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**Factors affecting the anthelmintic efficacy of cysteine
proteinases against GI nematodes and their
formulation for use in ruminants**

By

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**Thesis submitted to the University of Nottingham for the
degree of Doctor of Philosophy**

October 2012

ABSTRACT

Gastrointestinal (GI) nematodes are important helminth pathogens responsible for severe losses to livestock industries and human health throughout the world. Control of these infections relies primarily on chemotherapy; however there is rapid development of resistance to all available classes of anthelmintic drugs, and therefore new alternative treatments are urgently required.

Plant cysteine proteinases (CPs) from papaya latex, pineapple fruit and stem extracts have been demonstrated to be effective against GI nematodes of rodents, chickens, pigs and sheep. The current study extended evaluation of different plant extracts and the factors affecting the efficacy of papaya latex supernatant (PLS) as an anthelmintic against GI nematodes in a mouse model system and formulation and delivery for use in ruminants.

The study started with purification and concentration of CPs in PLS using different methods to determine which of them would provide high yield of CPs. It was found that concentration by dialysis provided a high yield of active enzyme in PLS. Storage of PLS at -20⁰C and -80⁰C retained more active enzymes for prolonged period of time than at ambient temperature and 4⁰C. Motility assay conditions showed to have no influence on enzyme activity. While the *in vitro* experiment results showed significant detrimental effect of pineapple fruit extract, stem bromelain and little effect of kiwi fruit extract against *Heligmosomoides bakeri* motility. *In vivo* experiments showed less efficacy of these enzymes than expected when compared with PLS.

The first factor to be assessed in this study was the effect of fasting on the anthelmintic efficacy of PLS. The results showed that PLS was equally effective in reducing worm burdens whether mice were fasted before treatment or not, and by avoiding fasting the side effects of treatment were minimized. Comparison of efficacy in a range of mouse strains indicated that efficacy varied between mice of different genotype. At the dose used, the treatment was most effective in C3H mice ranging from 90.5% to 99.3% in reducing worm burdens and less effective in NIH3, CD1 and BALB/c strains (7.9%, 36.0% and 40.5% reduction respectively). However, host sex and body size were shown not to have any influence on the anthelmintic efficacy of PLS. Since CPs are particularly sensitive to pH, variation between mouse strains in gut pH was investigated but no significant differences in pH were found along the GI tract of the poor (BALB/c) and high responder mice (C3H) to PLS treatment and concurrent administration of the antacid cimetidine also did not improve efficacy.

The study also explored the potential of formulation and delivery of PLS as an anthelmintic drug for ruminants. *In vitro* studies involving both immediate and slow release dosage formulations simulating the physiological conditions (pH, temperature and peristaltic movement) in the GI tract of the animal were conducted. In the slow release experiments, two hydrophilic matrices were tested, the xanthan gum and hydroxypropyl methylcellulose (HPMC) (both Methocel-LVCR and Methocel-CR). Methocel-CR provided better slow release results compared to the others. In the immediate release experiments 3 disintegrants (Primojel, L-HPC and Ac-Di-sol) were investigated and Ac-Di-Sol[®] was found to produce the faster immediate drug release rate. Preliminary *in vitro* studies also showed that PLS was highly effective against equine GI nematodes.

Finally the empirical findings in this study provide useful information for improvement of formulation and delivery of these naturally occurring plant-derived enzymes for treatment of intestinal worm infections in humans and livestock, while achieving maximum efficacy and minimal side-effects.

ACKNOWLEDGEMENT

First and foremost I would like to express my sincere gratitude to my main supervisor Prof. Jerzy Behnke and my co-supervisors Drs. Martin Garnett and Colin Melia for their valuable and detailed feedback, input and ideas. I would like to thank Dr. David Buttle of University of Sheffield for advice and for answering my numerous questions on the biochemistry part of my research and to my internal assessor Dr. Ian Duce for giving useful feedback at my progression assessments.

This research would not have been possible without the studentship from Mkwawa University College of Education and the University of Nottingham for accepting to host my PhD. I am greatly indebted to Ann Lowe for her friendly technical advice and long hours selflessly spent helping me in mouse dissections and worm counts. I am also thankful to Tim Smith for assisting me in the preparation and viewing of SEM specimens, and Colin Wills and Christine Grainger-Boulton for teaching me how to use the facilities in the School of Pharmacy labs. Many thanks to people of the BMSU, particular Dave who was always ready to help me with anything I wanted in the animal house.

Massive thanks must go to my colleague Fadlul Mansur for his generosity and companionship throughout my study period. I would like also to thank everyone in our research group Andrew Phiri, Fawzia Shawesh, Amy Hall, Yadong Zheng and others not mentioned for their help and advice which made my life here easy.

I owe special thanks to my wife, Irene Kasambala for her enduring love, patience and the sacrifices she made for accepting the responsibility of taking care of the family while I was away for my study. My daughters: Mercy, Lulu and Happy, sorry for missing me all this time that I was away.

Finally I wish to dedicate this thesis to my parents who laid the foundation of my education using their meagre resources.

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ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AT	Ambient Temperature
BAPNA	Benzoly-arginyl-p-nitroanilide
CPs	Cysteinase Proteinases
CR	Control Release
DALYS	Disability-Adjusted Life Years
E-64	L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane
EDTA	Ethylenediaminetetraacetic acid
EPG	Eggs per gram
ER	Extended Release
FDA	Food and Drug Authority
FEC	Faecal egg counts
GI Nematodes	Gastrointestinal nematodes
GRAS	Generally regarded as safer
HBSS	Hanks' balanced salt solution
HPMC	Hydroxyproxylmethyl cellulose
IC ₅₀	Half maximal inhibitory concentration
IL	Interlukin
L-HPC	Low-Substituted Hydroxypropyl Cellulose
ODTs	Orally Disintegrating Tablets
PL	Papaya Latex
PLS	Papaya Latex Supernatant
SEM	Scanning Electron Microscope
STH	Soil Transmitted Helminths
TB	Tuberculosis
TGF	Transforming Growth Factors
Th1	Type 1 helper T cells
Th2	Type 2 helper T cells
USP	United States Pharmacopeia
XG	Xanthan gum

CHAPTER 1: INTRODUCTION

1.0 Summary

Infections with Gastrointestinal (GI) nematodes have severe effects on human health and cause serious economic losses in livestock farming. Cysteine proteinases (CPs) from papaya latex have been demonstrated clearly to be powerful anthelmintics against nematodes of rodents and sheep. Despite this finding, the enzymes have not yet been developed to a market level for use as anthelmintic drugs. This chapter provides a review of the importance of GI nematodes for human health and livestock production and their life cycles, current methods available for their control and the development of drug resistance. The chapter details the potential advantages of using CPs as novel anthelmintics for humans and livestock and its formulation and delivery system. It concludes with the objectives of the study which are:

1. To determine the best preparation method of PLS and storage conditions for long term survival of the enzymes.
2. To evaluate the anthelmintic efficacy of CPs from sources other than papaya latex including pineapple and kiwi extracts.
3. To assess the effect of fasting on the anthelmintic efficacy of papaya latex supernatant (PLS).
4. To examine whether the efficacy of PLS treatment is host genotype dependent and
5. To formulate and design an appropriate delivery system for CPs from papaya latex for use in humans and livestock.

1.1 Background

Parasites are important pathogens worldwide causing severe effects on human health and serious economic losses in the livestock industry. They are of 2 types, ecto- and endoparasites. Endoparasites are mainly the protozoa and the helminths (May, 2007). The protozoa are responsible for the majority of the mortality associated with parasitic infections, while helminths generally are non-life threatening but produce long-term (or chronic), debilitating diseases (Chan, 1997).

Among the helminths, GI nematodes are the most common parasites affecting more than 50% of the human population worldwide particularly in the tropics (Chan, 1997) and cause significant economic losses to the livestock industry (Nieuwhof, 2005). However, their impact on human populations is often neglected by public health authorities and authorities acting as sources of research funding, in contrast to protozoans (Boatin *et al.*, 2012). The major control method for limiting the extent of infection with these parasites relies primarily on the use of chemotherapy (Behnke *et al.*, 2008).

1.2 GI Nematodes of medical importance

The most common GI nematode species that infect humans are *Ascaris lumbricoides* (roundworm), *Ancylostoma duodenale* and *Necator americanus* (hookworms), *Trichuris trichiura* (whipworm), *Enterobius vermicularis* (pinworm) and *Strongyloides stercoralis* (threadworm) (Table 1). These species are referred to as 'Soil Transmitted Helminths' (STH). Most of them have direct life cycles (involved single host) where all pre-parasitic stages are found free-living in the environment

and their development may take place either inside the egg or after hatching. The eggs or larvae of these nematodes, with the exception of *E. vermicularis*, require a period of development in the soil to become infective before transmission to the human host. It is very common to have multiple species infections for example *A. lumbricoides* and *T. trichiura* species commonly co-occur in the same host (Booth & Bundy 1992).

Table 1.1: Human GI nematode parasites of worldwide importance

GI Nematode species	Group name	Number infected	Distribution	Transmission
<i>Ancylostoma duodenale</i> & <i>Necator americanus</i>	Hookworm	1.3 billion	Worldwide, mainly in tropical regions	Skin contact with contaminated soil
<i>Ascaris lumbricoides</i>	Roundworm	1.3 billion	Worldwide, mainly in tropical regions	Ingestion of eggs
<i>Trichuris trichiura</i>	Whipworm	1.05 billion	Worldwide, mainly in tropical regions	Ingestion of eggs
<i>Enterobius vermicularis</i>	Pinworm	209 million	Worldwide	Ingestion of eggs; occasionally inhaled
<i>Strongyloides stercoralis</i>	Threadworm	30 million	Worldwide, mainly in tropical regions	Skin contact with contaminated soil; autoinfection

Source: Stepek *et al*, 2006

Their life cycle starts by the female worms producing and releasing eggs into the intestine which then pass into the external environment via the host faeces. In most cases the first larval stage develops (L1) within the eggs. After hatching the L1 develops through four further stages (L2, L3, L4 and pre-adults) with each stage

preceded by a cuticular moult with an increase in size of the worm, until finally they develop into adult worms. The major routes of infections of STH are through oral ingestion of eggs, or skin penetration of larvae usually between the toes and fingers in case of hookworm. The larval stage L3 is the main infective stage in most species.

GI nematode infections are associated with many physical and mental symptoms of disease. These include anaemia, wasting, stunting, cognitive impairment and lowered educational achievement, all of which can significantly reduce the productivity and wage-earning capacity of adults (Guyatt, 2000). Chan (1997) estimated that the disease burden caused by the 3 most important GI nematode infections (hookworm, *Ascaris lumbricoides* and *Trichuris trichiura*) resulted in 39 million disability-adjusted life years (DALYs) being lost due to these parasites compared with 35.7 million lost due to malaria. These figures explain why it is necessary for the health care services to commit their scarce resources for controlling intestinal nematode infections (Stephenson *et al.*, 2000).

Anaemia

Anaemia occurs when the adult worms (hookworms in particular) attach to the villi of the small intestine, drawing blood into their intestines to obtain oxygen and food, causing abdominal discomfort, diarrhoea and cramps, anorexia, weight loss and ultimately anaemia (Muller, 2001). A study by Georgiev (1999) reported that hookworm infections in humans, especially in children, are one of the leading causes of iron-deficiency anaemia. It has been also observed that iron-deficiency anaemia is more serious in pregnant women infected with hookworms, than non-pregnant

infected women (Dreyfuss, 2000). Thus poor iron status and iron deficiency anaemia are characteristics of hookworm disease (Crompton and Nesheim, 2002).

Malnutrition

Ascaris, *trichuris* and hookworm disease are associated with malnutrition caused by reduced food intake, impaired digestion, diarrhoea, abdominal pain, malabsorption, and poor growth rate (Bethony *et al.*, 2006). Malnutrition is exacerbated by infection because the nematodes damage the intestinal mucosal epithelial cells whilst feeding, resulting in the prevention of nutrient absorption by the host. They also cause loss of appetite (anorexia) which results in lowered food intake. A study in Kenya and Indonesia showed a significant improvement after treatment with albendazole in the appetite of school children infected with the hookworms *T. trichiura*, and *A. lumbricoides*. The children gained more weight and improved in appetite significantly when compared with placebo groups (Stephenson *et al.*, 2000). Stunted growth caused by helminthiasis in early childhood may remain significant even in later childhood even after treatment of the worms (Moore *et al.*, 2001). A study in India reported significant improvement in absorption of vitamin A in patients with *Ascaris* immediately after treatment of the worms (Albonico *et al.*, 2008). Clinical deficiency in vitamin A contributes to increased morbidity (such as blindness) and mortality.

Cognitive function

Worm burdens are directly associated with poor school attendance and low cognitive function in school children due to the fact that helminth infections cause malnutrition, stunted growth and iron deficiency anaemia which in turn is associated

with a deficit in cognitive functioning (Simeon and Grantham-McGregor, 1990). However, it is not easy to provide correlation evidence between helminth infections and educational achievement due to difficulties in separating the effects of infection from other confounding variables (World-Bank, 1993). A trial conducted in Jamaica, involving school children with moderate to heavy loads of *T. trichiura* reported a significant improvement in auditory test of children after treatment of the worms (Nokes *et al.*, 1992). This suggests that *T. trichiura* infection affects certain cognitive functions which are reversible after treatment.

Secondary infection

Helminth infections are endemic in developing countries where other infectious diseases such as AIDS, malaria and tuberculosis are highly predominant (Hotez *et al.*, 2006). Thus helminth infections in humans impair the immune response to these serious infections, which are controlled by a Th1 immune response (Maizels *et al.*, 1993). Helminth infections also induce the production of the T regulatory cytokines, interleukin (IL)-10 and transforming growth factor (TGF- β), which are immunosuppressive and are believed to inhibit immune responses that protect against TB (Bentwich *et al.*, 1999). Studies in Zanzibar island in Tanzania indicated that early exposure of infants to GI Nematodes induced Th2 dominant immune responses (Wright *et al.*, 2009). The existence of Th2 immune responses in infected individuals may cause the individual to be unable to develop a protective Th1 response upon vaccination against TB and other diseases caused intracellular parasites (virus, bacteria and prozotoans). The presence of helminth infections increases the susceptibility of individuals to secondary infections requiring a Th1 immune

response for protection. Thus eradication of helminthic infections may have a major impact on both AIDS and TB in developing countries (Clerici and Shearer, 1994).

1.3 GI nematodes of veterinary importance

The most important GI nematodes that parasitize domestic animals belong to the family Trichostrongylidae. This family contains most of the economically important parasitic nematodes of grazing livestock including *Ostertagia*, *Teladorsagia*, *Trichostrongylus*, *Cooperia*, and *Haemonchus* species. The most pathogenic nematode of small ruminants is *Haemonchus contortus*. This is a blood-sucking parasite that attaches to the abomasal mucosa and causes anaemia and oedema, which can lead to death of the animals. It is threatening the survival and productivity of the sheep and goat farming industries throughout the world (Roberts, 2000, Waller, 2005).

Almost all Trichostrongylidae family nematodes have similar life cycles which start by adult female worms laying eggs which are passed out in the faeces. The eggs contain embryos which will develop into first stage larvae (L1). L1 and second stage larvae (L2) feed actively on bacteria, however the third stage larvae (L3) are dormant and enclosed by a protective, impermeable sheath (the retained L2 cuticle) but remain infective. The hosts are exposed to infection by ingesting L3s, as they graze on pasture. The next step in the host is exsheathment from the protective sheath. Exsheathment is immediately followed by movement of parasitic L3 to sites of preference where growth and development to the adult stage occurs (Johnstone, 2001).

Helminths are the most economically devastating internal parasites of livestock. They cause great losses to animal production mainly among ruminants with important economic consequences for livestock owners (Waller, 2003). The direct losses caused by these parasites are attributed to acute illness and death, premature slaughter and rejection of some parts of meat at meat inspection such as liver, heart, kidneys and some parts of muscles (Hansen and Perry, 1994). Indirect losses include a reduction of productive potential due to stunted growth, weight loss in young growing calves and late maturity of stock for slaughter (Swai *et al*, 2006). Although ruminants can be infected with tapeworms, their effects on animal performance is minimal compared to that of roundworms (Gadberry *et al*, 2009).

1.4 Control and treatment of GI nematodes

1.4.1 Use of Anthelmintics

Current methods for controlling nematodes both in human and livestock involve repeated dosing with synthetic anthelmintic drugs. There are three broad-spectrum anthelmintic classes available for treatment and control of GI nematodes and they differ according to their chemical structure and mode of action (Kaplan, 2004).

The first group are the benzimidazoles which include albendazole (used for treatment of threadworms, roundworms, whipworms, tapeworms and hookworms) and mebendazole (more effective against pinworms, roundworms and hookworms) for humans and thiabendazole for livestock. Benzimidazoles were initially discovered as anthelmintics in 1961. They are heterocyclic aromatic organic compounds synthesized by condensation of o-phenylenediamine with formic acid.

Their anthelmintic efficacy is due to their ability to compromise the cytoskeleton through a selective interaction with β -tubulin, inhibiting its polymerization and so interfering with microtubule-dependent glucose uptake by the parasite (Borgers, 1975).

The second group includes the imidazothiazoles (levamisole) and tetrahydropyrimidines (pyrantel and morantel) which were introduced in 1970s. These anthelmintics act as nicotinic receptor agonists by prolonging and overstimulating the excitatory nicotinic acetylcholine receptors on the body wall of nematodes causing blockade of the neuromuscular junctions, and muscle paralysis of the worms. The parasites are then unable to move in the intestinal tract and are swept out by the peristaltic movement of the intestine. Studies have found that levamisole, and related compounds, also cause spastic paralysis and egg-laying in *C.elegans* (Holden-Dye and Walker, 2007).

The third group of anthelmintics are the macrocyclic lactones (avermectins and milbemycins) which were introduced in the 1980s by Merck. The commercial available avermectins include ivermectin, abamectin, doramectin, eprinomectin, and selamectin. Avermectins are semi-synthetic derivatives of large macrocyclic lactone fermentation products of the micro-organism *Streptomyces avermitilis* (Haber, 1991). Their modes of action involve eliciting persistent paralysis of nematode pharyngeal and body wall muscles. They act by opening glutamate-gated chloride channels, increasing chloride ion conductance, leading to defects in neurotransmission and flaccid paralysis. It is this high affinity for nematode glutamate-gated chloride channels that correlates with its potent anthelmintic activity.

There is a fourth group of anthelmintics which are narrow spectrum drugs such as piperazine which was introduced in the 1950s and still used today in the treatment of thread worm infections in children. Salicylanilides, closantel and nitrophenols are used for treatment of blood feeding parasites (*Haemonchus contortus*) in small ruminants. Also in some countries organophosphates are still being used (Holden-Dye and Walker, 2007, Coles *et al.*, 2006).

Other new classes of anthelmintics which have recently been launched include emodepside introduced in 1990s for treatment of enteric worms in cats (Buttle *et al.*, 2011), monepantel labelled for use in sheep and introduced in the market as Zolvix[®] in 2009 and derquantel (in combination with abamectin) introduced in the market in 2010 as Startect[®] (Pfizer Animal Health) also labelled only for use in sheep (Kaminsky *et al.*, 2010, Hosking *et al.*, 2010b).

These anthelmintics are relatively cheap, safe and have very few minor side-effects. However, in treatment of humans very few are recommended for use by pregnant women, who are most at risk from the severe effects of worm infections such as iron deficiency anaemia, malnutrition, even death. It has been reported that thiabendazole has high efficacy against *Strongyloides stercoralis*, however side-effects with this drug are high including nausea, dizziness, weakness, anorexia, vomiting and/or headache and may occur in up to 50% of cases (Cook, 1986, Stepek *et al.*, 2006a).

1.4.2 Sanitation & hygiene

All human GI nematodes with the exception of *E. vermicularis* depend for transmission on environmental contamination with egg-carrying faeces. As a result, worm transmission is intimately associated with poverty, poor sanitation, and lack of clean water (Hotez *et al.*, 2007). Worm infections predominantly occur in rural areas, where social and environmental conditions in unplanned slums and squatter settlements of developing countries are ideal for their persistence. Hence the provision of safe water, good personal hygiene and high levels of cleanliness are essential for the control of helminth infection.

Treatment of all family members simultaneously is very important for the prevention of infections with *E. vermicularis* because the female worms of this species contaminate bed clothes with infective eggs while infected people sleep. Also dogs can act as reservoirs of important zoonotic parasites, particularly in areas with loose defecation. For example in India, viable eggs of *T. trichiura* and *A. lumbricoides*, which are believed to be host-specific for humans, were found in the faeces of dogs kept as pets and can be transmitted to humans (Traub *et al.*, 2005). Though treatment with anthelmintics can significantly reduce parasite burdens and improve growth and development of millions of children, chemotherapy alone is unlikely to prevent recurring infections with GI nematodes.

The best controlled approach for limiting infections with GI nematodes in humans is believed to depend on integrated strategies, involving the provision of clean water, better housing, good sanitation and hygiene, health education and improvement in the general nutritional status, and the use of anthelmintics. Such

integrated control strategies will prolong the efficacy of the current anthelmintic drugs and delay the onset of resistance (Albonico *et al.*, 1999). However, it is not possible to integrate these strategies in developing countries where poverty is the predominant limiting factor.

1.4.3 Rotational grazing (Livestock)

Rotational grazing involves periodically moving livestock to fresh pastures, to allow the previous pasture to regrow and parasites stage to die out. Rotational grazing practices may reduce the parasite burden in livestock; however, the method alone will not guarantee parasite eradication. The process involves moving more susceptible animals to a safe pasture. Always animals are dewormed prior to placement on a safe pasture; otherwise, the pasture can become contaminated immediately.

Sometimes rotational grazing can increase infection compared to continuous stocking. This may be because rotational grazing allows higher stocking rates than continuous grazing (Gadberry, 2009). Rotational grazing rather than conventional grazing combined with strategic deworming programs can still reduce infection with helminths. However rotational grazing depends on sufficient land to enable rotation, with some pastures remaining ungrazed for long enough to allow transmission stages to die which the majority of the farmers cannot afford (Behnke *et al.*, 2008). Therefore, the most applicable method for controlling helminths is the use of chemotherapy; however, this strategy is limited by the development of resistance of the parasites to the drugs.

1.5 Anthelmintic resistances of GI nematodes

In many parts of the world, the control of intestinal helminth parasites has primarily been based on the use of modern synthetic anthelmintic drugs. However, routine use and misuse has resulted in development of parasite resistance to the drugs by the nematodes and this has become a severe global problem for livestock farmers (Yue *et al.*, 2003, Coles *et al.*, 2006, Sissay *et al.*, 2006).

Resistance is the ability of parasites to survive the normal effective dose of an anthelmintic and pass this genetic ability to their offspring (Prichard *et al.*, 1980). During anthelmintic treatments, a small number of worms survive due to the presence of resistant genes that alter the site of attack of the drug in question and hence endow the parasite with resistance (Anson, 2009). The gene can be transmitted to subsequent generations, eventually creating a population of worms resistant to that kind of anthelmintic (Papadopoulos, 2008, Kaplan, 2004). Unless new anthelmintic drugs with different modes of action are introduced, worm resistance will continue throughout successive generations.

The first report of resistance to broad-spectrum anthelmintics (benzimidazoles) was in the 1960s. Subsequently, resistance was reported within a few years of the introduction of each drug group (Waller, 2006) and it has been reported in all major nematode species of goats and sheep, in particular *Haemonchus contortus*, *Teladorsagia (Ostertagia) circumcincta* and *Trichostrongylus* spp (Kaplan, 2004).

Resistance to all 3 classes of anthelmintics has been reported to be established in small ruminants throughout the world, especially in New Zealand, Australia and South Africa (Besier, 2007, Waller, 2003). Resistance has been also reported in developing countries such as Tanzania (Keyyu *et al.*, 2002), Kenya (Gatongi *et al.*, 2003) and Ethiopia (Sissay *et al.*, 2006) in Africa and in South America (Gill and Lacey, 1998).

A greater concern is fast development of multiple-anthelmintic resistance which has been reported also from various parts of the world, particularly from the major sheep-producing countries; Australia, South Africa, New Zealand and several South American countries (Kaplan, 2004). Multiple-drug resistance presents a serious problem to livestock owners leaving them with no alternative effective drugs for treatment of worm infections.

Despite the prevalence of parasitic worms and resistance, anthelmintic drug discovery is very slow. The reason is that the nations which suffer most from these tropical diseases have little money to invest in drug discovery or therapy (Geary, 2005). Although there are many anthelmintic products available in the market, all belong to one of the 3 classes introduced more than 30 years ago, and we are still constrained by our reliance on them. The lack of new drugs over the last 30 years might be an indication that it is not an easy process to develop anthelmintic drugs with different modes of action from those already in the market (Behnke *et al.*, 2008). However, new classes of anthelmintics have been introduced recently including monepantel (2009) and derquantel (2010) but these are approved only for use in sheep. Derquantel has already been shown to be less effective against

macrocyclic lactone-resistant *Haemonchus* worm isolates with only 18.3% reduction in faecal egg counts (Kaminsky *et al.*, 2010).

There are few reports of anthelmintic resistance amongst nematodes of cattle. This does not mean that there is no resistance but there is a general belief that resistance is not yet an important issue (Kaplan, 2004). Recent reports have indicated that resistance of worms like *Cooperia spp* in cattle has spread widely throughout the world (Mejia *et al.*, 2003, Loveridge *et al.*, 2003, Coles *et al.*, 2001). In view of these findings, anthelmintic resistance in nematodes of cattle might be more common than is generally considered to be the case.

It is believed that if the use of drugs is discontinued, a reversion to susceptibility of worms to anthelmintics will occur. However, in practice, increased susceptibility to anthelmintics does not seem to occur as readily as once thought, meaning that once resistance has been generated it becomes a long term problem. A study conducted in Tanzania (Keyyu *et al.*, 2002, Kaplan, 2004) indicated that nematodes of sheep were still resistant to albendazole even after ten years when the drug had been withdrawn from use. This occurs because worms carrying resistance alleles, although reduced in numbers, have a great selective advantage once the drug is reintroduced.

Resistance is not yet a significant problem in the control of GI nematodes of humans (Steppek *et al.*, 2006a). However, there are indications of human nematodes becoming resistant to benzimidazoles and to pyrantel. Studies in Pemba Island, Tanzania have shown that the efficacy of mebendazole against hookworms in school

children who were routinely given mebendazole has declined significantly within 5 years (egg reduction rate fell from 82.2% to 52.1%) (Albonico *et al.*, 2003). Also, in Mali mebendazole and pyrantel showed low efficacy against *Necator americanus* infections (Sacko *et al.*, 1999). Similarly, in North Western Australia, pyrantel lacked efficacy against *Ancylostoma duodenale* (Reynoldson *et al.*, 1997).

Currently, there are many programs introduced in developing countries based on the mass treatment of people with ivermectin or praziquantel, each combined either with mebendazole or albendazole. They have made a significant impact on the prevalence of intestinal parasitic infections in the regions where such treatment has been applied (Savioli *et al.*, 2004, Hotez *et al.*, 2007). In West Africa, control programs for onchocerciasis have resulted in reduced susceptibility of microfilariae of *Ochocerca volvulus* to ivermectin with high potential for drug resistance to occur (Geerts and Gryseels, 2001). This provides a vital warning for the continued use of anthelmintics as the sole method for controlling human GI nematode infections. Moreover there is increasing consumer demand for organic food rather than relying on chemically treated food, especial dairy products and meat (Behnke *et al.*, 2008).

1.6 The search for new alternative anthelmintics

The problem of resistance has led to the search for alternative or complementary methods of parasite control. There is potential for exploiting anthelmintics obtained from natural plant extracts present in fruits, leaves and seeds of plants, and which may have a different mode of action compared to the present classes of synthetic anthelmintics.

Medicinal plants have been used by indigenous people for centuries in the treatment of diseases including parasites of livestock and humans (Hammond et al., 1997, Waller et al., 2001). Some of the earliest known medicinal anthelmintics from plants include use of the oil of the chenopodium plant (*Chenopodium ambrosioides*) in the early 18th century by both the indigenous and European settlers in America, for the treatment of *Ascaris* infections (Waller et al., 2001). The dried, unexpanded flower heads obtained from *Artemisia* plants were used by Romans in the first century, for the treatment of *Ascaris*, *Enterobius* and tapeworm infections (Sollmann, 1957). *Ficus* latex has been used in Central and South America as an anthelmintic against *Trichuris*, *Taenia*, *Ascaris* and *Enterobius* (Hansson et al., 1986). In addition, papaya (*Carica papaya*) and pineapple (*Ananas comosus*) have been used to treat poultry, dogs, pigs and humans infected with intestinal parasites by the native people of Panama and South America (Behnke et al., 2008). Their extracts have been shown to be more effective than the current synthetic drugs (Keiser and Utzinger, 2008). However with the development of synthetic anthelmintics, use of these natural plant herbal remedies has declined (Waller et al., 2001). Since there is a rapid increase in resistance to synthetic drugs among the GI nematodes, it is important to go back to history and re-examine the efficacy of these traditional medicinal plants (Willcox et al., 2001) and seek ways to improve their efficacies, exploiting modern technologies.

CPs are proteolytic enzymes secreted by most tropical plants including papaya, fig, kiwi, pineapple and many other plants (O'Hara et al., 1995) as it is summarized in Table 1.2 below. The enzymes are available in different parts of the plants and they are involved in protein maturation, senescence, abscission, programmed cell death, fruit ripening and protein rebuilding in response to internal

and external stimuli (Grudkowska and Zagdanska, 2004). They are also involved in removal of damaged or unnecessary proteins and defend mechanisms against arthropod (Dussourd and Eisner, 1987) and parasite attacks (Konno *et al.*, 2004). These enzymes have been reported to possess strong anthelmintic effects against GI nematodes (Satrija *et al.*, 1994, Stepek *et al.*, 2004, Robbins, 1930).

The CP enzymes belong to the papain family (Martínez *et al.*, 2012) and structurally they are made of a single polypeptide chain of 25 kDa, which is folded to form a globular protein with two domains. There is a deep cleft in between the two domains where the substrates can bind, and where the active-site cysteine and histidine residues form a thiolate–imidazolium ion pair (Rawlings and Barrett, 1994) which mediates the hydrolysis of the peptide chain.

Their enzyme activity can be quantified based on titration using commercial available substrates, linked with the fact that they are irreversibly inhibited by L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64). E-64 is a class specific inhibitor for cysteine proteinases isolated from *Aspergillus japonicas*. It inhibits the CPs by blocking the active Sulphydryl residues (-SH), the thiol groups (Hanada *et al.*, 1978). Due to its fast reacting mechanism with CPs, it is regarded as an ideal active-site titrant. Kinetic studies have indicated that E-64 is an irreversible inhibitor of CP enzymes (Hashida *et al.*, 1980). Titration with cathepsin B resulted in a 1:1 proportional decrease in titratable thiol groups. The activity of cathepsin in increasing concentrations of E-64 indicates that E-64 reacts with an equimolar amount of active thiol of cathepsin B (Hashida *et al.*, 1980). The protocol for active site titration with E-64 was first developed by Barrett *et al.* (1981). The solutions of

papain and cathepsin enzymes were treated with a series of increasing concentrations of E-64 and assayed for activity. They found that the activity declined linearly with increasing amounts of E-64. It was possible for them to draw a straight line through the points to the abscissa. The intercept was taken to give the molar concentration of the enzyme and the slope of the line gave the specific activity of the enzyme in terms of molar concentration. The protocol was later adapted by Zucker *et al.* (1985) for papain, chymopapain and papaya proteinase III. Hence this protocol has been adopted in this study as a standard assay for the determination of the concentration of all the CPs used in this study.

However, factors such as pH, ionic strength and nature of the buffer can contribute to variation in assay readings. CPs require addition of a small amount of sulphhydryl reducing agents such as cysteine, dithiothreitol (DTT) or 2-mercaptoethanol to activate them before catalysis of the reaction (Wilk, 2001). These reducing agents convert reversibly inactive forms of enzymes to the active forms and protect their catalyzed essential thiol group from oxidation (Caygill, 1979). Thus the assays in this study were performed in the presence of cysteine in activating buffer (Barrett *et al.*, 1981) and EDTA for removal of any heavy metal which might inactivate the enzymes (Zucker *et al.*, 1985).

Table 1.2: Some plants containing CPs with potential anthelmintic activity

	Plant species	Enzymes contained
1.	Papaya	Papain, chymopapain, caricain, glycyI endopeptidase (Balls and Jansen, 1952)
2.	Fig	Ficin, ficain (Robbins, 1930)
3.	Pineapple	Ananain, fruit bromelain, stem bromelain and comosain (Rowan <i>et al.</i> , 1988)
4.	Kiwi fruit	Actinidine (Brocklehurst <i>et al.</i> , 1981b)
5.	Egyptian milkweed	Asclepain (Brockbank and Lynn, 1979)

These plants (papaya, pineapple and fig) have been used as medicinal plants by indigenous communities for treatment of GI nematodes since antiquity (Behnke *et al.*, 2008). For example the crude latex extracted from papaya trees (*Carica papaya*) was used successfully to treat ascarids, tapeworms, whipworms and hookworms in the early 19th century (Berger and Asenjo, 1940). The recognition that the anthelmintic activities in these plants are due to proteolytic cysteine proteinases has offered the possibility of utilizing these plants as anthelmintics (Stepek *et al.*, 2004). Robbins (1930) was the first person to report that the active anthelmintic principle of ficin to be an enzyme that damaged the cuticle of *Ascaris suum*, presumably by proteolytic digestion. Fresh pineapple juice was found to possess an enzyme, bromelain, which is similar to ficin, and which completely digested *A. suum* (Berger and Asenjo, 1939). In 1940 the active ingredients which digested worm cuticle from papaya latex (PL) preparations was described to be the enzyme papain (Berger and Asenjo, 1940). These three separate studies suggested a similar mechanism of action by rapid digestion of the *Ascaris* cuticle.

In vivo assessment of these plant enzymes was neglected until the 1980s when Hansson et al (1986) carried out a clinical trial involving 181 residents infected with one or more GI nematodes in South America to examine the effectiveness of latex from the *Ficus glabrata*. Upon oral administration a substantial reduction in the number of worms was achieved, although not evaluated statistically. Later Satrija et al (1994) reported a significant reduction in worm burden in pigs infected with *A. suum* and in mice infected with *H. bakeri* (Satrija et al., 1995) when treated with papaya latex.

Although encouraging results for the anthelmintic efficacy of natural plant cysteine proteinases were provided by these studies, it is not until recently, that their effect was confirmed and their mode of action was studied closely (Stepek et al., 2007a, Stepek et al., 2007c, Stepek et al., 2005, Stepek et al., 2006b). It was reported that the effects of these enzymes initially caused observable changes to the surface of the cuticle, leading to lesions, fractures, and eventually complete destruction of the cuticle and bursting of the worms. The mechanism was completely blocked by the presence of the CPs specific inhibitor E-64, and is dependent on the presence of free cysteine, in the incubation medium *in vitro* (Stepek et al., 2004).

The work was carried out *in vitro* using the latex of papaya, milkweed and figs, and extracts of pineapple fruit and stems, and the purified enzymes papain (papaya), chymopapain (papaya), and ficin (fig), and all caused detrimental effects on adult *H. bakeri* worms (Stepek et al., 2005). The more convincing evidence was the *in vivo* work with papaya latex (PL) using three rodent nematodes; *H. bakeri* (Stepek et al., 2007b, Satrija et al., 1995), *Protospirura muricola* (Stepek et al.,

2007a) and *Trichuris muris* (Stepek *et al.*, 2006b) and sheep nematode; *H. contortus* (Buttle *et al.*, 2011) where the results showed a significant reduction in worm burden and egg counts. However, kiwi fruit extract had no effect on *H. bakeri in vitro* (Stepek *et al.*, 2005) and young pineapple juice showed anthelmintic activity *in vitro* but not *in vivo* against *A. tetraptera* worms (Satrija *et al.*, 1995). Due to these contradictory results there was a need to reassess cysteine proteinases from pineapple and kiwi plant sources individually *in vitro* and if possible *in vivo* using *H. bakeri*, a natural parasite of mice and PL as a positive control.

1.6.1 Animal model used in this study

The majority of intestinal nematodes are specific to their respective hosts. Human GI nematodes or nematodes affecting domestic livestock cannot be investigated in rodents other than in exceptional models such as *Necator americanus* in hamsters. Experiments in domestic livestock are constrained with several difficulties, including high cost to set up appropriate facilities for their husbandry. The experiment may not be permitted unless the agents had been tested first in laboratory animals. Also some basic principles may need to be established first in laboratory rodents. Only then can the experiment be conducted in the animal species most appropriate for both relevance and sentience. Similarly human trials would not be permitted by ethics committees unless there was evidence of efficacy and safety from animal trials. The University of Nottingham has suitable facilities for rodent trials. For these reasons rodents and rodent nematodes were the best starting point for this research. Moreover, rodents are the lowest vertebrate group in which well characterised host-parasite relationships with minimal severity have been

developed. Therefore *Heligmosomoides bakeri* (*H. bakeri*) worms were used and the *in vitro* experiments were extended to horse nematodes.

1.6.2 Life cycle of model parasite – *Heligmosomoides bakeri*

H. bakeri is a natural parasite of *Mus* species (model of small intestinal nematodes with a developmental stage in the intestinal wall) and related to hookworms of humans (*Ancylostoma duodenale*) and trichostrongyloids of domestic animals such as *Haemonchus contortus* and *Teladorsagia circumcincta* of sheep. It is an elongated, cylindrical, unsegmented worm with tapering ends. Its body is covered with a tough cuticle and it has an internal cavity with a high internal hydrostatic pressure.

Its life cycle starts by an adult female worm laying eggs in the small intestine (Fig.1.1). These are passed to the outside environment through host faeces. The eggs hatch into first stage larvae 36-37 hours after being laid. The L1 larvae moult to the L2 larval stage 28-29 hours after hatching. After 17-20 hours the L2 undergo partial moulting giving rise to ensheathed L3 infective stage larvae. The L3 are active but non-feeding. When the L3 infect a mouse orally, they migrate to the small intestine, shed their sheath and penetrate into the intestinal mucosa and moult into L4. The worms then migrate back to the lumen and develop into the adult worms (Bryant, 1973). In general the life cycle, from egg to egg, takes about 13.5 days.

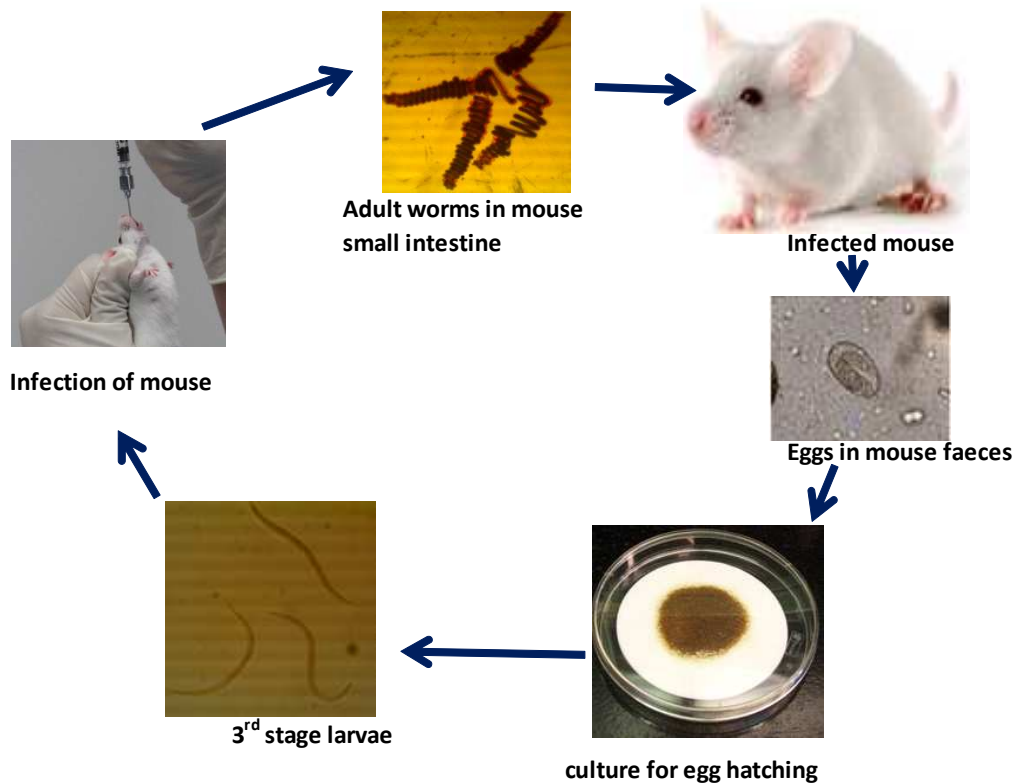


Figure 1.1: Life cycle of *H. bakeri* as maintained in the laboratory mice

The life cycle starts with collecting faeces from infected mice that are cultured for 7 days to allow the eggs to hatch and undergo a series of moults to infective L3 larvae. The L3 larvae are collected and stored in refrigerators for future infection. The infected mice develop the larvae into adult worms after 10 days when they start laying eggs which are expelled out in the faeces for a new life cycle.

1.7 Formulation and delivery of cysteine proteinases (CPs)

PL has been demonstrated to have an anthelmintic effect against nematodes of mice such as *H. bakeri* (Steppek *et al.*, 2007b, Satrija *et al.*, 1995), *Trichuris muris* (Steppek *et al.*, 2006b), *Protospirura muricola* (Steppek *et al.*, 2007a) and that of sheep, *Haemochus contortus* (Buttle *et al.*, 2011).

The results of Buttle *et al* (2011), indicated that for the PL to be more effective it requires an extended period of time for the active enzyme to damage the worms. This was demonstrated by the failure of a single dose in comparison with a regimen of 4 daily doses. This suggested that enzymes could show more efficacy if the contact time with worms was extended. Therefore for the enzyme to be safe and effective delivered for human and livestock GI nematode treatment, various formulation and delivery methods have to be reviewed before selecting the appropriate one.

1.7.1 Drug formulation

Drug formulation is the process in which different ingredients, active drug and excipients, are combined to produce a final medicinal product. It is the initial stage in the rational development of a dosage form (Undralla *et al.*, 2011) which then involves testing and modifying of the drug release profile, to provide optimum absorption, distribution and elimination characteristics. The overall aim is to improve efficacy and safety, as well as patient convenience and compliance. The formulated drug can be administered through a variety of routes including orally, nasally, buccally, rectally, topically or by inhalation. However, oral administration, in which therapeutically active substances are taken by mouth, remains by far the most popular route of drug delivery (Melia and Davis, 1989).

Different formulations and delivery systems have been employed to improve the efficacy and safety of the drugs in both humans and livestock. This includes a wide variety of liquid, semi-solid and solid based systems in formulations designed to be appropriate for their route of administration.

1.7.2 Liquid-based formulations

Liquid dosage forms commonly used for both human and livestock include oral solutions, suspensions and gel or pastes. They are prepared by dissolving or dispersing the active ingredients in aqueous or non-aqueous media. Liquid dosage forms are easier to swallow and usually faster acting than solid dosage forms as the active substances become available for absorption or contact with pathogens immediately after swallowing (Blackburn, 2010). In ruminants, the reticular groove closure allows oral solutions to flow directly into the abomasum by-passing the reticulo-rumen. This provides an immediate release of the drug into the abomasums and hence further down into the intestine (Grovm and Chapman, 1988). In cattle it can also be induced by administering sodium bicarbonate solution, or in sheep, by administering orally a copper sulphate solution or injecting intravenously a dose of lysine–vasopressin (Baggot, 2007). Liquid dosage forms provide a suitable means of drug administration not just to adults, but also to neo-nates and young animals (Parasrampur and Pitt, 2007).

Oral liquid drug formulations intended for livestock although convenient, require frequent dosing. This is demanding on the farmer when treating their animals, for example if they are on pasture they will need to frequently round up. They also pose a hazardous risk of dosing the liquid to the lungs instead of the stomach or may be spilled. They require careful measuring before administration, or have special storage or handling requirements such as refrigeration or shaking before use (Kockaya and Wertheimer, 2011). Drugs can have a bad taste which is difficult to mask. In the case of CPs used in the current study, there is a possibility of losing activity of the enzymes before being administered if they are in liquid form.

1.7.3 Solid based formulations

Oral drugs are more conveniently administered in solid form, as tablets, capsules, powder, premix or medicated blocks. Tablets are composed of one or more active substances usually with various excipients. These include diluents, binders, disintegrating agents, glidants and lubricants. They may also include substances capable of modifying the behaviour of the preparation in the digestive tract (Melia and Davis, 1989). Tablets have many advantages including easy self-administration and convenient, packaging, handling, and shipping, storing and having longer shelf life. The dosing is more accurate (single dose) and they are suitable for sustained release preparations. However, their preparation needs expensive machinery and some patients (e.g children and elderly people) may have difficulty swallowing them (Blackburn, 2010). Tablets are not fast enough for immediate action (injection is required) but they are highly convenient and therefore the most popular form of human medicine. However tablets are less popular for animals due to difficulties in administration as they require a special 'bolus gun' to make sure that they are placed deep enough to be swallowed by the animal.

Capsules are solid dosage forms in which the drug is contained in a water soluble outer shell they contain a mixture of the active and inactive ingredients. The most common type of shell is gelatin shells (hard or soft) which can be filled with powdered, pasty, or liquid medications. They are usually prompt release, but also can be enteric coated or sustained release. One advantage of a hard gelatin capsule for use in livestock is that it can be broken open and its contents spread over a food substance or into water before administration. Also it can mask the taste and odour of the active ingredient.

Powders and granules are mixed with other powdered excipients to produce final or aggregated products for oral administration. Powders and granules have better chemical stability than liquids and dissolve faster than tablets or capsules, because there is no need for disintegration. In livestock drugs which are in powder form or prepared granules can be easily added to the feed. The disadvantages of this method of administration are the unreliability in the amount of dose ingested by the animal. It needs the drug to have a wide margin of safety and palatability in the feed and that the animal must be feeding. Sick animals often eat less and are therefore less amenable to treatment with in-feed powder formulations (Baggot, 2007). This method can be improved by packaging of powders or granules in unit dose sachets. This provides convenience of drug administration for owners when the number of animals to be dosed is small. Drug powders are principally used prophylactically in feed, or formulated as a soluble powder for addition to drinking water or as milk replacer. Since PL is in powder form it would be possible for it to be added into feed, but it might not provide the expected results as its efficacy is dose dependent (Stepek *et al.*, 2006b) and hence its effectiveness will depend on the amount consumed by animals.

Premix and medicated blocks are specifically prepared for livestock. Premix is a homogeneously mixed product of active ingredient with feed at rates that range from few milligrams, to grams of drug per ton of feed. The common premix formulations administered to poultry, pigs, and ruminants, include coccidiostats, production enhancers, and nutritional supplements. Several factors need to be considered in the design of premix formulations and to ensure their satisfactory mixing in bulk feed. Premixes must always be diluted to the approved use level,

usually parts per million (ppm) [g/tonne (feed) or mg/L (water)], as appropriate for the animal species. For example IVOMEC® Premix[®] which is a free-flowing meal mixture containing 0.6% ivermectin for incorporation into animal feeding stuffs, is used for the treatment of pigs against mange mites, lice and worms.

Unlike the premix, medicated blocks are a compressed feed material that contain an active ingredient, such as a drug, anthelmintic, surfactant (for bloat prevention), or a nutritional supplement. It is commonly packaged in a cardboard box (<http://www.merckvetmanual.com>). Medicated blocks need to be non-toxic, stable, palatable, and of low solubility. Palatability can be improved by the addition of excipients like molasses. Since blocks are palatable, they need to be offered in a suspended position so that animals can lick, but not bite on the block, preventing the animals from eating large amounts at a time.

1.7.4 Tablet classification

As reported earlier tablets are by far the most popular among all solid dosage forms used for humans because of their convenience of self administration, compactness and easy manufacturing. According to United States Pharmacopeia (USP) tablets are classified based on the drug release rate either immediate release (Conventional) tablet or modified release tablet which includes extended and delayed release (Fig. 1.2).

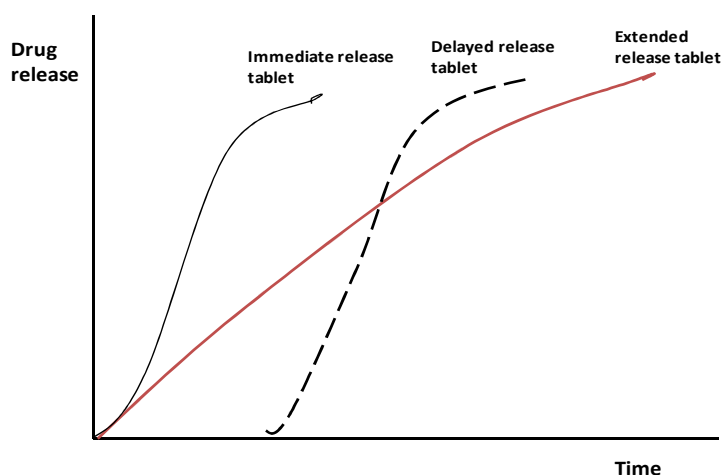


Figure 1.2: Drug release rate from different types of tablets

The figure shows the change in drug release rate patterns for immediate, delayed and extended release tablets.

1.7.4.1 Immediate release tablet dosage forms

In immediate release tablet, drug is intended to be released rapidly after administration, or the tablet is dissolved and administered as solution (Hyder and Sharma, 2011). The basic components of immediate release pharmaceutical tablets are active drug substance, diluents or filler, binders, disintegrants and lubricants (Fukami *et al.*, 2006).

In general, immediate release formulations are applied both to conventional tablets, which release the drug in the stomach and to those which are orally disintegrating tablets (ODTs) that dissolve in the patient's mouth. Usually a polymer or sugar coating is applied to increase patient compliance by creating a smooth, glossy finish that enables easier swallowing compared with the uncoated cores. Coating also provides mechanical protection and colour identification which helps patients differentiate between tablets when taking multiple medications (Bebber,

2011). In animals immediate release tablets are used to administer drugs to dogs and cats

Immediate release tablets disintegrate soon after swallowing as a result of the disintegrants within them. There are many disintegrants have been used but the most common are starch and cellulose and their derivatives (Govedarica *et al.*, 2011). Their mechanisms of action involve swelling or wicking, and these create either a disintegrating internal force or a wet network which dissolves soluble components of the matrix. Modern derivatives are often termed super-disintegrants (see below). These are effective at lower concentrations and impart greater disintegrating efficiency or mechanical strength to the tablet. They are chemically modified starches and cellulose, and are often obtained by crosslinking the polysaccharide/polymer chains and introducing carboxymethyl substituents (Zhao and Augsburger, 2005). Some common superdisintegrants used in this study are described below:-

1. Sodium Starch Glycolate (Primojel[®], Explotab[®]).

This is synthesized by cross-linking of the potato starch using an FDA approved starch esterifying agent in alkaline suspension, along with carboxymethyl substituents. The effect of crosslinking is to reduce both the water soluble fraction of the polymer and the viscosity of dispersion in water (Edge *et al.*, 2002). The carboxy methyl substituents elicit rapid water uptake by the polymer causing swelling with minimal gelling. The recommended concentration to be used is 2-8% with an optimum of 4%.

2. Low-substituted hydroxyl propyl cellulose (L-HPC[®]).

It is prepared from purified wood pulp cellulose and is insoluble in water. It rapidly swells in water to a degree dependent on the level of substitution, with grades LH-11 and LH-21 exhibiting the greatest degree of swelling (Kiyose *et al.*, 2007). Certain grades can also provide tablet binding properties while retaining disintegration capacity. The recommended concentration is 1-5%.

3. Croscarmellose sodium (Ac-Di-sol[®])

Croscarmellose sodium is cross linked carboxymethylated cellulose. It has high wicking and swelling with minimal gelling ability due to retaining its cellulose fibrous structure. Each fibre can act as a hydrophilic channel to facilitate water uptake into the tablet matrix and help increase the total water contact area with drug. The effective concentration is 1-3% for direct compression tableting and 2-4% for wet granulation (Zhao and Augsburger, 2005, Hyder and Sharma, 2011).

In selecting disintegrants there is no particular upper limit regarding the amount to be used as long as the mechanical properties of the tablet are compatible with its intended use (Mohanachandran *et al.*, 2011). However, it is sometimes necessary to evaluate different kinds of disintegrants in order to get a suitable disintegrant which combines excellent compatibility with disintegrating properties.

1.7.4.2 Enteric coated (delayed release) tablets

Enteric coated tablets are intended to release a drug after the tablet has passed through the stomach to other parts of the GI tract (Undralla *et al.*, 2011). Other

delayed-action tablets can be designed to release in the ileum or the colon. Enteric coating is aimed to protect the drug from gastric fluid in the stomach or *vice versa* and release the drug in the duodenum. Enteric coatings are used because some drugs are unstable in the low pH of the stomach (e.g, erythromycin) whilst others if released in the stomach may cause irritation, nausea or vomiting (e.g aspirin), Other drugs need to be delivered and released at high concentration in the intestine for absorption or local action.

The coatings used to produce enteric effects are natural modified polymers which are insoluble in acidic media but dissolve at neutral pH. The most commonly used are i) cellulose such as cellulose acetate phthalate, hydroxypropylmethyl cellulose acetate succinate and hydroxypropyl methylcellulose phthalate ii) polyvinyl acetate phthalate (PVAP) and iii) polymethacrylates such as Eudragit® L and S. All these types have the common feature of carboxylic or phthalic acids, sometimes in partially esterified forms, which remain unionised and insoluble in gastric acid but dissolve when the tablets leave the stomach as the pH increases and their substituent groups ionise (Chien, 1989). For this reason enteric coating is not suitable for drugs intended to be delivered in the stomach like the CP enzymes which are being developed to treat GI nematodes residing in the abomasums of sheep.

1.7.4.3 Extended release dosage forms

The United States Pharmacopoeia (USP) defines an extended release (ER) dosage form as one that allows at least 2-fold reduction in dosing frequency or a significant increase in patient compliance or therapeutic performance when compared with a conventional dosage form. ER dosage forms are used when it is desirable to have

drugs slowly and consistently released over an extended period of time, instead of all at once (Blackburn, 2010). The terms sustained-release, long acting, controlled-release, slow release or prolonged release have been used synonymously with extended release (Tiwari and Rajabi-Siahboomi, 2008) and although the exact meaning of these terms may differ in some respect, they all imply the gradual release of drug over a longer period of time than standard dosage forms. Oral tablets and capsules are the most common dosage forms that are formulated as extended-release (Alderman, 1984).

ER dosage forms have demonstrated to improve by maintaining consistent flow of drug throughout the given time period. The advantages of ER dosage forms include better control of blood level profiles, minimization of side effects and reduction in the number of dose administrations and (potentially) better patient compliance. Cost of medication is reduced as patients require fewer doses but this is often offset by a more prolonged period of formulation development and testing (Brennan *et al.*, 2007, Fernandes *et al.*, 2003).

1.7.4.4 Technologies used for ER in livestock

The most common technologies used to achieve controlled drug release in ruminants are intraruminal devices. According to the European pharmacopeia (2005) intraruminal devices are solid preparations containing one or more active substances intended for oral administration to ruminant animals and are designed to be retained in the rumen to deliver the active substance(s) in a continuous or pulsatile manner. Some intraruminal devices are intended to float on the surface of the ruminal fluid while others are intended to remain on the floor of the rumen or reticulum. For

continuous release, the intraruminal device is designed to release the active substance at a defined rate over a defined period of time. This may be achieved by erosion, corrosion, diffusion, osmotic pressure or any other suitable chemical, physical or physico-chemical means. Different types of intraruminal devices have been developed. These include erodible, reservoir, dispersed matrix and osmotic boluses.

i) Erodible boluses

These are boluses designed to dissolve or abrade under the mechanical action of the rumen. The boluses can be made from drug, carnuba wax, barium sulphate, polyethylene glycol and iron powder. Marston (1962) was the first person to develop an erodible bolus containing cobalt oxide (a trace nutrient for ruminants) and other diluents. The bolus was able to release cobalt throughout the grazing season. It was found that a density of greater than 2.0 was sufficient to prevent bolus regurgitation in cattle. Other examples include spanbolet[®] II which is available for controlled release of sulphate drugs, and release of oxytetracycline and S-methoprene from erodible boluses. Cardinal (1997) has recommended that erodible devices are best utilized for delivery of nutrients than drugs, where therapeutic indices are usually much broader.

ii) Reservoir systems

One of the earliest successful products for the treatment of parasitic infestations once per season was the Paratect flex[®] bolus (Pfizer, USA). This product was designed to provide continuous administration of the anthelmintic morantel tartrate. The system is composed of a stainless steel cylinder which is about 4 inches in length and 1 inch in diameter. It is capped at each end with porous polyethylene disks impregnated

with cellulose triacetate. This creates a reservoir within the cylinder which is filled with a mixture of drug plus polyethylene glycol. Drug release occurs over 90 days, making it highly effective in season-long control of GI roundworms (Borgsteede, 1983).

iii) Dispersed matrix

A dispersed matrix device is defined as one in which the active ingredient is dispersed within a non-biodegradable polymer matrix. It is designed to treat cattle with prophylactic doses of an anthelmintic intended to prevent the establishment of GI worms throughout the entire grazing season (Rothen-Weinhold et al., 2000). The device is in the form of a large sheet consisting of a trilaminate design wherein a core matrix containing a 50:50 mixture of morantel tartrate (anthelmintic) and ethylene vinyl acetate (EVA) and is coated on both of its outer surfaces (but not the edges) with a layer of pure EVA which is impermeable to the drug. The sheet is rolled up and adhered by water soluble tape to form a cylinder for administration. Release of drug occurs via diffusion within water filled channels of the porous core matrix to the uncoated edges. In the rumen, the adhesive dissolves and the device unrolls and its dimension increases preventing it from being regurgitated. This technology has been commercialized and is sold in Europe under the trade name of the Paratect Flex[®] bolus.

iv) Osmotic boluses

Osmotically controlled drug delivery systems utilize osmotic pressure as a driving force for controlled delivery of the active agent. It consists of an outer semi-permeable membrane enclosing a metal density element at one end, an osmotic

energy source at the other end, and the core containing the drug in the centre. In contact with the aqueous fluids, it absorbs water at a rate determined by the fluid permeability of the membrane and osmotic pressure of core formulations. Absorption of water through the semi-permeable membrane causes expansion of the bolus, which drives the drug through the exit port (Verma *et al.*, 2002).

The technology is appropriate for the delivery of parasiticides, insecticides, nutritional supplements, antibiotics, growth promoters, repartitioning agents and oestrus suppressants. It can provide controlled delivery of a drug for up to one year in the rumen of cattle and sheep. The technology was originally developed by ALZA for the OROS[®] for water soluble drugs and Push-Pull[®] systems for highly aqueous insoluble drugs (Cardinal, 1997). This effort resulted in development of the IVOMECS SR Bolus[®] which delivers the parasiticide ivermectin to cattle for 135 days (Rothen-Weinhold *et al.*, 2000).

v) Pulsatile boluses

In treatment of cattle or sheep with anthelmintics, the main concern is the development of resistance. One of the methods used to minimize this problem is through pulsed dosing, wherein therapeutic levels are administered on a regular basis, followed by periods of no drug delivery. Holloway (1982) designed a device which has a series of drug compartments separated by degradable cellulosic partitions. Each partition degrades on exposure to ruminal content. As successive partitions degrade, drug is released periodically.

vi) Systems to by-pass the rumen

These oral delivery systems are intended to pass through the rumen without releasing the drug and then the drug is released in the lower GI tract in areas such as the abomasum. One application for this technology is the delivery of methionine, which is degraded by exposure to the microbial flora of the rumen. To prevent drug release in the rumen, polymers are sometimes used that prevent the dissolution of the coating in the rumen fluids, similar to preparation of enteric coated dosage forms for human applications. Such coatings lead to significantly higher blood levels of methionine, which lead to great increase in wool production from treated animals (Cardinal, 1985).

vii) Hydrophilic matrices

Many current extended drug release systems are based on hydrophilic polymers which swell forming a surface 'gel' barrier of hydrated polymer, which retards drug release. The mechanism of drug release is complex but it is based on the diffusion through and erosion of the gel (Tiwari and Rajabi-Siahboomi, 2008, Melia, 1991). Water diffuses into the matrix, resulting in polymer chain relaxation with volume expansion, wherein the incorporated drug dissolves and diffuses or is eroded out of the system (Siepmann *et al.*, 2002, Li *et al.*, 2005).

The perceived advantages associated with hydrophilic matrices over other ER technologies, include simple formulation, a wide range of drug release profiles, use of existing tableting processes and the low cost of polymers, which are generally regarded as safe (GRAS). Many swellable polysaccharides are available, allowing flexibility for the needs of an individual formulation. Hydrophilic matrices are

erodible and therefore, unlike inert matrices, they reduce in size and dissolve as they pass through the GI tract. Swellable matrices are also suitable for other administration routes, including buccal, vaginal and rectal delivery (Melia, 1991).

The hydrophilic matrices utilise three groups of retarding agents, a) Cellulose derivatives which include methylcellulose, hydroxyethylcellulose, hydroxypropylmethylcellulose (HPMC) and sodium carboxymethylcellulose, b) Non-cellulosic gums and polysaccharides include guar gum, xanthan gum, sodium alginates, pectin, chitosan and modified starches and c) other swellable materials such as carbomers, copolymers of acrylic acid, and polyethylene oxide.

1.7.4.5 Factors to be considered in ER matrix formulation

Drug solubility, intrinsic dissolution rate and dose are the most important factors to be considered in the design of ER matrices and they play a major role in the eventual drug release profile. Tiwari and Rajabi-Siahboomi (2008) reported that to formulate ER dosage form with high dose and extreme solubility is very challenging because the drug dissolves within the gel layer and diffuses out into the matrix very easily and this is common with HPMC hydrophilic matrices. In this case, use of high viscosity grade HPMC (Methocel K4M CR, K15M CR OR K100MCR) tends to generate consistency of diffusion-controlled systems. In addition hydrophilic matrix formulation with high amounts (doses) of drugs is particularly challenging because of the small quantity of polymer required to limit the size of the tablet to one that is swallowable.

Polymer levels are a key factor in controlling the drug release rate. Use of 20-50% w/w is recommended for HPMC and 20% for xanthan gum, although 5% of xanthan gum has been reported to provide sustained release of an insoluble drug such as ibuprofen into gastric fluid (Melia, 1991). More prolonged extended drug release profile is achieved with finer particle size polymers because hydration rate is faster and this has led to the marketing of specific ER grades of HPMC. The effect of tablet compression force on drug release is minimal when tablets are made with sufficient strength and optimum level of polymers are used (Velasco *et al.*, 1999). Tablets with smaller size require higher polymer content because of their higher surface area to volume ratio and thus shorter diffusion pathway.

Coating of hydrophilic matrices with water-soluble polymers does not usually alter the extended drug release profile, but water insoluble polymers (e.g ethylcellulose) may modulate the drug release profile. The same also applies to fillers and other excipients if used. However, the effect depends on the drug, the polymer level and level of excipient (Levina and Rajabi-Siahboomi, 2004). Since the majority of ER dosage forms are designed to remain in the GI tract over a prolonged period (Williams *et al.*, 2009), the formulation of CPs in this dosage form would be appropriate as the enzymes will have enough time to damage the worm cuticle. However various matrices need to be evaluated before selecting the suitable one.

1.8 Hypothesis, Aims and Objectives of the study

The work described in this thesis is based around the central hypothesis that plant derived cysteine proteinases have anthelmintic activity and that this can be harnessed to produce an effective medication for use in livestock and humans. As with all

biological materials, the anthelmintic efficacy of plant derived CPs is likely to be affected by a variety of host intrinsic and extrinsic factors, and the formulation in which the drugs are made available to hosts will also affect their efficacy. This thesis is concerned with evaluating the principal factors in each case in order to refine and improve the delivery and prospects for effective removal of worms from infected animals.

Objective 1

The first objective of this study was to determine the preparation method of PLS and storage conditions for long term survival of the enzymes. Since it was not clear which method of preparation would provide high yield of CPs in PLS and under what storage conditions would maintain the stability of the active enzymes for prolonged period of time.

Objective 2

The second objective of this study was to evaluate the anthelmintic efficacy of CPs from sources other than papaya latex, such as pineapple fruit, stem and kiwi fruit extracts. In earlier studies it has been shown that cysteine proteinases have powerful anthelmintic effects on GI nematodes by removing more than 90% of the parasites in mice (Steppek *et al.*, 2005, Steppek *et al.*, 2007b, Satrija *et al.*, 1995) and in sheep (Buttle *et al.*, 2011). However throughout the studies the source of the enzymes used was from papaya latex. The effectiveness of CPs from other sources of related plants has been little studied. .

Pineapple fruit and stem extracts have only been studied *in vitro* and been shown to be effective against rodent nematodes (Stepek *et al.*, 2005). This justifies investigation of their effectiveness *in vivo*. On the other hand kiwi fruit extract was shown to have no detrimental effect on worms; however it belongs to the same papain C1 family CPs. There was a need therefore to verify why it behaves differently from other CPs by repeating the *in vitro* trials under more refined conditions.

Objective 3

The third objective was to assess the effect of fasting on the anthelmintic efficacy of papaya latex supernatant (PLS). This is because in earlier studies with CPs, a period of food deprivation was routinely employed before administration of CPs, but there has been no systematic evaluation as to whether this does actually benefit the anthelmintic efficacy of PLS or not. The information will be helpful in formulating the optimal protocol for delivery of the drug to animals or humans, in order to obtain maximum efficacy against the worms with the lowest possible dose.

Objective 4

The fourth objective was to examine whether the efficacy of PLS treatment is host genotype dependent. In earlier work with CPs, only two strains of mice were tested. These were the inbred male C3H strain (Stepek *et al.*, 2007a, Stepek *et al.*, 2007b) and female BALB/c strain (Satrija *et al.*, 1995). There has been no systematic study comparing different mouse strains of known contrasting genotype. If the efficacy of PLS is dependent on host genotype, differences in efficacy might be

expected between animals within breeds on farms where the therapy has to be applied. Thus the extent of variation arising from genetic differences between animals is an important issue to be assessed.

Objective 5

The last objective was to formulate and design an appropriate delivery system of the CPs from papaya latex to humans and livestock. As it has been stated early CPs from PL have been demonstrated to have high efficacy against GI nematodes. There is a need therefore to develop the enzyme preparations into an acceptable medication that can be sold commercially to end users. However, for it to be acceptable for commercial use, it has to be formulated into a safe, convenient and compliant way of delivery to animals or humans. Thus there was a need to find out the most suitable ways of delivering the drug to animals particularly sheep. In this study two delivery methods were evaluated:

- **Extended release hydrophilic matrix system.** The enzyme mixed with polymer was made into tablets and *in vitro* assessed for its slow release. The idea behind this was that the tablet should settle in the rumen where it should act as a reservoir dissolving the tablet in the rumen fluid and slowly releasing the enzyme to the lower gut. The major advantage of this mechanism is that large amounts of the drug can be given to animals at a time while extending the release time in this way reducing the frequency of drug administration. Two hydrophilic matrix polymers, xanthan gum (XG), and hydroxypropyl methylcellulose (HPMC) both low and high viscosity were investigated based on their ability to induce slow release while maintaining the enzyme activity.

- **Immediate drug release system.** It was not clear whether the slow extended release system would provide the optimum desired drug release rate, and so it was necessary to have another formulation equivalent to liquid dosage form but with minimum drug administration problems. In this case tablets with three types of disintegrants primojel, L-HPC and Ac-Di-sol were evaluated to determine their effectiveness in inducing fast release of CPs from these tablets.

CHAPTER 2: MATERIALS AND METHODS

2.0 Summary

This chapter summarizes the general materials and methods used for enzyme preparations, *in vitro* and *in vivo* assessment of the anthelmintic efficacy of the cysteine proteinases (CPs) and the papaya latex supernatant (PLS) formulation and delivery experiments. Details of the exact experimental design for each individual experiment are explained in relevant sections of each experimental chapter.

2.1 Enzyme preparations

The enzymes used for both *in vitro* and *in vivo* experiments were PLS from *Carica papaya* (containing a mixture of papain, chymopapain, glycyI endopeptidase and caricain), stem and fruit bromelain from the stem and fruit (respectively) of the pineapple plant (*Ananas comosus*) and actinidain from kiwifruit (*Actinidia chinensis*) prepared as explained below. All these preparations were stored at -80°C until required. Upon thawing the concentration of active cysteine proteinase was determined by active-site titration with the cysteine proteinase specific inhibitor E-64, and their concentrations were adjusted according to the exact requirements of the experiments.

2.1.1 Papaya latex supernatant

PLS was prepared by dissolving 4 kg of *Carica papaya* spray-dried latex purchased from Enzymase International S.A (Brussels, Belgium) in 12 litres of water. Since the spray dried latex is hazardous to the respiratory system (Flindt, 1978), the process

was carried out in a fume cupboard. The preparation was centrifuged at 17,700 x g at 4°C (Beckman model J2-21 centrifuge Rotor, UK) and the pellet was discarded. The supernatant was concentrated to a third of its original volume by placing it in dialysis tubing with MW cut-off 3,500 (SpectraPor 45 mm diam.) over polyethylene glycol 20,000. The concentrated PLS was aliquoted into individual vials and stored at -80°C. Throughout this study the preparation is referred to as the papaya latex supernatant (PLS).

At each step of the purification the molar concentration of the active cysteine proteinase was measured by active site titration with L-trans-epoxysuccinyl-leucylamido (4-guanidino) – butane (E-64) (Sigma-Aldrich and Apollo Scientific Ltd, UK) with 4 mM L-cysteine as a reducing agent and α -N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) was used as the synthetic substrate for PLS enzymes. The initial activity in 4 kg was 70 mmol of active enzyme. However, this amount was reduced by about half due to loss of activity during processing and freezing at -80°C. The estimate of lost activity is based on the assessment of the activity remaining in the individual thawed vials.

2.1.2 Pineapple fruit and Kiwi fruit extracts

Pineapple fruit extract (containing fruit bromelain) and kiwi fruit extract (containing actinidain) were prepared from pineapple fruit and kiwifruit juices respectively. The ripe fruits were purchased from Sainsbury supermarket, U.K and were peeled. Their fruit portion was cut into small pieces and crushed in a laboratory juicer (Philips juicer HR 1861) and filtered. The filtrate was then subjected to vacuum filtration to remove the remaining fine debris (the process was conducted under ice whenever

possible to avoid enzyme autolysis). The filtered juice was divided into two portions. The first portion of the juice was directly concentrated by dialysis using MW cut-off 3,500 dialysis tubing (SpectraPor 45 mm diam.) over polyethylene glycol 20,000. The concentrated juice was aliquoted into vials and stored at -80°C for future use.

The second portion of the juice was slowly mixed with 2/3 of its volume of cold acetone for enzyme precipitation. The acetone mixture was filtered using fresh filter paper and the waste filtrate was discarded. The collected precipitate was further washed with 100 ml of acetone. Then the filter paper was gently removed and kept in the fume cupboard to allow the acetone to evaporate and the filter paper to dry out. The dried precipitates were scrubbed and dissolved into 100 ml of 1 mM EDTA, dialysed and kept at -80°C.

2.1.3 Stem bromelain

Stem bromelain was obtained from Hong Mao Biochemical Co. Ltd, Thailand, kindly given as a gift. This was a dried stem pineapple juice preparation. Two hundred grams of stem bromelain were dissolved in 1 litre of distilled water and centrifuged at 17,700 x g at 4°C and the pellets were discarded while the supernatant was dialysed, aliquoted into vials and stored at -80°C. Individual vials were thawed and assessed for enzyme activity before use.

2.1.4 Active site titration

The concentration of active cysteine proteinase (CPs) from different preparations were determine by active-site titration adapted from Barrett *et al.* (1981) and Zucker

et al. (1985). The samples were titrated with increasing concentrations of the cysteine proteinase specific inhibitor, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64). The enzyme solution (10 μ l) was added to 250 μ l of activating buffer (0.2 M Na₂H/NaH₂PO₄ buffer/16mM L-cysteine, pH 6.85). From 0 to 100 μ l of 20 μ M E-64 were added to 11 tubes (at either 5 or 10 μ l increments) and 1mM EDTA (Ethylenediaminetetraacetic acid) was added to prevent reaction of enzyme with heavy metals and to make up a total of 1ml of final assay volume. The mixture was vortexed and incubated at 40°C for 15 min to allow the E-64 to bind with enzyme active sites. Depending on the source of the CPs 12.5 or 25 μ l of 10mM benzoyl-arginyl-p-nitroanilide (BAPNA) (Bachem Ltd, UK) substrate were added to PLS, carbobenzyloxyarginylarginyl-*para*-nitroanilide (Z-Arg-Arg-pNA) (Bachem Ltd, UK) to stem bromelain and Z-Phe-Arg-pNA (Bachem Ltd, UK) to pineapple fruit bromelain and actinidain solutions. Each of these substrates can be easily cleaved by its specific enzyme to release a yellow 4-nitroaniline compound, the concentration of which can be determined by spectrophotometry. After 15 minutes of incubation with the substrate the reaction was stopped by the addition of 1 ml of 0.1 M stopping buffer solution (sodium chloroacetate/ 0.2M sodium acetate buffer). The concentration of 4-nitroaniline released was then determined at 410 nm (Mole and Horton, 1973). Since E-64 inhibition results in a molar 1:1 stoichiometric inactivation of CPs, a linear decrease in the concentration of 4-nitroaniline released with increase in concentration of E-64 resulted. The intercept of the line with the abscissa was the molar concentration of the enzyme.

2.2 *In vitro* experiments

2.2.1 Parasites

The parasites used throughout this study were the rodent specific GI nematode, *Heligmosomoides bakeri*. The parasites have been maintained at the University of Nottingham in laboratory mice since 1976. For the *in vitro* studies BKW mice were purchased from B & K Universal Ltd, UK at 5 weeks of age and were infected at 6 weeks of age by oral gavage with a suspension of 300 *H. bakeri* L3 larvae in 0.2 ml of purified water. From day 14 post infection worms were already matured enough for use. Faecal samples were collected from the mice for egg counts to establish whether the mice were successfully infected with the parasites before the animals were sacrificed. The mice were then killed by exposure to increasing concentrations of CO₂ and dissected. Their small intestines were removed in their entirety and opened longitudinally with a pair of blunt-ended dissecting scissors. The intestines were placed into a Petri dish containing pre-warmed Hanks' Balanced Salt Solutions (HBSS) and placed in bench incubator at 37°C for about 10 min to allow the worms to detach from the intestine. Adult male and female worms were picked out with the help of fine forceps under the microscope and washed in pre-warmed HBSS before being used for motility assays.

2.2.2 Effect of CPs on the motility of adult worms

Adult male and female worms were transferred to 48-well plates (1 worm/well) containing HBSS (without phenol red), pH7.2 and 16mM L-cysteine and one of the following enzyme preparations: 0-3000 µM PLS, which was used as a positive control, 0-4000 µM stem bromelain, 0-150 µM pineapple fruit extract and 0 - 50 µM

kiwifruit extract. The control wells were incubated in parallel and contained no enzyme, no inhibitor, no cysteine, or enzyme which had been pre-incubated with E-64, in combinations as specified.

The worms were incubated at 37⁰C for 2 h and their motility was recorded every 15 minutes using a standard motility scale from 0–5 adapted from Stepek *et al.* (2005) where 0 was given to completely motionless worms not responding to manual stimulation, 1- movement only when prodded, 2 – active only in the tip ends, 3 – slowly spontaneously active, 4 - more active and 5 - highly active.

2.2.3 SEM for worms exposed to variety of plant CPs.

Adult *H. bakeri* worms were incubated in 48 well plates containing either 200 μM PLS+L-cysteine, 200 μM stem bromelain+L-cysteine, 150 μM pineapple fruit and 50 μM kiwi fruit extract+L-cysteine or HBSS. At 0, 30, 60, and 90 min worms were removed from each well plate and placed into bijoux containing 2.5% glutaraldehyde. After 1 h the glutaraaldehyde was carefully pipetted off and replaced with 0.1 M phosphate buffer and left for 1 h. The worms were then prepared for scanning electron microscope (SEM) by removing the phosphate buffer and fixing them in 1% osmium tetrachloride for 1 h (this was done in the fume cupboard). The osmium was removed and the worms were washed 3 times with distilled water and dehydrated in increasing ethanol concentration from 30-100%. The specimen were transferred to a metal basket boat (care was taken not to allow the specimen to dry) and placed into a critical point drying (CPD) apparatus in which ethanol was replaced by CO₂ and the specimens were dried with their features intact. The dried specimens were then

carefully mounted on disc stubs with quick-drying silver paint (Agar Scientific Limited, Essex, UK) and sputter coated with gold before examination in SEM.

2.3 *In vivo* experiments

2.3.1 Parasites

The source of the parasites used for *in vivo* experiments came from BKW mice previously infected with *H. bakeri* worms (between 14-30 days post infection). Mice were placed on a grid in the cage suspended over moistened tissue paper placed in the bottom of the cage in the evening and left overnight. The next day morning faecal samples were collected and brought into the laboratory and mixed with about equal amounts of activated carbon charcoal pellets. Distilled water was added and gently mashed into a paste, care being taken not to make it too wet. Then the paste was mounted at the centre of a damp 11 cm filter paper in a plastic Petri dish (Fig 1.2). The Petri dishes were then placed in a plastic container lined with moistened tissue papers and covered with a lid but not tightly leaving a space to allow exchange of air and were then incubated at room temperature for 7 days. This period was sufficient for eggs hatching and to enable the larvae to complete their free-living development stages to the infective L3 stage. The L3 larvae descend from the faecal slurry onto the filter paper and accumulate below it. These accumulated L3 larvae were washed off with distilled water from the underside of the filter paper and were collected into a clean bottle, which was covered and kept at 4°C in preparation for infection of mice.

2.3.2 Preparation of *H. bakeri* L3 larvae for infection

The number of fresh L3 larvae required for mice infections was estimated in 0.1 ml aliquots pipetted from the larval suspension placed on magnet stirrer and being gently stirred. Each 0.1 ml sample was emptied from the syringe, drop by drop onto a clean Petri dish, and the larvae in each drop were counted with the help of a tally counter under a dissecting microscope. The total number of larvae was doubled to obtain the number of larvae in 0.2 ml required for mouse oral gavage. If the number of suspended larvae in a 0.1 ml sample did not reach the required concentration for infection, the larvae were left to settle down for some time and an appropriate amount of supernatant was drawn off to concentrate the remaining larvae and then more aliquots were counted to confirm that the required concentration had been achieved. If the number of suspended larvae was more than the required number, a suitable amount of water was added to dilute the larval suspension. Although mice can tolerate quite heavy *H. bakeri* worm burdens it was necessary to be extremely careful in choosing the appropriate dose of larvae to avoid distress to the animals. In this study the doses of larvae used for infection did not exceed 300 L3, this level of infection being associated with a minimal stress in accordance with the Animal Procedure Acts (1986).

2.3.3 Animals

Eight strains of mice were employed including BKW (B&K Ltd, UK), C3H (Charles River Ltd, UK and some from Harlan, UK), NIH (Harlan, UK), NIH swiss (Harlan, UK), C57/BL (Charles River Ltd, UK), BALB/c (Charles River Ltd, UK), CBA/ca (Charles River Ltd, UK) and CD1 (Harlan, UK). The animals were purchased at the

age of 5 weeks and infected at 6 weeks of age. They were ear notched to enable identification and kept in standard polypropylene cages (5 mice per cage). The animals were provided with standard laboratory rodent food pellets and water *ad libitum* with the exception of experiments that involved food restriction. All the animal procedures were carried out under Home Office Project Licence 40/2942 under the regulations of the Animal Scientific Procedures Act 1986. The health of the animals was assessed based on physical appearance and body weight, in accordance with the terms of this licence that required avoidance of exceeding the moderate banding (as defined by the Home Office ASPA, 1989), and animal care and welfare was implemented throughout the experiment.

2.3.4 Faecal egg count (FEC)

On day 14, 16, 18, 21, 23 and 25 post infection faecal samples from the infected mice were collected by placing individual mice in a plastic cage for about 1 h. The mice were provided with water but not food. An amount of faeces approximating to 1 g was collected from each mouse into pre-weighed universal tubes. The faeces + the universal tubes were weighed and 10 ml of saturated salt (NaCl) were added. The pellets were allowed to soak and break up for 1 h using an Intelli-Mixer RM-2L (EMLMI Ltd, Latvia). The mixture was agitated and poured quickly through a sieve (5 diameters, 800X800 μm square apertures) and washed with a further 50 ml of NaCl salt solution for egg floatation. The number of eggs present was counted using a 2-chamber McMaster slide. The eggs that float within the designated grid lines of each chamber (with a volume of 0.15 ml) of the McMaster slide were counted under a microscope. The number of eggs per gram (EPG) was calculated using the modified McMaster egg count technique (Behnke and Parish, 1979) as follows:

$$EPG = \frac{\text{Number of Eggs Counted}}{\text{Weight of Faeces}} \times \frac{\text{Total volume of NaCl}}{\text{Volume Counted}}$$

2.3.5 Worm counts

On day 25 post infection mice were killed by exposure to increasing concentrations of CO₂ and the intestines were removed in their entirety. These were opened longitudinally with a pair of blunt-ended dissecting scissors. Each intestine was placed into a net made from gauze netting in a 50 ml beaker containing pre-warmed (37⁰C) HBSS and kept in a water bath for about 4 h to allow the worms to dislodge from the intestine. The female and male worms present in the net and those that had collected at the bottom of the beaker were counted under a dissecting microscope to provide a worm burden assessment.

2.4 PLS formulation and delivery experiments

2.4.1 Materials

Magnesium stearate was obtained from BDH Laboratory Supplies (Dorset, UK), freeze dried PLS prepared from the stock purchased from Enzymase International S.A (Brussels, Belgium), Ac-di-Sol (croscamellose sodium) was a kind gift of FMC BioPolymer AS (Billingstad, Norway), Primojel (sodium starch glycolate) a kind gift of DWV-Fonterra Excipients GmbH & Co.KG (Goch, Germany) and L-HPC 32 (Low- Substituted Hydroxypropyl Cellulose) was also a kind gift from Shin-Etsu Chemicals Co., Ltd (Tokyo, Japan). Other chemicals were Xanthan gum from CP Kelco, (Liverpool, UK) and Hydroxypropyl methylcellulose (HPMC) including

Methocel-CR and Methocel-LVCR premium grades, were a kind of gift from Colorcon Ltd (Dartford, UK). All other materials used for preparation of activating and stopping buffers were routinely available laboratory analytical chemicals.

2.4.2 Tablet manufacture

Different proportions of freeze dried PLS with either a hydrophilic polymer (XG or HPMC) or disintegrant powders were mixed and blended in a Y-cone blender (Neco, London, UK) for 9 min. Then 0.5% w/w of magnesium stearate was added as a lubricant to prevent the mixture from sticking to the machine and mixed for a further 1 min. These powder blends were compressed into 19 mm diameter, round, flat-faced tablet using a Manesty F3 single punch machine (Manesty, Liverpool, UK) operated manually.

2.4.3 Evaluation of tablet properties

Basic properties of the tablets including uniformity of mass, tablet hardness and friability were measured. The uniformity of tablet weight was determined as the relative standard deviation of 10 randomly selected tablets. The tablets were weighed individually using an analytical balance Kern ALS 120-4N (Kern & Sohn GmbH, Balingen, Germany). Then the individual weight was compared with an average weight. According to the European Pharmacopoeia (2008), no tablet should deviate from the average weight by more than 5 percent.

In each formulation 10 tablets were picked randomly for hardness testing. The tablet crushing load, which is the force required to break a tablet by compression

in the radial direction was determined using the tablet hardness tester C50 (Engineering systems (NOTTM) Ltd, Nottingham, England). Then the average value was calculated.

Friability was determined by placing 10 tablets of each formulation in a TAR 10 friabilator (Erweka GmbH, Heusnamm, Germany) and operating the drum for 4 min and 25 rpm. Friability was determined as the average loss in weight of the tablet due to fracture or abrasion. Friability less than 1% was considered as normal. Friability was calculated using the following formula:

$$\text{Friability} = [(Initial\ weight - Final\ weight)/Initial\ weight] \times 100$$

2.4.4 *In vitro* drug release studies

In vitro dissolution tests were used to simulate physiological conditions in the GI tract. Drug release from the tablets was determined using an USP apparatus 2 (paddle) in 900 ml of 50 mM phosphate buffer medium pH 6.8 at 100 rev. min⁻¹ and 37±0.5°C. At specified time intervals 3 ml of sample were withdrawn from the dissolution vessel using a plastic syringe attached to a filtered stainless steel cannula. The volume of sample taken was replaced back by fresh medium in order to maintain the volume of the medium. Then the content of protein (drug) release was quantified by UV spectrometry using a WPA light wave II Spectrophotometer (biochrom Ltd, Cambridge, England) at 280nm in a 10 mm path length quartz cuvette. The absorbance values were converted into protein concentration using the linear portion of the standard curve.

In order to explore the whole dose, 8 normal tablets of 19 mm in diameter were stacked together to form a bolus with weight about 15 g (Fig. 2.1). The tablets were then assessed for drug released as usual using USP apparatus 2 but because the tablets were too big to dissolve in a 900 ml vessel, then all the vessels from the dissolution tester were removed. The water bath of the dissolution tester was then used as the vessel for dissolution of the drug. In this case 16 litres of 50 mM phosphate buffer were employed. Samples were withdrawn as explained above.

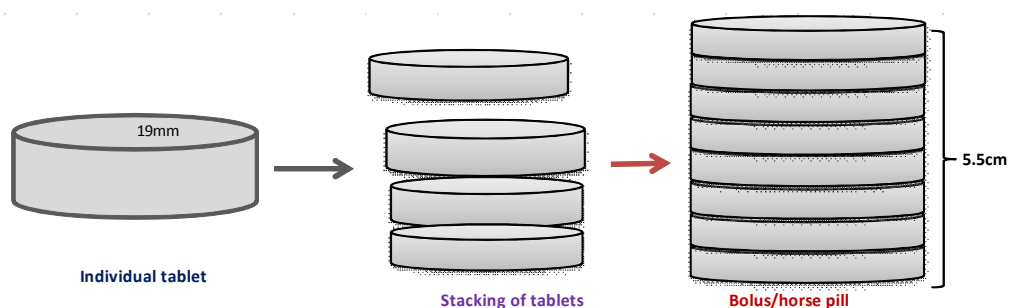


Figure 2.1: Preparation of ruminal bolus by sticking together individual tablets.

2.5 Statistical analysis

In vitro motility experimental data (raw data) and FEC transformed data $\text{Log}_{10}(\text{FEC}+25)$ were analysed using repeated measures general linear model (rmGLM) in SPSS (version 19.0). To assess whether there was a significant decrease in worm mortality and faecal egg counts over time, time after introduction of experimental treatments was fitted as the within-subject factor. To determine whether the decrease in mortality or FEC was significant different among treatments then treatments (concentrations of pineapple fruit extract, PLS, kiwi fruit extract and stem bromelain) were fitted as between-subject factors. When the data did not meet the

requirements of Mauchley's Test of Sphericity, Huynh-Feldt adjustment to the degrees of freedom was used to interpret significance on the side of caution.

However, to assess the different treatment effects on *in vivo* intestinal worm counts 1, 2 or 3-way ANOVA (depending on the experimental design) was used and significance was checked at 0.05 and 0.01 levels of probability among the various treatments. Statistical models were mostly fitted to raw data, rather than transformed data (although in some cases these were also implemented), because in most cases these conformed to their requirements of parametric tests. All parametric models were assessed for goodness of fit by R^2 and their residuals were checked for normal distribution. The IC_{50} for the motility assays was determined using curve fitting-statistical software GraphPad Prism 6. The data were analyzed using a non-linear regression model and sigmoid dose-inhibition curves were fitted using $\text{Log}_{10}(\text{inhibitor})$ vs. response – Variable slope (Four parameter dose-response curve) equation.

Model: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + \exp(S(\text{Log}IC_{50} - X)))$

The bottom was constrained to a constant value of 0 (minimum motility) and the top was constrained to upper limit of 5 (maximum motility).

CHAPTER 3: PREPARATION AND PROFILING OF PLS

3.0 Summary

This chapter deals with the preparation and profiling of papaya latex supernatant (PLS). The purification and profiling of PLS were conducted by centrifugation, dialysis and concentration over PEG, freeze drying and storing at different temperature conditions. Enzyme activity was assessed by active site titration. The results indicate that concentration by dialysis remains the best way of preparing the enzymes. There was a gradual decrease in enzyme activity for PLS stored at ambient temperature as after 1 week of storage only 27.6% of the active enzyme remained while the PLS stored at 4°C retained 27.9% of the active enzymes after 1 year. However, PLS kept at -20°C and -80°C retained its activity at more than 80% of the original level after 1 year. Freezing and thawing were found to have had no effect on enzyme stability. Moreover it was shown that there was no decline in enzyme activity over a period of up to 2 h during typical motility assays as described in Chapter 4. The freeze dried PLS that was stored at 4°C for 1 year retained more than 80% of its active enzymes compared with that stored at ambient temperature which retained only 40% of its original active enzymes.

3.1 Introduction

Like any other proteins, the extraction and purification of cysteine proteinases (CPs) is highly dependent on their unique physical and chemical properties, such as solubility, charge, hydrophobicity and binding affinity (Grodzki and Berenstein, 2010). Different methods have been employed for extraction of the CPs from plants, whereby the initial steps involve extraction of the latex or juices from the plants of

interest, followed by purification and concentration of the enzymes. Among the common methods used in purification and concentration of the enzymes are salting out (Grodzki and Berenstein, 2010), organic solvent fractionation (Merrill and Fleisher, 1932) chromatography (Bertsch, 1985, Ehle and Horn, 1990), concentration by ultra-filtration (Jiang *et al.*, 2004), electrophoresis (Ros *et al.*, 2002), dialysis (Monti *et al.*, 2000) and freeze drying (Roy and Gupta, 2004, Maa and Prestrelski, 2000).

In most cases, the processes of extraction and subsequent treatment have been shown to be detrimental to enzyme structure and/or its biological activity and these changes have been attributed to many different factors including temperature, pH, ionic strength, redox potential, autolysis, heavy metals, mechanical stress, damage of thiol groups and dilution effects (Arakawa *et al.*, 2001). The effect of temperature on enzyme activity has been described by two thermal parameters: the Arrhenius activation energy, which defines the effect of temperature on the catalytic rate constant K_{cat} (Peterson *et al.*, 2007) and the thermal stability which describes the mechanism of inactivation of enzymes at higher temperatures (Erarslan and Kocer, 1992). Exposure of enzyme outside the active pH range, is known to affect the state of ionization of either acidic or basic amino acids in that particular enzyme causing the structure of the active binding site to change and thereby resulting in an inability of the substrate to bind to the enzyme. These changes can lead to irreversible loss of enzyme activity (Nicholas and Stevens, 2002).

Autolysis is another factor that affects enzyme stability by causing degradation of enzymes through the activity of endogenous proteinases. The problem

has been reported to be accelerated by exposure of the enzymes to oxygen (Balls and Thompson, 1940) causing the active cysteine residue which is prone to oxidation, to form either disulphide bonds or oxidized species (Wilk, 2001). However, autolysis can be minimized by lowering the temperature to inhibit the action of proteinases or addition of proteinase inhibitors during extraction (Yu *et al.*, 1993). The oxidation process can be catalysed also by the presence of heavy metal ions (e.g Cd^{2+} , Pb^{2+} and Hg^{2+}) which react with sulphhydryl groups or transition metals (e.g Cu^{2+} or Fe^{2+}) which promote formation of complexes with unreactive oxygen molecules. The effect of heavy metals on proteolytic enzymes was first reported by Krebs in 1930 (Kozima, 1957) who found that Zinc, cadmium and mercury inhibited the hydrolysis of papain. However, inclusion of a low concentration of EDTA in the assay can remove any heavy metal through chelation (Zucker *et al.*, 1985).

In extraction of plant CPs, papain is the most studied and its process of extraction has been well established. Balls *et al.* (1940) were the first to develop a process for isolating and purifying papain from fresh papaya latex. However, due to the scarcity and high cost of fresh papaya latex the process was later modified by Kimmel and Smith (1954) by using commercial dried papaya latex and this method has become now the classic method for papain extraction.

The extracted enzymes need to be stored in such a way that the enzyme activity is maintained for a long period of time. However storage of enzymes depends on their stability and on when and how they will be used later on. For long term storage enzymes are generally advised to be frozen at either -20°C or -80°C or freeze dried. However during freezing and freeze drying the enzymes are exposed to

low temperature and formation of ice and potential mechanical disruption by ice crystals (Arakawa *et al.*, 2001) which may lead to inactivation. Cold denaturation has been documented clearly for several proteins and enzymes (Privalov, 1990) and the effect can be demonstrated during freeze-thawing while at high temperatures, enzymes are subjected to oxidation, resulting in loss of activity. Therefore, it was important to ascertain the best storage conditions for PLS that would allow extracted enzymes to be maintained for the experiments reported later in this thesis, over prolonged periods of time without significant loss of enzyme activity.

Aims and objectives

The first objective of the experiments reported in this chapter was to determine which method of concentration of papaya latex (e.g. centrifugation, dialysis or freeze drying) would provide high yields of CPs in PLS.

CPs in PLS are in their natural conditions, unlike purified enzymes, but it was not known if the latex has any particular role in protecting the enzyme during long term storage. Therefore, the second objective of this chapter was to assess the stability of CPs in PLS stored at different temperatures (ambient temperature, 4⁰C, -20⁰C and -80⁰C).

Freezing in solution and freeze-drying are the most commonly used methods for the long-term storage of enzymes but these processes have been reported to be detrimental to many enzymes, although the exact consequences of these treatments depend on the nature of the protein (Arakawa *et al.*, 2001). This means that survival of enzyme activity following this treatment depends to a large extent on the protein

in question. Thus the third objective of this study was to determine the effect of freeze-thaw cycles and freeze drying on the activity of the CP enzymes in PLS.

Determination of the anthelmintic efficacy of PLS against GI nematodes involves *in vitro* assays in which the motility of the worms is monitored over a period of 2-3 hours. However, it was not known whether the conditions used in these assays would influence the enzyme activity, perhaps leading to rapid loss of enzyme activity within the period of observation. So the fourth objective of this chapter was to determine how active and for how long the enzymes retained activity after introduction into the assay conditions, during a typical period of observation of worm motility.

3.2 Experimental design and Results

3.2.1 Experiment 1: Preparation of PLS

This experiment was carried out to concentrate CPs in PLS using various methods of purification including centrifugation, dialysis and freeze drying. The source of CPs was the refined spray dried papaya latex powder (Enzymase, Belgium). The powder is prepared by the Enzymase Company by mechanical filtration of freshly collected latex from the *Carica papaya* tree. The process involves several filtration steps under low temperatures with final spray drying. In this experiment 4 kg of powder was mixed with 12 litres of water for 1 h and the product was centrifuged, the pellets were discarded while the supernatant was collected. The supernatant was dialyzed and concentrated by placing it into dialysis tubing with MW cut-off 3,500 (SpectraPor 45 mm diam.) over polyethylene glycol 20,000. The dialyzed PLS was

frozen at -80°C . A portion of the frozen dialyzed PLS was taken for freeze drying, a process of removing water from frozen samples by sublimation and desorption (Roy and Gupta, 2004). The dried sample was reconstituted subsequently to its original volume for determination of enzyme activity. The amount of active enzyme present in each stage of purification was determined by active site titration with E-64 in the presence of L-cysteine.

3.2.2 Experiment 1: Results

The results are shown in Fig. 3.1 and these indicated that the centrifuged PLS prepared from refined spray-dried papaya latex showed comparable active enzymes to the original un-centrifuged material ($1.475\ \mu\text{M}$ and $1.385\ \mu\text{M}$ respectively). This indicates that the spray dried papaya latex had a high level of active enzymes. However, after dialysis there was a decrease in the amount of active enzymes by 30% remaining with $0.975\ \mu\text{M}$ when compared to the original un-centrifuged solution. The decrease in enzyme activity was continued further in the freeze dried PLS to 44% ($0.775\ \mu\text{M}$).

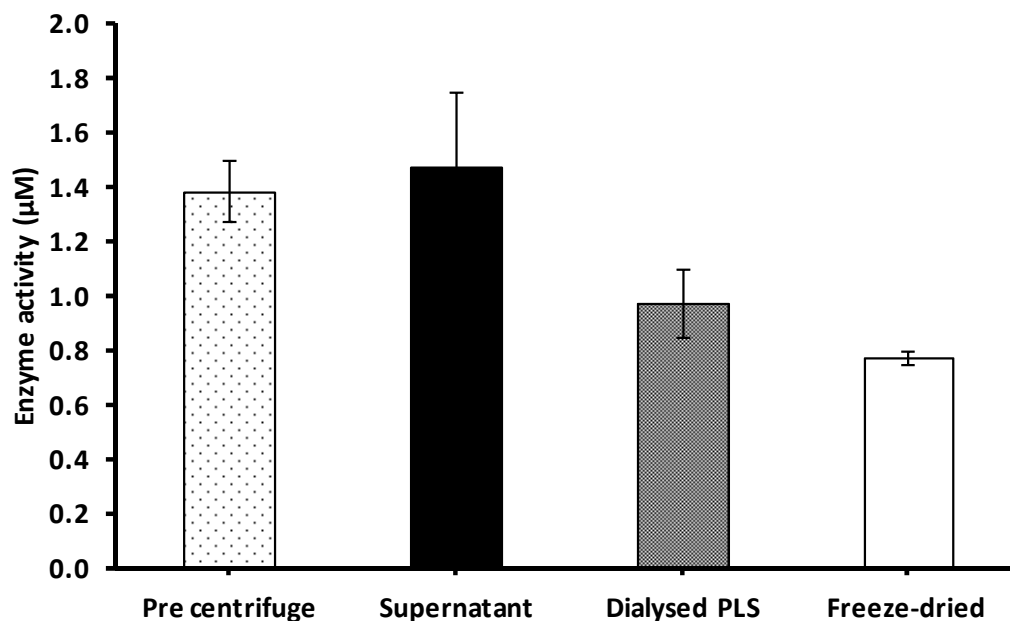


Figure 3.1: Amount of active enzyme present in different preparation methods

The graph shows variation in enzyme activity of papaya latex extracts that have undergone different treatments before active site titration. In this case the starting pre centrifuge solution was a raw preparation of papaya latex. The supernatant was the supernatant fluid after centrifugation of the raw preparation at 17,700 x g. The dialysed PLS was the supernatant fluid which has been concentrated by dialysis to remove extra water and small molecule impurities. The freeze dried PLS was the powder of freeze dried supernatant (which had undergone dialysis) and reconstituted to the original volume with water. The values are the mean of active enzymes (3 replicates) in nmol with standard errors.

3.2.3 Experiment 2: PLS profiling at different temperature storage conditions

This experiment was carried out to determine the most suitable storage condition for PLS that would retain as much of the active enzymes for as long a period of time as possible. Since CPs in PLS are still in their natural form, their stability and capacity to retain enzyme activity at various temperatures was determined. To achieve this objective PLS was aliquoted into individual containers and stored at a range of temperatures and conditions including at ambient temperature (22°C), in refrigerator

(4⁰C) and freezer (at -20⁰C or -80⁰C) for periods of 1, 4, 24 and 48 weeks, before being assessed for the amount of active enzymes remaining.

3.2.4 Experiment 2: Results

The results showed that there was a progressive decline in amount of active enzymes of the PLS kept at some temperatures but not at all (Fig.3.2). PLS kept at ambient temperature there was an abrupt decrease in active enzymes, immediately after 1 week of storage, the amount of active enzyme went down to 27.6% and after 1 month the activity was further reduced to 23.5% of the original active enzyme. After 1 year almost all the active enzymes were gone (only 1.3% remained). PLS kept at 4⁰C retained the active enzymes for a longer time; after 1 year it had remained with 27.9% of the active enzymes. For PLS stored at -20⁰C or -80⁰C more active enzymes were retained after 1 year 82.4% and 79.4% respectively of the original level was still active (Fig. 3.2, for numerical data see Appendix 3). Thus, as expected, the activity of PLS is strongly influenced by the temperature at which it is being stored.

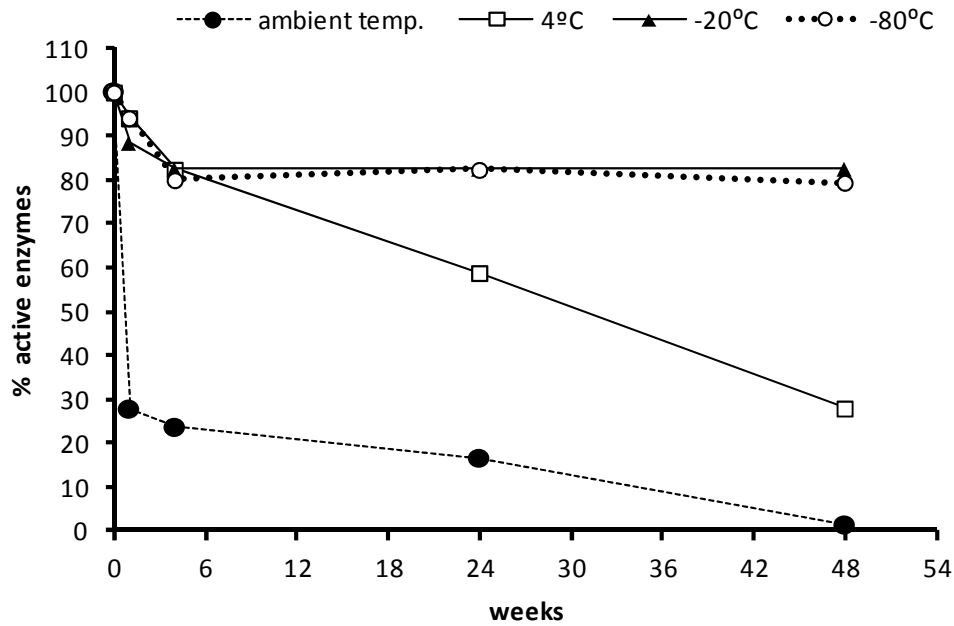


Figure 3.2: PLS activity at different storage conditions over time

The figure presents the percentage in amount of active enzymes present in 10 μl of the original sample of PLS stored at different temperature conditions (ambient, 4°C, -20°C and -80°C) assessed at different time intervals.

3.2.5 Experiment 3: Effect of freezing and thawing on PLS enzyme activity

This experiment was made to follow up the results of Experiment 2 which indicated that when PLS was stored at -20°C or -80°C it had retained more enzyme activity compared with PLS stored at higher temperatures. However, it is recognized that a critical phase for the survival of protein integrity and enzyme activity is the process of freezing and thawing from very low temperatures i.e. freeze-thaw cycles. This was an important consideration in the case of CPs because the enzymes were to be stored for the experiments conducted in the following chapters of this thesis and there was no published data on the stability of CPs during cycles of freezing and thawing. Therefore, this experiment was carried out to determine whether repeatedly freezing

and thawing of PLS would have an influence on enzyme activity. The dialyzed PLS was first frozen at -80°C and after 1 month the sample was thawed to ambient temperature (22°C). One ml of sample was taken from the thawed PLS (freeze-thaw 1) and tested for enzyme activity. The sample was re-frozen at -80°C for another 1 month and was thawed again to room temperature and a second 1 ml was taken from sample (freeze-thaw 2). This cycle was repeated 5 times yielding 5 freeze-thaw cycles. Each time the sample was thawed, and tested for enzyme activity.

3.2.6 Experiment 3: Results

The stability of enzyme activity due to repeated freeze-thaw cycles was assessed based on percentage reduction in amount of active enzymes in 1 ml assay (Fig.3.3). The first, second, third, fourth, and fifth freeze-thaw cycles reduced enzyme activity by 19.7%, 27.6%, 26.4%, 20.5% and 25.3% respectively when compared with the activity of the original sample. From these results it appears that there was little further reduction in enzyme activity after the first freeze-thaw cycle. This means that after the first freeze-thawed cycle the enzyme activity remains relatively stable and the number of freeze-thaw cycles have little impact on enzyme activity.

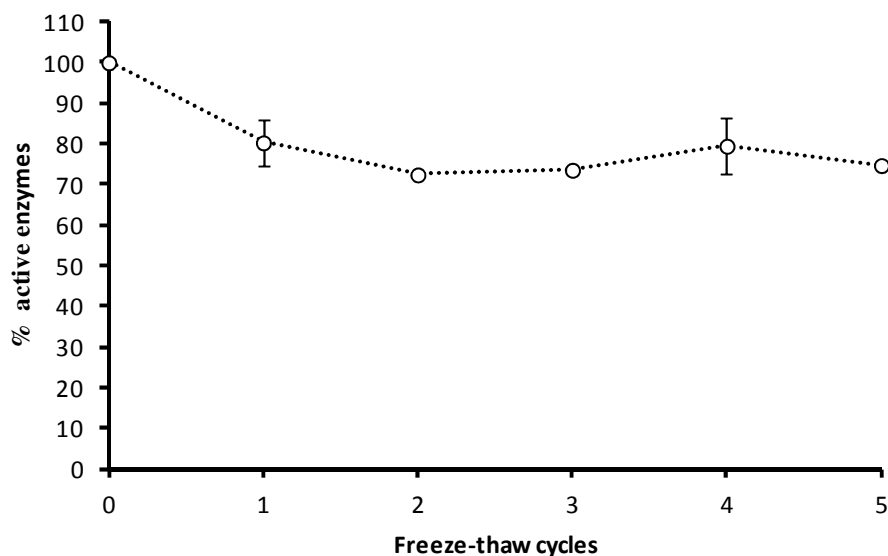


Figure 3.3: Effect of freeze-thawing cycles on PLS active enzymes

The figure shows the percentage change in amount of active enzymes for dialyzed PLS frozen at -80°C for 1 month and then thawed. The process was repeated 5 times and each time the amount of active enzymes remaining was determined. There was a reduction in enzyme activity at the first cycle and then the activity remained relatively constant in the succeeding cycles.

3.2.7 Experiment 4: Freeze dried PLS profiling at ambient temperature and 4°C

Enzymes and other proteins are said to be more stable in the solid state compared with solution (Roy and Gupta, 2004) and the freeze-drying process is perceived to be a gentle process for drying biologically active substances. Therefore, Experiment 4 was carried out to determine the post storage stability of the freeze-dried PLS at two storage conditions (ambient temperature and 4°C). These conditions were chosen based on the fact that the product being developed here is intended for use by farmers whose storage facilities are likely to be limited. Thus dialysed PLS was aliquoted into portions of 25 ml and sealed with perforated cling film. The samples were frozen

at -80°C before being placed into an SB4 freeze-dryer (Chemlab, England) and left overnight to freeze dry. The next day the freeze-dried PLS was taken out and tested for enzyme activity. Some portions of the freeze dried PLS sample were stored at ambient temperature (22°C) and the other portions were kept in a refrigerator at 4°C and then at different time intervals the samples were tested for enzyme activity.

3.2.8 Experiment 4: Results

The results are shown in Fig. 3.4 and these indicate that there was a decline in active enzymes immediately after freeze drying storage in both samples. However the decrease was higher in the sample stored at ambient temperature after 1 month (4 weeks), the active enzymes of PLS falling by 14.3% while that PLS kept at 4°C decreased only by 10% of the original total active enzymes. After 1 year (48 weeks) the freeze dried PLS stored at ambient temperature had retained only 57.1% of its original active enzymes whilst the PLS store at 4°C retained its active enzymes by more than 86.4%.

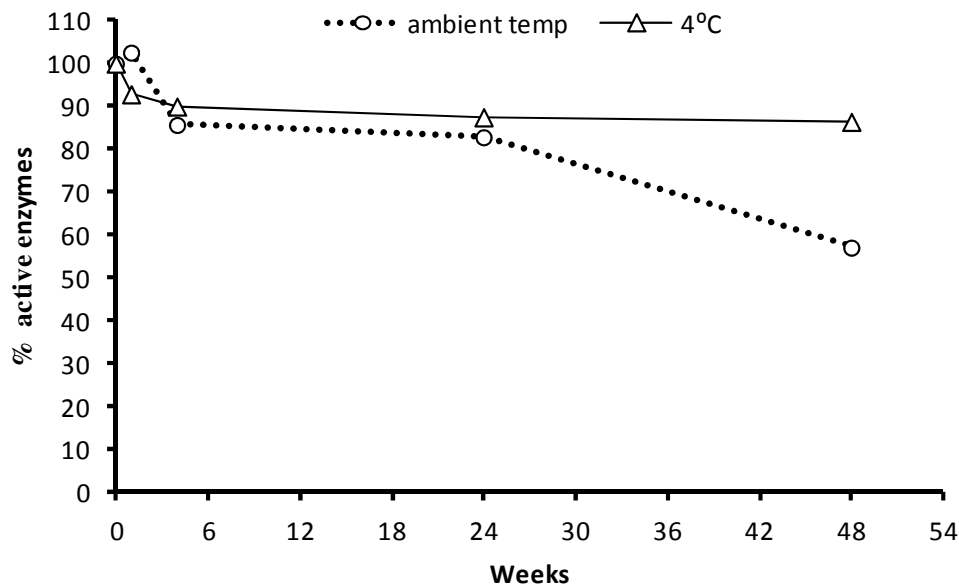


Figure 3.4: Freeze dried stored at different temperature conditions.

Freeze-dried PLS was stored at ambient temperature (O) and 4°C (Δ). The results showed that there was a gradual percentage loss in amount of active enzymes in PLS stored at ambient temperature than that stored at 4°C.

3.2.9 Experiment 5: Enzyme stability in motility assay

This experiment was aimed at determining the stability of CP enzymes during a typical motility assay. Since motility assays are carried out at 37°C, the mammalian body temperature that the worms require, it was important to determine the stability of CPs under these experimental conditions. In addition to the effect of temperature, the inclusion of Hanks' buffered saline solution (HBSS) and other ingredients in the incubation assay may have affected the stability of CPs and these had to be evaluated. To achieve these objectives PLS was incubated at 37°C in the HBSS and activating buffer. Then at 0, 15, 60 and 120 min a sample was drawn from the assay for the assessment of enzyme activity.

3.2.10 Experiment 5: Results

The results in Fig.3.5 indicate that the motility assay conditions have little/no influence on the enzyme activity. Initially there appeared to be a slight increase in the amount of active enzymes, particularly at first hour (113%), but then it remained stable until the end of the experiment, 2 hours later.

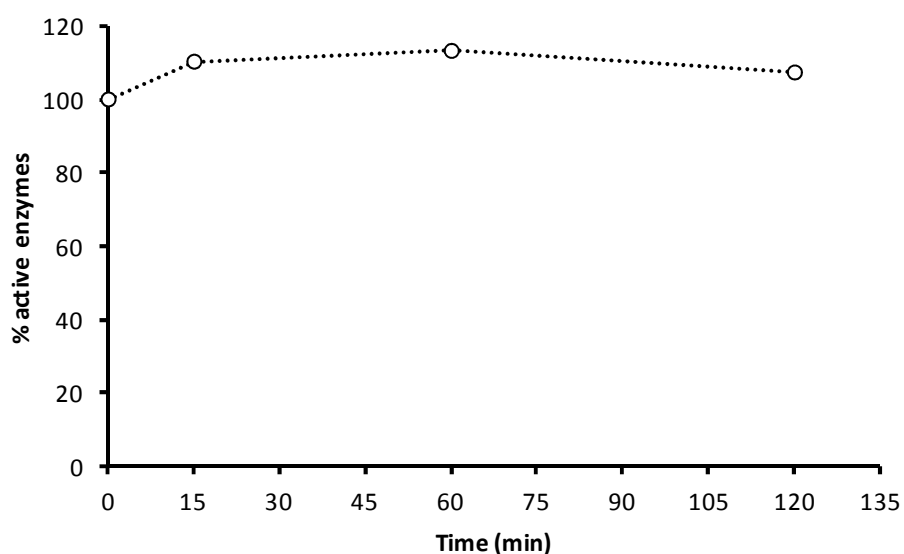


Figure 3.5: Enzyme stability in motility assay conditions

PLS was incubated under typical motility assay conditions and at 0, 15, 60 and 120 min a sample was taken for assessment of the amount of active enzyme. The amount of active enzymes remained stable throughout the incubation time over a period of 2 hours.

3.3 Discussion

PLS was concentrated in this study for the purpose of profiling, storage and to facilitate formulation into different sets of doses to be used for subsequent *in vitro* and *in vivo* experiments. The results have indicated that the amount of active enzyme in spray dried papaya latex solution and the supernatant was almost identical,

indicating that the original source, the sprayed dried PL, had less debris. This is in line with what Kimmel and Smith (1954) had reported in their study on the use of commercially available dried PL, that it contains sufficient amounts of the active enzyme for the crystallization of papain. There was a decrease in amount of active enzymes by 30% during dialysis process and further reduction was observed during freeze drying process (44%). This is due to the factor that most proteins become less stable against these stresses (Arakawa *et al.*, 2001).

It was also observed that there was a negative relationship between storage temperatures and enzyme activity. For example PLS stored at ambient temperature (about 22⁰C) rapidly lost its enzyme activity remaining with only 27.9% of the original active enzymes within 1 week of storage while that stored at -80⁰C had retained more than 94% of its active enzymes. The effect of temperature on enzyme activity has been widely documented and it is said to be associated with the oxidation of the functional Sulphydryl group in CPs (Balls and Thompson, 1940). This group in the protein can occur as a free Sulphydryl group or in disulphide linkages with other cysteinyl residues. During long term storage, these free Sulphydryl groups may be oxidized to the disulphide form. Then several different disulphide molecules can link together to form higher molecular mass aggregates which are less active or oxidation occurs to form cystine or even sulphinic, sulphonic or sulphenic acid, all of which represent essentially irreversible oxidation states (Arakawa *et al.*, 2001). Proteolytic enzymes like papain can also degrade themselves when in solution resulting in loss of activity. However, in this study the enzymes were still in the latex which is very similar to their natural conditions and it was assumed that the latex would prevent CPs from being exposed to oxidizing agents, thus preventing it from

being oxidized although the latex in this case seems to have had less protective effect at high temperatures. Devakate *et al.* (2009) also reported the same trend with a significant decrease in the enzyme activity of the CPs from pineapple plants stored at 4°C, 30°C and 60°C for 4 days to be 4%, 50% and 90% respectively.

Although the PLS kept in the refrigerator continued to lose enzyme activity, the extent of loss was slow, achieving 72% reduction after 1 year, in contrast to ambient temperature at which a similar loss had occurred within 1 week. However PLS stored at -20°C and -80°C decreased by only 20% after the entire period of 1 year. The decrease in activity of proteinase enzymes has also been reported by Hansson *et al.* (1986) who found that the fresh latex extracted from the ficus (*Ficus glabrata*) tree in Amazonia, fermented very rapidly at the ambient environmental temperature. However, when the latex was stored in a refrigerator it remained active for more than 4 months of storage, and when the latex was mixed with local brandy, *uguardiente*, sugared water (sugar cane juice or honey), orange juice or banana drink and kept in the refrigerator the enzyme activity was improved and remaining active for up to 9 months. This was probably due to alteration in protein-solvent interactions by the added substances (*uguardiente* and honey). Hale *et al.* (2005) also reported similar findings with diluted bromelain solutions which were found to lose enzyme activity by 50% within 24 h at ambient temperature but remained relatively stable for at least 1 week when in concentrated solution. It has been reported that many additives, such as sugars, certain amino acids, some amines, and glycerol, stabilize proteins against temperature and pH stresses (Wang *et al.*, 2009, Arakawa and Timasheff, 1983, Gerlsma and Stuur, 1974).

Balls *et al.* (1940) found that fresh latex was extremely powerful proteolytically, but it was easily inactivated by oxidation. It was predicted that air and other ingredients of the crude proteinases caused the rapid deterioration of the enzymes. They observed that the inactivation of enzymes was at first reversed by addition of reducing agents (hydrogen sulphide, hydrogen cyanide, and cysteine) but with continued exposure to oxidizing influences, the protein was no longer capable of reactivation after irreversible oxidation has occurred. This might be one of the reasons why the people of Amazonia kept the fresh Ficus latex preparation for 4-7 days before use (Hansson *et al.*, 1986), probably to provide time for the enzyme activity to fall to a desirable/acceptable level. It was observed that if the latex was taken still fresh it was associated with side effects but when taken after being stored for some days the side effects were reduced and it was more acceptable to the subjects that were treated.

Devakate *et al.* (2009) described the effect of temperature on inactivation rate of enzymes to be similar to the kinetics of the chemical reactions by the Arrhenius type of equation: $K = k_0 \exp(-E/RT)$, where k_0 is the numerical index representing the nature of the substance and characterizing the state of an object i.e. the presence of labile sites and bonds as well as their heat tolerance; E is the inactivation energy (kcal mol^{-1}), namely, the minimum energy that causes an irreversible change in the substance exposed to the temperature, R is a universal gas constant ($\text{kcal mol}^{-1} \text{K}^{-1}$) and T is temperature and (K) is the calculated inactivation energy.

Freezing is the most commonly used method for long term storage of enzymes and other proteins, however during this process enzymes are exposed to

critical stresses of low temperature and the formation of ice which can be damaging to protein structure. These cold temperatures have been reported to cause inactivation of many proteins which can be exaggerated further by repeated freezing and thawing (Privalov, 1990). Although it is recommended that samples for frozen storage should be dispensed and prepared in single-use aliquots so that once thawed the unused enzyme solution is discarded and not refrozen, this is not always practical. For example, for commercial purposes this process may not be practically feasible as single-usage is associated with additional costs of packing, room for storage and other inconveniences. However, the results of Experiment 3 show that freeze-thaw cycles have little influence on CPs enzyme activity after the first cycle. This implies that CPs do not need to be in single-use aliquots. Since PLS is a mixture of 4 proteinases (papain, chymopain, caricain and gycyl endopeptidase) which may have different stabilities, this suggests that a rapid loss of part of the enzyme activity in the first cycle may be due to instability of one of these components. For instance papain tends to be less stable than the other proteinases. The stability of the enzymes in the subsequent cycles may have been enhanced by the concentration of the PLS, the preparation having been dialysed and concentrated before being frozen. It has been reported previously that in many proteins, increasing protein concentration helps to increase the stability of proteins during freeze-thawing (Carpenter and Crowe, 1988). Another possibility may stem from the latex acting as cryoprotectant. This result is in accordance with several other studies which have investigated the effect of freeze-thaw cycles, including Hsing *et al.* (1989) who looked at the changes in the serum concentration of several hormones during 3 freeze-thaw cycles and only follicle stimulating hormone was found to have fallen in concentration by 3%. Comstock *et al.* (2008) studied the effect of repeated 10 freeze-thaw cycles on

concentrations of hormones in human plasma and serum. They found that repeated freezing to -70°C and thawing had no meaningful effects on plasma and serum concentration of hormones.

Another aspect which was investigated in this chapter was the effect of post freeze-drying storage stability of PLS. The results of Experiment 4 indicated that immediately after freeze drying there was a gradual fall in enzyme activity of the PLS stored at ambient temperature. Several factors have been reported to contribute to loss of enzyme activity of freeze dried powders (Roy and Gupta, 2004). The first factor is the way the freeze-drying process is carried out. It has been reported that the stability of freeze-dried powders depends critically on how the freeze-drying is carried out (Mazzobre *et al.*, 2001). The second factor is moisture content in the freeze-dried powder. Costantino *et al.* (1998) provided an example of how freeze dried proteins can become denatured in the presence of moisture during storage periods. A freeze dried protein at pH 7.3 with a moisture content of 40 g/100 g of protein stored at 37°C underwent aggregation resulting in diminished solubility. The aggregation was via thiol-disulphide interchange. However, when the same protein was freeze-dried from acidic solutions which inhibited the thiol groups from being ionized, the protein did not aggregate and the resultant solubility problem was solved. Arakawa *et al.* (2001) generalized that the inactivation mechanisms responsible for deactivation of freeze-dried powders at the storage stage are identical with those which have been known to operate in solutions (including mechanical stress, pH, ionic strength etc). This might be the reason why the PLS stored at 4°C retained more active enzymes than that stored at ambient temperature.

There was some concern that the motility assay conditions that were to be used in subsequent experiments might have an effect on the active enzymes present in PLS, with resultant deterioration of enzyme activity well short of the full experimental period. This is due to the fact that the assay contains different ingredients and is carried out at high temperature (37°C) which may have influenced the stability of enzyme activity. However, the results of Experiment 5 are reassuring, confirming that the enzymes remained stable under typical motility assay conditions throughout the entire period of 2 h used for motility assay experiments. The stability of CPs under the motility assay conditions may also be due to the short period of time used for assessment. Normally under these temperature conditions, enzymes lose their activity after a few days and not within a few hours, as reported here. Thus the typical assay duration is too short for any noticeable loss of enzyme activity.

CHAPTER 4: ANTHELMINTIC EFFICACY OF SOME PLANT CYSTEINE PROTEINASES

4.0 Summary

This chapter extends the *in vitro* and *in vivo* experiments examining the efficacy of plant cysteine proteinases (CPs), here focusing on pineapple and kiwifruit derived enzymes against the rodent nematode, *Heligmosomoides bakeri*. Additionally, PLS was tested against the equine nematode, *Strongylus vulgaris*. It was found that pineapple fruit extract and stem bromelain derived enzymes had significant *in vitro* detrimental effects on *H. bakeri* but in comparison there was little effect of kiwi fruit extract. Moreover, PLS had a marked detrimental effect on *S. vulgaris* which caused rapid loss of motility and digestion of the cuticle leading to death of the worms, thus demonstrating the anthelmintic efficacy of CPs from PLS against equine GI nematodes, *in vitro*. However, *In vivo* trials revealed far less efficacy of stem bromelain and pineapple fruit extract than expected from the *in vitro* experiments (24.5% and 22.4% reduction in worm burdens respectively) against *H. bakeri*. Scanning electron microscopy pictures indicated signs of cuticular damage for worms incubated in pineapple extract, stem bromelain and kiwi fruit extract but far less extensive when compared with those incubated in PLS.

4.1 Introduction

Cysteine proteinases from papaya latex have been thoroughly studied and confirmed to be efficacious against rodent, poultry, pig and sheep nematodes (Stepek *et al.*, 2006b, Stepek *et al.*, 2007a, Stepek *et al.*, 2007b, Mursof and He, 1991, Buttle *et al.*,

2011). However, little has been reported on the efficacy of CPs from other plant sources including pineapple and kiwi fruits.

Pineapple plants contain 2 main CP enzymes collectively called bromelain. The enzymes can be distinguished as either stem bromelain (EC.3.4.22.32) or fruit bromelain (EC 3.4.22.33) depending on its source in the plant. Other CPs found in pineapple stem are comosain and ananain (EC 3.4.22.31) (Rowan *et al.*, 1988). In most cases the term bromelain is used to mean crude pineapple CP enzymes. Bromelain has been used traditionally as an anti-parasitic agent in the Philippines and as a medicinal plant to reduce swelling, bruising, healing time and pain following surgery and physical injuries by different native societies of the world including South America, China and Southeast Asia (Gregory and Kelly, 1996, Chobotova *et al.*, 2010) and as a wound-debriding agent (Rowan *et al.*, 1990).

Bromelain is also sold in health food stores as a digestive aid and used as a non-prescription anti-inflammatory agent, particularly in large animals such as horses and cattle (Hale *et al.*, 2002, Gregory and Kelly, 1996). It has been reported to have both direct and indirect actions involving other enzymes like trypsin in exerting its anti-inflammatory effects. Moreover, bromelain has been proven to be capable of reducing oedema induced by cotton tissue, carrageenin and croton oil in various animal models (Smyth *et al.*, 1962). As a result of its anti-inflammatory effect bromelain has been found to reduce dramatically post operative swelling and pain by lowering the level of both plasma kinin and prostaglandins which play important roles as mediators of pain and inflammation (Leipner *et al.*, 2001). However, its mechanism of absorption from intestine into the body is not yet clearly known.

Lorkowski (2012) suggested to be through the mucosal barrier of the GI tract after oral dosing but how the enzymes survive in the blood in the presence of proteinase inhibitors is still not yet clear.

Bromelain has been demonstrated to have a variety of clinical benefits in therapeutic applications and it has been shown to be safe at high doses ($LD_{50} \geq 10\text{g/kg}$) for prolonged periods of time (Hale *et al.*, 2002). Clearly then, as was done with other CP family members from papaya and fig plants, and since bromelain has shown to digest worm cuticle *in vitro* (Berger and Asenjo, 1939; Stepek *et al.*, 2005), it was of interest to re-evaluate its anti-parasitic efficacy using the methodologies employed in the current project. In this chapter the anthelmintic efficacy of pineapple fruit extract and stem bromelain were investigated using *in vitro* and *in vivo* approaches and rodent GI nematodes to determine whether bromelain has potential as a novel anthelmintic, comparable to that now associated with PLS.

Another relatively unstudied CP is actinidain (EC 34.22.14) from the kiwifruit or Chinese gooseberry (*Actinidia chinensis*)(McDowall, 1970). Although the enzyme is used widely as a meat tenderizer (Christensen *et al.*, 2009), stabilizer of beer (Préstamo, 1995), for milk clotting (Katsaros *et al.*, 2010) and protein digestion (Kaur *et al.*, 2010) there is little published research on its medical applications. Stepek *et al.* (2005) reported this CP to have no detrimental effect on worms even though the enzyme contains the free Sulphydryl group essential for its activity (Kaur *et al.*, 2010) as do other members of the papain family such as ficin, papain and bromelain (Kamphuis *et al.*, 1985). It is not clear why the enzyme has been reported to behave so differently. Therefore, it was important to repeat the *in*

vitro experiments originally conducted by Stepek *et al.* (2005) under more standardized conditions to establish firmly whether this enzyme, actinidain, actually has any potential whatsoever as a novel anthelmintic.

To date studies have reported significant reductions in nematode parasite egg output and significant reductions in worm burdens of GI nematodes in monogastric animals such as rodents (Stepek *et al.*, 2006b, Stepek *et al.*, 2007a, Stepek *et al.*, 2007b), pigs (Satrija *et al.*, 1994), humans (Hansson *et al.*, 1986) and even in poultry (Mursof and He, 1991), as well multi-gastric animals, ruminants e.g. sheep (Buttle *et al.*, 2011) after treatment with papaya latex. However, no work has yet been conducted on parasites of horses, in which nematode infections are an economically important problem, especially with regard to the increase in anthelmintic resistance, which continues to grow as a problem in horse husbandry, especially in the racing and leisure industries (Little *et al.*, 2003).

GI horse nematodes, particularly the large strongyles, are the most important parasites of horse and are the most pathogenic, exerting a significant economic impact wherever horses are raised (McCraw and Slocombe, 1976). Of the three species of strongyles (*Strongylus vulgaris*, *Strongylus edentatus*, and *Strongylus equines*) *S. vulgaris* is the most pathogenic, often affecting 85-90% of horses particularly the yearlings (Slocombe and McCraw, 1973). For this reason, it is considered to be the most important of all equine endoparasites (Osterman Lind *et al.*, 1999). Horses become infected with these parasites through ingestion of L3 larvae which shed their protective sheath in the small intestine and penetrate into the mucosa and submucosa of the ileum caecum or ventral colon and moult to L4s. The

L4 larvae migrate to the anterior mesenteric artery and moult to immature adults which then return to the caecum and colon to complete maturation and begin producing eggs (Duncan, 1973). The migrating larvae cause great damage and inflammation to the mesenteric artery and its branches. Severe infection with strongyles causes colic, gangrenous enteritis, rupture of the intestines and even death (Duncan and Pirie, 1972, McCraw and Slocombe, 1976).

The main control and treatment of equine parasites relies on the use of chemotherapy including the benzimidazole family, pyrantel pamoate, ivermectin and oxibendazole. However, there is rapid development of resistance against these anthelmintics (Kaplan, 2004). Thus, PLS was tested to assess whether this source of plant-derived CPs could also be developed as an alternative anthelmintic against equine GI nematodes.

The aims of this chapter were: -

- i) To investigate *in vitro* and *in vivo* anthelmintic potential of pineapple fruit extract and stem bromelain against *H. bakeri*, a natural parasite of mice.
- ii) To assess the anthelmintic effect of actinidain from kiwi fruit extracts against *H. bakeri*.
- iii) To determine the *in vitro* anthelmintic efficacy of PLS against horse nematodes.

4.2 Experimental design and Results:

4.2.1 Experiment 1: The effect of pineapple fruit extract on worm motility

The molar concentrations or quantities of CPs used throughout this chapter are those derived from active-site titration with E64 (See Chapter 2 Section 2.1.4). Thus the various enzyme preparations are standardised in terms of the actual concentration of functional active sites, making experiments investigating enzyme activity absolutely comparable. The aim of this experiment was to determine the effect of pineapple fruit extract (fruit bromelain) on worm motility. Male and female *H. bakeri* worms were extracted from infected BKW mice and incubated individually in 48 well plates (1 worm/well) with 3 replicates per treatment in the following concentrations: 150 μM , 100 μM , 60 μM and 30 μM pineapple fruit extract + 16 mM L-cysteine dissolved in Hanks' buffered saline solution (HBSS). The control worms were incubated in HBSS+cysteine and 100 μM pineapple fruit extract without cysteine. The plates were incubated at 37⁰C and the motility of the worms was assessed by visual observation every 15 minutes for 2 h using a standard 0–5 motility scale adapted from Stepek *et al.* (2005) as explained in Chapter Two section 2.2.2.

4.2.2 Experiment 1: Results

Worms in pineapple extract with cysteine showed some reduction in motility after just 15 minutes of incubation. A rapid decline in motility was observed in worms incubated in higher concentrations of the enzyme. Although motility declined in all preparations, the extent of the decrease in motility was very low at low concentrations of pineapple fruit extract+cysteine. However, importantly, there was no decline in motility for worms incubated in pineapple extract without cysteine,

indicating that the effect of the pineapple extract depended on the presence of cysteine (Fig. 4.1), and there was very little loss of motility among worms maintained in Hanks' with cysteine.

To assess the changes in motility over time, the data were analysed using rmGLM, time being fitted as the within subject factor and the concentration of pineapple fruit extract as the between subject factor. Since the data did not meet the requirement of Mauchley's test of sphericity ($p < 0.001$), the Huynh-Feldt adjustment was used to aid interpretation of the data. There was a significant time-dependent reduction in motility which varied with different concentrations of the enzyme (2-way interaction between time and concentration of pineapple extract; $F_{12,6,67.1} = 3.855$, $p < 0.001$). The main effect of the concentration of pineapple extract was highly significant (between subject factor analysis; $F_{5,18} = 7.528$, $p < 0.001$).

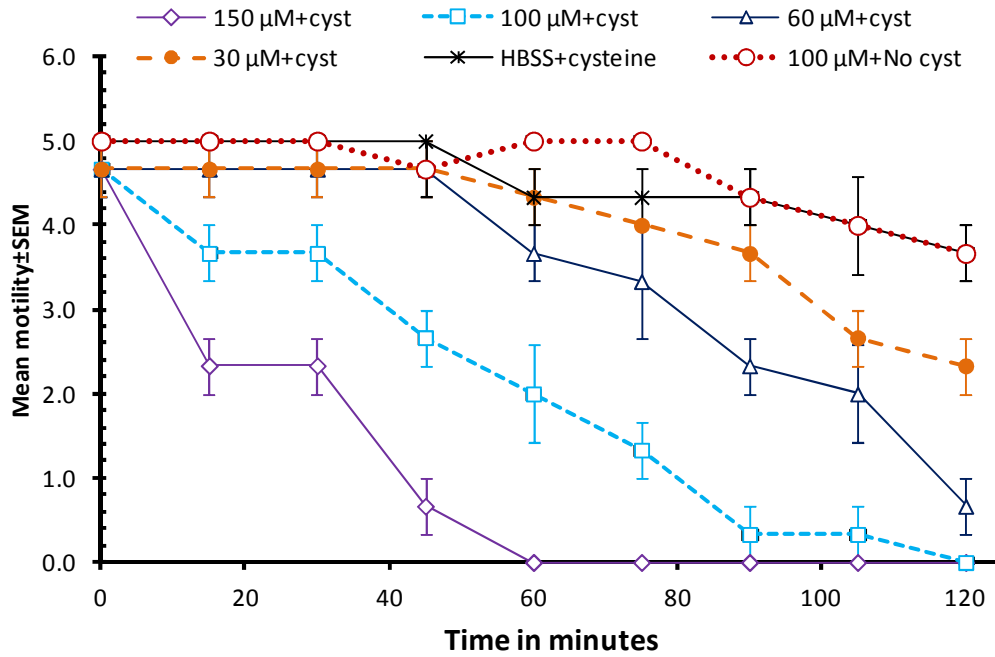


Figure 4.1: Motility change of the worm incubated in pineapple fruit extract over time

Adult *H. bakeri* were incubated individually in 48 well plates (mean± SEM, $n=3$) at 37°C for 2h in the following concentrations of active enzyme in pineapple fruit extract + 16mM L-cysteine: 150 μM , 100 μM , 60 μM and 30 μM with the control being HBSS + L-cysteine and 100 μM active pineapple derived enzyme without cysteine. The results indicated that there was a significant decline in the motility of worms incubated in pineapple fruit extract + cysteine over time.

4.2.3 Experiment 2: Effect of stem bromelain on the motility of *H. bakeri*

This experiment aimed at testing the *in vitro* effect of stem bromelain on worm motility. Stem bromelain was a kind of gift from Hong Mao Biochemical co. Ltd (Thailand) to Prof. Behnke. The enzyme was concentrated as described in Chapter 2 section 2.1.3. Adult *H. bakeri* worms were incubated in 48 well plates containing the following concentrations of stem bromelain + 16 mM L-cysteine 4000 μM , 2000

μM , 400 μM and 200 μM , while the control worms were incubated in 400 μM stem bromelain without L-cysteine and HBSS with L-cysteine.

4.2.4 Experiment 2: Results

The motility of the worms declined in all preparations, including the controls over the observational period of time. However, the loss in motility was significantly greater among worms exposed to stem bromelain+cysteine (Fig.4.2). The data were statistical analysed by rmGLM where time was fitted as within subject factor and different concentrations of stem bromelain as between subject factors. The results indicated a significant effect of time as the within subject factor ($F_{2,6,73.3} = 170.47$, $p < 0.001$) and the 2-way interaction between time and concentration of stem bromelain ($F_{25,9,73.3} = 6.713$, $p < 0.001$) indicated that the reduction in worm motility over time depended on the concentration of stem bromelain. The between subject factor analysis showed a significant main effect of stem bromelain concentration ($F_{6,17} = 17.049$, $p < 0.001$). As in the case of the enzyme in the pineapple fruit extract, worms incubated in stem bromelain without cysteine and those in Hanks' with cysteine showed only minor loss of motility over the 2h long incubation, compared with those incubated in stem bromelain with cysteine, indicating that the inhibitory effect is dependent on active enzyme and on the presence of cysteine (Fig.4.2).

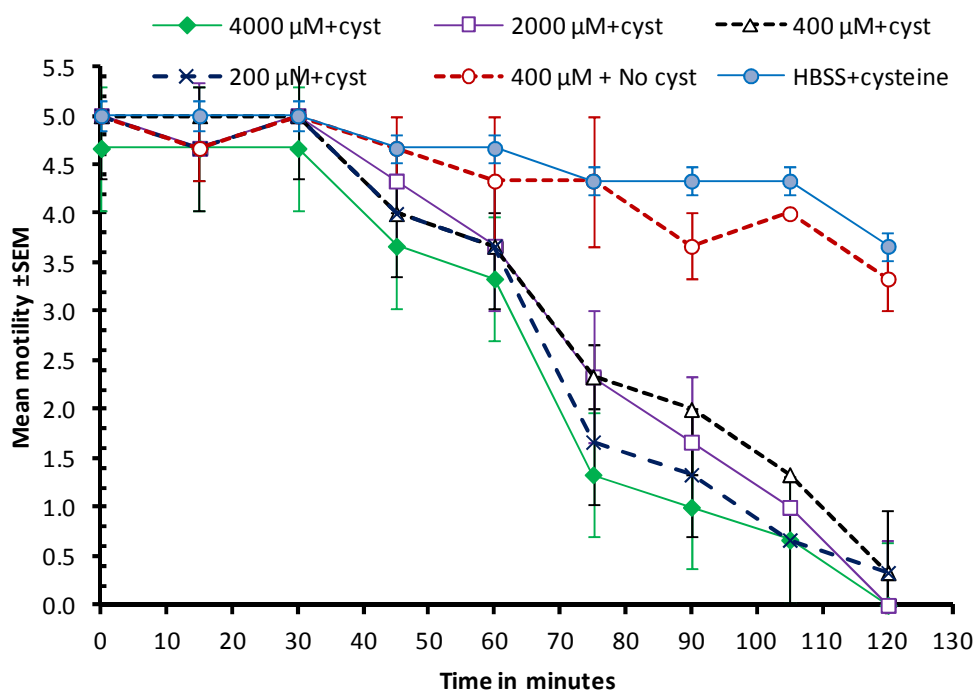


Figure 4.2: Effect of stem bromelain on worm motility

Changes in motility of adult *H. bakeri* worms incubated in stem bromelain (mean±SEM, n = 3) in the following concentrations with cysteine, 4000 μM, 2000 μM, 400 μM, 200 μM and 400 μM stem bromelain without cysteine and HBSS + 16 mM cysteine. The worms were incubated at 37°C for 2 h and a rapid decline in motility was observed in worms incubated in the stem bromelain + cysteine but the motility of worms in the control groups declined considerably more slowly.

4.2.5 Experiment 3: Effect of kiwi fruit extract on *H.bakeri* worm motility

The aim of this experiment was to assess the effect actinidain from Kiwifruit (*Actinidia chinensis*) which has been reported previously to have no detrimental effect on worm motility (Stepak *et al.*, 2005). The experiment was repeated to verify the original conclusion about the lack of anthelmintic activity with this CP. The enzyme was concentrated from fresh kiwifruit juice by dialysis as described in chapter 2 of materials and methods. However, the fruits seemed to contain less active enzymes with only 50 μM as the maximum possible concentration available for the

motility assay. Thus *H. bakeri* worms were incubated at 37°C for 2 h in 50 µM, 33 µM and 10 µM kiwifruit extract + 16 mM L-cysteine, and HBSS with and without cysteine.

4.2.6 Experiment 3: Results

Worms incubated in the highest concentration of kiwifruit extract+cysteine (50 µM) showed some loss of motility while worms in the lower concentrations and in control groups had little change in motility (Fig. 4.3). Analysis of changes in motility by rmGLM with time as the within subject factor gave a significant 2-way interaction (time and dose of enzyme, $F_{33,2,99,6} = 2.054$, $p < 0.001$) indicating that the changes in motility over time were dependent on the concentration of the enzyme. When concentration of kiwi extract was fitted as the between subject factor the results gave a significant main effect of concentration (the between subject factor analysis; $F_{5,15} = 7.238$, $p < 0.001$). There was no loss of motility among worms incubated in Hanks' with enzyme and in 50 µM enzyme without cysteine.

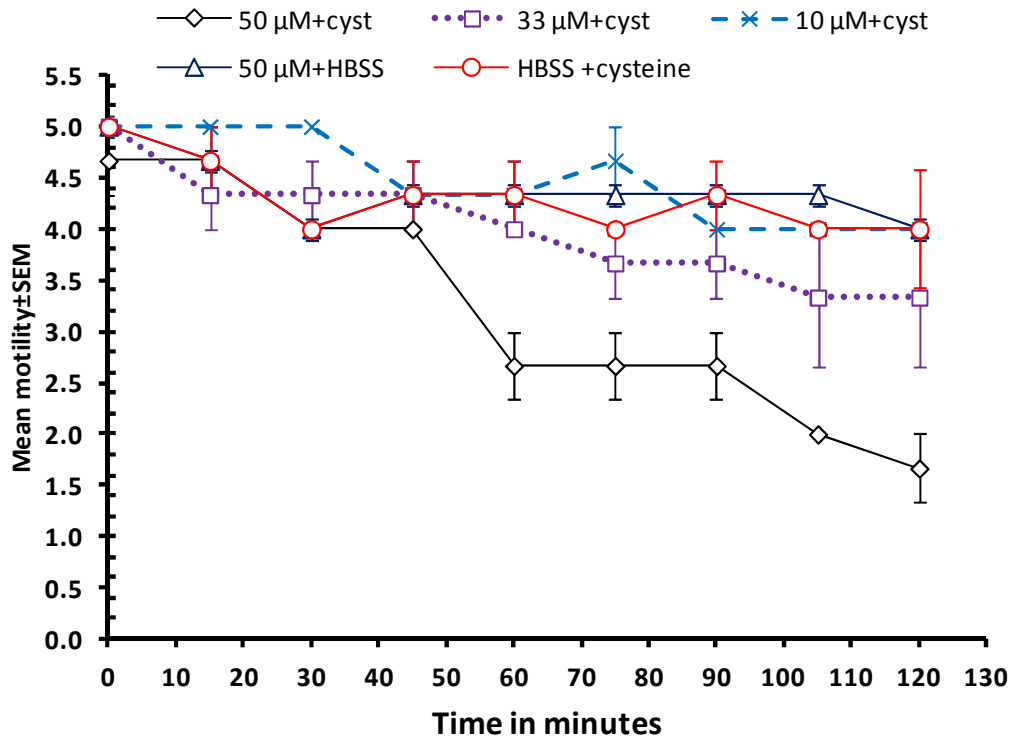


Figure 4.3: Effect of kiwi fruit extract on worm motility

Adult *H. bakeri* worms were incubated in different concentration of kiwi fruit extract (mean±SEM, n=3) i.e. 50 μM, 33 μM and 10 μM kiwi fruit extract with cysteine, and in 50 μM kiwi fruit extract without cysteine and in HBSS + cysteine at 37⁰C for 2 h. Their motility was recorded every 15 minutes. Worms in higher concentrations of kiwifruit extract+cysteine showed a more marked reduction in motility than worms incubated in low concentration of kiwifruit extract and in control groups.

4.2.7 Determination of IC₅₀ values for CPs derived from a variety of plants

Pineapple extract and stem bromelain were compared with PLS in their ability to inhibit the motility of worms by estimating their half maximal inhibitory concentration (IC₅₀) using dose-response relationships after 30 min of incubation with adult *H. bakeri* worms *in vitro*. Since the concentration of active enzyme (actinidain) in kiwi fruit extract was so low, the IC₅₀ for this enzyme was estimated after 2 h of incubation of worms in the extract. The IC₅₀ values were estimated by

GraphPad Prism 6.0 software using sigmoidal dose response regression. For PLS, pineapple extract and stem bromelain the IC₅₀ values estimated at 30 min. The values of the IC₅₀ for PLS and pineapple fruit extract were 39.65 µM and 81.95 µM respectively. However that of stem bromelain did not reach to the half of motility. While that of the kiwi fruit extract was estimated at 2 h and its value was 47.53 µM. According to these IC₅₀ values, PLS had the most potent effect in the motility inhibition assays followed by pineapple fruit extract and then stem bromelain (Fig. 4.4). However, it was difficult to compare the effect of kiwi fruit extract with that of other sources of CPs as its value was determined after 2 h of incubation when at this time worm motility in other CPs was reduced to almost zero motility.

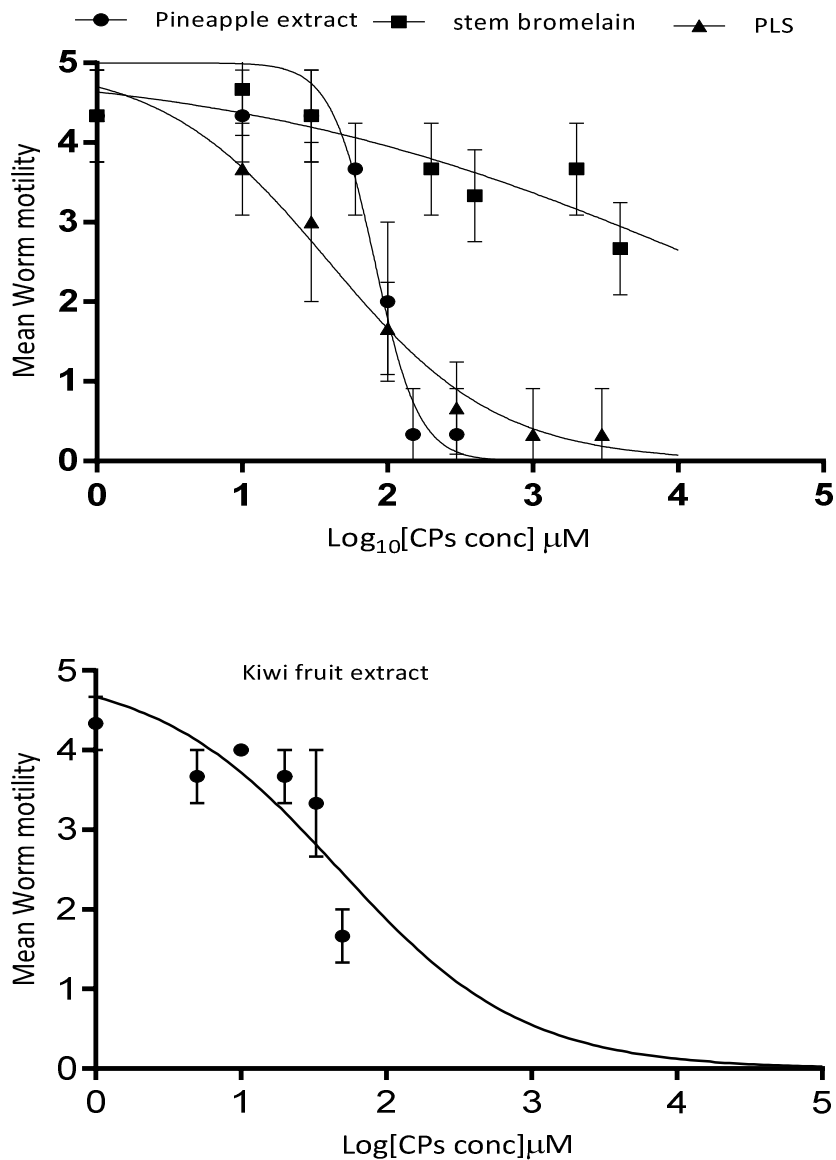


Figure 4.4: IC₅₀ values for CPs from different plant sources

The *in vitro* comparison of anthelmintic efficacy of plant derived cytochrome proteases (PLS, pineapple fruit extract, fruit bromelain and kiwi fruit extract) as determined by their ability to inhibit the motility of the worms. Sigmoidal dose-inhibition curves were fitted with an upper limit of 5 (maximum motility) and lower limit of zero (minimum motility) in GraphPad Prism (version 5). PLS was found to be more potent followed by pineapple fruit extract and then stem bromelain (excluding the kiwi fruit extract).

4.2.8 Experiment 4: SEM of worms incubated in CPs of pineapple and kiwi plants

In order to consolidate the results and to obtain visual information on the extent of the damage caused by CPs to the cuticle; adult worms were incubated in pineapple extract, stem bromelain, kiwi fruit extract and PLS. PLS was used as a positive control. At different time intervals (i.e. 0, 30, 60 and 90 min) the worms were fixed for scanning electron microscopy (SEM) to enable examination of the cuticular damage arising from exposure to CPs.

4.2.9 Experiment 4: Results

The results in Fig. 4.5 show the cuticular changes over the course of time for *H. bakeri* worms incubated in different CPs as seen under SEM at equivalent points along the body of the adult worms. Progressive cuticle damage was seen within 30 min for worms incubated in PLS, starting with the appearance of transverse wrinkles followed by blistering (at 60 min), finally digestion of the cuticle with expulsion of the worms' internal structures and contents (at 90 min). The same effect was seen in the worms incubated in pineapple extract and stem bromelain but not at the same intensity as that of PLS. However, the worms incubated in kiwi fruit extract (actinidain) only showed some initial signs of cuticular damage i.e. formation of transverse wrinkles along some parts of the cuticle after 60 min. This was probably due to the low concentration of active actinidain in the extracts used; 50 μM compared with 200 μM of PLS, 200 μM stem bromelain and 150 μM of fruit bromelain. In contrast, in the worms incubated in HBSS the cuticles remained intact throughout the experimental period with no sign of cuticular damage.

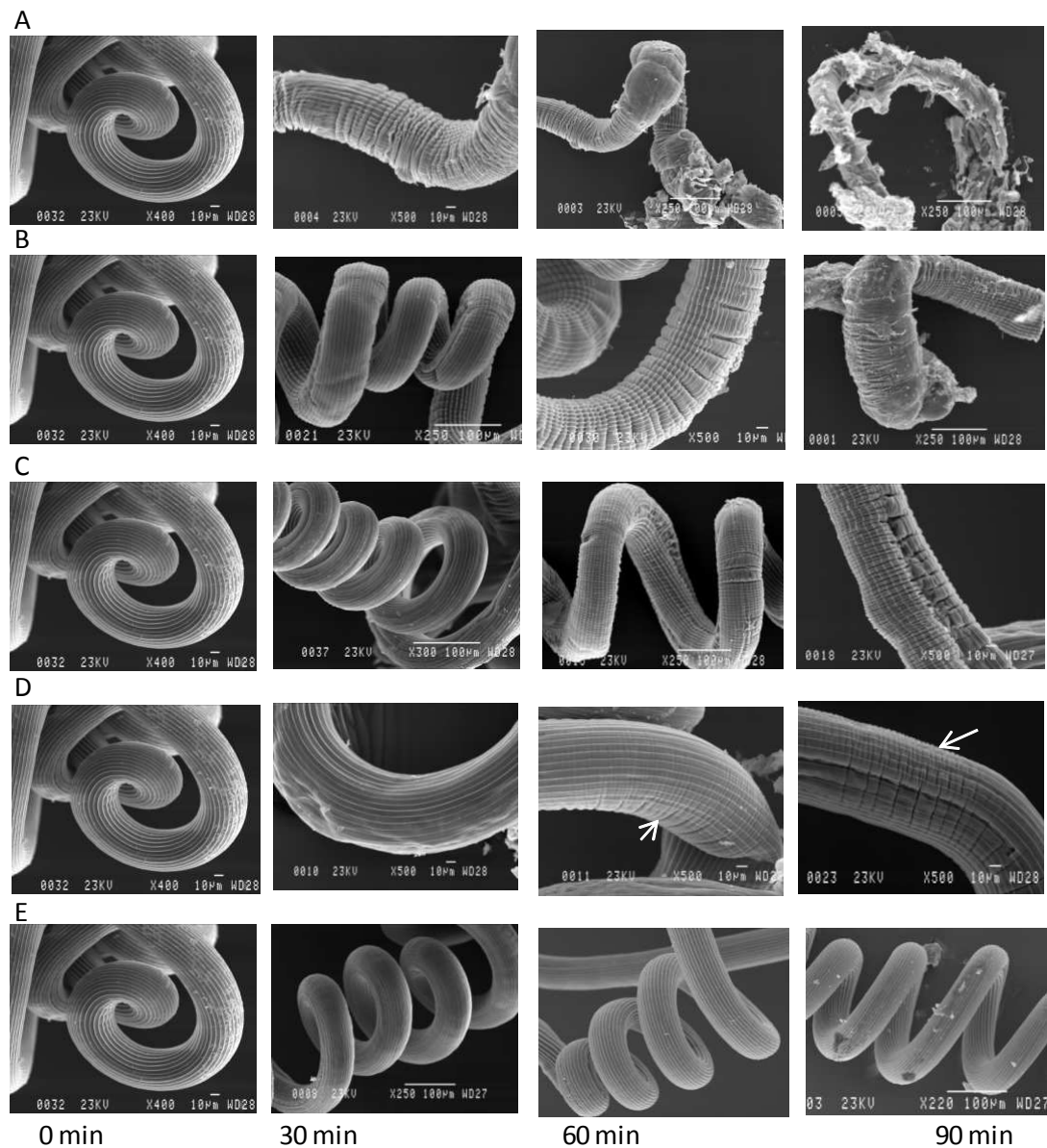


Figure 4.5: SEM of *H. bakeri* adult worms exposed to different sources of plant CPs

SEM of *H. bakeri* adult worms exposed to different sources of plant CPs *in vitro*. Clear evidence of damage to the cuticle was seen at 30 min for worms incubated in 200 μ M PLS (A) and at 60 min in 150 μ M fruit bromelain (B), 200 μ M stem bromelain (C) and only slightly changes in 50 μ M kiwi fruit extract (D) as indicated by the arrows. Note the transverse wrinkling leading to expulsion of the internal structures and contents. In contrast the worms incubated in HBSS (E) showed no detectable signs of cuticular damage with the longitudinal ridges remaining intact even after 90 min of incubation.

4.2.10 Experiment 5: *In vivo* efficacy of stem bromelain against *H. bakeri*

The aim of this experiment was to determine the efficacy of stem bromelain against *H. bakeri*, *in vivo*. Since stem bromelain has been used widely as a medicinal plant product and its concentration was higher than that of pineapple fruit extract or even that of PLS, the enzyme was tested *in vivo* to assess whether this source of CPs would be equally efficacious with PLS in expelling worms. To achieve this objective, 3 groups of 5 male C3H mice were infected orally with 150 *H. bakeri* L3 larvae on day 0. Mice were orally treated daily for 5 days from day 20 to day 24 post infection. Each group of mice received different treatments. The first group was treated with 240 nmol PLS in 0.2 ml of pure water, the second group was treated with 240 nmol stem bromelain in 0.2 ml water and the last group was given ultra filtered distilled water. Faecal egg counts (FEC) were conducted on days 14, 16 and 18 before treatment and on days 21, 23 and 25 during treatment. Autopsies of mice were carried out on day 25 post infection and the number of worms in the small intestine was counted.

4.2.11 Experiment 5: Results

The results (Fig. 4.6) show that FEC in the stem bromelain and the water groups remained steady whereas those in the PLS treated animals fell sharply soon after treatment began. The divergence in FEC between treatments over time was significant (2-way interaction between time and treatment in rmGLM with time as the within subject factor, $F_{3,99,23.97} = 7.65$, $p < 0.001$). There was also a highly significant overall difference between treatment groups (main effect of treatment $F_{2,12} = 25.754$, $p < 0.001$). Arithmetically, the anthelmintic efficacy of PLS was higher

than that of stem bromelain (92.93% and -13.85% reduction in FEC respectively). This reduction in egg output was due to reduction in the number of worms present and was consistent with the reduction in worm number 95.8% for mice treated with PLS and -1.9% for stem bromelain (worms in this group were completely unaffected and the mean worm burden was slightly higher than that of the control group). As with the FEC, the reduction in worm burdens was significant (main effect of treatment $F_{2,14} = 97.68, p < 0.001$).

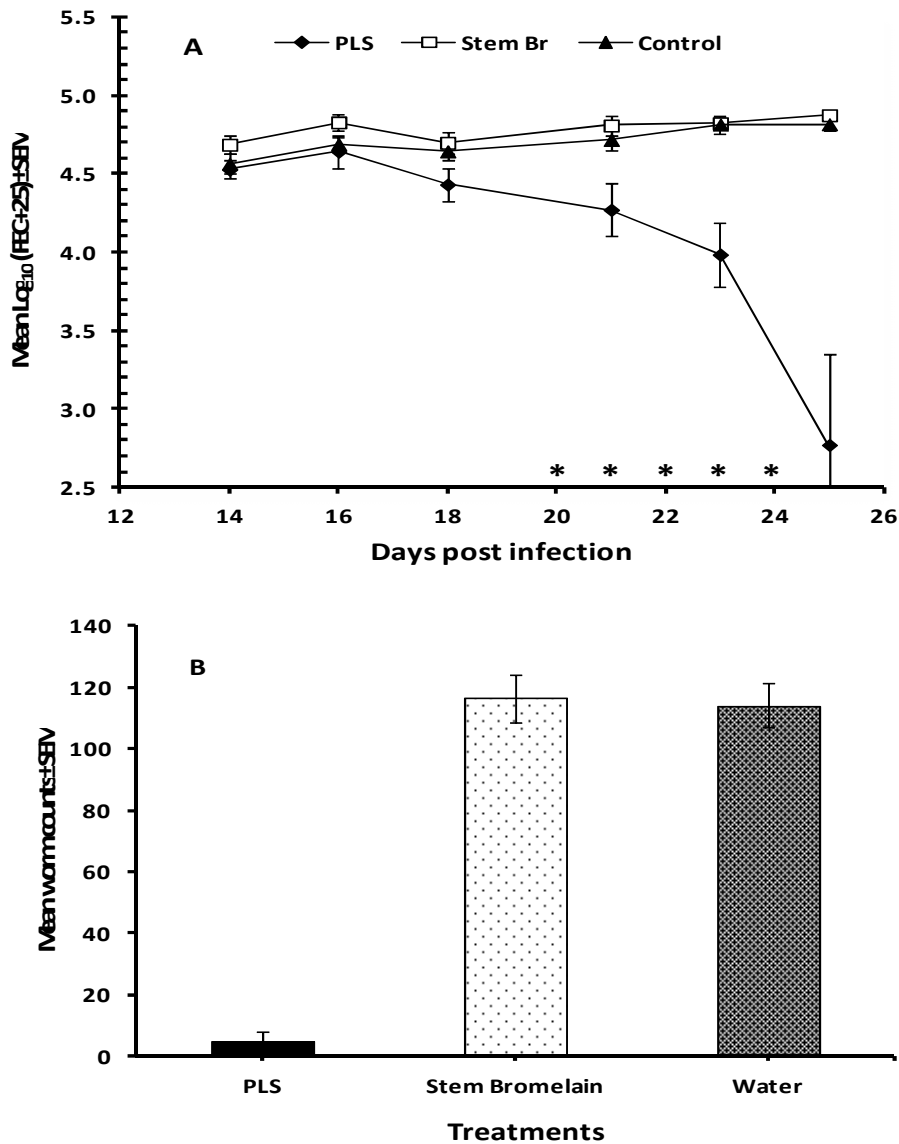


Figure 4.6: *In vivo* efficacy of stem bromelain against *H.bakeri* worms

Fifteen C3H mice were orally gavaged with 150 L3 larvae on day 0. Then the mice were divided into 3 groups, and the first group was treated with a standard dose of 240nmols PLS, the second group with 240nmol of stem bromelain in 0.2 ml water and the third groups received 0.2 ml pure water. There was a significant reduction in FEC and intestinal worm counts in the group treated with PLS. However, there was no reduction in FEC or worm counts for the groups treated with either stem bromelain or receiving water. *indicates day of treatment.

4.2.12 Experiment 6: *In vivo* efficacy of pineapple fruit extract and stem bromelain against *H. bakeri*

Since the results in Experiment 5 above showed no efficacy of stem bromelain *in vivo*, it was considered nevertheless worthwhile to repeat this experiment. However, this time pineapple fruit extract was included in order to complete the picture. Their efficacies were also compared with that attributable to PLS, in the extent of the reduction in FEC and worm burdens. To achieve this objective, 20 male C3H mice were infected orally with 150 *H. bakeri* L3 larvae on day 0. Then the mice were divided into 4 groups and on day 20 each group was given a different treatment daily for 5 days. The first group was treated with 240 nmol PLS in 0.2 ml of water, the second group was treated with 240 nmol stem bromelain, third group was treated with 60 nmol pineapple fruit extract and the last group was given water. Faecal egg counts (FEC) were conducted on days 14, 16 and 18 before treatment and on days 21, 23 and 25 during treatment. Autopsies of mice were carried out on day 25 post infection and the number of worms in the small intestine was counted.

4.2.13 Experiment 6: Results

It can be seen clearly in Fig. 4.7 that FECs in both pineapple extract and stem bromelain and in the control groups remained steady, as in Experiment 5, whereas those in the PLS treated animals fell sharply soon after treatment began. Statistically, analysis of FECs using log+25 transformed data indicated significant divergence between treatment over time (rmGLM with time as the within subject factor, 2-way interaction between time and treatment $F_{6,3,39,6} = 9.699, p < 0.001$). There was also a highly significant overall difference between treatment groups (main effect of

treatment $F_{3,19} = 19.023$, $p < 0.001$). Arithmetically, the anthelmintic efficacy of PLS was higher (94.5%) than that of pineapple extract and stem bromelain (14.0% and 21.9% reduction in FEC respectively). This reduction in egg output was attributable to the reduction in the number of worms present and was consistent with the reduction in worm numbers, 93.5% for mice treated with PLS, 24.5% for stem bromelain and 22.4% for pineapple extract. As with the FECs, the reduction in worm burdens was also significant (main effect of treatment $F_{3,20} = 50.932$, $p < 0.001$). *Post hoc* analysis by the Dunnett indicated that worm burdens in the treatment groups of mice (PLS, stem bromelain and pineapple fruit extract) were significantly different to those in the control group ($p < 0.001$, $p = 0.017$ and $p = 0.029$ respectively)

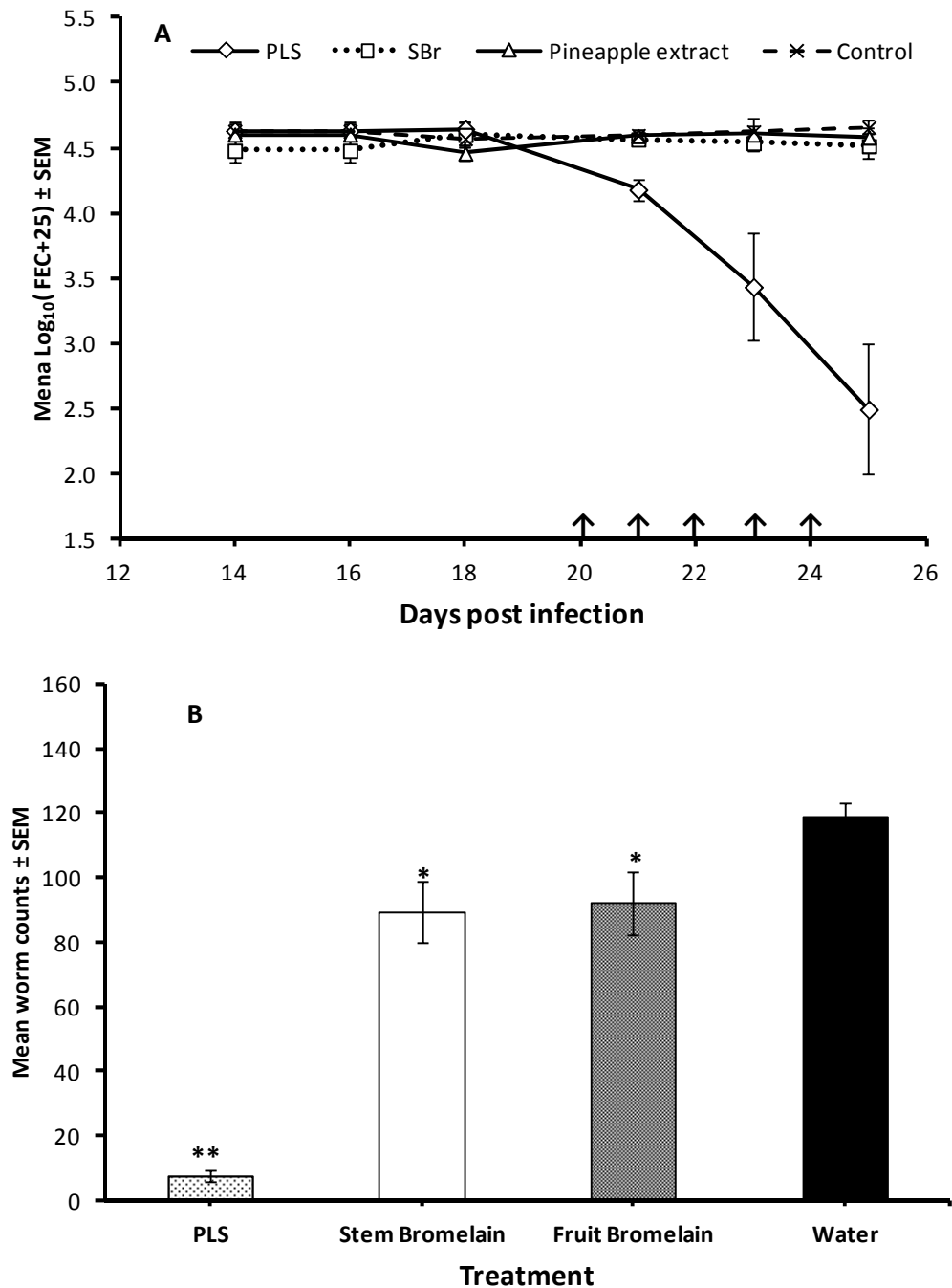


Figure 4.7: *In vivo* efficacy of fruit and stem bromelain against *H.bakeri*

The figure shows the response of mice to treatment with 240 nmol PLS, 60 nmol fruit and 240 nmol stem bromelain. Effect on FECs (A) and intestinal worm counts (B). Mice treated with PLS exhibited a sharp fall in both FEC and worm burdens whereas those treated with either pineapple extract or stem bromelain showed a considerably weaker effect. † indicates day of treatment, ** indicates significant at $p < 0.001$ and * significant at $p < 0.05$ level.

4.2.14 Experiment 7: *In vitro* assessment of PLS against horse nematodes

The aim of this experiment was to examine the *in vitro* efficacy of PLS against the horse nematode (*Strongylus vulgaris*). The worms were collected from naturally infected British horses culled at Turner's abattoirs - Nantwich, Cheshire. The worms were then kept in a flask containing horse caecal fluid and stored in a polyethylene box. The flask was kept in between bottles containing warm water to maintain temperature throughout the return journey to Nottingham. They were immediately transported back to the University of Nottingham (School of Biology) for *in vitro* assessment.

On arrival to School of Biology adult *S.vulgaris* worms were isolated from the caecal fluid and incubated in 48 well plates containing the following concentrations of PLS+16 mM L-cysteine: 3000 μ M, 1000 μ M, 300 μ M, 100 μ M, and 30 μ M while the control worms were incubated in 300 μ M PLS without L-cysteine and HBSS with 16 mM L-cysteine and 300 μ M PLS+cysteine+500 μ M E64.

4.2.15 Experiment 7: Results

The results showed that the motility of the worms incubated in PLS+cysteine declined rapidly compared with worms incubated in PLS without cysteine, HBSS+cysteine and PLS+cysteine+E64 (Fig. 4.8). The line graphs for worm incubated in 3000 μ M and 1000 μ M are not shown because the worms died within 15 minutes of incubation in PLS. However, the decline was most evident in worms incubated in higher doses of PLS+cysteine. The data were analysed by rmGLM where time was fitted as within subject factor and concentration of PLS as between

subject factor. Since, the data did not meet the requirement of the Machley's test of sphericity ($p < 0.001$), the Huynh Feldt adjustment to the degree of freedom was used. A time dependent reduction in motility was found in the presence of different concentration of PLS (rmGLM with time as the within-subject factor, interaction of time and PLS concentration $F_{38.3,108.4} = 15.937, p < 0.001$). The between subject factor analysis indicated significant main effect of concentration of PLS ($F_{6,17} = 57.139, p < 0.001$). Incubation of worms in Hanks' with cysteine but without enzyme, or in enzyme without cysteine did not result in loss of motility over the period of observation. The presence of E-64, the specific inhibitor of CPs, with enzyme and cysteine, blocked loss of motility completely.

The time at which, the PLS caused damage to the worm cuticle was also assessed under the light microscope. Clearly observable signs of cuticular damage were detected from 5-15 min of incubation impairing movement of the worms and eventually becoming so severe that the cuticle was ruptured releasing the internal structures (Fig. 4.9).

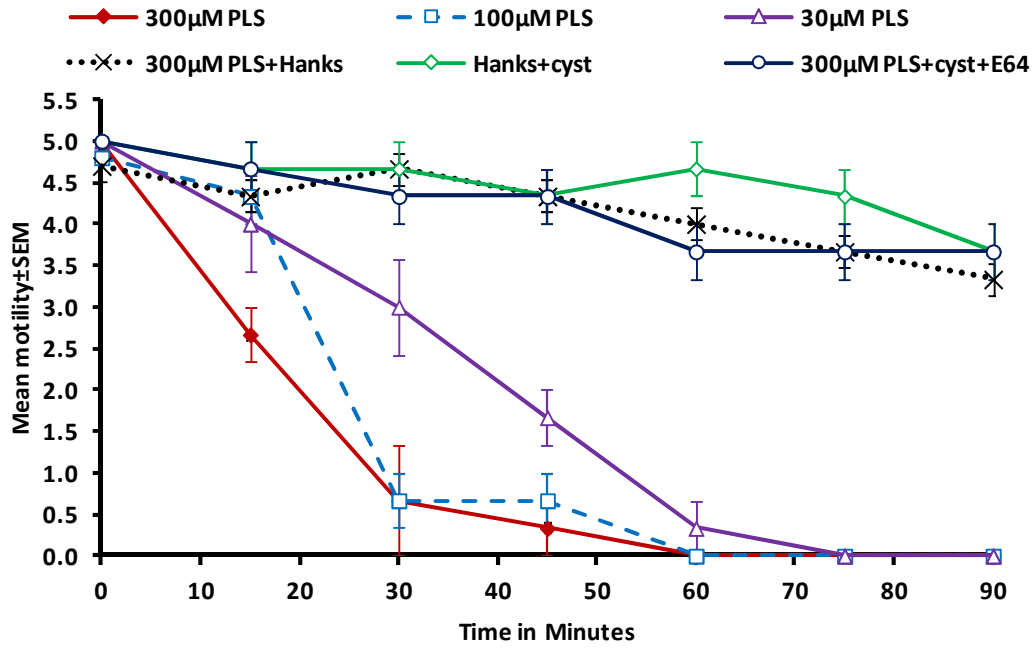


Figure 4.8: *In vitro* assessment of PLS against equine nematodes

S. vulgaris worms were incubated in 48 well plates (mean±SEM, n=3) at 37⁰C for 90 min in different concentrations of PLS (300µM, 100µM and 30µM) with cysteine, 300µM PLS + cysteine + E65, 300µM PLS +cysteine + HBSS + cysteine and their motility was recorded every 15 minutes. Worms in the PLS+cysteine showed a significant rapid reduction in motility compared with worms incubated in PLS without cysteine, HBSS+cysteine and PL+cysteine+E64 which remained active throughout.

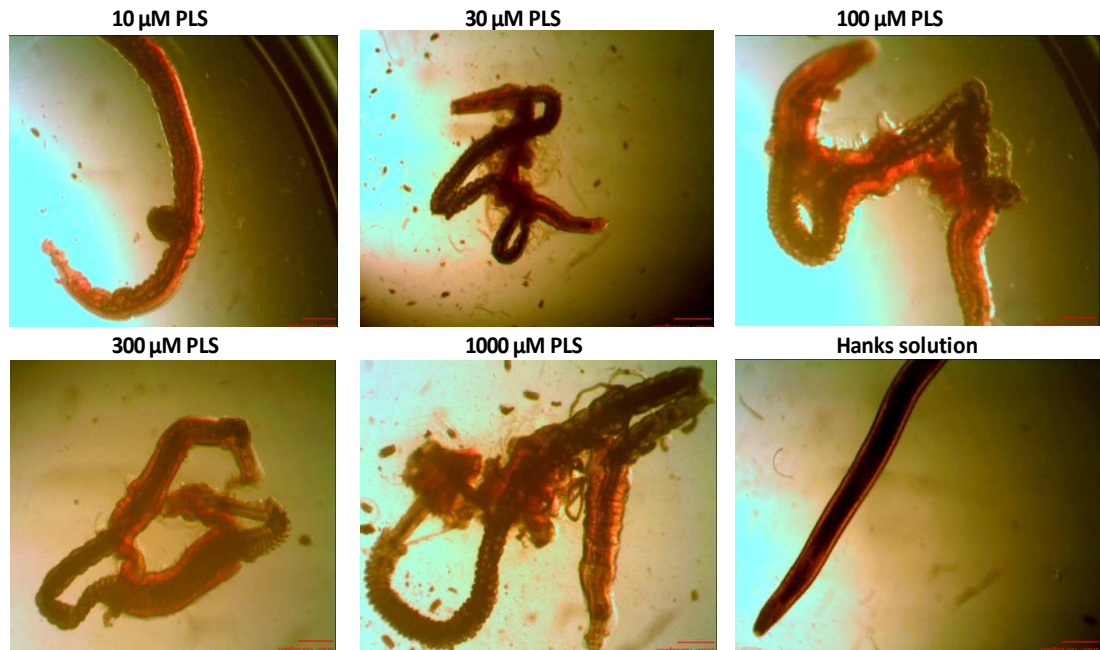


Figure 4.9: cuticular damage of horse nematodes incubated in PLS

Observable signs of cuticular damage to *S. vulgaris* worms after 15 min of incubation in different concentrations of PLS (10-1000 μM) as viewed by light microscopy. The signs of damage intensified as the concentration of PLS increased. However, there were no detectable signs of cuticle damage for the worms incubated in Hanks' solution. Scale bar = 2.65mm.

4.3 Discussion

In this chapter the *in vitro* anthelmintic efficacies of pineapple fruit extract, stem bromelain and kiwi fruit extract (actinidin) were assessed. As found in Experiment 4.1, pineapple fruit extract which is said to contain fruit bromelain, had detrimental effects on worm motility *in vitro*. The mechanism of reduced motility was identical to that reported by Stepek *et al.* (2005) which involved digestion of the cuticle as shown by the SEM pictures in Experiment 4. The decrease in worm motility was noted from 30 min of incubation in the 150 μM pineapple fruit extract. Similarly Berger and Asenjo (1939) reported complete digestion within 24h of *Ascaris*

lumbricoides from a hog's intestine, when incubated in freshly squeezed pineapple juice, which contrasted with worms incubated in heat-inactivated pineapple juice that remained active throughout the period of the experiment. The same trend on worm motility was also observed in stem bromelain preparations although not to the same extent as that of the pineapple fruit extract (Experiment 2). Even at high concentrations the inhibitory effect on motility of stem bromelain was still low when compared with that of pineapple extract. This can be supported by the IC₅₀ value which was did not reached 50% reduction in mortality for stem bromelain compared with 96.12 µM for the pineapple fruit extract. Moreover, the intensity of the cuticular damage was shown to be higher in pineapple extract than in stem bromelain (Fig. 4.5). On the basis of these results it can be argued that at identical active enzyme concentrations pineapple fruit extract is more efficacious than stem bromelain *in vitro*, although both enzymes are from the same plants but located in different parts of the plant. This may reflect the natural functions of the two enzymes in the plant and the nature of the target proteins in the organisms the plants have evolved to defend against. It is known that fruit and stem bromelains have very different substrate specificities (Rowan *et al.*, 1990) and are therefore unlikely to be targeting the same peptide bond(s) in cuticle proteins.

Kiwi fruit extract caused some detrimental effect on worm motility (Experiment 3) in contrast to reports in previous work. Stepek *et al.* (2005) reported no effect of actinidain from kiwifruit against parasitic nematodes *in vitro*. Although the motility reduction was not as high as in other CPs this might be due to the low concentration of the enzyme used, which was 50 µM and at this particular concentration even PLS shows little effect. Like any other cysteine proteinase,

actinidain from kiwi fruit contains a free Sulphydryl group essential for its activity and logically a similar effect on target proteins might be expected. However, the enzyme extracted from kiwifruit in this study was of low concentration making it difficult to concentrate to the desirable amount of the active enzyme. Other studies have also reported low yields of actinidain from kiwi fruits. For example Brocklehurst *et al.* (1981a) reported a yield of actinidain of less than 450mg/kg from fruit pulp compared with a yield of papain from papaya latex of about 40g/kg of the latex. Moreover, actinidain is more susceptible to oxidation than any other plant thiol protease (Kaur *et al.*, 2010). The enzyme has been reported also to vary remarkably in its activity depending on the growth stage of the fruit, postharvest storage and part of the fruit being tested (Lewis and Luh, 1988). There is also wide variation in the levels of actinidain within the genus, where fruits with low actinidain activity have been reported to lack actinidain precursors (Boyes *et al.*, 1997).

To confirm that kiwifruit extract had a detrimental effect against *H. bakeri* worms, the worms were incubated in the enzymes and examined by scanning electron microscope (SEM), and initial observable signs of damage on the surface of the worm cuticle started to appear after 1h of incubation which involved formation of transverse wrinkling unlike their counterpart worms incubated in Hanks' solution (Experiment 4). Since the kiwi fruit extract had been found to contain low active enzyme concentration, it is suggested that in the future work the worms are incubated for longer periods of time so that clear signs of cuticular damage could be detected.

In order to determine which of the CP source had the best potential for inhibiting worm motility, the results of the motility assays were compared using

dose-inhibition curves and their IC₅₀ values were estimated. Again, since the concentration of active enzyme in kiwifruit extract was low, its IC₅₀ was estimated after 2 h of incubation rather than after just 30 min of incubation as in other CPs. Based on the IC₅₀ values, PLS was found to be more effective in motility inhibition followed by pineapple fruit extract and then stem bromelain. For this reason the pineapple extracts and stem bromelain were assessed also *in vivo* (Experiments 5 and 6). The results of the *in vivo* experiment indicated less efficacy of these pineapple enzymes compared to that routinely obtained with PLS in comparable trials. In the case of the pineapple fruit extract, the lower efficacy may have been due to the lower concentration of the active enzymes administered to animals but the lack of a marked reduction in worm burdens is difficult to explain in the case of stem bromelain which was used at the same concentration as that of PLS. In this case it may be due possibly to host environmental effects, as for example with PLS which works well only in some strains of mice. Therefore, it is suggested that before concluding that pineapple CPs have less anthelmintic effect *in vivo* it is worth repeating these *in vivo* trials in different strains of mice or with different model parasites, particularly those residing completely in the lumen such as *Ascaris*. To some extent this result was disappointing given that bromelain is widely used as a natural plant derived medicine for other ailments and is conveniently available commercially.

It is not clear why some sources of plant CPs have anthelmintic effects against *H. bakeri* while others do not. Stepek *et al.* (2005) screened CPs from a variety of plant species *in vitro* and found that all were efficacious against *H. bakeri* but at different concentrations with the exception of actinidain which had no effect at all in her experimental systems. Brocklehurst *et al.* (1981a) compared the structure of

papain and actinidain and found that they differ in the interactions of the catalytic groups of their active centres. However, they have identical enzyme catalytic sites. This suggests that further biochemical studies of these enzymes are required to achieve a clearer understanding of their modes of action. If each of these enzymes has a higher affinity for a particular amino acid sequence in the target proteins, the differences in their efficacy as anthelmintics could be ascribed to the degree to which each is capable of attaching to the complementary site on the target proteins in the nematode cuticle.

This chapter also examined the *in vitro* effects of PLS against the blood feeding equine nematodes, *Strongylus vulgaris*. As expected, there was a significant rapid decline in motility of worms incubated in PLS. The mechanism of action was as reported in previous studies with the papaya and *Ficus* lattices causing observable changes on the surface of the cuticle, leading to lesions, fractures, and eventually complete destruction of the cuticle and bursting of the worms with release of the internal contents (Stepek *et al.*, 2005, Robbins, 1930). This experiment confirmed that CPs from PLS are able to digest equine nematodes and have potential as equine anthelmintics if their efficacy *in vivo* can be confirmed. The signs of cuticular damage were observed within less than 15 min., unlike with the rodent nematodes where signs of cuticular damage were reported between 15-30 min (Stepek *et al.*, 2005) at similar concentrations of active enzyme. This suggests that horse nematodes may be more susceptible to PLS, thus justifying the need for carrying out *in vivo* experiments in the future. If PLS is to be developed as an equine anthelmintic, as an alternative treatment for equine GI nematodes to the synthetic drugs currently used for treatment of horses, *in vivo* trials are the obvious next step.

CHAPTER 5: EFFECT OF FASTING ON THE EFFICACY OF PAPAYA LATEX AGAINST *H. BAKERI*, *IN VIVO*.

5.0 Summary

This chapter is based on the paper published in the *Journal of Helminthology* (2012) **86** (3):311- 316, first available online on 28 July 2011. In earlier studies of the anthelmintic activity of plant CPs, a period of food deprivation was routinely employed before administration of CPs, but there has been no systematic evaluation as to whether this does actually benefit the anthelmintic efficacy. Therefore we assessed the effect of fasting on the efficacy of CPs from papaya latex (PL) against *Heligmosomoides bakeri* in C3H mice. The source of CPs was from a refined, supernatant extract of PL with known active enzyme content. The animals were divided into three groups (fasted prior to treatment with PLS, not fasted but treated with PLS and fasted but given only water). The study demonstrated clearly that although food deprivation had been routinely employed in much of the earlier work on CPs in mice infected with nematodes, fasting has no beneficial effect on the efficacy of PLS against *H. bakeri* infections. Administration to fed animals will also reduce the stress associated with fasting.

5.1 Introduction

It is routine for some therapeutic drugs to be taken after a specified period of food deprivation. The rationale for this is based on the presence of food in the stomach influencing drug absorption and bioavailability by either physiological changes in the intestinal tract or physical or chemical interactions between food components, acid in the stomach, intestinal enzymes and drug molecules. Depending on the class of drug

and the resulting interaction, food has been reported to have reducing, delaying, increasing, or in some cases no effect on the absorption and bioavailability of different drugs when taken together with food (Toothaker and Welling, 1980, Welling, 1977).

In the case of orally administered antibiotics such as penicillin and amoxicillin, a 2h period without food is recommended because food reduces their bioavailability due to delayed stomach emptying (Welling, 1977). It is recommended that milk and other dairy products are avoided when taking tetracycline where absorption is inhibited because of chelation by calcium or magnesium present in milk (Dearborn *et al.*, 1957, Winstanley and Orme, 1989) or complexation with protein. Additionally, milk has the effect of the decreasing dissolution rate of the drug (Kohn, 1961). It is reported that demethyl chlortetracycline absorption was reduced by about 70% in the presence of skimmed milk whereas tetracycline bioavailability was reduced by 50 to 60% in the presence of whole milk (Welling *et al.*, 1977).

However, bioavailability of some drugs increases with food intake. One study found that serum concentrations of erythromycin in non-fasted individuals were 30-80% higher than in fasted individuals following a single dose oral administration of erythromycin (Toothaker and Welling, 1980). It was concluded that food caused delayed stomach emptying, thereby enabling greater amounts of the drug to dissolve in the stomach before passing into the small intestine for absorption. It has been found also that eating fatty food before treatment with some anthelmintics increases absorption, e.g. albendazole (Horton, 1997) mebendazole (Winstanley and Orme, 1989) and flubendazole (Munst *et al.*, 1980).

Therefore, whether to fast animals and humans before oral dosing with drugs has been an issue of some concern for a long time. In some cases fasting is recommended before oral chemotherapy, in others it appears to be unnecessary. In earlier studies with plant cysteine proteinases (CPs), a period of food deprivation was routinely employed before administration, but there has been no systematic evaluation as to whether or not this does actually benefit the anthelmintic efficacy. It is relevant that CPs do not need to be absorbed and anyway, are too large to be absorbed as such. They need to be available in the gut lumen to make direct contact with the parasite's cuticle. It could be argued that in the presence of food there would be an increase in substrate for the enzyme together with an increase in the volume of the GI contents, both of which could reduce efficacy of the enzyme on the nematode. However, fasting prior to administration appears to have been adopted through tradition rather than because of any convincing evidence that it actually enhances the efficacy of the CPs. The first example of food deprivation combined with oral treatment with CPs can be found among native people of South America where CPs in fresh latex of fig was taken early in the morning after fasting (Caldwell, 1929). This protocol was adopted by Caldwell & Caldwell (1929) in their clinical trial with higerolatex (latex from *Ficus laurifolia*) in treatment of trichuriasis in Alabama. Participants were given a light supper in the evening preceding the day of treatment and no breakfast was taken in the morning before the treatment. Hansson *et al.* (1986) applied the same protocol in their trial with the latex of *Ficus glabrata*, a traditional anthelmintic used in the Amazonian area. The same protocol was used by Satrija *et al* (1994) in assessment of the anthelmintic efficacy of papaya latex against *Ascaris suum* in pigs (using a single dose) where food was withdrawn the day of treatment and animals received only water. Then in the series of experiments by

Steppek *et al.* (2006, 2007a, b) food deprivation for a standard period of 6h was employed throughout the programme of research.

Because of the complexities involved, it is not easy to predict *a priori*, the exact nature of the interaction between food and drug efficacy in any particular case based solely on theory. In some cases, fasting may simply be based on tradition rather than on hard scientific evidence. Winstanley and Orme (1989) recommended that any development of new drugs should include assessment of food effects, including the benefits and disadvantages of fasting before treatment. Since there has been no systematic evaluation of the benefits of fasting prior to treatment of animals or people with intestinal worm infections with CPs, we undertook such a study. Here, we evaluate the effect of fasting on the efficacy of CPs from PL in mice infected with *H. bakeri*. The results should help to formulate optimal protocols for delivery of the drug to animals or humans, aiming to obtain maximum efficacy against the worms with the lowest possible dose and to minimise side-effects of treatment.

5.2 Experimental design and results

5.2.1 Experiment 1: Pilot study to determine effect of fasting

This experiment was a pilot run conducted to determine whether fasting enhanced the anthelmintic effect of papaya latex. Fifteen C3H mice were divided into three groups, each with five animals. The first and second groups of mice received orally 0.2 ml containing 240 nmoles of PLS for five consecutive days in total, beginning on day 20, while the last group (group 3) was given distilled water. Mice in groups 1 and 3 were fasted for 5 h before treatment (food was deprived at 07.00 hours and

restored at 12.00 hours on relevant days) while those in group 2 had access to food and water *ad libitum*. The fasting time interval was chosen because research has shown that this is an adequate time period to assure that the rodent stomach will be empty (Mittelstadt *et al.*, 2005). The mice were infected with 150 *H. bakeri* L3 larvae on day 0, faecal egg counts (FEC) were carried out on days 14, 16, 18, 21, 23 and 25, and mice were all killed on day 25 after infection for worm counts, one day after the last treatment.

5.2.2 Experiment 1: Results

The faecal egg counts from Experiment 1 are illustrated in Fig. 5.1. These show that egg counts declined in both PLS-treated groups of mice soon after the onset of treatment on Day 20, whereas those in the control group stayed steady, and even drifted upwards with days of experiment. Analysis by rmGLM was confined to the two PLS-treated groups to determine whether fasting resulted in better anthelmintic efficacy on $\log_{10}(\text{EPG} + 25)$ transformed data, fitting TIME (day on which faecal egg counts were carried out) as the within subject factor and TREATMENT (two levels, fasted or not-fasted excluding the not-treated, fasted control group) as the between-subject factor. There was no significant main effect of treatment (fasted versus not-fasted mice, $F_{1,8}=0.015$, $p=\text{NS}$).

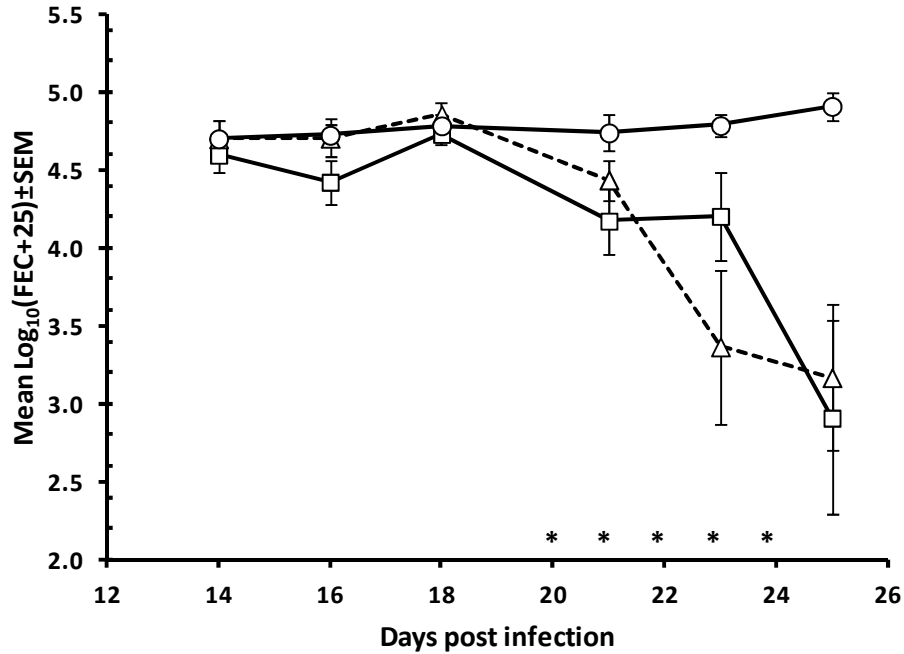


Figure 5.1: Faecal egg counts during the course of Experiment 1.

The result of control group is illustrated by open circles, fasted group by open squares and the not-fasted group by open triangles. Treatment with papaya latex commenced on day 20 after infection and was given then each day for 5 days. *indicates day of treatment

At autopsy on day 25, the control mice had a mean worm burden of 126.8 ± 24.88 , while the fasted, PLS-treated mice had 16.4 ± 6.41 worms and the non-fasted, PLS-treated mice had 14.0 ± 5.43 worms (Fig 5.2). Worm counts were analysed by one-way GLM, fitting treatment group (either two or three levels as above). There was a highly significant overall effect of treatment ($F_{2,12} = 18.1, p < 0.001$), reflecting the difference between the control group and the two PLS-treated groups, but no difference between the latter two (fasted versus not-fasted).

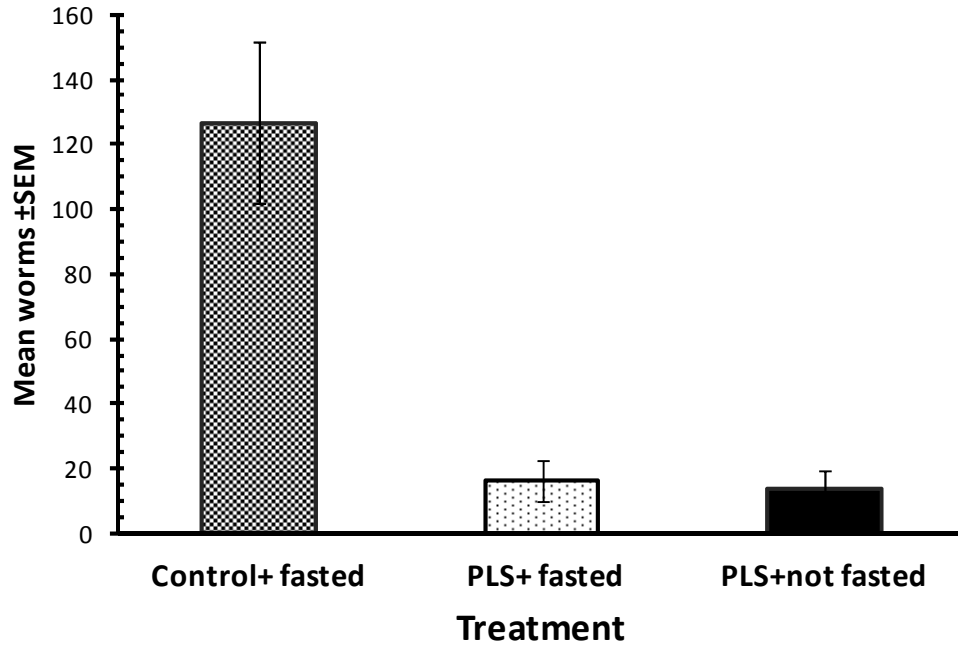


Figure 5.2: Worm counts at the end of Experiment 1.

The figure illustrates the intestinal worm recovery after 5 days of treatment with PLS prior to mice being either fasted or not-fasted. The worm count was assessed on day 25 post infection.

5.2.3 Experiment 2: Effect of fasting on the anthelmintic efficacy of PLS

Since in Experiment 1 worm burdens were only assessed after 5 daily treatments with PLS, a further experiment was carried out, in two blocks, to establish whether fasting had any additive effect with PLS earlier, after 1, 3 or 5 daily treatments. In block 1, 45 mice were divided into three groups, each group having 15 animals. The first group of mice was fasted for 5 h prior to treatment with 0.2 ml containing 240 nmoles of PLS on day 20 after infection, while the second group of animals was treated with the same dose of PLS but not fasted. The third group was the control group, with the animals being orally gavaged with 0.2 ml of ultra-filtered distilled water and, as for group 1, fasted for 5 h before oral dosing. Treatment was repeated daily for 5 days. After the first, third and fifth treatments five mice from each group

were killed for worm counts, so depending on the day on which the animals were killed for autopsy; they received 1, 3 or 5 treatments. Faecal egg counts were conducted on days 14, 16 and 18 (pre-treatment) and on days 21, 23 and 25 (during treatment) of the experiment on the groups destined for culling at the end of the experiment. The number of worms remaining in the intestines of treated and untreated mice was counted on days 21, 23 and 25 post-infection. Block 2 of this experiment was designed similarly, except that a total of 72 mice were used in three groups of 24, with eight mice culled at each time point. Another difference is that the 2 blocks were carried out at different times.

5.2.4 Experiment 2: Results

The data were analyzed first with both blocks included in the statistical model. The key hypothesis being tested was whether fasting was essential for treatment to be efficacious, and hence the analysis was confined first to the two PLS-treated groups (fasted and not-fasted) excluding the non-treated, fasted control group. Best-fit models were adopted after first exploring models with raw worm counts, and $\log_{10}(\text{worm count} + 10)$ transformed data, checking R^2 values, and the residuals for approximately normal distribution. Three-way GLM (fitting TREATMENT group (either two or three levels as above and Block two levels, block 1 or block 2 and DURATION three levels, 1, 3 or 5 days of treatments with PLS). However, the results indicated that there was a significant difference between the two blocks (3-way GLM, main effect of block $F_{1,66}=12.7$, $p=0.001$) and therefore the results have been illustrated separately for each block in Fig. 5.3A and B (which also shows worm burdens in the control untreated group for reference).

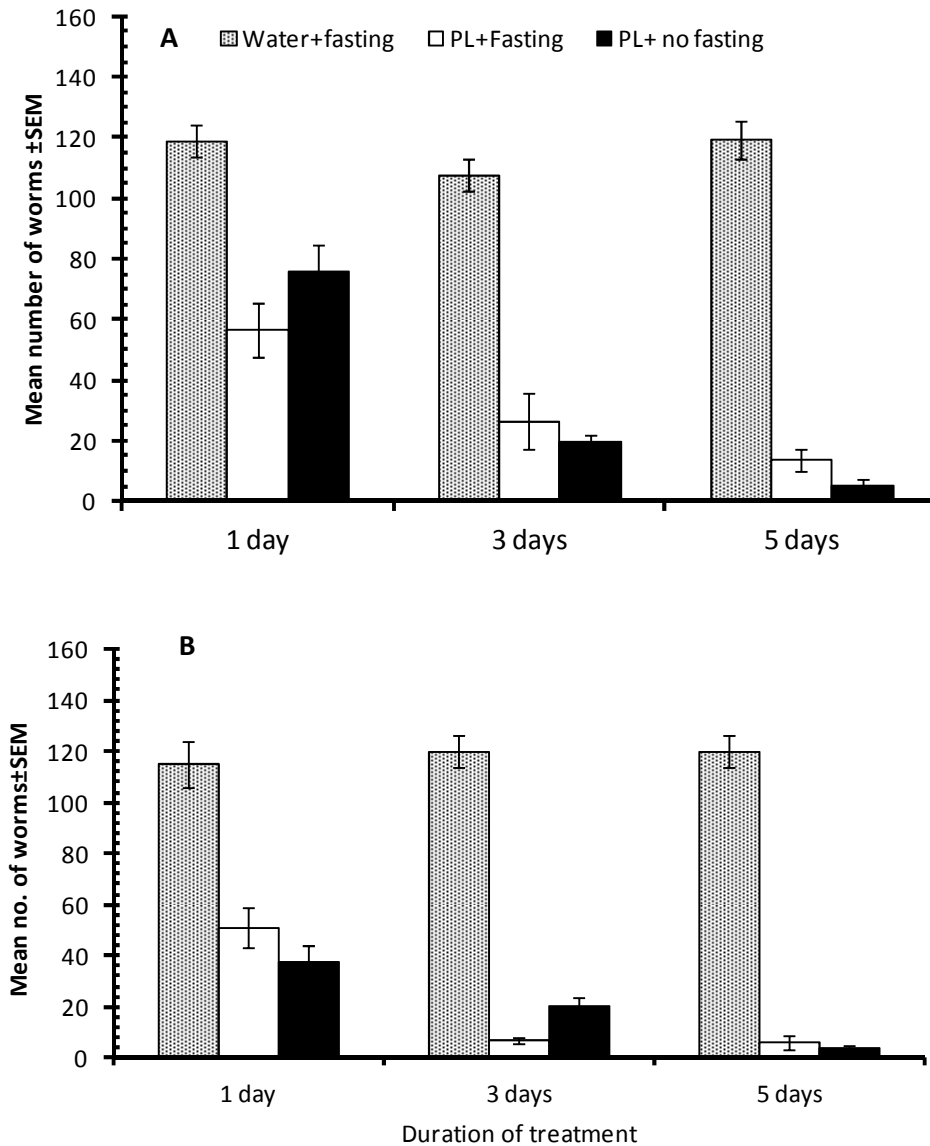


Figure 5.3: Effect of fasting or not-fasting on worm recovery of Experiment 2.

Effect of fasting or not-fasting on worm recovery after 1, 3 or 5 days of treatment with PLS. The results of block 1 are illustrated in A, and those of block 2 in B. The mice were treated either for 1 day, 3 days or 5 days and killed for worm counts on days 21, 23 and 25 post infection respectively.

As can be seen, and with the difference between blocks taken into account, overall there was no consistent difference in worm counts between the fasted or not-fasted treated groups (main effect of TREATMENT (fasting versus non-fasting, excluding the non-treated control group), $F_{1,66} = 0.009$, $P = \text{NS}$). There was a huge

effect on worm burdens with increasing duration of treatment (main effect of DURATION, $F_{2,66} = 76.0$, $P < 0.001$), and this differed to some degree between the two blocks of the experiment and between whether mice were fasted or not fasted (three-way interaction, BLOCK x DURATION x TREATMENT(fasted versus non-fasted), $F_{2,66} = 5.7$, $P = 0.005$), but there was no consistent effect in favour of either fasted or non-fasted animals.

As a final step, and to establish firmly that treatment with PLS was effective, albeit *a posteriori*, we fitted a three-way GLM but included the control water-treated, fasted group of mice this time. Not surprisingly there was a huge overall effect of TREATMENT (main effect of treatment (control, fasted plus PLS and non-fasted plus PLS), $F_{2,99} = 404.1$, $P < 0.001$), which is clearly apparent in Fig. 5.3 in both blocks of the experiment by the difference in worm burdens between the control water-treated, fasted group versus the two PLS-treated groups irrespective of whether the mice were fasted or not. With the data from both experiments combined, and relative to the control group, the reduction in worm burdens of the fasted mice was 54.4%, 87.3% and 92.8% after 1, 3 and 5 rounds of treatment, and for the not-fasted group the corresponding values were 55.2%, 82.9% and 96.2%, respectively.

Faecal egg counts were also carried out in each of these two blocks (Fig. 5.4) and these were broadly similar to those in Fig. 5.1 of Experiment 1, indicating that prior to treatment all mice had similar faecal egg counts, and that towards the end of the experiment they reflected the differences in worm burden between groups.

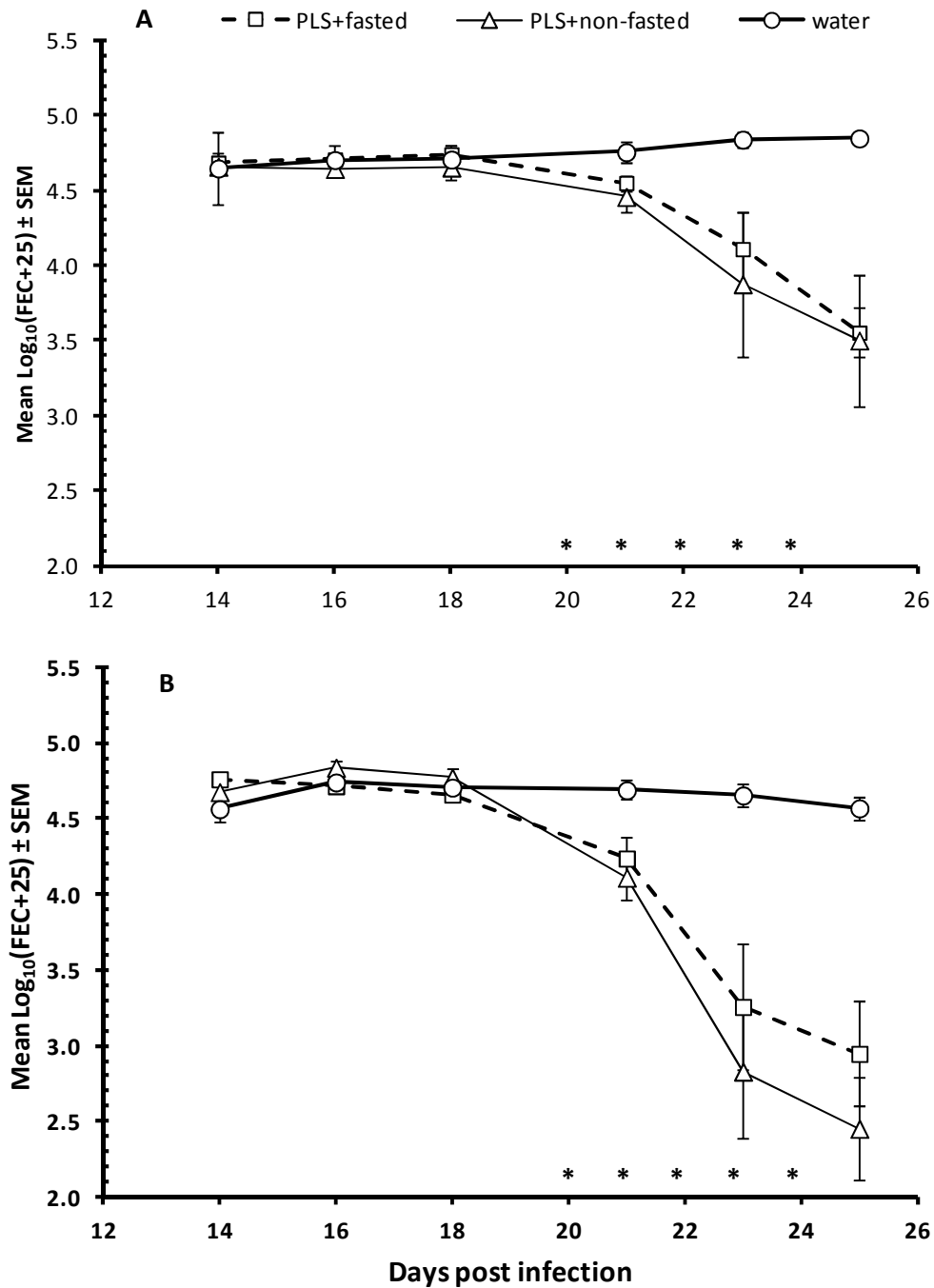


Figure 5.4: Faecal egg counts during the course of Experiment 2.

FEC results of block 1 Fig 5.4A and those of block 2 in B. The control group is illustrated by open circles, fasted group by open squares and the not-fasted group by open triangles. FEC was carried out on day 14, 16 and 18 (pre-treatment) and on day 21, 23 and 25 (during treatment). Treatment with PLS commenced on day 20 after infection and was given each day for 5 days. *indicates day of treatment.

5.3 Discussion

This chapter has demonstrated conclusively that the efficacy of PLS did not depend on fasting the animals prior to oral dosing (at least for the period of 5h that was explored here). Our results on the efficacy of PLS are in agreement with those of Satrija *et al.* (1995) who demonstrated anthelmintic activity of PL against *H. bakeri* worms in mice treated without food deprivation, but who did not evaluate the benefits or disadvantages of fasting *per se*.

In previous studies (Steppek *et al.*, 2007b) assessing the anthelmintic efficacy of PL against *H. bakeri*, mice were treated daily over 7 days with 313 nmoles of crude PL, preceded each time by food deprivation for 5–6 h. These experiments gave a 79% reduction of worm burdens. However, the present experiment has demonstrated that just three treatments, over three consecutive days with 240 nmoles of PLS without food deprivation was sufficient to eliminate 82.9% of the worms. Therefore, the current protocol, based on a refined extract of the active enzyme compartment of PL and less intervention (in that only three doses with less active enzyme content/dose were given), achieved comparable efficacy to that observed previously with seven doses (Steppek *et al.*, 2007b). This is likely to be beneficial for the wellbeing of the treated animals, reducing stress and the likelihood of side-effects.

Although food deprivation prior to oral dosing with CPs has been employed routinely in much of the earlier work on CPs in mice infected with nematodes, the present study has demonstrated clearly that fasting has no beneficial effect on the efficacy of PLS against *H. bakeri*. The presence of food typically affects a drug

through effects on absorption, but in this case the active ingredients work within the gut rather than relying on absorption. The results from this work are still surprising, as it would be expected that the presence of food could reduce the activity of the enzyme by dilution in gastrointestinal chyme and competition with other potential substrates for the enzyme. The maintenance of activity in the presence of food suggests that there must be other compensating factors influencing activity, which may include an increased residence time of the enzyme within the gut due to the increased volume of the gastrointestinal contents.

It can be concluded that there is no need in the future to fast the animals prior to treatment and this will also help to avoid any adverse side-effects of treatment, since food deprivation, even for limited periods of time, can cause stress and discomfort. Treatment for just 3 days at the active enzyme doses employed here is sufficient to eliminate a substantial worm burden from mice. These findings will be helpful for the research currently being undertaken to improve the formulation and delivery of these naturally occurring plant-derived enzymes for use as environmentally sound, organic therapies for the treatment of intestinal worm infections in humans and livestock, while achieving maximum efficacy and minimal side-effects.

**CHAPTER 6: INTRINSIC FACTORS CAUSING VARIATION IN THE
ANTHELMINTIC EFFICACY OF PLANT DERIVED
CYSTEINE PROTEINASES: HOST GENETICS, HOST SEX,
BODY SIZE AND INFECTION DOSE**

6.0 Summary

Eight strains of mice (BALB/c, CBA/ca, BKW, C3H, C57/BLK, CD1, NIH and NIH Swiss) were used to assess whether the anthelmintic efficacy of PL varied between mouse strains and therefore whether there is an underlying genetic basis influencing the degree to which PL is effective in removing *H. bakeri* worms from treated animals. The mice were first treated with 330nmol crude papaya latex (PLX) in 0.2ml pure water and then in the consecutive experiments were treated with 240nmol papaya latex supernatant (PLS) in 0.2 ml water. The results indicated wide variation of response in different strains of mice to PL medication. At the dose used, the treatment was most effective in C3H mice ranging from 90.5% to 99.3% reduction in worm counts and least effective in NIHS, CD1 and BALB/c strains (7.9%, 36.0% and 40.5% respectively). However, host sex and cimetidine treatment were shown not to have any influence on the anthelmintic efficacy of PLS. There was also no significant difference in efficacy between mice treated with the standard dose of 240nmol and those treated with adjusted doses based on mouse body weight (8µmol/kg body weight) in two strains, BKW and NIH. Physiologically there was no significant difference in pH changes along the GI tract, trypsin concentration and PLS activity of the poor (BALB/c) and high responder mice (C3H) to PLS treatment. It was also shown that the enzyme activity of PLS is not irreversibly inactivated even at low pH of 1.2. Finally, in responder mouse strains the efficacy of treatment was

not affected by the intensity of the worm burden, all worm burdens being removed with equal success.

6.1 Introduction

Epidemiological/pharmacological observational studies have shown that there is wide between-patient variability in the response to standard doses of drug therapy (Bosch, 2008). Such variation may range from failure to respond to a drug to life-threatening adverse reactions. These between-individual differences in response to standard doses may be attributable to the nature and severity of the disease being treated, nutritional status, individual age and race, organ function, concomitant therapy and illness. Although these factors are important, genetic differences between individuals within otherwise uniform subsets of the population (i.e. same racial background, age, sex etc.) are thought to be perhaps the most important factor in influencing the efficacy and toxicity of medications (Evans and Johnson, 2001). For many medications, these inter-individual differences are due in part to polymorphisms in genes encoding drug metabolizing enzymes, drug transporter and/or drug targets. This genetically determined variability among individuals in drug response defines the research area known as pharmacogenetics, where historically clinical observations on inherited differences in drug effects were first documented in the 1950s (Alving *et al.*, 1956).

The role of pharmacogenetics is to identify the network of genes that govern an individual's response to drug therapy with the ultimate goal of providing a stronger scientific basis for selecting the optimal drug therapy and dosages for each patient. It is also suggested that these genetic insights should be used as guidelines

for the discovery and development of new medications (McLeod and Evans, 2001). However, most of the literatures available for pharmacogenetics are based on the drugs absorbed in the blood and very few for drugs which act directly in the lumen of the intestine. Evans and Relling (2004) tried to summarize different genes controlling drug metabolizing enzymes and their influence on drug effects in humans. Only two drugs were found to have direct effects in the lumen. These drugs include omeprazole (an antacid treatment) which is used for treatment of peptic ulcers and heartburn and Irinotecan for treatment of colon and rectal cancer. These medications, however, have different modes of action when compared with PLS which directly attacks the parasitic cuticle.

In earlier chapters it has been shown that PLS has powerful anthelmintic effects on *H. bakeri* removing >90% of the parasites after 5 doses, and even 68% after just a single dose. Throughout the study, the mice used were males from the inbred C3H strain, which had been used by Stepek *et al.* in earlier work (Stepek *et al.*, 2007a, Stepek *et al.*, 2007b), whereas Satrija *et al.* (1995) used female mice of the BALB/c strain to assess the anthelmintic activity of papaya latex against *H. polygyrus* (currently known as *H. bakeri*). However, there has been no systematic study comparing mouse strains of known contrasting genotype.

Unlike drugs that are absorbed by the host, orally administered cysteine proteinases mostly stay in the lumen of the intestine where they act on worms directly. In this case therefore, between-host variation in drug efficacy could arise from polymorphic genes that influence the exact physiological conditions within the intestine, such as pH, host digestive enzyme concentrations, time of passage of food

through the gut, etc. The concentration of drug in the intestinal site where it is required to target worms may also vary due to differences in body size and weight, and hence proportionally differences between strains in the size and volume of the intestine.

Genetic differences between individuals that affect their susceptibility to a drug or affect the way the drug works on a targeted infectious organism can be considered as an intrinsic factor since genetic differences originate from within the host. Another prominent intrinsic factor is host sex. Male and female mammals differ markedly in the hormones that control their sex and reproductive cycles and these in turn have marked effects on their physiology. The differences in response to medication between males and females seem to be caused by steroid sex hormones (oestrogen, testosterone and progesterone) which can induce or inhibit the expression of Cytochrome P450 genes responsible for secretion of Cytochrome P450 enzymes in the liver (Cristofol *et al.*, 1998). Cytochrome P450 enzymes are involved in metabolism of exogenous toxic substances as well as endogenous substances (Kubota *et al.*, 2011, Finnstrom *et al.*, 2002). Variations in cytochrome P450 enzyme metabolism are seen prominently in the breakdown of medications. Depending on gene expression, drugs can be metabolized quickly or slowly. If a cytochrome P450 enzyme metabolizes a drug slowly, the drug stays active for longer and less is needed to get the desired effect. A drug that is quickly metabolized is broken down sooner and a higher dose might be needed to be effective. It is reported that Cytochrome P450 enzymes account for 70 – 80% of the enzymes involved in drug metabolism (<http://ghr.nlm.nih.gov/gene/Family/cyp>). Finnstrom *et al.* (2002) found a higher expression of CYP1B1 (a major P450 gene) in females than in males, a finding that

is consistent with its known role in the metabolism of oestrogens. Other factors which differentiate between males and females in drug responses include body weight (males weigh more than females), yet few drugs are dosed based on body weight. Additionally gastric acid secretion and its movement are lower in females than in males (Meibohm *et al.*, 2002, Henriksson *et al.*, 1986). Another relevant difference between the sexes is the higher percent body fat in females compared with males, which can increase the distribution of lipophilic drugs in females (Jochmann *et al.*, 2005). Also excretion of drugs through the kidneys is slower in females than in males because of the lower glomerular filtration rate (Anderson, 2008) and slower creatinine clearance rate due to testosterone which induces increased muscle metabolism in males (Meibohm *et al.*, 2002).

There are many examples of differences between the sexes in their susceptibilities to particular infections (Kiyota *et al.*, 1984, Alexander and Stimson, 1988, Bundy, 1988, Poulin, 1996) as also in the metabolism of and efficacy of administered drugs (Gokbulut *et al.*, 2009, Wolf *et al.*, 2000). Although sex difference in pharmacotherapy has been identified, only a few drugs exhibit differences, e.g. verapamil, beta-blockers and selective serotonin reuptake inhibitors (Meibohm *et al.*, 2002). It was important therefore to determine whether worm infections in mice of both sexes were equally susceptible to treatment.

Apart from the genetic host related factors, intensity of worm infection has been also reported to influence the efficacy of anthelmintics. Van Lieshout *et al.* (1999) reported a significant decrease in cure rate of praziquantel with increasing egg counts in individuals infected with *Schistosoma mansoni* before treatment in Senegal.

The same level of treatment failure was also observed in individuals treated with oxamniquine (Reis *et al.*, 2006). Recently a significant reduction in efficacy of albendazole against *Trichuris trichiura* with increase in intensity of infections has been reported (Levecke *et al.*, 2012). Due to these variations in efficacy of different types of anthelmintics there is also a need to determine whether the efficacy of PLS is affected by the intensity of worm burden.

Therefore the objective of this chapter was first to determine whether the efficacy of PLS treatment is host genotype dependent, by comparing inbred strains of mice which have defined genetic differences. Inbred mouse strains are expected to be >99% homozygous at all genetic loci, as a result of brother-sister mating for at least 20 generations and each strain has had a different selection history (<http://jaxmice.jax.org>). Thus within strains no significant genetic variation is expected, but between strains major differences in alleles are known to exist throughout the genome. Livestock on farms usually comprise well known and productive breeds but although breeds differ genetically between each other, within breeds there is still considerable genetic variation, allowing farmers to further select breeding stock and to improve breed quality with respect to productive traits such as meat, milk, wool etc. If the efficacy of PLS is dependent on host genotype, differences in efficacy might be expected between animals within breeds on farms where the therapy will be applied in the future. Thus the extent of variation arising from genetic differences between animals is clearly an important issue to assess.

A second objective of the chapter was to determine whether worm infections in male and female mice were equally susceptible to treatment with PLS.

The third objective was to examine the effect of cimetidine, an antacid which blocks acid in the stomach that might be inhibiting the activity of the PLS which is susceptible to pepsin degradation at low pH.

The fourth objective was to assess the degree to which genetic differences between strains might be attributable to differences in host body weight.

And finally, since recent data has shown that efficacy of anthelmintics can be dependent on the intensity of infection (Levecke *et al.*, 2012) the efficacy of treatment with CPs was assessed in two strains of mice that differ widely in their sensitivity to CPs, carrying three different levels of infection.

6.2 Experimental design and results

6.2.1 Experiment 1: Treatment of different strains of mice with crude papaya latex

This experiment was conducted by Stepek *et al* in 2004 and analyzed by WL. It comprised five strains of mice, three being inbred (BALB/c, C3H and CBA/ca) and two outbred (BKW and CD1). For each strain 10 mice were used with the exception of CD1 where 16 mice were used. All mice were infected with 200 *H. bakeri* L3 larvae on day 0. Then the mice in each strain were divided into two groups each with 5 mice, except CD1 which comprised groups of 8 mice. The first group of mice in each strain was treated with 330nmol crude papaya latex (PLX) in 0.2ml water daily for 7 days and the second group was given 0.2ml of ultra filtered pure water, both

treatments starting on day 19. All mice were fasted for about 6 hours prior to treatment but had *ad libitum* access to water. Faecal samples were collected on day 14, 16, 18 (pre treatment) and on day 19, 21, 23 and 25 (during treatment) post infection for quantification of parasite eggs and the mice were autopsied on day 25.

6.2.2 Experiment 1: Results

The results indicated that different strains of mice responded differently to treatment (Fig 6.1 and 6.2). In terms of faecal egg counts (FEC) BALB/c strain showed 75.3% reduction, CBA/ca mice had 82.1% reduction and BKW strain had 69.4% while CD1 strain had 56.6% reduction. On the other hand the C3H strain showed the greatest change in FEC amounting to 97.8% reduction, considerably greater than in any other strain (Table 6.1).

Statistically FEC were analysed by repeated measure GLM on \log_{10} (FEC+25) transformed data with time after infection as the within – subject factor, treatment (PLX or distilled water) and strains of mice as the between subject factors. The results indicated that there was a significant 2-way interaction of time* treatment (within subject measure; $F_{6,264} = 45.31, P < 0.001$) and a main effect of treatment (between subject measure; $F_{1,44} = 52.13, P < 0.001$). There was also a significant 2-way interaction between treatment*strains ($F_{4,44} = 5.14, P < 0.05$). The 3-way interaction between time*treatment*strain was also significant ($F_{24,264} = 5.55, P < 0.001$) and again as expected there was a significant overall effect of time (main effect of time, $F_{3,3,264} = 22.88, p < 0.001$).

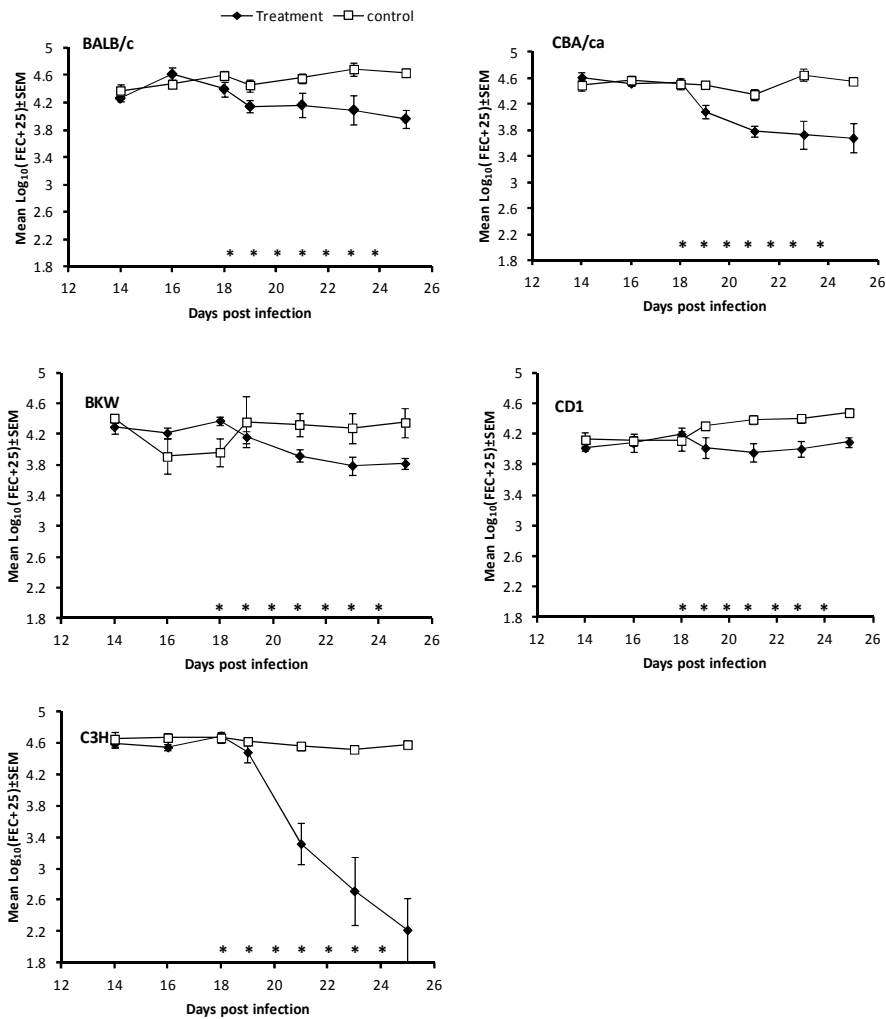


Figure 6.1: Faecal egg counts in different strains of mice treated with PLX

The figure shows the response of different strains of mice to PLX against *H.bakeri* measured in terms of average eggs/gram of faeces/mouse. Five strains of mice (BALB/c, CBA/ca, BKW, CD1 and C3H) each were divided into two groups. The first group was treated with 0.2ml of PL and the second group given distilled water, each group having 5 animals. First mice were orally gavaged with 150 L3 larvae and then treated for 7 days from day 19 post infection. Faecal egg counts were conducted on day 14, 16 and 18 (pre treatment) and on day 19, 21, 23 and 25 (post infections). The graphs show a progressive decline in egg counts with increased days of treatment. The decline is high in C3H and CBA strains for the groups treated with PLX (93.8% and 82.1% respectively) compared to those receiving water. Other strains experienced less reduction in worm burden (75.3%, 69.4% and 56.6% for BALB/c, BKW and CD1 respectively). *indicates day of treatment.

As in FEC the worm count data also indicated great variation in the extent of the reduction in worm burdens due to treatment between different strains of mice. The strain with the highest response to treatment was C3H (92.1%) and lowest response was observed in BALB/c and CD1 (40.3% and 36.0% respectively). Analysis by 2-way GLM using raw data gave a significant effect of treatment, $F_{1,46} = 189.34$, $p < 0.001$ and a significant interaction between strain and treatment $F_{4,46} = 7.13$, $p < 0.001$ indicating that the reduction in worm counts varied significantly between different strains of mice (Fig 6.2). *Post hoc* analysis by the Tukey indicated that post treatment worm burdens in C3H mice were significantly different to those in BALB/c and CD1 strains ($p < 0.05$ and $p < 0.001$ respectively).

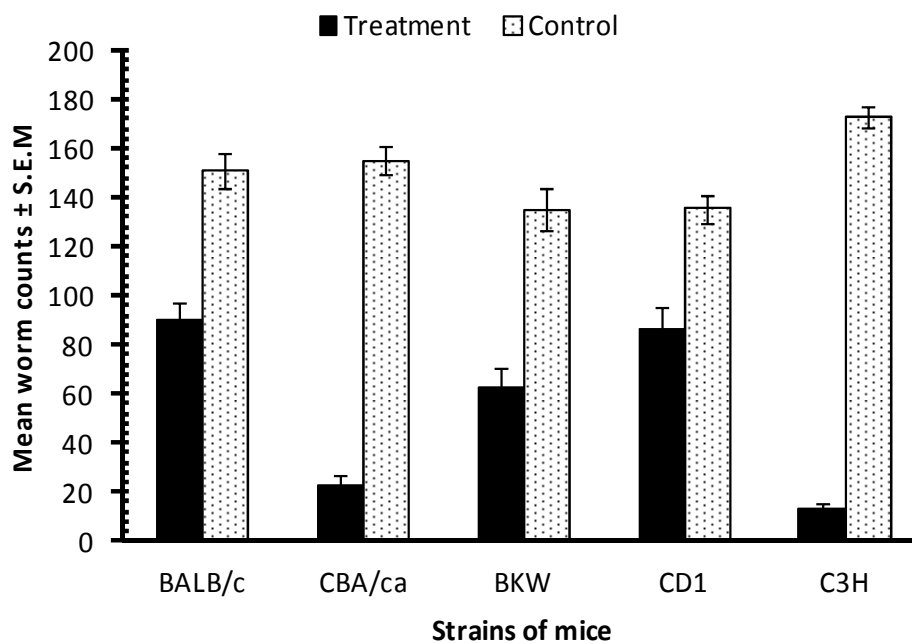


Figure 6.2: Intestinal worm count in different strains of mice treated with PLX

Intestinal worm burdens were assessed on day 25 post infection. Worm burdens from the treated and untreated group (n=5) of each strain are indicated by the different bars with standard errors of the means. The figure shows that C3H and CBA strains had a greater degree of reduction in worm burdens following treatment with PLS than other strains.

Table 6.1: Percentage reduction in FEC and worm counts in various strains of mice

	Experiment 1		Experiment 2		Experiment 3						Experiment 4		Experiment 6	
Mice strain	FEC	worm	FEC	worm	FEC		Worm		Combined sex		FEC	Worm	FEC	Worm
					M	F	M	F	FEC	worm				
BALB/c	75.3	40.3	5.0	47.2	-	-	-	-	-	-	-	-	-	-
BKW	69.4	53.6	-	-	-	-	-	-	-	-	-	-	69.9	60.1
CBA/c	82.1	85.4	-	-	-	-	-	-	-	-	-	-	-	-
CD1	56.6	36.0	-	-	-	-	-	-	-	-	-	-	-	-
C3H	97.8	92.1	97.1	95.9	96.1	90.6	93.1	88.5	92.5	90.6	99.3	98	97.0	91.9
C57/BLK	-	-	64.1	84.9	-	-	-	-	-	-	-	-	-	-
NIH	-	-	33.2	59.0	73.9	77.6	63.3	46.2	75.6	55.7	50.7	88	-	-
NIHS	-	-	-	-	-	-	-	-	-	-	-	-	38.2	7.9

Experiment 1: treatment of different strains of mice with PLX, Experiment 2: Treatment of additional strains of mice with papaya latex supernatant, Experiment 3: Response of male and female mice to PLS treatment, Experiment 4: Treatment of NIH mice with PLS based on body weight and Experiment 6: Response of BKW mice to PLS treatment based on body weight. FEC mean faecal egg counts

6.2.3 Experiment 2: Treatment of additional strains of mice with PLS

The second experiment extended the first by the addition of further strains of mice, and it differed in that in this case PLS was used rather than PLX. In this case two additional strains of mice were used C57/BLK and NIH, both inbred. BALB/c and C3H strains were used as negative and positive controls respectively (as they had shown contrasting results in Experiment 1, C3H showing a marked reduction in both FEC and worm counts while BALB/c a relatively poor change in both parameters). The experimental design was the same as that of the first experiment with the exception that mice were infected with 150 *H.bakeri* larvae instead of 200. Also they were treated with 240nmol PLS in 0.2 ml water daily for 5 days unlike in the first experiment when mice were treated with PLX for 7 days.

6.2.4 Experiment 2: Results

The results showed different responses to treatment in different strains of mice, as observed in Experiment 1. The effect of strain on the anthelmintic activity of PLS as assessed by FEC was analysed statistically by repeated measures GLM on $\log_{10}(\text{FEC}+25)$ transformed data, with time after infection as the within-subject factors, and treatments and strains as the between subject-factor in the full factorial model. The analysis was carried out only for four days of treatment as BALB/c were killed after 4 days of treatment, because of their deteriorating condition. The results indicated that the main effect of treatment was significant ($F_{1,36} = 9.274, p < 0.005$). The 3-way interaction time*treatment*strains was also significant (within subject analysis $F_{12,128} = 4.257, p < 0.005$). Based on arithmetic means there was great variation in the extent of FEC reduction, with reduction being weakest in BALB/c

(5%), intermediate in NIH and C57/BLK (33.2 & 64.2% respectively) and most intense in C3H (97.1%) mice (Fig 6.3). A *Post hoc* Tukey HSD analysis indicated a significant decline in FEC in C3H and C57/BLK mice ($p<0.001$ and $p<0.005$ respectively) when compared with BALB/c.

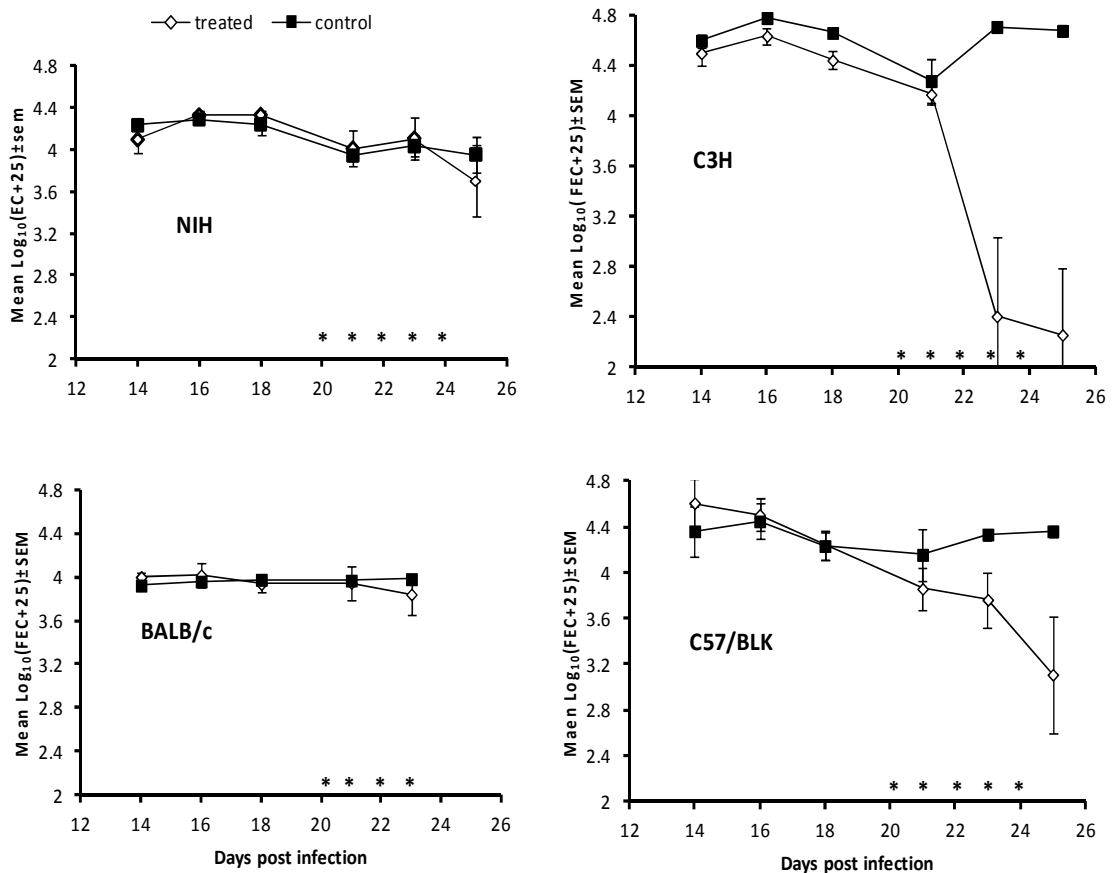


Figure 6.3: Mean FEC in different strains of mice treated with PLS.

The figure shows the mean FEC over time in 4 strains of mice (NIH, C3H, BALB/c and C57BLK). All 4 strains of mice were first orally gavaged with 150 L3 larvae and then each strain was divided into 2 groups, treated with PLS and those given distilled water for 5 days from day 20 post infection. FECs were conducted on day 14, 16, 18, 21, 23 and 25 post infections. The faecal egg counts decreased with increased days of treatment and the decline was more marked in C3H (97.1%) compared to the other strains (64.1 for C57/BLK, 33.2% for NIH and 5% BALB/c. *indicates day of treatment.

In terms of changes in worm burden due to treatment, marked variation between strains of mice was also observed, with reduction following treatment with PLS being greatest in C3H (95.9%) followed by C57/BLK (84.9%) then NIH (59.0%) and least in BALB/c mice (47.2%) (Fig 6.4). Analysis by 2-way GLM indicated a significant effect of treatment, $F_{1,32} = 481$, $p < 0.001$ and significant interaction between treatment and strain of mice $F_{3,32} = 6.16$, $p < 0.002$ indicating that the outcome of treatment depended significantly on strain of mice being treated.

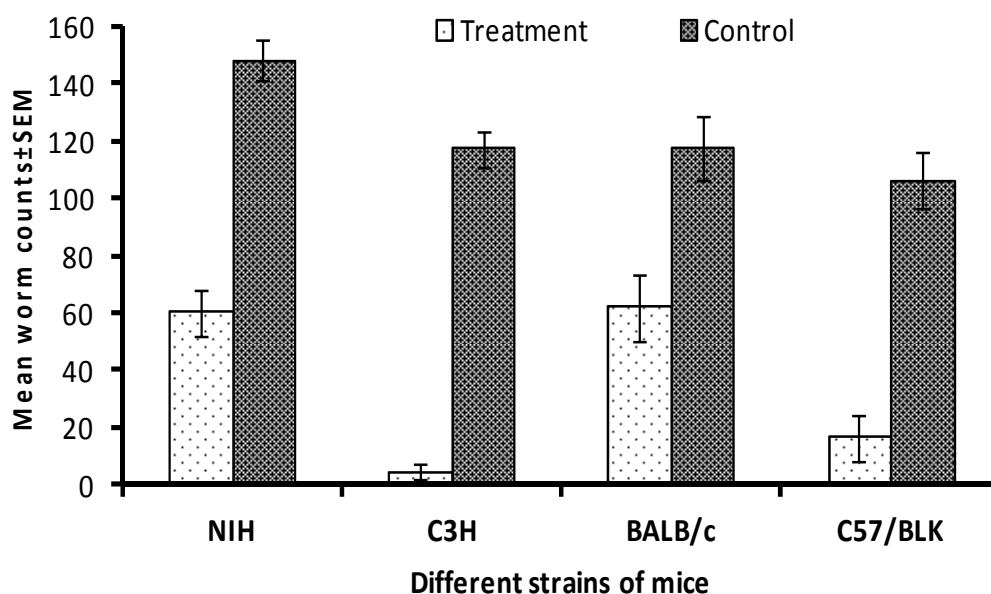


Figure 6.4: Intestinal worm count in different strains of mice treated with PLS

Mice were infected with 150 L3 larvae on day 0 and then treated with 8 $\mu\text{mol/kg}$ (240nmol/mouse) PLS once per day for 5 days from day 20 post infection. The worm burdens were assessed on day 25 post infection. The intestinal worm burdens of each strain, both groups treated with PLS and those receiving distilled water, are shown. Mice receiving PLS in each strain showed a significant reduction in intestinal worm burdens ($F_{1,9} = 10.364$, $p < 0.012$) compared with those given water.

6.2.5 Experiment 3: Response of male and female mice to PLS treatment

The objective of this experiment was to compare the response to treatment of male and female mice from a high and a lower responder strain. In this case two strains of mice were employed: C3H as the high responder and NIH as an intermediate responder, both inbred mice. The intermediate NIH strain was used rather than as originally intended the BALB/c because BALB/c mice showed intolerance to PLS in Experiment 2 and therefore in accordance with the 3Rs principle, NIH mice were selected as the contrasting strain to minimise suffering. In each strain 20 mice were used (10 male and 10 female). All mice were infected with 150 *H.bakeri* larvae on day 0. Then the mice in each strain were divided into two groups each with 10 mice (5 males and 5 females). The first group of mice in each strain was treated with 240nmol PLS in 0.2 ml water daily for 5 days and the second group was given 0.2ml of ultra filtered distilled water. Faecal samples were collected on day 14, 16, 18 (pre treatment) and on day 21, 23 and 25 (during treatment) post infection and mice were autopsied on day 25 for intestinal worm counts.

6.2.6 Experiment 3: Results

The results (Fig 6.5 & 6.6) indicate a different response to treatment between the strains as found in the previous experiments. For combined data of both male and female mice, C3H mice showed a better response in terms of reduction of both FEC and worm burden (92.5% and 90.6% respectively) due to treatment than NIH strain with 75.6% reduction in FEC and 55.7% reduction in worm burden (Table 1).

The Data for FEC were analysed by 4-way rm GLM with strain, sex and treatment as between subject factors and time as the within subject factor. Statistically, the results showed a significant main effect of treatment ($F_{1,32} = 37.54$, $p < 0.001$). The 3-way interaction time*treatment*strain was also significant ($F_{2,9,93,4} = 3.186$, $p < 0.005$). However, the main effect of sex was not significant and no interactions involving sex were significant.

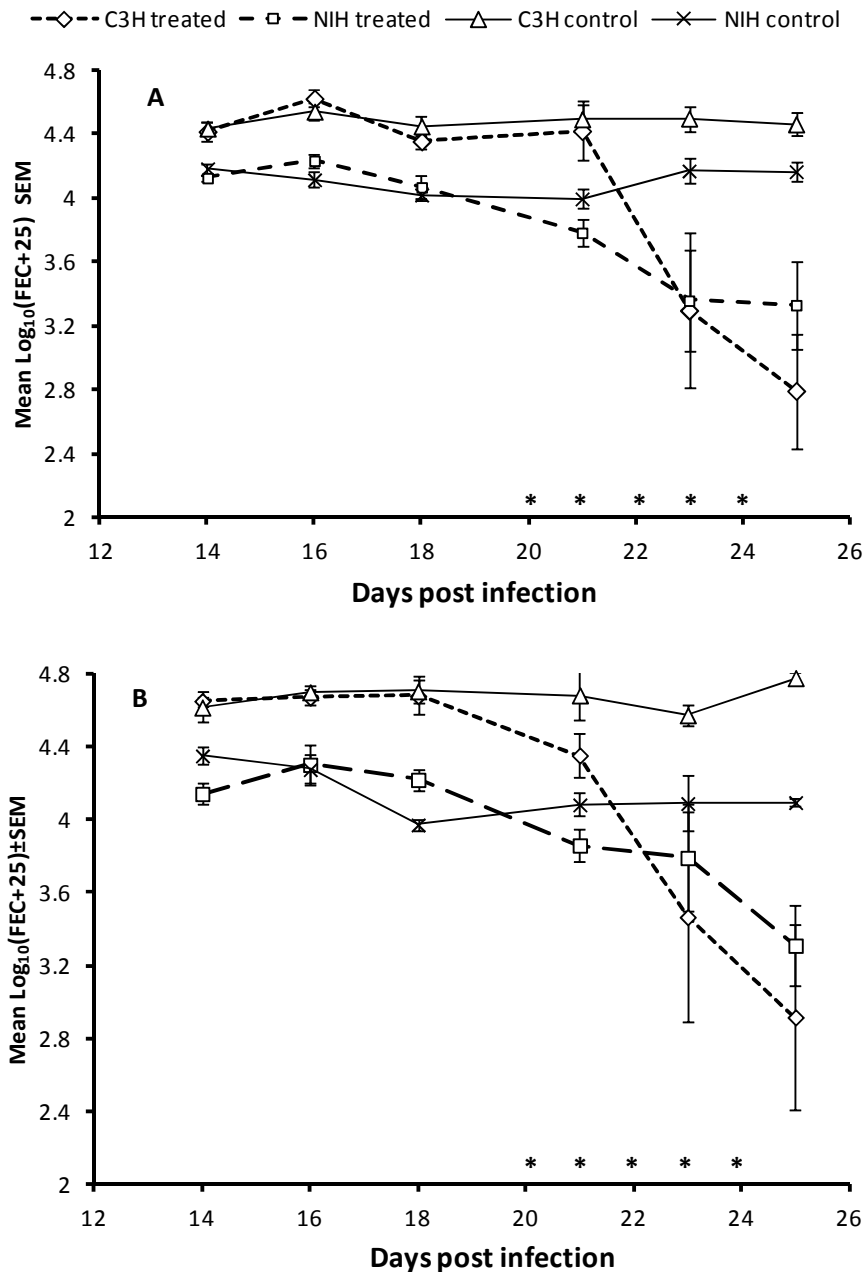


Figure 6.5: Mean FEC in male and female mice treated with PLS

Both strains (C3H & NIH) of mice were first orally gavaged with 150 L3 larvae on day 0 and then each strain was divided into 2 groups. The first group was treated with PLS and the second group was given distilled water for 5 days from day 20 post infection. FECs were conducted on day 14, 16, 18, 21, 23 and 25 post infections. The faecal egg counts decreased with increase in days of treatment and the decline was more marked in C3H mice irrespective of sex compared with NIH strain. A) FEC in male mice and B) in female mice. *indicates day of treatment.

Analysis of worm burdens by 3-way GLM indicated a significant effect of treatment, $F_{1,32} = 155.726$, $p < 0.001$ and a significant interaction between treatment*strain of mice $F_{1,32} = 25.044$, $p < 0.001$ but no significant interactions between treatment*sex ($p = \text{NS}$) and no main effect of sex, indicating that the efficacy of PLS does not depend on the sex of mice being treated in either strain, whether good responder to PLS therapy or intermediate.

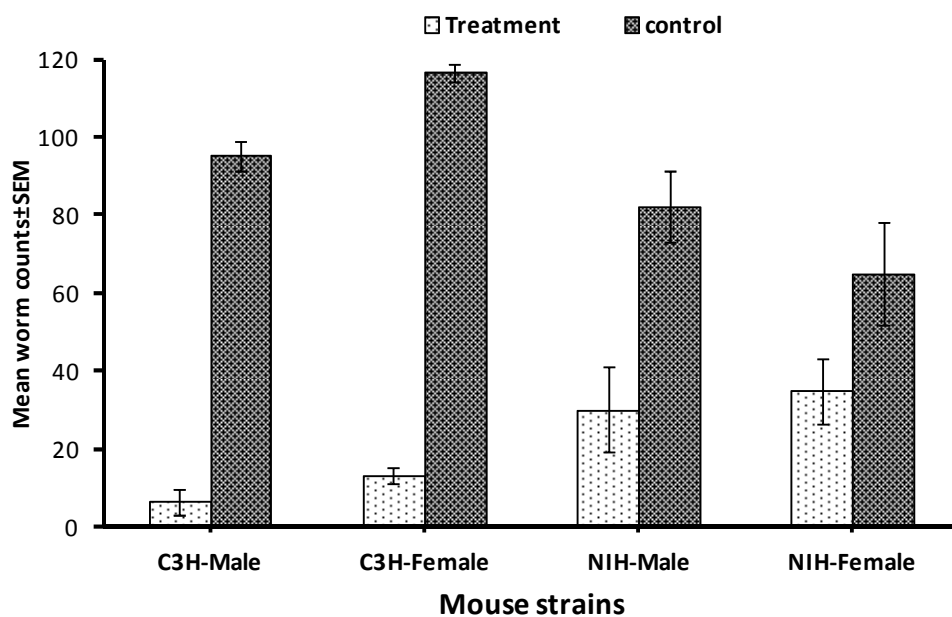


Figure 6.6: Intestinal worm count in male and female mice treated with PLS

Treatment details are as for Fig 6.5. The worm burden was assessed on day 25 post infection. The intestinal worm burden of each group treated with PLS and those receiving distilled water are shown, with the sexes of each strain illustrated separately. Mice receiving PLS in each strain showed a significant reduction in intestinal worm burdens ($F_{1,32} = 155.726$, $p < 0.001$) when compared with those receiving water. However the decline was significantly higher in C3H than in NIH mice indicated by the treatment*strain interaction ($F_{1,32} = 25.044$, $p < 0.001$).

6.2.7 Experiment 4: Treatment of NIH mice with PLS based on body weight

The aim of this experiment was to assess the efficacy of PLS based on mouse body weight. In this case NIH mice were used as they have been shown to have great variation in body weight and to vary in their response to treatment between individuals of the same age when compared with C3H mice. Thus 25 mice were used, 15 being NIH and 10 C3H mice. The 15 NIH mice were then divided into three groups of 5 mice each. One group of mice was treated with 0.2ml of the standard dose of 240nmol PLS regardless of their difference in body weight and the second set of mice was treated with an adjusted dose based on body weight of mouse (i.e. 8 μ mol/kg body weight). In this case the amount of PLS used was adjusted each day according to the weight of the mouse. It was assumed that the amount of PLS required depends on the body weight of the mouse and hence differences in amount of drug administered arise if body weight is not taken into account. The last group was gavaged orally with ultra-filtered distilled water. While the C3H mice were used as positive controls and divided into 2 treatments, the first group was treated with a standard dose of 240nmols of PLS and the second group was given distilled water. This experiment was conducted simultaneously with Experiment 7.

6.2.8 Experiment 4: Results

The results (Fig 6.7) showed indistinguishable responses of mice to treatment with PLS whether given as a standard dose of 240nmol/mouse or adjusted daily for host body weight to comprise 8 μ mol/kg body weight. There was a 50.7% FEC reduction for mice treated with the standard dose and 63.2% for those treated based on body weight. In terms of worm burden there was a reduction of 88% for mice treated with

the standard dose and 85.1% for those treated based on their body weight. Although the mice treated according to daily body weight showed a slightly higher figure for the reduction of FEC, when statistically tested by repeated measure GLM on $\log_{10}(\text{FEC}+25)$ transformed data, there was no significant difference between the 2 groups ($F_{2,10} = 0.042, p = 0.958$).

In terms of worm burden one-way ANOVA was used to analyse the data and this indicated a significant difference between groups of mice treated with PLS and those given water ($F_{2,14} = 65.75, p < 0.001$). *Post hoc* analysis indicated no significant difference between mice treated with the standard dose and those treated with an adjusted dose based on their body weight (Tukey test; $p = 0.944$). The C3H mice which were used as positive controls showed the expected superior response to treatment with a 99.3% reduction in FEC and 98% for worm burdens. The NIH had body weight variation of 25 g to 35 g.

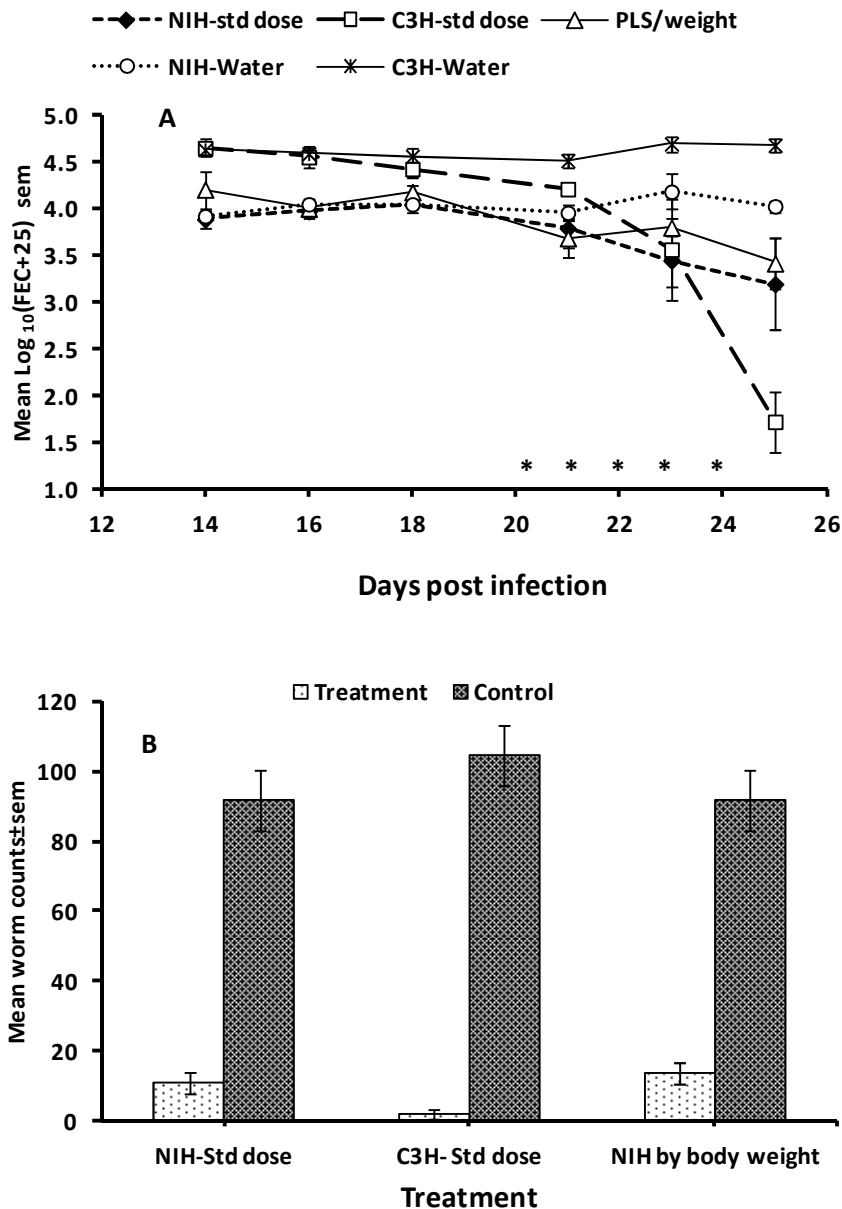


Figure 6.7: FEC and worm count in NIH mice treated based on body weight

NIH mice were first orally infected with 150 L3 larvae on day 0 and then divided into 3 groups. The first group of mice was treated with the standard dose of PLS for 5 days, the second group was treated based on adjusted body weight dose and the third group was given distilled water. Two groups of C3H mice were also included, the first group being treated with the standard dose of PLS and the second group was given water. FECs were conducted on day 14, 16, 18, 21, 23 and 25 post infections and worm counts on day 25. Both FECs (A) and worm counts (B) decreased significantly in the PLS treatment groups and there was no significant variation between the treated groups. *indicates day of treatment.

6.2.9 Experiment 5: Response of NIH Swiss mice to PLS treatment

In this experiment NIH Swiss (NIHS) mice were tested for their response to treatment with PLS. This strain is an outbred strain from which the inbred NIH strain was developed. The latter was selected as intermediate responder to compare with the C3H mouse response to PLS treatment. The aim of the experiment was to examine how this outbred strain responds to treatment with the standard dose of PLS when compared with C3H. It was expected that they would show a similar response to treatment if there was not any underlying genetic effect. This experiment was carried out simultaneously with Experiment 6 so that the same positive control C3H mice could be used in both experiments in order to minimize the number of animals used. In this case 10 NIHS mice were divided into 2 groups, the first group was treated with the standard dose of PLS and the second group was given distilled water.

6.2.10 Experiment 5: Results

The results indicate that NIHS mice experienced only a 37.3% reduction in FEC and 7.9% reduction in worm burdens, while C3H showed 97.0% reduction in FEC and 91.9% worm burden (Fig 6.8). Statistical analysis based on repeated measure GLM on $\log_{10}(\text{FEC}+25)$ transformed data showed a significant main effect of strain ($F_{1,15} = 22.742$, $p < 0.001$) and the 2-way interaction between treatment*strains was also significant ($F_{1,15} = 8.601$, $p < 0.01$). However there was no significant main effect of treatment ($F_{1,15} = 0.006$, $p = 0.938$) nor a significant interaction between time*treatment*strain ($F_{3,8,57.26} = 0.924$, $p < 0.453$).

Analysis of worm burdens by 2-way GLM indicated a significant main effect of treatment, $F_{1,15} = 45.594$, $p < 0.001$ and interaction between treatment*strain of mice $F_{1,15} = 35.983$, $p < 0.001$ but no significant main effect of strain $F_{1,15} = 3.715$, $p = 0.073$.

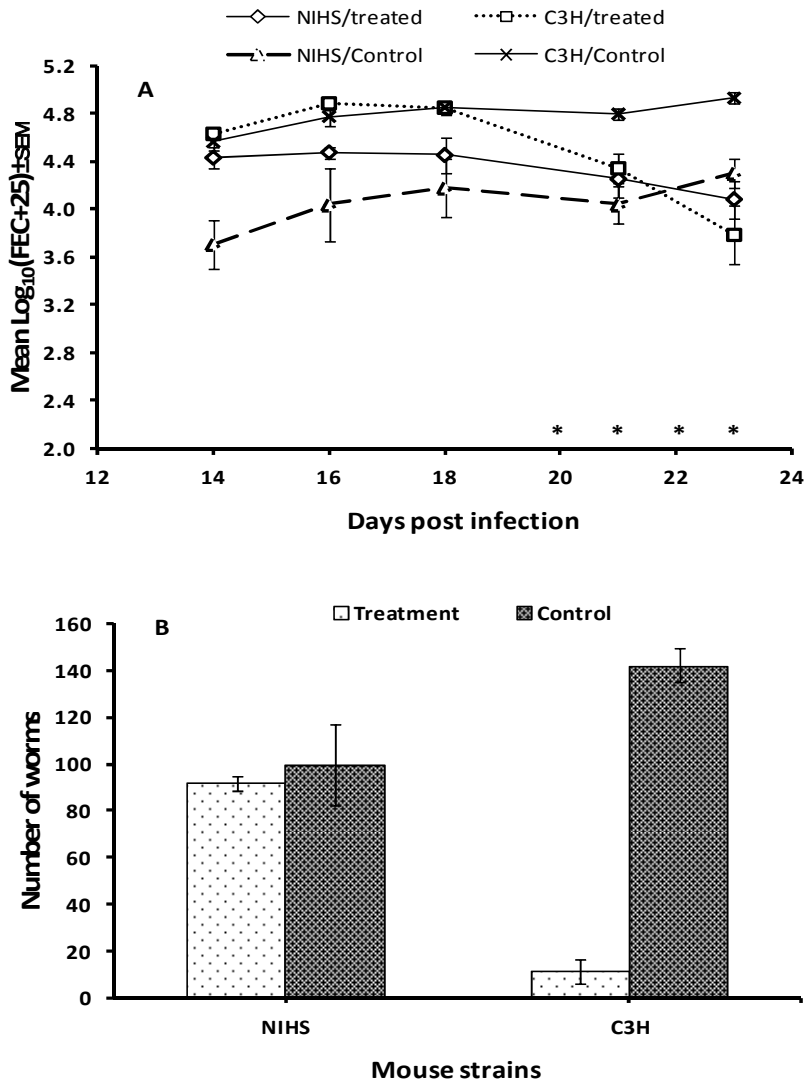


Figure 6.8: FEC and intestinal worm count in NIHS mice treatment with PLS

NIH swiss and C3H strains infected with 150 L3 on day 0 were divided into 2 groups for each strain (those treated with standard dose of 240nmol of PLS and those given 0.2ml distilled water). FEC were conducted on day 14, 16, 18, 21, 23 and 25 and worm burden on day 25 post infections. The results indicated significantly higher reduction in both FEC and worm burden in C3H strain compared with the NIHS.

6.2.11 Experiment 6: Response of BKW mice to PLS treatment based on body weight

This experiment was a repetition of Experiment 4 which aimed at assessing the efficacy of PLS based on mouse body weight. In this experiment BKW mice were used, instead of the NIH mice which had been used in the previous experiment but showed low variation in body weight and hence less deviation in response to treatment. BKW mice were chosen because they are heavier than NIH mice and are outbred with expected greater variation in body weight. Thus they were expected to show more deviation in response to treatment within the same group than the inbred strains. As usual C3H mice were used as the positive control.

Therefore 25 mice were used, 15 BKW and 10 C3H mice. All mice were first infected with 150 L3 *H. bakeri* then the 15 BKW mice were divided into 3 groups of 5 mice each. The first group was treated with the adjusted dose based on body weight of the mouse as explained in Experiment 4, the second group was treated with the standard dose of 240nmol PLS in 0.2 ml pure water and the third group was gavaged orally with ultra-filtered distilled water. The C3H mice were divided into 2 treatment groups, the first group was treated with a standard dose of 240nmols of PLS and the second group was given distilled water.

6.2.12 Experiment 6: Results

In this experiment, treatment was only continued for 4 days instead of the normal 5 days treatments. This is because some mice in the treatment groups showed some signs of distress with one of them having a distended abdomen. Although all the

animals were still within the moderate band of our Home Office project licence, it was suspected that the distressed animals might develop the same problem of distended abdomen, causing distress and suffering to the animals. For this reason the animals were culled one day before the planned end of the experiment while 3 of them were terminated 2 days before as they showed earlier signs of distress. One of the mice was from the group treated with a standard dose and 2 from the group treated based on body weight. The data from these mice were excluded in the final analysis as they had received fewer doses. Only the C3H mice were left until the end of the experiment as they did not appear to develop similar symptoms in this or any of the previous experiments in which they had been used.

The results (Fig 6.9) showed that the mice treated with a standard dose had a high reduction in the FEC (62.5%), in fact greater than those treated based on body weight (38.2%). However, when statistically tested by repeated measures GLM on \log_{10} (FEC+25) transformed data between the 2 groups (analysis confined to the two treatments in BKW mice), there was no significant difference between treatments ($F_{1,5} = 3.32, p = 0.128$). However, when BKW and C3H mice treated with the standard dose were analyzed by repeated measure GLM on \log_{10} (FEC+25) transformed data, the results showed a significant main effect of treatment ($F_{1,15} = 22.464, p < 0.001$) and the main effect of strain ($F_{1,15} = 23.58, p < 0.001$). The 3-way interaction time*treatment*strain was also significant ($F_{4,60} = 4.207, p < 0.005$) but there was no significant interaction between strain and treatment ($F_{1,15} = 0.221, p < 0.645$).

In terms of effects on the worm burdens there was a reduction of 60.1% for mice treated with the standard dose and 56.7% for those treated based on their body weight. One-way ANOVA was used and indicated no significant difference between the 2 groups of mice treated with PLS based on body size and those treated with the standard dose ($F_{1,8} = 0.081, p=0.7841$). But when the two strains, BKW (treated with standard dose) and C3H were analysed by 2-way GLM. There was a significant main effect of treatment ($F_{1,16} = 186.76, p<0.001$) and an interaction between treatment*strain ($F_{1,16} = 15.905, p<0.001$). The C3H mice which had been used as positive controls showed the expected greater response to treatment with a 97.0% reduction in FEC and 91.9% in worm burden. BKW mice had body weight variation was between 28 g to 40 g.

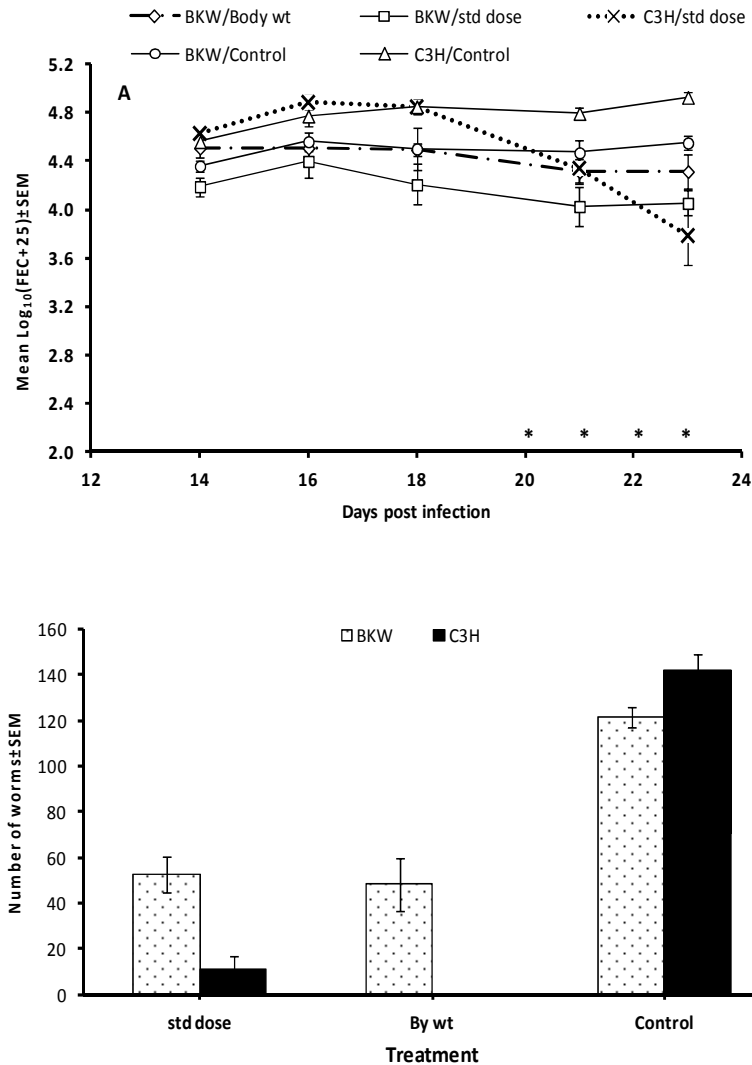


Figure 6.9: FEC and worm counts in BKW mice treatment based on body weight

BKW and C3H mice were orally gavaged with 150 L3 on day 0. Then BKW mice were divided into 3 groups (those treated based on body size, treated with standard dose of 240nmol of PLS and those given 0.2ml distilled water), while C3H mice were divided into two groups (those given standard dose and those given distilled water). FECs were conducted on day 14, 16, 18, 21, 23 and 25 and worm burdens on day 25 post infections. There was a significant reduction in FEC (A) and intestinal worm counts (B) in the treatment groups when compared with control group. However, the reduction was not significantly different between the group treated based on mouse body size and that given the standard dose. *indicates day of treatment.

6.2.13 Experiment 7: Effect of cimetidine on the efficacy of PLS

The objective of this experiment was to assess the effect of cimetidine on the efficacy of PLS and whether cimetidine had any effect on worm burden. In this experiment 10 NIH mice were divided into two groups, the first group was given cimetidine 15 minutes prior to being treated with 0.2ml of the standard dose of 240nmol of PLS while the second group was only given cimetidine. This treatment was intended to block the acid secreted in the stomach which is assumed to inhibit the activity of the PLS. Based on 3Rs principle this experiment was carried out at the same time as Experiment 4 so as to use the same control groups (NIH & C3H treated with standard dose of 240nmol and those given water).

6.2.14 Experiment 7: Results

The results of this experiment showed no difference between mice treated with PLS alone and those given cimetidine prior to being treated with PLS (Fig 6.10). There was a 50.7% FEC reduction for mice treated with only PLS and 56.3% for those given cimetidine prior to being treated with PLS. In terms of worm burdens there was a reduction of 88% for NIH mice treated with PLS alone and 80.5% for those administered with cimetidine before being treated with PLS. In C3H mice the reduction was 99.3% in FEC and 98% for worm burdens.

FEC data were analysed by rm GLM with treatment as the between subject factor and time as the within subject factor. The results showed a significant main effect of treatment ($F_{3, 14} = 7.141, p < 0.04$). However, there was no significant main effect of cimetidine ($F_{1, 27} = 2.769, p = 0.108$) or 3-way interaction, time*PLS

treatment*cimetidine ($F_{3.04, 81.97} = 0.808, p = 0.494$). Analysis of worm burdens by 2-way ANOVA indicated a significant main effect of PLS treatments ($F_{1,16} = 143.814, p < 0.001$) but no significant main effect of cimetidine ($F_{1,16} = 0.021, p < 0.886$) or interaction of PLS*cimetidine ($F_{1,16} = 1.107, p < 0.308$). This implies that the efficacy of PLS is not influenced by cimetidine administration.

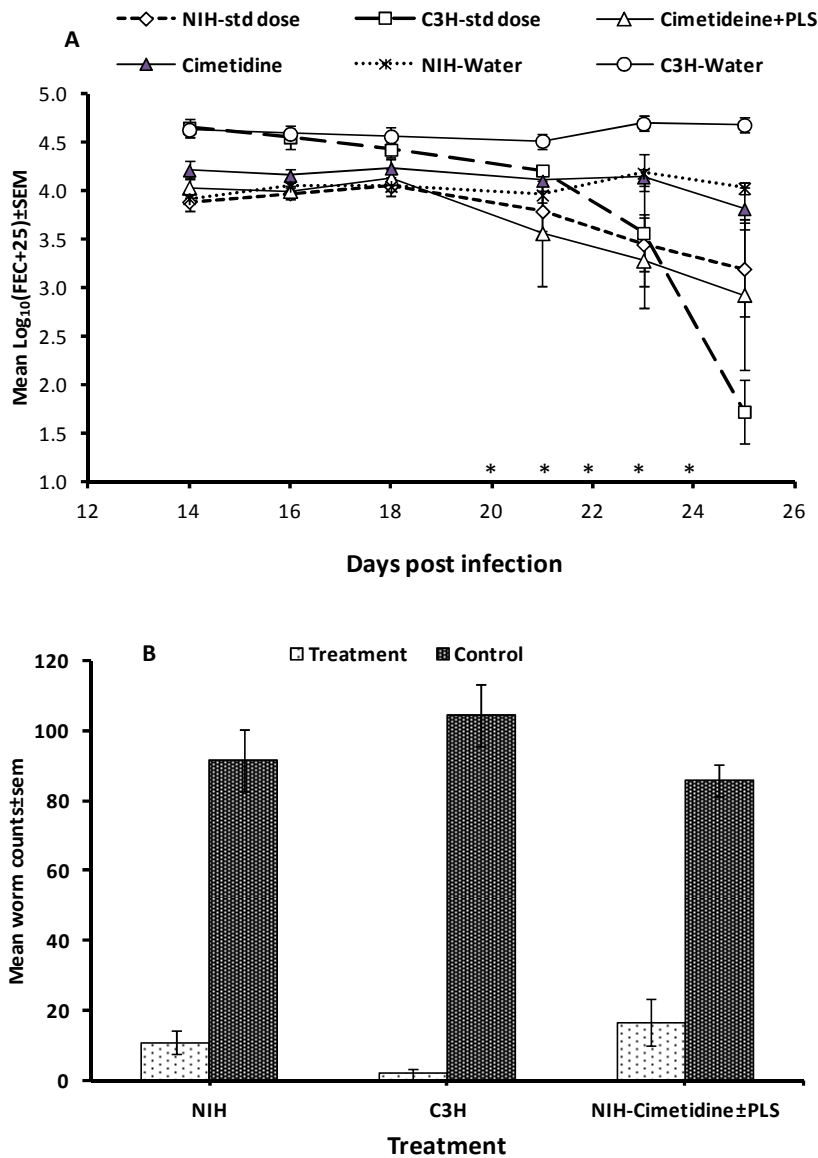


Figure 6.10: FECs and worm burdens in mice given cimetidine before treatment

NIH mice were orally gavage with L3 larvae on day 0. Then mice were divided into 4 groups, 2 treatment groups (those treated with standard dose of 240nmols and those given cimetidine 15 minutes prior to being treated with 240nmol of PLS) and 2 control groups that received cimetidine alone and another group given distilled water. C3H mice comprised 2 groups, those treated with standard dose of PLS and those given water. There was significant reduction in FEC (A) and intestinal worm counts (B) in the treatment groups when compared with those receiving either cimetidine alone or water. However, there was no significant reduction between the 2 treatment groups (PLS and cimetidine + PLS).

6.2.15 Experiment 8: Enzyme survivability at low pH.

This experiment was carried out to determine whether the CPs at low pH are permanently damaged by either the the low pH itself or by pepsin secreted in the stomach. To achieve this objective PLS was diluted in a ratio of 1:10 either in i) a saline solution made with 0.85% NaCl at pH 7, ii) saline solution + HCl at pH 1.2, iii) saline solution + HCl + 1% pepsin at pH 1.2 or iv) saline solution + HCl + 1% pepsin at pH 3.2 (mimicking the mouse stomach environment). All the solutions were made in duplicate and incubated at 37°C for 2 hours and then tested for enzyme activity. The absorbance values were then converted into concentrations by multiplying with molar extinction coefficient of $8800\text{M}^{-1}\text{cm}^{-1}$ for papain (Mole and Horton, 1973). Under these assay conditions 1 unit of enzyme activity was defined as that which produced 1 nmol min^{-1} of product.

6.2.16 Experiment 8: Results

The results shown here are the percentage enzyme recovery when compared to that incubated in saline without pepsin at pH 7 and were 93.3% for enzyme incubated at pH 1.2 with pepsin, 92.0% for that incubated at pH 1.2 but without pepsin and 89.1% for the enzyme incubated at pH 3.2 with pepsin. Examining these results it is evident that the amount of enzyme activity recovered under the different conditions tested was almost similar to that when PLS was incubated at pH 7 which is the optimum pH for CPs activity. This means that if the enzyme was inactivated at pH 1.2 then it was reactivated subsequently in the activity assay after being incubated with L-cysteine.

6.2.17 Experiment 9: Measurements of pH of the GI tract of mice.

This experiment was conducted to test whether the reduced efficacy of PLS in some strains of mice could be due to lower intestinal pH, with resultant inhibition of the enzyme activity against the nematodes. Thus there was a need to determine whether there was any difference in pH levels along the GI tracts of the poor and high responder strains of mice.

A group of 10 mice (5 BALB/c and 5 C3H) was used but unfortunately, 1 mouse in C3H group died before the start of the experiment thus leaving 4 mice for the actual experiment. All mice had free access to food and water. One mouse at a time was killed and immediately dissected, after which the intestinal tract was removed and divided into sections: stomach, upper small intestine (duodenum), lower small intestine (Ileum), caecum and colon. A small cut was made in the middle of the gut wall in each section in which a 6 mm electrode of a pH meter was inserted and the pH was read when stability was achieved. Measurements were made in sequence from the stomach to the colon. The electrode was rinsed with distilled water between each reading. The same procedure was repeated on all mice and it took about 3-5 minutes for the whole procedure on each animal to be completed.

6.2.18 Experiment 9: Results

The pH values of the different GI sections of both BALB/c and C3H are shown in Figure 6.11. As expected the lowest pH value was seen in the stomach of both strains BALB/c (3.53) and C3H (3.54) and then pH increased steadily in the duodenum to colon. The lower part of small intestine had slightly higher pH values than any other

parts of the GI tract (6.83 for BALB/c and 6.61 for C3H). Statistically the pH varied significantly along the GI tract sections ($F_{4,28} = 93.232, p < 0.001$), however there was no significant difference in the variation observed in pH between the two strains of mice ($F_{1,7} = 2.159, p < 0.185$), even the interactions between pH change in different sections of the GI tract and the strains being not significant ($F_{4,28} = 0.32, p < 0.862$). Therefore, it is clearly evident that the pH in particular regions of the intestine and how this changes along the length of the mouse GI tract does not differ between these two strains of mouse.

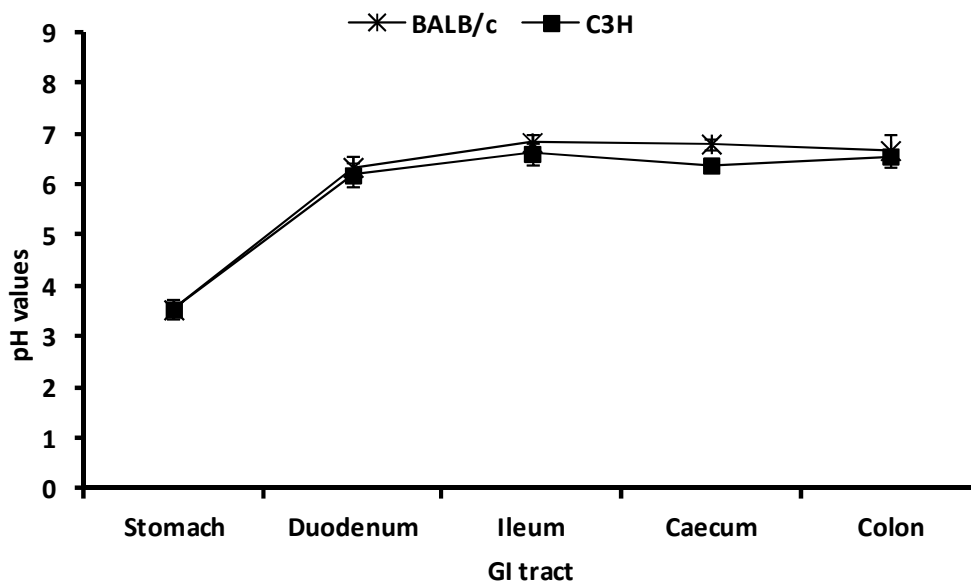


Figure 6.11: Measurements of pH along the mouse GI tract

The figure shows the change in pH along the GI tract (stomach, duodenum, ileum, caecum and colon) of the 2 strains of mice (BALB/c and C3H).

6.2.19 Experiment 10: Enzyme activity along the mouse GI tract

This experiment aimed at assessing the activity of CPs along the GI tract in poor (BALB/c) and higher responder (C3H) strains of mice. To achieve this objective 12

uninfected mice (6 from each strain) which had free access to both water and food were administered orally with 240 nmol PLS in 0.2 ml pure water and then one mouse from each strain was killed, by cervical dislocation, after the following time intervals: 10, 20, 60, 90, 120 and 180 minutes. At each time interval, entire GI tract from the stomach to the large intestine was removed from each mouse, briefly washed in a Petri dish of PBS and then divided into 5 sections: the stomach, the upper half of small intestine (duodenum), lower half of small intestine (ileum), caecum and the large intestine (colon). These sections were opened longitudinally and washed individually with 1 ml of PBS before the contents were filtered using muslin cloth.

The enzyme activity in each section over a 3h period was determined by activity assays using BAPNA as the substrate with and without E-64. The E-64 was used in order to distinguish the activity of the CPs and that of trypsin and other GIT enzymes, which can also cleave BAPNA. To ensure complete inactivation of CPs 2.0 nmol E64 in 100 μ l water was incubated with 1.6 nmol PLS enzyme solution in 10 μ l water. The following equation was used to calculate the hydrolysis units (U) of BAPNA: $U = A(410\text{nm})/\text{min} \times 1000 \times 1/8800$, where $8800\text{M}^{-1}\text{cm}^{-1}$ is the *p*-nitroaniline molar extinction coefficient and 1 unit of enzyme activity was defined as that which produced 1 nmol min^{-1} of the product.

6.2.20 Experiment 10: Results

The results indicated that there was a similar pattern of enzyme activity variation along the GIT of both strains (BALB/c and C3H) of mice. In both strains the activity

was initially higher in the stomach but rapidly declined after 90 min with only negligible levels of enzyme activity remaining thereafter. Whereas the activity of the enzyme in the small intestine rose steadily in the first 60 min and then started to decline rapidly by 90 min, a relatively low level of activity was observed particularly in the upper small intestine. At the same time the activity appeared to increase steadily in the caecum and colon, although in BALB/c the levels of activity in the lower intestine remained higher until 120 min. In short, the enzyme activity in both strains of mice decreased gradually in the stomach and small intestine over the first 90 min and then increased steadily in caecum and colon, so that by 180 min the majority of the enzyme activity was only in the large intestine particularly in the caecum (Fig 6.12).

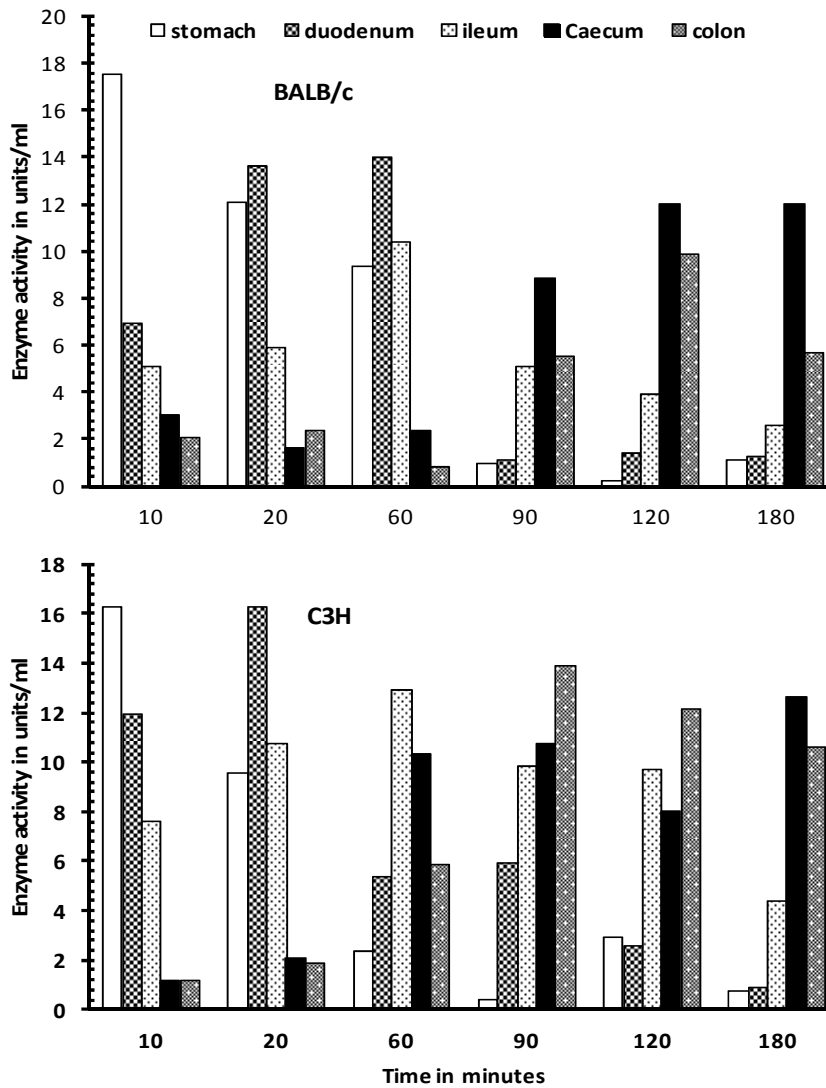


Figure 6.12: Enzyme activity along the mouse GI tract

The figure shows the variation in enzyme activity along the GI tract (stomach, duodenum, ileum, caecum and colon) of the 2 strains of mice (BALB/c and C3H) after different time intervals. Enzyme activity in both strains of mice declined rapidly in stomach and intestine over time but increased steadily in the caecum and colon.

6.2.21 Experiment 11: Determination of trypsin concentration in mouse intestine

The aim of this experiment was to determine the activity of trypsin in the GIT of the poor and higher responder strains of mice. It was possible that in poor responder

mice trypsin is secreted at higher levels than in responder strains, thus causing greater digestion of PLS and degradation of the enzyme with resultant reduction of the activity of PLS. To achieve this objective eight untreated mice were killed (4 BALB/c & 4 C3H) and the entire GI tract from the stomach to the large intestine was removed from each mouse, briefly washed in a Petri dish of 20mM tris buffer and then divided into 5 sections: the stomach, the upper half of the small intestine (duodenum), lower half of the small intestine (ileum), caecum and colon. As usual the sections were opened longitudinally and washed individually with 1 ml of tris buffer before the contents were filtered using muslin cloth.

The activity of trypsin was determined by activity assay using BAPNA as substrate. Briefly 10 μ l of enzyme solution was added to 12.5 μ l of 100 mM BAPNA solution and then 977.5 μ l of 20 mM tris.HCl pH 7.5, 5 mM CaCl₂ buffer were added to make 1 ml of the assay. The production of p-nitroaniline was measured by spectrophotometry at 410nm. BAPNA hydrolysis units, which are equivalent to enzyme activity, were calculated using the same producer as in Experiment 10. Stomach readings were used as negative control.

6.2.22 Experiment 11: Results

As expected, high activity was found in the small intestine of both strains of mice particularly in the lower part of the small intestine (Fig. 6.13). Statistical analysis by one-way GLM showed that there was no significant difference between the two strains ($F_{1,6} = 0.462$, $P = 0.522$). These results indicate that the patterns of trypsin activity are similar along the length of the intestines of both strains of mice, and

hence variation in secretion of trypsin is not likely to be an explanation for the difference in the efficacy of PLS between these two strains of mice.

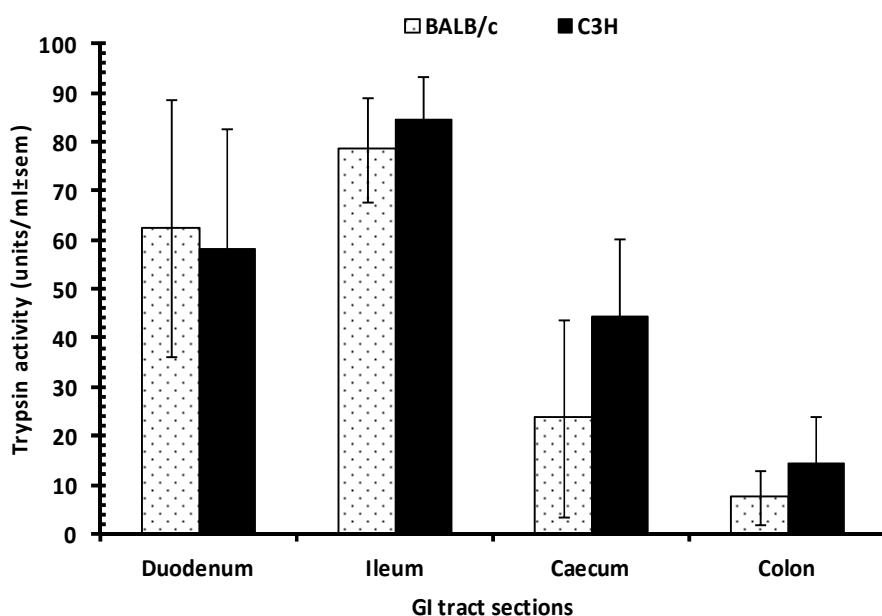


Figure 6.13: Trypsin activity along the GI tract of the 2 strains of mice

The variation in the amount of trypsin along the intestine of BALB/c and C3H strains of mice.

6.2.23 Experiment 12: Effect of worm burden intensity on the efficacy of PLS

This experiment was aimed at assessing the effect of worm intensity on the efficacy of PLS. In this experiment, 2 strains of mice were used, BALB/c, which had been excluded previously from experiments because the mice had shown less tolerance to PLS and C3H which has been used all the time as positive control. Since they had been used only once (Experiment 2) and then showed such marked side effects of treatment, it was necessary to conduct at least one more experiment with this strain to provide confirmatory evidence of their susceptibility to side effects. Also this strain was used here because it had earlier been shown to be a very poor responder to PLS even though the mice in this strain have lower body weight than C3H mice, and

hence one might anticipate a proportionally higher concentration of the active drug in the intestine. However, in this experiment much care was taken to ensure that the mice experienced minimal signs of intolerance to PLS and hence side effects. This involved more thorough cleaning of the oral gavage needle before and after each treatment to avoid the remaining PLS in the needle irritating the oesophagus, and thereby causing reflux of the PLS from the stomach.

The experiment employed 60 mice, 30 from BALB/c strain and another 30 from the C3H strain. The mice were divided into 3 groups of 20 animals (10 mice from each strain). Each group of 20 was infected with a different dose of L3 larvae of *H. bakeri* (50, 150 or 300 L3). Then the ten mice of each strain within each intensity category were divided into 2 groups of 5, one of which was treated with a standard dose of PLS and the other was given distilled water. Because of logistic limitations, faecal egg counts were only carried out for referencing in the groups of mice infected with 150 L3 larvae.

6.2.24 Experiment 12: Results

As earlier the BALB/c mice were less tolerant to PLS treatment, although this time not as seriously as before but nevertheless one mouse had to be culled as it lost substantial amount of weight exceeding the 20% moderate band limit allowed by the Home Office on our project licence. Another mouse was accidentally trapped by the bedding materials and dislocated the neck thus it also had to be terminated prematurely. One mouse from the control group was excluded from the analysis as it had a very low worm infection of only 8 worms out of the 300 L3 infected. All these 3 mice were from the BALB/c strain. Therefore the results reported herein are based

on 57 mice instead of 60 mice which were initially infected with different levels of worm intensity.

The results showed that in the reference group (mice infected with 150 L3) there was a 99.1% reduction in FEC for C3H and -16.0% for BALB/c mice. In terms of worm burdens, mice infected with 50 L3 larvae had 89.9% and 23.6% reduction in worm burden for C3H and BALB/c respectively. Those infected with 150 L3 larvae had 89.4% and 2.8% for C3H and BALB/c respectively. While those infected with 300 L3 larvae had a reduction of 91.7% and 14% for C3H and BALB/c respectively (Fig 6.14).

Statistically FEC were analysed by repeated GLM on $\text{Log}_{10}(\text{FEC}+25)$ transformed data with strain and treatment as between subject factors and time as the within subject factor. The results showed a significant main effect of treatment ($F_{1,15} = 7.086, p < 0.05$). However, the main effect of strain was just outside the threshold for significance ($F_{1,15} = 4.457, p = 0.052$). The 3-way interaction time*treatment*strains was significant ($F_{2,5,36,85} = 7.413, p < 0.001$).

Analysis of worm burdens by 3-way GLM showed a significant main effect of treatment ($F_{1,45} = 89.521, p = 0.001$), strain ($F_{1,45} = 89.521, p = 0.001$), worm burden intensity ($F_{2,45} = 117.428, p = 0.001$) and interaction of treatment*strain*worm intensity ($F_{2,45} = 6.181, p = 0.004$). Mice were also monitored for the pathological effects of treatment by observing hair coat and taking daily body weight. All the animals were active and healthy throughout the period of treatment,

except the mouse which lost more than 20% body weight and was terminated immediately before the end of the experiment.

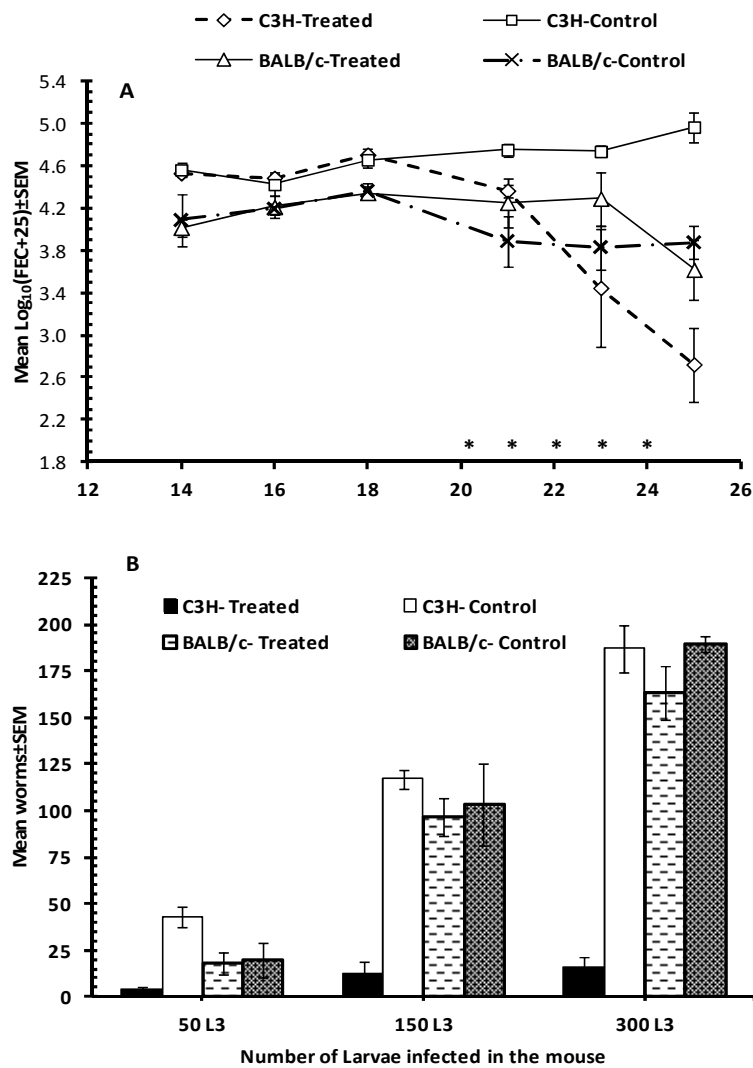


Figure 6.14: FEC and worm counts for mice infected with different intensity of L3 larvae

BALB/c and C3H mice were gavaged orally with different intensities of L3 larvae, 50L3, 150L3 and 300L3 on day 0. Then the mice were divided into 2 groups, those treated with standard dose of 240nmol and those given distilled water. FECs (A) were conducted on groups of mice infected with 150L3 on day 14, 16, 18, 21, 23 and 25 and worm burdens were assessed in all groups on day 25 post infection. There was significant variation in reduction of FECs and intestinal worm counts (B) between the 2 strains of mice. *indicates day of treatment.

6.3 Discussion

The results of the experiments presented in this chapter indicate clearly that mouse strains differ in the extent to which a standard dose of PLX or PLS removes worms from the intestines of mice and that the efficacy of PLS does not vary between the sexes of the animals being treated. Moreover, the difference between strains is not dependent on their body weights and efficacy in the poor responder strains does not improve when stomach acidity is blocked by cimetidine, and is not dependent on the intensity of the worm burden or on the levels of secreted trypsin.

In the first experiment C3H and CBA/ca strains were shown to have more marked responses to treatment with PLX than the other strains and the same observation was seen in Experiment 2 with regard to the C3H strain of mice. The reason for C3H and CBA/ca having similar responses to PLX and PLS treatment might be due to the fact that these 2 strains are closely related, originating from the same stock. They were developed from crossing Bagg albino female and a DBA male by Strong in 1920 followed by selection for high incidence of mammary tumours in the case of C3H and low incidence of mammary tumours for CBA/ca (<http://jaxmice.jax.org>).

BALB/c mice which are also an inbred strain had a relatively poor response to drug treatment in Experiment 1 with only a 40.3% reduction in worm counts. This led the strain to be used as a negative control in Experiments 2 and 11, where the results, this time using PLS rather than PLX were of a similar magnitude. Although in Experiment 2 the strain was found to be less tolerant to PLS as evidenced by some

toxicity and this became especially apparent towards the end of the period of treatment. The PLS treated mice showed loss of coat integrity and some loss of body weight although still within the recommended 20% moderate band by the Home office restrictions on our Animals Act licence. However, due to concerns about their wellbeing the mice in this group were terminated one day before the end of the experiment. For this reason the strain was excluded from the next experiment. However, the strain was used again in Experiment 11 where improvement in administration of treatment resulted in better tolerance of the mice to PLS.

The response of BALB/c mice to PLS on the other hand concurs with the prediction that individuals with likelihood of low drug efficacy are also likely to express high drug toxicity, whereas individuals with a high probability of drug therapeutic efficacy are likely to have low probability of drug toxicity (Evans and Johnson, 2001). Thus BALB/c mice responded poorly but showed some level of drug toxicity, in contrast to C3H mice which had a high response to treatment with minimum drug side effects. Inter-patient variations in response to medication have been reported also by other researchers (Wood, 2001) who found that individual patients responded differently to standard doses where a drug had toxic effects in some patients but failed to produce the expected therapeutic effect in others.

To some extent this result conflicts with that of Satrija *et al.* (1995) who reported a reduction in worm burden in female BALB/c mice of 84.5% after 3 days of treatment with papaya latex. Perhaps the sex of the mice had contributed to a better response to treatment than in the male mice which had been used earlier in the

experiments reported herein. Satrija *et al.* (1995) had used female mice, a factor which needed to be further investigated.

In this case a third experiment was conducted, where both male and female mice of a high and an intermediate responder strain (C3H & NIH respectively) were used. The results clearly indicated that the sex of the mice played no role in explaining variance in data and hence the efficacy of PLS is not dependent on host sex. Host sex has also been found to have no influence even in conventional synthetic anthelmintics (Hosking *et al.*, 2010a). In their paper, Hosking *et al.* (2010) reported no statistical differences between male and female sheep treated with half doses of monepantel but a significant difference between breeds. This strongly argues that host sex is not a factor affecting the efficacy of intestinal anthelmintics including PLS.

Another possible reason for varying efficacy between strains of mice might be host weight. The standard treatments in Experiments 1, 2 and 3 involved giving a dose of PLX or PLS delivered in a volume of 0.2 ml. The amount of PLS was not adjusted in accordance to the exact weight of individual mice and since some strains are heavier than others, it could have been that the heavier strains actually received a lower dose of drug on a per kg basis, especially in the site in the intestine where contact with worms was required. Although most drugs are recommended to be administered on a dose per kilogram body weight basis, there are few data showing that weight is an important factor in dosing to achieve a desired drug effect. Thus in Experiments 4 and 6, the efficacy of PLS was compared between mice treated with the standard dose (240 nmol per mouse in a volume of 0.2 ml) regardless of their

body weight variations and those treated based on body weight-adjusted dose (8 μ mol per kg body weight). However, the results showed no significant difference in the response of mice to PLS treatment between the 2 groups. Even when an outbred strain with greater variation in body size of the individuals within the same group was used, the result was the same (Experiment 6).

Since the mice used in this experiment were of the same age, it was expected that their body weight should be more or less similar within strains. The presence of variation could be due to a different degree of fat deposition between individuals and strains (obesity). Therefore, another approach may be to use lean body weight (total body weight minus excess weight due to obesity/fat or, in the case of a lean individual, actual body weight) instead of using total body weight as the content of the GI does not change with fat deposition (Kirking *et al.*, 1985). However, it is not easy to get lean body weight from live animals. Although lack of variation in body weight to have influence on PLS efficacy was contrary to our expectation, nevertheless it was a useful finding because the dosing of mice with PLS can now be simplified to a standard dose regimen of 240 nmol PLS/mouse in 0.2ml water and the exact dose a mouse received does not have to be adjusted for its exact body weight on the day of treatment.

In these experiments it was expected that the actual doses delivered, as reflected in weight of drug/kg body weight, would vary between strains when their weights were taken into consideration. Larger mice with larger intestines should experience a greater dilution of the standard volume of drug. In the first three experiments a standard dose was delivered to each mouse irrespective of their

weight. However, Experiment 4 and 6 showed that this is an unlikely explanation for variation in efficacy of PLS between strains, because NIH and BKW mice, the intermediate and variable responders, did not differ in their response when the dose given was adjusted for daily changes in body weight and a consistent daily dose (not varying in terms of weight of drug/kg body weight) was delivered over the entire period of treatment.

The genetic explanation may hinge on genetically controlled differences in the physiological environment in the intestine, including for example pH in the stomach and the duodenum, gut food transit times, host digestive enzymes etc. pH is known to damage CPs at low values reflecting acidic conditions (Huet *et al.*, 2006). Therefore, the ultimate challenge in this area of research was to identify ways in which the poor responders can be made to be more sensitive to drug treatment. One possible way was to combine antacid treatment with PLS. For this reason one set of mice was given cimetidine prior to being treated with the 0.2ml standard dose of 240nmol of PLS, in order to block acid in the stomach which might be inhibiting the activity of the drug. This experiment was encouraged by the results of (Huet *et al.*, 2006) who reported that papaya proteinases incubated with pepsin at low pH became unstable and easily degraded irreversibly by pepsin.

The hypothesis tested here was that the weaker responding strains may have lower stomach acidity which inactivates the PLX and PLS to a greater extent than in C3H mice. So it was assumed that most of the PLS might have been affected by the low pH of the stomach and less was available for exerting worm damage in the small intestine. However, Experiment 7 showed that blocking stomach acidity by

concurrent treatment with cimetidine did not improve efficacy in the intermediate responder strain. In contrast, Stepek *et al.* (2006a) found that administration of cimetidine improved the efficacy of PLS against adult female *P. muricola* worms in the stomach. In this case cimetidine neutralized the stomach pH, maintaining it at approximately 7.0, close to the optimal pH for cysteine proteinase activity. But, as shown here, neutralising stomach acidity did not change the efficacy of a standard dose of PLS in the small intestine.

It is possible that any inactivation of PLS by acid in the stomach is reversed in the small intestine and not permanently blocked as has been reported in previous studies (Huet *et al.*, 2006). Thus the role of cimetidine has little influence after enzymes have passed the stomach as their activity will be restored due to the increase in pH. This might be the reason why the activity of the PLS in the small intestine against *H. bakeri* in NIH mice did not improve with the administration of the cimetidine. The recovery of PLS after restoration of neutral pH was clearly seen in Experiment 8 where the enzyme incubated at pH 1.2 for 2 h showed the same level of activity as that incubated at pH 7, when transferred subsequently to optimal incubation conditions used for assessing activity in the colorimetric assay. Hale *et al.* (2005) reported a similar result with proteolytic activity of bromelain toward the Z-Arg-ArgpNA and Bz-Phe-Val-Arg-pNA substrates which was not inactivated by exposure to low pH conditions (PBS, pH + pepsin) for 2 h at 37°C.

It was considered then that differences between strains in the normal variation in pH along the GI tract might have contributed to the difference in response of the poor and high responder to PLS treatment. However, the results of Experiment 9

showed no significant difference in change in pH along the GI tract between the 2 strains. As expected the lowest pH values were observed in the stomach, being around 3.5 in both strains, slightly lower than that reported by (McConnell *et al.*, 2008) in fasted BALB/c mice (4.04). An interesting observation was that both strains of mice had a pH around 6.5 in the small intestine which is almost the same as the optimum pH for CPs activity for worm damage. However, the mice responded differently to PLS treatment suggesting that there may be some other factors affecting the efficacy of PLS in different strains of mice other than pH.

One of the possible factors might be variation between mouse strains in the transit time of the orally administered PLS along the GIT. It was thought that the length of exposure of the worms to PLS may also be a crucial factor because *in vitro*, the worms were consistently exposed to latex for the whole of the incubation period lasting up to 2 h. So the ineffectiveness of the PLS in the poor responder strains might be due to rapid passage of the contents of the enzyme in the small intestine. However, the results in Experiment 10 indicate that the rate of enzyme passage in the small intestine between the poor and higher responder mice was similar. In both strains it took about 90 min to empty all the enzymes from the small intestine. The same trend of enzyme activity of PLS has been reported earlier by Stepek *et al.* (2007a).

Since the result of Experiment 10 showed the enzyme can remain in the small intestine for more than 1 h, it was hypothesized next that poor responder mice might be secreting excess trypsin which might be degrading the CPs faster than possibly lower levels in responder strains. However, the results in Experiment 11 showed that

the concentration of trypsin in the poor and higher responder was similar. That means that if trypsin has any effect on the CPs then both strains are likely to be affected to the same extent.

Another possible factor was worm burden intensity. It was considered possible that as the worm burden increases the effectiveness of the PLS decreases due to the higher worm burden. Nevertheless, the results of Experiment 12 indicated that for the high responder strain, the extent of worm reduction increased with increased worm intensity. Although in the poor responder there was no clear trend with respect to efficacy of treatment, the worms seemed to have established better at higher intensity than at lower intensity.

Although, it has been reported in this chapter that the strain of mice has great influence on efficacy of PLS, the cause of variation is not yet known. Therefore further research is required. It is well known that the GI tract of animals provides a reducing environment for CP activation, so it may be important to try to administer the pre activated CPs with cysteine to poor responder strain to compare their response with that of inactivated CPs.

Alternatively, variation in the extent of the mucosal embedding activity of the adult *H. bakeri* between mouse strains may serve to protect the worms against the PLS activity more so in some strains of mice than others. This needs to be investigated further either by comparing *H. bakeri* with a free lumen intestinal residing nematode or by investigating the nature of mucosal layer in poor and high responder mice.

CHAPTER 7: FORMULATION AND DELIVERY OF CYSTEINE

PROTEINASES FOR THE TREATMENT OF RUMINANTS

7.0 Summary

This chapter explores potential formulations and delivery systems for cysteine proteinases (CPs) from papaya latex supernatant (PLS) as an anthelmintic drug for ruminants. *In vitro* studies involving both immediate and slow release dosage forms were conducted. In the slow release experiments, two hydrophilic matrices were tested, containing xanthan gum and hydroxypropyl methylcellulose (Methocel-LVCR and Methocel-CR). Methocel-CR provided better results compared to the others. In the immediate release experiments three disintegrants (Primojel, L-HPC and Ac-Di-sol) were investigated. Ac-Di-Sol was found to produce faster immediate drug release tablets. L-HPC and primojel had less effect. Ruminal boluses with a mean length of 5.5 cm, diameter of 19 mm and a weight of 16 g were prepared by sticking normal tablets together. This allowed dosing of the correct amount for treatment of sheep.

7.1 Introduction

The importance of CPs as anthelmintic drugs has been discussed in previous chapters of this thesis. Since, they are intended to be used in livestock; they need to be formulated in such a way that they will be effective in eliminating the target nematode infections. These cause serious economic losses in ruminant animals and the most affected animals in the livestock farming industry are sheep, cattle and goats.

The same methods and techniques used for the development of human drug dosage forms are applicable to the development of veterinary drug dosage forms (Klink *et al.*, 1998). Unlike in humans, livestock sector drug formulation involves developing a dosage form that minimizes the time and cost associated with the mass treatment of flocks/herds. In addition it has to focus on animal welfare, ease of administration and human safety. The most common drug delivery routes in livestock include injections, feed additives, ruminal boluses or horse pills (large tablets), and topical pour-on medicines. In comparison, drug formulation for companion animals is dictated by animal owners, and is based on the safety of the drug and convenience of delivery (Ahmed and Kasraian, 2002).

The majority of anthelmintic drugs available as human dosage forms were initially developed for use in veterinary practice in this way. Yet, some types of dosage forms have restricted use and are only suitable for use either in humans or certain animal species. Due to the physiological variations between species, each veterinary treatment needs to be specifically designed for the animal that is to be treated. The design aims to maximise the effectiveness of the drug in the target animal (Rothen-Weinhold *et al.*, 2000).

A series of papers published by Stepek *et al* and that of Buttle *et al.* (2011) have demonstrated that CPs from papaya latex have a strong anthelmintic effect against GI nematodes in rodents and sheep. The enzymes required 5-7 daily doses to cause a substantial reduction in worm burden. An extended/controlled drug delivery system would be the most appropriate method for delivery of the enzymes to ruminant animals. The main reasons for developing controlled drug release systems

for ruminants are to reduce the stress that results from restraining, handling and dosing animals, and to reduce the cost in terms of labour and time. Controlled release systems are more convenient to administer than repeated dosing, and enable the quantity of the drug administered to be known, in contrast to the administration of the drug in drinking water or food. The dosage forms also reduce human exposure to veterinary compounds that are unsafe to health (Medlicott *et al.*, 2004).

Ruminants (sheep, goats and cattle) are livestock of great economic importance that spend much of their life grazing in open fields. Thus they impose great challenges for daily drug dosing. A controlled drug delivery system is the most appropriate form for these animals which have unique physiological features that provide important opportunities for these drug delivery systems. Their stomach is composed of four chambers, the rumen, the reticulum, the omasum and the abomasum. The rumen is the largest compartment and it can be viewed as a large fermentation tank (Cardinal, 1985). The mean feed particle retention time in the rumen of cattle is 60 - 80 hours and that of sheep is 25 – 40 hours depending on the nature of the diet (Baggot, 1988). This region can therefore offer opportunities for long-acting drug delivery systems, provided that a suitable method is employed to prevent the device from either being regurgitated along with food or moving down the GI tract. This can be achieved by using heavy devices with sufficient density if the bolus is not heavy enough to settle down or by producing devices that expand in physical dimensions on entry into the rumen (Rothen-Weinhold *et al.*, 2000).

Different methods of controlled drug delivery in ruminants have been discussed in chapter one including erodible systems, reservoir and matrix devices.

The release rates from these systems are variable and therefore suitable for nutrients or mineral supplements. Reservoir and matrix devices provide better control of the delivery rates and are more reliable for active ingredients with relatively high aqueous solubility. This technology may also be applicable to papaya latex supernatant (PLS) which is very soluble in aqueous solutions.

Based on previous study PLS has been demonstrated to have high efficacy against sheep GI nematodes particularly *Haemonchus contortus* which had 98% reduction in worm burden (Buttle *et al.*, 2011). The treatment required a total amount of 15 g of PLS which was administered in oral solution for 4 daily doses. However, this dosing was too involving and posed a risk to the animals. There is a need to minimize the number of doses if possible to 1 or 2 doses while maintaining the total amount of dose of 15 g. This can be achieved through the use of extended drug release.

Therefore this chapter is concerned with testing the hypothesis that the release of CP can be controlled and either slowed through the use of hydrophilic matrices or accelerated through the use of disintegrants. Although hydrophilic matrices are applied to small molecules it was expected that they would have the same effect with a protein drug. The concept is that CP incorporated in a ruminal bolus would settle in the sheep rumen, dissolving its contents into the rumen fluid and slowly releasing the enzyme to other parts of the lower gut. Thus the rumen will act as a reservoir for slow release of the medicine in the GIT. Two hydrophilic matrix polymers, xanthan gum (XG) and hydroxypropyl methylcellulose (HPMC)

were assessed based on their ability to induce slow release while maintaining the enzyme activity.

XG was selected based on its remarkable ability to produce a large increase in the viscosity by adding a small amount of gum. This allows a large quantity of drug to be incorporated into the tablet. It is also reported to be very stable under a wide range of temperatures and pH (Talukdar *et al.*, 1996) and its mechanism of drug release is through polymer hydration.

HPMC is by far the most common cellulose ether used in swellable-soluble matrices. It is a water-soluble hydrophilic, non-ionic cellulose ether that gels and is stable over a wide range of pH 3.0–11.0 and is enzyme resistant (Li *et al.*, 2005). It is also low in toxicity and tablet matrices are easy to manufacture. HPMC is available commercially under the trade name METHOCEL (Dow Chemical Company, USA). The mechanism of drug release is through surface erosion of hydrated matrix.

It was also thought that if extended release using simple hydrophilic matrix tablets cannot provide sufficient slow release, then the tablets will be wrapped as a roll in cardboard tubes. The main idea was actually to reduce the rate of tablet hydration through a physical barrier which would eventually be degraded and fall apart in the rumen with time and therefore more prolonged release. This may be a convenient way of administering a large dose.

Since it is not known at this stage whether the slow release system will provide the optimum desired drug release rate, it was necessary to have alternative formulations equivalent to a liquid dosage form but with minimum drug administration problems. In this case fast release tablets with three types of disintegrants Primojel, L-HPC and Ac-di-Sol were evaluated to determine their effectiveness in inducing fast release of CPs from these tablets.

In the work described in this chapter the enzyme was incorporated into different hydrophilic polymer matrices and disintegrants then made into tablets with a 19 mm diameter. Seven to eight tablets were then stuck together to form a roll of tablets of more than 15 g by weight. Throughout the text that follows, this roll is referred to as a 'ruminal bolus' or simply 'bolus' and it was used for *in vitro* drug slow and fast release assessment.

7.2 Experimental design and Results

7.2.1 Experiment 1: Effect of xanthan gum on drug slow release

This experiment was carried out as a pilot study to determine the slow release profiles of the drug alone from a hydrophilic matrix tablet. Previous dosing experiments on sheep had shown PLS had very hydrophilic macromolecules which took some time to dissolve at high concentrations (Buttle *et al.*, 2011). This would indicate whether hydrophilic matrices are able to provide slow release of CPs. Since the experiment was just a pilot, the tablets were manufactured using a normal tablet punch die of 9 mm and the tablets were formulated using different proportions of xanthan gum. The formulation contained 0%, 10%, 15%, 20%, 25% and 30% of

xanthan gum. In each category, one tablet was assessed for drug release by determination of the amount of protein release in dissolution tests.

7.2.2 Experiment 1: Results

The results indicate the tablet made of PLS alone released the drug very quickly; within 10 minutes the tablet was completely dissolved (Fig 7.1). However tablets made with xanthan gum had slower drug release and the effect intensified with increase in amount of xanthan gum. Although some tablets had completely dissolved, the amount of drug release was low which suggested that xanthan gum might be affecting the enzyme availability. Also the results show that tablets prepared above 20% inclusion level of xanthan gum did not change the drug release profile (Fig 7.1).

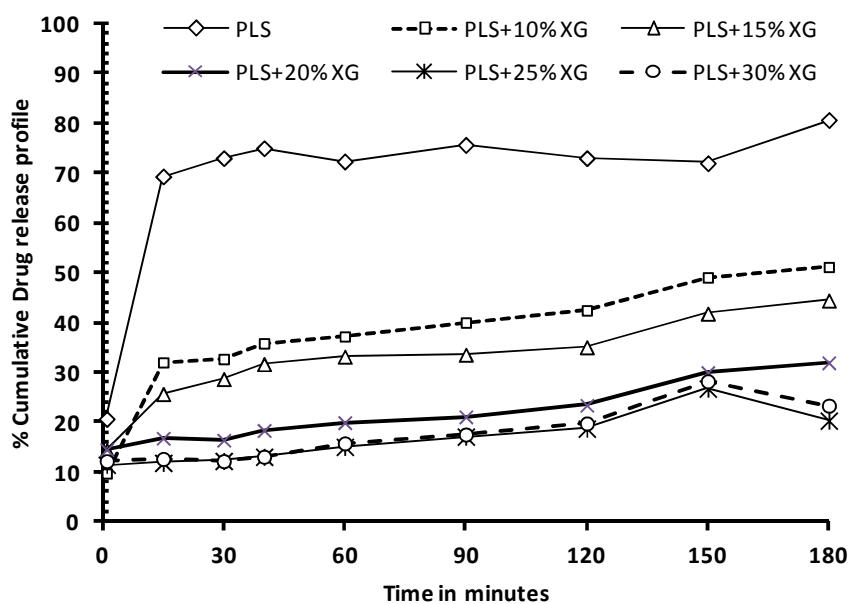


Figure 7.1: Effect of different levels of XG on drug release profile over time

Tablets made with different concentrations of xanthan gum. Dissolution test was done using USP apparatus 2, 100 rev.min⁻¹ and 900 ml of 50 mM phosphate buffer with pH 6.8, 37±0.5°C. The results show that as the amount of xanthan gum in the hydrophilic matrices increases the rate of drug release decreases.

7.2.3 Experiment 2: Screening of more hydrophilic matrix polymers

Since the drug released from tablets loaded with xanthan gum was lower than expected even for the tablets which were completely dissolved we thought it is better to try other hydrophilic matrices for comparison. Hydroxypropyl methylcellulose (HPMC) was selected including both methocel-LVCR and Methocel-CR. Methocel-CR is of high viscosity and methocel-LVCR is of low viscosity. These matrices were then mixed with papaya latex powder and made into tablets. At this time the tablet punch size was changed instead of 9 mm we used 19 mm. This size was the maximum diameter for preparation of sheep boluses to facilitate the delivery of the dose of PLS needed for effective therapy. Three formulations with different proportions of matrices were prepared: the first one contained xanthan gum, the second methocel-CR and third methocel-LVCR. Each set of matrices contained 0, 10%, 15%, 20%, 25% and 30% of hydrophilic polymer (Table 7.1). Throughout the study dissolution tests were carried out in triplicate.

Table 7.1: Composition of tablets used for controlled release rate

Ingredients(% w/w)	Formulation 1	Formulation 2	Formulation 3
Drug (PLS)	69.5-99.5	69.5-99.5	69.5-99.5
Xanthan gum	0-30	-	-
Methocel-CR	-	0-30	-
Methocel-LVCR	-	-	0-30
Magnesium Stearate	0.5	0.5	0.5

7.2.4 Experiment 2: Results

The results indicate that as the amount of polymer was increased, drug release became slower and a similar profile was seen in all the 3 types of polymer (Fig. 7.2). The exception was that above 20% the high viscosity grade of HPMC did not change the drug release profile with an increase in the amount of polymer.

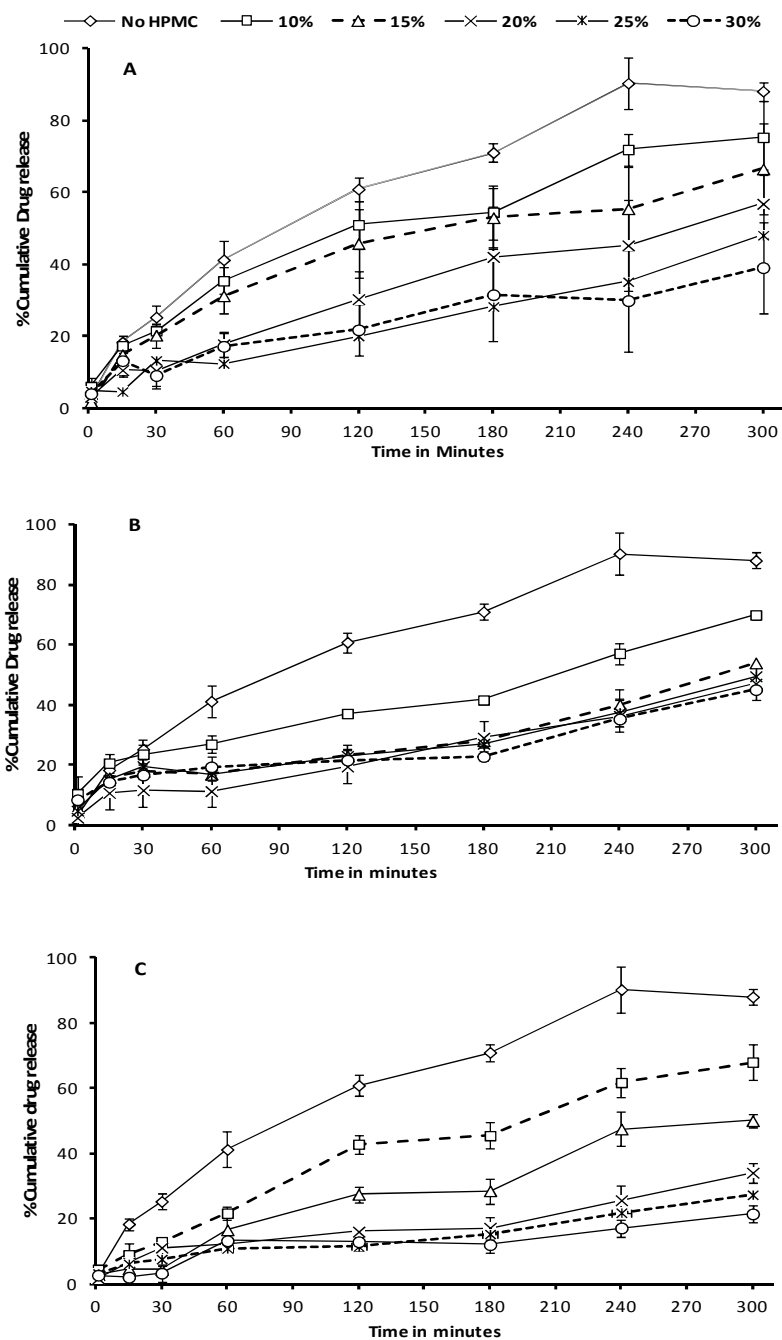


Figure 7.2: Effect of various hydrophilic matrices on drug release profile over time

Tablets made with different concentrations of A) Methocel-LVCR B) Methocel-CR and C) xanthan gum. Dissolution test was done using USP apparatus 2, 100 rev.min⁻¹ and 900 ml of 50 mM phosphate buffer with pH 6.8, 37±0.5°C. Mean±SD (n=3). The results show that as more amount of hydrophilic matrix was used the rate of drug release decreased.

7.2.5 Experiment 3: Enzyme active site titration for slow release tablets

Having demonstrated that hydrophilic matrix tablets do show a slow release of proteins from these formulations, it was then necessary to demonstrate that following tableting and release that the enzyme in solution was in an active form. Therefore this experiment was carried out by dissolution testing tablets with 30% polymer in 900 ml of phosphate buffer and a tablet with no polymer. The experiment was done in triplicate for consistency and the solutions were left overnight to ensure that all the tablets had been completely dissolved. The following day enzyme activity was determined by active site titration by titrating a fixed concentration of enzyme against varying concentrations of its inhibitor E-64. The same experiment was carried out with PLS powder to determine if tableting had an effect.

7.2.6 Experiment 3: results

The percentage of active enzyme present in tablets made of different polymer hydrophilic matrices was calculated as shown in appendix 5. It was found that tablets loaded with PLS alone contained 14.7% of active enzyme, while tablets loaded with PLS + 30% xanthan gum had 11.6% active enzyme, on the other hand tablets loaded with PLS + 30% methocel-CR had 14.5% active enzyme and tablets loaded with PLS + methocel-LVCR had 12.6% active enzyme. Based on these results, methocel-CR was found to have retained more of the active enzyme than any other matrix, almost the same amount as that of the tablet loaded with PLS alone. Therefore methocel-CR was selected for the ruminal bolus slow drug release experiment. The results also showed that both powder and tablets had almost similar amount of active enzymes

14.9% and 14.7% respectively. However the experiment compared the powder and tablet composed of only PLS.

7.2.7 Experiment 4: Use of disintegrants to enhance fast drug release

At this moment it was not clear whether the slow drug release would provide better results for delivery of the intended dose to sheep for 4 days. Therefore there was a need to carry out an alternative experiment which involved a fast drug release system. This would be one way to enable an instant delivery of some drug dose in addition to slow delivery at some later time points because even the tablets made with PLS alone had slow drug release when compared with normal small drug molecules. It was thus necessary to accelerate the release by using disintegrants. In this case three disintegrants (Primojel, L-HPC and Ac-Di-Sol) were chosen based on their properties as explained in Chapter 1. Three formulations were prepared and made into tablets. Then the dissolution test was carried out to determine which disintegrant had the greatest impact on accelerating the drug release profile.

Table 7.2: Composition of tablets used for fast release studies

Ingredients (% w/w)	Formulation 1			Formulation 2			Formulation 3		
Drug (Papaya latex)	97.5	94.5	89.5	97.5	94.5	89.5	97.5	94.5	89.5
Ac Di-Sol	2	5	10	-	-	-	-	-	-
Primojel	-	-	-	2	5	10	-	-	-
L-HPC	-	-	-	-	-	-	2	5	10
Magnesium Stearate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

7.2.8 Experiment 4: Results

The compositions of the tablets used in this experiment are shown in Table 7.2. Formulation 1 contained 2%, 5%, or 10% of Ac-Di-Sol, Formulation 2 contained 2%, 5%, or 10% of Primojel and Formulation 3 contained 2%, 5%, or 10% of L-HPC disintegrants. Drug release rate was expected to increase with increased percentage of disintegrants. The results in Figure 7.3 showed that 2% disintegrant in all formulations had no effect, but 5% and 10% disintegrants successfully accelerated the drug release rate when compared with the control tablets which contained no disintegrant. Of all 3 formulations Ac-Di-sol had the greatest effect, thus it was selected for further investigation.

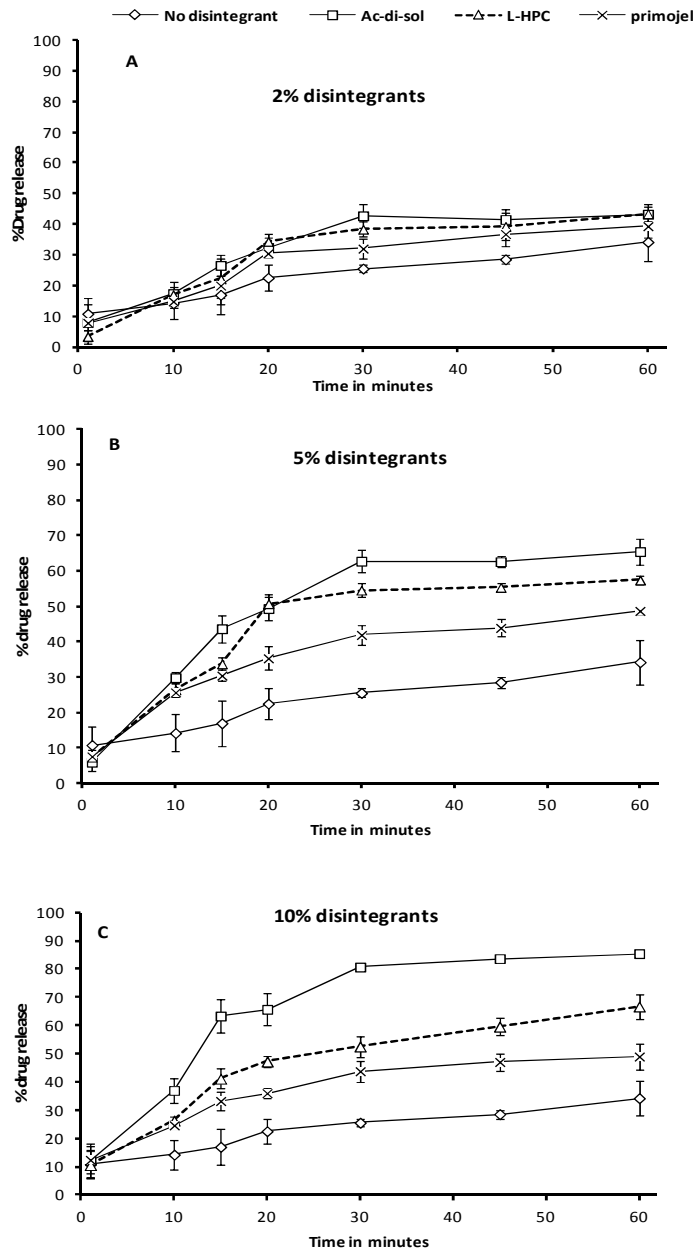


Figure 7.3: Effect of different disintegrants on drug release profile over time

Matrices made with different concentrations of disintegrants A) 2% B) 5% and C) 10%. Matrices were dissolution tested using USP apparatus 2, $100\text{rev}\cdot\text{min}^{-1}$ in 900ml of 50 mM phosphate buffer with pH 6.8, $37\pm 0.5^\circ\text{C}$. Mean \pm SD (n=3). Results showed that 2% disintegrant was ineffective, but that 5% and 10% disintegrant successfully accelerated the drug release rate when compared with tablets with no disintegrant. Of the three disintegrants, Ac-Di-sol had the greatest effect.

7.2.9 Experiment 5: Amount of Ac-Di-Sol required for fast drug release rate.

Based on the above experiment Ac-Di-Sol was found to have the best result in accelerating fast release. However it was not known what amount was enough to provide the maximal release rate. Therefore this experiment was carried out to determine the required concentration of Ac-Di-Sol in the tablets. The experiment was started with 5% Ac-Di-Sol because 2% had already shown to have no effect in Experiment 4. Therefore tablets loaded with PLS and 5 -10% of Ac-Di-Sol were manufactured and then assessed for *in vitro* dissolution test.

7.2.10 Experiment 5: Results

Although the results in Figure 7.4 showed little increase in drug release rate with variation in composition of Ac-Di-Sol from 5-10%, 10% of Ac-Di-Sol could provide the fastest release rate. Thus the 10% Ac-di-Sol was considered to be enough to provide the required fast release rate as we did not want to go beyond 10% disintegrant as it would decrease the amount of PLS which could be incorporated into the tablets.

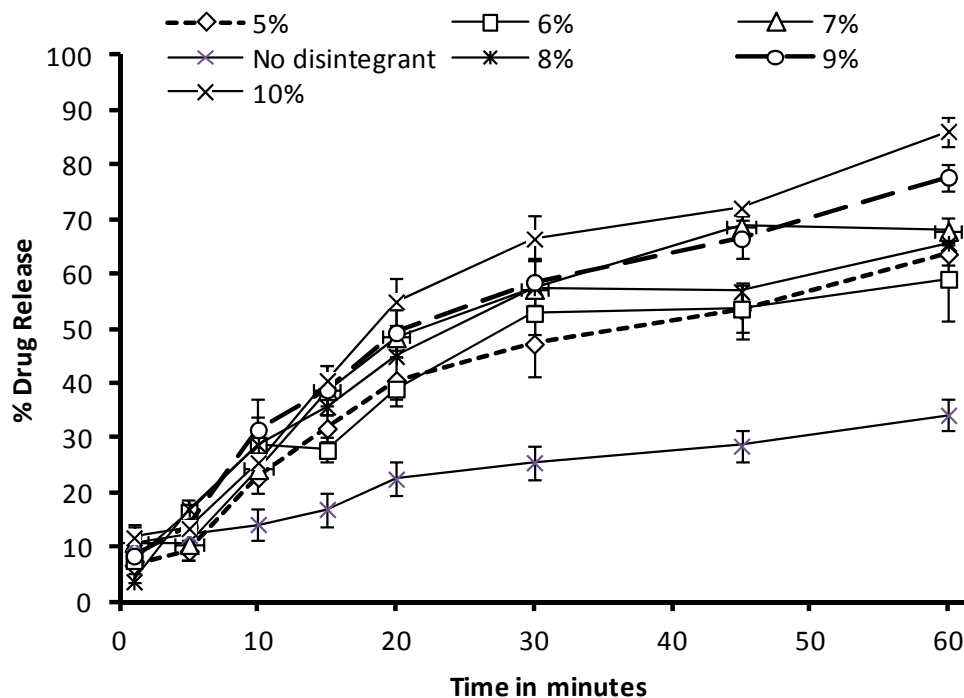


Figure 7.4: Effect of Ac-Di-Sol content on the drug release profile with time

Tablets made with different concentrations of Ac-Di-Sol disintegrant (5-10%) were tested for dissolution in USP apparatus 2, 100 rev.min⁻¹ and 900 ml of 50 mM phosphate buffer with pH 6.8, 37±0.5°C. Mean±SD (n=3). Results indicated little increase in drug release rate with increase in the amount of Ac-Di-Sol.

7.2.11 Experiment 6: Effect of Ac-Di-Sol on enzyme activity

Since Ac-Di-Sol was selected to provide fast drug release rate, it was also necessary to assess how this disintegrant affected the activity of the enzyme. To achieve this, an experiment was carried out by dissolving 3 tablets loaded with PLS+10% disintegrant and another 3 tablets loaded with PLS alone into 900 ml of phosphate buffer. The solutions were left overnight at 4⁰C to provide sufficient time for any reaction between the disintegrant and the enzymes to take place. The solutions were then tested for enzyme activity as explained in the materials and methods section.

7.2.12 Experiment 6: Results

The same procedure as used in Experiment 3 was also used here to calculate the percentage enzyme activity. The results indicated that tablets with 10% Ac-Di-Sol had 21.2% active enzymes compared with 28.7% active enzymes present in tablets made with no disintegrants. This means that Ac-Di-Sol disintegrant was able to retain more than 74% active enzyme.

7.2.13 Experiment 7: Effect of methocel-CR and Ac-Di-Sol bolus drug release rate

This experiment was intended to utilize the whole dose of 15 g of PLS. This has been recommended by Buttle et al. (2011) as a suitable dose to eliminate nematodes in sheep, however it requires 5 days of treatment. Thus the purpose of the experiment was to try to minimize the number of treatments from 5 to a treatment at a single dosing point, possibly with a number of different boluses. The experiment was carried out with 3 formulations. Formulation 1 comprised tablets loaded with PLS + 15% methocel-CR, formulation 2 composed of PLS + 10% Ac-Di-Sol and formulation 2 had tablets loaded with PLS alone which was used as control. The amount of 15% methocel-CR loaded in the tablet was chosen based on the fact that the amount had provided better results in Experiment 2. While 10% Ac-Di-Sol was considered to be enough to enhance fast release there was still some possibility of adding more, but our interest was to minimize the amount of excipient in order to incorporate more PLS. Then 7-8 normal tablets loaded with PLS + 15% methocel-CR, PLS+10% Ac-Di-Sol disintegrant and those loaded with PLS only were stuck together to form a roll of tablets referred to as a ruminal bolus. Then the *in vitro*

dissolution studies were performed as explained in the materials and methods section. The bolus had an average size of 19 mm in diameter, 5.5 cm in length and average weight of 16 g.

7.2.14 Experiment 7: Results

The results in Figure 7.5 indicated that the bolus with 15% methocel-CR matrix had a slower release rate than bolus with 10% Ac-di-Sol disintegrant and that with PLS alone. Surprisingly the tablets with Ac-Di-Sol disintegrant showed the same effect as that of PLS alone. Both completely dissolved within 1.5 h, implying that Ac-Di-Sol had little effect on drug. Moreover the drug released by Ac-di-Sol had only 75% drug release after complete dissolution when compared with 100% released from bolus loaded with PLS alone. On the other hand the bolus with 15% methocel-CR released only 30 % of its drug content after 3 h of dissolution, and the tablet remained undissolved.

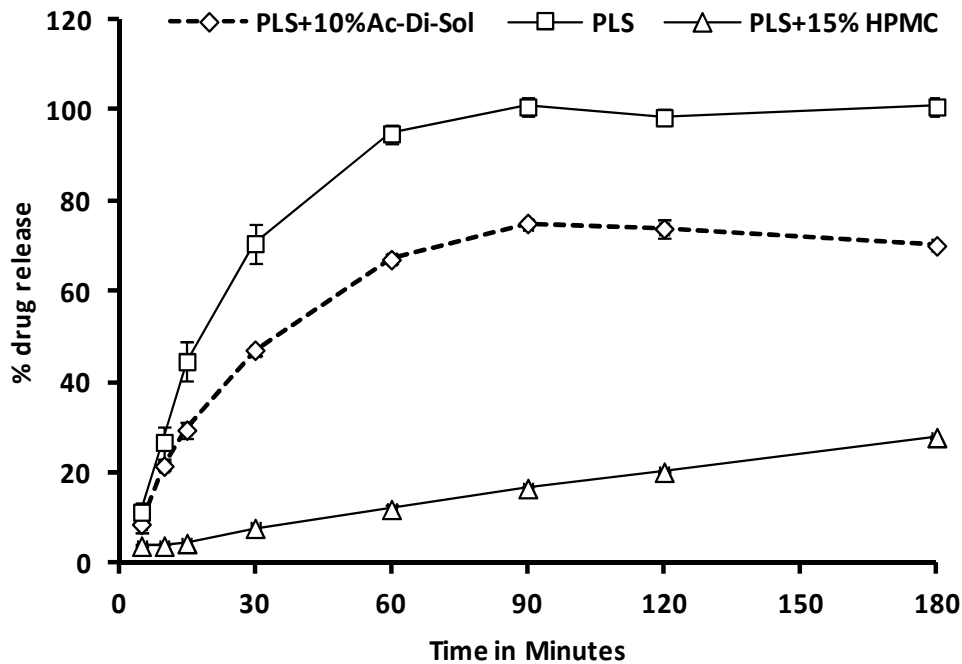


Figure 7.5: Drug release profile over time from ruminal bolus

Tablets loaded with PLS alone, PLS + 10% Ac-Di-Sol and PLS + 15% Methocel-CR were tested for Dissolution using USP apparatus 2, 100 rev.min⁻¹ and 16 litres of 50 mM phosphate buffer with pH 6.8, 37±0.5°C. Mean±SD (n=3). Results indicated that bolus loaded with PLS + 10% Ac-Di-Sol has the faster drug release and bolus with PLS + 15% Methocel-CR had the lowest release as expected.

7.2.15 Experiment 8: Effect of methocel-CR on bolus slow drug release

Since methocel-CR was shown to release only 30% of the drug after 3 h of dissolution in Experiment 7 and most of the tablet was not dissolved. There was a need to determine how long it would take for the bolus to have a complete dissolution. The experiment was intended to determine the exact time the methocel-CR was able to induce the slow drug release rate from the bolus. The boluses were manufactured in the same way as in Experiment 7. Two formulations were prepared;

Formulation 1 had tablets loaded with PLS alone while formulation 2 had tablets made with PLS + 15% methocel-CR. The boluses were tested for dissolution. Since the boluses did not have a complete dissolution within the same day then the experiment was left to run overnight until 9:00 am in the next day starting from 10:00 am in the previous day. Thus total time used in dissolution test was about 23 hours.

7.2.16 Experiment 8: Results

The results in Figure 7.6A indicated that the bolus loaded with PLS alone had a complete dissolution within 1 h while the bolus loaded with PLS + 15% methocel-CR matrix had a slower release rate; even after 7 h it had released only 40% of its drug (Figure 7.6B). This led to the dissolution system to be left on overnight and when tested after 23 h of dissolution it was found that more than 95% of the drug has been released. Although it is not easy to tell the exact time when the maximum drug release in the slow release bolus was achieved, it can be extrapolated and estimated to be around 18 h.

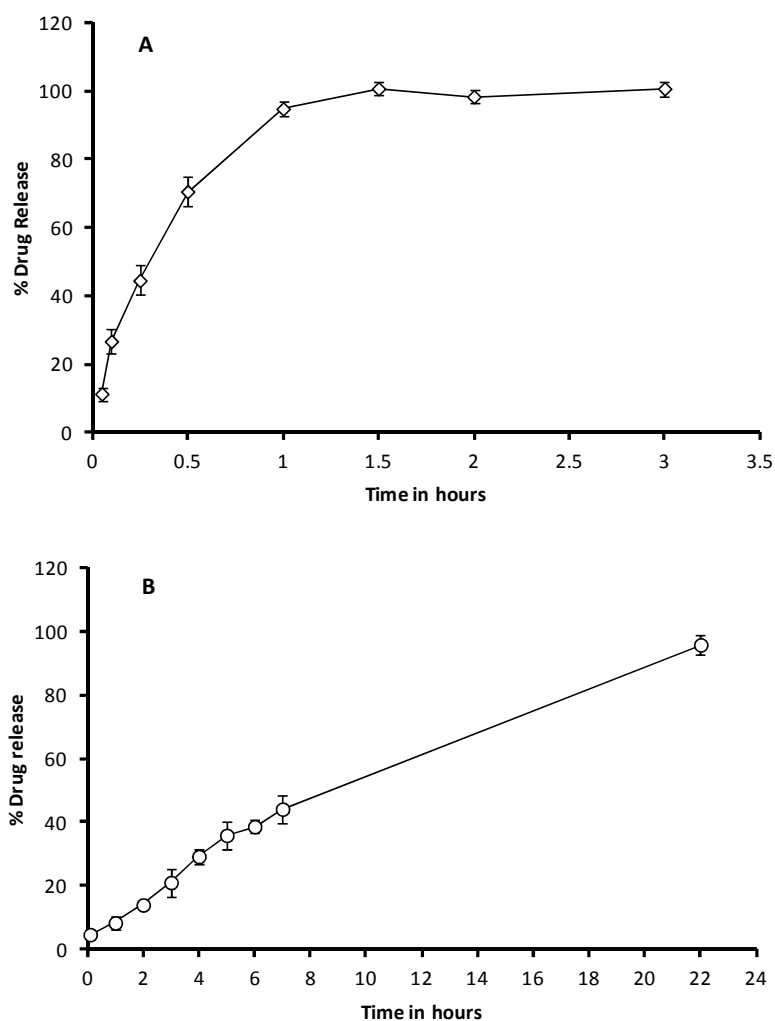


Figure 7.6: Effect of Methocel-CR on bolus slow drug release

Tablets loaded with PLS alone and PLS + 15% Methocel-CR were tested for dissolution using USP apparatus 2, 100 rev. min⁻¹ and 16 litres of 50 mM phosphate buffer with pH 6.8, 37±0.5°C. Mean±SD (n=3). Results indicated that bolus loaded with PLS alone had released its drug completely within 1 h while bolus loaded with PLS + 15% Methocel-CR had only released 40% of its drug after 7 h of dissolution.

7.2.17 Experiment 9: Physical properties of tablets

Finally it was necessary to assess the properties of the bolus produced. However due to logistical reasons it was not possible to determine the properties of bolus as a

whole, thus the physical properties of bolus was assessed based on its individual tablets used to prepare them. The physical properties of the manufactured tablets were assessed as described in the Materials and Methods and the results are summarized in Table 7.3.

Table 7.3: Physical properties of the 3 formulations of the tablets

Tablet	Weight (g)	Hardness (kg)	Friability (%)
PLS	2.25±0.03	13.78±0.39	2.8±0.03
PLS + Ac-di-Sol	2.18±0.03	13.61±0.40	1.4±0.01
PLS + Methocel-CR	1.99±0.04	19.79±0.26	1.5±0.01
Mean±SD (n =10)			

The formulated matrix tablets met the European pharmacopoeia requirement of uniformity of weight (not exceeding 5% of the average weight) and tablet hardness was also within acceptable limits. Only tablets loaded with PLS alone were tested for disintegration time and all the tablets disintegrated within 15 minutes. However all tablets prepared from the 3 formulations failed the friability test as their friability was higher than 1%, the highest acceptable limit. It can also be seen in these results (Table 7.3) that tablets formulated without polymer were twice as friable as tablets from other formulations. This is probably because polymers might have contributed to the compaction of tablets making them less friable.

7.3 Discussion

Results in this chapter have shown that both hydrophilic polymers XG and HPMC (Methocel-CR and Methocel-LVCR) had the same effects in providing slow release

of the CP enzymes from tablets. As expected the drug release rate decreased with increase in the matrix concentration up to 20%, whilst above this level, polymer content had very little effect on the drug release profile. These results are in agreement with previous studies (Ford et al., 1985, Velasco et al., 1999, Ford et al., 1991) which reported that one of the most important factors affecting the rate of release of a drug from polymer matrices is the drug: polymer ratio. An increase in polymer concentration may cause an increase in the viscosity of the gel as well as the formation of a gel layer with a longer diffusion path. This decreases the effective diffusion coefficient of the drug and therefore a reduction in the drug release rate.

Based on the results, a 15% polymer level was considered to be the optimum amount required for slow release of CP. This amount is less than the amount recommended by the manufacturer of 20-50% inclusion level in the tablets for slow drug release rate. This may be due to the fact that CPs are large molecules and their size and hydrophilicity may have been an impediment to fast release.

Xanthan gum and HPMC have a similar chemical structure but xanthan gum has some advantages over HPMC which include absence of an initial burst, high drug retarding ability and better flowability. However, the most common cited limitation of its use is the ionic influence on drug release behaviour (Talukdar *et al.*, 1996). On the other hand HPMC is by far the most common cellulose ether used to form hydrophilic matrices probably due to the fact that it has fast gel formation of a strong viscous gel to control drug release. The major disadvantage with HPMC is that drug release from this matrix does not follow time independent kinetics.

Since both polymer matrices had the same effect on slow drug release profiles they were then assessed on their ability to retain enzyme activity. It was found that xanthan gum had a negative influence on enzyme activity, in comparison with HPMC which had no influence on enzyme activity. The enzymes in tablets loaded with HPMC remained active at a similar level to that observed in tablets loaded with PLS only. Among the two HPMC excipients, methocel-CR had no effect on the enzyme activity at all probably due to the fact that this polymer has a higher viscosity which might have a strong effect on drug diffusion out of the polymer matrix. Thus it was selected for further investigation.

The possible reasons for low enzyme activity in the tablets with xanthan gum might be due to the formation of protein-polysaccharide complexes. Xanthan gum is a bacterial coat polysaccharide and it has been reported that protein can form complexes with polysaccharides by either hydrogen bonding or by covalent bonding. The interaction of hydrogen bonds has been demonstrated in the formation of coacervates between gelatin and pectin (Schmitt *et al.*, 1998). Covalent bonding has been reported by the chemical reaction of amino groups of the proteins and carboxylic groups from the polysaccharides (Stainsby, 1980). This bonding is said to be irreversible and very stable to pH and ionic strength changes (Hill and Mitchell, 1995). Most of the anionic polysaccharides like potato starch, pectin, xanthan gum, carrageenans and others can combine with proteins to form either covalent, electrostatic, hydrophobic or non-polar complexes depending on the structure and chemical properties of the components of the potential complexes (Lii *et al.*, 2003). Some studies have reported complex formation between xanthan gum and gelatine or ovalbumin (Schmitt *et al.*, 1998, Lii *et al.*, 2002). The reaction is due to the presence

of the anionic carboxylic group of the β -D glucuronic acid moiety in xanthan gum which potentially forms either ionic or covalent complexes with proteins.

In further work, tablets formulated with 2% of the three disintegrants (Ac-di Sol, LHPC and primojel) were shown to have no effect on the rate of drug release rate from tablets. In fact the 2% inclusion was selected based on the amount recommended by the manufacturers which is between 2-4%. The failure of the 2% incorporation into the tablets to have any impact on the drug release rate may be due to the fact that CP is a protein and might have a self inducing slow release effect when compared to small molecule drugs. For the tablets formulated with a high concentration of disintegrants (5% and 10%), Ac-Di-Sol was shown to have a higher effect than L-HPC and primojel in accelerating fast drug release. The same results have been reported by Zhao and Augsburger (2005) who found that dissolution of aspirin was faster in tablets with Ac-Di-Sol than tablets with primojel. However when further investigated with a ruminal bolus, this disintegrant was found to have less effect than expected, something that needs further research. The poor performance of Primojel may be due to its chemical nature which is a carboxymethylated potato starch which commonly forms complexes with proteins. Probably in this study it behaves similarly with CP resulting in a lower amount of CP being released into the dissolution medium.

The results also indicate that the effect the Ac-Di-Sol disintegrant on drug release rate increases with an increase in concentration of the disintegrant and 10% was considered enough for fast drug release rate although the effect was not clearly manifested with the ruminal bolus. The enzyme activity of the tablets loaded with

this disintegrant was found to possess only 74% active enzyme compared with the tablet with no disintegrant which had 100% active enzyme of the drug release. The possible reason could be the same as that of the primojel, that some of the enzyme might have become involved in complex formation with the disintegrant.

These tablets are being prepared for sheep treatment and in order for treatment to be successful the tablets need to be settled in the rumen and releasing the drug slowly to the lower part of the GI tract. To assimilate this mechanism, 7-8 tablets were stuck together so that the whole dose of 15 g PLS can be administered at once. Three types of formulations were used, these including tablets with 15% Methocel-CR, 10% Ac-di-Sol and tablets with PLS alone. This range of formulations were chosen because it was unclear how the larger bolus tablets would behave, and we anticipated that we would need both immediate availability of some drug with the remaining drug released slowly over a number of days.

Among the three formulations, boluses containing 15% Methocel-CR were found to have a slower drug release rate than boluses with Ac-di-Sol and PLS alone. It was observed that the drug was released through erosion and the 15% of the matrix seemed to be sufficient to induce slow release, 5% lower than the amount recommended by the manufacturer (20-50%). This may be due to either the large size of the bolus as bigger size tablets require less polymer content because of their lower surface area to volume ratio (and long diffusion pathway), or due to large protein size of the CPs thus contributing to slow release. While the 10% of Ac-Di-Sol considered to be necessary was above the recommended amount by the manufacturer of 2-4% this again may be due to the need to overcome the self induced

slow release by the CPs. However, it might need more of the disintegrants than is considered here for best results.

Finally the formulated ruminal boluses met the European pharmacopoeia requirement of uniformity with the exception of friability which exceeded the recommend amount of weight loss. There is a common problem with most medicinal plant extract tablets not meeting all the relevant quality standards. Some studies have even reported friability up to 15%. For example tablets made from AM-1 (a freeze dried decoction of powdered root extracts of shrubs used as anti-malaria in Nigeria) had friability of 15% even after using 5%w/w starch gel binder, the friability still remained above the highest acceptable limit (Builders *et al.*, 2011).

The *in vitro* experiments have found that HPMC (in particularly Methocel-CR) with a concentration of 15% is enough to provide a slow drug release rate although not to the target of 3 - 4 days so further experimentation is required to produce formulations with an even slower rate of release. This can be achieved by administering the matrices in cardboard tubes or by wrapping the matrices together with rice paper that would create a dosage form with a greater volume:surface area ratio resulting into more prolonged drug release.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

8.0 Summary

This chapter gives a general overview of the combined results of chapters 3-7 and their significance. It highlights the major findings and suggests areas for further future work. For more detailed and specific consideration of the results, the reader should refer to the results and discussion sections of the individual chapters in this thesis.

8.1 General discussion

Although the anthelmintic efficacy of plant derived cysteine proteinases have been extensively studied (Steppek *et al.*, 2006b, Satrija *et al.*, 1994, Stepek *et al.*, 2005, Stepek *et al.*, 2007a, Buttle *et al.*, 2011), there still remained a need to assess in more detail some of the possible variables/factors that may influence the efficacy of PLS against GI nematodes in rodent models. It was important to carry out such investigations to gain a broader understanding of the possible sources of variation in efficacy and to improve formulation and delivery systems of the enzymes for use in humans and livestock.

First, we initiated this project by purifying and concentrating the cysteine proteinase enzymes (CPs) in spray dried papaya latex using different methods to determine which of them would provide high yields of CPs. The best method was then adapted as a standard preparation of CPs to be used in *in vitro* and *in vivo* studies. It was found that concentration by dialysis provided a high yield of active

enzyme in PLS. We were also interested to find out which storage condition will provide long term stability of the CPs in the PLS. The results of our experiments demonstrated clearly that CPs can remain more active at low temperature (-20°C and -80°C) and freeze-thawing cycles have no effect on the enzymes' activities. The effect of temperature on enzyme activity has been widely documented (Balls and Thompson, 1940). At higher temperatures it is said that the free Sulphydryl groups in CPs are oxidized to the disulphide form or even sulphinic, sulphonic or sulphenic acid, all of which represent essentially irreversible oxidation states (Arakawa *et al.*, 2001). The stability of CP enzymes in cold temperature may have been enhanced by the concentration of the PLS and the latex acting as cryoprotectants. It has been reported that in many proteins, increasing protein concentration helps to increase the stability of proteins during freeze-thawing (Carpenter and Crowe, 1988). Also we were not sure if the enzymes would survive under the typical motility assay conditions employed in our experiments. To answer this question PLS were incubated in Hanks' buffer saline solution at 37°C for 2 h, under typical motility assay conditions, and we found that the enzymes remained stable throughout the entire period of the experiment without losing activity.

To obtain more understanding of the anthelmintic efficacies of kiwi fruit extract (actinidain), pineapple fruit extract (fruit bromelain) and stem bromelain, a number of *in vitro* and *in vivo* experiments were carried out. A previous *in vitro* study (Stepek *et al.*, 2005) with kiwi fruit extract failed to identify any anthelmintic effect although the enzymes in kiwi fruit are known to belong to the same group of the papain family. Nevertheless, it was important to repeat these experiments under more standardized conditions to establish firmly whether this enzyme, actinidain,

actually has any potential as a novel anthelmintic. To confirm this, worms were incubated in kiwi fruit extract and examined by scanning electron microscopy (SEM) for any sign of cuticle damage. We found that actinidain in kiwi fruit had little anthelmintic effect: only initial signs of cuticle damage were observed towards the end of the experiments. The biggest problem which we encountered with this enzyme was that of low active enzyme concentration. Our target was to try to concentrate the enzyme at least up to a 200 μM concentration so that it would be comparable to that of PLS. At this particular concentration PLS provides better results in the worm motility reduction assays compared to lower concentrations. However, the working concentration we managed to achieve from kiwi fruit extracts was only 50 μM , a concentration at which even the PLS has been shown to have a poor effect on motility (Stepek *et al.*, 2005). Based on this result it was not possible to conclude with any degree of certainty about the relative potency of the anthelmintic effect of actinidain. Further research is needed, either by devising novel ways to extract the enzymes from kiwi fruit to achieve a higher yield of active enzyme, new methods to concentrate the active enzyme more efficiently or alternatively prolonging the incubation time of the worms in the enzyme to enhance the extent of the damage. If the enzyme does require prolonged exposure to worms to achieve a comparable effect to that associated with PLS, it is unlikely to have much future as a novel anthelmintic, because peristaltic movements of gut contents are likely to flush the enzyme through the sections of the gut where worms reside before the enzyme has sufficient opportunity to exert its damage to the cuticle of the resident parasites.

Other enzymes which were assessed for anthelmintic effects included Bromelain, from both pineapple fruit and stem. Like kiwi fruit extract, with

pineapple fruit extract we only achieved a relatively low active enzyme concentration with a maximum concentration of 300 μM , far lower than that obtained with stem bromelain which we were able to concentrate even more effectively than PLS, achieving concentration levels of 8000 μM . In motility assay assessments stem bromelain and PLS were set at the same concentration (200 μM) expecting that the both would have the same level of inhibitory effect on worm motility. However the results indicated that stem bromelain had a significantly poorer effect on motility of the worms, even less than that of pineapple fruit extract at a 150 μM concentration. *In vivo* trials with both stem bromelain and pineapple fruit extract were disappointing showing much lower efficacy compared to PLS. It was not clear why some CPs have anthelmintic effects while others have a poorer or no effect. This opens an avenue for future research to investigate the cause of their variations. One likely explanation is that each of these enzymes target a slightly different sequence of amino acids in the target proteins, and it may be that the cuticular proteins of the nematodes used in this project did not have complementary amino acid sequences for the ineffective CPs.

Another major aspect considered in this thesis centred on the factors influencing the anthelmintic efficacy of PLS against GI nematodes in a mouse model system (Chapter 5 and 6). The preparation and delivery of any anthelmintic in an experimental system entails many variables which need to be explored and standardised to achieve best results. Many of the likely sources of variation have not yet been thoroughly assessed.

The first factor to be examined was the effect of fasting. It was important to assess this factor because in earlier studies with CPs, a period of food deprivation

was routinely employed before administration, but there was no systematic evaluation as to whether or not this does actually benefit the anthelmintic efficacy. So an experiment was carried out with 2 groups of mice where the first group was fasted for 5 h prior to treatment with PLS and the second group was treated without being fasted. The results (Chapter 5) demonstrated clearly that PLS was equally effective in reducing worm burdens whether the animals were fasted before treatment or not. This led to the conclusion that fasting has no beneficial effect on the efficacy of PLS and it was found that by avoiding fasting some of the adverse side-effects of treatment were reduced or even avoided entirely. Moreover, since food deprivation, even for limited periods of time, can cause stress and discomfort to animals, the non-fasted animals would have experienced less trauma during these trials. However, to some extent these results were surprising, as it was expected that the presence of food would reduce the activity of the enzyme by dilution in gastrointestinal chyme and competition with other potential substrates for the enzyme. The maintenance of efficacy of PLS in the presence of food suggests that there must be other compensating factors influencing activity, which need to be further investigated. One of the possible explanations is that the presence of food increases food residence time thus offering more time for the CPs to make and maintain contact with the worms, leading to increased worm damage.

The second factor which was examined in this study was the sex of the host. Male and female mammals are known to differ markedly in the hormones that control their sex and reproductive cycles and these in turn have marked effects on their physiology. The cause of the differences in response to medication between males and females seems to be caused by steroid sex hormones (oestrogen,

testosterone and progesterone) which can induce or inhibit the secretion of Cytochrome P450 enzymes in the liver (Cristofol et al., 1998), enzymes which are involved in metabolism of exogenous toxic substances (Kubota et al., 2011, Finnstrom et al., 2002). However, the results (Chapter 6) clearly indicated that variation in anthelmintic efficacy of PLS was not due to host sex. Host sex has also been reported to have no influence even in conventional synthetic anthelmintics (monepantel) (Hosking *et al.*, 2010a).

Another factor which was considered to cause variations in response to treatment among different strains of mice was host genotype. This factor was considered due to the fact that under natural conditions animals of different breeds or species vary in response to medication. Such variation may range from failure to respond to a drug to life-threatening adverse reactions (Bosch, 2008). To determine whether the efficacy of PLS treatment is host genotype dependent a range of mouse strains were compared including both inbred and outbred strains. It was found that efficacy of PLS varied significantly between mice of different genotype. At the doses used, the treatment was most effective in C3H mice and least effective in NIHS, CD1 and BALB/c mice in reducing worm burdens.

Different causes of variations in response to treatment were hypothesized and tested. One of the possible causes of variations in efficacy of PLS between strains of mice was hypothesized to be host weight. Throughout the study mice were treated with a standard dose of 240 nmol of PLS in a volume of 0.2 ml regardless of their variations in body weight. The concentration of PLS was not adjusted in accordance to the exact weight of individual mice and since some strains are heavier than others,

it could have been that the heavier strains actually received a lower dose of drug on a per kg basis. We carried out an experiment to compare the efficacy of PLS between mice treated with the standard dose of 240 nmol per mouse regardless of their body weight and those treated based on body weight-adjusted dose (8 μ mol per kg body weight). We found that there was no variation in response to treatment between the two treatment groups of mice. This result means that administration of a standard dose to mouse strains varying in body weight without compensating for body weight was an unlikely explanation for the cause of variation in efficacy of PLS between mouse strains.

Since CPs are particularly sensitive to pH, variation between mouse strains in gut pH was investigated but no significant differences in pH changes were detected along the GI tract when the poor (BALB/c) and high responder mice (C3H) were compared. Both strains had the lowest pH values (3.5) in the stomach and pH value around 6.5 in the small intestine, which is within the optimum pH range of CPs activity for worm damage. Lack of variation in pH values between the two strains of mice suggests that there must be some other factors affecting the efficacy of PLS in different strains of mice other than acid or pH.

It was also thought that the length of exposure of the worms to PLS may be a crucial factor because in *in vitro* experiments (Chapter 4), worms were consistently exposed to latex for the whole of the incubation period lasting up to 2 h. So the ineffectiveness of the PLS in the poor responder strains was hypothesized to be caused by the rapid passage of the contents of the enzyme in the small intestine. We carried out an experiment (Experiment 10 of Chapter 6) to compare the amount of

enzymes in different regions of the intestine at different time intervals after dosing and it was found that there was no significant variation in the rate of enzyme passage between the poor and higher responder mice.

Since the results indicated that PLS can remain in the small intestine for more than 1h, it was then hypothesized that poor responder mice might be secreting excess trypsin which might be degrading the CPs faster than possibly lower levels in responder strains. However, when compared the amount of trypsin secreted into the small intestine of poor and higher responder mice, as measured by enzyme concentration, was found to be very similar in the 2 strains. That means that if trypsin has any effect on the CPs then both strains were likely to be affected to the same extent.

Another factor that was hypothesized to be the source of some of the variation in response to treatment was worm infection intensity. In this study it was considered that in response to increasing worm burdens the effectiveness of the PLS should fall. Nevertheless, the results of Experiment 12 (Chapter 6) indicated that for the high responder strain, the extent of worm reduction increased with increased worm intensity, although in the poor responders there was no clear trend with respect to efficacy of treatment, the worms having established better at higher intensity than at lower intensity.

The main challenge in this study was to identify ways in which the poor responders might be made to be more sensitive to drug treatment. One possible way was to combine antacid treatment with PLS. For this reason one group of mice was

given cimetidine prior to treatment with PLS, in order to block acid in the stomach which might be inhibiting the activity of the drug. The hypothesis tested here was that the weaker responding strains may have lower stomach acidity which inactivates the PLS to a greater extent than in high responder mice (C3H mice). So it was assumed that most of the PLS might have been affected by the low pH of the stomach and less was available for exerting worm damage in the small intestine. However, Experiment 7 (Chapter 6) showed that blocking stomach acidity by concurrent treatment with cimetidine did not improve efficacy in the intermediate responder strain (NIH).

In the work described in this thesis, when investigating the causes of variation in host response to treatment with PLS, we focused exclusively on the physiological aspects of the host, neglecting the potential impact of the variation which might arise by possible different behaviour patterns of the parasite in different hosts. One of the parasitic behavioural aspects which still needs to be investigated is the extent of the mucosal embedding activity of the adult *H. bakeri* between mouse strains, which may serve to protect the worms against the PLS activity more so in some strains of mice than others. This can be investigated either by comparing *H. bakeri* with a free lumen intestinal residing nematode or by investigating the nature of the mucosal layer in poor and high responder mice.

It is also well known that the GI tract of animals provides a reducing environment for CPs activation. Based on this fact we assumed then that CPs were equally activated in all strains of mice after oral administration with PLS. This assumption may be unrealistic, considering the genetic variation between different

strains of mice. It might be that the poor responder mice failed to provide a full activation environment for CPs thus providing little chance for the enzymes to damage the worm cuticle. It is therefore important for future work to try to administer the pre-activated CPs to poor responder strain and to compare their response with that of inactivated CPs.

One of the objectives of this study was to explore the possibilities of formulating PLS as an anthelmintic drug to be used in ruminants. To achieve this *in vitro* studies were conducted. These studies involved both immediate and slow release drug dosage forms simulating the physiological conditions in the GI tract of the animal. In the slow release experiments, two hydrophilic matrices were tested, the xanthan gum and hydroxypropyl methylcellulose (HPMC) (both Methocel-LVCR and Methocel-CR). It was found that Methocel-CR provided better results compared to the others. In the immediate release Ac-Di-sol was found to produce the faster immediate drug release rate. Although Methocel-CR was able to provide a slow drug release rate, it was not slow enough to meet our target of 4 days. Further future work is required to produce formulations with slower release rate. This might be achieved by administering the matrices in cardboard tubes or by wrapping the matrices together with rice paper that would create a dosage form with a greater volume: surface area ratio resulting in more prolonged drug release. When Ac-Di-Sol was used to enhance fast release rate in the bolus the result (Chapter 7) was inconclusive and this also requires further investigation. One difficulty in this area of research is that it is extremely difficult to devise suitable formulations (with respect to volume and size) for experimental testing in mouse models. Therefore, if these

formulations are to be tested it will have to be in small ruminants such as sheep in the first instance, rather than in mice.

Finally we tested the anthelmintic efficacy of PLS on an equine GI nematode, *Strongylus vulgaris*. Strongyles have been reported to be the most pathogenic parasites of horses exerting a significant economic impact wherever horses are being raised and maintained, and allowed to graze on contaminated pastures (McCraw and Slocombe, 1976). Moreover, the problem has been worsened due to the development of resistance to most of the available chemotherapies. Therefore, it was important to test the effective of PLS as an alternative novel anthelmintic. Preliminary *in vitro* results (Chapter 4) revealed that PLS was highly effective against these nematodes. This provides a new opportunity for future investigation including *in vivo* experiments.

8.2 Conclusions

In summary we can conclude that freezing PLS either at -20°C or -80°C provided long term storage of more than 1 year without major loss of activity and freeze-thaw cycles had no effect on enzyme activity. Moreover motility assay conditions have no detrimental effect on enzyme activity over the specified period of assessment.

It has been clearly demonstrated that allowing animals to fast before drug treatment has no beneficial effect on the anthelmintic efficacy of PLS. The study has also indicated that there is considerable variation in response to treatment with PLS

between different strains of mice. However the cause/s of variation is/are not yet known, which opens the door for much possible further research.

The *in vitro* experiments for drug formulation have provided a better result with HPMC (in particularly Methocel-CR) which can be used to induce slow drug release rate although not slow enough to achieve our nominated estimated target of 3-4 days, and this emphasizes the need for further research in this area also. On the other hand 10% concentration of Ac-Di-Sol was sufficient to provide a fast release rate for individual tablets but its effect on the bolus was insignificant. Moreover, the preliminary *in vitro* results on the efficacy of PLS against equine GI nematodes shed some light for the potential development of PLS as an equine anthelmintic drug.

Finally the empirical findings in this study provide useful information for improvement of formulation and delivery of these naturally occurring plant-derived enzymes for treatment of intestinal worm infections in humans and livestock, while achieving maximum efficacy and minimal side-effects. I hope that in the years ahead my findings can be exploited to develop and refine plant derived proteinases as novel therapies for the treatment of intestinal worm infections.

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APPENDICES

Appendix 1: Paper published from the work in this thesis

Appendix 2: General Solutions

a) 4 x Assay Buffer Solution

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 8.28g

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 21.49g

Dissolved in 300 ml distilled water and made up to a final volume of 500 ml with water and adjusted the pH to 6.85 with NaOH.

b) Activating Buffer

4 x Assay buffer + 16 mM L-cysteine (made fresh each day of experiment)

c) Hanks' Buffer Saline Solution with Phenol red

Stock solution 1

NaCl 168.0 g

KCl 8.0 g

KH_2PO_4 4.0 g

0.2% Phenol red 200 ml

Made up to 2 litres with distilled water

Stock solution 2

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 3.92 g

$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 2.0 g

Made up to 2 litres with distilled water

Working Hanks:

110 ml of solution 1 were added to 110 ml of solution 2 and made up to 1 litre with water. The pH was adjusted to 7.2 with 1M of NaOH.

d) Hanks' buffer Saline solution without phenol red**Stock solution 1**

NaCl	42.0 g
KCl	2.0 g
KH ₂ PO ₄	0.5 g
Na ₂ HPO ₄ (anhydrous)	0.5 g
Dissolved in 500 ml of distilled H ₂ O	

Stock solution 2

CaCl ₂ .2H ₂ O	0.925 g
MgCl ₂ .6H ₂ O	0.5 g
Dissolved in 500 ml distilled H ₂ O	

Working Hanks:

55 ml of stock 1 were added to 55 ml of stock 2, made up to 500 ml with water and the pH was adjusted to 7.2 with few drops of NaOH

e) Stopping Buffer Solution

Sodium chloroacetate	5.82 g
Sodium acetate (anhydrous)	8.2 g

Made up to 500 ml with distilled water and the pH was adjusted to 4.3 with acetic acid

f) Phosphate Buffered Solution for Drug Dissolution Test

NaH₂PO₄·2H₂O 31.2 g in 1 litre of dH₂O (Stock1)

Na₂HPO₄·12H₂O 71.7 g in 1 litre of dH₂O (stock2)

25.5 ml of stock 1 were added to 24.5 ml of stock 2 and made up to 100 ml with distilled water.

g) 0.15M Phosphate Buffer for SEM:

Na₂HPO₄ 8.52 g in 400 ml dH₂O

NaH₂PO₄·H₂O 2.07 g in 100 ml dH₂O

400 ml NaH₂PO₄ added to 100 ml NaH₂PO₄ to obtain pH of 7.2

h) Gutaraldehyde 2.5%

2.2 ml glutaraldehyde + 19.8 ml phosphate buffer (usually x 2).

Appendix 3: Amount of active enzymes in different storage conditions

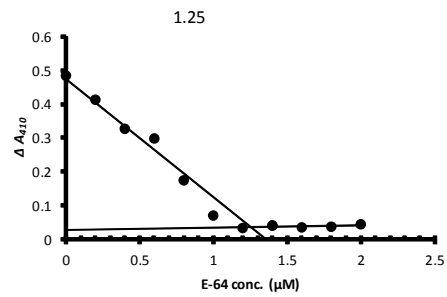
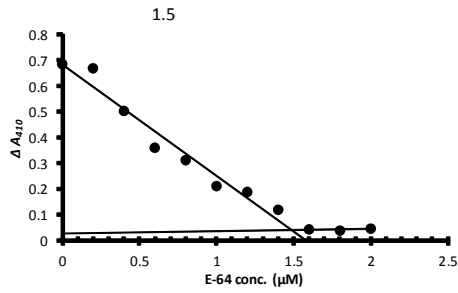
Conditions	1 week	4 weeks	24 weeks	48 weeks
room temp	23.3 (27.6)	20(23.5)	14(16.5)	1.1 (1.3)
4 ⁰ C	80(94.1)	70(82.4)	50(58.8)	23.8(27.9)
-20 ⁰ C	75(88.2)	70(82.4)	70(82.4)	70(82.4)
-80 ⁰ C	80(94.1)	68(80)	70(82.4)	67.5(79.4)

Figures outside the bracket are amount of active enzymes in nmol and those in bracket are percentages of the amount of active enzymes remaining.

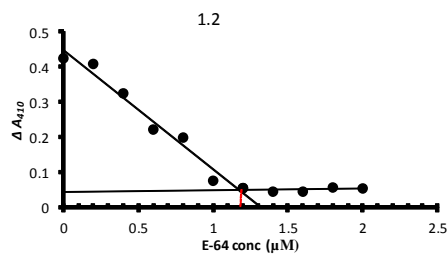
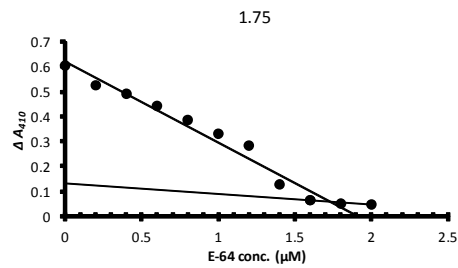
Appendix 4: Active site titration curves for determination of active enzymes in PLS

a) PLS processing

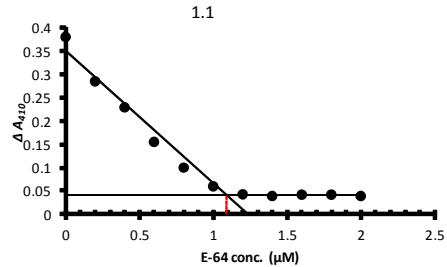
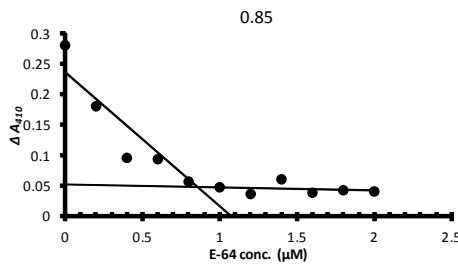
PL Solution before centrifuge 1:100



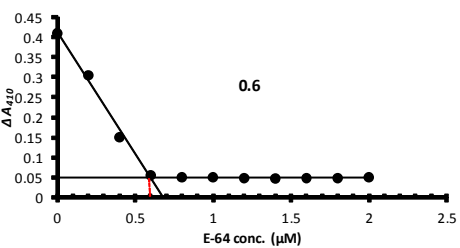
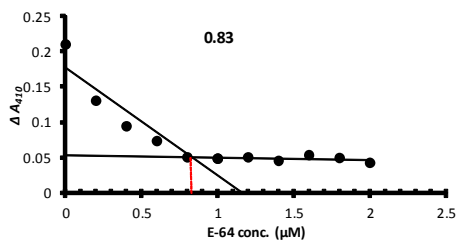
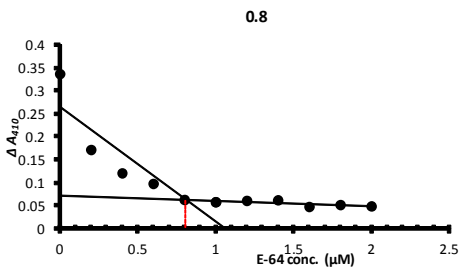
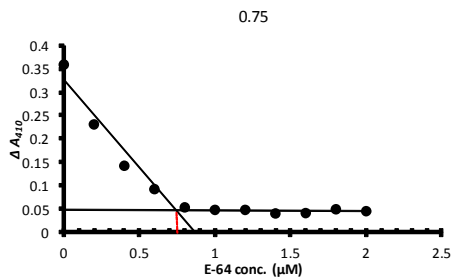
PLS after centrifuge 1:100



Dialysed PLS 1:100

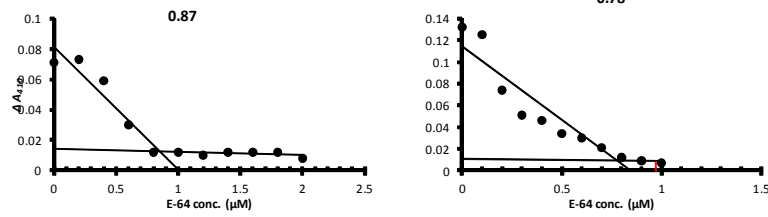


Reconstituted Freeze-dried PLS 1:100

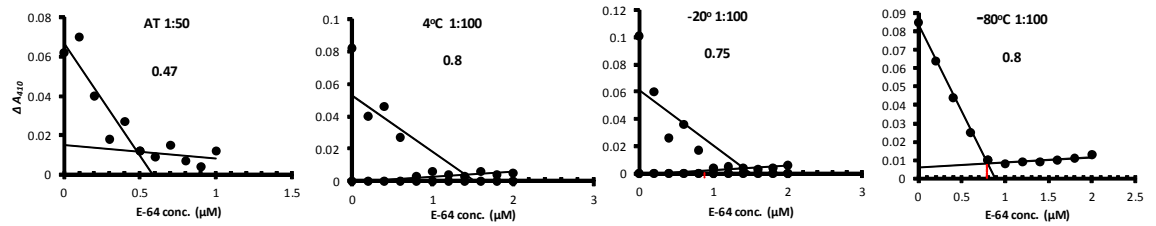


b) PLS storage at various temperature conditions

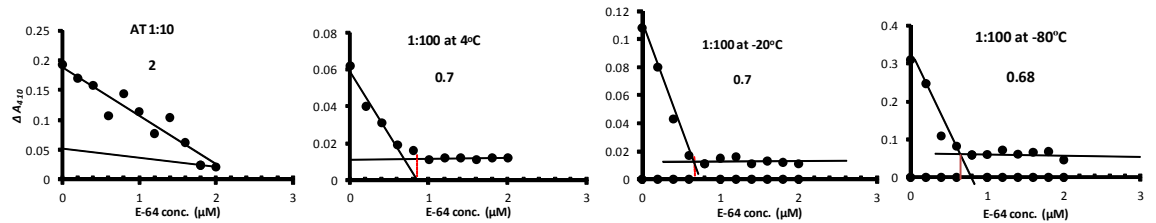
Day 0 1:100



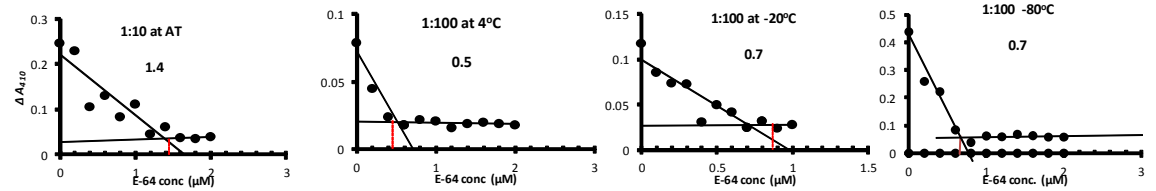
After 1 week



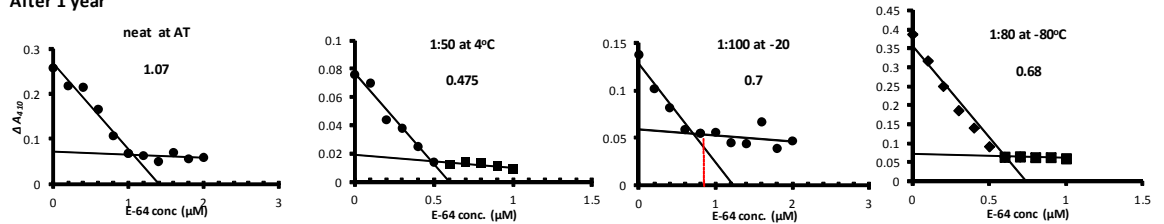
After 1 month



After 6 months

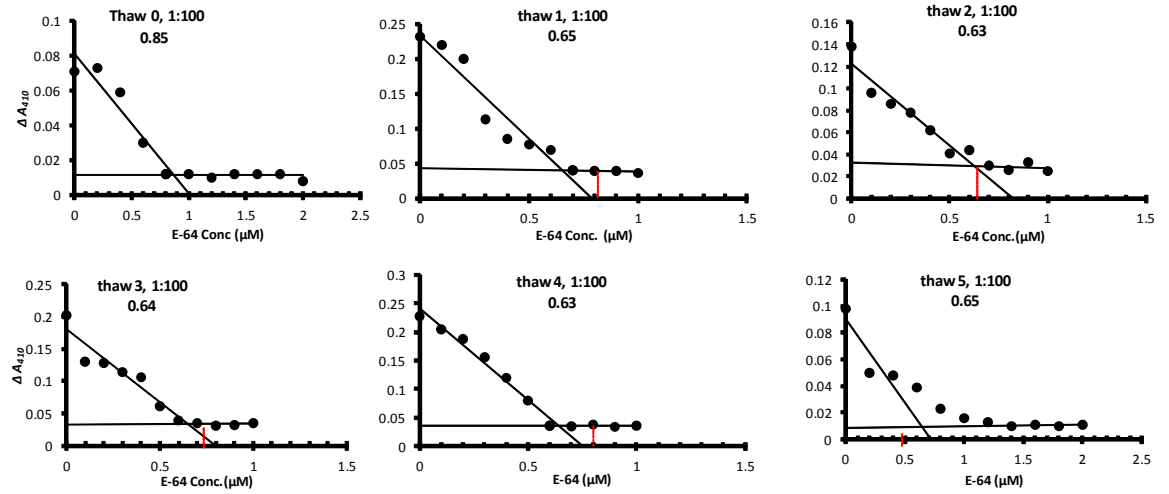


After 1 year

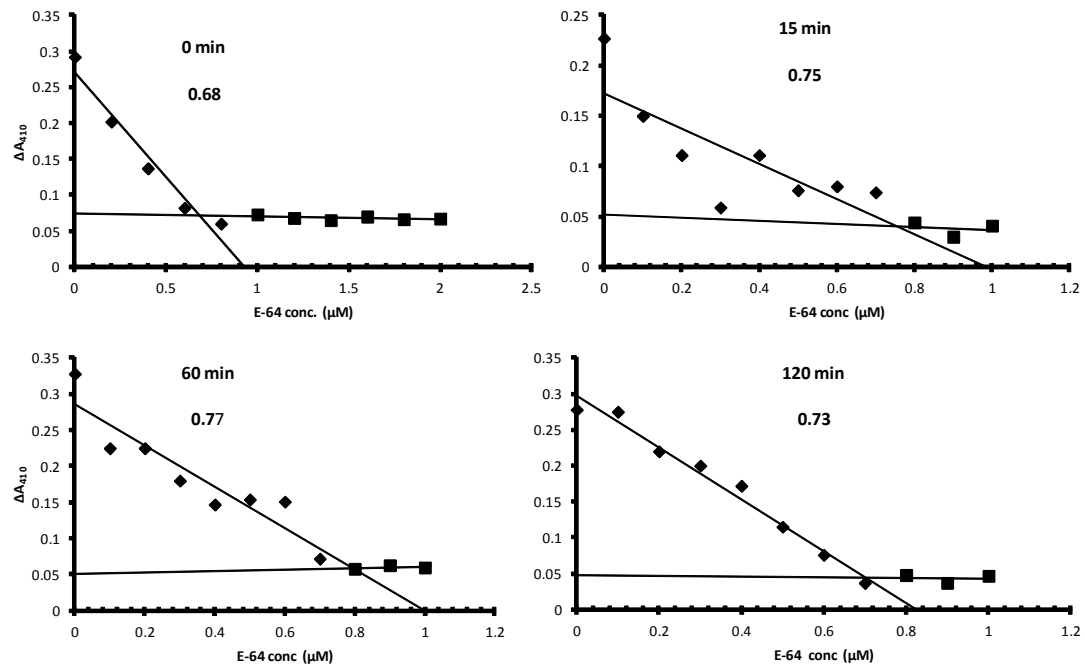


c) Effect of freeze-thaw cycles

Effect of freeze-thaw cycles

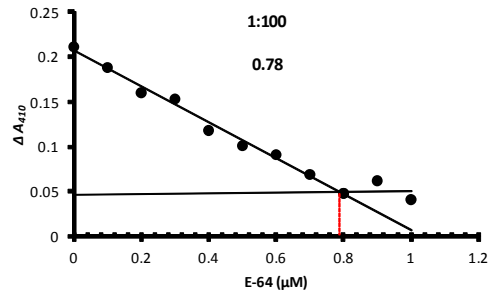
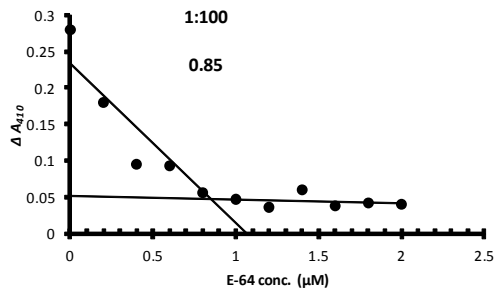


d) PLS stability in motility assays

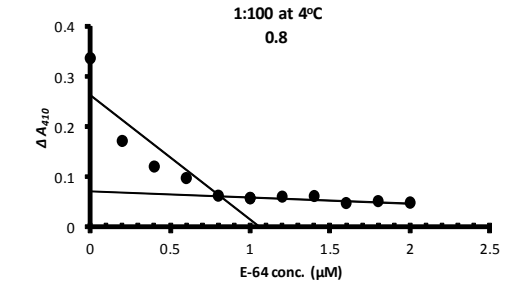
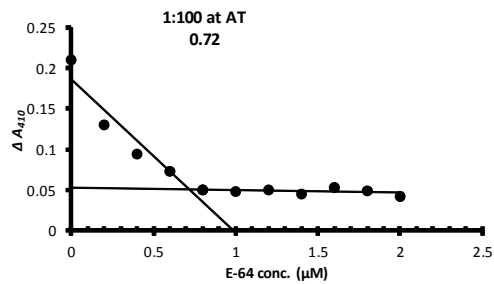


e) Stability of post freeze-dried PLS at ambient temperature (AT) and 4°C

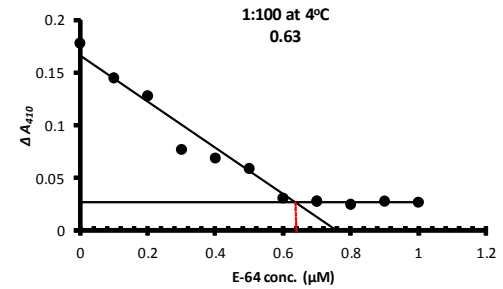
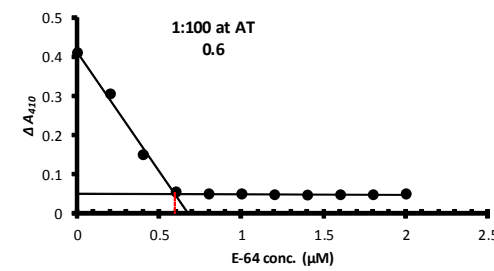
Day 0



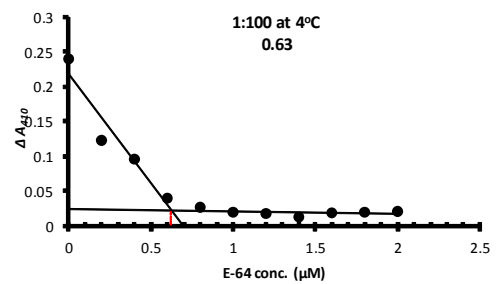
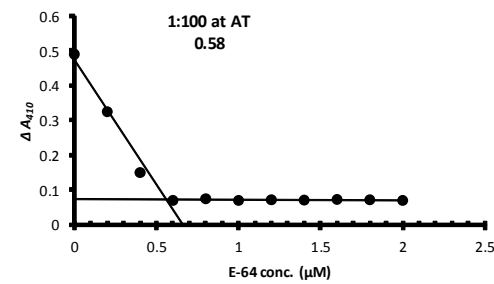
After 1 week



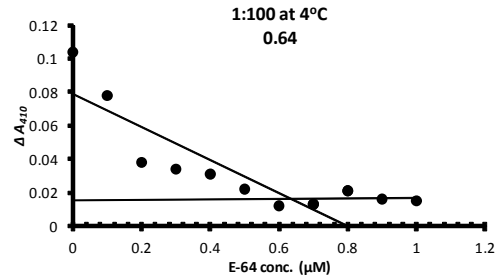
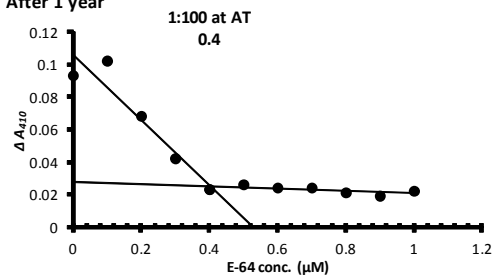
After 1 month



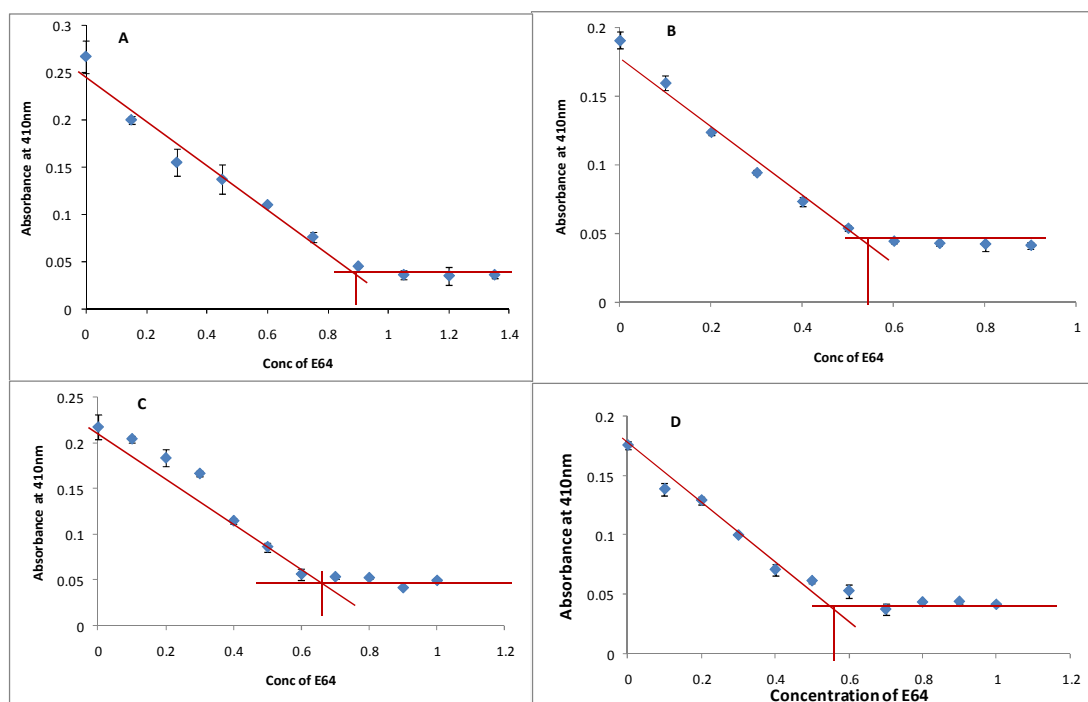
After 6 months



After 1 year



Appendix 5: Calculations of percentage active enzyme in tablets



The figures in this appendix show each point representing the mean absorbance readings at 410 nm of titration of a fixed concentration of enzyme against varying concentrations of the inhibitor E-64. The percentage amount of active enzyme present in each tablet was then calculated as follows. Since 20 μ M of E-64 was added at increment of 10 μ L then 0.2nmol of E64 was added per increment. Because molar inhibition ratio is 1:1, the baseline point represents the amount of E64 which is equivalent to the amount of active enzyme present in 100 μ l of the enzyme solution. Thus results show that the amount of active enzyme in 100 μ l is 0.9nmols for tablet with no matrix (Fig. 2A), 0.65nmols for tablet with Methocel-CR (Fig. 2C), and 0.55nmols for both tablets with Methocel-LVCR and xanthan gum (Fig. 2 B and D).

Since the molecular weight of the enzymes in papaya latex is 25,000g then 1nmol would be 25 μ g and 0.9nmol contains 22.5 μ g (amount of active enzyme in 100 μ L). However the initial weight of the tablet was 1.4g (1400mg) dissolved in 900ml then

total amount of enzyme in 100 μ L is 153.2 μ g (excluding 0.5% of excipient). Therefore, the percentage active enzyme in the tablet was 14.7% ($22.5/153.2 \times 100$). Based on this procedure the percentage amount of active enzymes present in the other tablets was also calculated and found to be 11.6%, 14.5% and 12.6% for xanthan gum, Methocel-CR and Methocel-LVCR respectively.

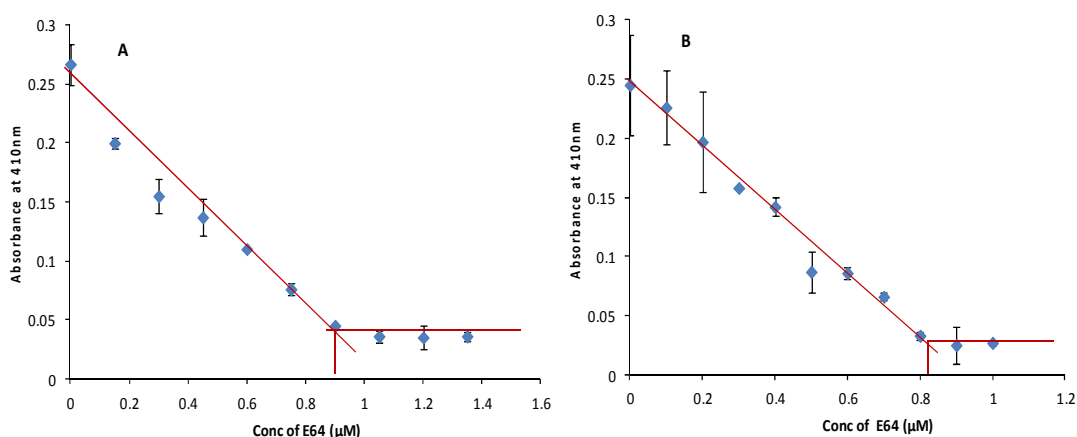


Figure 5: Active site titration curves of PLS with and without Ac-di-Sol disintegrant

Tablets loaded with A) PLS alone and B) PLS and 10% Ac-Di-Sol. Each tablet was dissolved in 900ml of phosphate buffer then left overnight and tested for enzyme activity. The graph represents the mean \pm SEM (n=3) absorbance readings at 410 of titration level of the inhibitor E64 against a fixed concentration of enzyme. The results indicate that tablet made with 10% Ac-Di-Sol retained 74% enzyme activity when compared with tablet made with PLS alone.