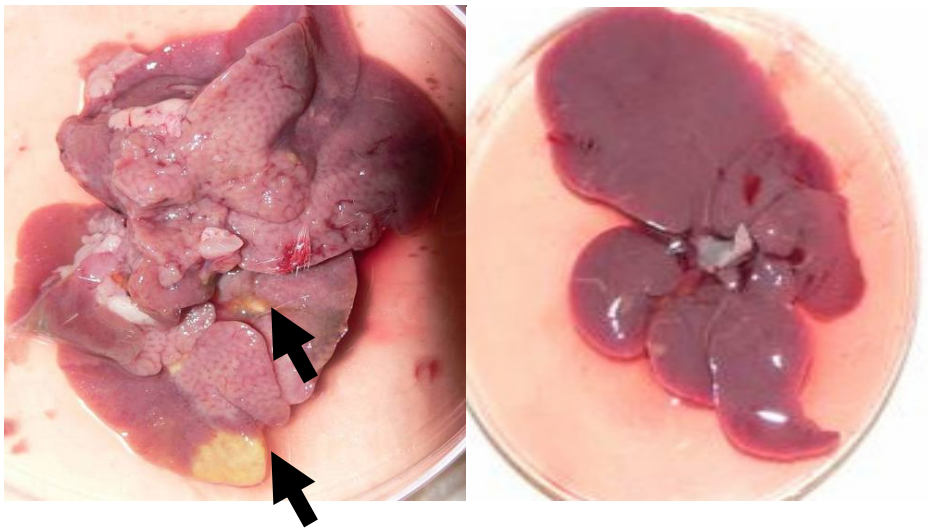
Figure 4.3. Diagrammatic representation of the percentage reduction in fluke counts in the vaccinated and control rat groups.

The reduction in worm burden in vaccinated groups is shown as the percentage protection induced by the vaccine. The blue bar indicates the maximal protection seen in any rat within a group.

4.3.2. Anti-pathology effects of *Fasciola* protein vaccination

A representative control Quil A and protein vaccinated rat showed hepatic and biliary stage pathological signs presented in Figures 4.4 and 4.5. Table 4.5 represents the liver damage score analysis in control and immunised rats. Adjuvant Quil A only administered rats showed significant hepatic damage, with severe lesions on the liver surface after *F. hepatica* infection. Necrotic tracts with severe inflammation of the liver lobules were observed. Calcified granulomas were noticed on the entry point of flukes at hepatic tissues (Figure 4.4A). Severe hepatitis with loss of normal lobular structure was also observed. Severe hyperplasia and dilatation of the bile duct was found (Figure 4.5).

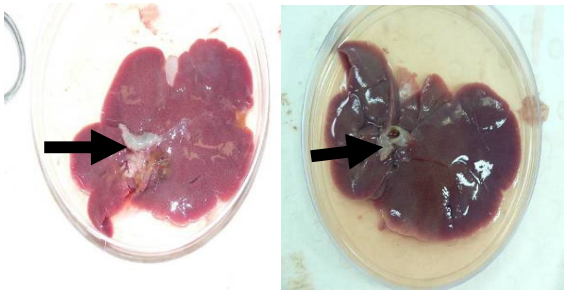
Liver damage scores in the protein vaccinated rats were significantly lower compared to control groups ($P<0.05$). Rats vaccinated with Cathepsin L1g, vaccines containing cathepsin B and L1g and the cocktail vaccine of three antigens showed approximately the same mean liver damage index (1.9) and this damage was confined to five percent of the liver surface. In rats vaccinated with cathepsin B alone or cathepsin B and L5, the livers were macroscopically healthy and had a soft and pliable structure (Figure 4.4.B) compared to control rat livers.



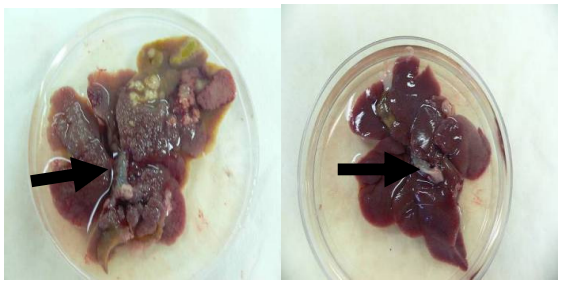
A **B**

Figure 4.4. Liver pathology in control and vaccinated rat livers at necropsy after 8 weeks of challenge infection. Two representative livers are shown.

Photograph **(A)**. Control rat liver. *F. hepatica* causes calcified granulomas at the entry point of flukes (indicated by bold arrows). Hepatic lopes were enlarged with necrotic lesions (liver damage score 4). Photograph **(B)**, Cathepsin B vaccinated rat liver showed a healthy, pliable and soft glassy appearance (liver damage score 1).



A B



C D

Figure 4.5. Comparison of rat *Fasciola* infection in control and vaccinated rats. Photograph **(A)**, and **(B)** shows hyperplasia and dilatation of bile ducts. In **(B)**, the bold arrow represents a cut section of the bile duct in which the fluke was collected; photograph **(C)** shows extensive hepatic necrosis with bile duct enlargement in the control rat liver (liver damage score 4); photograph (**D**), Macroscopic view of cathepsin B and L5 combined vaccine administered rat's liver where the bold arrow indicate the reduction in size of bile duct compared to control rat group.

Table 4. 5. Macroscopic liver damage score analysis in control and immunised rats

Vaccinated group	Score 0	Score 1	Score 2	Score 3	Score 4	Mean liver damage score ± standard deviation
Control	-	1	-	2	5	3.3 ± 1.0
Cat B	2	4	1	1	-	1.2 ± 0.7
Cat L5	3	3	-	2	-	1.3 ± 1.1
Cat L1g	0	5	-	3	1	1.9 ± 0.9
Cat B/ L5	6	-	1	1	-	0.8 ± 0.9
Cat B/ L1g	-	3	2	1	1	1.9 ± 1.0
Cat L1g / L5	2	3	-	3	-	1.6 ± 1.1
Cat B/ L1g / L5	1	3	2	1	-	1.9 ± 1.3

No livers were found to have the highest score for damage, therefore score 5 has not been included in this table. All the mean values of rat liver lesions from the immunised groups were significantly lower than in the control rats ($P<0.05$).

4.3.3. Effect of vaccination on the size of recovered flukes

The length and width of flukes represent the extent of maturation and growth. The mean length and width of the flukes from each vaccinated group is shown in Table 4.6 and presented graphically in Figure 4.7. Rats vaccinated with the cocktail vaccine containing cathepsin B and L5 yielded flukes that were significantly smaller in body length and width than those isolated from control groups and other vaccinated rat groups ($P<0.01$). Flukes recovered from rats vaccinated with this cocktail vaccine showed a highly significant percentage reduction in body length (83%) and width (79%) of flukes than the control group ($P<0.01$). As shown in Figure 4.6, both cathepsin B and cocktail vaccine cathepsin B/L5 vaccines induced a reduction in the length and width of recovered flukes and this leads to a reduction in liver pathology. Cathepsin B, L5 and L1g cocktail vaccine induced a similar reduction in size to that induced with the cathepsin L1g/L5 cocktail vaccine.

A **B** **C**

Figure 4.6. Morphometric dimension analysis of *F. hepatica* recovered from control and vaccinated rats bile ducts and size comparison with flukes recovered from human bile ducts.

Photographic slide **(A);** A representative large size fluke from a control rat and a smaller fluke from a protein vaccinated rat; photograph **(B)**, The serial reduction in the size of flukes from left to right were arranged to show the vaccination effect on the length and width of recovered flukes. The larger flukes (first two flukes) were extracted from Quil A adjuvant only vaccinated rats, second two recovered from cathepsin L1g rats, third two from cocktail vaccine containing cathepsin B/L1g administered rats and the last two from cathepsin B as well as cathepsin B/ L5 vaccinated rats (one fluke from each). Representative flukes are shown. Photograph **(C)** was taken from , and used here for a comparison of flukes recovered from the control rat infection with those from a case of human fasciolosis. Similar sized flukes were obtained.

Table 4.6. The mean length and width of flukes from each vaccinated and control group

Vaccine group	Mean body length and standard deviation (mm)	Mean body width and standard deviation (mm)
Control	23.6 ± 3.41	10.48 ± 3.49
Cat B	7.86 ± 2.72	4.33 ± 1.58
Cat L5	10.58± 2.15	4.82 ± 1.50
Cat L1g	12.15 ± 3.18	5.50 ± 1.19
Cat B/L5	4.00 ± 2.50	2.25 ± 1.38
Cat B/L1g	9.53 ± 3.50	5.61 ± 2.02
Cat L1g/L5	11.00 ± 5.16	5.40 ± 2.24
Cat B /L1g/ L5	11.07± 4.15	5.53 ± 1.80

All the mean values of fluke length and body width from the immunised groups were significantly lower than in the control rats (P<0.05).

4.3.4. Reduction in the wet weight of recovered flukes

Cathepsin protein immunised rats showed a significant reduction in the wet weight of recovered flukes compared to control rats (Table 4.7 and Figure 4.8). The percentage of reduction in the wet weight of flukes in the vaccinated animals ranged from 52 to 76.1 %. The wet weight of flukes recovered from groups receiving cathepsin L5 and B was also significantly lower compared to those receiving cathepsin L5 and cathepsin L1g. They were also significantly lower compared to groups receiving the combined vaccine containing cathepsin L1g and L5, as well as the cocktail of cathepsin B, L1g and L5.

Table 4.7. The mean wet weight with standard deviation of flukes from immunised rats. All protein vaccinated groups had significantly reduced wet weight compared to the control group ($P < 0.05$)

Immunised groups	Mean wet weight (mg)
Control	120.57 ± 31.1
Cat B	44.06 ± 27.8
Cat L5	50.00 ± 17.2
Cat L1g	68.65 ± 29.7
Cat B/L5	28.87 ± 18.7*
Cat B/L1g	45.06 ± 21.6
Cat L1g/L5	57.93 ± 24.8
Cat B/L1g/L5	58.15 ± 32.9

* indicates that the mean wet weights of flukes from cocktail of cathepsin BL5 vaccinated rats were significantly different to the wet weight of flukes from all other immunised and control rats ($P<0.05$).

4.3.5. Immune response

4.3.5.1. IgG antibody responses

The protein vaccinated and Quil A vaccinated rat sera IgG responses against cathepsin B, cathepsin L5 and cathepsin L1g from blood samples taken at the time of challenge and culling are presented in Figure 4.9. All the vaccines induced significantly antibody titres compared to the control group ($P<0.05$). The IgG antibody responses in vaccinated rat sera attained their peak at the time of challenge infection and then dropped at the time of necropsy.

4.3.5.1.1. Cathepsin B specific antibody responses

The cathepsin B specific IgG antibody responses were analysed in rat groups which were vaccinated with cathepsin B, including the cocktail vaccine groups. From these four vaccine formulations, vaccination with cathepsin B alone induced the highest antibody responses. The cocktails of cathepsin B/L1g and the three proteins produced the lowest anti-cat B titre. After 8 weeks of challenge infection, cathepsin B specific antibody responses decreased in vaccinated rat groups, but increased in control rats compared to the time of challenge infection.

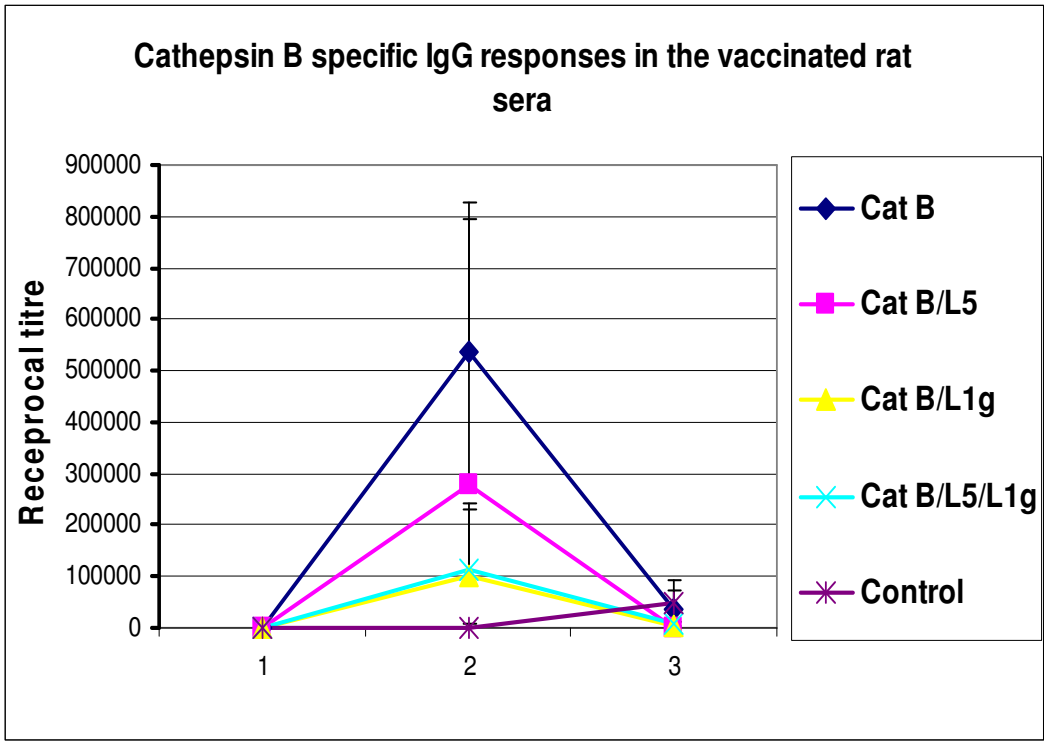
4.3.5.1.2. Cathepsin L5 specific antibody responses

Cathepsin L5 specific antibody analysis was performed for the groups that were vaccinated with cathepsin L5, including the cocktail vaccine groups. These four vaccines induced a significantly higher anti-cathepsin L5 antibody response compared to controls. Cathepsin L5 vaccination induced the highest anti-cathepsin L5 response, and the cocktail of three proteins evoked the lowest. After eight weeks of infection, antibody responses dropped. At this time, the control rats showed

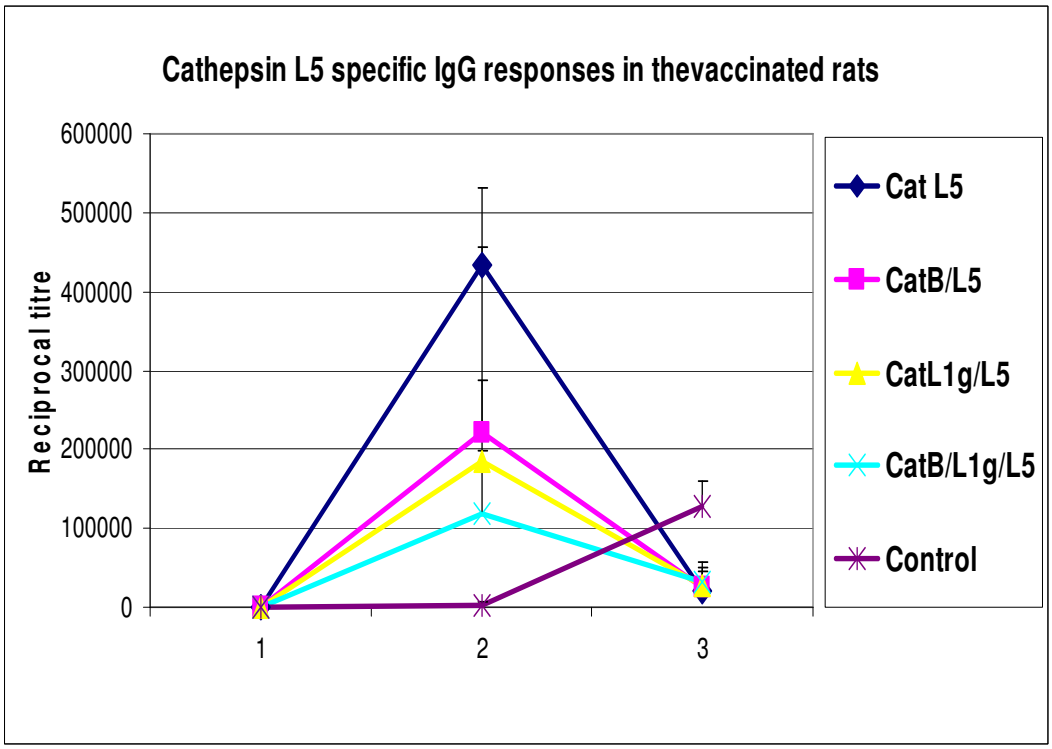
elevated anti-cathepsin L5 antibody responses compared to antibody responses of anti-cathepsin B and cathepsin L1g.

4.3.5.1.3. Cathepsin L1g specific antibody responses

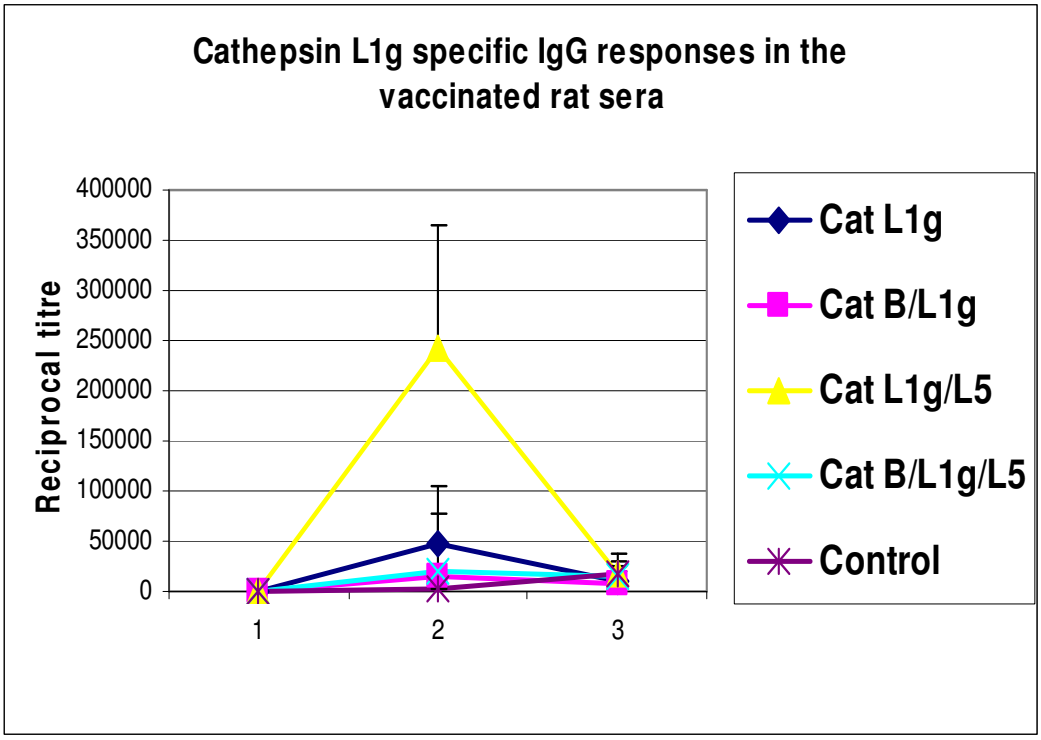
Interestingly, the cocktail of cathepsin L1g/L5 vaccine induced higher anti-cathepsin L1g responses than control rats. As shown in Figure 4.9 (C), the cocktail of cathepsin B/L1g and cocktail of cathepsin B/L5/L1g elicited low anti-cathepsin L1g antibody titres in vaccinated rats. At the time of necropsy, the cathepsin L1g specific antibody titre decreased as did anti-cathepsin B and cathepsin L5 antibody responses.



A. Cathepsin B



B. Cathepsin L5



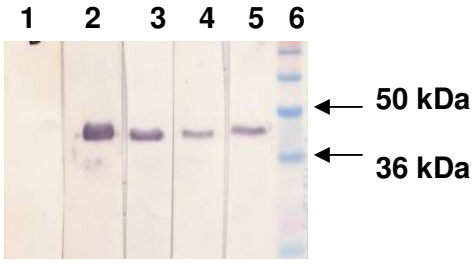
C. Cathepsin L1g

Figure 4.7. The recombinant antigen-specific IgG antibody responses in vaccinated rats. (A) cathepsin B; (B) cathepsin L5; (C) cathepsin L1g.

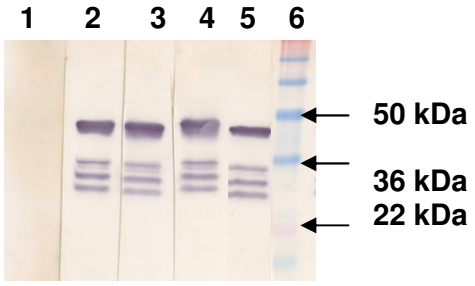
Point 1 indicates basal immunity (prior to vaccination), 2 refers to the time of challenge infection, and 3 refers to time of culling of rats.

4.3.5.2. Immunoblot analysis of vaccinated rat sera

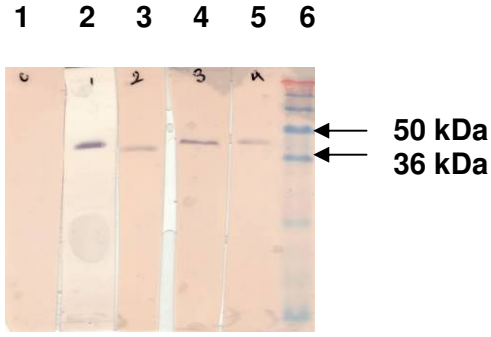
The immunoblot analyses of pooled sera from vaccinated and control rats are presented in Figure 4.8. The vaccinated rat pooled sera showed reactivity towards pro-cathepsins B and L1g. Cathepsin L5 specific rat sera showed positive reactivity to pro-cathepsin L5 and mature cathepsin L5. From Figure 4.9, (B), the four reactive bands indicate sequentially activated protease. Cathepsin L5 is autoactivated during storage and SDS-PAGE (Luke Norbury, personal communication, RMIT University, Melbourne). Quil A vaccinated rat sera showed no reactivity to these three antigens.



A



B



C

Figure 4.8. Immunoblot analysis of recombinant antigens probed against pooled sera from vaccinated rat sera.

A. Yeast expressed cathepsin B was separated by 12.5% SDS-PAGE and transferred to nitrocellulose strips. The immunoblots of cathepsin B were incubated with pooled sera from each group. Strip 1, Quil A adjuvant only; strip 2, cathepsin B; strip 3, cocktail of cathepsin B/L5; strip 4, cocktail of cathepsin B/L1g; strip 5, cocktail of cathepsin B/L5/L1g; strip 6, pre stained protein marker.

B. Yeast expressed cathepsin L5 was separated by 12.5% SDS-PAGE and transferred to nitrocellulose strips. The immunoblots of cathepsin B were incubated with pooled sera from each group. Strip 1, Quil A adjuvant only; strip 2, cathepsin L5; strip 3, cathepsin B/L5; strip 4, cocktail of cathepsin L5/L1g; strip 5, cocktail of cathepsin B/L5/L1g; strip 6, pre stained protein marker.

C. Yeast expressed cathepsin L1g was separated by 12.5% SDS-PAGE and transferred to nitrocellulose strips. The immunoblots of cathepsin B were incubated with pooled sera from each group. Strip 1, sera from Quil A adjuvant only; strip 2, cathepsin L1g; strip 3, cocktail of cathepsin B/L1g; strip 4, cocktail of cathepsin L1g/L5; strip 6, cocktail of cathepsin B /L5/L1g; strip 5, pre stained protein marker.

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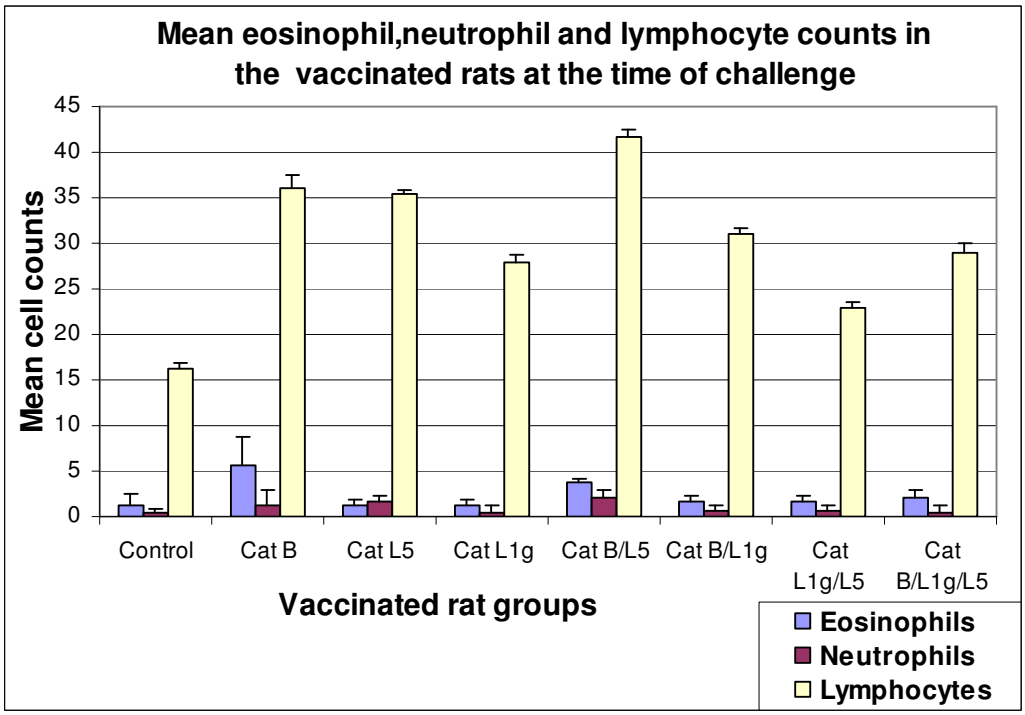
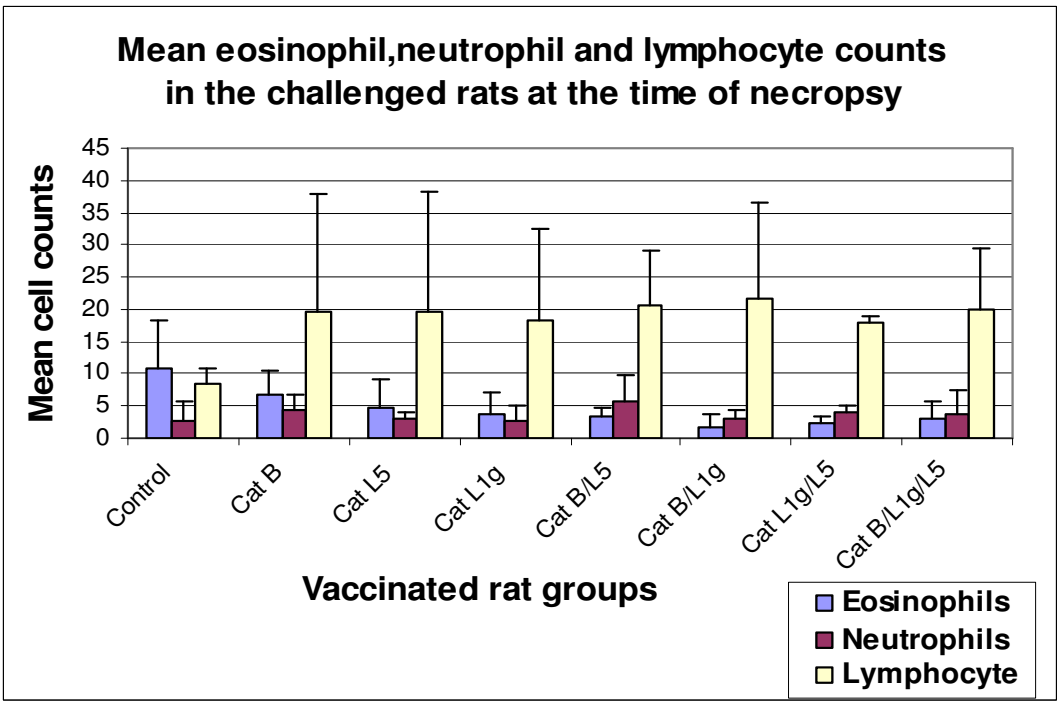
4.3.6. Peripheral leukocyte responses during vaccination and infection

Total leukocyte counts were monitored two weeks after the third vaccination and eight weeks after the challenge infection. The lymphocyte, neutrophil and eosinophil responses are shown in Figure 4.9. An increase in lymphocyte counts was observed in all vaccinated rat groups. A high eosinophil response was observed in rats vaccinated with cathepsin B and cocktail vaccine cathepsin B/L5 compared to control rats. The highest eosinophil responses were detected in the control rat group 8 weeks after challenge. High levels of neutrophil responses were observed in all cocktail vaccinated rat groups.


Mean eosinophil,neutrophil and lymphocyte counts in the vaccinated rats at the time of challenge
Mean cell counts
45
40
35
30
25
20
15
10
5
0
Control
Cat B
Cat L5
Cat L1g
Cat B/L5
Cat B/L1g
Cat L1g/L5
Cat B/L1g/L5
Vaccinated rat groups
Eosinophils
Neutrophils
Lymphocytes

A


Mean eosinophil,neutrophil and lymphocyte counts in the challenged rats at the time of necropsy
Mean cell counts
45
40
35
30
25
20
15
10
5
0
Control
Cat B
Cat L5
Cat L1g
Cat B/L5
Cat B/L1g
Cat L1g/L5
Cat B/L1g/L5
Vaccinated rat groups
Eosinophils
Neutrophils
Lymphocyte

B

Figure 4.9. Leukocyte profile of vaccinated and control rats.

A, Mean eosinophil, neutrophil and lymphocyte count of vaccinated and control groups at the time of challenge; **B,** Mean eosinophil, neutrophil and lymphocyte count of vaccinated and control groups eight weeks after challenge infection.

4.3.7. The relationship between the parasitic profiles of vaccination outcome

The number of flukes recovered from vaccinated animals is considered to be the primary parameter which determines the success of helminth vaccines . All of the vaccine preparations used in this rat trial elicit protective immune responses against experimental rat infection. To assess the relationship between various protective efficacy parameters, correlation and R square values were analysed as described by Raadsma *et al.* (2007). The following statistical analysis of anti-pathological and parasitological parameters was performed against fluke burden of each vaccinated rat using the SPSS 15.0 for Windows statistical software package. The correlation analysis of various protective efficacy parameters with fluke counts is shown in Figure 4.10.

4.3.7.1. Fluke burden versus liver damage score

The mean values of fluke recovery and mean value of hepatic lesion score showed a positive correlation for the control and each vaccinated group. The correlation between these parameters was highly significant at a level of 0.0039 and the R square value was 0.775.

4.3.7.2. Fluke burden versus wet weight of flukes

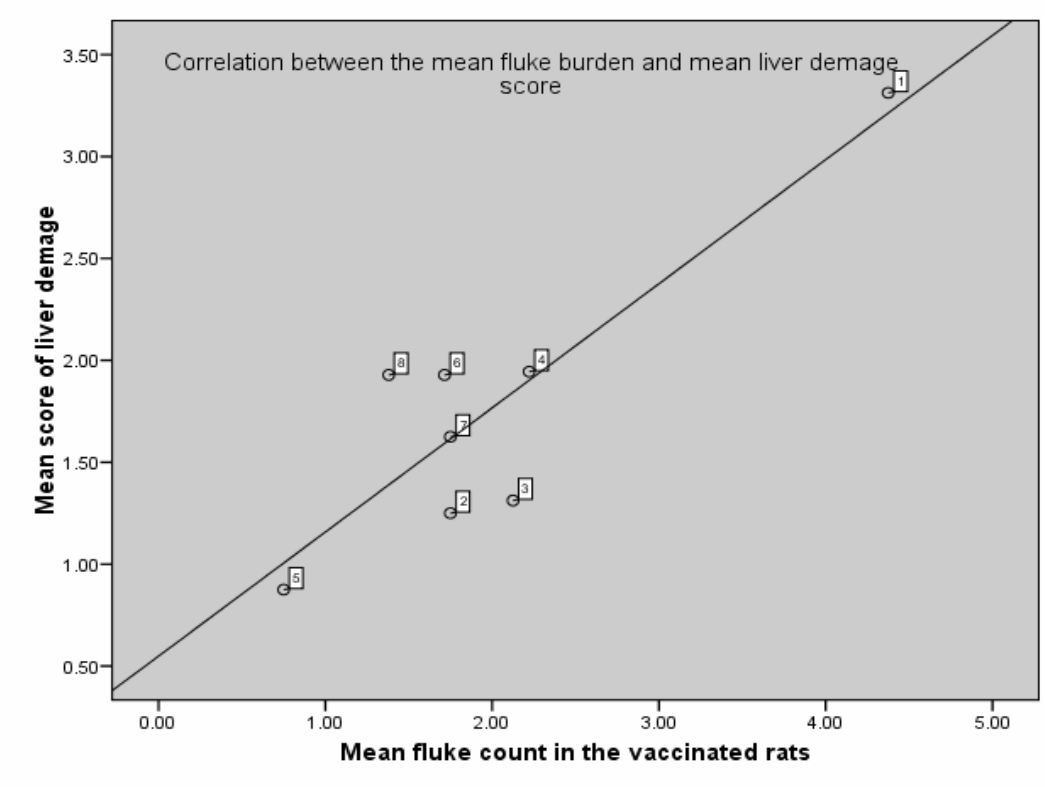
The mean value of fluke burden and wet weight of flukes was positively correlated and significant at a level of $P = 0.00031$ with an R square value of 0.901.

4.3.7.3. Fluke burden versus fluke body length and width of flukes

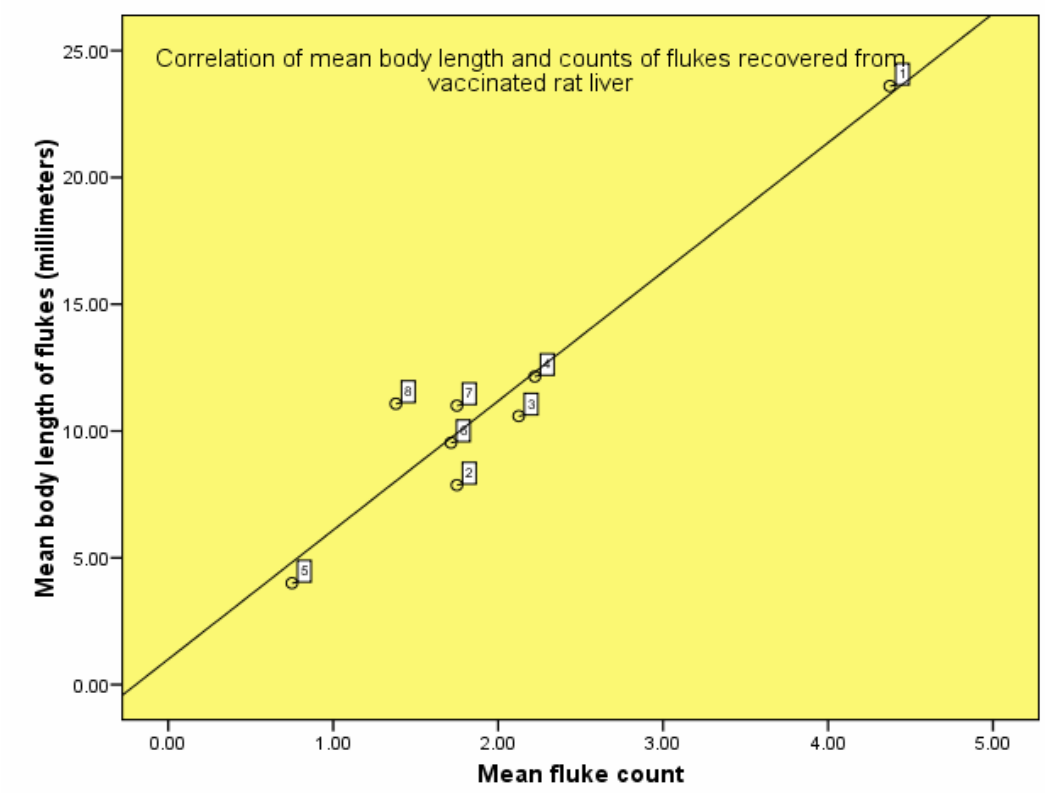
Both fluke body length and width were positively correlated with fluke recovery from the vaccinated and control groups. Both showed statistically significant correlation with fluke burden (*P* values of body length and width = 0.0001 and 0.00051, R square value = 0.923 and 0.884 respectively).

4.3.7.3. Fluke burden versus leukocyte count

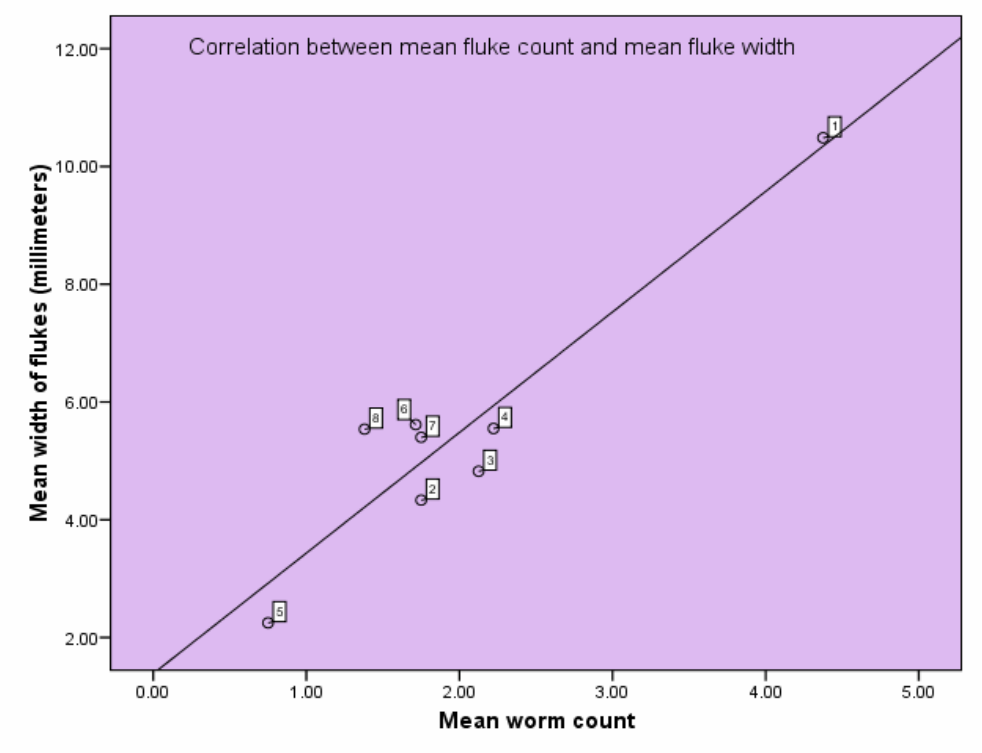
Both parameters were positively correlated (R square value =0.292).


Correlation between the mean fluke burden and mean liver demage score
Mean score of liver demage
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1.00
2.00
3.00
4.00
5.00
Mean fluke count in the vaccinated rats

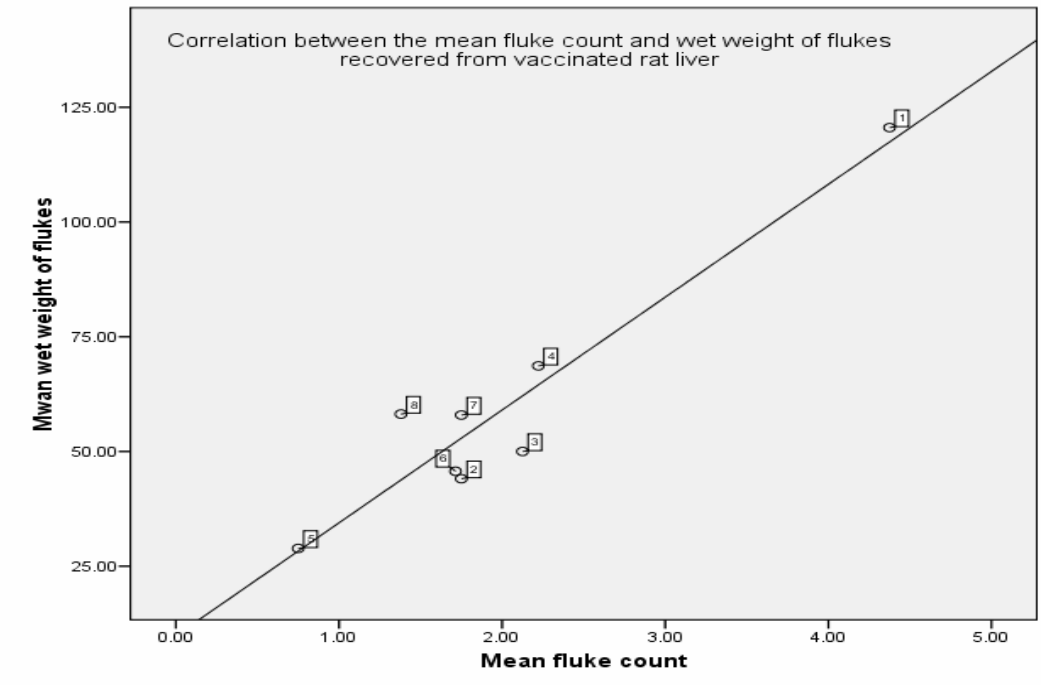
A


Correlation of mean body length and counts of flukes recovered from vaccinated rat liver
Mean body length of flukes (millimeters)
25.00
20.00
15.00
10.00
5.00
0.00
0.00
1.00
2.00
3.00
4.00
5.00
Mean fluke count

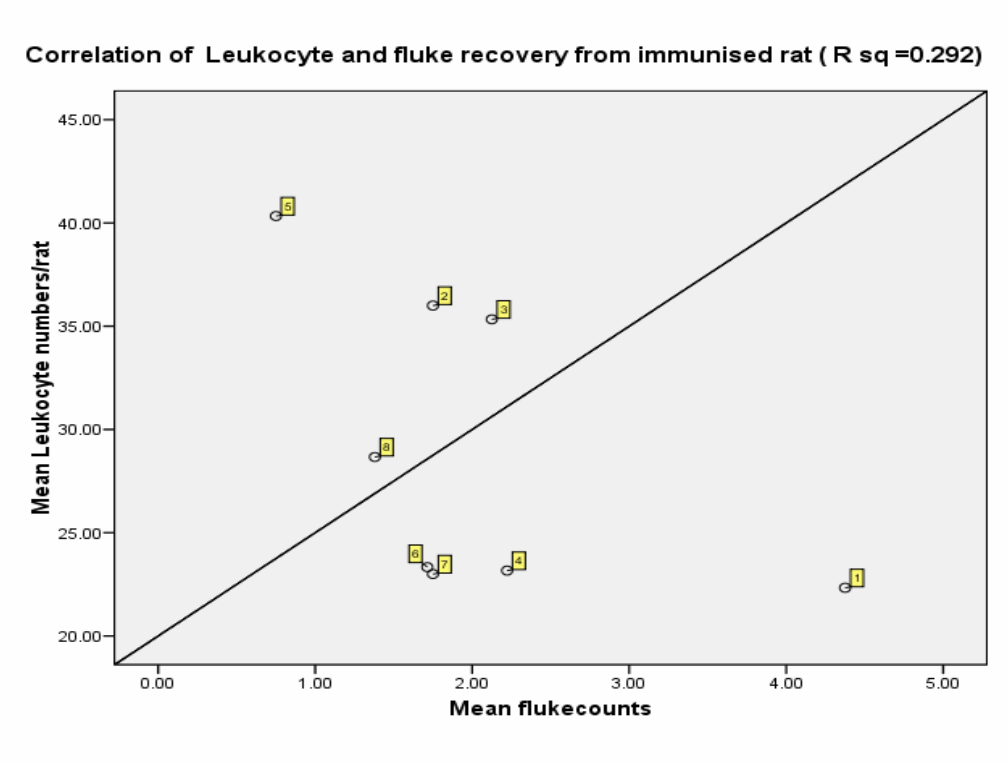
B


Correlation between mean fluke count and mean fluke width
Mean width of flukes (millimeters)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
1.00
2.00
3.00
4.00
5.00
Mean worm count

C


Correlation between the mean fluke count and wet weight of flukes recovered from vaccinated rat liver
Mwan wet weight of flukes
125.00
100.00
75.00
50.00
25.00
0.00
1.00
2.00
3.00
4.00
5.00
Mean fluke count

D



F

Figure 4.10. Correlation analysis of protective efficacy parameters of vaccination with fluke burden of vaccinated and control rats.

A. The correlation between mean fluke counts and mean liver damage score of vaccinated and control rats; **B,** The correlation between the mean fluke burden and mean body length of flukes from vaccinated and control rats; **C**, The correlation between the mean fluke numbers and mean body width of flukes from vaccinated and control rats; **D**, The correlation between the mean fluke numbers and mean wet weight of flukes from vaccinated and control rats: **F,** The correlation between the mean fluke burden and mean leukocyte count per rat.

For all graphs: 1 indicates control; 2, cathepsin B vaccinated rats, 3, cathepsin L5 vaccinated rats; 4, cathepsin L1g vaccinated rats; 5, cocktail of cathepsin B/L5; 6, cocktail of cathepsin B/L1g; 7, cocktail of cathepsin L1g/L5; 8, cocktail of cathepsin B, L1g and L5.

4.4. Discussion

4.4.1. Fluke stage specific antigens

Cathepsin proteases are involved in many essential processes in flukes, such as tissue invasion and immune depletion in the host. These proteolytic enzymes have been considered as potential vaccine candidates. Although many vaccine studies have been performed to develop a vaccine against *F. hepatica* (McManus & Dalton, 2006; Spithill *et al.*, 1997), the commercial reality has been hampered for the following reasons:

1. The identification of protective immunity-inducing antigens and large scale recombinant production from an appropriate expression host (Dalton *et al.*, 2003a; Spithill *et al.*, 1997).

2. Immune suppressive activity induced by fluke ES material in the mammalian host which leads to non-protective Th2 (immunopathological reaction) immune responses (Jefferies *et al.*, 1997; Meeusen *et al.*, 1995; Meeusen & Piedrafita, 2003).

3. Each stage in the *Fasciola* life cycle contains the same genes, but only those genes needed for that stage are expressed (Heussler & Dobbelaere, 1996; Irving *et al.*, 2003; Knox & Redmond, 2006; Meemon *et al.*, 2004). Each stage of fluke development occurs at a different location in the host and different antigens are secreted by the fluke (van Milligen *et al.*, 2000; van Milligen FJ *et al.*, 1998). Stage specific antigen expression was demonstrated by Tkalcevic *et al.* (1995).

Most vaccine research has concentrated only on target antigens of adult stage flukes (McManus & Dalton, 2006; Van Milligen *et al.*, 1999; van Milligen *et al.*, 2000). There are very few reports of juvenile fluke secreting proteins, however it is known that cathepsin B is a major component of the ES material of juvenile *F. hepatica* (Creaney *et al.*, 1996; Wilson *et al.*, 1998) and stage and tissue specific cathepsin B mRNAs were identified in *F. gigantica.*

The rationale behind the experiment described here was to test each of the three cathepsin antigens independently, and as multi-valent vaccines. It is known that at least two of these (cat B and cat L1g) are expressed in the juvenile stage and cathepsin L5 in the adult stage. Although vaccination with cathepsin L will induce antibodies that should cross-react with most cathepsin L's, including any that are expressed in the juvenile stage, the inclusion of cathepsin B allows these vaccines to induce immunity that more strongly targets the juvenile stage of the flukes.

4.4.2. Protective efficacy of yeast expressed proteins

A recombinant form of these vaccine candidates was expressed in yeast which produces these eukaryotic antigens in a form more similar to the native enzyme than can be produced by standard procedures in *E. coli* cells (Law *et al.*, 2003; Smooker *et al.*, 2000). After vaccination with these proteins, the induced anti-cathepsin L and cathepsin B antibodies are believed to reduce the proteolytic activity of *F. hepatica* ES material in the liver and bile duct (Dalton *et al.*, 1996; Mulcahy *et al.*, 1998; Mulcahy *et al.*, 1999). The results of this study have demonstrated that a cocktail of adult and juvenile fluke secreting antigens resulted in an 83% reduction in fluke burden compared with the control group. In addition, a remarkable reduction in the fluke body width and length and a significant reduction in liver pathology were

observed. Absolute maximal individual protection was observed after cathepsin B and all cocktail vaccines administered. There were 6 rats with zero flukes in protein vaccinated groups.

The juvenile *F. gigantica* somatic extract contains cathepsin B which is localised to the caecal epithelium in both metacercariae and juvenile stages . As cathepsin B and cathepsin L1g are present in the juvenile stage, responses directed to these should have their protective effects on the immature stage of flukes before they enter into the bile duct.

A recent report revealed that RNA interference of NEJ cathepsin B and cathepsin L significantly reduced transcript levels and encoded proteins in the gut. The authors of this study have highlighted that the cathepsin B and cathepsin L enzymes are essential for NEJ penetration of gut and silencing of either protein showed abnormal locomotion and reduction in penetration of rat intestine. Therefore, RNA interference with the function of either enzyme reduced the worm virulence. In these protein vaccine trials, we have used a total of 60 µg of yeast purified protein (20 µg for each injection) and we predict that these protein vaccines may induce immune responses that at least partly inhibit the flukes before they reach the hepatic parenchyma.

F. hepatica cathepsin L1 cleaves IgG antibodies which would prevent the antibody mediated attachment of eosinophils to juvenile flukes (Smith *et al*., 1993a; Smith *et al*., 1994). van Milligen *et al*. (1999) demonstrated that protection against *F. hepatica* infection in rats was highly correlated with IgG1 antibodies in serum and eosinophils in gut tissue. Among the vaccinated groups, cathepsin B and the cocktail of cathepsin B and L5 induced high eosinophil and neutrophil counts.

In addition, these two vaccine preparations induced high IgG responses. However, the antibody mediated eosinophil and neutrophil responses may have some detrimental effect on fluke growth and maturation in the vaccinated rats.

4.4.3. Rats are an ideal model for assessing the *Fasciola* infection

In human liver fluke infection, migratory and adult stage flukes correspond to two clinical stages of infection, the hepatic and biliary stages. A few millimetres in length, juvenile flukes cause severe necrotic lesions and necrotic cavities resembling tunnel-like tracts. Entry of flukes to the liver creates calcification of hepatic lobes, whereas adult flukes secreting proteolytic enzyme cause thickening of the bile duct wall and dilatation of the bile duct lumen in control rats.

The macroscopic lesions of control rats clearly mimic the human hepatic and biliary stage natural infection. In the control group, the mean body lengths were 23 mm and mean body width was 10 mm. In a human infection, the adult fluke from bile duct measures 20-40 mm in length and 8-13 mm in width. The body length and width of the flukes extracted from control rats matches with size of flukes from human bile duct. Valero *et al*. 2006 have confirmed that the rat is a highly suitable model for assessing human infection. In addition, adult fluke reside at the distal part of the bile duct resulting in dilatation of the bile duct. Therefore, Sprague Dawley rats are an ideal model for assessing the pathogenicity of human fasciolosis.

4.4.4. Juvenile, immature and adult fluke antigen vaccine efficacy

In an early cocktail vaccine study, cathepsin L2 and Haemoglobin combined vaccine showed 72.4 % protection against challenge infection . The cathepsin L5 has 80% sequence identity with cathepsin L2 (Smooker *et al*, 2000) and vaccination in rats elicits high antibody responses, 50% reduction in fluke burden and 59% reduction in wet weight of flukes compared to control rats.

F. gigantica cathepsin L1g showed moderate IgG responses compared to cathepsin B and cathepsin L5, 49% reduction in fluke counts and 43% reduction in wet weight of flukes compared to control rats. Conversely, cathepsin B vaccination in rats induced the highest antibody responses, 59% reduction in fluke counts and 63% reduction in wet weight of flukes compared to control rats. These results suggest that cathepsin B elicits better protection than either cathepsin L5 or cathepsin L1g.

The protective efficacy of cathepsin L1g was low compared to cathepsin B. *F. gigantica* shows lower infectivity than *F. hepatica* in Wister rats . Irving *et al*. (2003) demonstrated that *F. gigantica* and *F. hepatica* diverged about 19 million years ago, however, there is still significant sequence identity between orthologous proteins of the two species.

The additive effect of the three antigens (cocktails of cathepsin B, cathepsin L5 and cathepsin L1g) might have been expected to give a greater reduction in fluke counts, body length and width, but the lesser protective efficacy of this vaccine may be due to the lower amount of each protein (6.6 µg of each individual protein out of 20 µg protein/ dose). Therefore, if as these data suggest cathepsin B has the

greatest protective effect, the lower effective dose in the last group may not be sufficient for high levels of protection.

4.4.5. The effect of vaccination on pathology and parasitism

Liver damage is always associated with morbidity as well as mortality from infection in ruminants . The pathological consequences of NEJ migration lead to high mortality in sheep as well as morbidity in cattle (Martinez-Moreno *et al.*, 1997; Meeusen *et al.*, 1995). Our results show that vaccination with cathepsin B, cathepsin L5 or the multivalent cathepsin B/L5 results in less liver damage than in control rats.

Reduction in the size of flukes in vaccinated animals is a feature of protective immunity because it demonstrates a deficiency in their normal physical development . Estuningsih *et al*, (1997) tested native glutathione S-transferase, cathepsin L, paramyosin, fatty acid binding protein (FABP), and an *E. coli* expressed FABP in Brahman cattle. In cattle vaccinated with native FABP, there was a 31% reduction in fluke burden and up to 36% reduction in the average wet weight of the flukes.

In this study, the reduction in fluke size and number in vaccinated rats is reflected in a reductuion in liver pathology. It may be that a fluke vaccine does not need to induce sterile immunity, but reduce the pathology to a level that is tolerated by the animal.

4.4.6. Recombinant proteins induces leukocyte cell changes

Spithill *et al*, (1997) have hypothesized that fluke ES materials inhibit antibody-mediated attachment of eosinophils, neutrophils and macrophages to the tegument of flukes. Jefferies *et al*. (1997) reported that inhibition of superoxide release was observed in human and sheep activated neutrophils by *F. hepatica* ES material.

The ES material has superoxide dismutase activity (inhibition of release of neutrophil superoxide free radicals) and its expression was shown to be higher in *F. hepatica* than *F. gigantica* (Piedrafita *et al*., 2000; Piedrafita *et al*., 2007). Natural immunity against infection is thought to be via antibody-mediated cellular cytotoxic mechanisms. In this study, the peripheral eosinophil counts tend to increase in all protein vaccinated rats at the time of challenge and neutrophil responses tend to increase in cocktail vaccinated rats. There was increase in lymphocyte responses at time of challenge, but it starts declining at the time of necropsy. The induced protective immunity in rats may act *via* these same immunological events during this time period.

4.5. Conclusion

The results of this study showed that juvenile antigen cathepsin B induces stronger immune protection than the adult fluke antigen cathepsin L5. Cocktails of juvenile and adult stage *Fasciola* recombinant proteins elicited the highest protective immunity against experimental infection. This combination of antigens by its ability to reduce the parasite load and size in a rat model represents a step forward in the development of a vaccine against this disease and now needs to be tested in ruminants.

Chapter 5

Immunological characterisation of responses induced by DNA vaccine vectors encoding *F. hepatica* cathepsin B

5.1. Introduction

Juvenile and immature stages of *F. hepatica* produce ES material and cathepsin B is found to be a major part of this material (Tkalcevic *et al.*, 1995; Wilson *et al.*, 1998). The major action of this cathepsin B is proposed to be assisting in the excystment and migration of young flukes into host tissues (Creaney *et al.*, 1996; Tkalcevic *et al.*, 1995). Therefore, *F. hepatica* cathepsin B is considered a potential vaccine candidate against *Fasciola* infection. In the previous chapter recombinant cathepsin B was evaluated as a recombinant protein vaccine, and shown to induce protective responses in rats. Work described in this chapter evaluates a different form of delivery.

DNA immunisation is a novel technology in the field of vaccine development and DNA vaccines have been shown to activate both cellular and humoral immunity against infectious parasitic diseases (Smooker *et al.*, 2004). DNA vectors encoding antigen present major advantages over protein vaccines in terms of protein processing, safety and stability .

Many DNA vaccine constructs have been shown to induce Th1 type responses via intramuscular delivery, as inferred by IFN-γ cytokine and IgG2a antibody production in vaccinated animals. Intradermal DNA vaccination generally generates Th2 responses

with IL-4 and IgG1 production in vaccinated animals (Kennedy *et al.*, 2006; Smooker *et al.*, 1999). In order to increase the effectiveness of DNA vaccines, a number of vaccine strategies have been employed to increase the immunological potency of the antigen.

One strategy has been to use secretory vaccine vectors. For example, plasmid DNA encoding secretory and cytoplasmic *F. hepatica* glutathione S transferase , fatty acid binding protein and cathepsin L5 (Smooker *et al.*, 2001) were investigated in mouse trials, and the encoded antigens evoked greater immune (humoral) responses when in secreted form.

Another strategy involves the use of a chemokines that improves the immunogenicity of poorly immunogenic antigens by targeting them to antigen presenting cells (APCs) via chemokine receptors. MCP3 has been evaluated by cancer biologists and virology researchers as an anti-cancer cytokine and chemo-attractant of leukocytes (Ben-Baruch *et al.*, 1995; Lanzavecchia & Sallusto, 2007; Rosati *et al.*, 2005).

CTLA-4 encoding plasmids are one of the most attractive DNA vaccine vectors which attempts to target antigen to APCs in the lymph node by virtue of affinity of the protein for CD80/86 (B7-1 and B7-2) on such APCs (Boyle *et al.*, 1998; Boyle *et al.*, 1999; Drew *et al.*, 2001). Vaccination with constructs encoding CTLA-4 fusion proteins can induce strong antibody responses and provides a novel generic DNA vaccine for the development of therapies against a wide range of diseases (Huang *et al.*, 2000; Jia *et al.*, 2006; Rainczuk *et al.*, 2003). Targeting of APCs by CTLA-4 encoding ovalbumin was performed in pigs via gene gun delivery. This DNA vaccination induced elevated anti-ovalbumin- IgG, IgA, IgG1 and IgG2 antibody

responses. A large-scale DNA vaccination trial was performed with sheep to investigate whether an antigen targeted by CTLA-4 enhanced and accelerated the humoral immune response (Chaplin et al., 1999). CTLA-4 mediated targeting and CpG motifs enhance immunogenicity in DNA prime/protein boost strategy in sheep against Fasciola infection was demonstrated by Kennedy et al. (2006).

In work described in this chapter, the humoral and cellular immune responses to various DNA vaccines encoding *F. hepatica* cathepsin B protease were investigated. This panel of constructs was analysed for *in vitro* expression with COS-7 cells and *in vivo* with BALB/c mice via the intramuscular route.

5.2. Materials and Methods

5.2.1. *F. hepatica* DNA vaccines encoding cathepsin B

Cloning of cathepsin B and the construction of DNA vaccines was performed by Dr. Peter Smooker and colleagues (Law *et al*., 2003), and unpublished data. The constructs to be tested are as follows:

pVR1012 Cat B. Secretion of cat B using the native signal peptide.
pVR1020 Cat B. Secretion of cat B using the TPA signal peptide.
pMCP3 Cat B. Secretion of cat B fused to murine MCP-3.
pCTLA-4 Cat B. Secretion of cat B fused to murine CTLA4.

The coding region for procathepsin B was inserted into the DNA vaccines. The exception was for pVR1012 cat B, where the full coding region, including the native cat B signal peptide, was inserted. For fusion protein constructs, the cat B formed the C-terminal part of the fusion.

5.2.2. Giga plasmid preparation and purification of DNA vaccines for immunisation

The control plasmid VR1012 and the four DNA vectors encoding the pro-mature part of cathepsin B were purified from one litre of *E. coli* culture using an endotoxin free plasmid Giga kit (Qiagen) according to the manufacturer's instructions. Freshly transformed DH5α cells containing one of the five plasmids of interest were grown on Luria-Bertani (LB) plates supplemented with 100 µg/mL of ampicillin or 50 µg/ml of kanamycin. A single colony was inoculated into LB broth and grown at 37^0 C with vigorous shaking at 200 rpm overnight. This culture was diluted (1:1000) into one

litre of LB broth and grown for 16 hours prior to plasmid purification (see section 2.4.2.1). The purified DNA was diluted in endotoxin free 0.9% saline solution at a concentration of 1 mg/ml. Plasmid integrity was assessed by restriction enzyme digestion and agarose gel electrophoresis.

5.2.3. Analysis of COS-7 cell expression of cathepsin B from DNA vaccine vectors

In vitro expression of cathepsin B from DNA vaccines was evaluated by transfecting COS-7 cells (a kind gift from Kimberly Dynon, Melbourne University, Parkville) using the lipofectamine reagent according to the manufacturer's instructions. DMEM medium supplemented with 10% new born calf serum (NCS) was used for COS-7 cell growth. The day before transfection, cells were seeded in 6 well sterile tissue plates in 2 mL complete medium. Cells were incubated in a 5 % CO_2 humidifier at 37^0C until the cells attained 80% confluency. For each transfection, 4 µg of plasmid was added to 100 µL of DMEM medium without NCS and 20 µL of lipofectamine LTX reagent. The mixture was incubated for 5 minutes at room temperature and this was added to COS-7 cells. After incubation at 37^0C for 24 hours, one mL of complex DMEM medium with NCS (10 %) was added. After incubating for a further 48 hours at 37^0C, the cells were washed with PBS and growth media without NCS and grown for a further 24 hours. After harvesting of COS-7 cells, the supernatant was concentrated using an Amicon ultra filtration unit (cut off value 15 kDa) and 20 µL of highly concentrated supernatant and/or yeast expressed cathepsin B was used for western blotting.

5.2.4. Purification of recombinant cathepsin B from yeast cells

The expression and purification of cathepsin B from *S. cerevisiae* BJ 3505 cells proceeded according to Law *et al.* (2003), as described in Chapter 3. The purified cathepsin B was used for ELISPOT assays, ELISA, IgG avidity assays and western blotting.

5.2.5. DNA vaccination protocol

Groups of five 6-8 week old BALB/c female mice were immunised with VR1012 as the control, VR1012 cat B, VR1020 cat B, MCP3 cat B and CTLA-4 cat B as vaccines. The plasmid DNA was administrated three times at two week intervals via an intramuscular injection to the thigh. Mice received 100 µg of DNA in 100 µL of 0.9% endotoxin free saline solution (50 µL each thigh) on days 0, 14, 28. Mice were bled on days 28, 42, 56 and 70.

5.2.6. Indirect ELISA

Indirect ELISA was performed as described previously by Smooker *et al*. (2001). The sterile ELISA plates (96 well) were coated with recombinant cathepsin B at 5 µg/ mL in carbonate bicarbonate buffer pH 9.6 and incubated overnight at 4^0C (see section 2.5.2). After blocking, sera from individual mice were serially diluted (1:100), loaded onto the plates and incubated at 37^0C for 2 hrs under gentle shaking. The bound antibodies were detected using anti-mouse HRP conjugated IgG (1:3000 dilution), followed by the addition of TMB substrate. The reaction was stopped by

adding 2M sulphuric acid. Reciprocal titres were calculated as the dilution that yielded an OD_{450} absorbance of 0.2.

5.2.7. Antibody avidity assay

An antibody avidity assay was performed as described by Kang *et al*. (1998) and Rainczuk *et al*. (2003b) with the following modifications. Yeast expressed cathepsin B (5µg/mL) was used to coat 96 well plates. The sera collected from individual mice were added to all wells according to their antibody titre value (OD450 absorbance of 0.2) and incubated for one hour at 37^0C, followed by the addition of an increasing concentration of urea to 0, 1, 2, 3, 4, 5, 6 and 7 M and further incubation for 30 minutes at 37^0C. The humoral responses were assessed using anti-mouse HRP conjugate and developed as described for the ELISA above.

The relative avidity was calculated by setting the absorbance reading at OD450 at zero molar urea concentration as 100%, with the values at increasing urea concentrations expressed as a percentage of this value .

5.2.8. *In vitro* splenocyte culture

In vitro culturing of spleen cells and ELISPOT assays was performed as described previously by Bachtiar *et al*. (2003). Vaccinated mice were sacrificed at week 10 (4 weeks after the third vaccination). Spleens were extracted from two animals in each group, crushed, cells washed two times using RPMI medium and incubated in ACK lysis buffer (see section 2.5.1) for 5 minutes. Cells were washed in 1mL RPMI medium and suspended at a concentration of $1x\ 10^6$ cells/90 µL.

5.2.9. IL-4 ELISPOT assay

Methanol treated 96 well multi screen plates (Millipore) were coated with 100 µL of 5 µg/mL of anti-mouse interleukin-4 overnight, followed by washing with PBS-Tween 20, blocking with 5% skim milk in PBS for 2 hours and a further washing step with PBS-Tween 20. Splenocytes ($1X10^6$) were then added to the wells. Splenocytes were stimulated with 250 µg/mL of cathepsin B or concanavalin A. Cultures were incubated at 37^0C in a 5% CO_2 humidifier for 21 hours. Following washing with PBS, biotinylated rabbit polyclonal anti- IL-4 in PBS was added to wells and incubated at room temperature for 2 hours. After washing, strepavidin-alkaline phosphatase was added and incubated at room temperature for 1 hour. After washing three washes with PBS-Tween 20 and two washes with sterile Milli Q water, the plates were developed with ELISA substrate solution (see section 2.5.1) and spots were counted using a dissection microscope. Experiments were performed in triplicate and results were expressed as the mean number of cytokine secreting cells per 10^6 splenocytes.

5.2.10. SDS-PAGE and western blotting

COS-7 cell expression of cathepsin B and induction of antibody in mice was evaluated by western blotting. For COS-7 cell expression of cathepsin B, the cell lysates and supernatants were collected. Cell supernatants were concentrated using Amicon ultra filtration membranes. SDS-PAGE was performed (see section 2.4.4.6) and proteins were transferred to nitrocellulose membranes (see section 2.4.4.7.2). Western blots were probed with rat anti-cathepsin B antibodies (1:100) and followed by anti- rat alkaline phosphatase conjugated secondary antibodies (1:100) and reactive antibodies were visualised using western blot developing solution (see section 2.4.4.1).

For assessing the cathepsin B specific antibodies in pooled sera of vaccinated mice groups, cathepsin B was separated (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking, the membrane was probed with antisera (1:100 dilution) from all vaccinated groups and then probed with anti-mouse Alkaline phosphatase conjugated (1:2000 dilution) and then finally developed by western blot developing solution (see section 2.4.4.1).

5.2.11. Statistical analysis

For all analyses of antibody titre, antibody avidity and ELISPOT assays, mean± standard deviation was calculated and data were analysed using SPSS software.

5.3. Results

5.3.1. DNA vaccine vectors, COS-7 cells expression and vaccine purification

A diagrammatic representation of the DNA vaccine vector constructs is shown in Figure 5.1. To ensure integrity of the purified plasmid, VR1012 and VR1012 cat B were digested with *BamH* I/*Not* I, VR1020 cat B and MCP3 cat B with *BamH* I alone and CTLA-4 cat B with *Mlu* I. The restriction enzyme digestion of the panel of vectors yielded the approximately 1 kb fragment encoding cathepsin B (actual size depending on the construct) after agarose gel electrophoresis (Figure 5.2).

In order to confirm the functional expression of antigens, the VR1012, VR1012 cat B, VR1020 cat B, MCP3 cat B and CTLA-4 cat B constructs were examined for protein secretion from COS-7 cells. The five plasmid DNAs were delivered into COS-7 cell lines by liposome mediated transfection. Western blotting was used for detection of protein in the culture medium from COS-7 cells transfected with DNA vectors (Figure 5.3).

Analysis of western blots probed with cathepsin B specific rat sera revealed the secretion of proteins from COS-7 cells in each case. The recognition of 36 kDa cat B and 50 kDa fusion protein bands was observed. There was no reactivity observed when COS-7 cells were transfected with empty vector.

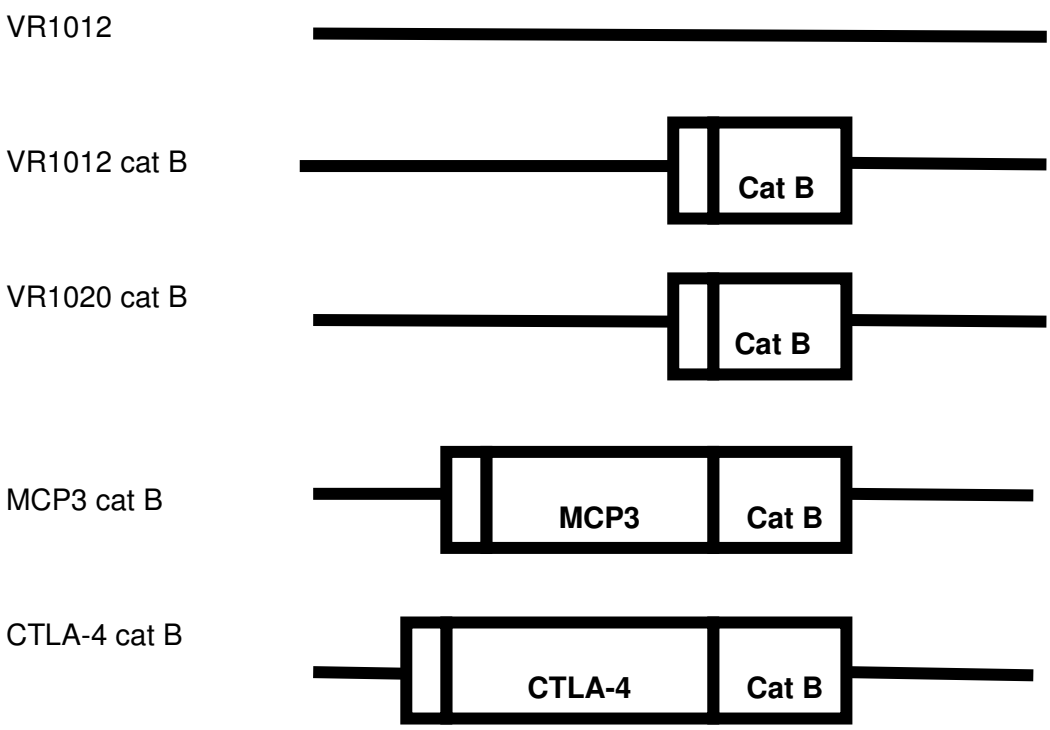


Figure 5.1. Diagrammatic representation of the DNA vaccine vector constructs VR1012, VR1012 cat B, VR1020 cat B, MCP3 cat B and CTLA-4 cat B.

The respective coding regions are detailed as rectangles (cat B) and signal peptide as small rectangles. VR1012 cat B has the native cat B signal peptide. VR1020 cat B has the TPA signal peptide encoded in the vector. MCP3 cat B and CTLA4 cat B have their own signal peptides.

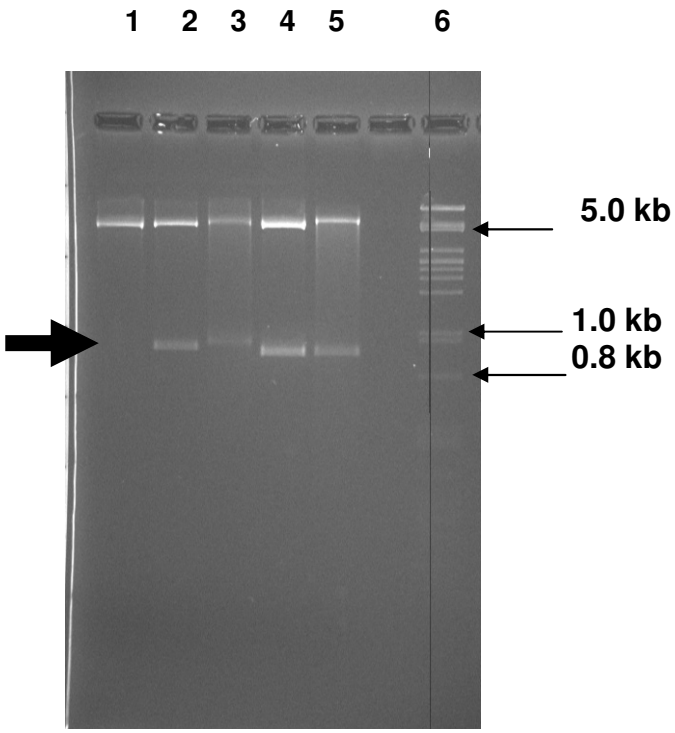


Figure 5.2. Restriction digestion analysis of purified DNA vaccine vectors encoding cathepsin B analysed by agrose gel electrophoresis.

Lane 1, *BamH* I/*Not* I digested VR1012; lane 2, *BamH* I/*Not* I digested VR1012 cat B; lane 3, *BamH* I digested VR1020 cat B; lane 4, *BamH* I digested MCP3 cat B; lane 5, *Mlu* I digested CTLA4 cat B; lane 6, *Pst* I lambda marker. The DNA fragment carrying the cathepsin B coding sequence is arrowed

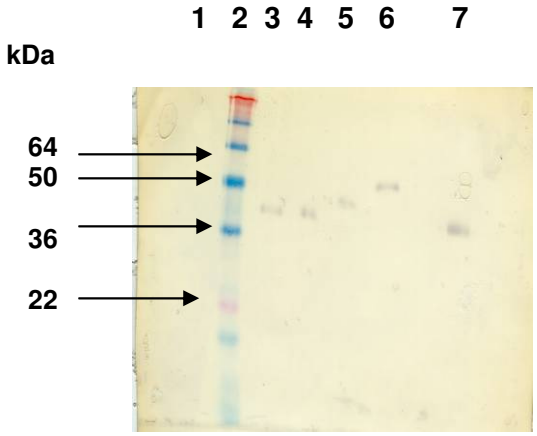


Figure 5.3. Western blot detection of encoded proteins secreted from COS-7 cells transfected with DNA vaccine vectors.

Lane 1, VR1012 transfected cell supernatant; lane 2, pre-stained protein marker; lane 3, VR1012 cat B cell supernatant; lane 4, VR1020 cat B cell supernatant; lane 5, MCP3 cat B cell supernatant; lane 6, CTLA4 cat B cell supernatant; lane 7, yeast expressed cathepsin B as a positive control.

5.3.2. Analysis of humoral immune responses

5.3.2.1. IgG responses

F. hepatica cathepsin B specific antibody titres induced after vaccination of BALB/c mice with DNA vaccines are presented in Figure 5. 4. Sera were sampled at weeks 4, 6, 8 and 10. At every time point all groups of animals vaccinated with cat B encoding constructs showed enhanced antibody titres when compared with control vectors. All four vaccines generated antibody responses by week four after the initial dose, but showed varied kinetics of the humoral response.

Mean antibody titres generated in mice immunised with the CTLA-4 targeted construct were significantly higher ($P<0.05$) than all other constructs at week 4. There was no significant difference between the mean titres of any of the 4 test groups at weeks 6 and 8. However, at week 10 the mean antibody titres in sera of mice vaccinated with CTLA-4 tagged cat B was again higher than VR1012 cat B and MCP3 cat B, but not VR1020 cat B. Therefore it appears that the CTLA-4 fusion construct generally induces titres faster than other constructs, and ultimately induced a higher overall titre. The early induction of humoral responses to CTLA-4 containing DNA vaccine constructs was previously noted in BALB/c mice (Boyle *et al.*, 1998).

5.3.2.2. Avidity of IgG responses induced by DNA vaccines encoding cathepsin B

An ELISA based IgG avidity assay was performed. The percentage of bound antibodies at increasing concentrations of urea was calculated, relative to the bound level in zero urea (figure 5.5). The gradual increase in urea concentration results in

the disruption of bound immune complexes. The rapid drop in titre observed for the VR1012 group (ie: the negative control) reflects the non-antigen specific nature of binding in this group. Although the MCP3 cat B sera showed a higher percentage of binding to antigen compared to other groups, the difference between the four vaccine groups was not significant.

5.3.2.3. Immunoblotting

Pooled serum (week 10) from all vaccinated mice within a group was used to probe yeast expressed cathepsin B by western blotting (shown in Figure 5.6). Sera from all groups vaccinated with cathepsin B constructs was able to recognise the protein. No signal was detected for control vaccine sera. In these semi-quantitative blots, MCP3 and CTLA-4 construct showed stronger reactivity than other vaccinated groups.

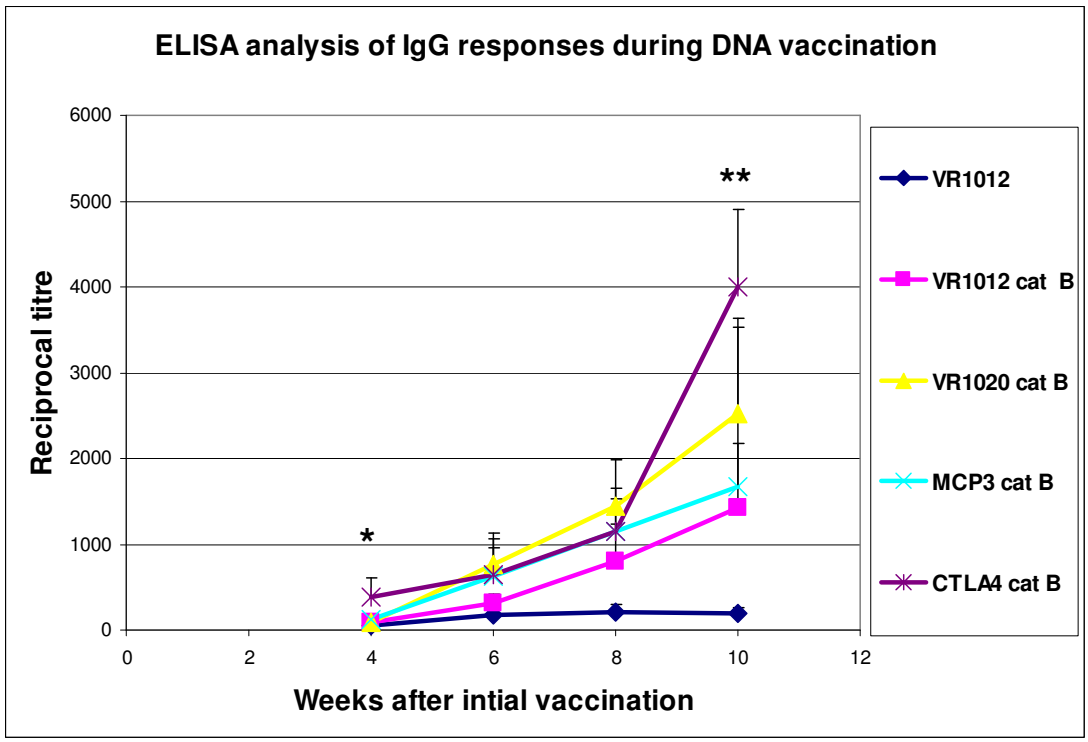
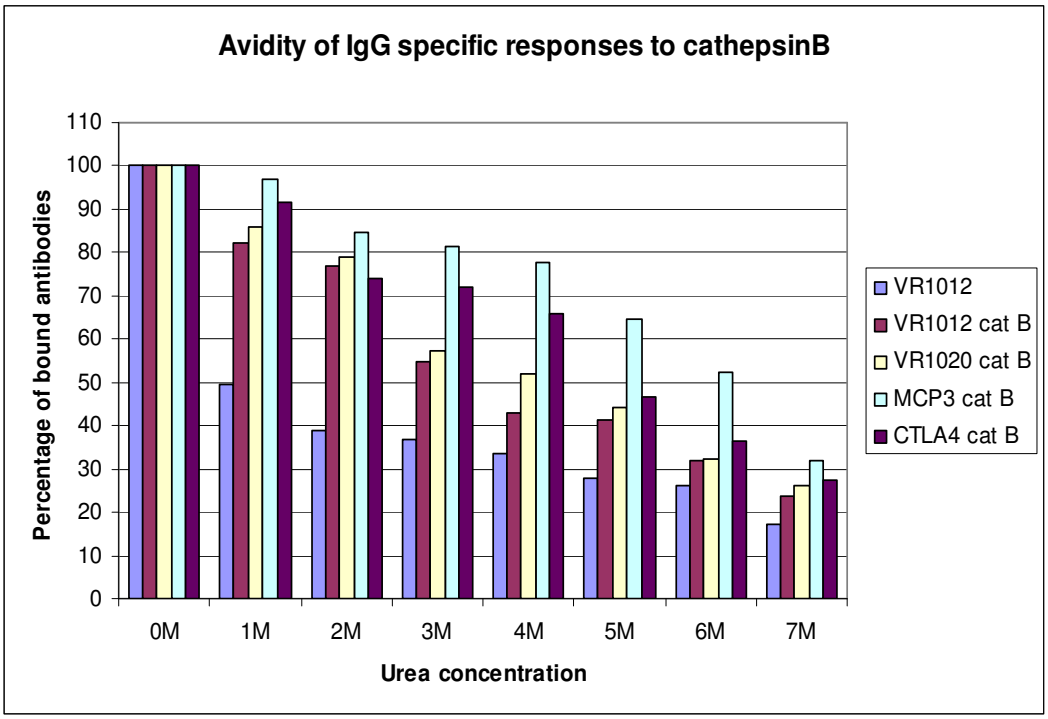
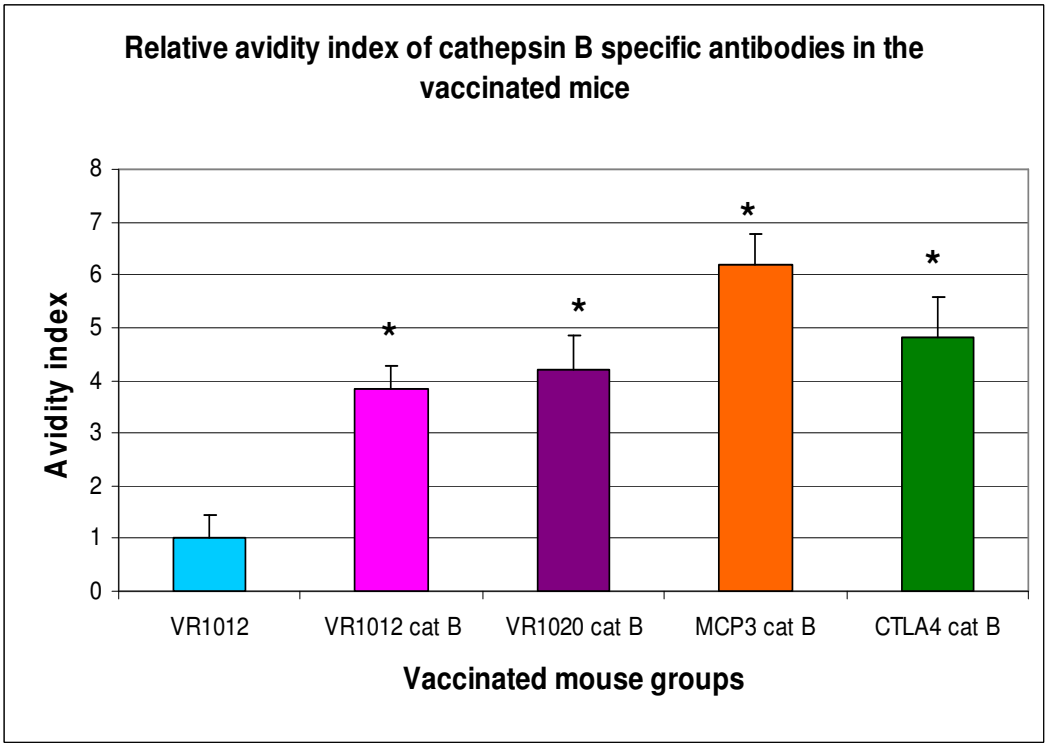


Figure 5. 4. Cathepsin B specific IgG antibody responses in vaccinated mice at bi-weekly intervals from weeks 4 to 10 after the first vaccination.

At every time point, all vaccinated groups with cat B encoding constructs showed enhanced antibody titres when compared with control vectors (P<0.05). * indicates that the mean antibody titre of CTLA-4 cat B vaccinated mice is statistically higher other vaccinate groups at week 4 (P<0.05). ** indicates that the mean antibody titre of CTLA-4 cat B vaccinated mice is statistically higher than VR1012 cat B and MCP3 cat B, but not VR1020 cat B at week 10 (P< 0.05).


Avidity of IgG specific responses to cathepsinB
Percentage of bound antibodies
110
100
90
80
70
60
50
40
30
20
10
0
0M
1M
2M
3M
4M
5M
6M
7M
Urea concentration
VR1012
VR1012 cat B
VR1020 cat B
MCP3 cat B
CTLA4 cat B

A


Relative avidity index of cathepsin B specific antibodies in the vaccinated mice
Avidity index
8
7
6
5
4
3
2
1
0
*
*
*
*
VR1012
VR1012 cat B
VR1020 cat B
MCP3 cat B
CTLA4 cat B
Vaccinated mouse groups

B

Figure 5.5. Measurement of the avidity of IgG antibody responses to cathepsin B.

(A). The percentage binding relative to 0 M urea is shown plotted against increasing urea concentration.

(**B**). Relative avidity index of anti-cathepsin B specific IgG antibodies in immunised mice**.** The mean and standard deviation of avidity index are presented here. The relative avidity index was calculated as the urea concentration required to reduce the binding percentage to 50%. All the vaccinated groups showed significantly higher indeces compared to control (* $P < 0.05$)**.**

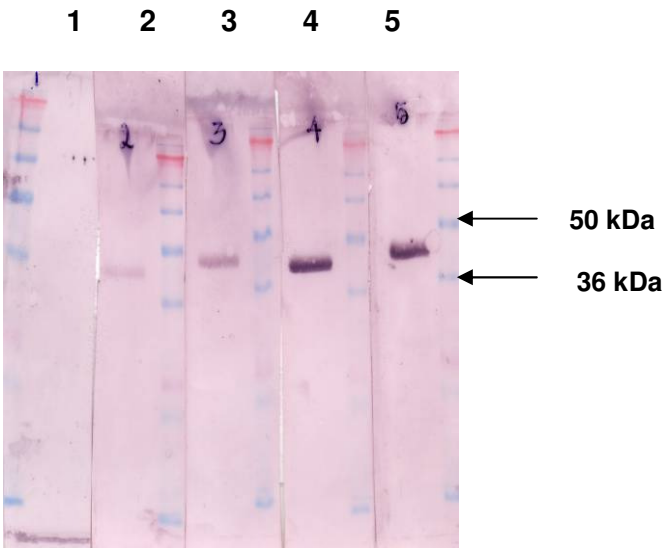


Figure 5.6. Western blot of yeast expressed cathepsin B protein probed with pooled sera of vaccinated mice.

Sera from mice vaccinated with: Strip 1, VR1012; strip 2, VR1012 cat B; strip 3, VR1020 cat B; strip 4, MCP3 cat B, strip 5, CTLA4 cat B sera. The individual strips contain See Blue pre-stained protein markers. Each individual immunoblot shows reactivity except the control DNA vaccinated sera.

5.3.3. Cellular immune responses

Cellular memory induced after cathepsin B DNA immunisation was estimated using the ELISPOT assay to measure the numbers of cathepsin B specific IL-4 cytokine secreting cells. Figure 5.8 shows the numbers of IL-4 secreting splenocytes from vaccinated mice.

Vaccination with any of the plasmids encoding cathepsin B induced highly significant IL-4 cytokine secretion ($P<0.005$) compared to the control groups. Interestingly, the native signal peptide carrying construct (VR1012 cat B) vaccinated mice produced the highest level of IL-4 cytokine production. However, there was no significant difference between the numbers enumerated from any group vaccinated with a cathepsin B encoding construct.

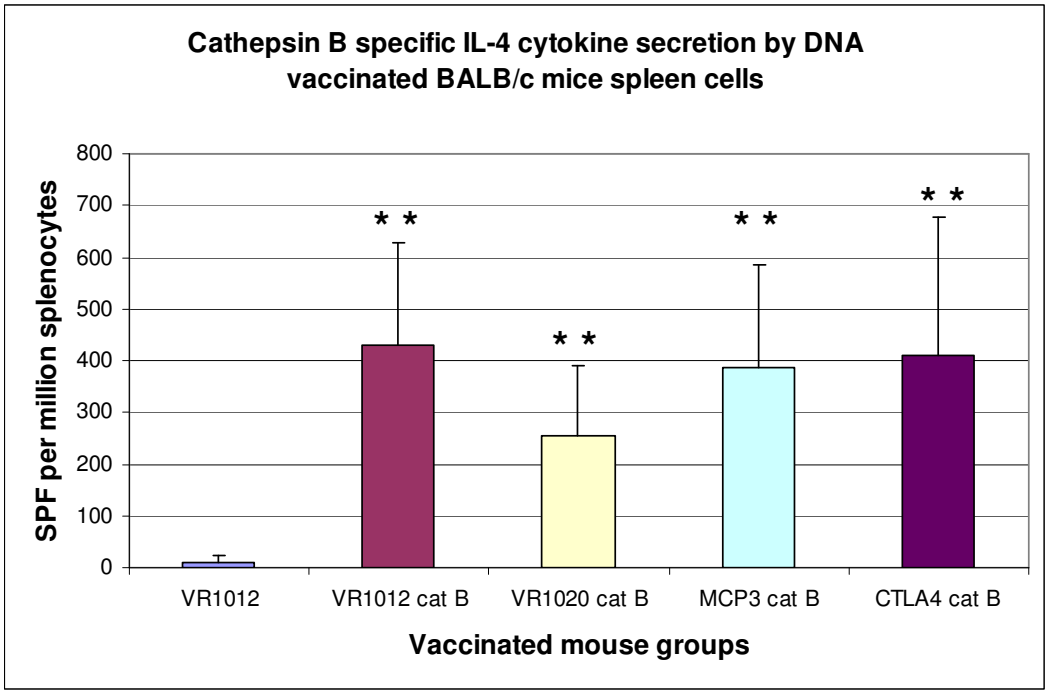


Figure 5.7. Quantitative determination of IL-4 secreting spots (ELISPOTs) in vaccinated groups (P < 0.01).**

The mean and standard deviation are presented here. Each vaccine test group yielded significantly higher numbers of IL4 secreting cells than the control (SPF - spot forming T cells).

5.4. Discussion

5.4.1. Expression of cathepsin B protein

The DNA vaccines described here encode cathepsin B in a variety of forms, however all are designed to be secreted after expression in the host, which should facilitate the induction of humoral immunity. As shown in Figure 5.3, each of the expressed proteins can be detected in the supernatant after culture of transfected COS-7 cells, demonstrating secretion. The detection of protein in the VR1012 catB medium indicates that the native cathepsin B signal peptide is able to function in these cells. This has also been previously demonstrated for another *Fasciola* protein, cathepsin L (Smooker *et al* 2001).

5.4.2. Immunogenicity of the DNA vaccines

This study demonstrates that delivery of *Fasciola* antigen via DNA vaccine vectors induces humoral and cellular responses in BALB/c mice. Each of the four DNA vaccine vectors tested elicited cathepsin B specific antibody responses as well as cell-mediated memory responses.

5.4.3. Humoral responses to cathepsin B

Humoral responses were induced by each of the four test constructs after intramuscular vaccination of mice. This was expected, as the COS-7 transfection analysis indicated that *in vivo* expressed protein should be secreted from cells and be available for the priming of B cells. The kinetics of antibody induction was different between the four constructs, with CTLA-4 cat B inducing a strong response at the earliest time point measured (4 weeks) compared to the three other

constructs. This confirms what has been seen in several studies- one of the major advantages of using CTLA-4 fusion constructs is the early induction of immune responses (Boyle *et al* 1998; Drew *et al*., 2001). Although the responses at weeks 6 and 8 were not significantly different between the test groups, at week 10 the cathepsin B antibody response in the CTLA4-targeted group had again increased relative to VR1012 cat B and MCP-3 cat B. The CTLA-4 targeting antibody efficiency mainly relies on the size and antigenicity of encoded protein. For example, a CTLA-4 construct encoding a *Taenia ovis* antigen showed 30 fold higher immune responses than non-targeted DNA vaccination.

It has been previously shown that high avidity binding of CTLA4 to its receptor requires dimerization (Boyle *et al* 1998; Drew *et al*., 2001). The highest Ab responses were obtained when CTLA4 and cathepsin B was present via both a targeting ligand and a dimerization moiety was required for enhancement. The dimerization is critical for the enhancement effect and this is probably due to the fact that dimers bind with much greater avidity than monomers.

There are a number of reports explaining the speed and magnitude of IgG antibody induction in mice vaccinated with secretory vaccine vectors (Boyle *et al*., 1997; Inchauspe *et al*., 1997; Jia *et al*., 2006; Rainczuk *et al*., 2003; Smooker *et al*., 2001). A previous report by Smooker *et al*. (2001) showed that after DNA vaccination with constructs encoding liver fluke antigens, antibody responses peaked (1/2000) at week 8 and remained high for 20 weeks. In this study, constructs encoding liver fluke FABP only induce antibodies when delivered in a form that will secrete FABP from the host cell. Antigen availability for B cell priming is an essential factor in designing DNA vaccinations for the induction of humoral responses (Boyle *et al*., 1997).

In these experiments, the MCP-3 construct did not induce increased responses over those constructs in which unfused cathepsin B was secreted. Other studies (Biragyn

et al., 1999; Rainczuk *et al.*, 2003) have shown increased responses. The reason for the failure of MCP-3 in these experiments is unknown, and is presumably related to the specific combination of antigen and chemokine that is expressed.

One way of characterising antibody responses is to estimate the antigen-specific affinity and avidity of antibodies. As an indicator of antibody avidity an IgG antibody ELISA with urea as a denaturing agent was used. The relative avidity can be calculated as the urea concentration required dissociating 50% of the antibody-antigen complex. The relative avidities are shown in Figure 5.6. This clearly shows the sharp drop in binding in the control group, which is obviously reflective of non-specifically bound antibody. One interesting finding was that the relative avidity of MCP3- cat B induced antibodies appeared higher than those induced by other vaccines, as inferred by a urea IgG ELISA (Figure 5.6). Rainczuk *et al.* (2003) tested the relative affinity of DNA vaccines (malarial MSP4-5 fused with MCP-3 or CTLA-4) and found that the avidity of both these constructs was comparable.

5.4.4. T cell responses

F. hepatica ES material is proposed to induce Th2 polarising activity during the chronic stage of natural infection (McManus & Dalton, 2006; Spithill *et al.*, 1997). Smooker *et al. (*2001) observed Th2 dominant responses after vaccination of plasmid-encoded (secreted via the native signal peptide) VR1012 cat L5 in BALB/c mice.

The induction of IL4 by fluke infection in mice has been well documented by many groups (Brady *et al.*, 1999; Cheever *et al.*, 1994; Da'Dara *et al.*, 2006; O'Neill *et al.*, 2000). As stated above, the migratory juvenile and adult liver fluke ES material elicits Th2 responses (Meeusen *et al.*, 1995; Mulcahy *et al.*, 1998). IL-4 acts as a

potent stimulus for Th2 differentiation in mice (Abbas *et al*., 2002). Each of the vaccines encoding cathepsin B tested here induced significant numbers of splenocytes secreting the IL-4 cytokine, indicating good induction of Th2-like memory responses. In *L. amazonensis* experimental infection, there is observed a low level of IFN-γ and high levels of IL-4 and also IgE antibodies. The combined effects lead to an immunopathological reaction and the development of inflammatory granulomas in the liver (Stanley & Engwerda, 2007). The secreted cathepsin B from juvenile flukes may be responsible for the formation of necrotic granulomas (immunopathological and inflammatory lesions of the liver) of hepatic lobes, due to the induction of cytokines. However, this needs to be confirmed by further studies.

Chen *et al*. (2005) has investigated the protective effect of a cocktail vaccine containing a murine IL-4-encoding cDNA and *S. japanicum* cathepsin B encoding DNA in mice. The percentage of reduction in worm burden was 43.2 % and the egg reduction rate was 76.6% over cathepsin B DNA vaccine alone. In future work, murine IL-4 DNA as an adjuvant may increase the induction of immune responses to *F. hepatica* cathepsin B in this model system.

5.5. Conclusion

Cathepsin B delivered as a DNA vaccine can be secreted from mammalian cells and induce humoral and T cell responses in mice. The results of this investigation demonstrate that DNA vaccines have potential to activate both humoral and cellular immunity against a juvenile fluke target antigen, and that fusion with CTLA-4 is a mechanism that may augment the responses somewhat.

Chapter 6

Expression and refolding of recombinant *F. gigantica* cathepsin L1g protease expressed in *E. coli*

6.1. Introduction

As has been described previously, the cathepsin L-like proteases of *Fasciola* spp. are considered as immunodiagnostic antigens and vaccine candidates (Dalton *et al.*, 2003b; Spithill *et al.*, 1997; Yamasaki *et al.*, 1992). Cathepsin L1g expression and purification is required for exploring further applications of its immunodiagnostic potential, enzyme kinetics and vaccine studies. In work described in chapter 3 of this thesis, yeast BJ3505 cells expressed *F. gigantica* cathepsin L1g protease in a soluble form, but the yield was low.

E. coli is still the most widely used expression host because of ease of handling and low cost of production, despite limitations such as the lack of ability to perform post-translational modifications and over-expression into insoluble aggregates . Yamasaki *et al*. (2002) have expressed *F. gigantica* procathepsin L and pro-peptide deleted cathepsin L in *E. coli* and inferred that the pro-region of the protease is essential for refolding of functional protease. Kesik *et al*. (2007) expressed a cysteine protease as inclusion bodies in *E. coli* and this protein was used for enteral vaccination of rats against fasciolosis. The major problem associated with expressing *Fasciola* cathepsins in bacteria is the inability to correctly form disulphide bonds and hence refolding is necessary. For large-scale production of the *F. gigantica* procathepsin L that we have expressed in yeast, bacterial expression is desirable.

In vitro refolding conditions using fractional factorial screens have been successfully used to improve the yield of soluble human pro-cathepsin to support crystallographic efforts (D'Alessio *et al.*, 1999; Kramer *et al.*, 2007; Tobbell *et al.*, 2002; Willis *et al.*, 2005). In this chapter, an efficient procedure to obtain relatively high quantities of functionally active *F. gigantica* cathepsin L1g from *E. coli* BL21 cells was investigated.

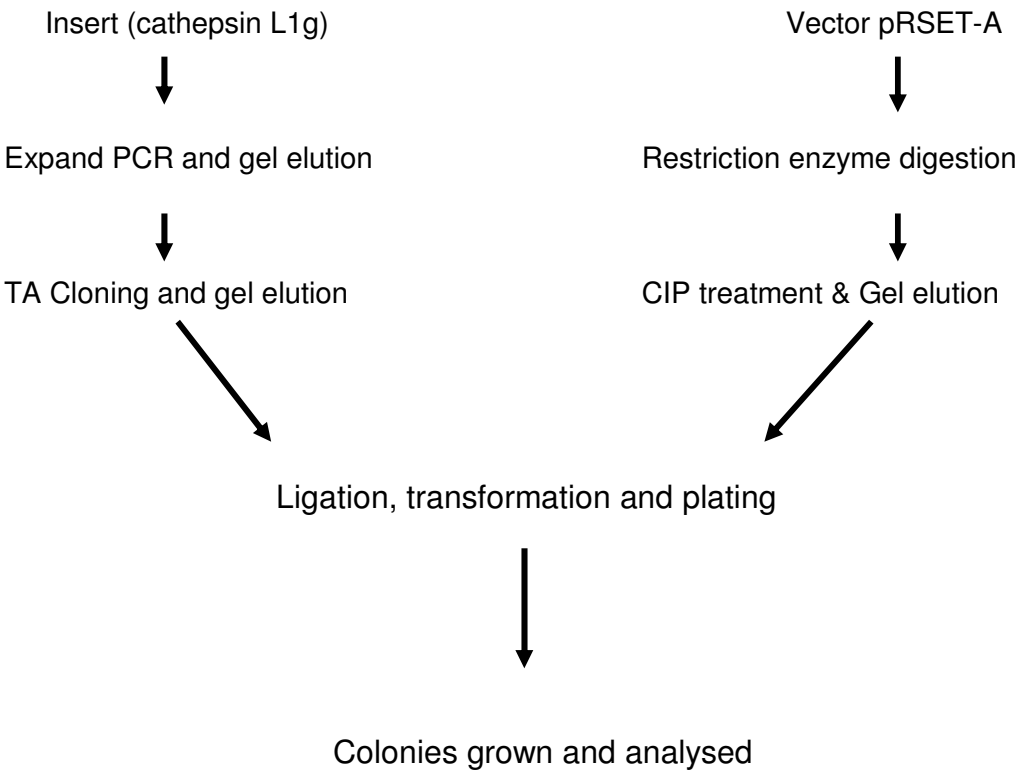
6.2. Materials and Methods

6.2.1 Cloning and construction of plasmid pRSET-A catL1g

6.2.1.1. Expand PCR

The 945 bp fragment containing cDNA of *F. gigantica* cathepsin L1g cloned into pGEMT was a kind gift from Dr. Rudi Grams (Thamasaat University, Thailand) and was used as a template for the construction of the expression vector. The cloning procedure was presented Figure 4.1. The coding region of cathepsin L1g protease was amplified by the expand polymerase chain reaction (Expand-long template PCR) according to the procedure given below. The oligonucleotide primer F_1-(5'-ATA GGA TCC AAC GAT GAT TTG CAT GAA TGG AAG CGA-3') and primer R_1–(5'-ATA GGA TCC TCA CGG AAA TCG TGC CAC CAT-3') were synthesised by Gene Works, Melbourne, Australia. Oligonucleotide F_1 was used as the upstream primer that introduced a *BamH* I restriction site (underlined above). Oligonucleotide R_1 used as the downstream primer that also added a *BamH* I restriction site to the amplified DNA.

Figure 6.1. Overview of the cloning procedure



The expand PCR programme for amplification of the cathepsin L1g DNA segment was as follows:

(Procedure adopted from Roche diagnostics)

Number of cycles		Temperature	Duration
1x	(denaturation)	93^0C	2 minutes
10x	(denaturation)	93^0C	10 seconds
	(annealing)	50^0C	30 seconds
	(elongation)	68^0C	7 minutes
15x	(denaturation)	93^0C	10 seconds
	(annealing)	53^0C	30 seconds
	(elongation)	68^0C	7 min+ cycle elongation for 20s for each cycle
1x	(extension)	68^0C	10 minutes

The 50 µL expand PCR reaction volume has

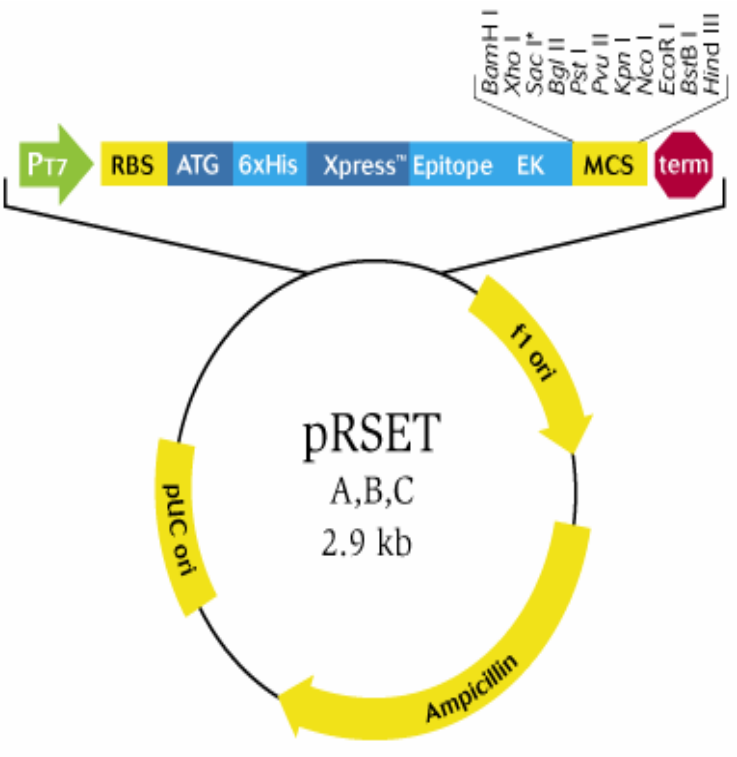
Template (cathepsin L1g) DNA	x µL (up to 300 ng)
Forward primer (up to 300 nM)	3 µL
Reverse primer (up to 300 nm)	3 µL
dNTPs	0.2 mM
Expand buffer II	5 µL
Expand polymerase	0.75 µL

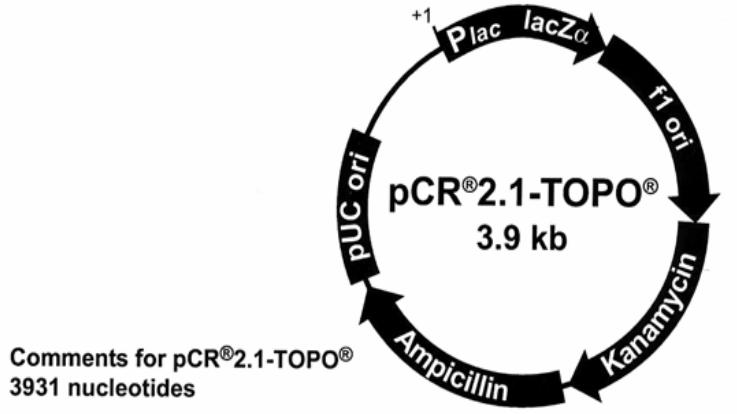
6.2.1.2. TA cloning

The PCR product was electrophoresed (0.8% agarose gel) and the band was eluted using the QIA quick gel extraction kit. Following quantification, the purified PCR product (945 bp) was ligated into the pCR 2.1 (Figure 6.2) vector using T_4 DNA ligase. The reaction was allowed to proceed overnight at 16^0 C (see section 2.4.3.2)**.** The ligated product was used for transformation of *E. coli* DH5α competent cells. Transformed cells were grown on Luria-bertani (LB) medium plates containing 100 µg/ml ampicillin, IPTG and X-gal, to allow blue/white colony screening (see section 2.4.3.3.2). Plasmid DNA was purified from white colonies. Successful ligation was confirmed by restriction digestion with *BamH* I and agarose gel electrophoresis.

6.2.1.3. pRSET- A cloning

To insert the *F. gigantica* cathepsin L1g fragment into the multiple cloning site (MCS) of the pRSET-A vector, the plasmid was digested with *BamH* I, dephosphorylated with calf intestinal phosphatase (refer 2.4.3.1), then ligated to *BamH* I digested, gel eluted cathepsin L1g insert from pCR 2.1. The resulting construct encodes pRSET-A catL1g that includes the coding region for the pro-mature region of the protease. Plasmid DNA was transformed into *E. coli* strain BL21 (DE3) pLysS competent cells.





Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Figure 6.2. Schematic map of vector pRSET and pCR 2.1 (the picture adopted from: https://catalog.invitrogen.com/productImages/2100/2042_thumb.GIF and http://www.mtsu.edu/~rseipelt/web4460c/12224020.jpg).

6.2.1.4. DNA sequence analysis and confirmation of correct orientation of pRSET-A cat L1g

Correct orientation of the insert was confirmed by restriction digestion with *EcoR* I and agarose gel electrophoresis. DNA sequencing was performed to confirm the integrity of the construct. The double stranded plasmid DNA was sequenced by the Big dye terminator cycle sequencing analysis system (Monash University, Clayton, Australia) (see section2.4.3.3.3). Sequencing results were analysed using clone manager software (SE Central).

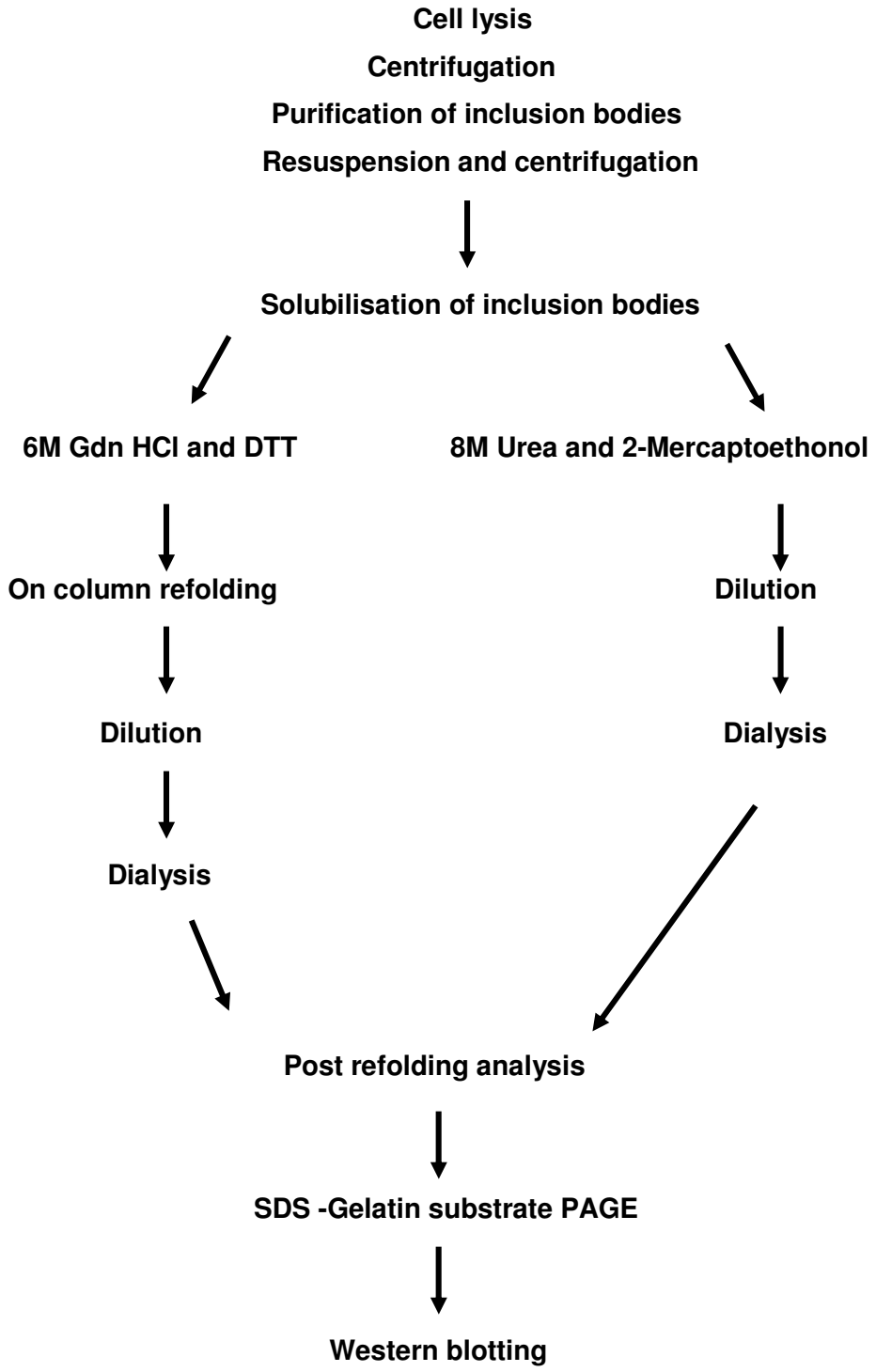
6.2.2. Cathepsin L1g protease expression and refolding

Two pathways were taken to assess the purification and re-folding of the protease (Figure 6.3)

6.2.2.1. Expression of Cathepsin L1g and collection of inclusion bodies

Transformed *E. coli* BL21 (DE3) pLysS was grown in one litre of LB broth supplemented with ampicillin (100 µg/mL) at 37^0C with 200 rpm continuous shaking. The bacterial culture was grown until the absorbance at 600 nm reached 0.4 and was followed by induction with 1 mM IPTG. Hourly expression was assessed by SDS-PAGE. The culture was harvested by centrifugation at 15,000 g for 15 minutes at 4^0C. The supernatant was discarded and the pellet was stored at -80^0C overnight.

Figure 6.3. Schematic outline for purification and refolding of cathepsin L1g protease



6.2.2.2. Bacterial cell lysis, washing and harvesting of inclusion bodies

Cell pellets were resuspended in 10 ml of lysis buffer (see section 2.4.4.4) and incubated for 30 minutes at room temperature with gentle shaking. The cell lysate was disrupted by several sequential freeze/thaw cycles. Before the final cycle, 5 units/ml of DNase I was added to the lysate, which was then centrifuged at 15,000 g for 30 minutes at 4^0C in a BECKMAN high speed centrifuge to collect the insoluble pellet and resultant supernatant was discarded. The remaining aggregated pellet was rinsed two times in PBS pH 8.0 containing low concentrations of the non-ionic detergent Triton X -100 (1%).

6.2.2.3. Solubilisation of inclusion bodies

Once collected, the inclusion bodies were solubilised in one of two buffers. The first was the inclusion body solubilisation buffer, containing urea (see section 2.4.4.4) (Cabrita & Bottomley, 2004; Middelberg, 2002). One gram of wet pellet was dissolved in 10 ml of solubilisation buffer. Urea-solubilised inclusion bodies were stirred for 2 hours at room temperature and centrifuged at 15,000g for 30 minutes at 4^0C. The supernatant was syringed through a 0.45 µm PVDF sterile filter membrane and stored at -20^0C. The second buffer was guanidine HCl (Section 2.4.4.4). Guanidine solubilised inclusion bodies were incubated for 2 hours under shaking at 4^0C and then centrifuged, filtered and stored at -20^0C as above. Further processing of the resolubilised inclusion bodies was then undertaken, either by first utilising affinity chromatography, followed by dilution/dialysis, or directly to the

dilution/dialysis step. The two methods were tested as it was unknown how pure the inclusion bodies are, so it was hypothesized that an affinity step may be required.

6.2.2.4. Step-wise urea gradient chromatography of urea solubilised cathepsin L1g

Step-wise urea gradient Ni^{2+}- NTA chromatography was performed as described by Cabrita and Bottomley (2004) with some modifications. The urea solubilised protease was mixed with Ni-NTA resin by gentle shaking for 60 minutes at room temperature (Batch chromatography mode). This mixture was then applied to a disposable column and the flow-thorough was collected for SDS-PAGE analysis. Urea was removed slowly at room temperature using a 10 ml urea gradient buffer (see section 2.4.4.4). The final wash containing no urea was supplemented with 50 mM imidazole. Finally, the bound protease was eluted with 400 mM imidazole. Eluted fractions were collected in 1.5 ml eppendorf tubes and stored at -80^{0}C.

6.2.2.5 Dilution and dialysis

Dilution and dialysis was performed based on published methods for human pro-cathepsin L (D'Alessio *et al.*, 1999; Kramer *et al.*, 2007) and liver fluke cathepsin L (Kesik *et al.*, 2007; Yamasaki *et al.*, 2002). Eluted fractions of affinity purified protease or Guanidine hydrochloride solubilised cathepsin L1g protease were slowly added drop wise (100 µl/minute) into 100-fold renaturation buffer (see section 2.4.4.4) and stirred for 24 h at 4^{0}C. The diluted resuspension was concentrated 100-fold by a VIVAFLOW 50 tangential ultra filter membrane. The concentrated refolded proteins were again dialysed against 50 mM Na_2HPO_4, pH 8.0 containing 0.5 M NaCl. The dialysed proteins were desalted by an Amicon ultra centrifugal filter (10

kDa), centrifuged at 15,000 g for 30 minutes at 4^0C and the supernatant was stored at -80^0C.

6.2.2.6. Protease concentration assay

Cathepsin L1g concentration was estimated using the Bradford method, Coommassie Blue G 250 was used as the dye reagent to measure the absorbance at 600 nm. Bovine serum albumin (BSA) was used to create the standard curve (see section 2.4.4.2).

6.2.2.7. SDS-PAGE analysis

The expressed, column purified and refolded proteases were analysed by 12.5% SDS-PAGE. The recombinant protease was resuspended in SDS-PAGE sample buffer. Gel electrophoresis was performed in Mini-protean Electrophoresis cell (see section 2.4.4.6**).** The gels were stained with Coomassie Brilliant Blue R 250 and destained using 10% acetic acid and 10 % ethanol.

6.2.3. Substrate (Gelatin) gel electrophorsis for protease activity analysis

Cathepsin L1g proteolytic activity was determined as described by Kesik *et al*. (2007) and Naggie and Bentley (1998). The refolded proteases were loaded onto 12.5% SDS-PAGE with a separating gel that contained 0.5% gelatin (see section 2.4.4.6).

6.2.4 Anti-His and anti-cathepsin L western blot analysis

Recombinant cathepsin L1g protease was separated on a 12.5% SDS-PAGE gel and transferred to a nitrocellulose membrane (see section 2.4.4.7.2). The membrane was incubated with anti-His monoclonal antibody, or anti-cathepsin L antisera (1:4000 and 1:2000 dilution respectively) overnight at 4^0C, washed three times in TBS, incubated with anti-mouse alkaline phosphatase (1:4000 and 1:2000 dilution) and visualised using western blot developing solution containing the NBT/BCIP substrate.

6.3 Results

6.3.1 Plasmid construction

The expand long template PCR protocol was followed. Primer F_1 contained sequences for a *BamH* I site and first eight codons of the cathepsin L1g cDNA fragment (starting from the pro-sequence). Primer R_1 contained the sequence for a *BamH* I site and the last seven codons of cathpsin L1g, including the stop codon. The expand PCR produced a single amplified 945bp DNA fragment as shown in Figure 6.3. After ligation sequentially into pCR2.1 and pRSET-A, clones were analysed by restriction digestion. The restriction enzyme *BamH* I digestion of miniprep plasmid purification products of pCR 2.1 and pRSET-A cathepsin L1g gave an approximately 945 bp fragment (shown in Figure 6.4 and 6.5).

Two miniprep products of pRSET-A cat L1g (pRSET-A cat L1g-8 and pRSET-A cat L1g-14) showed the correct orientation after digestion with *EcoR* I (Figure 6.6). During the pilot-scale expression of these two positive constructs in BL 21 cells, pRSET-A cat L1g-14 showed high expression and yield, whereas the other clone did not. The nucleotide sequence of the first clone was obtained and found to agree with that previously obtained from this clone (Luke Norbury, personal communication). All further expression work was performed with this clone.

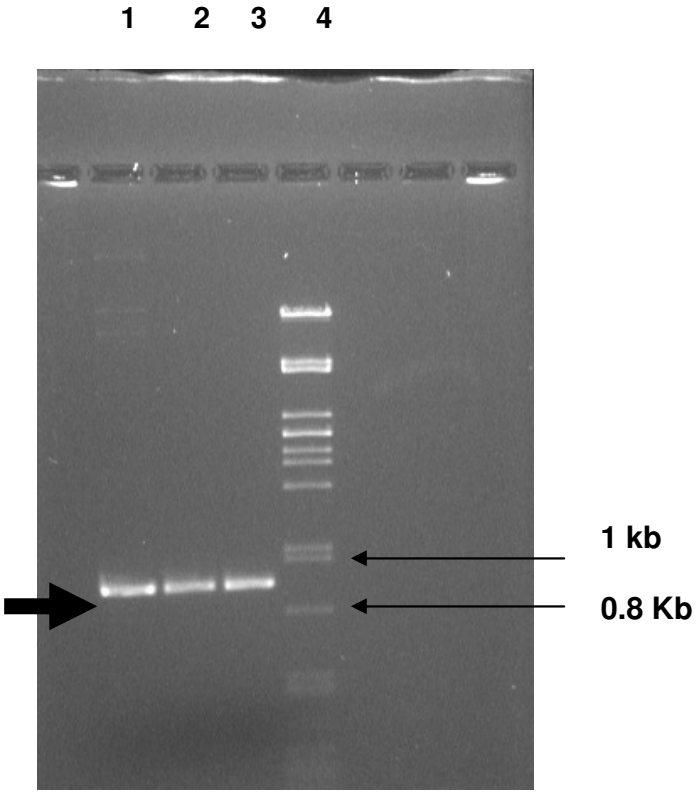


Figure 6.4. Expand PCR products amplified from cDNA encoding cathepsin L1g analysed in an agarose gel.

The 945 bp cathepsin L1g amplified fragment is indicated by the bold arrow in lane 1, 2 and 3; lane 4, *Pst* I lambda marker. This amplified fragment was eluted and utilized further for TA cloning.

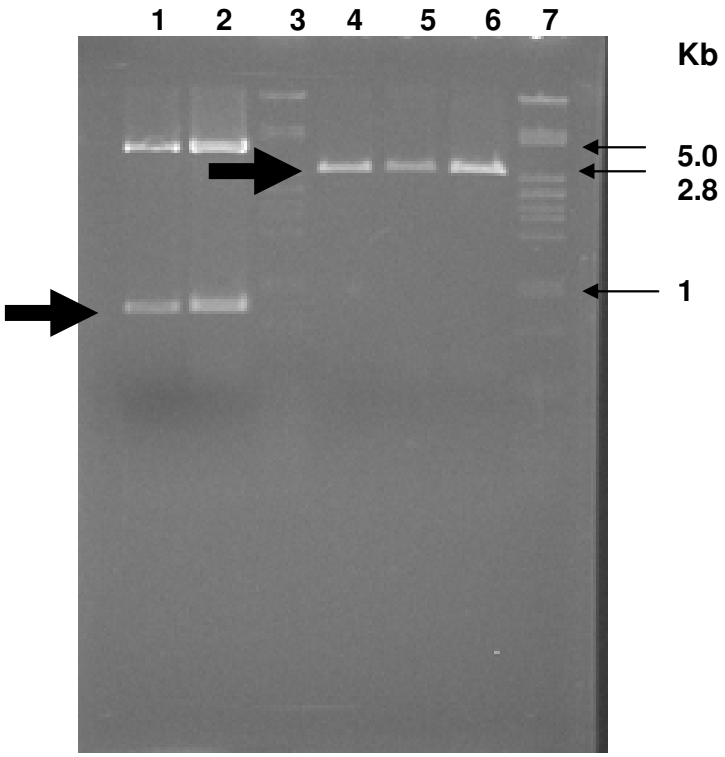


Figure 6.5. DNA gel electrophoresis of *BamH* I digested pCR2.1 (3.9 kb) miniprep products and CIP treated pRSET-A vector.

Lane 1 and 2, *BamH* I digested miniprep products of the pCR2.1 vector (indicated by the bold arrow) containing the cathepsin L1g coding sequence (indicated by the bold arrow); lane 3 and lane 7, *Pst* I lambda marker; lane 4, lane 5 and lane 6 *BamH I* digested and CIP treated pRSET-A (2.8 kb size band-referred in bold arrow).

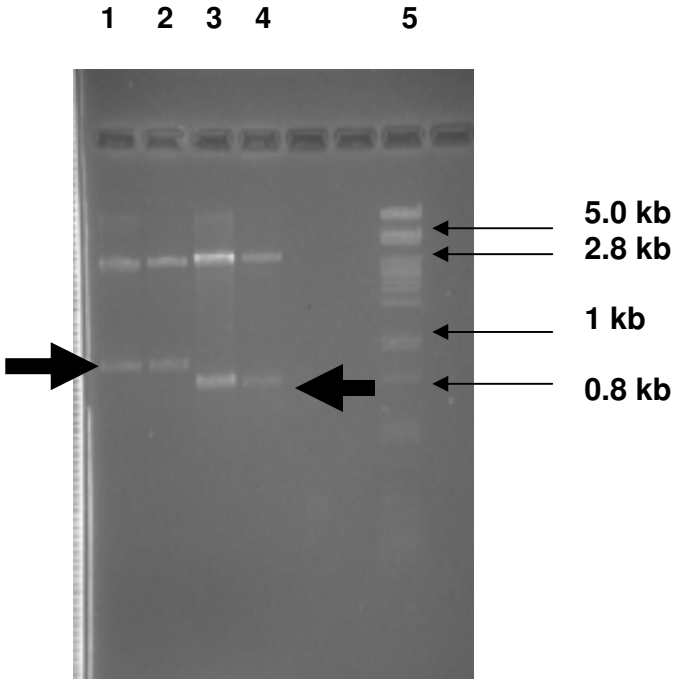


Figure 6.6. DNA gel electrophoresis of *BamH* I and *EcoR* I digested pRSET-A containing *F. gigantica* cathepsin L1g.

Lane 1 and lane 2, *BamH* I digestion of pRSET-A containing *F. gigantica* cathepsin L1g; lane 3 and lane 4, *EcoR* I digestion of pRSET-A containing *F. gigantica* cathepsin L1g; lane 5, *Pst* I lambda marker.

6.3.2 Expression, inclusion body preparation and solubilisation

The recombinant protease was expressed well in *E. coli*. The bacterial cells from an induced culture were lysed under non-denaturing conditions. After removal of cell debris by centrifugation, the protein was analysed by SDS-PAGE (Figure 6.7). The best yield was attained when cells were harvested 3 hours after induction with 1 mM IPTG. Recombinant protein showed an approximate molecular weight of 40 k Da, which corresponds to the size of *F. gigantica* cathepsin L1g pro-protein with the N-terminal extension. After purification, cathepsin L1g protease concentration was 8.4 mg/litre from the urea solubilised inclusion bodies and 5.6 mg/litre from the guanidine hydrochloride solubilised inclusion bodies.

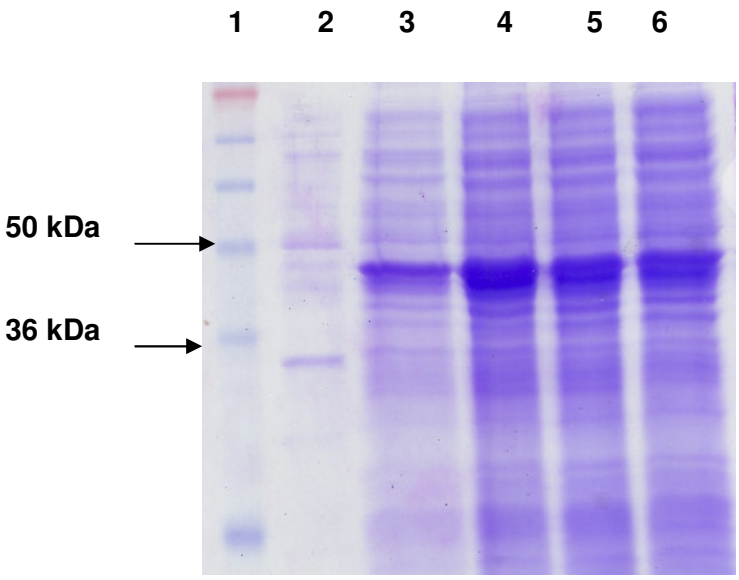


Figure 6.7. SDS-PAGE analysis of expressed recombinant proteins from *E. coli* BL21 (DE3) cultures.

After induction, 1 mL aliquots of culture were taken at hourly intervals. Cells were collected by centrifugation and resuspended in phosphate buffer (see section 2.4.4.4). 20 µ L of protein solution was mixed with SDS sample buffer and the samples electrophoresed, after which the gel was stained with Coomassie blue. Lane 1, marker; lane 2, cell lysate before IPTG induction; lanes 3- 6, lysates from 1 to 4 hours post-induction.

6.3.3 Ni- NTA column purification of urea solubilised cathepsin L1g

Immobilised metal affinity chromatography (IMAC) was applied for the purification of cathepsin L1g protease that had been solubilised with urea. Denatured cell lysate was incubated with Ni-NTA resin slurry to allow binding of $(His)_6$-cathepsin L1g. In order to remove urea from this protease, the column-loaded mixture was washed with decreasing concentrations of urea. The final wash (no urea) was supplemented with 50 mM imidazole to displace contaminant proteins that are loosely bound to Ni-NTA agarose and protein was eluted with 400 mM of imidazole (Figure 6.8). The proteinase yield was assessed by Bradford assay and was 3.8 mg / litre of culture. The eluted fractions had no digestive activity with gelatin gel, indicating that despite the removal of urea from the protease, it was not refolded into an active conformation (not shown). These results suggested it was necessary to proceed to dilution and dialysis.

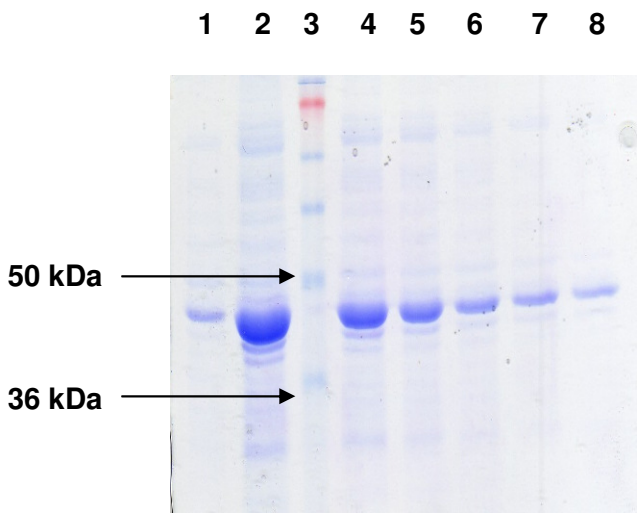


Figure 6.8. SDS-PAGE analysis of fractions of protein samples eluted with 400 mM from urea gradient Ni-NTA column chromatography.

20 µL of eluted fractions were mixed with SDS sample buffer and run on 12.5% SDS-PAGE (Coomassie stained). Lane 1-2, 4-8, fractions 1-7. Lane 3, See blue pre-stained protein marker.

6.3.4 Refolding by Dilution and Dialysis

The column purified protease and/or guanidine hydrochloride denatured proteins were slowly diluted into 100-fold refolding buffer with gentle stirring (Drop wise dilution method) at 4^0C. The buffer pH was modified to 10.5 and the redox couple millimolar concentration (GSH and GSSG) was increased from 1:0.1 to 4:0.4. However, several experiments were performed to optimise the redox couple ratio, to determine the ratio that resulted in optimal activity as determined by good gelatin digestive activity.

Urea and guanidine hydrochloride are potent aggregation suppressing agents used in solubilisation buffers, whereas L-arginine mono hydrochloride has been found to be an efficient anti-aggregation agent in refolding buffer. The one litre diluted protease was concentrated to 10 ml by VIVA FLOW 50 tangential ultra filters (cut off value 10 kDa). The concentrated protein was dialysed against NaCl (one step) by using dialysis tubing (cut of value 10 kDa). Proteins were then desalted by an Amicon diafilteration (10 kDa). The renatured cathepsin L1g was run on SDS-PAGE along with yeast BJ3505 cells expressing cathepsin L1g. Both proteins had an approximate molecular weight of 40 kDa in a single band (Figure 6.9). This result indicates that the purity of the inclusion bodies is high, as the protein in lane 2 had not been subjected to affinity chromatography.

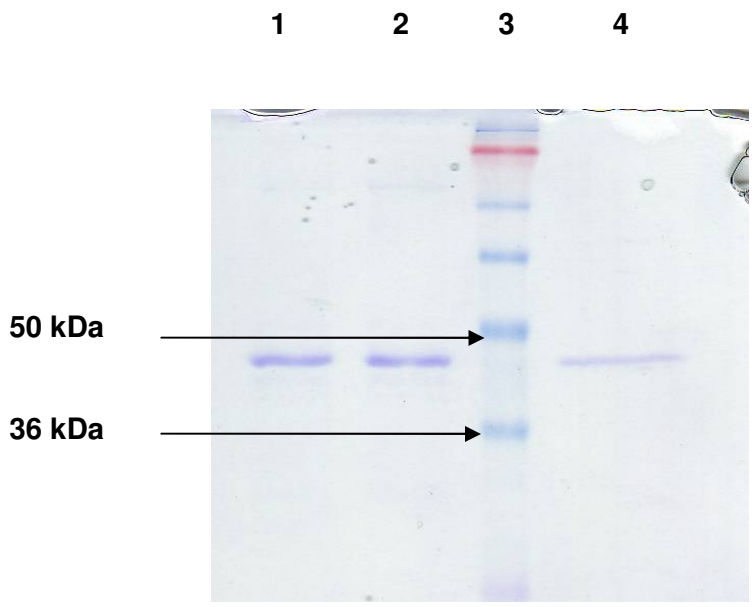


Figure 6.9. Commassie SDS-PAGE analysis of refolded and yeast expressed cathepsin L1g.

0.5 µg of protein was mixed with SDS-sample buffer run on 12.5 %SDS- gelatin PAGE and stained with Coomassie Blue R250. Lane 1, refolded cathepsin L1 (Urea denatured and step-wise urea chromatography purified); lane 2, refolded cathepsin L1g (GdnHCl denatured); lane 3, markers; lane 4, yeast-expressed cathepsin L1g.

6.3.5 Proteolytic activity assay

The proteolytic activity of refolded cathepsin L1g was assayed under acidic conditions (pH 4.5), measured by digestive activity against gelatin substrate SDS-PAGE and is shown in Figure 6.10. In this gel, yeast expressed cathepsin L1g was used as a positive control. The activity was viewed under a light box which revealed digestion of gelatin protein by both refolded and yeast expressed cathepsin L1g. Two prominent bands are seen corresponding to the pro-mature and mature proteins respectively. This indicates that some auto-processing has occurred prior to electrophoresis.

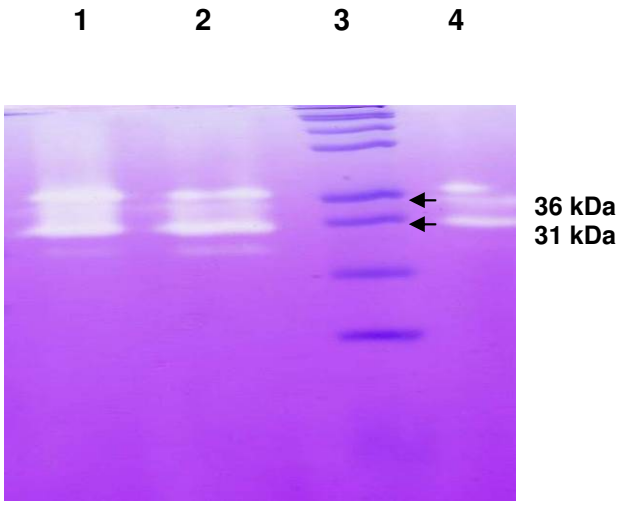


Figure 6.10. SDS-Gelatin PAGE protease activity assay.

1 µg of protein was mixed with non- reducing SDS-PAGE sample buffer and run on 12.5 %SDS-gelatin PAGE. Lane 1, refolded cathepsin L1g (Urea denatured and step-wise urea chromatography purified); lane 2, refolded cathepsin L1g (Gdn HCl used for denaturation); lane 3, markers; lane 4, yeast–expressed cathepsin L1g. Proteolytic activity can be observed in both the *E. coli* and yeast-expressed samples.

6.3.6. Western blotting of expressed and refolded cathepsin L1g

The immunoreactivity of pRSET-A cathepsin L1g was assayed against an anti-His monoclonal antibody (pRSET-A vector encodes six histidine residues for Ni-NTA column purification) as well as an anti-cathepsin L antibody. Reactivity with anti-His antibodies at a band size of 40 kDa indicated the pro-cathepsin L1g at this molecular weight (Figure 6.11). The *E. coli* expressed protein from positive clone pRSET-A-14 showed anti-His reactivity with the predicted size of cathepsin L1g whereas clone pRSET-A-8 expressed a protein with a lower molecular weight than expected, indicating a probable C-terminal truncation (sequence analysis of this clone was not performed, as the pRSET-A-14 clone was shown to be correct). Western blot strips containing renatured cathepsin L1g gave reactive bands identical to yeast expressed cathepsin L1g indicating that cathepsin L1g expressed from *E. coli* BL21 was immunologically cross-reactive (Figure 6.12).

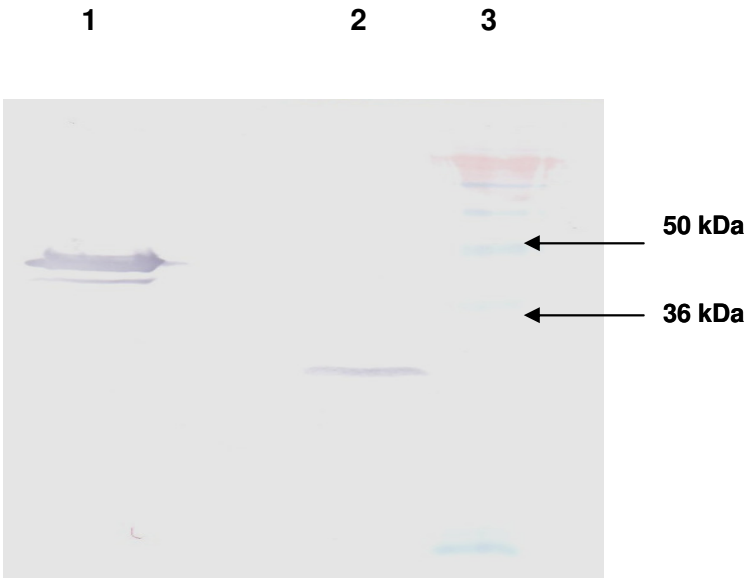


Figure 6.11. Western blot of pRSET-A cathepsin L1g protease probed with an anti-His 6 antibody.

20 µL of whole cell lysate was mixed with SDS-PAGE sample buffer, run on 12.5 % SDS- PAGE and transferred to nitrocellulose membrane. The strip was probed with an anti-His antibody. Lane 1, postive clone 14 expressed cathepsin protein; lane 2, postive clone 8 expressed protein; lane 3 See blue pre-stained protein marker.

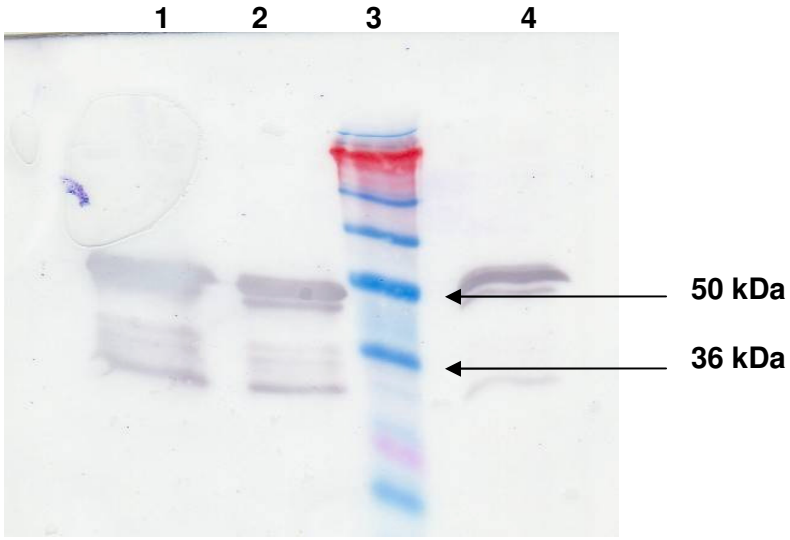


Figure 6.12. Western blot analysis using mouse anti-cathepsin L sera.

1 µg of purified protein was mixed with SDS sample buffer, run on 12.5 % SDS-PAGE and transferred to nitrocellulose membrane. The strip was probed with anti-cathepsin L sera. Lane 1, refolded cathepsin L1g (Urea denatured and step-wise urea chromatography purified); lane 2, refolded cathepsin L1g (GdnHCl used for denaturation); lane 3, markers; lane 4, yeast-expressed cathepsin L1g. Two reactive bands corresponding to the pro-protein and mature protein can be observed.

Table 6.1. Purification summary of liver fluke cathepsin L1g

Purification step	Protein concentration mg/litre
1. Total bacterial lysate	10
2. Urea denatured extract	8.4
3. Gunanidine hydrochloride denatured extract	5.6
4. Column purified protease fractions	3.8
5. Refolded protease (Both methods)	2.5

6.4 Discussion

One of the potential barriers for subunit protein vaccine projects, structural genomics studies and production of therapeutic proteins is the successful production of soluble, biologically active proteins. To achieve this, researchers often need to follow a complex and cumbersome procedure. In addition, soluble expression of eukaryotic proteins is generally less successful compared to prokaryotic proteins . This is particularly so when bacteria are used as the host. Insoluble pellet formation is a common theme in recombinant protein technology. To fully exploit the production capacity of cells, efficient strategies for further processing have to be developed for each protein.

Cathepsin L1g contains cysteine residues and requires disulfide bond formation for correct folding of the protease, but expressed insoluble proteases consist of misformed disulfide bridges due to over expression. These misfolded proteases are not active. In order to utilize these proteins fully, refolding into an active form was pursued.

6.4.1 Protein purification and column chromatography

The recombinant liver fluke protease can be expressed by *E. coli* BL21 (DE3) at high levels in inactive aggregates and can be purified as the pro-mature enzyme. Cathepsin L1g was also expressed in yeast strain BJ3505, but the yield was very low. The main aim of this study was to develop an expression system to produce large quantities of refolded, biologically active parasite protease for vaccine studies. Recombinant protease requires denaturants to promote solubilisation and two efficient denaturants utilized in this study were urea and guanidine hydrochloride. At high concentrations (10 M urea or 6 M guanidine hydrocholoride), protein solubility

is greatly improved . However, such solubilised protein is neither active nor suitable for vaccination without further processing.

After solubilisation by denaturants, the protease must be refolded before it can become active. On column refolding (OCR) has been given much importance in recent years for purification of *E. coli* expressed heterogeneous proteins (Jungbauer & Kaar, 2007; Middelberg, 2002). Immobilised metal affinity chromatography has opened new prospects for efficient purification of proteins with hexa-histidine tags. However, active cathepsin L1g was not achieved by using stepwise urea gradient chromatography, perhaps because on the column the purified cathepsin L1g may not form the correct pattern of disulphide bridge. To achieve the correct disulphide exchanges, further processing need to be performed (dilution and dialysis).

6.4.2 Refolding by dilution and dialysis

Redox pairs are supplemented in the refolding buffer in order to attain the correct formation of disulfide bonds. Optimisation of redox pair concentration was performed and modified to 4 mM GSH: 0.4 mM GSSG (From 1 mM GSH: 0.1 mM GSSG). Renaturation was performed at alkaline pH to promote thiolate anion exchange formation and disulfide exchanges .

Suppressing aggregation nuclei without altering protease concentration, yield and purity has been achieved by adding an aggregation inhibitor to the refolding buffer. L- arginine is considered an important aggregation inhibitor because it destabilizes incorrectly folded proteins by reducing improper interactions within parts of polypeptide chains. L-arginine (0.7 M) was used in this study to destabilize and increase the solubilisation of folding intermediates (Buchner *et al.*, 1992; Gu *et al.*, 2001). The dialysis buffer was changed to pH 8.0 for enhancing stabilisation of the pro-mature form and preventing auto-activation during processing.

6.4.3 Protease activity assay

Renaturation attained a yield of 50% and proteins showed activity in gelatin substrate SDS-PAGE. This assay is considered to be a selective method because it permits the detection of protease activity that is stable in the presence of SDS.

6.5. Conclusion

The results of this study suggest that denatured cathepsin L1g was refolded only by the dilution and dialysis renaturation method and not by the on column refolding procedure. This study has resulted in a simple but highly efficient procedure for the optimal refolding and purification of biologically active cathepsin L synthesized from *E. coli* with a high yield. As there was little dicernable difference between the purity of the final protease that had been affinity purified, or simply solubilised in guanidine, the later method is preferable. After refolding, the protease is active and immunoreactive with anti-cathepsin L sera.

Chapter 7

Concluding remarks

7.1. Expression of stage-specific *Fasciola* proteases and their evaluation in vaccination trials

Liver flukes go through complex developmental and morphological changes from metacercariae to the adult stage in mammalian hosts. Different immune responses are induced at each stage of fluke infection in the host (Mulcahy *et al.*, 1999; van Milligen *et al.*, 1998). The interplay between flukes and the host immune system induces specific immunomodulatory mechanisms, partly due to fluke ES material which also ensures fluke survivability in the mammalian host . An understanding of the host-parasite interactions can assist in vaccine development.

Based on Knox *et al*. (2001), vaccine development can be classified into three levels in the laboratory, such as target antigen selection (for example cathepsin L and cathepsin B cysteine proteases), antigen production (such as yeast and *E. coli* expression and purification) and vaccine potential analysis (for example protein and DNA vaccination in rats and mice). The advances in genomics as well as proteomics lead to the identification of many protective vaccine candidates against *Fasciola* infection (Dalton *et al.*, 2003a; Hillyer, 2005; McManus & Dalton, 2006; Meeusen & Piedrafita, 2003; Smith & Zarlenga, 2006; Spithill *et al.*, 1997).

7.2. Recombinant *Fasciola* protein production

Cysteine proteases are considered to be essential for the life cycle and virulence of the liver fluke parasite (Carmona *et al.*, 1992; Carmona *et al.*, 1993; Dalton & Heffernan, 1989; Smith *et al.*, 1993a; Wijffels *et al.*, 1994b; Wilson *et al.*, 1998).

Vaccine trials with native cathepsin L cysteine proteases and other vaccine candidates showed protective immune responses in many reports (Dalton *et al.*, 1996; Dalton & Mulcahy, 2001; Spithill *et al.*, 1997; van Milligen *et al.*, 2000; Wijffels *et al.*, 1994b). The main drawback of this approach is the limited availability of the parasite material and that isolated proteins undergo proteolytic degradation (Roche *et al.*, 1997). The main challenge at present is to develop recombinant protein vaccines with similar efficacy. Recombinant *Fasciola* cysteine proteins have primarily been produced in yeast (Dowd *et al.*, 1997; Law *et al.*, 2003; Roche *et al.*, 1997; Smooker *et al.*, 2000) or in *E. coli* (Kesik *et al.*, 2007; Yamasaki *et al.*, 2002). These recombinant cathepsin proteins were subjected to vaccine trials against challenge infection, although very few reports has been reported in ruminants .

One aim of this project was to confirm the expression and purification of cathepsin B, cathepsin L1g and cathepsin L5 proteins from yeast. In this investigation, the expressed proteins were purified from yeast supernatant using Ni-NTA affinity chromatography and purity was confirmed using SDS-PAGE analysis. The results of this study showed that yeast BJ 3505 cells secreted high levels of cathepsin proteins compared to the previous reports of Dowd *et al.* (1997) and Roche *et al.* (1997). Cathepsin B and cathepsin L1g showed the same pattern of migration as a single band, whereas cathepsin L5 was found as a doublet in SDS-PAGE. This indicates that during processing cathepsin L5, but not L1g or B, can undergo auto-processing. Immunoblotting results showed that a cathepsin L monoclonal antibody recognised cathepsin L5, cathepsin L1g, *F. hepatica* native cathepsin L and *F. hepatica and F. gigantica* ES material. Recombinant cathepsin B was detected by cathepsin B vaccinated rat sera. These proteases were therefore considered suitable for vaccine studies.

7.3. The protective efficacy of expressed *Fasciola* proteins

The crude proteins of adult and juvenile stage *F. hepatica* have been tested for the induction of protective immunity in rats. Results show that juvenile proteins showed high protection against challenge infection (van Milligen *et al.*, 2000). Prior to analysing the protective efficacy of recombinant proteins in ruminants (which is the most appropriate means of testing vaccine efficacy), this investigation has focussed on testing three recombinant antigens (two from juvenile parasites) in a rat model.

In addressing this issue, eight vaccines were formulated in Quil A adjuvant, 4 of which were cocktail vaccines containg 2 or 3 antigens. Quil A adjuvant was used as this is an acceptable adjuvant in the veterinary vaccine industry, unlike Freund's adjuvant which can only be used in experimental settings. The ideal standard for anti-fluke vaccine development is reduction in fluke numbers in vaccinated animals compared to control animals . This was observed. The influence of recombinant vaccines on the fluke body size and wet weight are also considered to be important indicators of protection in vaccinated animals (Nambi *et al.*, 2005; Ramajo *et al.*, 2001; Valero *et al.*, 2006). Most of *Fasciola* vaccine experiments have correlated protection against challenge infection with immunological parameters (Kesik *et al.*, 2007; Mulcahy *et al.*, 1998; Mulcahy & Dalton, 2001; Wedrychowicz *et al.*, 2003; Wedrychowicz *et al.*, 2007). There are few reports of the anti-pathological effects of recombinant protein vaccines (Kesik *et al.*, 2007; Nambi *et al.*, 2005). The results of this investigation have demonstrated that pathological lesions of the liver (hepatic stage and biliary stage) are an important indicator of the protective efficacy of administered vaccines in rats (pictorial presentation, chapter 4).

Vaccination with cathepsin B generated rapid and maximal antibody titre (reciprocal titre of 10^5) and a 59% reduction in fluke counts compared to control rats. *F. gigantica* cathepsin L1g which showed moderate IgG responses compared to cathepsin B and cathepsin L5 induced a 49% reduction in fluke counts. Cathepsin L5 vaccination elicits high antibody responses and 50% reduction in fluke burden

compared to control rats. These findings suggest that each of the cathepsin proteases can induce a protective effect in rats. Furthermore, stunting of fluke growth the resultant reduction in liver damage in vaccinated rats was seen as a protective effect of recombinant cathepsin protein vaccination against parasitism and pathogenicity caused by the challenge infection.

A biphasic effect on IgG antibody responses in vaccinated rat serum was also observed. The protein specific IgG antibody in vaccinated rats peaked at time of challenge and then had declined at the time necropsy.

In general, immunisation with cocktail vaccines elicited higher levels of protection as compared to vaccination with a single antigen (Dalton *et al.*, 1996; Estuningsih *et al.*, 1997; Wijffels *et al.*, 1994b). In agreement with previous observation, in these studies it was observed that the percentage of protection ranged from 57 to 83% depending upon the combination of antigens used. The percentage of maximal protection with individual rats was 100% in all cocktail and cathepsin B vaccinated rats (in other words, these groups each contained rats with no flukes). The results of the study suggested that a cocktail of adult fluke cathepsin L5 and juvenile cathepsin B vaccination showed the highest percentage of protection against *F. hepatica* metacercariae challenge, not only reflected by reduced fluke recovery and size of flukes, but also reduced intensity of liver lesions in rats vaccinated with these combinations.

Results of this study matches with previous observation of Kesik *et al*. (2007). The vaccination of *E. coli* expressed (CPFhW) cysteine proteins (400 µg) in Sprague Dawley rats induced 80% protection against challenge infection . However, our results contradict the observation of Reszka *et al*. (2005) who demonstrated that a low percentage of the protection (18%) was induced by yeast expressed cathepsin L (125 µg of FhCL3) in Wistar rats.

Piedrafita *et al*. (2007) suggest that *in vitro* killing of *F. hepatica* occurs in the first 24 to 48 hours of infection and is mainly by antibody mediated effector cells (eosinophils, and macrophages) releasing nitrous oxide. *F. hepatica* cathepsin L proteinase prevents the adherence of eosinophils to newly excysted juveniles . Spithill *et al*., (1997) have hypothesized that fluke ES materials inhibit antibody mediated attachment of eosinophils, neutrophils and macrophages to the tegument of flukes. In this investigation, the recombinant protein vaccination induced peripheral eosinophilia, and increased neutrophil and lymphocyte responses in vaccinated rats at the time of challenge infection. At the time of necropsy, the eosinophil counts had tended to decrease and there is an increase in the neutrophil counts in the vaccinated rats. The evoked protective immunity in rats may act via these same immunological events during this time period. The corollary of previous observations suggests that natural killing of juveniles in rats may not confer protection to a subsequent infection. However the protective immunity elicited by recombinant protein vaccination appears to evoke effector and memory responses against infection.

7.4. Novel vaccination strategies for delivery of *Fasciola* antigen

The application of parasite DNA vaccines in ruminants has been much less promising due to the lack of immune responsiveness against administered vaccines . The access of antigens to APCs appears to be rate-limiting step in the generation of immune responses to DNA vaccines. DNA vaccine targeting either the lymph node (CTLA-4) or chemo-attractant (MCP3) represents an attractive immune enhancing feature for targeting antigen to APCs and lymph nodes. Juvenile and immature fluke cathepsin B has been detected within 5 weeks of infection (Law *et al*., 2003). Considering the immature fluke cause extensive liver damage during this

period, a vaccine target against the early period of infection would be helpful to reduce the intensity of liver lesions.

The third aim of this thesis was to determine *F. hepatica* cathepsin B specific immune responses in mice vaccinated with a variety of constructs encoding cathepsin B. Each construct was designed to secrete cathespin B, as this will maximise the humoral response. Previous DNA vaccination studies by Smooker *et al*. (2001) demonstrated *in vitro* expression from a DNA vaccine encoding cathepsin L5 and the subsequent induction of anti-cathepsin L5 specific IgG, IgG1, IgG2a, IgG2b and IgE antibodies in BALB/c mice. As a continuation of the above findings, firstly the supernatants of DNA vaccine vectors transfected COS-7 cells were subjected to the immunoblotting. The supernatants of cells transfected with the four vectors encoding protein were recognised by cathepsin B vaccinated rat sera. After vaccination of mice, humoral and T cell responses were elicited.

In this investigation the CTLA-4 DNA vaccine shows a more rapid increase in antibody titre in BALB/c mice compared to other DNA vaccines in mice. However, the increase in titre by either of the targeting molecules (MCP3 or CTLA4) was non-existent or modest. The ELISPOT results showed significantly higher cathepsin B specific IL-4 production in all test groups over the control group. However, there was no significant advantage of one construct over another. A recent study by Ingale *et al*. (2008) shows T cell responses during *F. gigantica* infection was a Th2 type responses and no IFN-Y was detected during the early phase of infection of calves. High levels of IL-4 production and the resultant Th2 response lead to an immunopathological reaction and development of inflammatory lesions in the liver. However, the expression of cathepsin B in the early stage of fluke infection (Law *et al*., 2003) and their ability to digest a range of molecules including immunoglobulin, gelatin and albumin also suggests its possible role in directly causing hepatic damage.

In the rat vaccine trial, cathepsin B vaccination induced high peripheral eosinophilia. High IL-4 production was observed from DNA vaccination. These two observations suggested that an allergic type of immune reaction was induced in vaccinated animals. The presence of *Fasciola* specific IgE antibody and eosinophil responses is a good indicator of acquired immunity which has been demonstrated elsewhere (Hagan *et al*., 1991; Meeusen *et al*., 1995; Meeusen & Balic, 2000).

It appears that the addition of a targeting sequence to the DNA vaccine construcst has not markedly enhanced immunogenicity. This is possibly a protein- specific limitation, whereby the constructs encoding cathepsin B fusion proteins are inefficiently expressed or secreted *in vivo* (although they were efficiently secreted from COS-7 cells), or perhaps the fusion proteins are unstable or poorly taken up by cells. The results of this study demonstrate that CTLA-4 fusion proteins may be the best way for any future delivery of cathepsin B as a DNA vaccine.

7.5. Enhancing the functional activity and refolding of *Fasciola* target antigen from *E. coli* expressed inclusion bodies

The fourth aim of this work is to produce a functionally active form of *F. gigantica* cathepsin L1g from *E. coli* BL 21 cells expressed in inclusion bodies. This was undertaken due to the very poor expression of this protease in yeast (compared to other cathepsin L's and cathepsin B). The recombinant cathepsin L1g refolding process involves denaturation of inclusion bodies, renaturation and functional activity analysis. Two efficient solubilising agents used in this study were urea and guanidine hydrochloride. It was found that urea denaturation followed by affinity chromatography did not result in active protein, and therefore this step could be omitted. Indeed, the purity of solubilised proteins with or without the affinity step was

similar, hence the easiest procedure to obtain active protein was to collect inclusion bodies, solubilise and apply directly to the re-folding buffer. In this was, active cathepsin L1g (as determined by gelatin digestion) that was immunoreactive was obtained.

7.6. Future work

The protective efficacy of *E. coli* expressed *F. gigantica* cathepsin L1g and yeast expressed cathepsin L1g should be investigated further, as *E. coli* expressed (CPFhW) cysteine proteins showed 80% protection against *F. hepatica* challenge infection. The immune activation of yeast expressed cathepsin L (FhCL3) was previously compared with baculovirus expressed proteins against challenge infection in Wister rats. Hence, the immune responses induced by proteins expressed in various expression systems (yeast, *E.* coli and baculovirus system) should be investigated in ruminants.

The protective immunity of DNA vaccine encoding cathepsin B against challenge infection needs to be explored in a challenge model. The results of this current investigation (chapter 6) showed IL-4 responses in vaccinated mice. However, mice are not a suitable challenge model, and these DNA vaccines could be tested in a sheep trial.

The protective efficacy of these yeast expressed stage specific *Fasciola* antigens needs to be investigated in target species, for example sheep and cattle. This is considered as the most appropriate means of testing its vaccine efficacy. Considering the results obtained of vaccine studies in rats (chapter 4), combinations of these recombinant proteins may be effective vaccines when delivered in an acceptable veterinary adjuvant in large animals.

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