MODULATED ENZYME EXPRESSION IN GASTROINTESTINAL NEMATODES

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Declaration

I declare that this thesis was composed by myself,
and all the work described is my own
unless otherwise stated.

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Abstract

Gastrointestinal nematodes cost the farming industry millions of pounds a year. Control is largely dependent on the use of anthelmintics but widespread resistance to these drugs is now developing. Attention has, therefore, turned to the development of novel methods for parasite control. Recently, research has focused on the identification of parasite antigens which stimulate protective host immune responses with the aim of vaccine development. Parasite enzymes are appropriate targets for this strategy but there is some evidence that parasites can 'adapt' enzyme expression in response to host immune attack while the degree to which enzyme expression is influenced by parasite strain divergence is largely undefined. These factors could have considerable implications for the development of subunit vaccines and novel anthelmintics.

The first part of this study characterised the proteinases of the parasitic stages of *Teladorsagia circumcincta*, an important ovine abomasal nematode in the UK. Proteinases released during the *in vitro* maintenance of third or fourth stage larval as well as adult parasites were characterised on the basis of pH optima, molecular size and specific proteinase inhibitor sensitivity. Proteinases were released in a stage specific manner and metallo-proteinases predominated with the ability to degrade a variety of potential natural protein substrates. Immunological studies indicated that these proteinases were weakly immunogenic stimulating enzyme inhibiting antibody responses.

Enzyme expression in the ovine abomasal parasite *Haemonchus contortus*, including proteinases and superoxide dismutases, was examined in comparisons of 1) UK and Australian strains of the parasite and 2) parasites surviving in lambs vaccinated against homologous challenge, the vaccine being an integral gut membrane protein complex, and those from the corresponding challenge controls. In both comparisons marked differences in the level of total proteinase expression were noted.

The influence of the host on parasite enzyme expression was studied more closely in the rat/*Nippostrongylus brasiliensis* intestinal nematode infection system. Profound differences were demonstrated in the proteinase content of adult worm extracts, as judged by gelatin-substrate gel analysis, when parasites harvested from individual rats were compared following primary infection. Moreover, the intra-host environment also markedly influenced parasite superoxide dismutase isoenzyme expression. Similar effects were noted when worm populations harvested following primary and secondary infections were compared.

To facilitate the definition of the molecular events underlying variable parasite enzyme expression, a reverse transcriptase polymerase chain reaction (RT-PCR) assay for quantifying total SOD mRNA transcript production in *N. brasiliensis* was developed. *N. brasiliensis* SOD encoding cDNAs were isolated and their nucleotide sequences determined, these analyses indicating that sequence variants were contiguously expressed in the adult parasite. The RT-PCR assay was developed using primers designed to anneal to all the known SOD transcripts and then used to quantify SOD encoding mRNA production in a variety of different parasite populations and these data compared with total enzyme activity determinations. Highly significant correlations were obtained indicating that SOD mRNA transcript levels were indicative of active enzyme production.

In conclusion, the work described in this thesis provided further evidence to support the view that parasites can modulate enzyme expression in response to the

changing intra-host environment. The development of an RT-PCR approach with the potential to quantify enzyme expression in a gene specific manner will facilitate experiments to define whether or not altered enzyme expression is primarily due to genotypic or phenotypic variation in the parasite population and provides the sensitivity to examine individual worms.

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Abbreviations

Ab	antibody
AChE	acetylcholinesterase
Ag	antigen
APS	ammonium persulphate
ATCI	acetylthiocholine iodide
ATP	adenosine triphosphate
bp	base pairs
cDNA	complementary deoxyribonucleic acid
°C	degrees celcius
cm	centimetres
$CuSO_4$	copper sulphate
DAB	3,3' diaminobenzidine tetrahydrochloride
d(d) ATP	2'(3'-di) deoxyadenosine 5'-phosphate
d(d) CTP	2'(3'-di) deoxycytidine 5'-phosphate
d(d) GTP	2'(3'-di) deoxyguanosine 5'-phosphate
d(d) TTP	2'(3'-di) deoxythymidine 5'-phosphate
d(d) NTP	2'(3'-di) deoxynucleotide 5'-phosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminotetra-acetic acid
E/S	excretory/secretory
FEC	faecal egg counts
g	gravitational force
GI	gastrointestinal
gm	grams
GSH-px	glutathione peroxidase
GST	glutathione s-transferase
HCl	hydrochloric acid
HPTI	human placental thrombin inhibitor
hr	hours
HRP	horse-radish peroxidase
IFN	interferon
Ig	immunoglobulin
INT	iodo-nitro-tetrazolium
IPTG	isopropylthio-β-galactoside
IU	international units
IVR	*in vitro* released
Kb	kilobase
KCN	potassium cyanide
kDa	kilodaltons
kg	kilogrammes
KH_2PO_4	potassium dihydrogen phosphate
K_2HPO_4	potassium hydrogen orthophosphate
L	litre
L1	first larval stage
L2	second larval stage

L3	third larval stage
L4	fourth larval stage
L5	fifth larval stage
LDH	lactate dehydrogenase
M	molar
mA	milliampere
mg	milligrams
$MgCl_2$	magnesium chloride
MHC	major histocompatability complex
mm	millimetres
ml	millilitre
µg	microgrammes
µl	microlitres
µm	micrometres
mRNA	messanger ribonucleic acid
N_2	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
ng	nanogram(mes)
O_2	oxygen
OD	optical density
%	percentage
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PAMS	phenazine methosulfate
PCR	polymerase chain reaction
PGE	parasitic gastroenteritis
pH	$-\log_{10}$ (hydrogen ion concentration)
p.i.	post infection
PmsF	phenyl metho sulfyl fluoride
psi	pounds per square inch
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodocyl sulphate
SL1	*trans*-spliced leader 1
SL2	*trans*-spliced leader 2
SOD	superoxide dismutase
spp	species
TAE	tris-acetate/Na_2EDTA
TBE	tris-borate/Na_2EDTA
TBS	tris buffered saline
TBST	TBS with Tween
TCA	tri-chloroacetic acid
TE	tris/Na_2EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
tris	tris (hydroxymethyl) amino methane
Triton X-100	octylphenoxypolyethoxethanol
U	units

UV	ultra violet
V	volts
v/v	volume to volume
v/s	versus
W	watts
w/v	weigh per unit volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

Nucleotide	One letter symbol code
adenosine	A
cytidine	C
guanosine	G
thymidine	T
inosine	I
unknown	X

Amino Acid Designations

	3 letter designation	1 letter symbol
Alanine	ALA	A
Arganine	ARG	R
Asparagine	ASN	N
Aspartic acid	ASP	D
Cysteine	CYS	C
Glutamic acid	GLU	E
Glutamine	GLN	Q
Glycine	GLY	G
Histidine	HIS	H
Isoleucine	ILE	I
Leucine	LEU	L
Lysine	LYS	K
Methionine	MET	M
Phenylalanine	PHE	F
Proline	PRO	P
Serine	SER	S
Threonine	THR	T
Tryptophan	TRP	W
Tyrosine	TYR	Y
Valine	VAL	V

CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

Trichostrongylus, Ostertagia, Teladorsagia and *Haemonchus* genera are members of the family Trichostrongylidae. They are roundworms which reside in the intestine and abomasum of sheep, cattle and goats, causing reduced growth rate and poor carcass quality in infected animals (Urquhart, Armour, Duncan, Dunn and Jennings, 1991). The disease syndrome is referred to as parasitic gastroenteritis (PGE) and results in considerable economic losses world-wide (Brunsden, 1980). Intensive grazing systems used in many countries have dramatically increased the level of exposure of grazing ruminants to gastrointestinal (GI) nematodes and have resulted in a growing incidence of PGE (reviewed Holmes, 1987). In heavy infections, mortality is an important cause of economic loss but considerable losses accrue from moderate infections due to reduced performance and the cost of anthelmintic therapy. PGE often results in inappetance, reduced utilisation of nutrients, loss of plasma proteins and abnormal nitrogen balance (Coop, Sykes and Angus, 1977) with anaemia and diarrhoea evident depending on the species of parasite present (reviewed Holmes, 1987). Control involves strategic use of anthelmintics as it is virtually impossible to graze animals on clean pasture in intensive farming systems. Unfortunately anthelmintic resistance is now a serious problem in ovine nematode populations throughout the world (Conder and Campbell, 1995). As yet immunological control is not an option, but novel methods for control are continually being sought.

Parasite excretory/secretory products (E/S) may stimulate host protective immune responses and are therefore attractive targets for either chemotherapeutic or immunological intervention because of their putative importance to maintenance within the host. The parasitic stages of the Trichostrongylidae are closely associated with the gut surfaces and parasite enzymes, most notably proteinases, have been ascribed a variety of functions such as nutrition (Thorson, 1956), penetration of host tissue barriers (Matthews, 1977 and 1984; McKerrow and Doenhoff, 1988), evasion of host immune responses (Chapman and Mitchell, 1982; Leid, Suquet and Tanigoshi, 1987; McKerrow and Doenhoff, 1988; Smith, Dowd, McGonigle, Keegan, Brennan, Trudgett and Dalton, 1993) and as contributory factors in the development of the immunopathology of helminth infections (Brophy and Prichard, 1992). Other enzymes

such as free-radical scavenging enzymes and acetylcholinesterase may contribute to the evasion of host immune responses (reviewed Knox, 1994). There is some evidence however, to suggest that parasites can 'adapt' enzyme expression in response to host immunological attack, (Ogilvie and Hockley, 1968; Knox and Jones, 1992). Furthermore, between parasite populations there may be considerable strain divergence in the range of enzymes expressed (Karim, Hoare and Trees, 1991; Echevarria, Gennai and Tait, 1992). These factors could have considerable implications for the development of antiparasitic subunit vaccines and novel anthelmintics.

This chapter will focus on GI nematodes in ruminants with specific reference to *Teladorsagia circumcincta* and *Haemonchus contortus*, subjects of the present study and both economically important parasites in the UK. The importance of parasite enzymes in research and development of control procedures is also discussed as well as highlighting the information available on the ability of parasites to modulate their enzyme expression.

1.1.1 **Phylogeny**

The class nematoda contains many parasite species which infect both plants and animals. These parasites cause considerable disease and hardship world wide, by either infecting the host directly or by destroying important food sources. The class nematoda can be divided into 2 subclasses, the aphasmidia and the phasmidia. The phasmida is further subdivided into 6 orders which are distinguish from the aphasmidia by the presence of caudal sense organs (reviewed Smyth, 1988). The strongylida is one of the six phasmidia orders and is in turn divided into a number of superfamilies and families of which the Trichostrongylidae and Heligmosomatidae are members (Smyth, 1988). Members of the Trichostrongylidae family are very important pathogens of domestic animals. For example *T. circumcincta*, *H. contortus*, *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, *Nematodirus battus* and *Cooperia* species. *Nippostrongylus brasiliensis* is a rodent intestinal nematode often used as a model for the study of gastrointestinal infections, and is a strongylida nematode belonging to the family Heligmosomatidae (Smyth, 1988).

T. circumcincta is a member of the subfamily Ostertaginae, a classification based on differences in the morphology of male worms through the arrangement of spicules and genital cones (Gibbons and Khalil, 1982). The Ostertaginae contain genera of veterinary importance including Ostertagia, Teladorsagia and Marshallagia. The nomenclature of Ostertaginae became complicated in the early 1980s when it was proposed that a number of the species were not true individual species in their own right but polymorphs of each other (Litchenfels, Pilitt and Lancaster, 1988). Several groups of researchers tried to resolve the issue using interbreeding experiments, morphology (Durette-Desset, 1989) and enzyme studies (Gasnier, Carbet and Suarez, 1993). As a result of these studies *Ostertagia circumcincta* was renamed *Teladorsagia circumcincta* on the basis of the arrangement of its bursa rays (Durette-Desset, 1989, Gasnier, Carbet and Suarez, 1993).

1.1.2 **Life Cycle of the Trichostrongylidae**

GI nematodes of sheep have a direct life cycle. The generalised life cycle of the Trichostrongylidae is described here, and summarised in Figure 1.1. Generally, the eggs are passed in the faeces of an infected host and under favourable conditions of temperature (approximately 20°C) and humidity the eggs hatch, releasing the first larval stage (L1; Urquhart *et al*, 1991). After a period of intense feeding on microrganisms, the first moult to the second larval stage (L2) occurs, the larvae at this stage are still present in the faeces. The faeces gradually break down, with the release of larvae and the L2 moult into the infective third larval stage (L3), retaining the cuticle of the L2 as a protective sheath (Urquhart *et al*, 1991). The L3 do not feed but migrate to the top of blades of grass, where they are ingested by feeding livestock. If the environmental conditions are not suitable for development, i.e. cold or drought, the pre-parasitic stages can stop their development for a number of months although eggs and larvae are less able to survive hot dry weather than extremes of cold and wet (Donald, 1968).

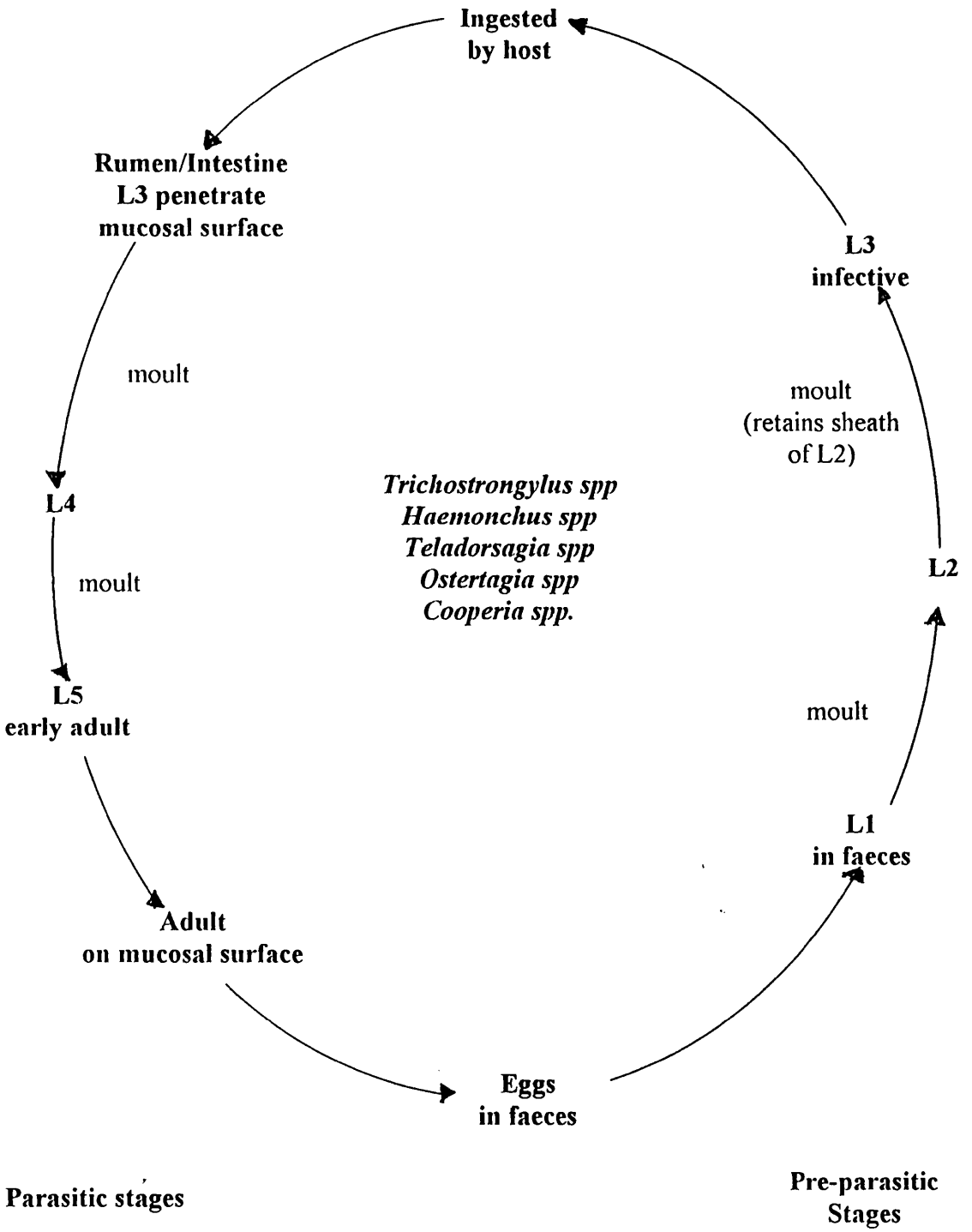
Once ingested the larvae exsheath in the rumen and penetrate the gastrointestinal mucosa at their predilection site. After about 4 days the larvae moult to become early fourth stage larvae (L4) which then undergo a period of rapid growth and differentiation before a further moult to become fifth stage larvae (L5) which are young

adult worms (Urquhart *et al*, 1991). Develpoment through to L5 usually takes place around 8-9 days after infection. The adult parasites then mature and are found in and around the mucosal surface from about 12-16 days after ingestion. Eggs are usually present in the faeces from about 21 days post infection (Urquhart *et al*, 1991).

In some cases the parasitic larvae may arrest their development during the early stages of infection. A number of factors may stimulate this cessation in development such as the climatic conditioning of the free-living larvae (i.e. exposure to cold or drought), a high density of adult worms or the initiation of a local host immune response (reviewed Miller, 1984).

Figure 1.1

Generalised Life Cycle of the Trichostrongylidae



1.1.3 **Epidemiology**

Pasture contamination by GI nematodes is dependent on type of pasture, climatic conditions and stocking rates (Crofton, 1957). Young animals are predominantly infected as immunity usually only develops at about 6 months of age.

A number of studies have been carried out on the worm burdens of sheep in Scotland which have indicated that each species of parasite follows a definite seasonal pattern. *T. circumcincta* has been found to be the most common abomasal nematode in the spring and early summer (Crofton, 1954; 1957; Thomas, 1956; Connan, 1968), accounting for up to 90% of the total worm burdens (Reid and Armour 1975b), while *H. contortus* occurred sporadically in small numbers during this time, (Crofton, 1954 and 1957; Connan, 1968). *T. vitrinus* was the most prevalent nematode found in the small intestine. The highest level of infection with this species was found to occur during the late summer months (Crofton, 1954 and 1957; Connan, 1968). *N. battus* however appeared to be one of the first species to establish an infection each year although the numbers were consistently low (Crofton, 1954; 1957; Thomas, 1956; Connan, 1968). It is important to note that there was always a number of species present at any one time.

Studies by Crofton (1954;1957), Connan (1968) and Reid and Armour (1975a and b) also reported evidence of the 'spring rise' in faecal egg counts in ewes, a well documented event in which the faecal egg count of ewes rises dramatically in the spring. This phenomena has been attributed to the maturation of previously inhibited larval stages, the development of recently ingested larvae and changes in environmental conditions enabling eggs and larvae which over wintered on the pasture to resume their development (Crofton, 1954; 1957). It is also thought that the ewe's immunological response is temporarily relaxed, due to the stresses of the winter followed by lambing, lactation and subsequent weaning, (Crofton, 1955; Reid and Armour 1975 a and b). Later on in the year however, the ewes were again resistant to infection (Reid and Armour, 1975b).

1.1.4 **Parasitic Gastroenteritis (PGE)**

The disease syndrome is caused by a number of co-infecting species but is often subdivided on the basis of the principle causative nematodes.

Ostertagiosis: Parasites of the genus Teladorsagia and Ostertagia are often referred to as "brown stomach worms", due to their colour, and are a major cause of parasitic gastroenteritis (PGE), in ruminants in Scotland and other temperate areas of the world. In sheep, *T. circumcincta* and less frequently *Teladorsagia trifurcata* are responsible for the disease Ostertagiosis. The disease is classified into two types, with Type I Ostertagiosis being seen mainly from mid summer until autumn when the greatest numbers of L3 are found on the pasture, (Thomas and Waller, 1975). If the L3 are ingested after October, many become arrested in development for several months, which can give rise to type II Ostertagiosis when the larvae mature in late winter or spring (Urquhart *et al*, 1991). Clinical signs usually manifest as a marked loss of weight and intermittent diarrhoea. If a lamb does not die initially from infections, it is often affected for a long time, and the animal becomes uneconomic to maintain (Urquhart *et al*, 1991). The disease syndrome is particularly severe in lambs as they are not yet immune to the parasite, and the resultant check in growth prolongs the time required to reach market weight while carcass and fleece quality are reduced leading to overall economic losses (Coop, Sykes and Angus, 1977). Studies by Thomas and Waller (1975) and Coop, Sykes and Angus (1977), showed that low levels of continual infection with *T. circumcincta* can result in reduced carcass weight of infected sheep even in the absence of clinical symptoms. Infections with Ostertagia cause a depression in appetite, and, often, diarrhoea which are linked to large numbers of larvae being found on pastures, (Coop, Sykes and Angus, 1977; McLeay, Anderson, Bingley and Titchen 1973; Thomas and Waller, 1975). Infections with *T. circumcincta* can result in a reduction of food intake of 20% in infected lambs (Coop, Sykes and Angus, 1977). The aetiology of the anorexia is unclear, but studies have indicated that parasite induced alterations in gastrointestinal motility and digesta flow may reduce voluntary food intake (reviewed Holmes, 1987). In addition, loss of plasma protein across the damaged abomasal mucosa, interference with the post absorptive metabolism of protein and the

utilization of metabolisable energy affect the hosts ability to maintain weight gain (Coop, Sykes and Angus, 1977; McLeay *et al*, 1975). Elevated, abomasal pH and serum pepsinogen levels, associated with GI nematode infections, affect the hosts ability to absorb nutrients due to the reduction in protein uptake across the damaged mucosa (McLeay *et al* 1973; Urquhart *et al*, 1991). Decreases in the deposition of fat, proteins, skeletal calcium and phosphorus and increases in body water as a percentage of body weight are also common features of ovine Ostertagiosis (Holmes, 1987) Alterations in electrolyte balance due to increases in sodium and potassium concentrations and profound diarrhoea can bring about organ failure, resulting in the death of an animal (Holmes, 1987). The milk output of experimentally infected ewes has also been shown to be reduced compared to that of control animals (Thomas and Ali, 1983).

Haemonchosis: This disease syndrome is caused by the parasitic nematode *H. contortus*. Like *T. circumcincta* it has a world wide distribution but it is the most important parasite in tropical and subtropical climates. Sheep, cattle and goats are all affected by parasites of the genus *Haemonchus*, although the parasite is most commonly found in sheep and goats. The worm inhabits the abomasum, the adult males are red in colour and the females spiral red and white. *H. contortus* is the most pathogenic of the GI nematodes commonly infecting ruminants due to the blood feeding habits of the late larval and adult stages. The most important type of infection, economically, is that of sheep in good condition and feeding on good pasture, being exposed to a massive infestation (Urquhart *et al*, 1991). Haemonchosis may also occur in sheep in poor condition failing with an infection that would not normally cause any problem. Raising the level and duration of infection and reducing the amount of nutritional protein available to the animal also affects the animals general health, which in turn affects it's voluntary food intake (Abbot, Parkins and Holmes, 1986). A sudden depression of the protein content of the diet can cause a serious fall in resistance of an animal and permit progression of a latent infestation (Urquhart *et al*, 1991). It also exacerbates the disease which causes serious hypoproteinaemia due to the blood loss caused by the worm. Acute haemorrhagic anaemia can occur due to the feeding habits of the worms and haemoglobin concentration is reduced in infected animals compared

to controls (Thomas and Ali, 1983). The normal affects of PGE are evident in Haemonchosis, but diarrhoea is not normally present.

Trichostrongylosis: Parasitic nematodes causing the disease Trichostrongylosis are round worms which infect the proximal small intestine of sheep. The adult worms are small, slender pale reddish-brown worms less than 1 cm long. (Soulsby, 1968). *T. vitrinus* is one of the principle causative nematodes of PGE within Scotland while *T. colubriformis* is more common world-wide (Urquhart *et al*, 1991). Common features of PGE i.e. diarrhoea, reduced carcass and fleece quality manifest during Trichostrongylus infections (Sykes and Coop, 1976; Sykes, Coop and Angus, 1979).

1.1.5 **Pathology**

Once a lamb has ingested *T. circumcincta* L3 the larvae develop into adults in the gastric pits causing swelling, infiltration of mononuclear cells and areas of fibrosis, with the replacement of specialised secretory cells by un-differentiated mucous cells (McLeay *et al*, 1973; Coop, Sykes and Angus, 1977). In Trichostrongylus infection villous atrophy and as a result mucosal flattening is evident in the small intestine, (Jackson, Angus and Coop, 1983) and anaemia is evident in Haemonchus infections due to their blood feeding habits (Smyth, 1988). Lymph flow increases to areas of chronic inflammation and abomasal damage caused by the parasites (Smith, Jackson, Jackson, Dawson and Burrells, 1981). A number of other features typical of pathology due to abomasal nematodes have been reported such as thinning of the fundic abomasal mucosa (McLeay *et al*, 1973), hypertrophy of the mucosa, lesions (Coop, Sykes and Angus 1977), hypoalbuminaemia and an increase in abomasal pH, (Thomas and Waller, 1975). As increasing numbers of glands are affected, significant reductions in the level of hydrochloric acid in the rumen occur, causing the pH to rise from around pH 2 to about pH 6, (Thomas and Waller 1975).

Developing larvae are usually the most pathogenic stage of GI nematode infections (except in Haemonchosis) and there is a direct correlation between a rise in serum pepsinogen levels and pathology (McLeay *et al*, 1973). As a result of these findings a number of studies have noted that serum pepsinogen levels are a more

accurate indicator of abomasal damage than faecal egg counts, (Coop, Sykes and Angus 1977; Thomas and Waller, 1975).

1.2 CONTROL METHODS

At present, control measures rely on grazing management, in conjunction with prophylactic chemotherapy for the treatment of existing infections. With increasing levels of parasite resistance to the anthelmintics (Conder and Campbell, 1995), new drugs and other methods are being sought with which to prevent and control the levels of infections. Ultimately it is to be hoped that vaccines will be developed to control PGE by stimulating the host natural immune responses, but to achieve this it is necessary to identify and characterise the parasite antigens which stimulate protective immunity.

1.2.1 Grazing Management

The principle aims of pasture management are to control or prevent contamination of pasture and prevent infection of the most susceptible animals. However labour, cost and the availability of land must be taken into account before extensive grazing regimes are considered. As mentioned earlier pregnant ewes and lambs less than 6 months old are most susceptible to GI nematodes and, in Scotland, pasture contamination is at its highest during the spring through to the autumn, (Reid and Armour, 1975a and b). To ensure that pastures are worm-free it would be necessary to move animals at least every 3 weeks to prevent reinfection, (Crofton, 1955) and to leave pastures ungrazed for a year to ensure that any residual populations have died off (Thomas, 1956). This relies on all the larvae developing at the same rate, however different species of GI nematode infect the pasture at all times and species prevalence changes during the grazing season. Nowadays, however, with intensive grazing systems farmers do not often have enough land available to allow for this. Grazing the land with other species such as cattle, or growing hay or silage on the field can be a viable option, but care must be taken as a number of GI nematodes can infect other hosts (Morley and Donald, 1980).

1.2.2 **Anthelmintics**

Development of novel anthelmintics is dependent on the identification of i) biochemical differences in the metabolism of the host and parasite, ii) the parasites defence mechanism against the host iii) its mechanisms for nutrient uptake and iv) evolutionary divergence of enzymes and functional pathways (reviewed Wang, 1984; Knox, 1994). In helminths, chemotherapy is directed toward the microtubule structure and the nervous system, success being largely due to the evolutionary differences of the host and parasite, (reviewed Wang, 1984).

Three types of anthelmintic are currently used to control GI nematodes of ruminants. The levamisole and morantel family are cholinergic agonists and cause muscle contraction due to the depolarisation of sodium ion channels at the muscle synapses. Resistance to levamisole has been detected world wide, and is believed to be due to a reduction in the number of acetylcholine receptors in resistant populations, (reviewed Prichard, 1990). Benzimidazoles cause disintegration of parasite cellular microtubule complexes, by binding to tubulin (reviewed Prichard 1990; Conder and Campbell, 1995). Resistance has been detected world wide and is believed to be due to genetic changes in β-tubulin reducing the binding efficacy of the drug (Prichard 1990; Conder and Campbell, 1995). This reduction in binding efficiency found in resistant populations appears to be due to a loss of 2 β-tubulin isoenzyme 1 alleles (Prichard, 1990; Conder and Campbell, 1995). Sequence analysis discovered that there was a different amino acid in one of the β-tubulin isoenzymes of resistant *H. contortus* compared to susceptible (Kwa, Veenstra and Roos, 1994). It is not known however, whether this is the cause of resistance or a genetically linked factor (Conder and Campbell, 1995).

Ivermectin is believed to bind to glutamate-gated chloride channels, resulting in similar effects as the levamisoles (Cully, Vassilatis, Liu, Paress, Van der Ploeg, Schaeffer and Arena, 1994). Cases of resistance to ivermectin by *H. contortus* and *T. circumcincta* have also been reported world wide, (Prichard, 1990; Shoop, 1993). however the mechanisms of resistance remain unknown.

The problem of anthelmintic resistance is growing. It is due to some parasites in a population being able to tolerate a chemical, while others can not and it's spread is

due to there being a greater proportion of resistant compared to susceptible parasites in a population, (Prichard, 1990; Shoop, 1993). Attempts to control the spread of resistance must be carefully managed, as selection for resistance and not susceptibility may result (Prichard, 1990). Simply increasing the dose rate is not always economically feasible and it can result in even greater resistance and increased levels of toxicity. Effective chemotherapy is dependent on the parasite being exposed to the drug for as long as possible (Hennessy, 1993). Moving recently treated animals to clean pasture also selects for and not against resistance, as any surviving worms are the ones contaminating the pasture (Prichard, 1990). Control of resistance therefore is dependent on the minimal necessary usage of anthemintics at the recommended dosage.

1.2.3 **Biological Control**

Increasing awareness of the potentially detrimental effects of drug residues in the environment and consumer concern about food contamination have resulted in the investigation of biological control procedures.

Soil dwelling fungi: *Nematophagous* fungi are widespread in the soil and feed on free-living nematodes as well as the free-living larval stages of parasitic nematodes (Waller and Larsen, 1993). The fungi can survive in the ruminant GI tract and, hence, can be incorporated in the feed of animals. The fungi are then dispersed in the faeces at the same time as the parasitic eggs where they thrive under the same conditions as the developing larvae (Waller and Larsen, 1993). In cattle the experimental use of feeding the fungus *Duddingtonia flagrans* had encouraging results. It was found that the presence of the fungi prevented severe clinical signs in cattle grazed on contaminated pasture and significantly reduced the level of larval infectivity on herbage (Gronvold, Wolstrup, Larsen, Henriksen and Nansen, 1993; Nansen, Larsen, Gronvold, Wolstrup, Zorn and Henriksen, 1995). This mode of control is attractive because the fungi thrive in the same environment as the larvae require for development and therefore parasite resistance to the fungi seems unlikely. At this stage however, it does appear that this method would need to be used prophylactically in conjunction with anthelmintics to

control livestock infections as the fungi do not kill adult parasitic worm present in the infected host (Waller and Larsen, 1993).

Genetic resistance: The differing abilities of individuals to cope with infectious disease is attributed to the genetic control of immune responses to infection (reviewed Wakelin, 1985; Kennedy, 1990). Resistance to helminth infections is primarily due to the major histocompatability complex (MHC) and MHC-linked genes as well as other background genes which control the responses to infection (Kennedy, 1990). Many of these differences in an individuals ability to respond to infection are inherited leading to the possibility of breeding for resistant animals. Success in this way has been achieved by breeding sheep resistant to helminth infections (Windon, 1990). Laboratory studies have also demonstrated that a number of factors such as the region of intestine occupied by the parasite, the survival of adult worms in a primary infection, the rapid expulsion of worms following a secondary infection and the number of maturing larvae all varied in different species of mice infected with *Trichinella spiralis*, (Wakelin, 1985). MHC genes have a role in the regulation of T cell function, a major mechanism in immunity to helminth infections. Resistance to infection appears to be genetically determined, as measured by comparing parasite establishment, duration of infection and elimination from individuals of a particular host species (Wakelin, 1985). In mice a single gene linked to the MHC has been found to control the rapid expulsion of secondary infections of *T. spiralis* (Wakelin, 1985). It is however, important that the effects of other background genes controlling effects of the host immune system are not ignored (Kennedy, 1990).

Dietary control: Interactions between nutrition and parasitism are important because they can affect the ability of the host to respond to infection. For example, in *Ostertagia ostertagi* infected calves being fed either a high or low protein diet, the recipients of the lower level protein diet were less resistant to infection (Mansour, Dixon, Rowan and Carter, 1992). A low protein diet raised levels of anti-parasite antibody and was correlated with higher faecal egg counts compared with calves on a high protein diet (Mansour *et al*, 1992). Similar effects were noted with *Trichuris muris* infections (Michael and Bundy, 1992). Studies by Suttle, Knox, Jackson, Coop

and Angus (1992) demonstrated that the establishment or maintenance of nematode infections can be inhibited by depriving the parasite of copper by incorporating molybdenum in the diet of young animals. Although copper depletion is detrimental to the health of the host, it does provide further information on the host/parasite relationship by indicating the relevance of copper to the survival of the parasite.

Vaccination is also a means of biological control and will be discussed in section 1.3. after describing the host immune responses to parasitism.

1.3 IMMUNITY

Protective immunity to ovine GI nematodes appears to be most marked in animals over 6 months of age and infestations of GI nematodes in healthy non-reproducing lambs in their second and subsequent grazing years are rare. The acquisition of immunity appears to be dependent on i) the size of larval challenge; ii) the age of the host; iii) the host's genetic make up and iv) the host's nutritional status.

Much research on the mechanisms of immunity towards GI nematodes have been carried out in the laboratory but the precise mechanisms which effect their elimination remain unclear. Worm expulsion is facilitated by both the humoral and cellular arms of the immune response, but the relative importance of each varies depending on the site of infection and the parasite involved. Helminth parasites differ from protozoa and microbial pathogens in that they are large non phagocytosable objects and immune response directed against helminths either results in parasite death, expulsion or concomitant immunity. The following sections provide an overview of what is presently known regarding the interactions between the host immune system and nematode parasites with emphasis on ruminant abomasal parasites.

Size of Larval Challenge: Immunity to *T. circumcincta* infestations, reviewed Smith (1988) appeared to be related to the size of the larval challenge. Under experimental conditions, if the challenge dose was reduced from a single dose of 50,000 L3 per lamb to 1,000 L3, no lymphocyte proliferation response or elevation of parasite specific IgA levels were detected in the gastric lymph and functional immunity was reduced (Smith, 1988). When lambs were exposed to a trickle infection of 2,000 L3 per day, they

became highly immune to homologous challenge infection exhibiting elevated IgA and pepsinogen concentrations although little increase in numbers of lymphocytes and IgA containing cells was detected (Smith, 1988). Similar results were observed with *H. contortus* in sheep (Jackson, Miller, Newlands, Wright and Hay, 1988).

Age: Studies have indicated that cellular and IgA responses are less well developed in young lambs (Duncan, Smith and Dargie, 1978), by the inability to achieve protection against homologous challenge when vaccinating lambs less than 6 months old although some degree of protection was achieved with lambs older than 6 months. These authors suggested that although young lambs can respond to viral infections they are unable to mount a protective (IgA) immune response to complex nematode antigens and the abomasum may be slow to develop the capacity to produce anti-parasitic IgA. Later studies by Smith, Jackson, Jackson and Williams (1985) compared immunological unresponsiveness in $4^1/_2$ and 10 month old lambs infected with *T. circumcincta.* These studies indicated that young lambs were not as efficient in mounting an antigen specific mucosal immune response as were older lambs. An increase in the numbers of basophils and IgA containing cells were detected 6 days post infection (d.p.i) in $4^1/_2$ month old lambs, although there was a large between sheep variation, arrested larvae and a decrease in length of worms harvested from the $4^1/_2$ month lambs was also recorded, however the degree of resistance was less than that detected in 10 month old lambs (Smith *et al*, 1985).

The influences of genetic make up and nutritional status have been discussed in the previous section.

1.3.1 **Humoral Responses**

Studies to determine the mechanisms of expulsion of GI nematodes in ruminants indicate that IgA is the main immunoglobulin involved. Duncan, Smith and Dargie (1978) indicated that resistance to *H. contortus* infections in lambs older than 9 months was associated with increased IgA in the abomasal mucosa and increased parasite specific IgG levels in the serum. Lambs infected and challenged with *H. contortus* demonstrated a 20-40 fold elevation of parasite-specific antibody levels compared with

uninfected controls (Gill, Husband and Watson, 1992). In this experiment IgA, IgG_1 and IgM levels were all raised by 28 d.p.i., however IgA was the most abundant antibody. The peaking of IgA levels also coincided with the appearance of eggs in the faeces which may have resulted from the proliferation of immunoglobulin containing lymphocytes in response to the adult E/S products in the sub mucosa. Gill, Husband and Watson (1992) concluded that IgA and IgG_1 may be produced locally and play a role in host defence against *H. contortus* infections. In *T. circumcincta* infections IgA levels peaked 4-6 d.p.i. and dropped off 3 days later (Smith, Jackson, Jackson and Williams, (1983). The faster response and decline of IgA antibodies in *T. circumcincta* infections is in contrast to that described for *H. contortus* (Gill, Husband and Watson, 1992), however, it may be an indication that antibody responses against *T. circumcincta* are not parasite specific which may also explain the lack of protection achieved when trying to vaccinate lambs against this parasite (Smith *et al*, 1983).

Research on Schistosome infections have demonstrated that IgE levels are elevated in immune hosts and low in those susceptible to reinfection (reviewed Hagan, 1993), however there is some controversy over this theory and other workers (eg. Pritchard, 1993a) believe that increased IgE levels observed during helminth infections are not parasite specific. It, however, seems likely that IgE is involved in the mechanisms of worm expulsion through hypersensitivity reactions e.g. mast cell degranulation and by promoting antibody dependent activation of macrophages and granulocytes. Although IgE appears to be involved in some way in worm expulsion the precise mechanisms are not fully understood and other host immune mechanisms are probably more important (Hagan, 1993).

In some parasitic infections incoming infective larvae are rapidly expelled from immunologically primed hosts and this is termed 'rapid expulsion'. Most work on this form of worm expulsion has focused on laboratory infections of *T. spiralis* and *N. brasiliensis* (reviewed Miller, 1984). The precise mechanisms are unclear although it has been suggested that the secretion of mucous (Lee and Ogilvie, 1981) and antibody binding to invading larvae may impair their penetration of the mucus layer around the epithelial tissue or the antibodies may bind to the mucus itself, entrapping the larvae as they enter (Carslile, McGregor and Appelton, 1990).

In the laboratory passive transfer of resistance has been attempted with a number of GI nematode infections such as *N. brasiliensis, T. spiralis, T. colubriformis, N. dubius* and *Trichuris muris*, and some degree of protection against parasite challenge was observed (Wakelin, 1978). Sarles and Taliaferro (1936) successfully managed to prevent *N. brasiliensis* from establishing an infection in a naive host by immunising the rat first with sera/plasma taken from a previously infected and immune animal. The same results were not attainable with *T. spiralis* and the authors concluded that passive protection results are inconsistent (Love, Ogilvie and McLaren, 1976). Attempts however to immunise lambs against *T. circumcincta* by intravenous infusion of large amounts of immune lymph, containing IgA, have however been unsuccessful (Smith, 1988). These results indicate that although parasite specific antibody responses are involved in protective immunity, other effect mechanisms such as cellular and inflammatory responses are also required (Miller, 1984). The role of these other immunological arms will be reviewed more fully in the following sections.

1.3.2 **Inflammatory Mediators**

Components of the inflammatory response are also linked to the expulsion of GI nematodes, but there has been much controversy regarding the role of inflammatory mediators in worm expulsion and three separate mechanisms have been proposed (reviewed Rothwell, 1989). Firstly, it is thought that an increase in the permeability of the intestinal mucosa may result in the infiltration of large amounts of anti-worm antibody which attaches to the worm in the GI tract, causing expulsion. Secondly, expulsion of GI nematodes is thought to be brought about by a deterioration of the parasites microenvironment as a result of inflammatory responses, stimulated by mediator release. Finally, mediators themselves may have a direct adverse effect on worms (Rothwell, 1989).

The immediate hypersensitivity reaction is initiated by the exposure of large numbers of mast cells and globule leukocytes, coated with parasite-specific IgE, to parasite antigens (Wakelin, 1985). Subsequent exposure to parasite antigens results in degranulation of the sensitised cells, releasing proteinases (sheep mast cell protease, SMCP), histamine (Smith, 1988), serotonin, prostaglandins and leukotrienes into the

interstitial fluid and lymph (Rothwell, 1989). These inflammatory mediators are then thought to increase the permeability of the mucosal epithelium allowing plasma proteins, antibodies and mast cells to pass into the mucous layer (Smith, 1988; Rothwell, 1989). Anaphylactic antibodies are most likely to be involved in the expulsion of worms from immune animals as mast cell and basophil numbers increase in the mucosa as a result of an increase in mucosal permeability (Yakoob, Holmes and Armour, 1983).

The inhibition of *T. circumcincta* larval development in immune animals may be due to hypersensitivity reactions. Smith, Jackson, Jackson, Williams and Miller (1984) demonstrated that inflammatory mediators were detected 24-48 hours post infection in immune sheep in which worms were stunted by 5 d.p.i. It has been postulated that if cells rapidly and selectively returned to the mucosa of origin, they may be involved in cell mediated damage which may arrest development of the parasite between days 2-5 d.p.i. (Smith *et al*, 1984).

In some parasitic infection the host has the ability to limit nematode infections by a 'self cure' response. During this process established adult worm populations are expelled by the hosts immune response to subsequent larval challenge (Duncan, Smith and Dargie, 1978). This process appears to be initiated by hypersensitivity reactions (Miller, 1984, Rothwell, 1989).

1.3.3 **Cellular Responses**

The presence of a lymphocyte response against GI nematodes appears to be involved, in part at least, in worm expulsion and development of immunity. In *T. circumcincta* infected lambs elevated levels of lymphocytes from 2 d.p.i., which peaked at 4-5 d.p.i. and decreased to preinfection levels at 7 d.p.i., were observed (Smith *et al*, 1983) and basophilic proliferation was also recorded (Smith *et al*, 1983). At their peak these cells were present in numbers more than 16 times the base level (Smith *et al*, 1983), indicating their putative importance in worm expulsion. The involvement of lymphocytes and basophils in the development of protective immunity to *T. circumcincta* was further supported by adoptive transfer experiments with gastric lymphocytes in genetically identical sheep (Smith, Jackson, Jackson, Graham and

Williams, Willadsen and Fehilly, 1986). In this experiment partial immunity to *T. circumcincta* was transferred from immune to susceptible sheep (Smith *et al*, 1986; reviewed Smith, 1988). The highest levels of immunity were transferred during the increased basophil response. Significantly less immunity, judged by the stunting or loss of worms, was transferred before or after the basophil response (Smith *et al*, 1986). The role of lymphocytes in worm expulsion was also investigated by Dineen and Adams (1971). In their experiments neonatal thymectomy and long term lymph drainage prevented the normal expulsion of *T. colubriformis* from the guinea pig due to the removal of thymus dependent and long term circulating lymphocytes.

Studies have also indicated that mast cells may have a role in protective immunity in GI nematodes (Smith *et al*, 1986). Mast cell deficiency however, has little or no effect on the expulsion of *N. brasiliensis* in rats (Rothwell, 1989), indicating that different effector arms are important in different infections. Concurrent infection with *N. brasiliensis* and *Strongyloides ratti* provided further support for this observation, indicating that expulsion of *N. brasiliensis* was associated with globlet hyperplasia whereas intestinal mastocytosis was involved in expulsion of *S. ratti* (Nawa Ishikawa, Tsuchiya, Horii, Abe, Khan, Bing-shi, Itoh, Ide and Uchiyama, 1994). The initial mechanism of immunity in both parasites, however, is under the control of T cells (Nawa *et al*, 1994).

Eosinophils are a major component of the cellular infiltrate in parasitised tissues and have phagocytic and secretory activities (reviewed Rothwell, 1989). Studies have demonstrated that eosinophils are found in their greatest numbers around and in closest contact with dead worms and *in vitro* studies have also demonstrated that eosinophils attach to helminth surfaces and extrude their contents into them (Rothwell, 1989). Eosinophil attachment however, has not been demonstrated with GI nematodes, and as a result it is thought that eosinophils are unlikely to have a direct anti-worm effector role in expulsion from the GI tract (Rothwell, 1989).

1.3.4 **T Cells and Cytokines**

T helper cells have a central role in eliciting specific antibody responses, in generating cell mediated immune responses and recruiting and activating macrophages and

granulocytes. They are not able to respond to antigens unless antigens are processed and presented to T cells in association with Class II MHC molecules, macrophages or B lymphocytes or other antigen presenting cells (APCs; reviewed Nussler and Thomson, 1992).

Cytokines are the intracellular communication chemicals which are produced by a variety of cells. They produce a variety of effects on the cells of the immune system and mediate events such as inflammation, oxidative burst, antibody formation and activation of T and B lymphocytes and natural killer cells (reviewed Finkelman and Urban, 1992). Interleukin (IL) -3, IL-4 and IL-5 appear to be important in the stimulation of mast cells, IgE and eosinophil responses and appear to be associated with immune response to helminth parasites (Finkelman and Urban, 1992).

The role of cytokines in *Trichuris muris* infections has been investigated extensively (Finkelman and Urban, 1992; Else, Hultner and Grencis, 1992; Else, Finkelman, Maliszeweski and Grencis, 1994). The variability in resistance to infection in different strains of mice was linked to CD_4 T helper (Th) cells mediating cytokine production (Else *et al*, 1994). In studies it was found that the cytokines produced by Th1 cells (IL-2, IL-3 and IFN γ) were increased in mice susceptible to infection, whereas a Th2 response (IL-4, IL-5, IL-9 and IL-10) was present in mice resistant to infection (Else *et al*, 1994). The same authors found that if a Th1 response was prevented by blocking the production of IFN γ, then a susceptible mouse became resistant to infection, in contrast blocking IL-4 production turned a resistant animal into one susceptible to infection (Else *et al*, 1994). In other infections however, Th1 cytokine responses have been associated with immunity (reviewed Finkelman and Urban, 1992). These authors suggested that different species of parasite may trigger different cytokine responses and that different strains of host may also vary in their sensitivity to these Th1 or Th2 cytokine responses. IgE also appears to be involved in stimulating production of either Th1 or Th2 cells (Else *et al*, 1994), increased IgE levels in mice stimulated the production of Th2 lymphocytes, important in resistance to infection and low levels of IgE appear to be involved in a Th1 response, occurring in mice susceptible to *Trichuris muris* infections.

1.3.5 **Reactive Oxygen Intermediates (ROI)**

ROI are thought to have a role in host immunity, and have been detected in a number of cells including macrophages and eosinophils (reviewed Rothwell, 1989). ROI are thought to cause worm expulsion by either i- altering the intestinal environment by damaging intestinal cells and rendering it unsuitable for parasite maintenance or ii- by damaging the worms directly, reducing levels of protective enzymes (Smith and Bryant, 1989b) and inhibiting lipoprotein synthesis, causing DNA damage and lipid peroxidation in membranes (Nussler and Thomson, 1992). Generation of ROI by activated phagocytes in *N. brasiliensis* infections has been demonstrated to occur earlier and levels were greater in heavy infections (Smith and Bryant, 1989a). The susceptibility of different life cycle stages to *in vitro* killing by granulocytes and oxidant mediated killing also appears to be dependent on the levels of superoxide dismutase (SOD) and other antioxidant enzymes (Lightowlers and Rickard, 1988). Comparison of rodent infections with *Nematospiroides dubius* and *N. brasiliensis* have implied a role for antioxidant enzymes including SOD in the longevity of infections (Smith and Bryant, 1986). Higher levels of antioxidant enzymes were detected in *N. dubius* compared to *N. brasiliensis*, indicating a possible reason for *N. dubius* infections persisting while *N. brasiliensis* infections are expelled (Smith and Bryant, 1986;1989a and b). In *N. brasiliensis* infections SOD levels have been demonstrated to decrease while infections progress although rising antioxidant levels were detected in the host intestine (Batra, Srivastava, Gupta, Katiyar and Srivastava, 1993). In *Schistosoma mansoni* infections parasite antioxidant levels increase as the parasite matures (Nare, Smith and Prichard, 1990). It therefore appears that antioxidant enzyme levels play a role in worm expulsion and lower levels of these enzymes may explain self-cure of *N. brasiliensis* infections.

1.3.6 **Immunity to *N. brasiliensis***

A lot of early work on the mechanisms of immunity to GI nematode infections was carried out using the rodent gastrointestinal nematode *N. brasiliensis*. Ogilvie (1965) stated that egg production by *N. brasiliensis* declined because host immunity develops

and not because the female worms could no longer produce eggs. Attempts to confer protection to naive rats by passive immunisation with antiserum from *N. brasiliensis* infected rats had limited success (Jones, Edwards and Ogilvie, 1970; Ogilvie and Jones, 1971; Ogilvie 1972), and it was concluded that a number of mechanisms were responsible which only elicited worm expulsion when they occurred together (Jones, Edwards and Ogilvie, 1970; Ogilvie and Jones, 1971). It was proposed that in the intestinal mucosa of rats infected with *N. brasiliensis*, an IgE response associated with mast cell discharge due to a hypersensitivity reaction, and the leakage of protective antibodies into the lumen may result in worm expulsion (reviewed Ogilvie and Jones, 1971; Murray, Robinson, Grierson and Crawford, 1979). This response was thought to be brought about by the release of vasoactive compounds by the cellular response (mast cells) opening up intracellular pathways in the mucosal epithelium which enhanced it's permeability to host protective antibodies (Murray *et al*, 1979), although the exact mechanisms are unknown.

1.2.8 **Parasite Evasion of Host Immune Responses**

Parasites appear to have developed specific evasion strategies to aid their survival in immunocompetent hosts. For example, the expression of stage-specific antigens which have variable antigenic properties (Maizels, Bundy, Selkirk, Smith and Anderson, 1993). Several potential evasion strategies have been proposed. For example, invading protozoal and helminth parasites can continually shed surface components which can result in the removal of antibody from the parasite surface or, shed parasite surface components may impair or suppress the function of lymphocytes, macrophages and granulocytes (Leid, Suquet and Tanigoshi, 1987; Lightowlers and Rickard, 1988). In addition, constant exposure of the host to parasite antigen in quantity can induce immunological unresponsiveness or tolerance (Maizels *et al*, 1993) and Schistosomes have been demonstrated to acquire host antigens such as blood group antigens, α_2-macroglobulin and host major histocompatability antigens, preventing the activation of the immune response (Leid, Suquet and Tanigoshi, 1987). Other evasion strategies proposed include the expression of repeated antigenic determinants, and the release of enzymes which can detoxify or degrade immune effector responses (Leid, Suquet and

Tanigoshi, 1987). Parasites contain and secrete a variety of enzymes which protect them from oxidative attack by free radicals and other toxic components including a variety of drugs (Leid, Suquet and Tanigoshi, 1987; Knox, 1994). Leid, Suquet and Tanigoshi (1987) suggested that there was a direct correlation between the level of these enzymes and the virulence of the parasite.

As more information becomes available, it is clear that parasite enzymes play a central role in the evasion of host anti-parasite immunity. Secreted proteinases can degrade host immunoglobulin and the degradation products themselves can influence cell-mediated immune responses (Auriault, Ouissi, Torpier, Elsen and Capron, 1981; Kumar, 1993) and they may inactivate cytotoxic mediators. Parasites contain a variety of proteinase inhibitors, some of which may be secreted (MacLennan, 1995) and may block immune effector mechanisms which are mediated by proteinases such as complement activation or impair neutrophil and lymphocyte function (Leid, Suquet and Tanigoshi, 1987).

Finally many important parasite proteins are encoded by multigene families which may provide a degree of antigenic and functional diversity within a parasite population. To date multigene families have been identified which encode for cysteine proteinases (Pratt, Armes, Hagerman, Reynolds, Boivenue and Cox, 1992; Heussler and Dobbelaere, 1994) and SOD expression (Knox and Liddell, personal communication) in *H. contortus*, observations which may indicate the presence of a number of enzymes with the same function, which may enable the parasite to preferentially 'switch' on or off enzyme expression in response to it's environment. The identification of differing parasite isoenzymes in parasite populations is also evidence of genetic variation within parasite populations (Nadler, 1990) which may effect parasite survival within the host.

1.4 VACCINATION

As mentioned in the previous section, host immunity can develop against nematode infections, indicating that immunological-based control should be a feasible method of preventing and reducing the spread of parasitic infections.

1.4.1 **Irradiated Larval Vaccines**

To date, the only commercially available vaccines against nematodes of ruminants are live attenuated vaccines, produced by irradiation of *D. viviparus* larvae (Jarrett, Jennings, Martin, McIntyre, Mulligan, Sharp and Urquhart, 1958), for use in cattle and *Dictyocaulus filaria* in sheep and goats (Sharma, Bhat and Dhar, 1988). An immune response is directed toward the larvae, priming the host for any subsequent infections in the field. Continual exposure to the parasite keeps the host immune system primed and irradiation of the larvae in the vaccine prevents them from maturing into adults. Smith, Jackson and Jackson (1982) attempted to vaccinate sheep with irradiated larvae of *H. contortus* and *T. circumcincta* with limited success. Two vaccinations with 10,000 *T. circumcincta* larvae, followed by a challenge dose of 10,000 larvae, reduced the worm burdens of lambs by approximately 50% compared with *T. circumcincta* challenged controls. Vaccination of lambs with irradiated *H. contortus*, followed by challenge doses of *H. contortus* or *T. circumcincta* also reduced worm burdens (86% and 50% respectively) compared to the respective challenge controls. (Smith, Jackson and Jackson, 1982). The authors concluded that a degree of cross protection between *T. circumcincta* and *H. contortus* could be achieved by vaccination with *H. contortus* larvae. The degree of cross protection however, was not substantial and it appears that for some unknown reason *T. circumcincta* is less antigenic than *H. contortus*. Similarily vaccination with irradiated *T. colubriformis* protected lambs older than 6 months (Gregg, Dineen, Rothwell and Kelly, 1978). Although encouraging results have been obtained, the lack of protection achieved in younger lambs and the large amount of irradiated L3 required for each vaccination, this method is impractical for commercial development (Emery and Wagland, 1991).

1.4.2 **Parasite Antigens**

Parasite antigens can be isolate from 3 different sources, somatic/tissue proteins, cuticular/surface proteins and proteins which are excreted/secreted/released by the parasite (reviewed Emery and Wagland, 1991; McKeand and Knox, 1995). Antigen vaccines appear to be the way forward in developing helminth vaccines and these are

dependent on the identification of immunogenic components. The identification of these antigens would allow for the subsequent development of recombinant vaccines, allowing for the mass production of the antigen from a small amount of original antigen (Emery and Wagland, 1991; McKeand and Knox, 1995). This type of vaccine could be produced in one of two ways: i) the use of conventional antigens which are recognised by the host immune response during a normal infection; and ii) 'covert' antigens which are not recognised during the course of a normal infection (reviewed Emery and Wagland, 1991; McKeand and Knox, 1995). Problems with conventional antigens could be encountered if mutations occurred in the genes encoding these antigens which were selected for in the parasite population (reviewed Emery and Wagland, 1991). As a result the vaccine could then become ineffective. Covert antigens on the other hand do not stimulate the host immune response during the normal course of an infection and, as a result, immunity would need to be maintained by repeated vaccination, (Emery and Wagland, 1991). Other important factors to be considered in the design of vaccines is the formulation and route of immunisation as maximisation of the immune response is vital to prevent infection.

1.3.3 **Vaccination Using Somatic Parasite Extracts**

Vaccination against *H. contortus* using covert antigens from the gut of the parasite has provided promising results. Munn, Greenwood and Coadwell (1987) and Munn (1993) described the protective effects of contortin, which is present in the lumen of the parasite's intestine from early in the L4 stage and loosely associated with the luminal surface of the plasma membrane of the parasite's intestinal epithelium. During infection contortin is bathed in the hosts blood and as a result it was thought that it would be readily exposed to circulating antibody responses which may bind directly or trigger binding of complement causing lysis of plasma membranes. In protection trials, Clun Forest and cross bred lambs were immunised with a total of 20 mg of purified contortin in PBS and an equal volume of Freunds complete adjuvant through a single dose either 28 or 12 days before challenge or by two vaccinations 28 and 12 days before infection with 25,000 *H. contortus* L3 (Munn, Greenwood and Coadwell, 1987). In the experiment there was wide variation in the egg counts from vaccinated lambs and these

were not significantly reduced compared to the infected controls. Total worm counts however of vaccinated lambs were significantly lower than that of challenge control animals and a primary antibody response directed toward the protein was detected in the sera of the all the vaccinated lambs at death. This response was found to be stronger in the lambs vaccinated 28 days before infection (Munn, Greenwood and Coadwell, 1987). Further work by Smith (1993) attempted to immunise sheep with integral membrane proteins from the dissected guts of *H. contortus*. In these experiments, substantial immunity was conferred against *H. contortus*, although cross protection against *T. circumcincta* or *N. battus* infection was not observed (Smith, 1993). These results suggest that there are antigenic differences in contortin proteins expressed on the gut surface between the species of parasite and/or that these proteins in other parasites may not be exposed to immunoglobulin in the same way as *H. contortus* (Smith, 1993).

Another covert antigen of *H. contortus*, H11 has been identified and promising results have been obtained in vaccination trials (Tavernor *et al*, 1992a; Tavernor, Smith, Langford, Graham and Munn, 1992b; Munn, 1993). H11 (110 kDa doublet) is an integral membrane protein expressed in the intestinal microvilli of parasitic stages. Vaccination with H11 resulted in a mean reduction in faecal egg output of >80% and an 83% reduction in total worm burdens, with proportionally fewer female worms in the vaccinated lambs, compared with the challenge control animals (Tavernor *et al*, 1992a and b; Munn, 1993). These immunised animals had elevated serum antibody levels which correlated with protection (Tavernor *et al*, 1992a and b). Recombinant H11 has now been expressed and is undergoing field trials (Munn, 1993). Investigations on the suitability of other integral membrane proteins for use in vaccines are, however, ongoing. *Haemonchus* galactose containing glycoprotein complex (H-gal-GP) has been identified (Smith, Smith and Murray, 1994) and, although it appeared to be less effective in reducing worm burdens compared with H11, reductions in egg counts were comparable (Smith, Smith and Murray, 1994).

Protection has been achieved in the laboratory by using crude extracts of GI nematodes as antigens, (Murray *et al*, 1979; Munn, 1993). Attempts to confer protection against *T. circumcincta* in lambs has had some success. The level of protection was however, very dependent on the type of extract (i.e. surface antigens of

somatic antigen) and the type of adjuvant used (Wedrychowicz, Bairden, Tait and Holmes, 1992). The antigens responsible for the protective effect also remain unidentified and at the moment this is not a viable vaccine option as 25μg/kg of L3 were required to prepare sufficient antigen to vaccinate each lamb, (Wedrychowicz *et al*, 1992). In addition, membrane proteins from adult *H. contortus* and *T. circumcincta* which have been selected by thiol-sepharose chromatography and enriched for cysteine proteinase activity (20 fold) have been found to confer protection in lambs. Vaccination of 3 - 4 month old lambs with extracts of these parasites gave a 53% reduction in worm burdens and >75% reduction in egg output of lambs exposed to a homologous challenge with 5,000 L3, compared to infected controls (Knox, Smith, Smith, Redmond and Murray, 1995).

1.4.4 **Vaccination Using Excreted/Secreted (E/S) Parasite Material**

The use of products released during *in vitro* parasite culture (E/S or IVR products) has been investigated for their suitability to induce a protective response. Cuticular and tegumental surfaces of some parasites are important sources of released antigens while others are released through specialised organs (reviewed Lightowlers and Rickard, 1988). Studies by Schallig, Leeuwen and Hendrikx (1994) on the E/S products of *H. contortus* demonstrated that these E/S products induced humoral and cellular responses in Texel sheep. Challenge infections produced an accelerated rise in the serum IgG_1 reaction with *H. contortus* E/S compared to uninfected controls (Schallig, Leeuwen and Hendrikx, 1994). The E/S proteinases of both L3 and adult *D. viviparus* are inhibited by purified antibody indicating the potential importance of parasite released antigens in the development of host immunity (Britton, Knox, Canto, Urquhart and Kennedy, (1992). In *D. viviparus* infections shared antigens between the L3 and adult parasite have been identified (Britton, *et al*, 1992) and there is evidence that sera taken from sheep infected with other Trichostrongylidae (i.e. *T. circumcincta*) also react, although weakly, with E/S from *H. contortus* indicating that the parasites may share antigens or antigenic epitopes (Schallig, Leeuwen and Hendrikx, 1994). These results could have useful implication for vaccine development.

As a result of these observations and similar observations in studies of many helminth infections over a number of years, a number of experiments have been carried out using parasite E/S products in an attempt to stimulate protective host immune responses. Savin, Dopheide, Frenkel, Wagland, Grant and Ward (1990) produced a recombinant version of a glycoprotein (ESgp30) found in the E/S products of adult *T. colubriformis* and used this to immunise guinea pigs against the parasite. Challenge of the 'vaccinated' guinea pigs with *T. colubriformis* resulted in a reduced worm burden compared to challenge controls. Other E/S components of adult *T. colubriformis* and L3 *T. circumcincta* have also been used to confer some degree of protection (30-60%) in sheep against homologous parasite challenge (reviewed Emery and Wagland, 1991).

1.3.5 **Recombinant vaccines**

Up until now the problem of limited availability of suitable parasite derived antigens has prevented further investigation of their suitability as vaccine candidates. However, with the development of recombinant DNA techniques for the expression of parasitic genes in prokaryotic and eukaryotic cells methods for the production of larger amounts of antigen of interest are now available. Several recombinant E/S antigens from *T. colubriformis* have been shown to confer a 30-60% protection against homologous challenge (Emery, McClure and Wagland, 1993). Recombinant DNA techniques have also been used to investigate the protective effects of antigens derived from *Schistosoma japonicum, Taenia spp., H. contortus* and the blood feeding tick *Boophilis microplus* and in all cases a significant degree of protection against homologous challenge was obtained (reviewed McKeand and Knox, 1995). All of these successful trials indicate that vaccination against some of the parasites, which are a major concern for the agricultural industry, is not too far into the future.

1.5 **PARASITE ENZYMES**

Parasite enzymes are thought to play a role in host invasion, feeding and adaptation to the host environment (Thorson, 1956; Matthews, 1984; Knox and Kennedy, 1988; McKerrow and Doenhoff, 1988; Britton *et al*, 1992). Acetylcholinesterase (AChE) has

been shown to be present in a number of parasites where it is believed to function as a biochemical holdfast, inhibiting local gut peristalsis and mediating immune responses (reviewed Pritchard, 1993b). Inhibitory antibody responses to *D. viviparus* AChE were demonstrated in calves which had either been exposed to a natural infection or had been vaccinated with DICTOL, indicating that AChE may induce protective immune responses (McKeand, Knox Duncan and Kennedy, 1994a). Parasite proteinases have been shown to degrade complement and immunoglobulin (Chapman and Mitchell, 1982; Leid, Suquet and Tangoshi, 1987; McKerrow and Doenhoff, 1988; Smith, Dowd, McGonigle, Keegan, Brennan, Trudgett and Dalton, 1993), and other enzymes such as the reactive oxygen metabolites superoxide dismutase and catalase have been identified in nematodes (Knox and Jones, 1992) where they possibly protect the parasite from the harmful effects of the host immune system.

1.5.1 **Proteinases**

Proteinases are enzymes that hydrolyse peptide bonds between the amino acids either within proteins (endopeptidases) or at the terminal (carboxyl or amino-) end (exopeptidases). Proteinases can be divided into four main classes namely serine, cysteinyl (or thiol), metallo and aspartyl. The class of proteinase depends on which functional amino acid residues are present within the active site. Proteinase release has been documented in several parasite species, where they have been ascribed a number of important roles in survival. Parasite proteinases have therefore been targeted as potential tools for serodiagnosis, anthelmintic therapy and vaccine development as well as for determining how the parasite functions within the host.

Many parasitic helminths have been demonstrated to release proteinases during *in vitro* maintenance (e.g. Howell, 1966; Matthews, 1977; Knox and Kennedy, 1988; Knox and Jones, 1990; Healer, Ashall and Maizels, 1991; Britton *et al.*, 1992). Proteinase release is often stage-specific (Knox and Kennedy, 1988; Robertson, Bianco, McKerrow and Maizels, 1989; Healer, Ashall and Maizels, 1991) and neutralising antibody responses to proteinases have been detected in animals which are immune to parasite challenge (Knox and Kennedy, 1988; Britton *et al.*, 1992). This is indicative of *in vivo* proteinase release. Helminth proteinases have been ascribed a variety of

functions including parasite nutrition (Thorson, 1956), penetration of host tissue barriers (Matthews, 1984; McKerrow and Doenhoff, 1988), evasion of host immune responses (Chapman and Mitchell, 1982; Leid, Suquet and Tanigoshi, 1987; McKerrow and Doenhoff, 1988; Smith *et al*, 1993) and as contributory factors in the development of the immunopathology of helminth infections (Brophy and Pritchard, 1992). Proteinases are also responsible for triggering or contributing to morphological changes in the parasite such as larval moulting (Lustigman, 1993) and facilitating parasite metabolism within the host (Knox and Kennedy, 1988). Stage-specific proteinase release has been studied in order to understand gene regulation during morphological transformation and provide information on the evolutionary origin of parasites (Davis, Nanduri and Watson, 1987). Differences in the levels of *in vitro* proteinase release by adult male and female *N. brasiliensis* and *N. battus* have been reported (Lumley and Lee, 1980). These sex specific differences may indicate the requirement for specific proteinases for egg production in female worms (Lumley and Lee, 1980).

Much research has focused on the potential importance of parasite proteinases as targets for chemotherapeutic intervention and vaccination (Wijfeels, Salvatore, Dosen, Waddington, Wilson, Thompson, Campbell, Sexton, Wicker, Bowen, Friedel and Spithill, 1994). A cathepsin L-like proteinase in the food vacuole of the malarial parasite *Plasmodium falciparum* has been identified which appears to catalyse the initial cleavage haemoglobin to facilitate nutrition for the parasite within the erythrocyte (reviewed McKerrow, 1989). Inhibition of this enzyme produces a dramatic abnormality in the food vacuole and parasite death then occurs if irreversible inhibitors are used (McKerrow, 1989). Proteinases can stimulate or suppress the host immune responses (reviewed Knox, 1994), for example proteinases from schistosomulae have been implicated in the modulation of the host immune response (McKerrow, 1989).

1.4.2 **Esterases and Acetylcholinesterases (AChE)**

Esterases hydrolyse uncharged substrates such as lipids or acetylcholine and are located throughout the tissues (Long, 1961). Esterases have been detected in *T. colubriformis* (Rothwell, Anderson, Bremner, Dash, Le Jambre, Merritt and Ng, 1976), *N. brasiliensis* (Edwards, Burt and Ogilvie, 1971) and *D. viviparus* (McKeand, 1992) and

elevated levels of non-specific esterase activity, esterases with a relatively broad substrate specificity, have been detected in *H. contortus*, *T. circumcincta* and *T. colubriformis* strains resistant to benzimidazoles (BZ) compared to parasites which were susceptible to the drug (Sutherland and Lee, 1992). Why esterase levels are increased is unknown, but it has been suggested that the enzyme is involved in the detoxification of BZ (Sutherland and Lee, 1992) or alternatively, BZ binding to tubulin may interfere with microtubule formation which may impair enzyme secretion (Lacey, 1988).

Cholinesterases hydrolyse esters of choline and carboxylic acids and are separated into two groups, true cholinesterases and pseudo-cholinesterases. AChE is a true type cholinesterase and hydrolyses acetylcholine (ACh) as a substrate (Long, 1961). AChE in parasites has a 'house keeping' role in modulating ACh levels in neuronal and neuromuscular junctions. Secreted AChE may have a number of possible functions, including modulating mucus secretion, inhibiting some anti-parasite immune responses and functioning as a 'biochemical holdfast' by inhibiting local gut peristalsis (reviewed Pritchard, 1993b). AChE has been detected in a number of nematodes and antibodies to AChE have been detected following infections in a number of host/nematode systems (Jones and Ogilvie, 1972; Rothwell *et al*, 1976; McKeand *et al*, 1994a). As a result AChE has been proposed as a potential immunogen, for use in serodiagnosis or vaccination (Jones and Knox, 1990; Knox, 1994). AChE release during *in vitro* maintenance has also been recorded for a number of parasites including the GI nematodes *N. brasiliensis*, *T. circumcincta*, *H. contortus*, and *T. colubriformis* (Ogilvie, Rothwell, Bremner, Schnitzer, Nolan and Keith, 1973) and, recently, evidence of AChE release by *D. viviparus* (McKeand *et al*, 1994) and *T. vitrinus* (Jones and Knox 1990; MacLennan, 1995) has been provided. Immunolocalisation studies have demonstrated that AChE in *N. brasiliensis* is concentrated in the subventral glands insinuating secretion (Nakazawa, Yamada, Uchikawa and Arizono, 1995). Moreover, *in vitro* release is implied by the demonstration of anti-parasite AChE antibodies in immune animals (Rothwell *et al*, 1976; McKeand *et al*, 1994a). McKeand, Knox, Duncan and Kennedy (1994b) noted that the isoenzymes of AChE in *D. viviparus* were antigenically different and suggested that this may allow parasite AChE to remain active in the face of antigenically specific immune responses which could possibly effect

parasite survival. Pooled E/S products were used in this study, however, so the presence of a number of antigenic isoenzymes may have been due to population variation in the AChEs expressed (McKeand *et al*, 1994b).

Resistance to levamisole-morantel anthelmintics has also been associated with raised levels of AChE in the parasite (Sutherland and Lee, 1992) further indicating the potential importance of AChE for parasite survival.

1.5.3 **Free Radical Detoxifying Enzymes**

Reactive oxygen intermediates (ROI), the most toxic being hydrogen peroxide (H_2O_2), hydroxyl (OH) and superoxide anions (O_2^-) are produced by the host immune cells undergoing oxidative burst and during normal metabolism in both the host and parasite (reviewed Callahan, Crouch and James, 1988). Potential extracellular sources of ROI in the GI mucosa are phagocytic cells such as eosinophils and activated neutrophils (Callahan, Crouch and James, 1988; Leid, Suquet and Tanigoshi, 1989; James, 1994). SOD and other detoxifying enzymes such as catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase are involved in neutralising the harmful effects of ROI by catalysing their detoxification to the harmless by-products water and oxygen.

Three major types of SOD exist in eukaryotes, manganese, iron or copper/zinc (Cu/Zn), determined by their metal ion dependency for activity (reviewed James, 1994). Several Cu/Zn SODs have been identified which possess N-terminal signal peptides or trans-membrane domains, indicating that these enzymes could be located in membranes or be secreted (James, 1994). SOD has been identified in a number of helminth parasites (Rhoads, 1983; Leid and Suquet, 1986; Sanchez-Moreno, Leon, Garcia-Ruiz and Monteoliva, 1987; Callahan, Crouch and James, 1990, 1991; Knox and Jones, 1992; Britton, Knox and Kennedy, 1994) and extracellular Cu/Zn SODs have been reported in helminths and been detected in *in vitro* culture fluids (Knox and Jones, 1992; Rhoads, 1983; Britton *et al*, 1994)). The secretion of SODs by filarial parasites has been suggested as an evolutionary adaptation to parasitism. However the role of SOD as a defence mechanism against phagocytic cells is still speculative (reviewed James, 1994).

Knox and Jones (1992) found that the larval stages of GI parasites tended to contain higher SOD levels than adult stages. These authors suggested that elevated SOD levels in the larval stages might be required to neutralise the effects of the host imflammatory response within the GI mucosa at the first stages of parasite invasion. Alternatively SOD may be required to detoxify free radicals generated by the increased oxidative metabolism during the rapid growth and development of the infecting parasite (Knox and Jones, 1992). In infections with *N. brasiliensis,* the expulsion of the parasite from the intestine of the rat has been attributed to a gradual decline in the antioxidant defences of the parasite and the progressive generation of ROI within the rat intestine as infection progresses (Batra *et al*, 1993). The same authors showed that H_2O_2 had the potential to damage the parasite and, as the parasite developed, their ability to neutralise ROI declined.

1.4.4 **Phosphatases**

In eukaryotic organisms, phosphatases are involved in nutrient transport across membrane barriers, digestion of nucleotides and in inhibition of the oxidative burst preventing the release of ROI (Long, 1961). Changes in the total level, as well as iso-enzyme profiles, of acid phosphatase were detected in homogenates of *N. brasiliensis* harvested from immune compared with those harvested from naive rats have been described (Edwards, Burt and Ogilvie, 1971). These authors attributed the changes in acid phosphatase production to degradation of the gut cells in the worm, in which acid phosphatase is located, due to immune mediated damage. In murine *Schistosoma mansoni* infections treatment with Praziquantel has been demonstrated to expose parasite acid phosphatase due to tegumental damage and antibody mediated inhibition of acid phosphatase increased the mortality of female *S. mansoni* in the presence of Praziquantel (Fallon, Smith, Nicholls, Modha and Doenhoff, 1994). These experiments are indicative of acid phosphatases playing an essential role in parasite maintenance and survival.

1.6. MODULATION OF PARASITE ENZYME EXPRESSION

There is evidence that nematode parasites may significantly modulate enzyme expression in response to different intra-host environments. Studies by Edwards, Burt and Ogilvie (1971) demonstrated that a proportion of a *N. brasiliensis* infective dose is able to survive immune expulsion from primed rats. Modifications in AChE expression led to these parasites being termed 'adapted' (Edwards, Burt and Ogilvie, 1971) and there is additional evidence that these 'adapted' parasites exhibited different SOD iso-enzyme profiles from the 'normal' parasite (Knox and Jones, 1992). These enzyme modulations are described in more detail in the introduction to Chapter 6.

Both AChE and SOD can facilitate evasion of host immune responses (Knox and Jones, 1992) and the their modified expression in 'adapted' parasites may contribute to their survival. The evolution of nematode parasites would be expected to be dependent on climatic influences on the free-living stages and host influences on the parasitic stages. Vaccination experiments carried out at Moredun have demonstrated that immunisation of lambs with parasite gut derived extracts protects lambs from subsequent homologous parasite challenge (Smith, Smith and Murray, 1994). Following this vaccination, analysis of adult *H. contortus* surviving in immunised lambs demonstrated elevated expression of cysteine proteinases compared to parasites harvested from challenge control lambs (Knox, Redmond and Jones, 1993). The authors suggested that elevated proteinase expression in response to a hostile intra-host environment could enhance parasite survival. In addition, the same authors noted they could no isolate a specific cysteine proteinase cDNA sequence (AC1) which had previously isolated and characterised from a USA strain of the parasite (Cox *et al*, 1990) suggesting that geographical strain divergence may have occurred. Cysteine proteinase expression in *Haemonchus* appears to be regulated by multigene families (Pratt, Armes, Hageman, Reynolds, Boisvenue and Cox, 1992; Knox *et al*, 1995) enabling the parasite to synthesise large quantities of proteinase rapidly or, to produce proteinases of differing substrate specificity or antigenicity although they may share the same function in the parasite which is essential to it's survival.

The results from these studies have indicated that there is some evidence for modulation of enzyme expression in parasites in response to a different or changing

host environment. These modulations may be under the control of genetic differences enhancing parasite survival or phenotypic differences due to differential expression of particular genes.

1.5 AIMS OF WORK DESCRIBED IN THIS THESIS

i) To characterise the *in vitro* released proteinases of the parasitic stages of *T. circumcincta* and to define whether or not these enzymes are recognised by antibody from immune lambs.

ii) Seek evidence for modulated enzyme expression in parasite populations with specific reference to a) geographically different strains of *H. contortus*; b) the effect individual hosts have on parasite enzyme expression, using the rodent intestinal nematode *N. brasiliensis*, and c) *H. contortus* and *N. brasiliensis* which have been exposed to, and survived, a protective host immune response.

iii) To obtain evidence for modulated enzyme expression, by developing techniques to monitor levels of specific enzyme gene expression.

Chapter Two

Materials and Methods

2.1 LABORATORY REAGENTS

2.1.1 Chemical Suppliers

Chemicals for general laboratory use were purchased from:

British Drug House Limited (BDH), Poole, Dorset, UK.

Difco, East Molesey, Surrey, UK.

Fisons Scientific Equipment, Leicestershire, UK.

Pharmacia Fine Chemicals, Uppsala, Sweden.

Sigma Chemical Company Limited, Poole, Dorset, UK.

Ultra pure agarose was obtained from Northumbria Biochemicals Limited, Nelson Industrial Estate, Cramlington, Northumberland, UK.

2.1.2 Enzymes

Enzymes and kits were supplied by:

Boehringer Mannheim UK (Diagnostics and Biochemicals) Limited, East Sussex, UK.

Sigma Chemical Company Limited, Poole, Dorset, UK.

2.1.3 Other Materials

Filter paper used was Whatman number 3 (3 MM), supplied by Whatman Limited, Maidstone, Kent, UK.

Hybond™-N nylon transfer membrane (0.45 μm pore) from Amersham, Little Chalfont, Buckinghamshire.

Schleicher and Schuell nitro-cellulose (0.45 μm) from Anderson and Company Limited, Surrey.

RX medical X-ray film was obtained from Fuji, Japan.

MSE (Loughborough, Leicestershire, UK) micro centaur microfuge and MSE Mistral 2000 centrifuges were used throughout.

2.2 SOLUTIONS

Unless otherwise stated, standard solutions were made with deionised distilled water and sterilised by autoclaving at 15 psi/15 min All glassware was washed and sterilised before use.

2.2.1 General Solutions

Phosphate buffered saline (PBS): A solution of 0.1 M phosphate buffer, pH 7.4 containing 0.9% (w/v) sodium chloride (NaCl).

***Tris buffered saline (TBS*):** 50 mM Tris-HCl buffer, pH 8.0 containing 150 mM NaCl.

TBS with Tween (TBST): TBST was prepared by adding 0.5 ml of Tween-20 to 1 L of TBS.

Sodium acetate buffers (pH 3.0 to pH 6.0): A 0.1 M aqueous solution of sodium acetate was prepared and adjusted to the desired pH with glacial acetic acid.

Phosphate buffers (pH 5.5 to pH 8.0): 0.1 M aqueous solutions of potassium hydrogen orthophosphate (K_2HPO_4) and potassium dihydrogen orthophosphate (KH_2PO_4) were prepared and then mixed in the appropriate quantities to give the desired pH.

Tris buffers (pH 7.5 to pH 9.5): After preparing a 0.1 M aqueous solution of Tris base the pH was adjusted with hydrochloric acid.

Sodium carbonate-bicarbonate buffers (pH 9.5 to pH 11.0): Sodium carbonate-bicarbonate buffer was prepared by mixing varying amounts of 0.1 M sodium carbonate (Na_2CO_3) with sodium bicarbonate ($NaHCO_3$) to give the required pH.

2.2.2 **Proteinase Inhibitors**

1,10 Phenanthroline: 200 mM stock solution in methanol.

Phenylmethylsulphonyl fluoride (PmsF): 50 mM stock solution in methanol.

Pepstatin A: 40 μM stock solution in methanol.

trans-*epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E 64):* An aqueous 800 μM stock solution.

Ethylenediaminotetra-acetic disodium salt (EDTA): A 0.5 M aqueous stock solution.

2.2.3 **Electrophoresis Solutions**

Acrylamide: A commercially available solution 30% w/v acrylamide/0.8% w/v bis acrylamide (Easygel, Scotlab) was used.

Ammonium persulphate: A 10% (w/v) aqueous solution was prepared fresh daily.

TEMED: Commercially available N,N,N',N'-tetramethylethylenediamine was used (Bio Rad cat no: 161-0801).

Separating gel buffer: 27.23 gm Tris base was dissolved in approximately 80 ml distilled water and the pH was adjusted to pH 8.8 with hydrochloric acid and made to a final volume of 150 ml with water.

Stacking gel buffer: 6 gm Tris base was dissolved in approximately 60 ml distilled water. The pH was adjusted to pH 6.8 with hydrochloric acid and made up to a final volume of 100 ml with water.

10% SDS (w/v): 2 gm of sodium dodecyl-sulphate (SDS) was dissolved in 20 ml of distilled water.

SDS-PAGE loading buffer: Loading buffer was prepared by mixing 4 ml distilled water; 1 ml 0.5 M Tris-HCl, pH 6.8; 0.8 ml glycerol; 1.6 ml 10% (w/v) SDS; 0.4 ml 2-β mercaptoethanol and 0.2 ml 0.05% (w/v) bromophenol blue.

Non-reducing SDS-PAGE loading buffer: As above but omitting the 2-β mercaptoethanol. Allows dissociation of the di-sulphide bonds without denaturing the proteins.

Native loading buffer: As for the reducing loading buffer, but omitting the 2-β mercaptoethanol and 10% SDS. This buffer allows the proteins to run in their native state.

5X SDS-PAGE electrode running buffer: A 5x stock solution of running buffer was made by dissolving 9 gm Tris base, 43.2 gm glycine and 3 gm SDS in a final volume of 600 ml distilled water. For electrophoresis, 60 ml of 5X stock was diluted in 240 ml of distilled water.

Coomassie blue stain: 1 gm Coomassie brilliant blue was dissolved in 300 ml of methanol and 100 ml acetic acid. The final volume was made up to 1 litre with distilled water.

Coomassie blue destain: 300 ml methanol, 100 ml acetic acid and 600 ml of distilled water were mixed together.

Diaminobenzidine tetra-hydrochloride (DAB): DAB (50 mg) was dissolved in 5 ml of methanol, made up to 50 ml with TBS and 100µl of 30% hydrogen peroxide added.

2.2.4 Solutions for DNA and RNA Manipulations

5X Loening 'E': A stock of 5X Loening 'E' buffer was made by dissolving 21.7 gm Tris, 23.4 gm sodium dihydrogen phosphate and 1.88 gm of EDTA in distilled water and making the final volume up to 1L.

Tris-acetate (TAE) buffer: 50 X stock was prepared by dissolving 242 gm Tris base in 500 ml distilled water. 57.1 ml glacial acetic acid and 100 ml of 0.05 M Na_2EDTA (pH 8.0) was added and the solution made up to 1 L with distilled water. 1X TAE was the working concentration, containing 40 mM Tris-acetate and 2 mM Na_2EDTA.

Bromophenol blue: An aqueous solution containing 0.25% (w/v) bromophenol blue, 40% (w/v) sucrose and 1 mM Na_2EDTA was prepared

Denaturation buffer: 100 ml of a 3 M NaCl stock solution and 100 ml of 1M NaOH were mixed together.

Neutralisation buffer: 100 ml of 3 M NaCl, 100 ml of 1M Tris-HCl (pH 7.2) and 0.6 ml of 0.5 M EDTA were mixed together.

20 X NaCl-citrate (SSC): A solution containing 3 M NaCl and 300 mM tri-sodium citrate was prepared in distilled water and adjusted to pH 7 with 5 M NaOH.

6% acrylamide/urea: 210 gm of urea and 72.5 ml of commercially available 40% acrylamide/bis (Easy Gel, Scotlab) were made up to 450 ml with distilled water. The solution was then deionised with 0.5 gm each of 'amberlite' resin IRC-50 (H) and IR-45 (OH), filtered and 50 ml 10X TBE added before making the final volume up to 500 ml with distilled water.

Sequencing gels: To 80 ml of 6% acrylamide/urea, 800 µl of 10% (w/v) APS and 40 µl of TEMED were added.

Tris-borate (TBE) buffer: A 10X stock solution was made by dissolving 121 gm Tris base, 7.4 gm EDTA and 53.4 gm boric acid in distilled water to a final volume of 1L, pH 8.3.

Tris-EDTA (TE) buffer: 10 mM Tris-HCl buffer, pH 8.0 was prepared, containing 1 mM Na_2EDTA (pH 8.0).

Sodium acetate: An aqueous solution of 3 M sodium acetate was adjusted to pH 5.2 with glacial acetic acid.

Ammonium acetate: An aqueous solution of 4 M ammonium acetate was adjusted to pH 6.4 with hydrochloric acid and sterilised by filtration (0.45 μm discs).

X-gal: A stock solution of X-gal was prepared by dissolving 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside in 1ml of dimethylformamide. The solution was stored at -20°C.

LB-broth: Bactotryptone (10 gm), bacto yeast extract (5 gm) and NaCl (10 gm) were dissolved in 800 ml of deionised distilled water. The solution was adjusted to pH 7 with hydrochloric acid and the final volume made up to 1 L with water. The solution was then sterilised at 15 psi/20 min

LB-agar plates: 15 gm/L of Bacto-agar were added to LB-broth, autoclaved and the solution was then cooled to 43°C and ampicillin (1:1000 dilution of 50 mg/ml stock) was added. The agar was then poured into 90 mm diameter sterile petri dishes (Bibby Sterilin Ltd., Staffordshire, UK) to give approximately a 0.5 cm covering. The plates were left to cool and set on a level surface prior to storage at 4°C. Before use, the plates were dried briefly at 37°C.

Ampicillin: A stock solution was prepared by dissolving 50 mg of ampicillin in 1 ml of deionised distilled water and then filter sterilised (0.45 μm filter) before storage at -20°C.

2.3 PARASITES

2.3.1 *T. circumcincta*

T. circumcincta worms were kindly provided by Dr W. D. Smith and his staff (Moredun Research Institute, Edinburgh, UK) as required.

Adults: Sheep abomasa were collected from the abattoir, slit open, and the contents tipped into warm PBS (37°C). The abomasal surface was gently washed in warm PBS and the washings added to the contents. These were then left for 30 min in a warm room (37°C), after which 1/4 of the fluid poured off. The contents and washings were then re-suspended and divided into 600 ml volumes. 300 ml of agar solution (5.4 gm agar in 300 ml PBS, 60°C) was then slowly added to the mixture with constant stirring. The agar and abomasum contents were then poured onto a wire mesh casing and left to set. After setting, the wire mesh casing was removed from the lid and placed in a funnel containing PBS. The worms were left to migrate out overnight at 37°C. The parasites were then collected from the bottom of the funnel and washed extensively in sterile PBS containing penicillin (500 IU/ml) and streptomycin (5 mg/ml). The parasites were then cultured overnight as described (Section 2.4.1) or blotted dry and stored at -70°C prior to homogenisation, (Section 2.5.2).

L4: 3 month old lambs were given an oral infection of 40,000 infective L3 from a pure isolate of *T. circumcincta* maintained at Moredun Research Institute. Parasites were harvested from the abomasum 7 days post infection (d.p.i.), blotted dry and stored at -70°C prior to homogenisation (Section 2.5.2).

L3: Infective L3 were obtained by culturing host faeces of hosts harbouring a pure experimental infection at 25°C and were a gift from Department of Veterinary Parasitology, Glasgow of University Veterinary School.

2.3.2 *N. brasiliensis*

L3: Faeces were collected from infected Wistar rats (3 months old) 6 days post primary infection when egg output was maximal. The rats were housed in cages with wire mesh floors which enabled the faeces to fall through onto wet paper towels below. A sample of faeces was retained for egg counts and the rest softened into a thick paste with water, and mixed well with activated charcoal (BDH, 0.85-1.7 mm granules). Faeces were then put onto wet filter paper, (Whatman No. 1, 70 mm; 1001 070) in a petri dish and incubated at 27°C.

L3 stage parasites were present in the petri dish cultures from day 4 onwards. The infective L3 were harvested from the faeces/charcoal mix by flooding the petri dishes gently with warm water (37°C) and then floating the petri dishes in a basin of warm water. To collect the parasites a plastic funnel with a stopper tap at the bottom was filled with water at 37°C and wire gauze placed in the funnel, this was lined with 'non fluffy' paper. The surface water from the petri dishes was then gently decanted on top of the wire gauze and paper and left for 1 hour to allow the larvae to migrate to the bottom of the funnel, after which they were collected, washed in 5 volumes of sterile PBS, counted and concentrated/diluted to the correct dosage for subsequent rat infections.

Adults: Adult worms were collected from rats 7 d.p.i.. The rats were culled in a mixture of halothane (4 U/L) and CO_2 (0.5 U) prior to cervical dislocation. The abdominal cavity was opened up, small intestine located and removed. The contents of the small intestine were gently squeezed out and the small intestine slit longitudinally before cutting it into small lengths. A simple Baerman apparatus, using gauze swabs (Millipledge, Nottingham) and cocktail sticks, containing the gut contents was then suspended in warm PBS and placed in hot room at 37°C for 4 hours. Adult parasites migrated into the PBS and collected at the bottom of the beaker. The parasites were harvested and washed in 5 volumes of sterile PBS containing penicillin/streptomycin (500 IU/ml and 5 mg /ml respectively), prior to determining the worm numbers. The worms were stored at -70°C prior to use.

'Adapted' worms: 'Adapted' worms were obtained by inoculating immune rats with 10,000 L3 subcutaneously 10 weeks post primary infection. Worms were harvested, as described above, 7 days post secondary infection.

2.4 PARASITE *IN VITRO* RELEASED (IVR) PRODUCTS

2.4.1 *T. circumcincta*

L3 (5000/ml; sheathed and exsheathed), L4 (135/ml) and adult (60/ml) parasites were maintained, with constant gentle agitation, for 16 h at 37°C in sterile RPMI 1640 medium supplemented with glucose (1% w/v), gentamycin (1 μg/ml), penicillin/streptomycin (500 IU/ml and 5 mg/ml respectively) and glutamine (10 μM). Parasite viability at the end of the culture period was confirmed on the basis of structural integrity and motility. The culture supernatant was concentrated 20 fold using Centricon-10 filters (Amicon Ltd., Stonehouse, UK) and centrifugation at 1,500 g (4°C) for 1 h using a MSE Mistral 6L centrifuge. Concentrated culture fluid was stored at -70°C prior to analysis. Culture fluids containing *in vitro*-released products are referred to as IVR. To check for bacterial contamination aliquots of culture fluid were streaked onto LB-agar plates in the absence of antibiotics and incubated overnight at 37°C.

2.4.2 *N. brasiliensis*

Adult parasites (500/ml) were maintained for 16 h at 37°C in sterile RPMI 1640, with constant agitation, as described in the above Section. Parasite viability was confirmed on basis of motility. IVR material was stored unconcentrated at -70°C for later use.

2.5 SOMATIC EXTRACTS

2.5.1 *H. contortus*

UK and Australian extracts: UK parasites were obtained from a strain maintained at Moredun Research Institute. The Australian parasites were a gift from Pitman-Moore Ltd, North Melbourne, Victoria, Australia. In both cases the parasites were treated in the same way.

Adult parasites (3 gm) were homogenised in 20 ml of ice-cold PBS using an electric pulse homogeniser (ultra-Turrack, T 25, Janke and Kunkel, IKA Laborteckik, Germany). The homogenate was centrifuged at 10,000 g (Beckman J2-21) for 20 min at 4°C. The supernatant (S1) was removed, and stored in aliquots at - 70°C. The pellet was then re-suspended in 0.1 % (v/v) Tween 20, re-homogenised (24,000 rev/min) and centrifuged for 20 min at 4°C and 10,000 g. Aliquots of the supernatant (S2) were then stored at - 70°C.

Vaccinated and control sheep: Adult parasites harvested from either unvaccinated control lambs from lambs vaccinated with H-gal GP (see Chapter 5) prior to challenge infection were homogenised on ice in 1% Triton X-100 in sterile PBS (0.15 gm/ml) over a 30 min period using a glass/glass hand homogeniser (Jencon Scientific, UK). The supernatant was microfuged at 9,000 g for 3 min and aliquots stored at - 70°C.

2.5.2 *T. circumcincta*

Adult *T. circumcincta* (0.5 gm) were homogenised in 2.5 ml sterile PBS using a glass hand homogeniser, (Jencon's Scientific, UK). The contents were homogenised on ice with 10 full turns of the homogeniser every 15 min over a 60 min period. The extracts were microfuged for 2 min at 9,000 g prior to aliquots being stored at -70°C until required.

2.5.3 *N. brasiliensis*

N. brasiliensis adults (1,000/ml) were homogenised in 1 ml of sterile PBS using a hand held micro homogeniser, (Biomedix, 2 West Avenue, Panner, Middlesex). The parasites were thawed on ice and broken up with 10 full turns of the homogeniser. The resultant extracts were microfuged at 9,000 g for 2 min and aliquots of the supernatant stored at -70°C until required.

2.6 PROTEIN TECHNIQUES

2.6.1 Total Protein Concentration

The bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) was used for determining the protein content of parasite material. Bovine serum albumin (BSA) was used as a standard. Stock solutions were diluted in PBS to create a standard curve in the estimated range of the samples, usually 0.1 to 2.5 mg/ml. Working reagent was made up according to manufactures instructions, 1 part reagent A (Copper solution) to 50 parts reagent B. 10 µl of standard/sample/ blank was added to 200 µl of working reagent, vortexed and incubated at 37°C for 30 min. The samples were cooled to room temperature before determining their absorbance at 570 nm using a Monarch microcentrifugal analyser (Instrumentation Laboratory, Warrington, UK). The samples were referenced to a water blank control and their protein concentration calculated by reference to the absorbance values obtained for the BSA standards included with each analysis.

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

SDS-PAGE, using a method based on that of Laemmli (1970), was commonly used to visualise parasite proteins. Polyacrylamide gels provide a matrix through which proteins migrate according to their molecular size and charge. Larger protein molecules are not able to migrate as fast as the smaller proteins and are found nearer the top of the gel. Reducing the percentage of acrylamide makes a less dense matrix

allowing larger proteins to migrate further through the gel and vice versa. The addition of the detergent, SDS, to the gels disrupts nearly all non-covalent interactions in native proteins giving a net negative charge, while β-mercaptoethanol reduces disulphide bonds within proteins. Therefore proteins migrate more or less according to their molecular size.

The separating gel, usually 7.5%, was prepared by mixing 2.5 ml separating buffer (1.5 M Tris-HCl, pH 8.8), 2.5 ml 30% acrylamide, 100 µl 10% (w/v) SDS and 4.85 ml distilled water. To initiate the polymerisation of the gel, 80 µl of a 10% (w/v) solution of ammonium persulphate (APS) and 20 µl TEMED were added just before casting the gel. After polymerisation, a stacking gel was also cast on top of the separating gel and comprised 2.5 ml stacking gel buffer (0.5 M Tris-HCl, pH 6.8), 1.3 ml 30% acrylamide (4%, final concentration), 6.1 ml distilled water and APS and TEMED as before. Gels were run using the Bio-Rad "Mini-Protean" gel system (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK) in 1X SDS-PAGE buffer at 200V for 45 min. Prior to electrophoresis, an equal volume of SDS-PAGE loading buffer was mixed with an equal volume of sample and the mixture boiled for 5 min. Molecular weight markers (Sigma; 205, 116, 97, 66, 45 and 29 kDa) were also fractionated to allow estimation of the protein sizes.

Coomassie stain: After electrophoresis, the gels were stained in Coomassie blue with constant agitation at room temperature for 20-30 min, rinsed briefly in distilled water and destained in Coomassie blue destain. Proteins appeared as blue bands on a clear background.

Silver stain: For low abundance proteins which were undetectable by Coomassie staining, the more sensitive Bio-Rad Silver Stain Plus kit, based on the method of Gottlieb and Chavko (1987), was used. After electrophoresis, the gels were fixed in 100 ml of methanol, 20 ml acetic acid, 70 ml of distilled water and 10 ml of 'fixative enhancer concentrate', for 20 - 30 min, rinsed in distilled water for 2 x 20 min and then stained. The stain, prepared fresh each time, comprised 35 ml of distilled water, 5 ml of 'silver complex' solution, 5 ml of 'reduction moderator' solution and 5 ml of 'image development reagent', added in order with constant stirring. Finally, while still stirring,

50 ml of development accelerator reagent were added to the beaker. The solution was then poured over the gel and gently agitated. When the desired intensity of bands was reached the staining solution was decanted off, and the reaction stopped by washing the gel in a 5% (v/v) acetic acid solution.

Western blotting: After electrophoresis the proteins were transferred to nitro-cellulose (0.45 μm) using the Bio-Rad Mini Protean Transblot system according to manufacturers instructions (modified method of Towbin, Staehlin and Gordon, 1979). Before transfer, the polyacrylamide gel and components of the system were equilibrated for 45 min in ice-cold Tris-glycine transfer buffer (25 mM Tris base, 192 mM glycine, pH 8.3). A sandwich was then made of sponge, Whatman 3 MM filter paper, gel, nitro-cellulose, Whatman 3 MM filter paper and sponge which was then encased in the holder with the nitro-cellulose toward the anode. The tank was then filled with ice cold Tris-glycine buffer and the proteins transferred for 1 hr at 100 mA.

After transfer the marker track was cut off the nitro-cellulose and stained in Coomassie as was the post transfer gel to ensure transfer of the proteins. The nitro-cellulose was then blocked for 1 hr in 4% (w/v) Marvel (Premier Beverages, Stafford) in TBST (Section 2.2.1) to minimise non specific antibody binding. The blot was then washed for 1 hr in 3 changes of TBST and incubated for 16 hr in a 1:400 dilution of rat serum to TBS (Section 2.2.1). After further washes in TBST the secondary antibody, anti-rat IgG Horse radish peroxidase (HRP, Sigma), diluted 1:400 with PBS, was incubated with the blot for 1 hr. The substrate DAB (Section 2.2.3) was then added to develop the blot. The colour development was stopped by placing the blot in distilled water.

2.6.3 **IgG Purification**

IgG was purified from sheep plasma through a pre-swollen protein G agarose column (1 ml; Boehringer Cat no: 1243233). Before each purification the column was washed through with 13 mls of sterile PBS and the plasma was spun at 9,000 g for 5 min to remove cellular debris. Plasma (1 ml) was then carefully laid on top of the column and incubated at room temperature for 1 hr. Unbound proteins were then washed from the

column with PBS until the absorbance of the eluate at 280 nm fell below 0.05. IgG was then eluted from the column with approximately 6 mls of 0.2 M glycine, pH 2.8 and the absorbance at 280 nm determined until it again fell, determining that all the bound IgG had been washed off. Immediately after elution, IgG containing fractions were neutralised by the addition of 1 M Tris-HCl (pH 7.5, final concentration 0.1 M). The column was then cleaned with 10 mls of PBS and the IgG fractions were pooled and concentrated using Centricon-3™ (Amicon) concentrators. Concentrated IgG was fractionated by 10% reducing SDS-PAGE gels and Coomassie stained to determine purity. IgG heavy chain was evident at approximately 50 kDa and the light chain approximately 20 kDa.

2.7 SPECTROPHOTOMETRIC DETERMINATION OF TOTAL ENZYME ACTIVITY

2.7.1 Proteinase Activity

The assay works on the principle of proteolytic cleavage of azocasein releasing the azo dye producing an orange colour in the supernatant following acid precipitation of undigested protein.

Proteinase activity at 37°C, with azocasein as substrate, was determined over a broad pH range (3.5 to 10.5) using the buffers described by Knox and Kennedy (1988). Usually, a sample (10 µl), or RPMI blank, was incubated overnight with 100 µl of buffer, 10 µl azocasein (5 mg/ml) and penicillin/streptomycin to final concentrations of 500 IU and 5 mg/ml respectively, after which the undigested protein was precipitated by addition of an equal volume of 5% trichloroacetic acid. Following incubation on ice for 30 min, the precipitated protein was pelleted by centrifugation at 11,000 g for 10 min. and the absorbance of the samples at 405 nm was determined using a Monarch microcentrifugal analyser. All the reactions were done in duplicate.

2.7.2 Ninhydrin Determination of Free α–Amino Nitrogen

This method enables proteolytic digestion of any protein substrate to be detected and, due to the high molecular adsorption of the coloured product, provides a more sensitive determination of the digestion of azocasein by the samples to be analysed. In this reaction the level of free α amino nitrogen released by the digestion of azocasein is directly proportional to the level of colour development, determined by measuring the OD of the test samples at 570 nm. The supernatants were harvested and the increase in free α-mains nitrogen was measured using ninhydrin reagent (Matthews, 1984) as outlined by Knox, Redmond and Jones, (1993).

Ninhydrin solution was prepared fresh daily (100 mls; 0.78 gm ninhydrin; 0.078 gm hydrundantin; 14.4 mls methoxyethanol). After dissolution 10.5 mls 4 M sodium acetate pH 5.5 and 75 mls 60% methoxyethanol in distilled water were added and, using the samples described in the previous Section, 25 µl sample added to 75 µl of acetate buffer pH 5.5 and 100 µl of ninhydrin solution. The samples were heated to 95°C for 15 min, cooled to room temperature and the absorbance read at 570 nm.

2.7.3 Acetylcholinesterase (AChE) Activity

Total AChE activity was determined using the technique described by Ellman, Courtney, Andres and Featherstone (1961). In this assay the substrate acetylthiocholine iodide (ATCI) is hydrolysed by AChE to thiocholine which in turn reacts with dithiobisnitrobenzoate to give a yellow coloured product.

The reaction mixture was prepared by mixing the following components in the described order. ATCI (2 µl, 21.67 mg/ml) was added to 50 µl of dithiobis-nitrobenzoic acid (DTNB, 37.6 mg in 10 mls of 0.1 M phosphate buffer pH 7 and 15 mg of sodium bicarbonate) and 300 µl 0.1M phosphate buffer pH 8. This was pre-warmed to 37°C prior to adding 5 µl standard/sample/blank. The change in absorbance was then monitored at 412 nm over 5 min at 37°C using a Monarch microcentrifugal analyser. The reaction was controlled by substituting water for sample. Enzyme activity was then determined and expressed in international units (IU):

$$\text{IU AChE activity/L} = 4662 \times \text{change in } OD_{412}/\text{min}$$

2.7.4 **Lactate Dehydrogenase (LDH) Activity**

The assay is based on the reduction of pyruvate to lactate with the coupled oxidation of NADH to NAD^+ in the presence of LDH. The oxidation of NADH results in a decrease in absorbance at 340 nm which is directly proportional to LDH activity in the sample. LDH determinations were performed using a commercial kit (Sigma Cat. no. DG1340-UV) and a microcentrifugal analyser.

To prepare Sample Start Reagent, 0.4 ml of reagent B (pyruvate) was mixed with 10 ml of reagent A (NADH and phosphate buffer, pH 7.5) and 250 µl, for each test sample and blanks, was aliquoted into the outer reagent/optical cuvette well of the rotor (IL-Instrumentation, Cat no: 06396500). For the test, 10 µl of sample was loaded into the inner sample well of the rotor, spun in a microcentrifugal analyser to mix the sample and reagent, incubated for 30 sec. and then the change in absorbance at 30°C was determined over 3 min. LDH activity was determined by the following equation:

$$\text{LDH (IU/L)} = 4180 \times \text{change in } OD_{340}/\text{min.}$$

One unit of LDH is defined as the amount of enzyme required to catalyse the formation of 1 µM/L of NAD per min.

2.7.5 **Superoxide Dismutase (SOD) Activity**

Xanthine in the presence of water and xanthine oxidase produces superoxide ions which react with the iodo-nitro-tetrazolium dye releasing the iodo dye producing a purple colour. SOD removes superoxide ions preventing colour formation logarithmically proportional to enzyme activity. The assay reagents were prepared as follows:

Iodonitrotetrazolium violet (INT; 0.253 gm in 15 mls 75% ethanol made up to 100 mls with distilled water), 5 mM Xanthine; (0.076 gm in 0.1 N NaOH made up to 100 mls with distilled water), 50 mM Carbonate buffer pH 10.2 (5.3 gm anhydrous Na_2CO_3 and 0.35 gm Na_2EDTA + 750 mls distilled water, adjusted to pH with 50 mM $NaHCO_3$, total volume 1 litre). The mixed substrate comprised 5 mls INT and 20 mls xanthine made up to 1 litre with carbonate buffer.

SOD standards (20 U/ml to 1.25 U/ml) were made up in serial dilutions from a commercial stock purchased from Sigma Chemical Company (Cat. no: S-2515).

Standard/blank/sample (25 µl) was added to 170 µl of mixed substrate and the reaction initiated by addition of 5 µl of xanthine oxidase (15 µl stock BCL Cat. no: 110-434) in 1 ml distilled water). The samples were vortexed and loaded into the reagent well of the centrifugal analyser rotor. The absorbance was determined (0 sec.) using the Monarch microcentrifugal analyser and this was repeated after incubation at room temperature for 30 min (30 min). SOD activity was calculated for the change in absorbance (30 min - 0) and was expressed in units (U) where 1 U was the amount of enzyme required to inhibit colour formation by 50% over the assay period.

2.7.6 **Glutathione Peroxidase (GSH-px) Activity**

Total GSH-px activity in parasite extracts was determined using the Randox kit (Ransel; Cat. no: RS 504) for glutathione peroxidase which is based upon the method of Paglia and Valentine (1967). Glutathione peroxidase (GSH-px) catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide and in a coupled reaction glutathione reductase (GR) and NADPH then reduce the oxidised glutathione (GSSG) with the NADP being produced with a resultant decrease in absorbance at 340 nm.

Using the components of the kit, 5 µl of sample was added to 250 µl of reagent and 10 µl of cumene hydroperoxide and the change in absorbance at 340 nm was then determined over 3 min. A reagent blank was also set up using distilled water instead of sample. The amount of GSH-px was then determined using the following equation:

$$\text{U/L of sample} = 8412 \times OD_{340}/\text{min}$$

2.8 **ISO-ENZYME ANALYSIS USING POLYACRYLAMIDE GELS**

2.8.1 **Gelatin-Substrate Gel Analysis**

7.5% SDS-PAGE gels were cast using a Mini Protean apparatus (Bio Rad™) as previously described in Section 2.6.2 except for the inclusion of 0.1% (w/v) gelatin in the separating gel. Sample was mixed with an equal volume of non-reducing loading buffer, loaded into the gel sample well, and electrophoresis was carried out at 4°C using standard electrode buffer according to the manufacturer's instructions until the dye

front reached the bottom of the gel. Following electrophoresis the gels were washed in 3 changes of 2.5% Triton X-100 for 30 min to remove the dissociating agent SDS which would inactivate the enzyme if left in the gel. The gel was then incubated at 37°C for 16 h in buffer of the appropriate pH for optimal enzyme activity. The gel was then stained in Coomassie blue followed by destaining in 10% acetic acid. Proteinase bands were identified as clear bands on a dark blue background.

2.8.2 **Inhibitor Sensitivity Analysis**

The effect of various class-indicating proteinase inhibitors (purchased from Sigma Chemical Company, U.K.) on enzyme activity was determined by pre-incubating sample with inhibitor for 30 min. at the following final concentrations prior to gelatin-substrate gel analysis:- phenylmethanesulphonylfluoride (1 mM, PmsF, serine), 1,10 phenanthroline (5 mM, 1,10 Phen, metallo), ethylenediaminotetra-acetic-acid (10 mM, EDTA, metallo), pepstatin (1μM, pepst, aspartyl), L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (10μM, E64, cysteinyl) as well as the cysteine proteinase activator dithiothreitol (0.5 mM, DTT). Inhibitor stock solutions (20x) were prepared and stored as recommended by Beynon and Salvesen, (1989). Prior to electrophoresis inhibitor was added to the samples and incubated on ice for 30 min. After electrophoresis, gels were sliced, washed thoroughly in 2.5% (v/v) Triton X-100 and the gel slices incubated overnight with buffer containing the appropriate inhibitor. Zones of proteolysis were visualised by Coomassie blue counter-staining. Reduced intensity of banding or total disappearance in the presence of inhibitors was indicative of proteinase class.

2.8.3 **Superoxide Dismutase (SOD) Activity**

Native polyacrylamide gels 5-7.5% were run under the same conditions as SDS-PAGE except that SDS was omitted from both the gel matrix and the electrode running buffer. After electrophoresis the gels were immersed in 5 mM nitroblue tetrazolium (NBT) for 15 min, the NBT was then poured off and the gel incubated in working buffer for 20 min containing 20 ml 0.1 M Tris pH 9; 84 μl TEMED (6.6 M) and 7.5 μl Riboflavin

(10 mgs/ml). The gel was then illuminated on a light box, SOD iso-enzymes were identified as clear bands on a purple background (Beauchamp and Fridovich, 1971).

2.8.4 **Peroxidase Activity**

As described previously native PAGE gels were run and stained for general peroxidase activity utilising a method devised by Dr D. P. Knox (personal communication). The stain was freshly prepared by dissolving 30 mg of DAB in 50 ml 0.1 M Tris HCl, pH 8 with the addition of 100 µl hydrogen peroxide (30% solution). Enzyme activity appeared as dark brown bands on a light background.

2.8.5 **Esterase Activity**

Native or non-reducing SDS gels were run as described previously prior to staining, based on the method of Grunder, Satori and Stormont, (1965). The stain consisted of 0.5 mls of a 2% (w/v) α-naphthyl acetate solution prepared in 1 ml acetone, 50 mgs fast blue RR salt dissolved in 10 mls PBS and made up to a final volume of 50 ml with distilled water. The gels were incubated at room temperature with constant agitation until dark brown bands appeared on a light background.

2.8.6 **Acetylcholinesterase (AChE) Activity**

This stain works on the principle of AChE cleaving ATCI in the presence of water to produce thiocholine. The thiocholine reacts with the ferricyanide producing ferrocyanide which, in turn, reacts with copper ions producing copper ferrocyanide, which is seen as reddish bands on the gel.

For AChE, native or non-reducing SDS polyacrylamide gels were run using standard conditions outlined above, the SDS being eluted prior to staining as described for proteinase gels. The stain was prepared by dissolving 10 mg acetylthiocholine iodide (ATCI) in 13 mls 0.1 sodium phosphate and then adding in order 1 ml 0.1 M sodium citrate (29 mg/10 mls), 2 mls 30 mM copper sulphate (75 mg/10 mls) and 2 mls 5 mM potassium ferricyanide (17 mg/10 ml) with constant stirring. The stain was

poured over gel while on a shaker and the zones of enzyme activity were visible within a few minutes being a red-brown colour (Karnovsky and Roots, 1964).

2.8.7 **Lactate Dehydrogenase Activity**

After electrophoresis in a 7.5% native polyacrylamide gel the gel was stained by immersion in a solution containing 0.24 g lithium lactate, 5 mg NAD+, 10 mg phenazine methosulphate (PAMS), 10 mg iodo-nitro-tetrazolium (INT) and 25 mg potassium cyanide (KCN). All the compounds were first dissolved in 0.1M Tris HCl, pH 7.5 before combining and making the final volume up to 50 ml with buffer. The potassium cyanide was added before incubating the gel in stain at 37°C until bands were evident (Gaal, Medgyesi and Vereczkey, 1980).

2.8.8 **Phosphatase Activity**

Gels, again 7.5% and native conditions, were run on ice with pre-chilled native electrode buffer as previously for enzyme analysis on gels. After electrophoresis for 45-50 min, the gels were incubated in 20 mg sodium α-naphthyl phosphate in 2 mls acetone, and either 18 mls 0.1 M sodium acetate pH 5 (acid phosphatase), or 0.1 M Tris-HCl pH 9 (alkaline phosphatase) for 10 min based on the method of Gomori, (1950). An aliquot of the staining buffer was then removed and 20 mg of fast blue RR added and mixed thoroughly, this was then added to the rest of the buffer containing the gel and mixed on a shaker. Dark brown bands on a pale background developed within a few min. The gel was then removed from the stain and the reaction stopped by washing in distilled water.

2.9 **PROTEIN DEGRADATION ASSAYS**

The effect of *T. circumcincta* IVR proteinases on proteins which would be present in the host environment was determined. Adult (20 μg) or L4 (5 μg) IVR products were incubated overnight (37°C) at the appropriate pH with 20 μl (10 mg/ml in assay buffer) of each of the following substrates derived from the bovine:- fibrinogen,

albumin, haemoglobin, plasminogen, complement and immunoglobulin G (IgG) (all Sigma). The effect of proteinase inhibitors, at the final reaction concentrations described earlier (Section 2.8.2), on degradation was also tested. For negative control reactions the parasite material was replaced with sterile water. The outcome of these reactions was assessed using 10% SDS-PAGE under reducing conditions (Laemmli, 1970).

2.10 NUCLEIC ACID MANIPULATIONS

2.10.1 mRNA Extraction from *N. brasiliensis*

Poly(A)$^+$ RNA (mRNA) was extracted from *N. brasiliensis* according to the instructions of the Quick Prep Micro mRNA purification kit (Pharmacia, Biotech) which is based on the selection of poly(A)$^+$ RNA by the affinity matrix oligo(dT) cellulose (Aviv and Leder, 1972). All reagents required were supplied with the kit.

Adult parasites (0.1 gm) which had been snap frozen in liquid nitrogen immediately upon harvesting were homogenised in 0.4 ml extraction buffer (guanidinium thiocyanate and N-Lauroyl sarcosine) using a micro-homogeniser (Jencons, Scientific) to form a uniform suspension. The homogenate was then diluted in 0.8 ml of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and thoroughly mixed, prior to being decanted into a 1.5 ml eppendorf. The homogenate and oligo (dT) cellulose suspension provided in the kit were microfuged separately at 9,000 g for 1 min. before the buffer from the oligo (dT) cellulose was pipetted off and 1 ml of the homogenate supernatant was added to the oligo (dT) cellulose. The sample and oligo (dT) cellulose were suspended by vortexing before mixing on a shaking tray for 5 min. followed by centrifugation for 10 sec. at 9,000 g after which the supernatant was pipetted off. High salt buffer (1 ml, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 0.5 M NaCl) was added to the homogenate/oligo (dT) suspension and mixed by pipetting before centrifugation at 9,000 g for 10 sec. and the supernatant removed by pipetting. This washing procedure was repeated for a total of 5 times after which low salt buffer (1 ml, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 0. M NaCl) was added, mixed by pipetting and microfuged for 10 sec. before pipetting off the supernatant. This process

was repeated once more. The oligo (dT) cellulose and parasite homogenate mix were then re-suspended in 0.3 ml low salt buffer and added to a Microspin™ column (provided in the kit) placed inside a 1.5 ml eppendorf and the top covered with Nescofilm™. The column and oligo(dT) cellulose/parasite suspension was then microfuged at 9,000 g for 5 sec. after which the column was removed and the contents of the eppendorf poured away. Low salt buffer (0.5 ml) was layered on top of the column, placed inside the eppendorf and spun again for 5 sec. and the eluate which collected in the eppendorf was poured off. This process was repeated three times. The column was then placed into a fresh eppendorf and 0.2 ml of the pre-warmed (65°C) elution buffer was added to the column and after at 9,000 g for 5 sec., and the eluate containing the mRNA was harvested. This process was repeated once more to give 0.4 ml of mRNA containing solution.

2.10.2 **Quantifying Recovered RNA**

The amount of mRNA recovered was estimated by determining the OD of a 1 in 5 dilution of the eluate at 260 nm using a Beckman DU spectrophotometer. The RNA concentration was calculated using the following formula:

$$RNA = Absorbance_{260} \times 40 \times \text{dilution factor}$$

The diluted sample was also scanned over the wavelengths 260 nm to 280 nm to ensure that an approximate 2:1 ratio in absorbance was obtained indicating that the RNA was not contaminated with protein.

2.10.3 **Nucleic Acid Detection in Polyacrylamide Gels**

7.5% polyacrylamide gels were made by mixing 2 mls 5X Loening E (Section 2.2.4), 2.5 mls bis acrylamide (0.8%/30%, v/v), 5.5 mls distilled water, 100 µl 10% w/v APS and 20 µl TEMED. A continuous gel was poured and a 1:10 ratio of bromophenol blue to nucleic acid was loaded. A 1 kilobase ladder was also run with the samples. Gels were run at 200 v until the blue dye front reached the bottom of the gel, after which

they were fixed in 10% ethanol: 0.5% acetic acid: water for 15 min. The gel was then stained in a silver nitrate solution (0.38 gm $AgNO_3$ in 200 ml of distilled water) for 15 min, rinsed very rapidly in 2 washes of distilled water before the nucleic acids were visualised by immersion in a solution containing sodium hydroxide (6 gm), formaldehyde (1.5 mls) and 200 mls of water with constant agitation. The colour development was stopped by decanting the developing solution and then adding a solution of sodium carbonate (0.75 g in 100 mls). The gel was then rinsed in distilled water. This staining protocol was described by Herring, Inglis, Ojeh, Snodgrass and Menzies, 1982.

2.10.4 **Precipitation of mRNA**

mRNA in 400 µl elution buffer was precipitated by addition of 10 µl glycogen solution (10 mg/ml), 40 µl of 2.5 M potassium acetate and 1 ml of ice cold 95% ethanol. The solution was stored at -70°C for 1-2 h to precipitate the RNA which was then pelleted by centrifugation at 9,000 g 4°C for 15 min. The ethanol was pipetted off and the pellet was then left to vacuum dry before dissolution in 10 µl of TE buffer.

2.10.5 **Synthesis of Complementary DNA (cDNA)**

Synthesis of DNA from mRNA for use in the reverse transcriptase PCR reactions was performed using the GIBCO BRL Superscript™ Preamplification System for First Strand cDNA Synthesis kit (Life Technologies). In this kit, first strand cDNA synthesis is catalysed by the Superscript II RNase H^- Reverse Transcriptase (Superscript RT), the RNase H^- preventing degradation of mRNA during the first strand reaction while the enzyme is stable in temperatures up to 50°C, allowing amplification from small amount of mRNA. All reagent required were supplied in the kit.

For each reaction, 10 µl of sample mRNA (1-5 µg) was mixed with 1 µl oligo (dT) primer and 1 µl of DEPC-treated water in a sterile 0.5 ml eppendorf, and incubated at 70°C for 10 min to denature the RNA and then placed on ice for 1 min. Primer annealing for cDNA synthesis was performed by adding, 2 µl of 10x PCR buffer, 2 µl of 25 mM $MgCl_2$ 1 µl 10 mM dNTPs and 2 µl of 0.1 M DTT to each

reaction, mixing and incubation at 42°C for 5 min. The synthesis of cDNA was then initiated by adding 1 µl (200 units) of Superscript II RT™ to each tube and incubating for a further 50 min. The reaction was then terminated by incubation at 70°C for 15 min followed by chilling on ice. After brief centrifugation, 1 µl of RNase H was added and the samples incubated at 37°C for 20 min to each tube to remove any residual RNA. During the synthesis procedure a control reaction was set up using RNA supplied. An aliquot of newly synthesised RT-DNA was then used directly in PCR amplification, and the rest stored at -20°C prior to use.

2.10.6 **Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

PCR amplifies a segment of DNA which is flanked by two regions of known sequence. In RT-PCR reactions, DNA is amplified directly from single stranded DNA linked to it's complementary RNA sequence, but in order to provide single stranded DNA template the RNA/DNA hybrid is denatured first by boiling. The temperature is then lowered to allow annealing of two complementary primers (directed to opposite strands of template DNA) to regions of known sequence which flank the segment of DNA to be amplified. The temperature is then raised again and new strands of complementary DNA are then synthesised by incorporating deoxyribonucleotides (dNTPs) present in excess in the PCR reaction. The synthesis of new strands of DNA is catalysed by the thermostable enzyme *Taq* DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* (Chien, Edgar and Trela, 1976). This process is then repeated 20-30 times producing many copies of DNA, based on the method of Saki, Scharf, Faloona, Mullins, Horn, Erlich and Arnheim, (1985).

All PCRs were carried out in a reaction volume of 50 µl. Usually 28 µl of sterile distilled water, 5 µl 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% (w/v) gelatin), 5 µl of each oligonucleotide primer (final concentration 0.2 mM), 1 µl of DNA (10 ng), 10 µl of stock dNTP's (2 mM, dATP, dTTP, dCTP, dGTP) and 1 µl (1 unit) of a 1:10 dilution of TAQ DNA polymerase (Boehringer) were added in order to give a final reaction volume of 50 µl. The reactions were mixed, centrifuged briefly and then overlaid with 50 µl of mineral oil to prevent evaporation of the reactions during the PCR cycles. The PCR annealing

temperatures varied depending on the primers used. Usually PCR amplification was performed by heating the samples to 95°C for 5 min for 1 cycle to break the RNA/DNA hybrid apart, then 20 to 30 cycles of 95°C for 1 min, 40°C for 2 min to allow specific annealing of the primer and 72°C for 3 min to allow extension of sequence from the site of the primer annealing. Finally a single cycle at 72°C for 8 min to maximise the number of full copies obtained. The PCR reaction was performed using a Hybaid Omnigene thermocycler.

The products from PCR amplifications were analysed either by agarose gel electrophoresis and ethidium bromide staining or by polyacrylamide gel electrophoresis and silver staining.

2.10.7 **Agarose Gel Electrophoresis**

Amplified PCR products were fractionated by horizontal slab gel electrophoresis using a Pharmacia GNA-100 mini gel and 20 ml or 40 ml gels (Pharmacia) with 1% (w/v) agarose gels (nucleic acid grade "ultra pure" agarose) in TAE. After heating to melt the agarose followed by cooling to approximately 55°C, ethidium bromide (0.5 μg/ml) was added and the gel poured on a flat surface. The ethidium bromide intercalated with the DNA allowing visualisation under UV light (302 nm). The gels were placed in the electrophoresis tank which was filled with TAE containing ethidium bromide (0.5 μg/ml). The PCR samples, mixed 9 to 1 with bromophenol (Section 2.2.4) and 1 Kb markers (GIBCO BRL, Life Technologies Ltd., Paisley, UK) as a molecular weight standard were loaded onto the gel and the gel was run at 80 V for 1 to 2 h depending on the desired separation of DNA fragments.

2.10.8 **Southern Blotting**

To transfer DNA from agarose gels to nylon membranes, a capillary blot was used based on the method of Southern (1975). After electrophoresis, the gel was placed in denaturation buffer for 30 min. The gel was then rinsed briefly in distilled water and soaked in neutralisation buffer for two 15 min washes. A capillary blot was set up by covering a solid support with 3 MM soaked in 10X SSC and stood in a reservoir of

10X SSC. The gel was then orientated so that the bottom of the gel was uppermost, providing the DNA with less distance to transfer across to the membrane. The gel was surrounded in Nescofilm™ to prevent accidental touching of the membrane and wick which would prevent transfer. The membrane (Hybond™), cut to the size of the gel was carefully placed on top of the gel and smoothed to remove trapped air bubbles. The membrane was then overlaid with 2 layers of 3 MM pre-soaked in 10X SSC, a stack of 3 MM blotting paper and paper towels to a height of approximately 10 cm. A glass plate was then placed on top as well as a weight. The blot was then left overnight. The following morning the stack of paper towels etc. was removed and the orientation of the tracks from the gel marked on to the membrane. To remove any fragments of agarose which may have stuck to the membrane was rinsed in 2X SSC for 5 min. The membrane was then allowed to air dry before wrapping in Saranwrap™ and irradiating the blot with UV (254 nm) to fix the DNA to the membrane prior to probing.

2.10.9 **Digoxigenin (DIG) Labelling of DNA**

The enhanced chemiluminescence (ECL) random primed labelling kit (Boehringer Mannheim Cat no; 1175 033) was used for labelling of DNA probes.

Digoxigenin is a steroid hapten which is coupled to dUTP for DNA labelling (DIG-11-dUTP) for use in hybridisations and is detected by either colour production or luminescence. DNA probes are labelled with DIG by random priming of hexanucleotides to denatured DNA. The complementary DNA strand (cDNA) is then synthesised by Klenow enzyme which uses the random oligonucleotides (hexanucleotides) as primers and a mixture of deoxyribonucleotides (dNTPs) containing DIG-11-dUTP for elongation, incorporating DIG into the newly synthesised DNA. The DIG DNA probe is then used as in standard hybridisation reactions and detected with anti-DIG-alkaline phosphatase antibody and visualised with the chemiluminescent substrate CSPD™ (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1^{3,7}] decan} 4-yl) phenyl phosphate) producing light at 466 nm which may be detected on X-ray film.

DNA (100 ng) was added to 10 µl of distilled water and denatured by boiling for 3 min and then placed on ice immediately. Using the components of the kit, 2 µl of hexanucleotide mix, 2 µl of dNTP's and 1 µl (2 units) of Klenow enzyme were added to the DNA, the components harvested at the bottom of the tube and incubated at 37°C for 2-16 h. The reaction was stopped by the addition of 2 µl 0.2 M EDTA and labelled DNA precipitated by addition of 2.5 µl 4 M LiCl and 75 µl pre-chilled (-20°C) 70% (v/v) ethanol and storage at -70°C for 30 min. After precipitation the labelled DNA was pelleted by centrifugation at 9,000 g for 15 min. The ethanol was removed and the DNA washed by re-suspension in 50 µl ice cold ethanol and subsequent centrifugation at 9,000 g for 5 min. The ethanol was removed and the DNA was air dried before dissolution in 100 µl of sterile distilled water.

2.10.10 **Detection of *N. brasiliensis* DNA using DIG**

Southern blot hybridisations were carried out using the enhanced chemiluminescent (ECL) detection kit (Boehringer Mannheim Cat no: 1363 514). The blot (Section 2.10.8) was blocked in 5 ml of DIG Easy Hyb in a Hybaid bottle (Hybaid, Teddington, UK) at 42°C for 2 h using a Hybaid rotating oven. The DIG- labelled DNA probe was denatured by boiling and immediately placed on ice for a few min before addition to 5 ml of pre-warmed DIG Easy Hyb. The blocking solution from the blot was poured off and the probe was added. Hybridisation was for 16 h at 42°C. After hybridisation the probe was removed and kept at -20°C for later blots. The blot was then washed for 15 min in 2X SSC, 0.1% SDS at 42°C and then two 30 min washes in 1X SSC, 0.1% SDS at 42°C. The higher the temperature and the lower the salt concentration of the washes, the more stringent the conditions.

The blot was then incubated in 1x blocking solution (DIG block diluted 1:10 in 0.1 M Maleic acid; 0.15 M NaCl, adjusted to pH 7.5 with solid NaOH) for 30 min, and then immersed in anti-DIG AP conjugate (1:10,000 dilution in blocking solution) and the blot incubated for 30 min. After washing twice for 15 min in washing buffer (maleic acid buffer plus 0.3 % v/v Tween 20), the blot was equilibrated for 5 min in detection buffer (0.1M Tris HCl, O.1 M NaCl; 50 mM MgCl2, pH 9.5) before adding the chemiluminescent solution (CSPD) on top of the membrane, after 5 min the excess

liquid was left to drip off, blotted briefly onto Whatman™ 3 MM and wrapped in SaranWrap™. After incubating the blot at 37°C for 15 min to enhance the luminescent reaction the blot was exposed to X-ray film for 10-30 min at room temperature.

2.10.11 **Purification of DNA from Agarose Gels**

After fractionation of the DNA sample in a 1.2 % w/v agarose gel, the band to be purified was excised and as much excess agarose as possible was trimmed away. The gel slice was then cut up into very small pieces and put into a weighed 1.5 ml eppendorf. 1 ml of NaI from the GENECLEAN™ kit was added to the DNA and the samples heated to 55°C until the agarose totally melted. GLASSMILK™ (5 µl) was added, mixed and the mixture left at room temperature for 5 min and then mixed again, spun for a few sec. and the NaI pipetted off. The pelleted GLASSMILK to which the DNA was bound was then washed 3X by re-suspension in 500 µl of NEW WASH™, spinning down and removing the supernatant each time. DNA was eluted into 10 µl of distilled water and the solution incubated for 3 min at 55°C. 1 µl of 10X TE buffer was then added to the sample and the purified DNA transferred to a fresh eppendorf and stored at -20°C (Vogelstein and Gillespie).

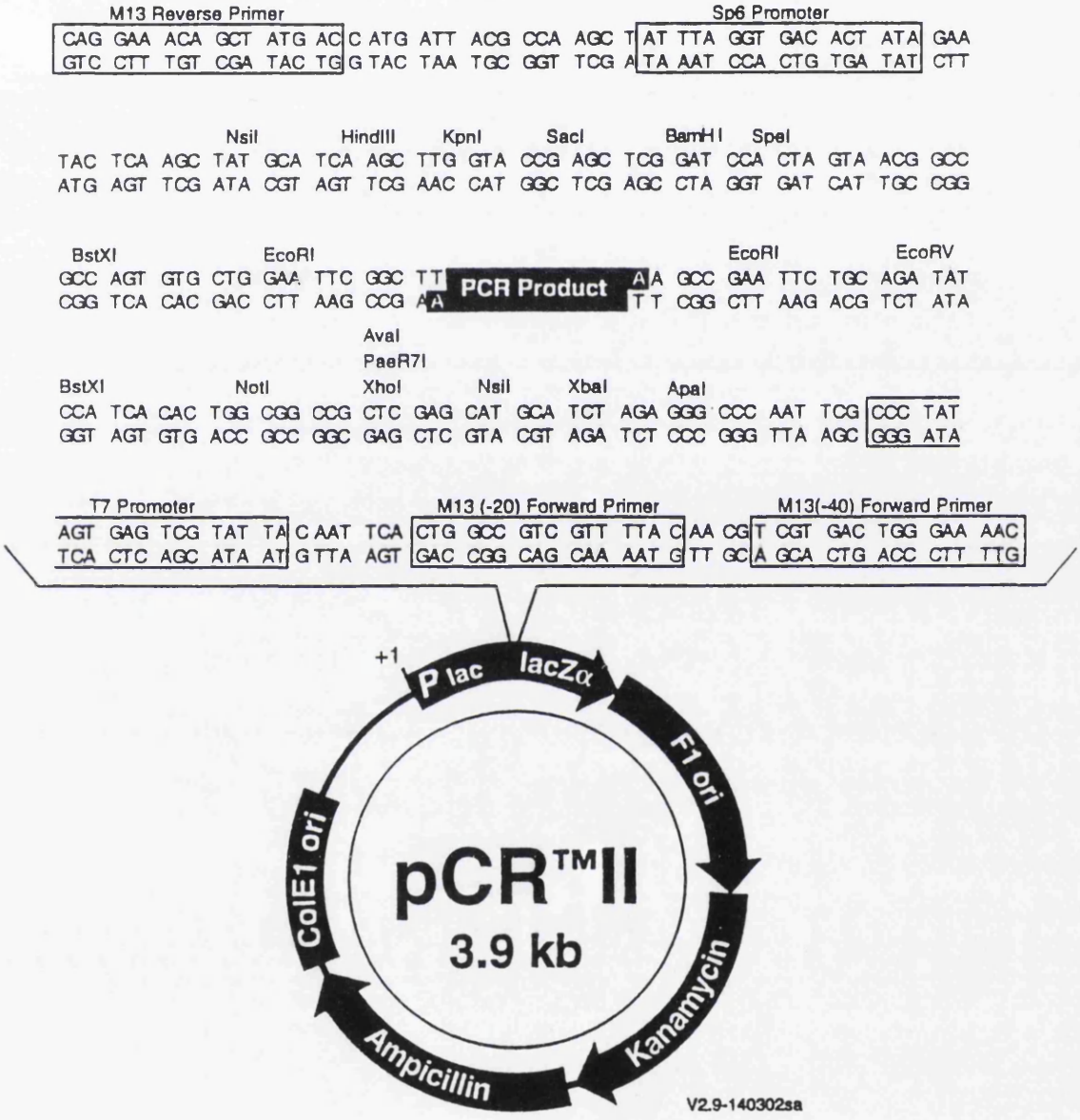
2.10.12 **Cloning of DNA Fragments into the Plasmid Vector pCR II**

The commercially available cloning kit TA Cloning Kit was used (Invitrogen). The vector pCR II (Fig. 2.1) is 3.9 Kb in size and contains 2 *Eco* R1 restriction sites, *lac* promoter region and M13 forward and reverse priming sites for use in sequencing of the insert DNA as well as kanamycin and ampicillin resistant genes.

Each ligation was set up using the components of the kit in the following order. Into a sterile 0.5 ml eppendorf were added 0.5 µl DNA ligase, 2 µl buffer, 2 µl GENECLEANED PCR product to be cloned, 1 µl of vector and distilled water to a final volume of 10 µl. The ligation mixtures were then incubated overnight at 12°C prior to storage on ice.

Figure 2.1

Map below of pCR II taken from Invitrogen kit



2.10.13 **Transformations**

Using the Invitrogen TA Cloning kit, frozen competent *E. coli* cells (50 µl aliquots per ligation) and β-mercaptoethanol were thawed on ice after which 2 µl of β-mercaptoethanol and 3 µl of the appropriate ligation reaction was added to the cells (Section 2.10.12). The reactions were incubated on ice for 30 min, 42°C for exactly 60 sec. and then put back on ice for 2 min SOC medium (450 µl) warmed to room temperature was added to each transformation and the vials incubated at 37°C for 1 hr. An aliquot (150 µl) of the transformation reaction was then spread onto an LB-agar plate containing ampicillin (50 µg/ml) and X-gal (40 mgs/ml). The plates were incubated overnight at 37°C. Colonies which have no insert (non-recombinant) grow blue, whereas colonies with inserted DNA (recombinant) grow as isolated white colonies due to insertional inactivation of the β-galactosidase gene product.

2.10.14 **Extraction of Plasmid DNA (Mini Preps)**

Single isolated white colonies were selected using aseptic techniques and inoculated into LB-broth and the cultures incubated with shaking (200 rpm) at 37°C overnight. Bacterial cells in a 1.5 ml aliquot of the culture were pelleted by centrifugation in a microfuge, the supernatant discarded and the pellet suspended in 100 µl of ice cold TEG (50 mM glucose, 25 mM Tris HCl pH 8.0, 10 mM EDTA), vortexed and incubated at room temperature for 5-15 min Then, 200 µl of freshly prepared alkaline SDS (0.2 M NaOH, 1% SDS) were added and the samples left on ice for 5 min before adding 150 µl ice cold potassium acetate (60 ml 5 M KAc, 11.5 ml glacial acetic acid and 28.5 ml distilled water) and centrifugation for 5 min Saturated phenol (GIBCO; 450 µl) was added and the samples vortexed and spun for 2 min after which the upper aqueous layer was transferred to a clean eppendorf, ethanol (900 µl) added, the samples vortexed and the DNA left to precipitate at room temperature. The samples were microfuged for 5 min to pellet the DNA, the ethanol pipetted off and the plasmid DNA pellet washed in ice cold 70 % ethanol. The pellet was then vacuum-dried before dissolution in 50 µl of TE buffer containing RNAase (20 µg/ml).

2.10.15 **Restriction Enzyme Digests**

The size of the inserted DNA was confirmed by digesting recombinant plasmid DNA with *Eco* R1. The vector pCR II has two sites for *Eco* R1 flanking the insert cloning sites, and after digestion, a fragment of the size of the original insert should be produced. Digests were carried out by incubating 5 µl of plasmid DNA with 1 µl of the restriction enzyme (1:10 dilution 10 U/µl), 1 µl of appropriate restriction enzyme buffer and sterile distilled water to a volume of 10 µl for $1^1/_2$ hours at 37°C. Digests were then analysed on a 1% agarose gel.

2.10.16 **Labelling of DNA for Sequencing**

DNA sequencing reactions were carried out using the T7 sequencing kit (Pharmacia) which utilises bacteriophage T7 DNA polymerase based on the method of Sanger, Nicklen and Coulson (1977). Chain terminating ddNTPs are incorporated into DNA similarly to dNTPs, however ddNTPs prevent further extension on the DNA leading to termination. Many fragments of DNA are then formed varying in length from the primer to the site of termination. The incorporation of radiolabelled dATP(^{35}S) then allows for the DNA sequences to be visualised by autoradiography once they have been run through a gel (Section 2.10.17). Primer annealing and sequencing reactions were carried out precisely as recommended in the kit instructions in the forward and reverse directions. The T7 DNA polymerase catalyses base-specific termination of primer-extension reactions. All reagents required were supplied with the kit except [^{35}S]-α-dATP which was purchased from Amersham (UK).

2.10.17 **Sequencing Gels**

A 6% acrylamide gel was prepared as described in Section 2.2.4. The gels were run using the S2 BRL apparatus (Gibco) at a constant power setting of 50 W. Prior to loading of the DNA, the gel was pre run for 30 min. Further loadings of the samples (4 µl denatured template) were done 2 and 4 h after the first loading was performed. The gel was run for a maximum of 5 h, after which it was fixed for 15 min in an aqueous

solution containing 10% (v/v) methanol and 10% (v/v) acetic acid. The gel was then dried onto Whatman 3 MM under vacuum. Dried gels were then placed into a X-ray film cassette containing film and left for 16 h before developing in the X-ograph compact 2 automatic developer to visualise the resolved DNA fragments.

2.10.18 **Computer Analysis of DNA Sequences**

DNA sequences were analysed using the Daresbury SEQNET computer databases (Daresbury Laboratory, Daresbury, Warrington, UK) and the University of Winsconsin Genetics Computer Group programs (UWGCG) available via the User Interface Group. Alignment to known DNA sequences in GENBANK and EMBL was carried out using the 'FastA' programme (PEARSON) and nucleotide sequences were aligned using 'PILEUP'.

2.10.19 **Recombinant Stocks**

Freshly grown LB-broth culture was stabbed (using a bacterial loop) into the bottom of a 2 ml tube containing 1.5 ml LB-agar. The culture was stored at room temperature in the dark. Glycerol stocks were made by pipetting 850 µl of fresh LB culture into 150 µl of sterile glycerol (15% v/v) and stored at -70°C.

CHAPTER THREE

CHARACTERISATION OF THE PROTEINASES OF *TELADORSAGIA CIRCUMCINCTA*

3.1 INTRODUCTION

The characterisation of parasite enzymes has enhanced the understanding of the host/parasite relationship, and has identified potential antigens for serodiagnosis, anthelmintic control and vaccination. The current literature on the potential importance of parasite proteinases for survival and maintenance within the host was reviewed in Chapter 1.

The parasitic stages of *T. circumcincta* are closely associated with the gastric glands and, hence, parasite excretory/secretory (E/S) products may be involved in stimulating host protective local immune responses. Proteinase release by the L3 and adult stages of this parasite has been demonstrated previously (Knox and Jones, 1990) although the enzymes were not characterised in detail. Similarly, proteinases released during *in vitro* maintenance of the closely related bovine parasite *Ostertagia ostertagi* have been partially characterised (de Cock *et al.*, 1993) and it would therefore be interesting to determine if proteinase expression by the ovine and bovine parasites are similar.

In this chapter, the somatic proteolytic enzymes of the adult worm and the *in vitro* released (IVR) proteinases of the parasitic stages of *T. circumcincta* were characterised on the basis of pH optima, molecular weight, and inhibitor sensitivity. Preliminary attempts at determining whether proteinases, released *in vitro* by the adult parasite were recognised by immune sera, were also made.

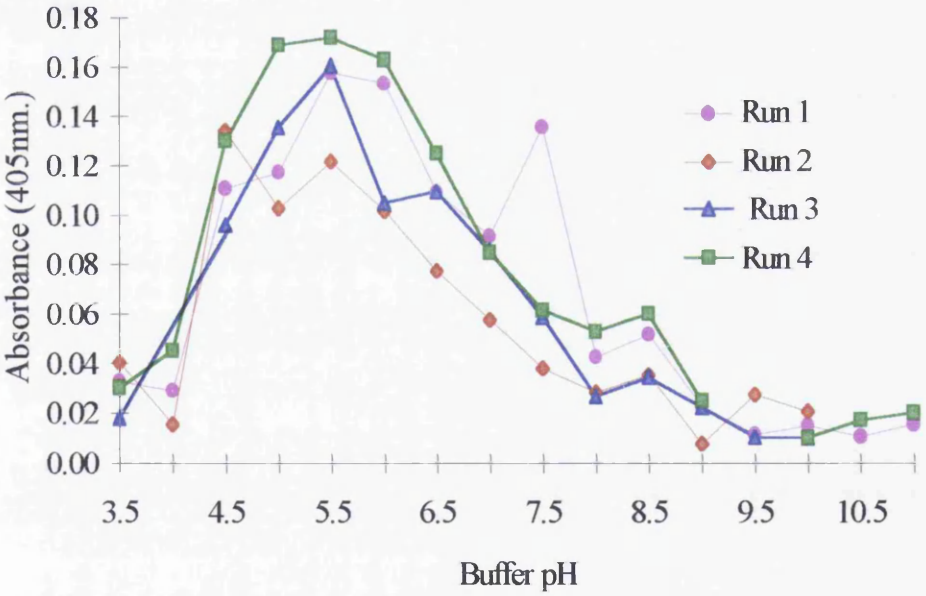
3.2 RESULTS

3.2.1 pH Optima of Somatic Extracts of Adult *T. circumcincta*

The degradation of azocasein by somatic extracts was determined over a pH range of 3.5 to 11, and was found to be consistently maximal at pH of 5.5 (Graph 3.1). Some variation in the pH profile was noted when separate batches of somatic extracts were compared, most notably in run 1 in which an extra peak of activity at pH 7.5 was observed. A much smaller peak of activity was also consistently observed at pH 8.5. When pH optima were assessed in gelatin-substrate gels, maximum resolution of comparative proteinases was noted at pH 6.

Graph 3.1

pH Optima of Azocasein Degradation by Somatic Extracts of Adult *T. circumcincta*



3.2.2 Gelatin Substrate Gel Analysis of Somatic Extracts of Adult *T. circumcincta*

Proteinases in extracts of adult parasites were examined to ascertain whether or not some of the IVR proteinases resulted from non-specific leakage of somatic enzymes

during culture. The results of this analysis are shown in Fig. 3.1. Somatic extracts were separated by 7.5% non-reducing SDS-PAGE gels containing 0.1% gelatin and the gels were incubated for 16 h in buffer at pH 6 in the presence and absence of 0.5 mM DTT as well as buffer alone at pH 8.5. Faint proteolysis was observed 107, 55 and 60 kDa at pH 6 (Fig. 3.1a). Enzymes active at pH 6 were stimulated by the sulphydryl reagent, dithiothreitol (DTT; Fig. 3.1a), producing a zone of activity from 60 to >205 kDa. At pH 8.5, in the absence of DTT, a doublet at approximately 55 kDa, activity at 97 and 116 kDa was evident as well as a smear of proteolysis at >205 kDa (Fig. 3.1a). Activity at pH 8.5 was reduced by DTT (not shown).

Fig. 3.1b demonstrates the effect of inhibitors on the proteinases active at pH 6 in the presence of DTT. E 64, a cysteinyl proteinase inhibitor, greatly reduced their activity, while activity was either unaffected or only slightly reduced by pepstatin, PmsF or EDTA, inhibitors of aspartyl, serine and metallo proteinases respectively. By contrast, activity at pH 8.5 (Fig. 3.1c) was most susceptible to a metallo inhibitor, EDTA, totally inhibiting activity at 116 kDa and reducing the intensity of proteolysis at >205 kDa.

Figure 3.1 **Somatic Proteinases of Adult *T. circumcincta***

7.5 % SDS-PAGE containing 0.1% gelatin were run under non-reducing conditions. The adult somatic proteinase profile is shown in Fig. 3.1a where, proteinase activity was demonstrated from left to right, at pH 6, pH 6 + 5 mM DTT and pH 8.5. The effect of inhibitors on the proteinases at pH 6 + DTT (Fig. 3.1b) and pH 8.5 (Fig. 3.1c) are also shown where C = control; Pepst = pepstatin, an aspartyl proteinase inhibitor; PmsF = a serine proteinase inhibitor; E 64 = a cysteine proteinase inhibitor; EDTA = a metallo proteinase inhibitor. Molecular weight markers in kilo daltons (kDa) are also shown.

Figure 3.1a

Somatic Proteinases of Adult *T. circumcincta*

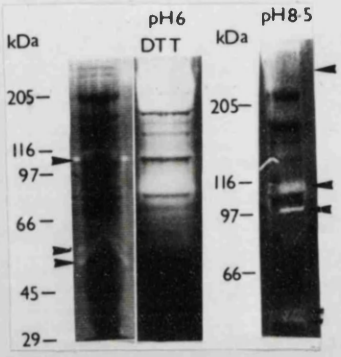


Figure 3.1b

Inhibition of Somatic Proteinases at pH 6 + DTT

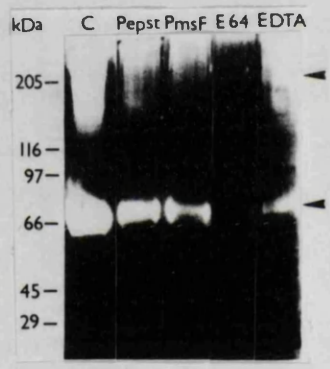
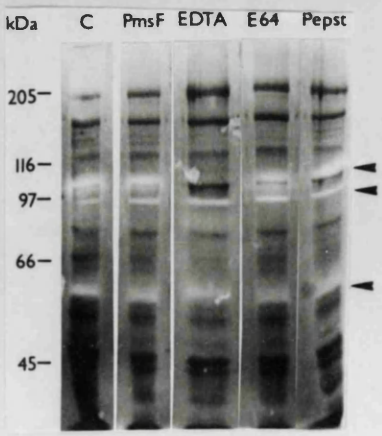


Figure 3.1c

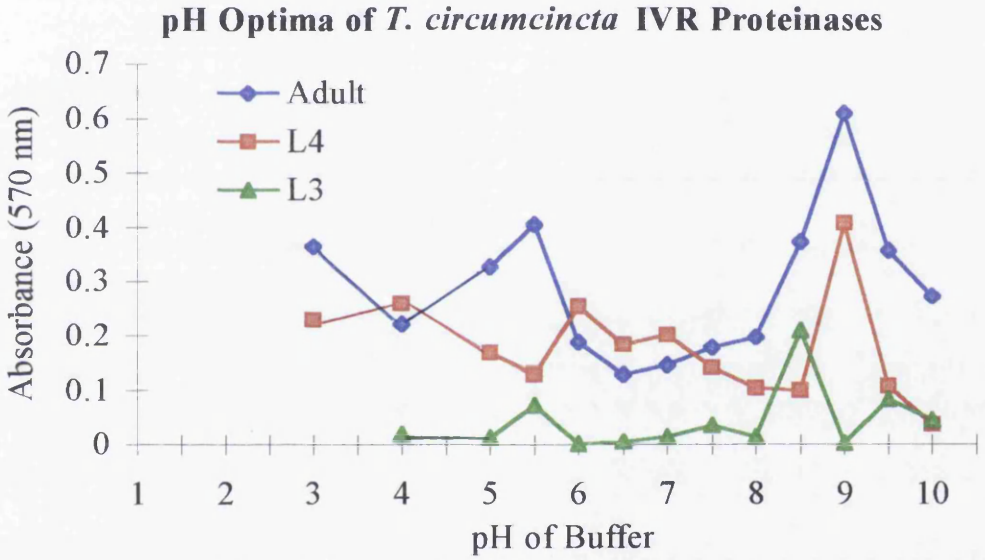
Inhibition of Somatic Proteinases at pH 8.5



3.2.3 pH Optima of IVR Products of the Parasitic Stages of *T. circumcincta*

Using the spectrophotometric azocasein assay, proteolytic activity was weak and variable between different batches of IVR (data not shown) and results shown here are the mean of 2 or 3 runs. Maximal proteolytic activity was consistently observed at pH 9 with IVR from L4 and adult parasites and at both pH 5 and pH 8.5 with L3 (Graph 3.2).

Graph 3.2



3.2.4 pH Optima of *T. circumcincta* IVR Products as Judged by Gelatin Substrate Gel Analysis

The pH dependency of IVR proteinases was also assessed using gelatin-substrate gels. Results of these analyses are shown in Figures 3.2A, B and C (adult, L4 and L3 IVR products respectively).

With adult IVR (Fig. 3.2A), several zones of proteolysis, at approximately 32, 47, 100 and > 205 kDa were evident at pH 9 which were also observed at pH 6, albeit less strongly. A single zone of proteolysis at 47 kDa was evident at pH 4. Generally, proteolysis was more marked at pH 9, in accord with the azocasein assay. Similarly,

the proteolytic profile observed with L4 IVR was more complex with increasing pH (Fig. 3.2B). With L3 IVR, high molecular weight (>205 kDa) activity was only evident at pH 5 while a 29 kDa zone of activity was uniquely observed at pH 9 (Fig. 3.2C). Activity at 40 kDa decreased with increasing pH.

3.2.5 **Molecular Size and Inhibitor Sensitivity of *T. circumcincta* IVR Products**

Adult IVR:- At pH 9 (Fig. 3.3a) the bands of proteolysis at approximately 47 kDa and a less obvious band at 75 kDa were completely inhibited by 1,10 phenanthroline (a metallo proteinase inhibitor), while activity at 32 kDa was reduced. Activities at 130 kDa, and an occasionally observed zone at 100 kDa, were abolished by PmsF. E64 and pepstatin did not inhibit proteolysis. The 47 kDa proteinase active at acidic pH (see Fig. 3.2A) was partially and inconsistently inhibited by E64 (not shown).

L4 IVR:- At pH 9 (Fig. 3.3b), several zones of proteolysis were evident. In L4 IVR prominent bands were observed at approximately 32, 47, 55, 60, 90 and 100 kDa. Proteolysis at 90 kDa was completely inhibited by PmsF and EDTA reduced the activity of the 55 and 60 kDa bands. The zone of hydrolysis at 29 kDa in the EDTA, E64 and pepstatin lanes was artifactual.

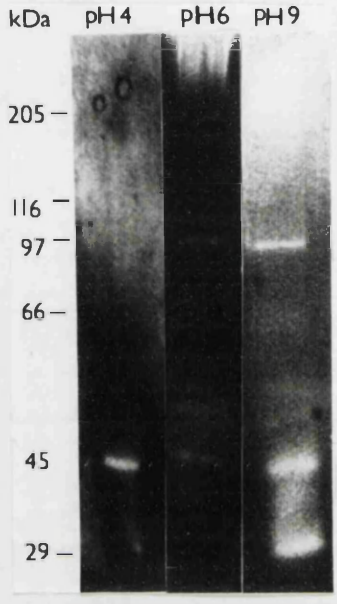
L3 IVR:- At pH 5.5, zones of proteolysis at approximately 45 kDa and 205 kDa were evident (Fig. 3.2C) and were enhanced in the presence of DTT. The prominent 29 kDa activity evident at pH 9 was completely abolished by 1,10 phenanthroline (not shown).

Figure 3.2 pH Optima Profiles of *T. circumcincta* IVR Products

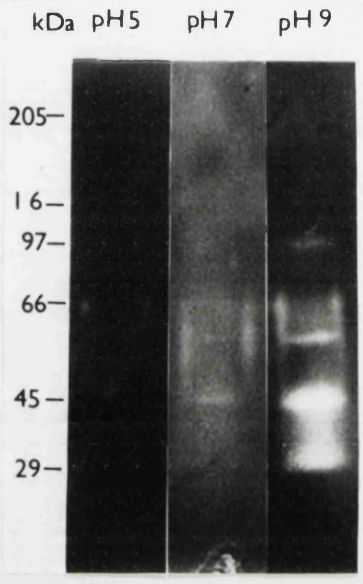
7.5 % SDS-PAGE containing 0.1 % gelatin run under non-reducing conditions showing the pH dependent proteinase profiles of adult IVR products at pH 4, pH 6 and pH 9 (Fig. 3.2A), L4 IVR products at pH 5, pH7 and pH 9 (Fig. 3.2B) and L3 (Fig. 3.2C) IVR products at pH 5, pH 5 in the presence of 5 mM DTT, pH 7 and pH 9. Molecular weight markers are shown in kilo daltons (kDa).

Figure 3.2

A) Adult IVR Proteinases



B) L4 IVR Proteinases



C) L3 IVR Proteinases

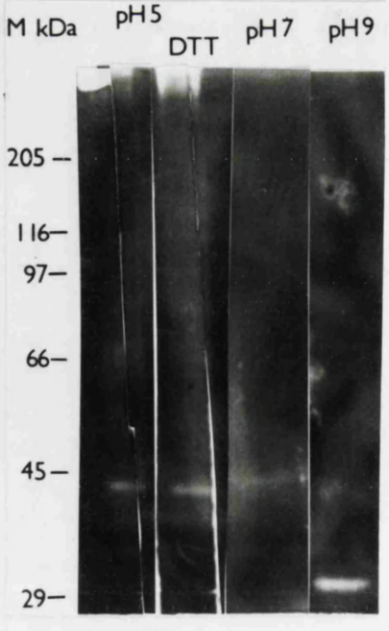


Figure 3.3 The Effect of Proteinase Inhibitors on *T. circumcincta* IVR Proteinases

7.5 % SDS-PAGE containing 0.1% gelatin were run under non-reducing conditions prior to Coomassie staining. The effect of proteinase inhibitors on adult (Fig. 3.3a) and L4 (Fig. 3.3b) IVR proteinases at pH 9 was assessed where C = control; PmsF = a serine proteinase inhibitor; Phen = 1,10 phenanthroline, a metallo proteinase inhibitor; EDTA = a metallo proteinase inhibitor; E 64 = a cysteinyl proteinase inhibitor and Pepst = pepstatin, an aspartyl proteinase inhibitor. Molecular weight markers (M) are shown in kilo-daltons (kDa).

Figure 3.3a

Effect of Inhibitors on Adult IVR Proteinases at pH 9

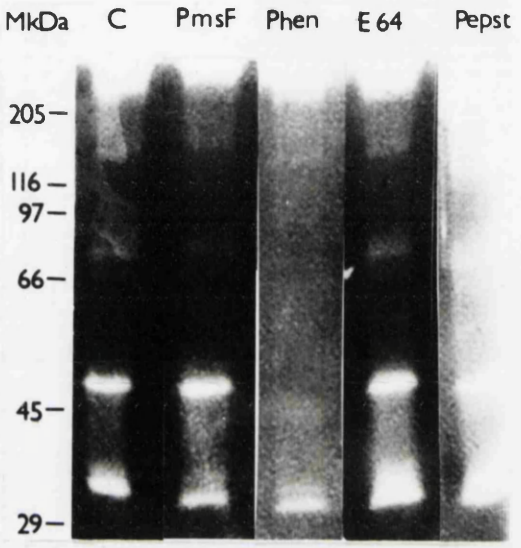
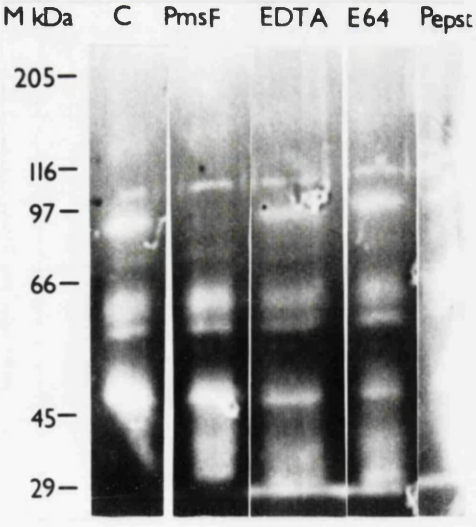


Figure 3.3b

Effect of Inhibitors on L4 IVR Proteinases at pH 9



3.2.6 **Degradation of Potential Natural Protein Substrates by Adult IVR Products**

Fibrinogen, plasminogen, albumin, immunoglobulin, complement serum components and haemoglobin, potential natural protein substrates, were incubated with IVR products from adult parasites to determine if any of these proteins were possible substrates for the parasite proteinases *in vivo*. The digestion products were analysed by 10% SDS-PAGE and compared to unincubated protein alone lanes.

Of the several natural protein substrates tested at pH 9 (Fig. 3.4a) only fibrinogen was degraded by adult IVR products. This was restricted to the α (63.5 kDa) and β (56 kDa) peptide chains. Degradation peptide products at 29 kDa and 31 kDa were clearly visible in adult IVR digests. All three fibrinogen peptide chains α, β and γ (47 kDa) were degraded at acidic pH, (Fig. 3.4b), yielding three degradation products below 29 kDa. This is in contrast to the degradation products at 29 kDa and 31 kDa observed at alkaline pH (Fig. 3.4a). At pH 4, degradation of plasminogen and albumin was also observed (Fig. 3.4b). The lower bands at approximately 52 and 41 kDa in the plasminogen lanes were breakdown products due to auto proteolysis.

The effect of several proteinase inhibitors on fibrinogen degradation at pH 9 (Fig. 3.5a) and pH 4 (Fig 3.5b) were tested. At pH 9, fibrinogen degradation was completely inhibited by 1,10 phenanthroline (Fig. 3.5a). Although at pH 4 (Fig. 3.5b), several peptides were evident in the 27 kDa to 35 kDa region, fibrinogenolysis was apparently impaired by pepstatin as judged by the re-appearance of the α, β, and γ peptide chains. In addition 1,10 phenanthroline also impaired degradation (Fig. 3.5b). At pH 4, degradation of plasminogen (Fig. 3.5c) and albumin (Fig. 3.5d) were inhibited by the aspartyl inhibitor, pepstatin.

Figure 3.4 Substrate Degradation by Adult *T. circumcincta* IVR Proteinases

10 % SDS-PAGE were run under reducing conditions prior to Coomassie staining. The degradation of a variety of protein substrates by adult IVR proteinases at pH 9 (Fig. 3.4a) and pH 4 (Fig. 3.4b). For each substrate, the left-hand lane is substrate incubated with IVR products, the right-hand lane is the substrate control alone. Molecular weight markers are shown in kilo daltons (kDa).

Figure 3.4a

Substrate Degradation by Adult IVR Proteinases at pH 9

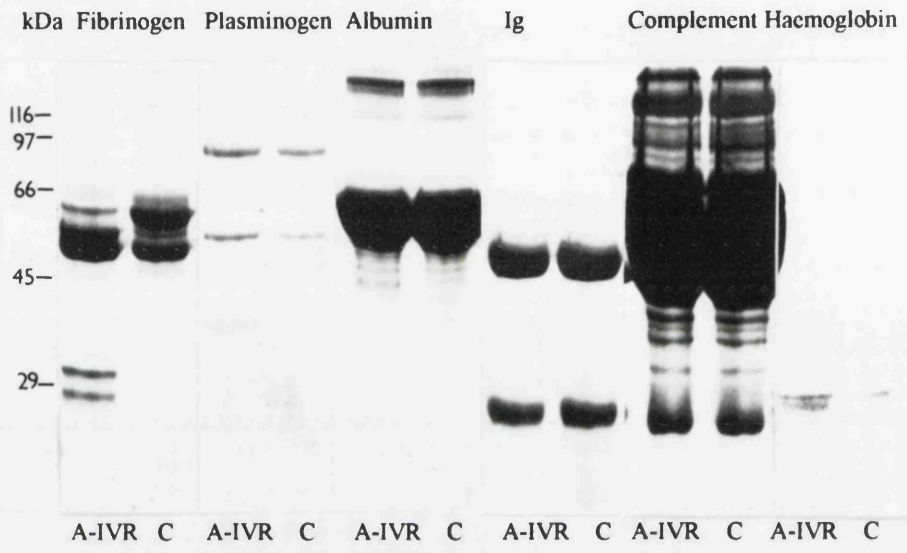


Figure 3.4b

Substrate Degradation by Adult IVR Proteinases at pH 4

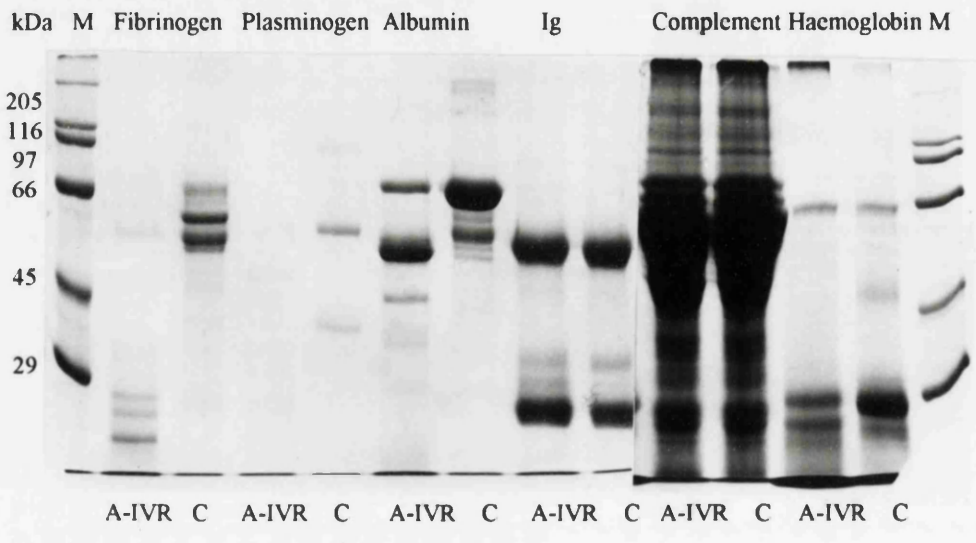


Figure 3.5 The Effect of Proteinase Inhibitors on Fibrinogen Degradation by Adult IVR Proteinases

10 % SDS-PAGE were run under reducing conditions prior to Coomassie staining and the effect of various proteinase inhibitors on fibrinogen degradation by adult IVR at pH 9 (Fig. 3.5a) and pH 4 (Fig. 3.5b) are shown.

From left to right in Fig. 3.5a: C = fibrinogen control; M = high molecular weight markers; A IVR C = adult IVR products + fibrinogen; PmsF = IVR products, fibrinogen and serine inhibitor; E 64 = IVR product, fibrinogen and cysteine inhibitor; 1,10 phen = IVR, fibrinogen and 1,10 phenanthroline, metallo inhibitor; Pepst = IVR products, fibrinogen and aspartyl inhibitor; EDTA = IVR products, fibrinogen and metallo inhibitor. In Fig. 3.5b: Fib C = fibrinogen control, all other lanes as Fig. 3.5a.

Figure 3.5a

Effect of Inhibitors on Fibrinogen Degradation by Adult IVR Proteinases at pH 9

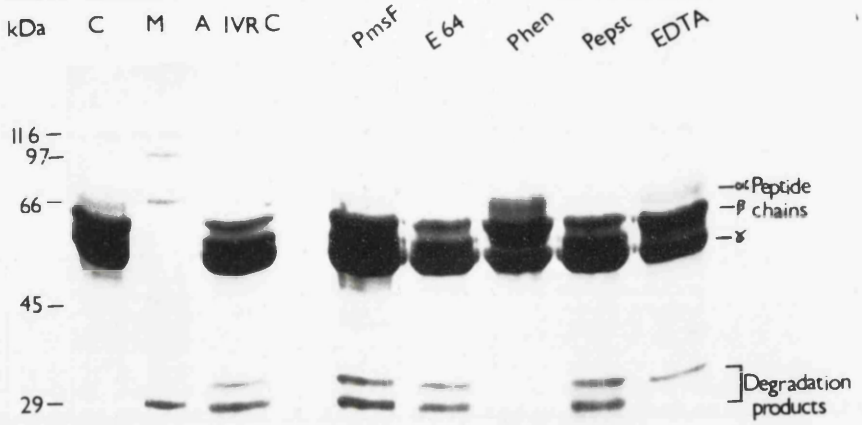


Figure 3.5b

Effect of Inhibitors on Fibrinogen Degradation by Adult IVR Proteinases at pH 4

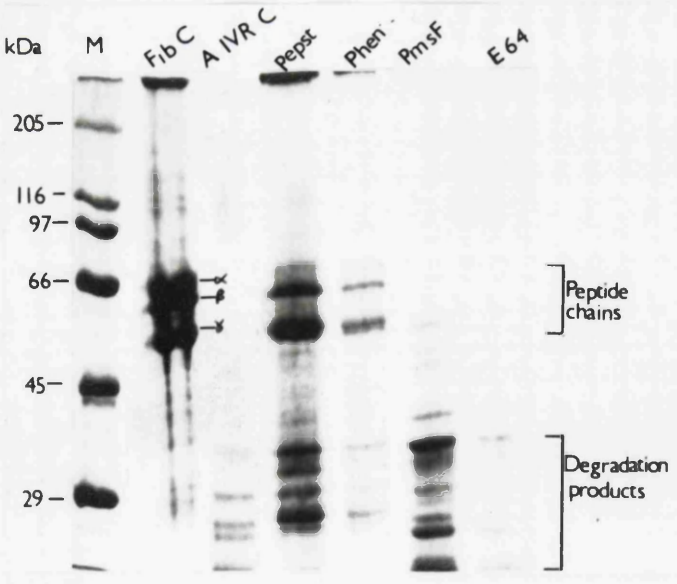


Figure 3.5 continued **The Effect of Proteinase Inhibitors on Degradation of Plasminogen and Albumin by Adult IVR Proteinase at pH 4**

10 % SDS-PAGE were run under reducing conditions and the effect of inhibitors on the degradation of plasminogen (Fig. 3.5c) and albumin (Fig. 3.5d) at pH 4 are shown. From left to right the lanes are; M = high molecular weight markers, C = substrate alone; Adult IVR = IVR products and substrate. The following are with IVR products and substrate: Phen = 1,10 phenanthroline, metallo inhibitor; E 64 = cysteine inhibitor; Pep = pepstatin, aspartyl inhibitor and PmsF, serine inhibitor.

Figure 3.5c

Effect of Inhibitors on Plasminogen Degradation by Adult IVR Proteinases at pH 4

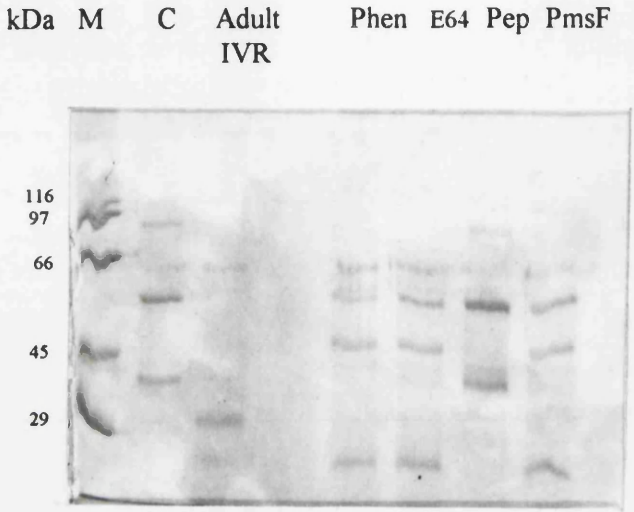
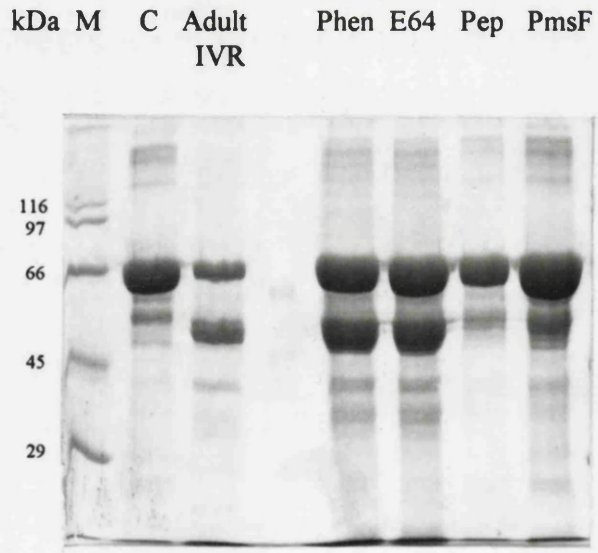


Figure 3.5d

Effect of Inhibitors on Albumin Degradation by Adult IVR Proteinases at pH 4



3.2.7 **Degradation of Potential Natural Protein Substrates by L4 *T. circumcincta* IVR Products**

Similarly to the results obtained with adult IVR products, fibrinogen was degraded by L4 IVR products at pH 9 although there was an indication that plasminogen was also degraded (Fig. 3.6a). However with the L4 IVR products only the α-peptide chain of fibrinogen was degraded and no degradation products were evident. At pH 4 fibrinogen, plasminogen and albumin were degraded. Again only the α fibrinogen chain was degraded and this time a product at <29 kDa was evident. Lack of material prevented studies on inhibitor sensitivity of these proteinases.

Figure 3.6 The Degradation of a Variety of Protein Substrates by L4 IVR Proteinases

10 % SDS-PAGE were run under reducing conditions prior to Coomassie staining. The degradation of a variety of protein substrates by L4 IVR proteinases at pH 9 (Fig. 3.6a) and pH 4 (Fig. 3.6b) are shown. For each substrate, the left-hand lane is substrate incubated with IVR, the right-hand lane is a substrate alone control.

Figure 3.6a

Substrate Degradation by L4 IVR Proteinases at pH 9

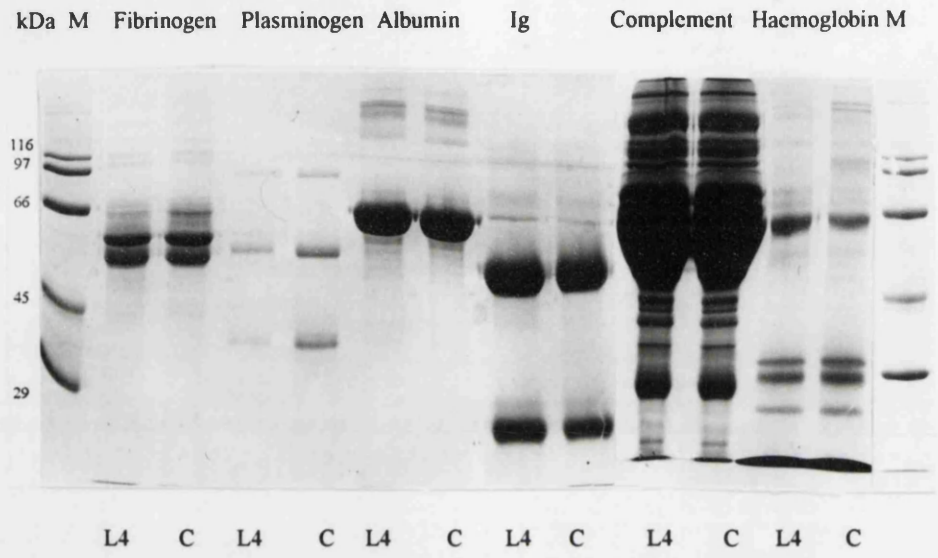
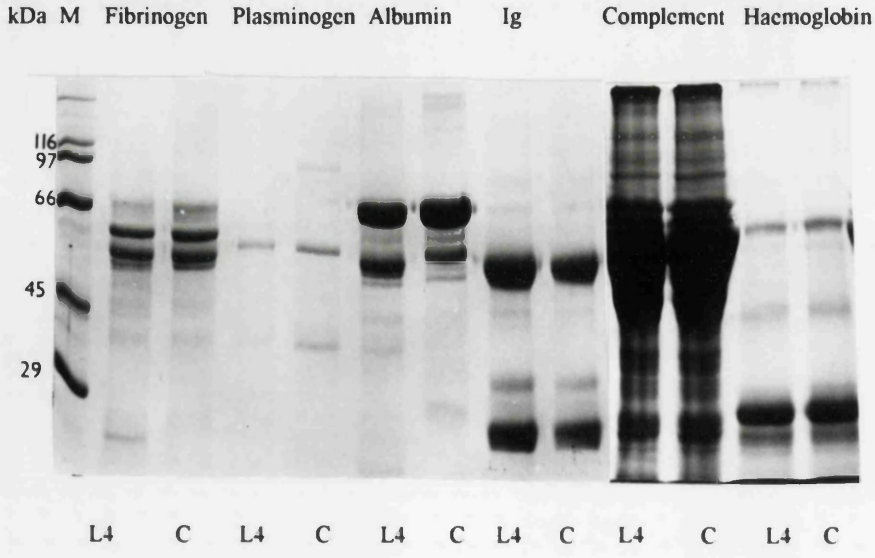


Figure 3.6b

Substrate Degradation by L4 IVR Proteinases at pH 4



3.2.8 **Inhibitory Effect of Immunoglobulin, Lymph and Plasma on Adult *T. circumcincta* IVR Proteinases**

Adult IVR products (5 μg) were incubated with either purified sheep IgG (10 μg), gastric lymph or plasma (10 μl), (Fig. 3.7) for 30 min. prior to separation through native 7.5% polyacrylamide gels containing 0.1% gelatin. Native gels were used to minimise the disruption of protein/antigen/antibody interactions which could occur in the presence of SDS.

IgG was purified from six sheep which were six months old at the start of the experiment. Four sheep (17, 20, 25 and 33) had prior exposure to a natural pasture infection. At the start of the experiment all the sheep were given anthelmintics to clear any existing infection. Sheep 33 was not re-infected. Sheep 17, 20 and 25 were then given two infections of 50,000 *T. circumcincta* L3, eight weeks apart, with a dose of anthelmintic four weeks after the primary infection. IgG was purified from plasma taken eight weeks after the secondary infection. Sheep 83 and 85 were housed worm-free from birth. Plasma (P11, 12) and gastric lymph (L11, 12) were taken from two, six month old sheep (11 and 12) which had been exposed to trickle infection of *T. circumcincta* L3, given anthelmintic and challenged with 50,000 *T. circumcincta* L3. The plasma and lymph were taken 8 and 7 days post challenge respectively. Plasma and lymph were also taken from sheep 13 and 19, which had been exposed to a trickle infection, followed by anthelmintic. The lymph and plasma from these animals was taken on day zero of the challenge infection. Sheep 27 and 29 were never exposed to *T. circumcincta*. No lymph was taken from these two animals..

The plasma for IgG purification was kindly provided by Dr M Stear, Division of Large Animal Clinical Studies, Glasgow University Veterinary School, the rest of the plasma and lymph was kindly provided by Dr L. Stevenson, Moredun Research Institute.

Purified plasma IgG from both naive sheep (Lanes 85 and 83), a sheep exposed to a natural infection for the first six months of it's life (33) and putatively immune sheep (Lanes 25, 20 and 17) had little or no inhibitory effect on adult IVR proteinases. However lymph from immune sheep (Lanes 11 and 12) and sheep previously exposed (13 and 19) totally inhibited activity (Fig. 3.7 lane, L11) and greatly reduced activity

(Fig. 3.7 Lanes, L12, L13 and L19). Plasma from the corresponding immune sheep from which lymph had been taken also had an inhibitory effect in Lanes P11, P12, P13 and P19. P27 and P29 were IVR products incubated in the presence of plasma from naive sheep. Here inhibition of activity was evident compared to the antigen only control lanes (C), however the reduction in activity is not as marked as Lanes containing plasma from immune and previously exposed sheep.

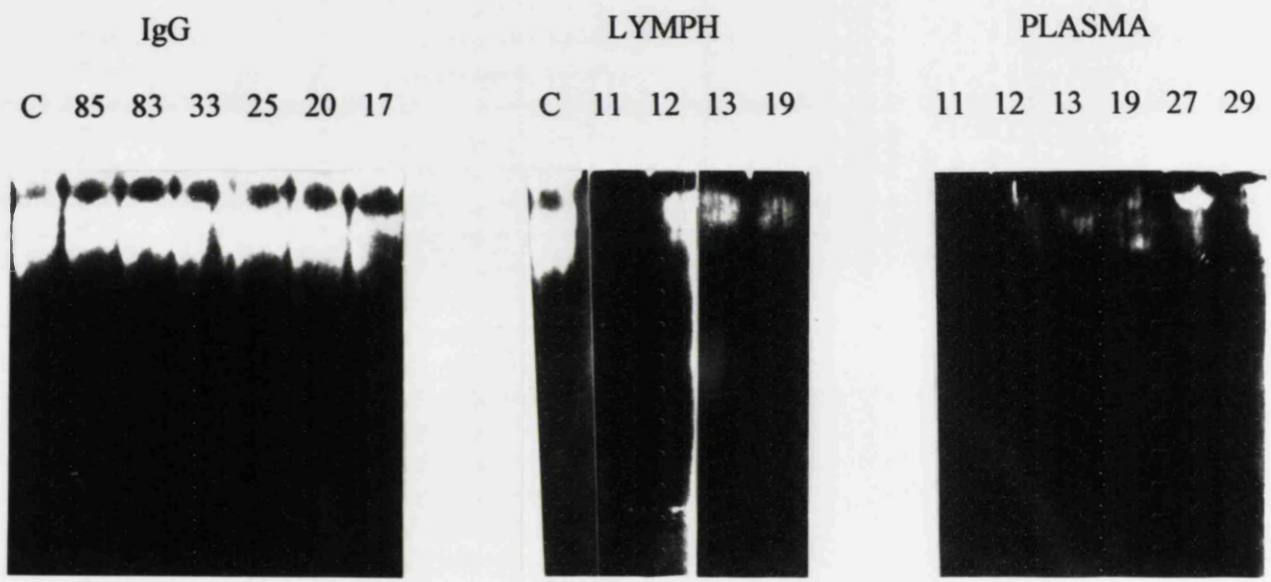
Figure 3.7 Incubation of Adult IVR Products with IgG, Lymph or Plasma

IVR products were incubated with purified IgG, immune lymph or plasma harvested from naive or immune sheep prior to electrophoresis through 7.5 % native polyacrylamide gels and Coomassie staining.

Lanes are as follows; C = IVR alone; 85 and 83, purified IgG from naive sheep; 33, 25, 20 and 17 purified IgG from infected sheep; Lymph 11, 12, 13 and 19, gastric lymph from immune sheep; Plasma 11, 12, 13 and 19, plasma from immune sheep and plasma 27 and 29 from naive animals.

Figure 3.7

Adult *T. circumcincta* IVR Products Incubated with either IgG, Lymph or Plasma



3.3 DISCUSSION

This study has demonstrated that proteinases are released by adult and larval *T. circumcincta* during *in vitro* maintenance. IVR products from various stages showed maximal activity at acidic (L3) or alkaline (L4 and adult) pH. In addition, stage-specificity was indicated when proteinases in adult and larval IVR were compared by gelatin-substrate gel analysis and was confirmed by their comparative sensitivity to class-specific proteinase inhibitors. Comparative analyses of adult IVR and somatic proteinases yielded markedly different profiles. This indicated that the proteinases detected in IVR were not likely to have resulted from non-specific "leakage" due to damage or viability of the cultured parasites. Finally, L4 and adult IVR proteinases exhibited contrasting abilities to degrade a panel of protein substrates.

The alkaline pH optimum observed is at variance with the acidic environment which exists in the ovine abomasum. However, the abomasal pH increases during parasite infection as a result of damage to the parietal cells and resultant reduction in hydrochloric acid release (Thomas and Waller, 1975). In addition, the micro-environment in the vicinity of the parasite at the mucosa may be radically different from that observed in the lumen due to local mucus secretion, immune effector cell responses and leakage of plasma proteins. Moreover, the choice of protein substrate used to determine enzyme activity can influence the pH optimum determined. For example, the relative proteinase activity present in extracts of *Dirofilaria immitis* and *Ascaris suum* showed considerable (up to 75%) variation depending on the protein substrate used for analysis and differed by up to 3 pH units (Maki and Yanagisawa, 1986).

Proteolytic analysis of the closely related bovine parasite, *O. ostertagi*, produced some very similar results to those described in this chapter. Azocasein degradation was maximal at pH 5-6 with adult somatic extracts and at pH 9 with the IVR products from the L4 stage (de Cock *et al*, 1993) These results confirm the results described here because Teladorsagia and Ostertagia species are closely related. Comparison of proteinase profiles from the L4 IVR products of the two species indicated that a proteinase of approximately 116 kDa, and two in the size range of 55-75 kDa appear to be present in both species. In addition inhibitor sensitivity analysis of

O. ostertagi L4 IVR products (de Cock *et al,* 1993) indicated that metallo and serine proteinases were present, similar to the findings observed with *T. circumcincta* L4 IVR products. These results indicate that these proteinases may be of particular importance to the host parasite relationship due to their apparent expression in both parasites and future analysis of the amino acid or nucleotide sequence may provide information on the evolutionary origins of the two parasites. As the L4 is the stage which invades the gastric pits of the host mucosa, it may be that these proteinases are important for allowing the parasite to establish itself, by enabling penetration of host tissue, providing the parasite with its nutritional requirements or by evading the host immune system.

Stage-specific proteinase release from *T. circumcincta* was indicated in the molecular size and inhibitor sensitivity analyses. While metallo-proteinases were predominant in adult IVR (Fig 3.3a), a serine proteinase was identified in L4 IVR products (Fig. 3.3b). The L4 IVR products also contained several other activities which could not be ascribed clearly to a particular class with the inhibitors used in the analysis. A different proteinase profile was observed with the L3 IVR (Fig 3.2c) where cysteine proteinases were predominant. These differing profiles may reflect the differing intra-host environments encountered by these various parasite stages and their different roles. It can be anticipated that the invasive L3 stage has, at least initially, a requirement for proteinases active at acidic pH to facilitate feeding and invasion in the highly acidic abomasum. The L4 lies within the mucosa where the environmental pH may be fairly neutral and although the adult parasite is found on the abomasal surface, the local pH might be markedly altered as a result of the pathogenic effect of the parasite and the inflammatory and immune response to the parasite.

Although stage-specific proteinase release during *in vitro* maintenance has been demonstrated for a variety of gastro-intestinal nematodes (Knox and Kennedy, 1988; Robertson *et al.*, 1989; Healer *et al.*, 1991 and de Cock *et al,* 1993), the function of these enzymes remains undefined. A metallo-proteinase with elastinolytic properties has been described in *Ancylostoma caninum* which may facilitate penetration of the host intestinal mucosa and the penetration of mucosal capillaries (Hotez, Le Trang, McKerrow and Cerami, 1985). The results of the protein degradation studies indicated that, at pH 9, adult IVR proteinases had specificity for the α- and β-peptide

chains of fibrinogen and that this activity was primarily due to metallo-proteinases (Fig. 3.5a). At acidic pH, all three fibrinogen peptide chains were degraded, and this was due to aspartyl proteinase activity (Fig. 3.5b). Although this class of proteinase was not detected by initial gelatin gel analysis, it has been noted that aspartyl proteinases are not readily visualised using this substrate gel combination (Dr D.P. Knox, personal communication). The future use of a substrate specific for aspartyl proteinase activity may enable this activity to be visualised in a gel system. Specific fibrinogen degradation at alkaline pH compared to the broad proteinase substrate specificity at acidic pH, observed in the present experiments, would suggest that alkaline IVR proteinases have an anticoagulant function although *T. circumcincta* is not an obligate blood-feeder. Degradation of plasminogen and albumin was also due to aspartyl proteinase activity, similarly indicating a possible anticoagulant function of the parasites proteinases. As to why the adult parasite appears to secrete proteinases with anticoagulant functions is a mystery.

Limited data indicated that L4 IVR contained at least one proteinase with similar properties to adult IVR products. Proteinases with an anticoagulant function would make more sense at the L4 stage as it is closely associated with the mucosa, the damage of which would expect to produce an influx of blood proteins such as fibrinogen, plasminogen and albumin. The adult parasite may also use proteins such as albumin, fibrinogen and plasminogen as they leak across the mucosa to the abomasum, not distinguishing them from another proteins in the local environment.

At acidic pH, adult IVR proteinases degraded a variety of proteins (Fig. 3.4b). It was notable that neither L4 nor adult IVR proteinases degraded complement or IgG, this is in contrast to the proteinases released by the closely related ovine abomasal parasite *H. contortus* (Dr D.P.Knox personal communication), and the ovine intestinal nematode *T. vitrinus* (MacLennan PhD thesis, 1995). While these observations may indicate that adult IVR proteinases are not required for evasion of these host immune responses, this conclusion is reached with caution in the light of the restricted set of protein substrates used. It is feasible, however, that the IVR proteinases may degrade other host proteins such as IgA and cytokines.

Attempts to demonstrate that the IVR proteinases of *T. circumcincta* were immunogenic in sheep infected with *T. circumcincta* proved difficult. The running of

native gelatin substrate gels with adult IVR products previously incubated in the presence of immune gastric lymph or plasma, however, produced evidence that host recognition did occur through the reduction or inhibition of proteolytic activity, (Fig. 3.7). This inhibition was very variable, an outcome which could reflect the comparative insensitivity of the technique used. Alternatively, the inhibition observed may have reflected individual host variation in circulating proteinase inhibitors such as α_2- macroglobulin or α_1-antitrypsin. Evidence of antibody-proteinase interactions has been provided in similar studies in *D. viviparus* (Britton *et al*, 1992) and *H. contortus* (Knox, Redmond and Jones, 1993). While no inhibition was observed with purified serum IgG it cannot be concluded that specific immunoglobulin is not involved because the serum tested may not have contained the specific local isotype i.e. IgA.

In summary, the present study has provided a comprehensive definition of the proteinases released *in vitro* by the parasitic stages of *T. circumcincta*. The data indicated that these proteinases were released in a stage-specific manner and this, combined with protein degradation studies, indicated that some enzymes may have very specific roles to play in parasite maintenance within the abomasum, however little evidence of host immune recognition to parasite derived components which were analysed here was obtained and requires further study.

Chapter Four

Biochemical Comparison of a UK and Australian Strain of *Haemonchus contortus*

4.1 INTRODUCTION

Enzymes have been implicated in parasite maintenance and survival within the host. For example, proteinases (Knox and Jones, 1990; Healer, Ashall and Maizels, 1991), superoxide dismutase (Knox and Jones, 1992) and AChEs (Ogilvie, Rothwell, Bremner, Schnitzerling, Nolan and Keith, 1973; Jones and Knox, 1990; Griffiths and Pritchard, 1994), have been ascribed particularly important roles at the host/parasite interface in GI nematodes (reviewed Knox, 1994). A number of studies have demonstrated immune recognition of parasite enzymes (Knox and Kennedy, 1988; Britton, Canto, Urquhart and Kennedy, 1993; McKeand *et al,* 1994b) and proteins released *in vitro* by *H. contortus* stimulate humoral and cellular immune responses in Texel sheep (Schallig, van Leeuwen and Hendrikx, 1994). Vaccination of sheep with a purified cysteine proteinase from *Fasciola hepatica* has also been demonstrated to reduce parasite burdens following homologous challenge (Wijffels *et al,* 1994) and McKeand, Knox, Duncan and Kennedy (1995) reported evidence of protection in guinea pigs *to D. viviparus* when vaccinated with parasite enriched AChE fractions. Similarly the parasite derived enzyme glutathione-S-transferase (GST) from *F. hepatica* has been demonstrated to significantly reduce parasite burdens in sheep (reviewed McKeand and Knox, 1995). Thus the possibility exists that future vaccine development may in part, be dependent on the use of parasite derived enzymes. There is however, evidence which may indicate geographical strain divergence in parasite enzyme expression. For example a cysteine proteinase (AC1) present in an American strain of *H. contortus* (Pratt *et al,* 1992) could not be detected in a UK strain of the same parasite species (Knox, Redmond and Jones, 1993). These differences may be due to genotypic divergence or may reflect phenotypic changes induced by the intra-host environment. The fact that such strains are different could have profound implications for the development of subunit anti-parasite vaccines using specific parasite products; for instance a vaccine developed on the basis of an enzyme expressed in one strain of parasite which is not present in other strains would render the vaccine ineffective.

In this chapter a number of enzymes expressed by a UK and Australian strain of *H. contortus* were compared. The enzymes studied were chosen because of their importance to the parasite and their possible role in the host parasite relationship. As

indicated above, there is evidence for strain divergence in parasite proteinase gene expression (Knox, Redmond and Jones 1993) and the same authors noted that proteinase expression was elevated in adult *H. contortus* exposed to host anti-parasite antibody responses. There is also evidence to suggest that profound differences may occur in proteinases released by parasites from different geographical locations (Karanu *et al*, 1993; Rhoads and Fettener, 1995). In addition, elevated levels of AChE, SOD and acid phosphatase as well as altered isoenzyme expression have been noted in *N. brasiliensis* harvested from rats which have previously experienced infection (Jones and Ogilvie, 1972; Edwards, Burt and Ogilvie, 1972; Knox and Jones, 1992). The potential importance of these enzymes to parasite survival suggest that these changes, in part, determine the longevity of parasite survival within the host.

In this chapter, proteinase, AChE, SOD and acid phosphatase activity was analysed in a comparison of UK and Australian strains of *H. contortus*. In these studies lactate dehydrogenase (LDH) activity was also determined in parasite extracts to act as a constituently expressed control enzyme. LDH is found in all glycolysing cells (Long, 1961) and enzymes involved in metabolising glucose are generally less variable than other enzymes (reviewed Nadler, 1990).

4.2 RESULTS

In all analyses comparable amounts of protein were run on each lane and samples were prepared as described in Chapter 2 (Section 2.5.1).

UK parasites used were from a parasite isolate which has been maintained at Moredun Research Institute for a number of years. On collection, parasites were stored in liquid nitrogen until required. The Australian strain was kindly provided by Dr Sue Newton, Pitman-Moore, Melbourne, Australia, and was snap frozen in liquid nitrogen and transported to the UK in the same medium. The parasite samples were prepared as described in chapter two with the S1 extracts containing water soluble proteins and the S2 extracts containing membrane associated proteins.

4.2.1 Proteins

7.5% non-reducing SDS-PAGE gels were run as described in Section 2.6.2, and stained for proteins with Coomassie blue. Non-reducing conditions were used for this analysis to be comparable to the gel conditions used in the analyses of proteinases and AChE 'isoenzymes'. Comparison of the S1 protein profiles of UK and Australian (Aust) samples indicated that they were very similar. In both extracts a major protein band was evident at 55 kDa and a number of relatively minor bands were present between 29 and 55 kDa as well as faint protein bands between 55 and 116 kDa. However, 3 prominent protein bands at 87, 116 and 205 kDa were present in the Aust S2 extract which were not evident in the comparable extract from UK *H. contortus* (Fig. 4.1). A major protein band at 55 kDa was again visible as were the numerous minor bands ranging in size from 29 to 55 kDa.

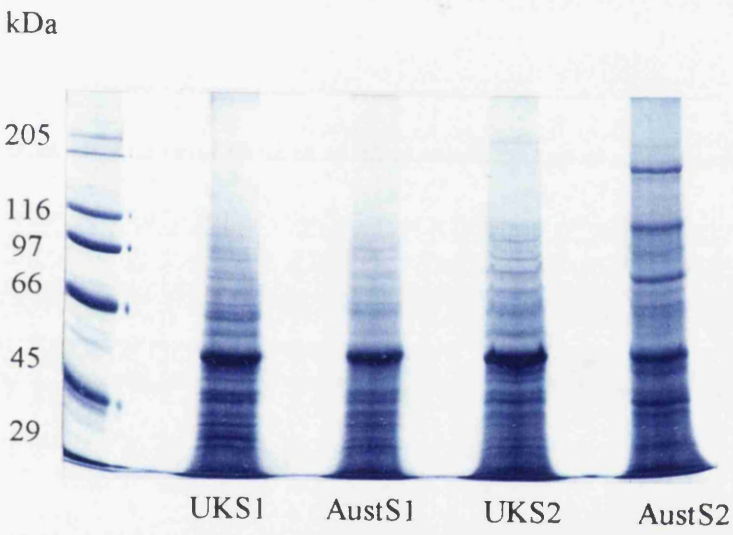
Figure 4.1 Non-reducing SDS-PAGE Analysis of the Proteins Present in Water-Soluble (S1) and Membrane Associated (S2) Extracts of UK and Australian Isolates of *H. contortus*

7.5% non-reducing SDS-PAGE of UK S1, Australian (Aust) S1, UK S2 and Australian S2 extracts stained with Coomassie blue.

Lanes are as follows: kDa = molecular weight markers in kilo-daltons; UK S1 = water soluble extract of UK *H. contortus* strain; Aust S1 = water soluble extract of Australian *H. contortus* strain; UK S2 = detergent extract, containing membrane associated proteins of UK *H. contortus* strain; Aust S2 = detergent extract, containing membrane associated proteins of Australian *H. contortus* strain.

Figure 4.1

Protein Profile of UK and Australian *H. contortus*



4.2.2 Comparison of Various Total Enzyme Activities in Pooled S1 and S2 Extracts of UK and Australian *H. contortus*

Proteinase, with azocasein as a substrate, AChE, SOD and LDH activities were determined spectrophotometrically at 30°C using a Monarch microcentrifugal analyser (Table 4.1) and expressed per mg protein and the methods described in Chapter 2. The S1 and S2 samples were pooled together for statistical analysis and enabled direct comparison of the enzyme activities of the two strains of parasite.

Table 4.1

Total Enzyme Activities from UK and Australian *H. contortus*

Enzyme	Pooled S1/S2	Mean	No. of Assays	Standard deviation	Significant Difference
Proteinases OD_{405} nm at pH 5.5	**UK**	57.3	n = 2	+/- 21.5	**$p < 0.05$**
	Australian	18.7	n = 2	+/- 5.6	
AChE IU $x10^{-3}$	**UK**	6.2	n = 5	+/- 5.3	**N/S**
	Australian	8.1	n = 5	+/- 4.7	
*** Superoxide Dismutase U**	**UK**	5.6	n = 2	+/- 4.0	**N/S**
	Australian	5.6	n = 2	+/- 6.0	
Lactate Dehydrogenase IU $x10^{-3}$	**UK**	5.5	n = 1	N/A	**N/A**
	Australian	1.4	n = 1	N/A	

Statistical analyses were performed using the Mann Whitney U test. Medians were considered significantly different at $p < 0.05$ level.

* S1 extract only; N/S = not significant; N/A = not applicable.

Proteinases: The total proteolytic activity of the pooled S1 and S2 extracts of UK and Australian strains was determined at pH 4, pH 5.5 and pH 8.5 as described in Section 2.7.1. Proteolytic activity in both strains was maximal at pH 5.5 and the activity at this pH of the UK strain was significantly elevated compared to the Australian ($p < 0.05$). At the other pHs tested (not shown) proteolytic activity in the UK parasites was also significantly different compared to the Australian worms.

AChE: Total AChE activity of the pooled S1 and S2 extracts for the two strains of *H. contortus* was determined as described in Section 2.7.3. Activity in the Australian strain was higher than that in the UK strain although this difference was not significant.

SOD: The SOD activity in the S1 extracts was determined on two separate occasions as described in Section 2.7.5. It was not possible to determine SOD activity in the S2 extracts due to the interference of endogenous enzyme in the extracts. The levels of activity observed in the two strains of parasite were not significantly different.

LDH: LDH activity was determined as described in Section 2.7.4. Activity was only determined on one occasion due to the limited amount of material available. Although no statistical analysis therefore could be performed, it appeared that the UK strain had markedly higher LDH levels than the Australian strain.

4.2.3 **Isoenzyme Expression in UK and Australian *H. contortus***

Proteinases: Proteolytic activity was detected by fractionating the samples (20 μg) on 7.5% SDS-PAGE gels containing 0.1% gelatin and incubating the gel for 16 h in 0.1 M sodium acetate buffer at pH 5.5 in the presence of the cysteine proteinase activator dithiothreitol (5 mM, DTT). Degradation of gelatin was then determined by staining the gel in Coomassie blue and destaining in 10% acetic acid as described in Section 2.

The most striking difference between the UK and Australian extracts was in the comparative levels of gelatin degradation observed. In both the S1 and S2 extracts from UK *H. contortus* degradation was greatly enhanced compared to the Australian extracts (Fig. 4.2). Proteolytic profiles of the two S1 samples were similar and exhibited a number of proteinases ranging in size from 45 to >205 kDa. A band at 40 kDa evident in both the UK and Australian S1 extracts appeared to be stronger in the Australian extract compared to the UK extract.

Proteolytic activity in the UK S2 extract was reduced compared to the S1 extracts and was very weak in the Australian S2 extract. Comparison of the profiles did not indicate any differences between the two strains although activity in the UK lane was again comparatively higher (Fig. 4.2).

Figure 4.2 Proteinase Profile of UK and Australian *H. contortus* at pH 5.5 + DTT

7.5% gelatin substrate gel analysis at pH 5.5 in the presence of 5 mM DTT of UK S1, Australian (Aust) S1, UK S2 and Australian (Aust) S2 extracts.

Lanes are as follows: kDa = molecular weight markers in kilo-daltons; UK S1 = water soluble extract of UK *H. contortus* strain; Aust S1 = water soluble extract of Australian *H. contortus* strain; UK S2 = detergent extract, containing membrane associated proteins of UK *H. contortus* strain; Aust S2 = detergent extract, containing membrane associated proteins of Australian *H. contortus* strain.

Figure 4.2

Proteinase Profile of UK and Australian *H. contortus* at pH5.5 + DTT

Esterases: The UK and Australian samples were fractionated in either 7.5% native polyacrylamide gels or in non reducing SDS-PAGE gels and stained for non specific esterase activity as described in Section 2.8.5.

Fractionation of the extracts under non-reducing conditions (Fig. 4.3a) produced a clearer isoenzyme profile than when the extracts were separated under native conditions (Fig. 4.3b). Using both procedures, differences in the esterase profiles of the UK and Australian strains were detected. Three zones of activity at 55, 66 and >205 kDa were visible in the UK S1 lane whereas 4 zones of activity at 29, 55, 97-116 and >205 kDa were detected in the Aust S1 extract (Fig. 4.3a). In the UK S2 extract, faint zones of activity were detected at 55 and 97-116 kDa while zones of activity were strongly evident at 29 and 55 kDa in the Aust S2 extract.

Differences in the profiles observed when the samples were run under native conditions were not as marked (Fig. 4.3b). Two slow migrating bands were evident in the UK S1 extract, while a diffuse faster migrating band was present in the Aust S1 extract. Comparison of the S2 extracts showed a smear of activity in the Australian lane which did not resolve into definite bands and single slow migrating band of activity in the UK extract (Fig. 4.3b)

Figure 4.3a and b **Comparison of Esterase Isoenzymes in Extracts of UK and Australian *H. contortus***

A 7.5% polyacrylamide gel of UK S1 and S2 extracts and Australian (Aust) S1 and S2 extracts was run under non-reducing (Fig. 4.3a) and native (Fig. 4.3b) conditions.

Lanes are as follows: kDa = molecular weight markers in kilo-daltons; UK S1 = water soluble extract of UK *H. contortus* strain; Aust S1 = water soluble extract of Australian *H. contortus* strain; UK S2 = detergent extract, containing membrane associated proteins of UK *H. contortus* strain; Aust S2 = detergent extract, containing membrane associated proteins of Australian *H. contortus* strain.

Figure 4.3a

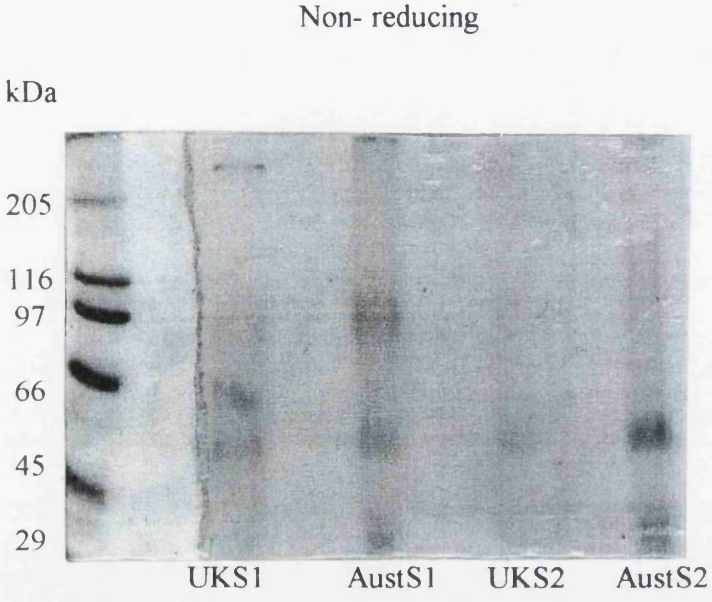
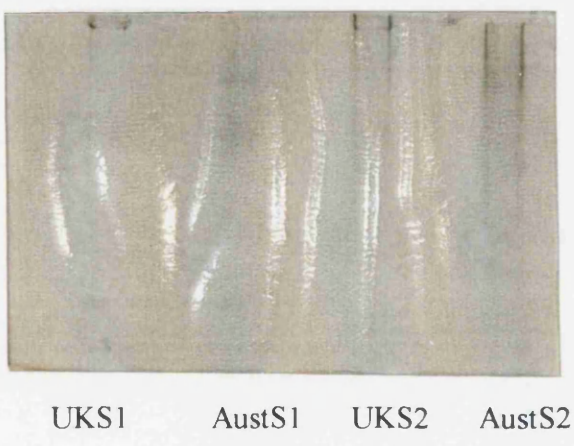


Figure 4.3b



SOD: SOD isoenzymes in the UK and Australian samples were detected by fractionating the extracts on native polyacrylamide gels before staining them in nitroblue tetrazolium by the method of Beauchamp and Fridovich (1971).

Two isoenzymes were detected in both the UK and Aust S1 extracts (Fig. 4.4a), a slow migrating light band at the top of the gel and a faster migrating one about one third of the way down. Variation was detected in the intensities of these isoenzymes, the slow migrating band in the Aust S1 extract being of a greater intensity compared to the UK band (Fig. 4.4a). The faster migrating band in the UK extract was more intense than the Australian band, although this difference was not as marked, while this band appeared to migrate slightly further in the Australian sample. There was no evidence of SOD activity in the S2 extracts.

Acid phosphatase: Acid phosphatase activity was detected by running the samples in 7.5% native polyacrylamide gels and staining based on the method of Gomori (1950).

Little activity was detected in the S1 extracts of the UK and Australian samples except for faint streaking down the lanes. Again in the S2 extracts activity did not resolve as distinct bands (fig. 4.4b) but smeared enzyme activity were detected in both samples. The zone in the Australian extract migrated slightly further than the UK sample.

Figure 4.4 SOD and Acid Phosphatase in Extracts of UK and Australian *H. contortus*

UK and Australian (Aust) S1 and S2 extracts of *H. contortus* were fractionated in a 7.5% native polyacrylamide gel which was then stained for SOD (Fig. 4.4a) or acid phosphatase (Fig. 4.4b) activity.

Lanes are as follows: UK S1 = water soluble extract of UK *H. contortus* strain; Aust S1 = water soluble extract of Australian *H. contortus* strain; UK S2 = detergent extract, containing membrane associated proteins of UK *H. contortus* strain; Aust S2 = detergent extract, containing membrane associated proteins of Australian *H. contortus* strain.

Figure 4.4a

Superoxide Dismutases in UK and Australian *H. contortus*

UKS1 AustS1 UKS2 AustS2

Figure 4.4b

Acid Phosphatases of UK and Australian *H. contortus*

UKS1 AustS1 UKS2 AustS2

4.3 DISCUSSION

This study indicated that there was some degree of enzymatic variation in geographically different strains of adult *H. contortus*. This variation was evident i) in a significantly higher level of proteolytic activity in the UK strain; ii) differences in esterase isoenzyme profiles and iii) variation in the intensity of SOD isoenzyme activities.

The protein profiles of the UK and Australian S2 extracts differed, while the S1 profiles were identical (Fig 4.1). Three extra protein bands were evident in the Australian S2 extract, compared with the UK S2. The significance of these differences could not be assessed as the proteins were undefined. It is unlikely that these differences were due to sample treatment because both samples were prepared at the same time and treated in exactly the same way.

Proteolytic activity was maximal at pH 5.5 and this was in agreement with previous studies, which demonstrated the abundance of cysteine proteinase activity in adult *H. contortus* (Pratt *et al*, 1992; Knox *et al*, 1993). Elevated proteolytic activity in the UK strain compared with the Australian strain was apparent using both spectrophotometric determination of total activity (Table 4.1) and gelatin-substrate gel analysis (Fig. 4.2). This difference in activity was found to be statistically significant at $p< 0.05$ level at the optimal pH 5.5. It is unlikely that the lower level of activity detected in the Australian extracts was due to degradation of the samples in transit as they were frozen immediately upon harvest, transported in dry ice and were kept frozen on receipt. AChE and SOD total activities were similar in the two strains indicating that the Australian samples had not deteriorated. No apparent variation was detected in the proteinase profiles observed although previous studies have identified a number of cysteine proteinase genes in *H. contortus* (Pratt *et al*, 1992), some of which may show pronounced strain differences (Knox *et al*, 1993). Multigene families are thought to encode cysteine proteinase expression in *H. contortus* (Pratt *et al*, 1992) and this may explain the difference in levels of proteinase expression between the two parasite strains if different strains have more affinity for expression of a particular gene.

The level of azocasein degradation observed here for the UK parasite strain was elevated in comparison with previous reports, whereas those of the Australian strain

were in the same range (Knox and Jones, 1990), the differences between previous and the present levels of total proteinase activity may be indicative of within population variation.

Variation in esterase profiles was evident when samples were fractionated in native or non-reducing polyacrylamide gels, more defined banding being evident with the latter technique. A comparison of the esterase profiles using native conditions was essential in case SDS irreversibly inhibited esterase activity. Under non-reducing conditions a more complex profile was evident in all extracts compared with that seen in native gels. Variation was evident in all the profiles, although a zone of activity at 55 kDa was present in all the extracts (Fig. 4.3a). Spectrophotometric determination indicated that AChE levels in the two strains of *H. contortus* did not differ significantly.

Differences in AChE isoenzymes and total enzyme levels have been observed in *N. brasiliensis* harvested from immunologically primed hosts compared to parasites harvested from hosts harbouring a primary infection (Edwards, Burt and Ogilvie, 1971). and in the esterases of *H. contortus* resistant and susceptible to benzimidazoles (BZ) have been observed (Sutherland, Lee and Lewis, 1988). Variation in propionyl esterase activity was also detected between South African and UK *H. contortus* (Echevarria, Gennari and Tait, 1992). Studies on the effect of oxfendazole and mebendazole on extracts of *N. brasiliensis* have shown that AChE levels are elevated in treated compared to untreated worms (Rapson, Lee and Watts, 1981). The increase in somatic enzyme activity was thought to be associated with disruption of microtubule formation in the parasite resulting in an accumulation of AChE inside the worm. Further studies showed that AChE levels in culture fluids were reduced in (BZ) treated parasites maintained *in vitro* compared with untreated worms while AChE levels in somatic extracts remained elevated (Rapson, Lee and Watts, 1981). These findings are indicative of the ability of parasites to modulate esterase and AChE function in response to influences of the host environment.

Although no differences were apparent in the SOD profiles of the two strains, differences were apparent in the quantity of the two isoenzymes present. The faster migrating band in the UK S1 extracts was more intense than the Australian S1 band. The opposite occurred for the slow migrating band. Total SOD activity in the two strains was not significantly different, while SOD activity described here was

consistently lower, except on one occasion, than the levels reported by Knox and Jones (1992). It is worthy of note that only two isoenzymes were detected in the present study compared to three recorded by Knox and Jones (1992). Considering the parasite strain and analytical techniques used were comparable these study differences are difficult to reconcile, however in light of results discussed in Chapter six, this may reflect within population variation of SOD isoenzyme expression.

The differences in proteolytic activity detected here could be due to either strain, effects of the host immune response on the parasite or the parasite responding differently to it's environment. Genetic variation can be between individuals, between strains or between species and this is brought about by genetic heterogeniety within the parasite population and is indicated by their ability to respond to selective pressures (Grant, 1994). Genetic variation in the response to infection by a host is well recorded and it influences the ability of the parasite to maintain an infection for variable periods of time (Wakelin, 1985). *H. contortus* enzymes are immunogenic and antibody responses to them inhibit their activity (Knox, Redmond and Jones, 1993). These host influences are likely to affect parasite enzyme expression as the parasite tries to maintain an infection, potentially, by up-regulating proteinase expression, switching on genes not usually expressed to synthesise antigenically different isoenzymes. Equally, it is clear that parasite AChEs (Rothwell *et al*, 1976; McKeand *et al* 1994b) and SODs (Britton *et al*, 1994) stimulate enzyme inhibiting antibody responses and that different individuals recognise different enzyme isoenzymes, a response that may be MHC restricted (McKeand *et al*, 1994b).

The amount of variation between strains appears to be dependent on the parasite and enzyme analysed. For example comparison of selected isoenzymes from British and Bangladeshi strains of the protozoal parasite *Eimeria tenella* indicated no variation in the six enzymes analysed but the profile of glucose-phosphate-isomerase in a Philippino strain did differ from other geographically separated strains (Karim, Hoare and Trees, 1991). There was some indication however, that one strain of *E. tenella* from Bangladesh was immunologically different from another Bangladesh strain and the UK strain. Vaccination of young birds with sporulated oocyst followed by homologous challenge resulted in protection, as judged by oocyst output, however, heterologous challenge with one of the Bangladesh strains did not protect the birds although the

other two strains did (Karim. Hoare and Trees, 1991). These findings are interesting as the British and Bangladeshi strains of *E. tenella* are exposed to different selection pressures. Apart from pronounced climatic differences, the same authors noted that in Bangladesh, until recently, chemotherapy was not used extensively to control infection in the poultry industry. In a similar experiment, a range of glycolytic enzymes and propionate esterases were compared between a UK and South African strain of *H. contortus* (Echevarria, Gennari and Tait, 1992). No differences were detected with the exception of propionyl esterase activity. These authors concluded therefore, that polymorphism between geographically separate parasite strains is at a low level (Eschevarria, Gennari and Tait, 1992). In this study however, different enzymes were analysed compared to glycolytic enzymes analysed by Karim, Hoare and Trees, (1991) and Eschevarria, Gennari and Tait, (1992) and it has been noted that higher levels of variation are found in enzymes which are not involved in glycolysis (Nadler, 1990).

While the work described in this chapter does indicate that some strain divergence may occur in parasite enzymes of potential importance at the host/parasite interface, the experiments would require consolidation before firm conclusions could be drawn. First, the differences noted here could simply reflect small sample size and it could be argued that similar differences could be evident in comparisons of *H. contortus* within the UK or Australia. To counteract this argument, larger individual samples from different locations within the two geographical regions could be compared. In addition, excretory/secretory enzymes active specifically at the host/ parasite interface, not studied here, are more likely to be divergent as a result of genotypic and phenotypic influences. Finally, the differences observed here could be due solely to host influences on enzyme expression. The genotype could be very similar but gene expression could be up or down-regulated by elements in the intra-host environment, particularly the host immune response. Different sheep breeds are farmed in different regions of the world and these breeds differ in their response to, and susceptibility to infection. Experiments where UK breeds are infected with Australian *H. contortus* and vice versa could help to clarify this.

The effects of host immunity on parasite enzyme expression and intra-population variation in expression of parasite enzymes is looked at more fully in Chapters 5 and 6.

CHAPTER FIVE

ANALYSIS OF SELECTED ENZYMES OF *HAEMONCHUS CONTORTUS* HARVESTED FROM VACCINATED AND CONTROL SHEEP

5.1 INTRODUCTION

As mentioned in the general introduction, a number of integral gut membrane proteins from adult *H. contortus* have been identified as potential targets for vaccine development. One of these, a protein complex, *Haemonchus* galactose-containing glycoprotein complex (H-gal GP) confers a high degree of protection against homologous challenge in lambs (Smith, Smith and Murray, 1994). Faecal egg counts of vaccinated lambs challenged with 5,000 *H. contortus* larvae were reduced by 93% compared to controls. Reduction in worm number (23 d.p.i) was not as marked but was significantly different from controls with a 52.9% reduction (Smith, Smith and Murray, 1994). H-gal GP contains a number of peptide components including aspartyl proteinase activity (D. P. Knox, personal communication). The proposed mechanism of vaccine action is antibody inhibition of enzyme activity leading to worm expulsion. If this is the case, why do some of the parasites survive the lethal host immune response? Results from the previous chapter indicated that there is a possibility that different strains of the same parasite could express different isoenzymes of the same enzyme. In this study, enzyme profiles of worms harvested from unvaccinated control sheep were compared with worms harvested from lambs vaccinated with H-gal-GP. This comparison was performed in an attempt to determine whether stimulation of protective host immune responses by vaccination could select for parasites genotypically or phenotypically capable of surviving this response, an outcome which could have serious implications for the development of parasite antigen vaccines. Identification of differences in levels of expression or changes in enzyme profile, of surviving worms from vaccinated animals may be the first indication of 'resistance' developing.

H-gal GP is made up of a complex of proteins fractionated from the intestines of adult *H. contortus* (Smith, Smith and Murray, 1994). After homogenisation, detergent extract proteins which bound to a peanut lectin column were eluted and used for immunisation (Smith, Smith and Murray, 1994). On 10% SDS polyacrylamide gels run under non-reducing conditions 4 major bands in the size range 230, 200, 190 and 45 kDa were evident (Smith, Smith and Murray, 1994).

The worms used in the enzyme analysis described in this chapter were taken from sheep immunised with either 500 μg or 200 μg H-gal GP complex and compared

with worms harvested from control lambs (Smith and Smith, 1996). The outcome of this trial is shown in Table 5.1. Clearly, there was considerable between group and, within a group.

The enzymes examined in this study included proteinases, esterases, acetylcholinesterase (AChE), acid phosphatase, superoxide dismutase (SOD) and lactate dehydrogenase (LDH). The enzyme activities were defined as total enzyme activity per mg homogenate protein, as well as 'isoenzyme' definition by gel fractionation and specific enzyme staining.

Table 5.1

Individual Worm Counts, Sex Ratios, Final Egg Counts and Eggs/Female of *H. contortus* Harvested from Vaccinated and Control Sheep.

Group	Antigen	Sheep	Total Worms	% Male	Final e.p.g.	E.p.g./ Female
1	500 µg SDS Hgal GP	1	6	100	9	N/A
		2	521	82	990	14.32
		3	358	60	243	1.69
		4	4222	52	13140	6.48
		5	1004	95	252	5.01
		6	1168	88	1116	7.96
		Mean	1213	79.5	2625	6.34
2	200 µg SDS Hgal GP	7	78.3	92	549	8.76
		8	329	53	207	1.33
		9	2426	75	3033	5.00
		10	1525	89	1260	7.51
		11	38	95	27	14.21
		12	48	50	216	9.00
		Mean	858	75.7	882	7.64
7	Controls	51	1654	50	2934	3.55
		52	5310	57	8766	3.84
		56	3026	48	8838	5.61
		57	626	41	2772	7.51
		58	3087	54	10701	7.53
		59	3330	56	12879	8.79
		60	2677	59	9190	8.36
		Mean	2816	52.1	8010	6.456

Note: Table of results adapted from Smith and Smith (1996).

Results in shading, worms not available for enzyme analysis. N/A = not available, e.p.g.= eggs per gram

5.2 RESULTS

5.2.1 Proteins

Parasites harvested from H-gal GP vaccinated lambs and from unvaccinated controls were homogenised in 1% Triton X-100 (0.15g/ml) and the homogenate centrifuged at 9,000g (Section 2.5.1). Aliquots (10 µl) were fractionated using 7.5% SDS-PAGE and reducing conditions and the gels subsequently stained with Coomassie blue. Typical results are shown in Figure 5.1.

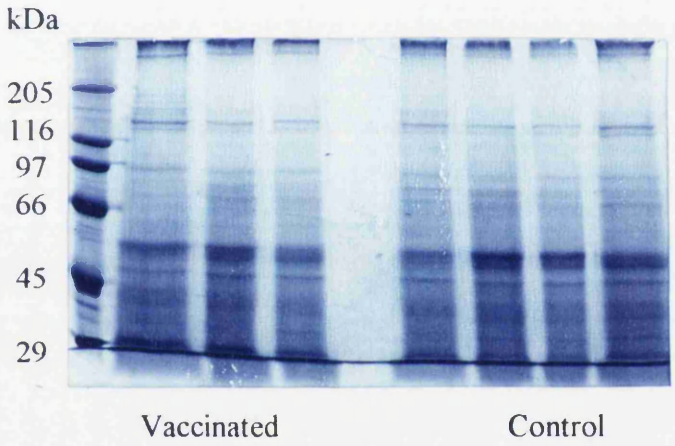
No obvious differences were noted in protein profiles of parasites harvested from vaccinates compared with those from controls. Furthermore, no obvious differences were observed when parasites taken from individual sheep of the same group were analysed.

Figure 5.1: Proteins of *H. contortus* Harvested from Vaccinated and Control Sheep

Extracts of worms were fractionated by 7.5% reducing SDS-PAGE and stained with Coomassie blue. From left to right, the first three protein lanes are of worms extracts from three different vaccinated sheep. The last four protein lanes are worm extracts taken from four different control sheep. kDa = protein markers in kilo-daltons

Figure 5.1

Protein Profiles of *H. contortus* Harvested from Vaccinated and Control Sheep



5.2.2 Spectrophotometric Determination of Enzyme Activity in Adult *H. contortus* Harvested from Vaccinated and Control Sheep

Proteinase, AChE, SOD and LDH activities of *H. contortus* homogenates from vaccinated and control sheep were determined spectrophotometrically at 30°C and the results are shown in Table 5.2. The values are expressed as activity per mg protein.

Table 5.2

Activities of Selected Enzymes in *H. contortus* Harvested from Vaccinated and Control Lambs

Enzyme (Activity/mg protein)	Sample	Median	No. of Assays	Standard Deviation	Significant Difference
Proteinases OD_{405} nm at pH 5.5	Vaccinated	20.97	n = 3	11.38	$p < 0.05$
	Control	6.60	n = 3	3.7	
AChE IU $x10^{-3}$	Vaccinated	6.4	n = 2	3.1	N/S
	Control	6.7	n = 2	3.4	
SOD U	Vaccinated	0.08	n = 2	0.01	$p < 0.05$
	Control	0.52	n = 2	0.10	
LDH IU $x10^{-3}$	Vaccinated	3.7	n = 1	N/A	N/A
	Control	4.8	n = 1	N/A	

Statistical analysis performed with Mann Whitney U Test

Proteinases: Proteolytic activity as judged by the degradation of azocasein at pH 5.5, was significantly higher ($p < 0.05$) in the worms harvested from vaccinated sheep compared with those from control sheep (Table 5.2). Azocasein degradation at pH 4

and pH 8.5 by *H. contortus* harvested from vaccinated sheep was also significantly higher than that observed in worms harvested from control sheep (not shown).

AChE: The level of AChE activity in the two groups of worms was very similar and significant differences were not observed. Both groups had an average activity of 7.2 x 10^{-3} IU/ mg protein.

SOD: SOD activity of the two groups was significantly different at $p < 0.05$ level. Control worms contained almost 7x more enzyme activity than worms from vaccinated lambs.

LDH: LDH levels were higher in the control group worms compared to the vaccinates but the difference was not significant.

5.2.3 Isoenzyme Profiles of *H. contortus* Harvested from Vaccinated and Control Sheep

Proteinases: 7.5% SDS-PAGE gels containing 0.1% gelatin were run under non-reducing conditions and the proteinases present in extracts of *H. contortus* harvested from vaccinated and control sheep were compared at pH 4 (Fig. 5.2A) and pH 5.5 (Fig. 5.2B), both in the presence of 5 mM DTT, and at pH 8.5 (Fig. 5.2C). At all three pH's tested enzyme activity was elevated in the worms harvested from vaccinated sheep compared with controls. Proteolysis was most marked at pH 5.5 (Fig. 5.2B). Although differences were noted in the intensity of degradation, no differences were evident in the proteinase profiles present in worms from the vaccinated and control sheep.

Figure 5.2: Proteinase Profiles of *H. contortus* Harvested from Vaccinated and Control Sheep

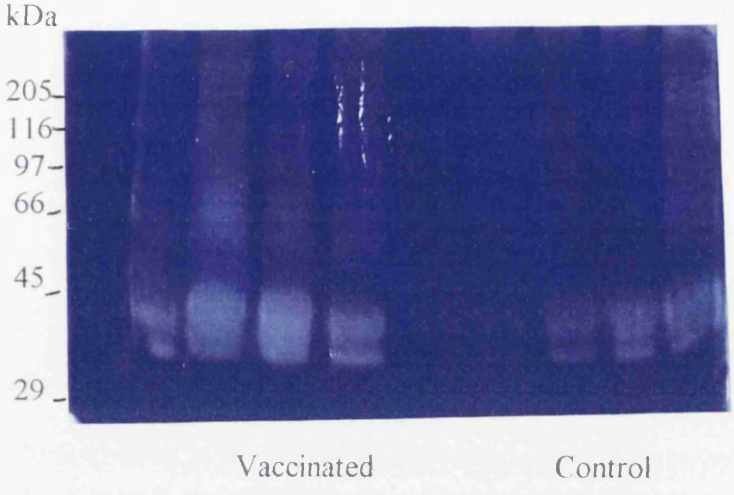
Worm extracts were fractionated in 7.5% gelatin-substrate gels under non-reducing conditions prior to incubation in buffer at pH 4 + 5 mM DTT (Fig. 5.2A), pH 5.5 + DTT (Fig. 5.2B) and pH 8.5 (Fig. 5.2C).

kDa = markers in kilo-daltons

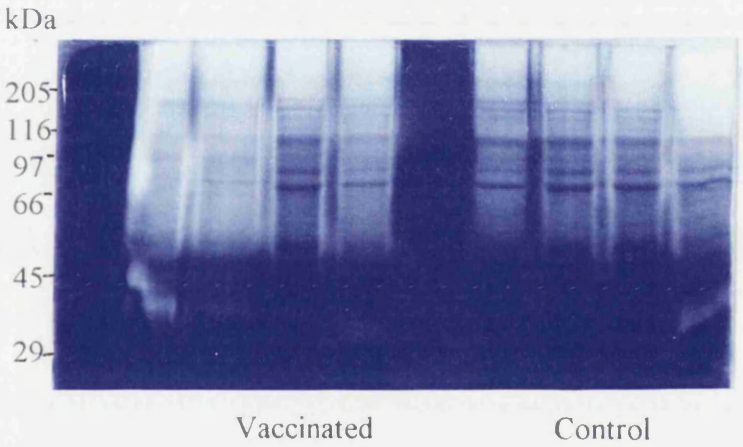
Figure 5.2

Proteinases of *H. contortus* Harvested from Vaccinated and Control Sheep

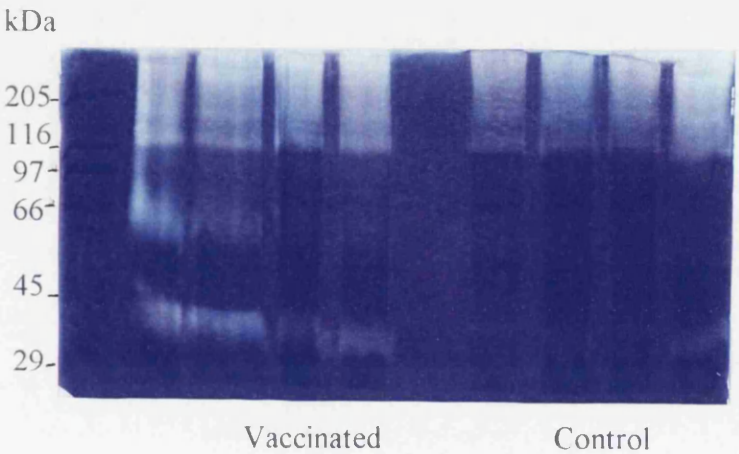
A) pH 4 + DTT



B) pH 5.5 + DTT



C) pH 8.5



Esterases: Extracts made from worms harvested from vaccinated and control sheep were separated in 7.5% native polyacrylamide gels before staining. Differences were not detected in either the relative abundance of the enzymes or in the isoenzyme profiles when the two groups were compared (Fig. 5.3a). However, variable profiles were noted when worms taken from individuals within either group were compared. The level of variation was not associated with one group more than another.

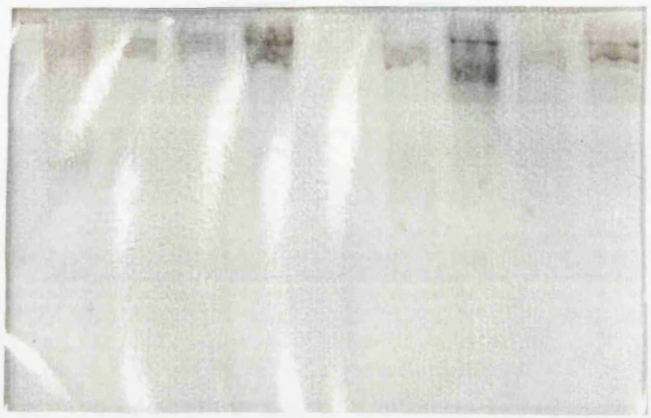
Phosphatases: Worm extracts were fractionated on 7.5% native polyacrylamide gels and stained for acid phosphatase (Fig. 5.3b). No variation in intensity of enzyme or isoenzyme profile was detected between worms from vaccinates or controls.

Figure 5.3 a and b: Esterases and Acid Phosphatases of *H. contortus* Harvested from Vaccinated and Control Sheep

Esterase (Fig. 5 3a) and acid phosphatase (Fig. 5.3b) activity in extracts of *H. contortus*. Parasite extracts were fractionated in 7.5% native polyacrylamide gels and enzyme activity visualised by specific staining as described in Sections 2.8.5 and 2.8.8 respectively.

Figure 5.3a

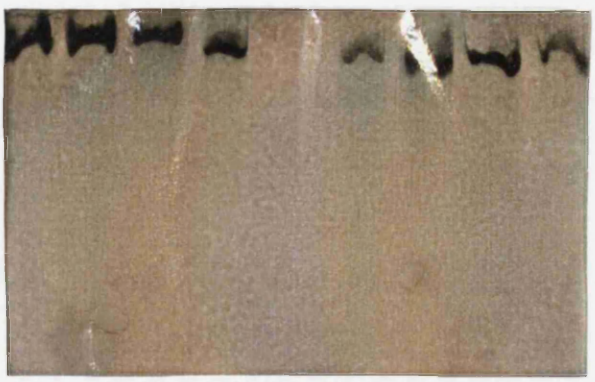
Esterases of *H. contortus* from Vaccinated and Control Sheep



Vaccinated Control

Figure 5.3b

Acid Phosphatases of *H. contortus* from Vaccinated and Control Sheep



Vaccinated Control

5.3 DISCUSSION

The results described in this chapter indicate that the parasites surviving in the vaccinated host had different enzyme expression levels from worms harvested those from unvaccinated controls. In particular, proteinase activity was 3-fold higher in vaccinated parasites while SOD activity in the same group was 7-fold lower than the corresponding activities in controls. These differences were emphasised by the observations that the levels of the constitutively expressed enzyme LDH (Nadler, 1991), as well as AChE, were very similar in the two groups. No differences were noted in the levels or profiles of individual isoenzymes visualised by zymogram analysis.

H-gal GP is a protein complex expressed on the microvillar surface of the intestinal cells in late larval and adult *H. contortus*. The nature and function of this complex is not fully defined but it does contain proteinase activity and is probably involved in digestion of the blood meal (Smith, 1993). The protection conferred to lambs by vaccination with H-gal GP is mediated by antibody which binds to the microvillar surface of the parasite gut and, presumably, impairs digestion (Smith, 1993). The observations, here, that proteinase activity is elevated in parasites surviving in vaccinated lambs compared to those from controls (Table 5.2, Fig. 5.2) support the preliminary findings of Knox, Redmond and Jones (1993). These authors noted that total proteinase activity was elevated in *H. contortus* harvested from lambs vaccinated with an integral membrane protein extract prepared from adult *H. contortus* although they also suggested that isoenzyme expression was different in vaccinated worms compared with controls. In the present study, there was no evidence for different isoenzyme expression in worms surviving in vaccinated hosts compared to controls suggesting that general proteinase expression was up-regulated as a phenotypic survival response as opposed to vaccination selecting for a population of worms which were genotypically 'fit' to survive in the vaccinated host. This response may not be sustainable if the protection experiment had been allowed to continue for a few more days, it is possible that these parasites would also have been expelled from vaccinated hosts.

Total SOD activity was also found to be significantly different between the two groups, being significantly higher in the worms from the control compared to the

vaccinated group. SOD isoenzymes were analysed but could not be clearly visualised here. This is because SOD staining protocols available at the time of this study depend on the equilibrium between O_2^- generation and SOD activity to be detected. The assay was developed in order to detect SOD activity in red blood cells and because SOD activity in parasite extracts is weak in comparison, the O_2^- generation at standard concentrations tends to swamp it. In this assay system, SOD activity is visualised by 'white' bands on a pale background, which are transient and difficult to see due to the comparatively lower levels of SOD in parasite extracts.

The difference in total SOD levels of the two parasite groups could reflect down regulation of cytosolic SOD and release of secretory forms of the enzyme by the parasite (James, 1994). *H. contortus* contains separate genes which encode the cytosolic (SODc) and putative extracellular (EC-SOD) forms of the enzyme. SODc is encoded by a single copy gene while EC-SOD is encoded by a multigene family, different members of which are expressed at the same time as judged by cDNA sequence analysis (S Liddell and D. P. Knox, personal communication). EC-SOD can be detected in culture media after *in vitro* maintenance of the adult parasites indicating an extra-corporeal role and enzyme expression is highest in the young adult (14-22 day old) parasite (S. Liddell and D. P. Knox, personal communication). It is possible that EC-SOD expression is up-regulated in response to a stressful environment for the parasite. This response may not be sustainable, a possibility which may explain why total SOD in vaccinates was lower than that in controls (Table 5.2). Serial kills over a number of days would help to define the dynamics of SOD expression in the controls and vaccinates as infection progresses and *in vitro* culture studies may indicate whether levels of SOD released by worms from vaccinated sheep are elevated compared with worms from controls. SODc or EC-SOD specific RT-PCR assays, such as that described in Chapter seven could provide discriminatory tools for this analysis.

The analyses in this study were performed on detergent extracts and H-gal GP proteinase activities were not specifically analysed because there were insufficient worms available from individual lambs to facilitate H-gal GP purification. It is possible that specific genotypic/phenotypic differences occur in this complex which might have been 'masked' in the total worm extracts used here. It is also worthy of note that the proteinases are up-regulated in sheep vaccinated with a vaccine which contains

proteinase activity. This is in contrast to other enzyme levels i.e. SOD and acid phosphatase. Thus, the proteinase up-regulation may be due to the presence of specific antibody to the proteinases present in the vaccine. Specific antisera are currently being raised to recombinant enzyme proteins equivalent to the aspartyl proteinase and neutral endopeptidase present in H-gal GP. These reagents could be used in future studies using western blotting to seek differences in protein expression between worms from control and vaccinated lambs, and determine whether the proteinase activity is up-regulated as a direct response to antibodies present that are specific to the aspartyl and neutral endopeptidase proteinase activity. In addition, because the DNA sequences encoding these enzymes are now known, it should be possible to develop RT-PCR assays, such as that described in Chapter seven, to measure RNA production which may be a more sensitive indicator of protein expression.

In summary, the results of this study did indicate that the portion of the challenge *H. contortus* infection surviving in H-gal GP vaccinated lambs showed different levels of proteinase and SOD expression compared to parasites surviving in control lambs. While the proteinase results indicated that survival was a phenotypic adaptation, this suggestion requires considerable experimental confirmation. For example, there may be sufficient variation in the specific genes encoding H-gal GP to confer antigenic variation in crucial 'lethal' epitopes required for worm expulsion to occur as a result of vaccination. If this is the case, it has serious previously unconsidered implications for the long term utility of 'hidden antigen' based vaccines. This possibility could be examined by comparing H-gal GP purified from worms surviving vaccination with those from control lambs. Moreover, this comparison can be conducted at the DNA level as gene probes for the individual peptide components of H-gal GP become available. DNA sequences as well as amino acid sequences could be compared.

As indicated above, the longevity of infection may be critical. If the ability to survive was due to genotypic fitness then infection should persist beyond the 23 days used in H-gal GP vaccination experiments. In addition, progeny of the surviving worms should be resistant to this vaccination regime. Neither of these possibilities needs to be 100% true. For example, a potentially lethal immune response which inhibits H-gal GP function may be masked by a phenotypic response where an enzyme with overlapping

activity can compensate for this loss. In addition, the antigenicity of H-gal GP may vary between individuals so that antibody may bind to different regions of the complex, inducing lethal, damaging or ineffective inhibitory changes. All of these possibilities are worthy of further analysis.

CHAPTER SIX

AN INVESTIGATION OF THE EFFECT OF THE HOST ON ENZYME EXPRESSION IN *NIPPOSTRONGYLUS BRASILIENSIS*

6.1 INTRODUCTION

The work described in the preceding two Chapters (4 and 5) provided an indication that parasite enzyme expression, particularly that of proteinases, could be influenced by the strain of the parasite and, to a greater extent by the intra-host environment. To extend these studies using the sheep/*H. contortus* host parasite system would have been expensive and time consuming in the context of the present research programme. Instead, the effect of the host on parasite enzyme expression was investigated using the rat/*N. brasiliensis* system, a well characterised model of GI parasitism. This system offered a number of advantages, including ease of use and relatively low experimental costs enabling the use of larger group sizes than would have been practical with the sheep. More importantly, there was evidence in the literature that parasite AChE, acid phosphatase (Edwards, Burt and Ogilvie, 1971) and SOD (Knox and Jones, 1992) was altered in *N. brasiliensis* surviving in the immunologically primed host compared to those from naive hosts. In addition, one report noted that the total proteinase and SOD activity was elevated in *N. brasiliensis* harvested from rats given a dietary molybdenum supplement which appeared to enhance the local anti-parasite inflammatory response (Knox *et al*, 1992).

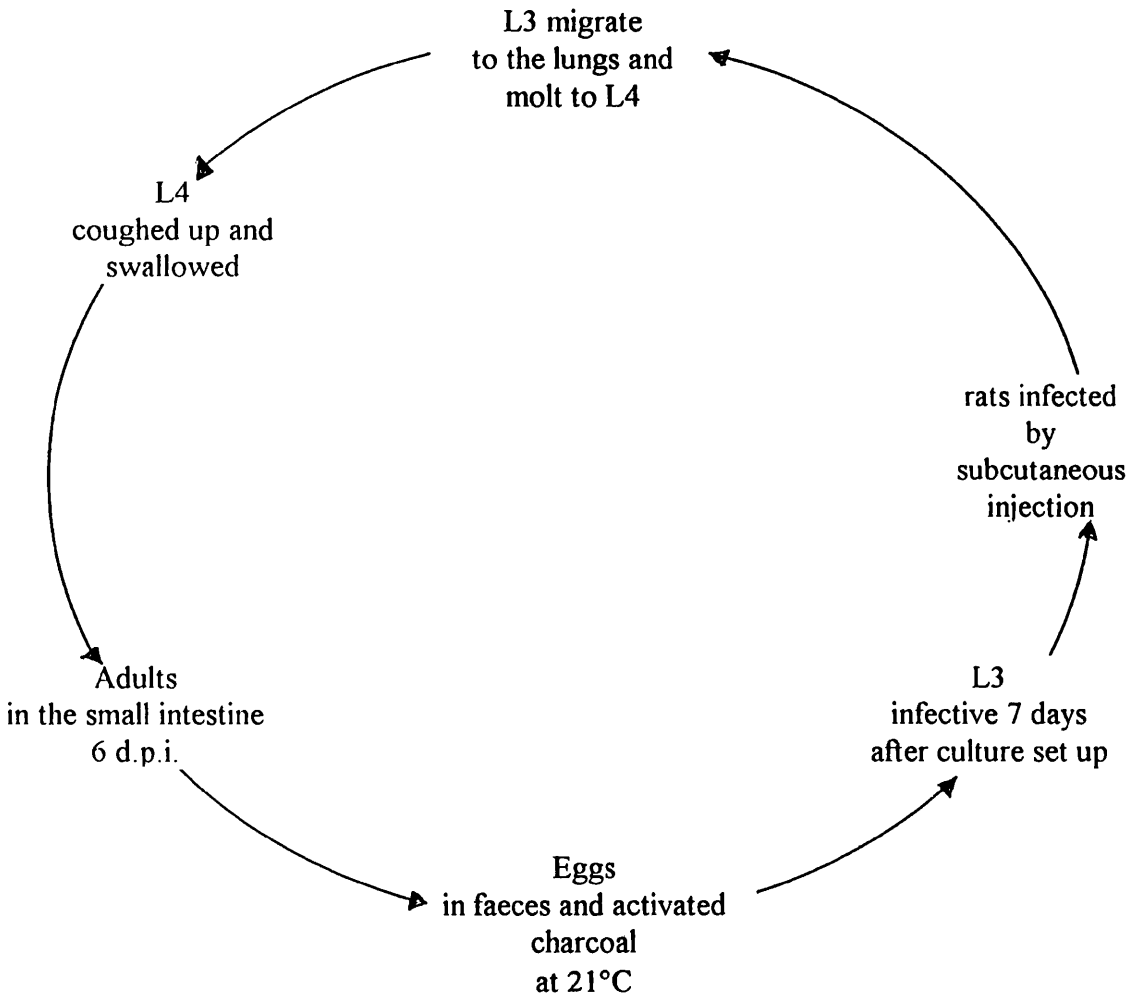
6.1.1 Life Cycle of *N. brasiliensis*

N. brasiliensis is a GI nematode of rats which is often used as a model for studying immunity to GI nematodes of medical and veterinary importance. The ease of maintenance in the laboratory, cost and the short life cycle of the parasite account for it's frequent use.

In the laboratory, *N. brasiliensis* eggs hatch into the L1 stage in about 18 to 24 hours (18-22°C). They then grow and moult to the L2 stage, usually within 48 hours before developing into infective L3 (4-5 days) and exsheathing. Infection in the laboratory is usually by subcutaneous injection after which they migrate through the tissues into the blood stream. After entry into the blood stream, the larvae are carried via the heart to the lungs within about 11-20 hours, (usually 15 hours). The larvae feed on blood, and differentiate before the moult to L4. The L4 are carried by ciliary action

up the bronchi and trachea, coughed up and swallowed before finally passing down the pharynx to the intestine. The larvae reach the intestine an average of 41 hours post infection, and then moult into adult males and females, where they mature mate and eggs are produced 6 days post infection (reviewed Smyth, 1988).

***N. brasiliensis* Life Cycle (Laboratory)**



6.1.2 'Normal', 'Damaged' and 'Adapted' *N. brasiliensis*

The ability of a small population of *N. brasiliensis* to survive in an immunologically primed host was first recorded by Ogilvie and Hockley (1968). The terms 'normal', 'damaged' and 'adapted' were then used to describe the ability of worms to survive the host immune response. 'Normal' worms were described as those taken from non-

immune rats which re-established successfully on transfer to an immunologically naive rat. 'Damaged' worms were so described because they were affected by immunity at the end of an initial infection and were rapidly expelled when transplanted into parasite naive rats. The term 'adapted' was used to describe adult worms taken from re-infected rats which rapidly recovered from the effects of immunity when transferred into a naive rat. These 'adapted' worms also had a greater ability to resist expulsion from rats than either 'normal' or 'damaged' worms (Ogilvie and Hockley, 1968; Edwards, Burt and Ogilvie, 1971). On the basis of the morphology of worm gut cells, 'damaged' parasites had noticeably fewer ribosomes compared to 'normals' and these were replaced by vacuoles, 'adapted' parasites were smaller than 'normals' had less vacuoles than 'damaged' worms and on transfer to naive rats the 'adapteds' reached normal levels of egg production (Ogilvie and Hockley, 1968). The morphology of the gut cells was felt to accurately indicate the extent to which the worms had been affected by immunity because changes in the structure of these cells appeared to correspond to changes in the infectivity and reproductive capacity of the worms (Ogilvie and Hockley, 1968). Changes were also noticed in the tissues of the worms but the authors were unable to determine which of these changes occurred first or whether they all occurred at the same time. The authors concluded that the presence of antibody may have had an effect on the ability of parasites to adapt, whereas the worms infecting normal rats which were developing an antibody response to the parasite were unable to adapt. The ability of 'adapted' worms to survive in an immunologically primed host was increased if the worms were acquired gradually as a trickle infection and appeared to be due to a mutual tolerance between the host and parasite (Jenkins and Phillipson, 1972a and b). Passive immunisation of rats also had little effect on the establishment of 'adapted' parasites (Ogilvie, 1972).

Comparisons of enzyme activity in 'normal', 'damaged' and 'adapted' *N. brasiliensis* suggested that there were profound metabolic differences in the parasite sub-populations. AChE levels were greatly increased and the isoenzyme profile markedly altered in 'damaged' and 'adapted' worms compared to 'normals'. AChE is present in all adult worms as three isoenzymes designated A, B and C, with additional bands B_1 and B_2 being seen occasionally (Edwards, Burt and Ogilvie, 1971). The levels of all five AChE Isoenzymes described above were greatly increased in 'adapteds'

compared to normals, however in 'damaged' worms although activity in bands B, B_1, B_2 and C was increased band A was markedly reduced compared to 'normals' (Edwards, Burt and Ogilvie, 1971). Increases in AChE production were thought to be related to antibody production (Jones and Ogilvie, 1972). Differences in acid phosphatase isoenzymes of 'damaged' and 'adapted' compared to 'normal' worms were also noted, two additional isoenzymes and an overall increase in enzyme activity being detected in the 'damaged' worms. 'Adapted' worms also exhibited an increase in enzyme activity and a marked smearing of activity from the origin of the gel, although the authors did not consider this to be an additional isoenzyme because it did not resolve as a clear band (Edwards, Burt and Ogilvie, 1971). These differences were thought to be linked to changes in the gut cells of the parasite.

The expulsion of *N. brasiliensis* from the rodent host involves, in part, an immediate hypersensitivity reaction in the gut mucosa with associated infiltration of various immune effector cells including mast cells and eosinophils (Miller, 1984). Parasite survival in the rodent gut has been correlated to the levels of free-radical detoxifying enzymes they contain (Smith and Bryant, 1986) and subsequent experiments (Smith and Bryant, 1989a and b) provided strong evidence that free radical exposure did affect parasite survival. Knox and Jones (1992) noted that SOD levels were three-fold higher in 'adapted' *N. brasiliensis* compared to 'normal' worms while adapted worms had a more complex isoenzyme profile. Therefore there is some evidence to suggest that the ability of a parasite to survive in a primed host may depend on the modulation of expression of key enzyme systems.

This Chapter describes the outcome of an experiment to examine the effect individual hosts have on parasite enzyme expression during a primary infection. In addition, attempts were made to compare parasite enzyme expression in parasites surviving in the immune host with those in naive hosts. The enzymes characterised included proteinases, peroxidases and a constitutively expressed enzyme lactate dehydrogenase (LDH; Long, 1961, Nadler, 1990), the activities of which had not been extensively described previously. AChE, acid phosphatase and SOD were also analysed to confirm previous findings and to allow comparison to previous studies.

6.2 RESULTS

For all the analyses described, equal protein loadings (20 μg somatic extracts; 5 μg IVR products) were used throughout to allow direct comparison of the results. All samples were prepared as described in Chapter two. For comparisons of worm populations harvested from individual rats, somatic extracts were prepared from the worms (7 d.p.i.) in 10 individual rats while IVR products were prepared from 7 day worms harvested from a further 15 rats. The number of 'adapted' worms harvested from individual rats was very low and, as a result, comparisons of enzyme activities in 'normal' and 'adapted' *N. brasiliensis* were carried out using pooled parasite populations.

Before these studies could commence there was a need to define the pH optima of somatic and IVR proteinases in both the azocasein assay and in gelatin-substrate gels because the information was not available in the literature.

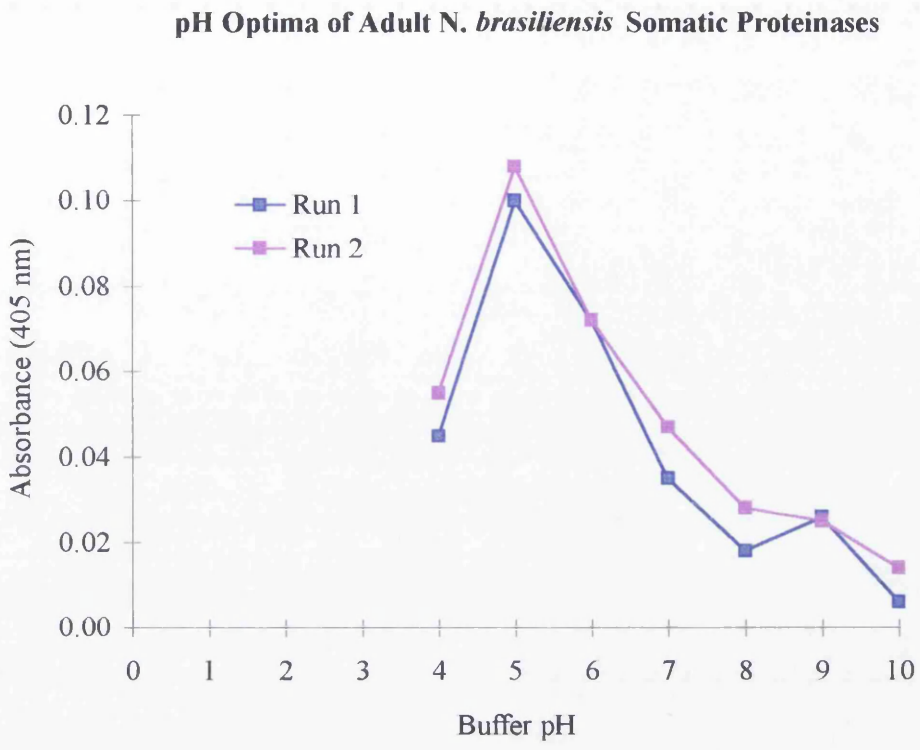
SECTION A: CHARACTERISATION OF PROTEOLYTIC ACTIVITY IN ADULT *N. BRASILIENSIS*

6.2.1 Spectrophotometric Determination of Azocasein Degradation by Adult *N. brasiliensis*

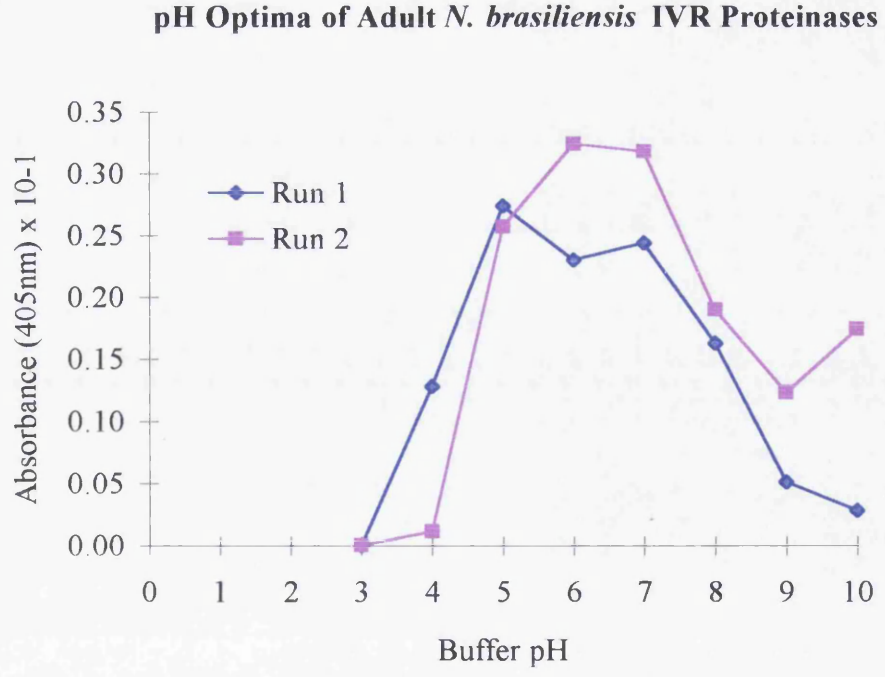
The optimal pH for degradation of azocasein by somatic extracts and IVR products of adult *N. brasiliensis* was determined spectrophotometrically using two separate worm batches over the pH range 3 to 10 and buffers described in Chapter 2.

Somatic extracts (Graph 6.1a) showed a relatively sharp pH optimum at pH 5 with only 50% maximal activity remaining at + or - 1.5 pH units. A small increase in activity was suggested at pH 9. In contrast, IVR proteinases were active over a relatively broad pH range with at least 50% maximal activity being evident between pH 4.5 and 8 (Graph 6.2). One batch of IVR showed a biphasic profile with peaks of activity a pH 5 and 7 while the other batch showed a single broad optimum between pH 6 and 7 and a hint of increasing activity at alkaline pH 10.

Graph 6.1a



Graph 6.1b



6.2.2 Gelatin Substrate Gel Analysis of Adult *N. brasiliensis* Proteinases

Degradation of gelatin, as judged using substrate gel analysis by adult *N. brasiliensis* somatic extracts (Fig. 6.1a and b) and IVR products (Fig. 6.1c and d) was determined over a broad pH range (Fig. 6.1a and c respectively) and at the optimal pH the proteinases were characterised on the basis of class specific inhibitor sensitivity (Fig. 6.1 b and d respectively). These analyses were carried out as described in Chapter two.

pH optima: Figure 6.1a shows that maximal activity in somatic extracts was evident at pH 10. Three proteinase between approximately 29 and 40 kDa were evident from pH 6 to 8, an additional low molecular weight proteinase (> 29 kDa) was evident at pH 9 as was 3 faint zones of proteolysis between 45 and 66 kDa. At pH 10 proteolytic activity of the isoenzymes just described was enhanced to such an extent that definite bands were difficult to distinguish, but the 3 proteinases in the region of 45 to 66 kDa were clearly visible as was an additional zone of activity at > 205 kDa.

The DTT enhanced activity of the 40 kDa band at acidic pH (Fig. 6.1a, pH 5 + DTT), however the proteinase profile was unaffected.

Optimal proteolytic activity for individual IVR populations was at pH 9 (Fig. 6.1c). Faint proteolysis at approximately 116 and 155 kDa was evident at pH 6. The intensity of these zones of proteolysis increased as pH increased up to pH 9, however at pH 10 the intensity of these proteinases was again reduced. Further analyses of IVR proteinases to determine their inhibitor sensitivity revealed a clearer, more complex profile. Proteinases at < 29 kDa, 45 kDa, 97 kDa and > 205 kDa were clearly defined while activity at 155 kDa was evident as a broad smear.

Inhibitor sensitivity: Pooled somatic extracts and IVR products were incubated in the presence of the four class differentiating proteinase inhibitors, 1,10 phenanthroline, PmsF, Pepstatin and E 64 prior to electrophoresis as described in Chapter 2 (Section 2.8.2).

In somatic extracts (Fig 6.1b) activity at approximately 60 kDa was inhibited in the presence of E 64 and pepstatin and reduced by 1,10 phenanthroline indicating that a number of proteinases of similar size may be active. Pepstatin and E 64 also greatly

reduced activity at approximately 29 kDa, however the 45 kDa zone was largely unaffected by any of the inhibitors tested.

With IVR products (Fig. 6.1d) a completely different inhibition profile from that above was observed. Activity at approximately 155 and > 205 kDa was inhibited by 1, 10 phenanthroline and the 45 kDa zone was slightly reduced. Activity at < 29 and 116 kDa was largely unaffected by any of the inhibitors used.

Figure 6.1 Gelatin Substrate Gel Analysis of Adult *N. brasiliensis* Proteinases

7.5% gelatin-substrate gels were run under non-reducing conditions. After electrophoresis the gels were sliced and incubated for 16 h in buffer of the designated pH (Adult somatic extracts, Fig. 6.1a, pH 4-10; Adult IVR, Fig. 6.1c, pH 3-10) or in pH 9 buffer containing class specific proteinase inhibitors (Adult somatic extracts, Fig. 6.1b; Adult IVR, Fig. 6.1d). In Fig. 6.1a, a slice of gel was also incubated at acidic pH (pH 5) in the presence of 5 mM DTT. Bands were visualised by Coomassie Blue staining followed by destaining in 10% acetic acid solution.

M = molecular weight markers in kilo-daltons (kDa). Phen = 1,10 phenanthroline, a metallo proteinase inhibitor; PmsF = a serine proteinase inhibitor; E 64 = a cysteinyl proteinase inhibitor and Pepst = pepstatin an aspartyl proteinase inhibitor.

Figure 6.1a

pH Optima of Proteinases in Somatic Extracts of Adult *N. brasiliensis* as Judged by Gelatin Substrate Gel Analysis

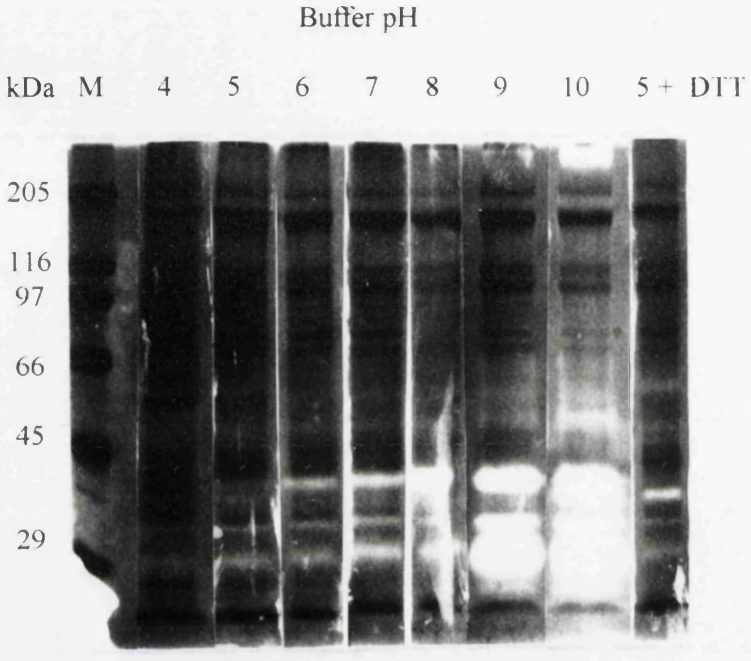


Figure 6.1b

Inhibitor Sensitivity Profile of Proteinases in Somatic Extracts of Adult *N. brasiliensis*

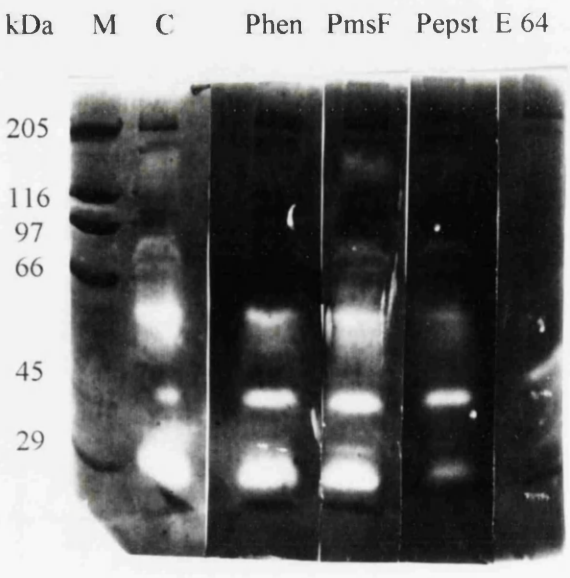


Figure 6.1c

pH Optima of Proteinases in IVR Products of Adult *N. brasiliensis* as Judged by Gelatin Substrate Gel Analysis

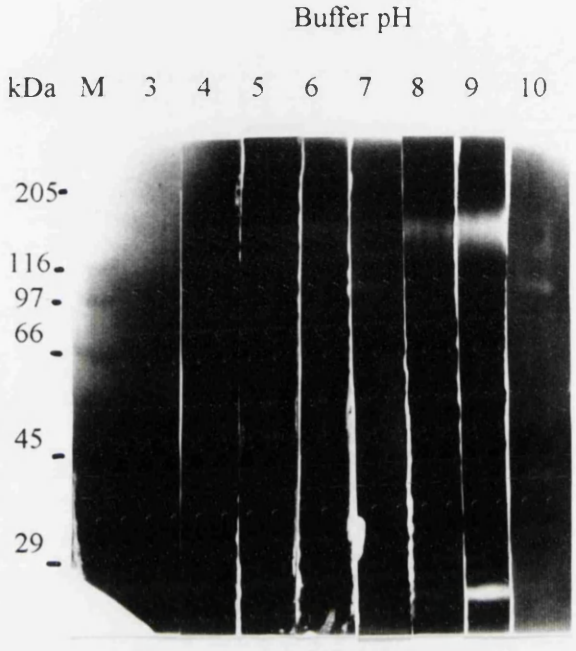
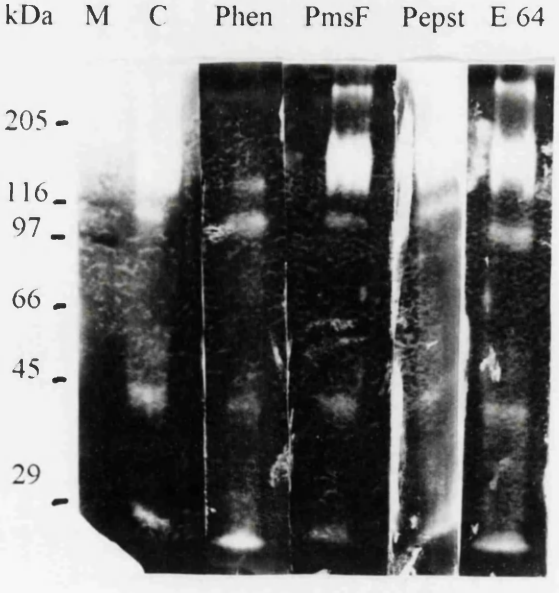


Figure 6.1d

Inhibitor Sensitivity Profile of Proteinases in IVR Products of Adult *N. brasiliensis*



SECTION B: BIOCHEMICAL ANALYSIS OF SOMATIC EXTRACTS AND IVR PRODUCTS OF ADULT *N. BRASILIENSIS* HARVESTED FROM INDIVIDUAL RATS

For the preparation of somatic extracts, 10 rats were infected with 3,000 *N. brasiliensis* L3 subcutaneously and adult parasites retrieved from the small intestine 7 d.p.i. as described in Section 2.5.3.

A further 15 rats were infected in the same way and the parasites harvested from the individual rats were washed 3x in sterile PBS and maintained overnight at 37°C in RPMI (1000 parasites/ml) containing antibiotics as described in Section 2.4.2. The culture fluid was carefully pipetted off and stored in aliquots at -70°C until analysed.

The analyses performed on both somatic extracts and IVR products were total protein, SDS-PAGE analysis of protein profiles, various spectrophotometric determinations of total enzyme activities as well as zymogram analysis of enzyme isoenzymes. The methods used are described in Chapter 2.

6.2.3 **Protein Profiles of *N. brasiliensis* Populations**

Worms harvested from 10 rats were homogenised separately. The extracts were fractionated under 7.5% non-reducing SDS-PAGE conditions and Coomassie stained. No obvious differences were observed in the protein profiles from these worms (Fig. 6.2a).

The culture fluid containing the *in vitro* released (IVR) products of worm populations harvested from 15 individual rats were fractionated under the same conditions as the somatic extracts, however after electrophoresis the gels were silver stained in order to detect the lower amounts of protein (Fig. 6.2b). No differences were observed in the protein profiles of the separate worm populations.

Figure 6.2 Protein Profiles of Adult *N. brasiliensis* Harvested from Individual Rats

Somatic extracts of adult *N. brasiliensis* harvested from 10 individual rats (Fig. 6.2a, lanes 1-10) were fractionated by 7.5% non-reducing SDS-PAGE and stained with Coomassie blue to visualise the proteins. IVR products of adult *N. brasiliensis* harvested from 15 individual rats (Fig. 6.2b, lanes 1-15) were fractionated under the same conditions as the somatic extracts and silver stained to visualise the proteins.
High molecular weight markers (M) were run to allow the approximate molecular weight of the proteins in figures 6.2a and b to be estimated.

Figure 6.2a

Protein Profile of Somatic Extracts from Adult *N. brasiliensis*

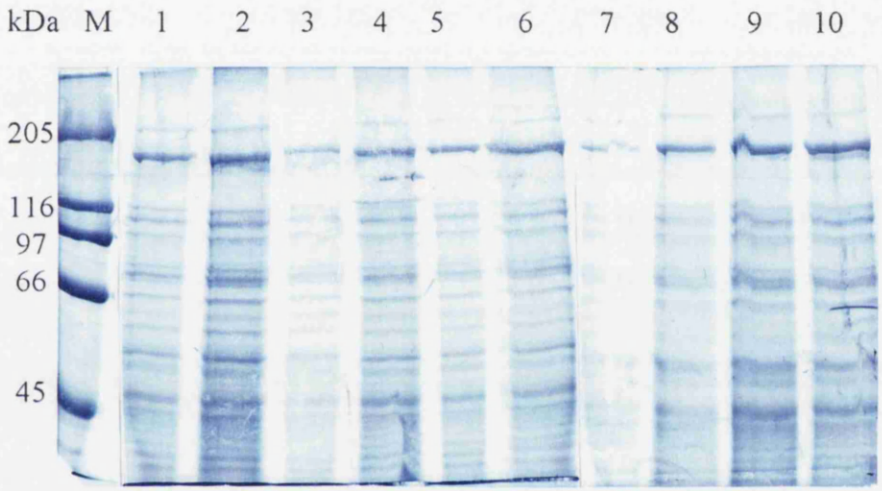
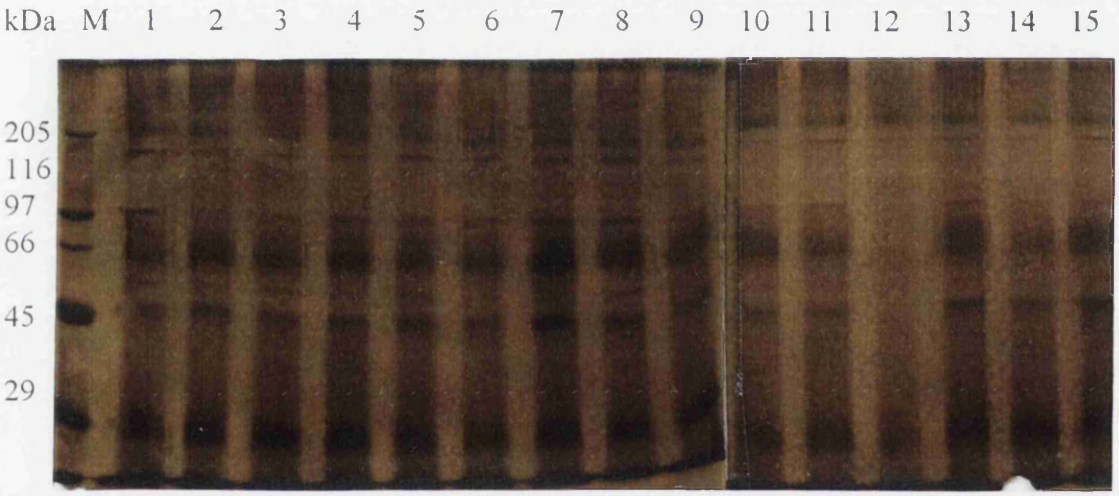


Figure 6.1b

Protein Profile of Adult *N. brasiliensis* IVR Products



SPECTROPHOTOMETRIC DETERMINATION OF ENZYME ACTIVITIES IN SOMATIC EXTRACTS AND IVR PRODUCTS OF WORM POPULATIONS FROM INDIVIDUAL RATS

All enzyme activities were determined as described in Chapter 2. Somatic enzyme activities were expressed per mg protein while IVR activities were expressed per 1000 worms per ml of culture fluid.

Enzyme activities in somatic extracts are summarised in Table 6.1a and those in IVR products in Table 6.1b.

Generally all enzyme activities in somatic extracts tended to be quite variable when individual worm populations were compared although it was notable that proteinase activity (mean 5.14, range 4.14 to 6.0) was less variable than AChE (mean 10.11, range 4.75 to 13.57) or SOD (mean 24.66, range 14.9 to 47.1). In addition, it was presumed at the start of this study that LDH would serve as a 'constitutively' expressed enzyme marker with little variation in activity when individual worm populations were compared. This was not the case and wide variation was noted (mean 0.0849, range 0.0551 to 0.1183).

When total AChE and SOD enzyme activities of IVR products were compared AChE levels were again, highly variable (mean 0.172, range 0.053 to 0.345) while SOD levels were less variable (mean 2.53, range 2.18 to 3.30) than in somatic extracts. It was notable that the IVR proteinase activity was highly variable in contrast to somatic levels.

Note: In tables 6.1a and b, n = number of assays

Table 6.1a

Enzyme Activities of Somatic Extracts of Worms from Individual Hosts

Rat No.	Proteinases OD_{405} nm pH 5 n= 2	AChE IU x 10^{-3} n = 3	Glutathione Peroxidase U n = 2	SOD U n = 3	LDH IU n = 2
	Mean	Mean	Mean	Mean	Mean
1	5.80	8.77	0.043	22.0	0.0698
2	5.28	10.17	0.028	25.6	0.0551
3	5.69	4.75	0.047	16.6	0.0793
4	6.00	8.27	0.042	14.9	0.0826
5	4.14	9.27	0.040	24.8	0.0611
6	5.55	13.55	0.058	18.9	0.1183
7	4.60	13.57	0.043	16.6	0.1033
8	4.71	11.92	0.049	19.0	0.1047
9	4.82	10.57	0.037	41.1	0.0754
10	4.88	10.31	0.055	47.1	0.0996
Mean (S.D.)	**5.14** (+/- 0.61)	**10.11** (+/- 2.62)	**0.044** (+/- 0.0087)	**24.66** (+/- 10.92)	**0.0849** (+/- 0.0207)

Table 6.1b

AChE and SOD Activity in IVR Products of Worms from Individual Hosts

Rat No.	Proteinases n = 2	AChE IU per ml n = 2	SOD U per ml n = 2
	Mean	Mean	Mean
1	0.0371	0.214	2.56
2	0.0274	0.325	2.42
3	0.0132	0.175	3.30
4	0.0067	0.205	2.63
5	0.0067	0.137	2.49
6	0.0078	0.093	2.23
7	0.0089	0.345	2.43
8	0.0072	0.102	2.77
9	0.0005	0.122	2.22
10	0.0308	0.168	2.70
11	0.0025	0.126	2.21
12	0.0114	0.053	2.18
13	0.0089	0.198	2.51
14	0.0048	0.119	2.59
15	0.0145	0.198	2.76
Mean & (S.D.)	**0.0271** (+/- 0.0293)	**0.172** (+/- 0.0081)	**2.53** (+/- 0.29)

6.2.4 Proteinases of Adult *N. brasiliensis* from Individual Hosts

Somatic extracts: Although equal amounts of protein were loaded in each lane, definitive differences were noted when the proteinase profiles of somatic extracts of worms harvested from individual rats were compared (Figure 6.3a). For example, proteinases at 29, 32, 36 and 55 kDa were evident in worm populations from rats 1, 2, 6, 8, 9 and 10. The 29 kDa proteinase was not evident in 3, 4, 5 and 7. In addition, the level of expression of the 36 and 55 kDa proteinases was also variable although present in all populations.

IVR products: In accord with the total proteinase estimations (Table 6.1b), IVR proteinase activity as judged by gelatin-substrate gel analyses, was very variable (Fig. 6.3b). Unlike the situation with somatic extracts when an isoenzyme evident in one population was completely absent in another, the same isoenzymes were evident in all 15 batches of IVR products compared, although the extent to which these proteinases were released during culture was highly variable between individual parasite populations (compare rat 4 with rats 1, 5, 6 and 7 for example, Fig. 6.3b).

Figure 6.3 Proteinase Profile of Somatic Extracts and IVR Products from Adult *N. brasiliensis* at pH 9

Somatic extracts (Fig. 6.3a, lanes 1-10) and IVR products (Fig. 6.3b, lanes 1-15) from adult *N. brasiliensis* were fractionated using 7.5% SDS-PAGE gels containing 0.1% gelatin using non-reducing conditions. After overnight incubation at 37°C in buffer at pH 9, zones of proteolysis were visualised by Coomassie blue staining. High molecular weight protein markers (kDa) were run on each gel as standards.

Figure 6.3a

Proteinase Profile of Somatic Extracts from Adult *N. brasiliensis* at pH 9

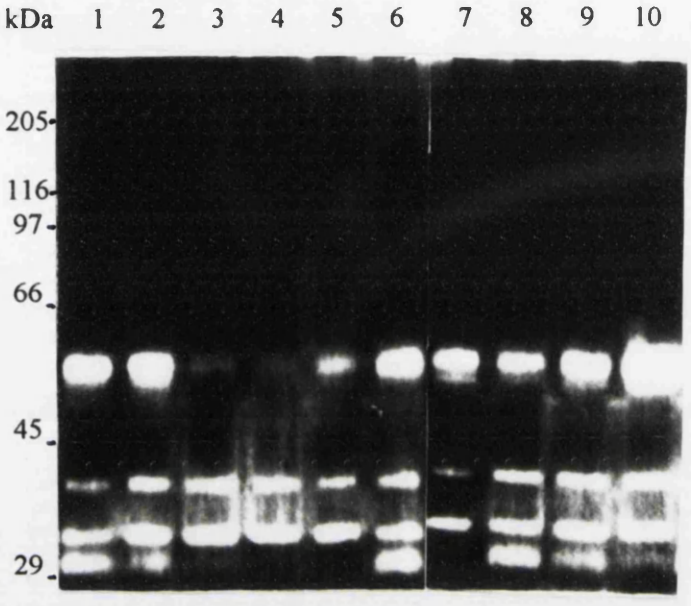
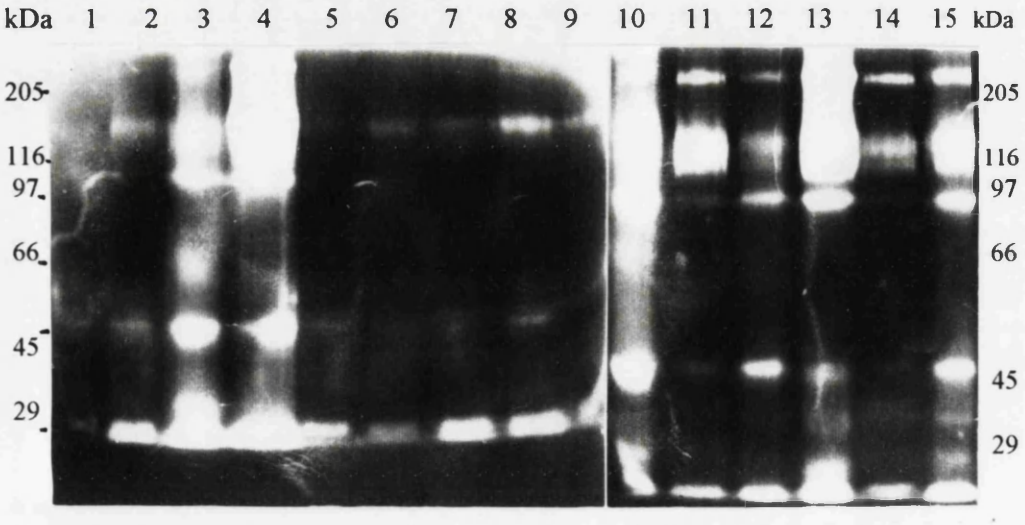


Figure 6.3b

Proteinase Profile of Adult *N. brasiliensis* IVR Products at pH 9



6.2.5 Esterases of Adult *N. brasiliensis* from Individual Hosts

General esterase Isoenzymes in somatic extracts (Fig. 6.4a and b) and IVR products (Fig. 6.5a and b) from adult *N. brasiliensis* as well as AChE isoenzymes in the same samples (Fig. 6.4c and d, Fig 6.5c and d respectively) were visualised by specific enzyme staining (see Chapter 2) after fractionation of the samples by native PAGE (Fig. 6.4a and c, Fig. 6.5a and c) or non reducing SDS-PAGE. (Fig. 6.4b and d, Fig. 6.5b and d)

Somatic extracts:- Using native PAGE, similar esterase profiles were observed when extracts of *N. brasiliensis* from individual rats were compared (Fig. 6.4a). The rapidly migrating, well defined zones of enzyme activity were particularly prominent while a faint zone of activity in the middle of each lane as well as a smear of activity which migrated slowly into the gel were also observed. Non-reducing SDS-PAGE gave a more sharply defined esterase profile but, again, no marked differences were observed when the esterase present in worm populations from individual rats were compared (Fig. 6.4b). A particularly prominent isoenzyme was present in all extracts at 62 kDa, with the possible exception of population 3 (Fig. 6.4b, lane 3) with weaker zones of activity at 47 and 44 kDa.

When AChE isoenzyme expression was analysed in the worm populations from individual hosts, again no distinct differences were noted in the profiles observed following native (Fig. 6.4c) or non-reducing SDS-PAGE (Fig. 6.4d). After native PAGE (Fig. 6.4c), the profile was very similar to the general esterase profile (Fig. 6.4a) with the exception that the upper band of the 3 rapidly migrating isoenzyme noted by general esterase staining (Fig. 6.4a) was not prominent here (Fig. 6,.4c). However, by contrast, the AChE isoenzyme profile visualised after non-reducing SDS-PAGE (Fig. 6.4d) was more complex than the corresponding general esterase profile (Fig. 6.4d). In particular, a relatively sharp band of activity at about 180 kDa as well as a diffuse zone of activity at 97 kDa were observed here (Fig. 6.4d) which were not evident in the general esterase profile (Fig. 6.4d).

IVR products: Again, no marked differences were noted when the esterases released by adult parasites from individual hosts were compared using native PAGE (Fig. 6.5a). However, subtle differences were evident after fractionation using non-reducing SDS-PAGE (Fig. 6.5b). In particular, a defined band of staining at approximately 120 kDa was strongly evident in lane 4 (fig. 6.5b) and weakly evident in lanes 13 and 14 (Fig. 6.5b). A strongly staining band at 58 kDa in lane 14 was only weakly evident or absent in the other lanes (Fig. 6.5b). No differences were noted when the AChE isoenzymes in IVR products were visualised after native (Fig. 6.5c) or non-reducing (Fig. 6.5d) PAGE.

Figure 6.4 **Esterase and AChE Isoenzymes in Somatic Extracts of Adult *N. brasiliensis***

General esterase (Fig. 6.4a and b) and AChE (Fig. 6.4c and d) isoenzymes in somatic extracts of adult *N. brasiliensis* were visualised by specific staining following fractionation of extract proteins by 7.5% native PAGE (Fig. 6.4a and c; lanes 1-10) or non-reducing SDS-PAGE after which the SDS was eluted by Triton X-100 washing prior to staining (Fig. 6.4b and d; lanes 1-10).

M = high molecular weight markers in kilo-daltons (kDa)

Figure 6.4a

Native PAGE of Somatic Extracts from Adult *N. brasiliensis*

Esterases

1 2 3 4 5 6 7 8 9 10

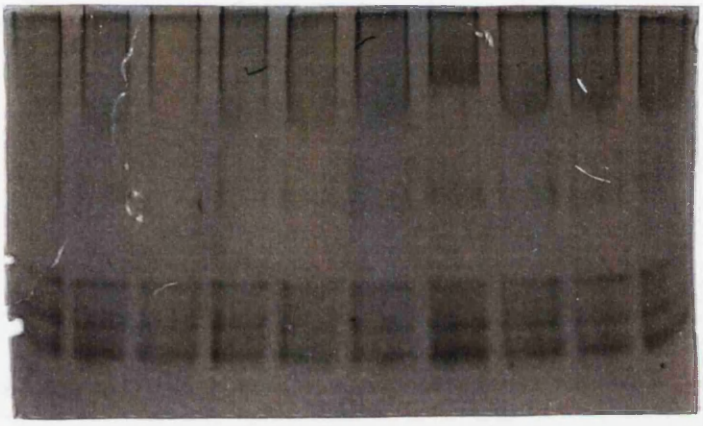


Figure 6.4 b

Non-reducing SDS-PAGE of Somatic Extracts from Adult *N. brasiliensis*

Esterases

kDa M 1 2 3 4 5 6 7 8 9 10

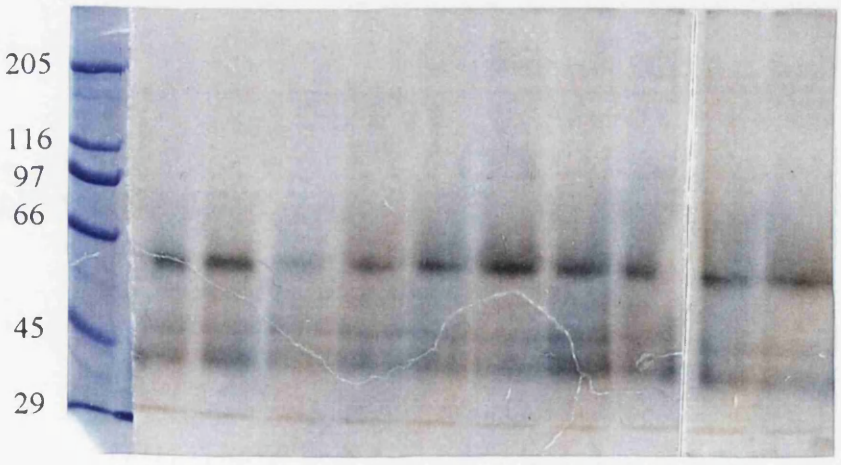


Figure 6.4c

Native PAGE of Somatic Extracts from Adult *N. brasiliensis*

Acetylcholinesterases

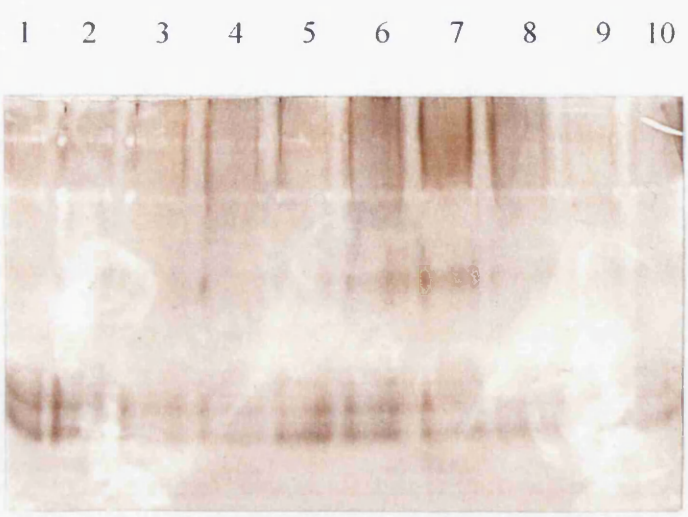


Figure 6.4d

Non-reducing SDS-PAGE of Somatic Extracts from Adult *N. brasiliensis*

Acetylcholinesterases

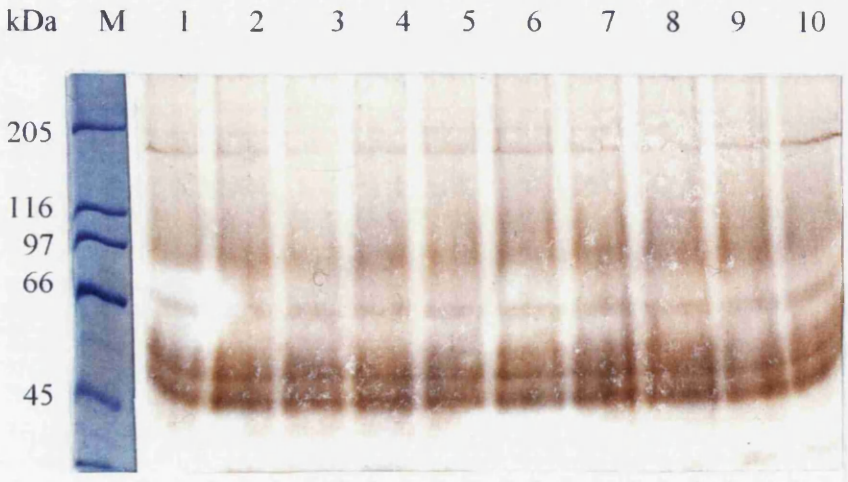


Figure 6.5 **Esterase and AChE Isoenzymes of Adult *N. brasiliensis In Vitro* Released (IVR) Products**

General esterase (Fig. 6.5a and b) and AChE (Fig. 6.5c and d) isoenzymes, in IVR harvested from populations of adult *N. brasiliensis* from individual rats, were visualised by specific staining following fractionation of extract proteins by 7.5% native PAGE (Fig. 6.5a and c, lanes 1-15) or non-reducing SDS-PAGE after which the SDS was eluted by Triton X-100 washing prior to enzyme staining (Fig. 6.5b and d, lanes 1-15). M = high molecular weight markers in kilo-daltons (kDa).

Figure 6.5a

Native PAGE of Adult *N. brasiliensis* IVR Products

Esterases

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

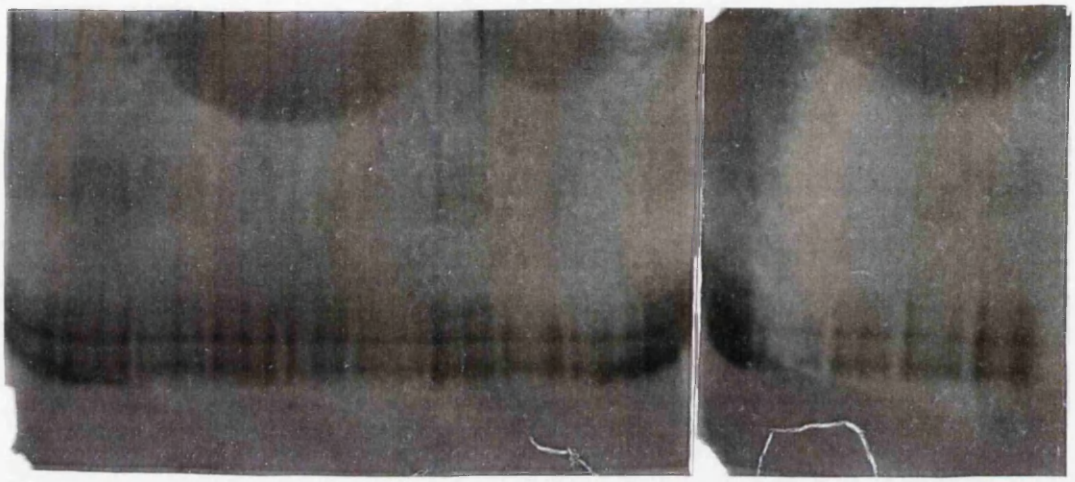


Figure 6.5b

Non-reducing SDS-PAGE of Adult *N. brasiliensis* IVR Products

Esterases

kDa M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

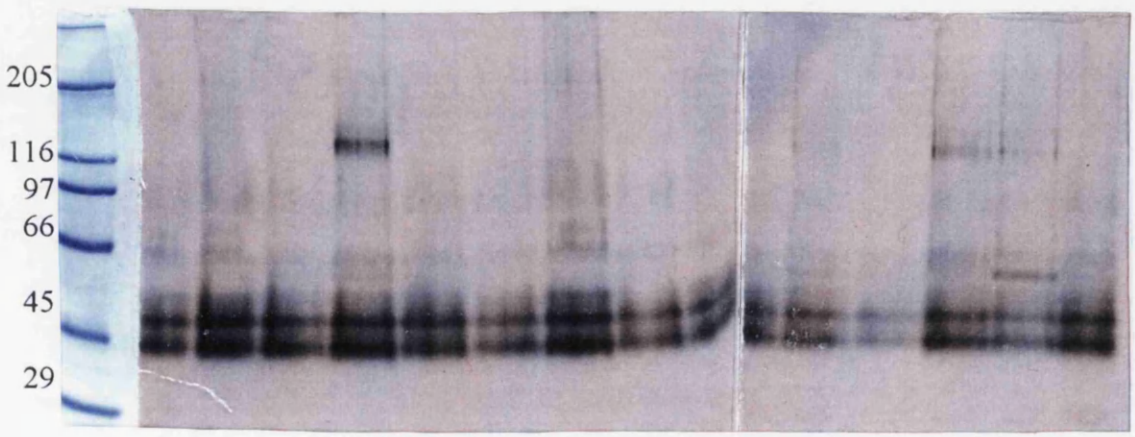


Figure 6.5c

Native PAGE of Adult *N. brasiliensis* IVR Products

Acetylcholinesterases

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

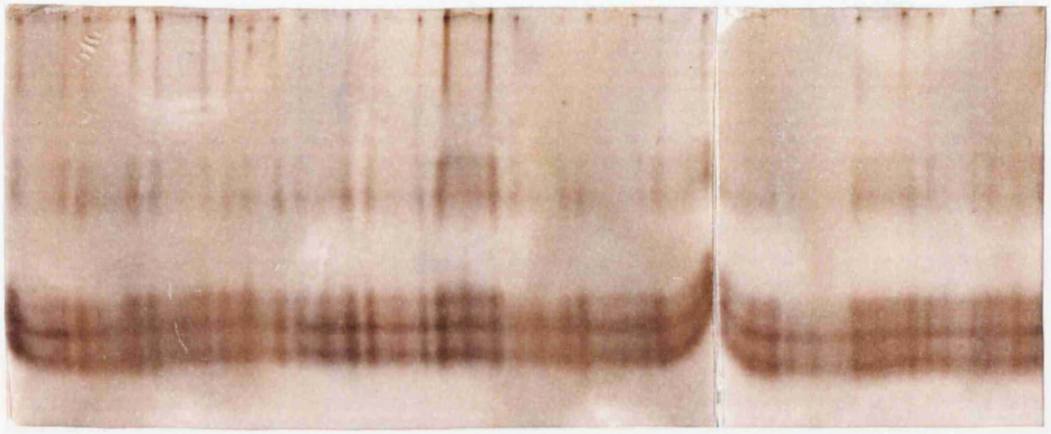
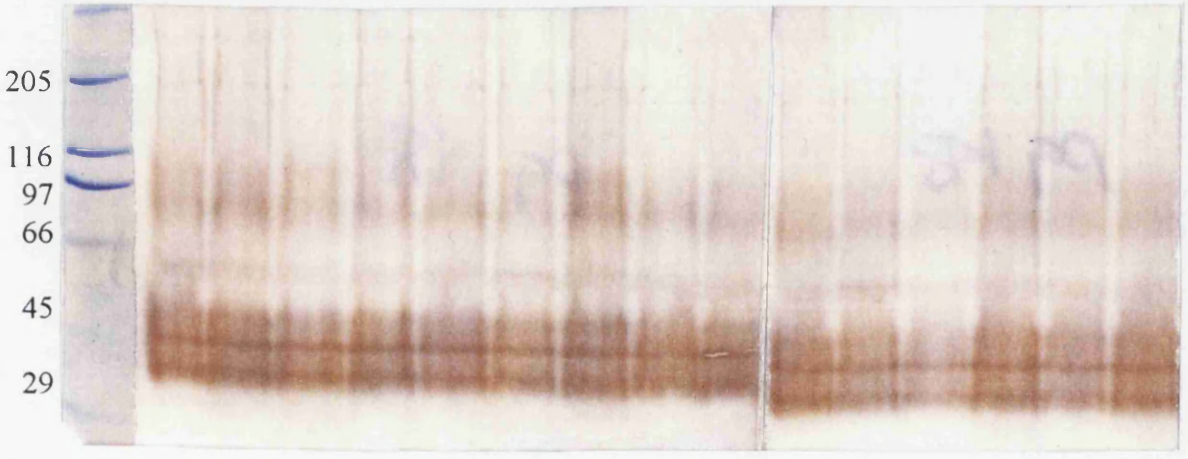


Figure 6.5d

Non-reducing SDS-PAGE of Adult *N. brasiliensis* IVR Products

Acetylcholinesterases

kDa M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



6.2.6 Superoxide Dismutases in Somatic Extracts of Adult *N. brasiliensis* Harvested from Individual Rats

SOD isoenzyme staining in somatic extracts was very faint and relatively transient making photographic reproduction impossible without specialised equipment. Therefore, the profiles observed are represented as a block diagram (Fig. 6.6).

Quite considerable variation in isoenzyme expression was noted in the worm populations from individual rats varying from three (lanes 3 and 6), the more usual four (lanes 1,4, 5, 7 and 8) or in some cases (lanes 2, 9 and 10) to five isoenzymes (Fig. 6.6). The intensity of the isoenzymes also varied but, usually, the slow migrating Isoenzymes were more readily visualised.

SOD activity in the IVR products (see Table 6.1b) was not strong enough to be visible using this detection system.

6.2.7 Peroxidase Isoenzymes from Somatic Extracts of Adult *N. brasiliensis*

Activity was restricted to a single slow migrating zone (Fig. 6.7) in all the somatic extracts. No activity was detected in the IVR products (not shown).

6.2.8 Lactate Dehydrogenase Activity in Somatic Extracts of Adult *N. brasiliensis*

There was no variation in the isoenzyme profile of lactate dehydrogenase in somatic extracts of adult *N. brasiliensis* from different individual rats. Two slow migrating isoenzymes were visible in all worm populations after fractionation of somatic extracts by native PAGE, followed by specific enzyme staining (Fig. 6.8). No LDH was detected in the IVR products (not shown).

Figure 6.6 Block Diagram of SOD Isoenzymes in Somatic Extracts of Adult *N. brasiliensis* Harvested from Individual Hosts

SOD isoenzyme profiles from somatic extracts of adult *N. brasiliensis* harvested from 10 individual rats were visualised by native PAGE followed by specific enzyme staining (Fig. 6.6, lanes 1-10).

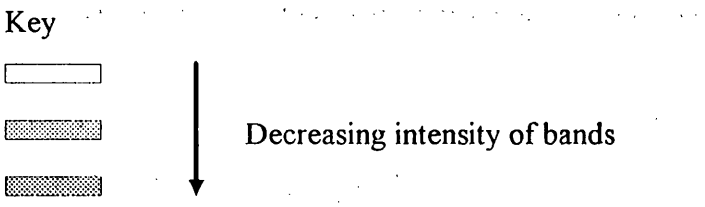


Figure 6.6

Block Diagram of SOD Isoenzymes in Somatic Extracts of *N. brasiliensis*

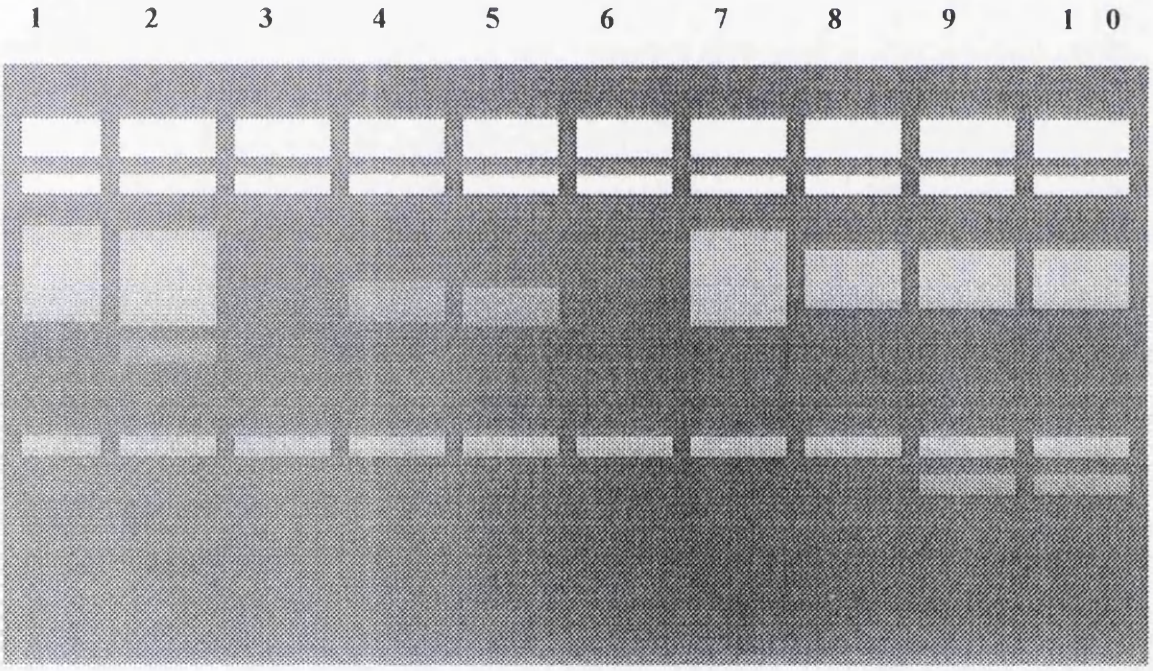


Figure 6.7 Peroxidase Activity in Somatic Extracts of Adult *N. brasiliensis*

Worm extracts (20 μg) from ten individual rats (lanes 1-10) were separated in 7.5% native polyacrylamide gel conditions and stained for peroxidase activity.

Figure 6.7

Somatic Extracts of Adult *N. brasiliensis* Stained for Peroxidases

1 2 3 4 5 6 7 8 9 10

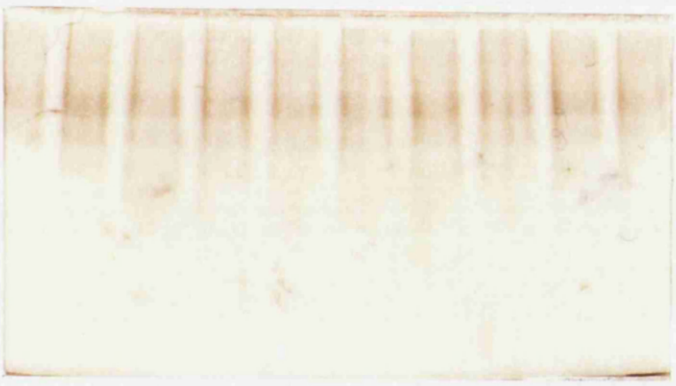


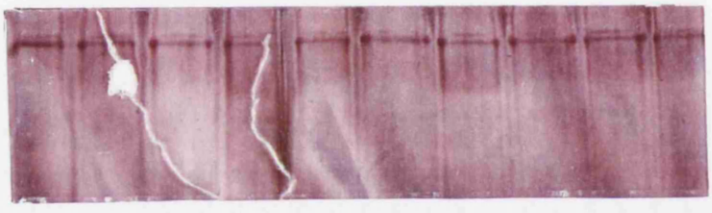
Figure 6.8 LDH Activity in Somatic Extracts of Adult *N. brasiliensis*

Worm extracts (20 μg) were fractionated using 7.5% native polyacrylamide gels prior to staining for LDH activity.

Figure 6.8

Somatic Extracts from Adult *N. brasiliensis* Stained for Lactate Dehydrogenase

1 2 3 4 5 6 7 8 9 10



6.2.9 Systemic Antibody Responses to Adult *N. brasiliensis* IVR Products in the Primed Rodent Host

Although no differences were observed in the protein profiles of IVR of the adult *N. brasiliensis* from 15 individual rats, the differences noted in the enzyme profiles described in the preceding Sections were noteworthy and posed the question as to whether different IVR preparations were antigenically distinct. To investigate this possibility, serum was harvested from 5 rats (7 d.p.i.) following a challenge infection (10,000 L3) eight weeks post primary infection and the sera pooled. The pooled serum was then used to probe a western blot of IVR products of the parasites from the 15 individual rats used previously for enzyme definition.

IVR products (5 μg) from the 15 *N. brasiliensis* populations which had been harvested from different rats were fractionated using standard methods by non-reducing 7.5% SDS-PAGE. After electrophoresis the proteins were blot transferred to nitro-cellulose as described in Section 2.6.2.

Antibody in the serum bound to IVR components of approximately 29 to 45 kDa in all samples analysed (Fig. 6.9). A prominent protein at approximately 66 kDa was inconsistently recognised in the different parasite populations by the rat sera. Compare lanes 1, 6, 8, 9 and 12 with 3, 4 and 7. The serum bound to a 100 kDa component in all but parasite population 12, whilst binding to this band was less in lanes 6, 8, 9 and 13, compare lanes 1-5, 6, 10, 11 and 13-15. In lane 1 the 66 kDa protein is absent although the 100 kDa band is recognised.

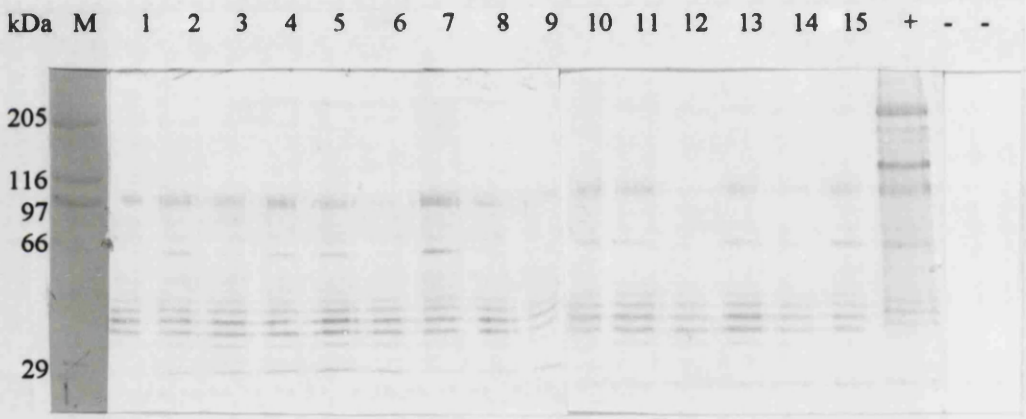
Figure 6.9 Western Blot of Adult *N. brasiliensis* IVR Products Harvested from Individual Hosts and Probed with Pooled Immune Rat Sera

Proteins of *N. brasiliensis* IVR products harvested from 15 different rats were fractionated using 7.5% non-reducing SDS-PAGE, blot transferred to nitro-cellulose and incubated with a pooled anti-*N. brasiliensis* sera preparation from 5 primed rats (lanes 1-15). The negative controls (lanes -) were prepared by probing IVR products of worms harvested from two of the 15 rats with a pool of normal rat sera. A positive control (+) consisting of an adult somatic worm extract probed with immune rat sera was also included.

M = high molecular weight markers in kilo daltons (kDa).

Figure 6.9

IVR Products from Adult *N. brasiliensis* Incubated with Rat Sera



SECTION C: A COMPARISON OF ENZYME ACTIVITY IN 'NORMAL' AND 'ADAPTED' *N. BRASILIENSIS*

SPECTROPHOTOMETRIC DETERMINATION OF ENZYME ACTIVITIES IN SOMATIC EXTRACTS

Enzyme activity for pooled extracts of 'normal' and 'adapted' adult *N. brasiliensis* was determined as previously described. Enzyme activities are expressed per mg protein and are shown in Table 6.3.

Only a very limited amount of 'adapted' parasite material was available and as a result, spectrophotometric analyses were limited to AChE and SOD to allow comparison to previous studies and the bulk of the parasite material was retained for zymogram analyses.

Table 6.2

Enzyme Activities of 'Normal' and 'Adapted' *N. brasiliensis*

Enzyme	**AChE** $IU \times 10^{-3}$		**SOD** U	
	Median	**Mean**	**Median**	**Mean**
Normal	0.0922	**0.0971** +/- 0.019 n = 4	10.6	**11.03** +/- 3.44 n = 4
Adapted	0.8475	**0.8965** +/- 0.081 n = 2	26.30	**27.9** +/- 7.3 n = 2

n = number of assays. Statistical analysis performed using the Mann Whitney U test.

AChE and SOD levels were significantly ($p < 0.05$, $p < 0.01$ respectively) higher in somatic extracts of 'adapted' compared to 'normal' worms.

6. 2 10 **Esterases in 'Adapted' and 'Normal' *N. brasiliensis***

Somatic extracts (pooled) of *N. brasiliensis* from 'normal' and 'adapted' populations were analysed by native PAGE and non-reducing SDS-PAGE were used and the gels stained for esterase and AChE as previously described in Chapter 2.

When native polyacrylamide gels were stained for esterases multiple zones of activity were detected in 'normal' and 'adapted' parasites. These zones of enzyme activity had similar migratory properties to those described previously (Edwards, Jones and Ogilvie, 1971), and where appropriate, the banding designations (A, B and C) ascribed by these authors to determine the AChE isoenzymes have been used (Fig 6.10a). A slow migrating zone of activity was evident in both parasite extracts. Band A, which migrated half way down the gel, was faintly visible in 'normal' worms and darkly stained in 'adapted' worms and appeared to be composed of 2 bands. Activity of bands B and C were of similar intensities in both 'normal' and 'adapted' parasites and, again, several bands were evident.

Non-reducing SDS-PAGE of 'normal' and 'adapted' extracts enabled better separation of the isoenzymes (Fig. 6.10c and d). A 66 kDa zone of activity which appeared to be composed of several bands was evident in the 'adapted' and not the 'normal' parasites (Fig. 6.10c) and probably corresponds to band A observed in native gels (Fig. 6.10c). Band C appeared to exist as a doublet in both extracts while band B was also evident as a 55 kDa doublet in the 'adapted' worm population (Fig. 6.10c), which probably correspond to bands B_1 and B_2 ascribed originally by Edwards, Jones and Ogilvie (1971).

6.2.11 **AChE in Adapted' and 'Normal' *N. brasiliensis***

In general, the AChE profiles (Fig. 6.10b and d) mirrored the esterase profiles (Fig. 6.10a and c), with band A being particularly strong in 'adapted' worm populations. Notably, the slow migrating activity evident under native conditions (Fig. 6.10a) and

the 55 kDa esterase activity evident under non-reducing conditions (Fig. 6.10c) were not visible after AChE staining (Fig. 6.10b and d respectively).

6.2.12 **Proteinases Present in 'Normal' and 'Adapted'** ***N. brasiliensis***

Proteinase expression in the 'adapted' parasites was greatly increased compared to the 'normals' with an intense zone of activity in the region 29 to 66 kDa and further bands of activity at approximately 90, 116, 155, 205 kDa and > 205 kDa (Fig. 6.10e). Similar banding profiles were seen in the normals when higher levels of protein were loaded and some of these bands are faintly visible in the figure (lane N, Fig. 6.10e), indicative of up-regulation of enzyme activity.

6.2.13 **Superoxide Dismutase Present in 'Normal' and 'Adapted'** ***N. brasiliensis***

SOD isoenzyme visualisation, following fractionation of extracts using native PAGE, was inconsistent and poorly defined although the general level of SOD counter-staining appeared higher in 'adapted' worms (not shown).

Figure 6.10 **Isoenzyme Profiles in Pooled Somatic Extracts of 'Adapted' and 'Normal' *N. brasiliensis***

All gels were 7.5% polyacrylamide gels using under either native PAGE (a and b) or non-reducing SDS-PAGE conditions (c, d and e).

After electrophoresis the gels were either stained for esterase (a and c) or AChE (b and d) or proteinase activity (e).

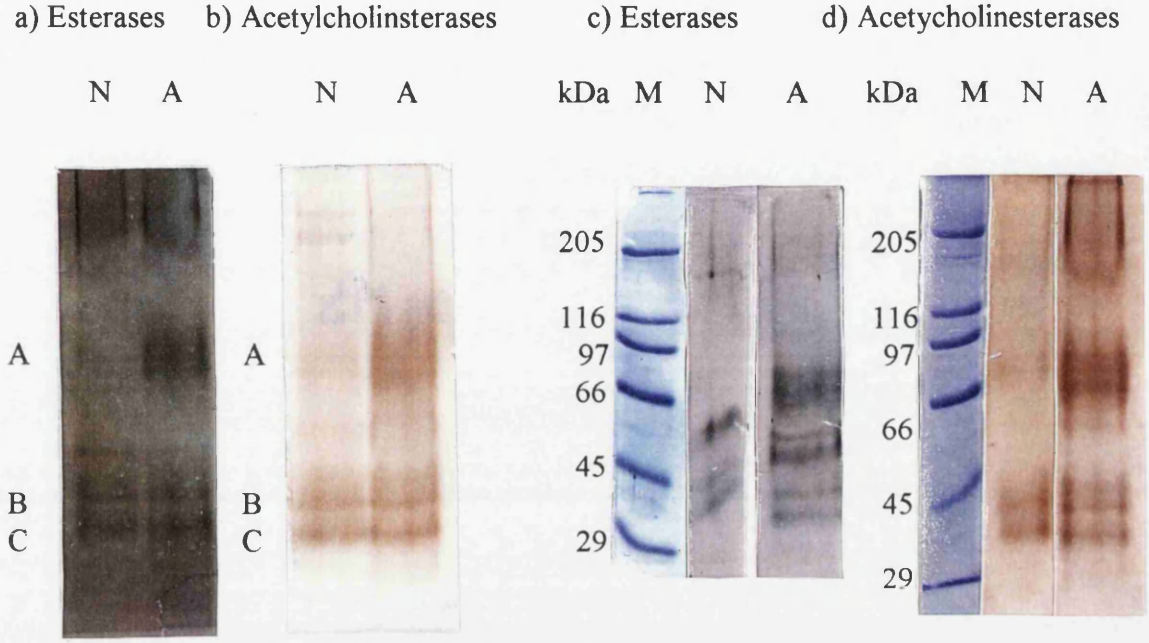
M = high molecular weight markers in kilo daltons (kDa); N = normal worm extract (5.5 μg); A = adapted worm extract (5.5 μg). Bands A, B and C at the side of Figs. 6.10a and b refer to AChE isoenzymes as designated by Edwards, Jones and Ogilvie (1971).

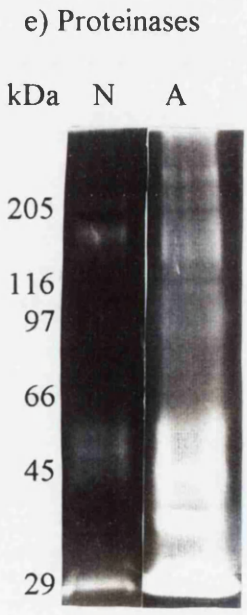
Figure 6.10

Somatic Extracts of Adapted and Normal *N. brasiliensis* Compared

Native PAGE

Non-Reducing SDS-PAGE





6.3 DISCUSSION

In the preceding two Chapters the effects which geographical origin and host immunity may impose on expression of selected parasite enzymes were discussed. In this Chapter, initial work concentrated on characterisation of adult *N. brasiliensis* proteinases in terms of pH optima, molecular size and inhibitor sensitivity. Following this characterisation, the *in vivo* effects of individual host on enzyme expression in adult *N. brasiliensis,* were examined. Results obtained demonstrated that the host has profound effects on parasite enzyme expression, particularly on parasite proteinases and SODs. Additional studies on 'adapted' and 'normal' adult *N. brasiliensis* provided further evidence of modulation of enzyme expression in parasites due to host effects. Parasite AChE, non specific esterases and SOD isoenzyme profiles were found to differ in 'normal' and 'adapted' *N. brasiliensis* as previously reported (Edwards, Burt and Ogilvie, 1971; Blackburn and Selkirk, 1992; Knox and Jones, 1992) and proteinase expression in 'adapted' parasites was elevated compared to 'normal' parasites although no definitive differences in proteinase isoenzyme profiles were observed.

The pH optima of proteolytic activity in somatic extracts of the adult parasite was determined spectrophotometrically using the substrate azocasein and by gelatin-substrate gel analysis, over a pH range of 3 to 10. Maximal proteolytic activity was substrate dependent, proteolytic activity using azocasein being optimal at acidic pH (Graph 6.1a), while optimal proteolysis was observed at pH 10 using gelatin-substrate gels (Fig. 6.2). Similarly, optimal proteolytic activity in adult IVR products was substrate dependent, gelatin degradation being optimal at alkaline pH (pH 9; Fig. 6.1c) while azocasein degradation was maximal at neutral pH (Graph 6.1b). Differences in the optimal pH's and in the proteinase profiles of somatic and IVR products indicated the presence of proteinases which may have different specificities *in vivo.* Differences in apparent proteinase pH optima when different substrates were used has been observed in other parasite species (Maki and Yanagisawa, 1986; Knox and Jones, 1990; Young, Knox and McKeand, 1995) and may reflect the effect of pH on the tertiary structure of the protein substrate thus exposing different peptide bonds which may be cleaved by quite distinct proteinases.

Adult *N. brasiliensis* somatic extracts contained several low molecular weight proteinases, less than 50 kDa, while, higher molecular weight activity was also observed at a high alkaline pH (Fig 6.2). These results contrast with those reported by Healer, Ashall and Maizels (1991). In these experiments (Healer, Ashall and Maizels, 1991), proteolytic activity of somatic extracts were determined at pH 7.6 and a number of proteinases greater than 50 kDa in size were recorded. Differences in the proteinase profile of IVR products were also noted when comparing results of the previous study (Healer, Ashall and Maizels, 1991) with those of the present study. Healer, Ashall and Maizels (1991) detected a single proteinase at 50 kDa in adult *N. brasiliensis* IVR products, contrasting with the relatively large number of proteinases detected in the present study. In the present study, pH optima definition of gelatinolysis (Fig. 6.1a and c) in somatic and IVR products of adult *N. brasiliensis* indicated that proteolysis was much more marked at pH 9 and 10 than at pH 7, but the proteinase profiles were essentially the same at all pH values tested. Therefore, the differences between the present data and those of Healer, Ashall and Maizels (1991) are likely to be due to parasite strain differences or different host influences or slight differences in experimental techniques.

Preliminary characterisation of adult *N. brasiliensis* somatic and IVR proteinases on the basis of class specific inhibitor sensitivity using gelatin-substrate gel analysis, indicated that adult somatic extracts contained aspartyl and cysteinyl proteinases as judged by the partial sensitivity of the proteinases to pepstatin and E 64 (Fig. 6.1b). Adult *N. brasiliensis* high molecular weight IVR proteinases were inhibited by 1, 10 phenanthroline, a metallo proteinase class inhibitor, although the lower molecular weight proteinases were largely unaffected by any of the inhibitors tested. Similarly, in the study by Healer, Ashall and Maizels (1991) activity in adult *N. brasiliensis* IVR products was diminished in the presence of 1,10 phenanthroline using a fluorogenic 7-aminomethylcoumarin-linked peptide as a substrate. These authors also demonstrated marked inhibition of adult somatic proteinases with 1, 10 phenanthroline using the same substrate. However, in gelatin-substrate gel analysis no specific inhibitor effect was noted on either somatic extracts or IVR products (Healer, Ashall and Maizels, 1991). It therefore seems likely that a number of proteinases of different proteinase class are present in both somatic and IVR products, some of which may be

of a similar size. The presence of a variety of proteinases may reflect the parasites ability to degrade a variety of substrates *in vivo* or modulate and or neutralise effects of the host immune response.

Infection of a group of rats of the same age and at the same time with the same population of L3 yielded some results which supported the hypothesis that the intra-host environment could markedly influence parasite enzyme expression. Variations in the proteinase and SOD profiles observed when individual worm populations were compared could be due to i) survival of specific genotypes in different individual hosts while other genotypes are expelled, dictated by the intra-host environment; ii) phenotype variation in the enzymes expressed in response to the intra-host environment and iii) allelic variation with no correlation to environment.

Variation in the parasites ability to express a proteinases or SOD may be dependent on the 'intensity of selection' by the host (Grant, 1994). Parasite populations then respond in different ways when the selection pressure is applied, both the response of the host and parasite being genetically determined (Grant, 1994). The variability in the responses of the parasites which originated from the same gene pool may reflect the different responses of the host, enabling a parasite genotype or allotype to survive better in one host than another.

Considerable variation was observed in the somatic and IVR proteinase profiles of parasites harvested from individual rats. Three out of the five proteinases observed in somatic extracts were inconsistently observed when the individual parasite populations were compared (Fig. 6.3a, lanes 1, 3 and 6) while no marked differences in total proteinase activity between populations was evident (Table 6.1a). By contrast proteolytic activity varied markedly in the IVR products of the different populations but these differences were related to level of activity and were not reflected in different Isoenzyme expression (Fig. 6.3b).

The variation in proteinase profile and total activity observed in this study between individual populations (Fig. 6.3a and b; Table 6.1a and b) may reflect differences in levels of proteinase gene expression or the expression of different genes in the individual parasite populations. *Fasciola hepatic* cysteine proteinase expression is, apparently, controlled by multi-gene families, differences have been detected in the steady state levels of mRNA present for some specific proteinases (Heussler and

Dobbelaere, 1994). *H. contortus* cysteine proteinase expression is also controlled by multi-gene families (Pratt *et al*, 1992). It is therefore possible that multigene families control proteinase expression to a greater or lesser extent in *N. brasiliensis* and this may account for the variable proteinase profiles observed in this study. This question can be addressed by isolating cDNAs encoding somatic and IVR proteinases and using these cDNAs as probes to seek evidence of multigene control using genomic southern blots.

The total activity (Table 6.1a) and isoenzyme profile (Fig. 6.8) of the free radical detoxifying enzyme, SOD, were also variable in somatic extracts of the adult parasite from individual rats. While low levels of SOD activity were detectable in IVR, this activity was too weak to be visualised by gel analyses and significant variation between parasite populations was not indicated. Nematode parasites contain both cytosolic and extracellular forms of SOD (Ou, Tang, McCrossman, Henkle-Duhrsen and Selkirk ,1995a Liddell and Knox personal communication) and evidence available to date suggests that the cytosolic form is encoded by a single copy gene, while the extracellular form may be encoded by a multigene family (Liddell and Knox, personal communication). If SOD expression is similarly controlled by more than one gene in *N. brasiliensis*, then differential gene expression may explain the variable SOD isoenzyme profiles noted here. These isoenzyme differences may also reflect the different 'fitness' status of the individual parasite populations. If the infection had been allowed to continue the parasites exhibiting the larger SOD levels may have maintained their infection for longer than the parasites with lower levels of enzyme. Age dependent variations in the metabolism of reactive oxygen species in parasites has been recorded, and the level of SOD in the parasite and it's release decreases markedly with age and appears to be correlated with worm expulsion (Smith and Bryant, 1989a; Batra *et al*, 1993).

No great differences were detected in the levels of another antioxidant enzyme, glutathione peroxidase (GSH-px; table 6.1b) or in isoenzyme profiles of undefined peroxidases in this experiment (Fig. 6.7). Previous work by Batra *et al* (1992), recorded a decrease in levels of SOD as infection progressed but they did not detect any marked alterations in levels of GSH-px. Similarly, a comparison of the free radical detoxifying enzymes of two parasites *Nematospiroides dubius* and *N. brasilienis*, in an

attempt to explain the ability of *N. dubius* infections to persist while *N. brasiliensis* are expelled by the host, found that although SOD levels were higher in *N. dubius* compared to *N. brasiliensis*, levels of GSH-px were comparable (Smith and Bryant, 1986). It was therefore concluded that GSH-px may not play as an essential role in parasite survival as SOD, and that levels of other enzymes or substrates such as glutathione-S-transferase or reduced glutathione, a free radical scavenger and the substrate for GSH-px, may have more of an effect on the parasite's ability to evade free radical attack (Smith and Bryant, 1986; Batra *et al.* 1992). It therefore seems pertinent to note that the lack of variation detected in total GSH-px levels (Table 6.1a) in this study may not reflect differences in the ability of a particular parasite population to neutralise free-radical attack. Other compounds not detected in this study, may have a more regulatory role in this process.

Non-specific esterases and AChE in somatic extracts of adult *N. brasiliensis* harvested from individual rats did not appear to be affected by the host in any marked way. Esterase and AChE isoenzyme profiles of somatic extracts were similar to those previously recorded (Edwards, Burt and Ogilvie, 1971; Blackburn and Selkirk 1992). In contrast levels of AChE in the IVR products of worms harvested from individual rats were higher than the levels detected in somatic extracts and differed widely depending on which rat they were harvested from (Table 6.2). This variation in level of enzyme released between batches of worms has been reported previously and is to be expected if the enzyme is being actively released by the parasite (Ogilvie *et al*, 1973). AChE isoenzyme analysis of adult *N. brasiliensis* IVR products did not reveal any alterations in profile (Fig. 6.5c and d), but variation was evident in the profiles of non-specific esterases when viewed under non-reducing conditions (Fig. 6.5b). AChE profiles of IVR products under native polyacrylamide conditions were similar to somatic extracts. and are in accord with the AChE profiles under native conditions reported by Blackburn and Selkirk (1992).

Adult *N. brasiliensis* somatic extracts were also stained for LDH, a constitutively expressed enzyme which did not exhibit any isoenzyme variation within the individual parasite populations (Fig. 6.8), however wide variation in levels of total enzyme activity were noted.

Variation in establishment and longevity of parasite infections in hosts has been recorded and it is well documented that parasite infections are over dispersed in hosts, a few host harbouring many parasites and many hosts harbouring a few parasites (Keymer and Slater, 1986). These differences have been linked to differences in the host immune response which may be under the control of genes of the major histocompatability complex (MHC) (Wakelin, 1985; Kennedy, 1990). The results described here strongly suggest that host influences do have an effect on parasite enzyme expression and, indirectly, that this could influence parasite survival within a population

Antibodies to AChE in *N. brasiliensis* infections have been detected indicating their possible role in eliciting protective immune responses by the host (Jones and Ogilvie, 1972; Nakazawa, Yamada, Uchikawa, Arizono, 1995) and dynamic enzyme expression could help the parasite to overcome the host immune response. No antibody recognition was observed with non-specific esterases, however AChE bands A, B and C were inhibited to varying degrees by immune rat sera (Jones and Ogilvie, 1972). The variation in inhibition of AChE bands was related to sera from different rats providing further evidence that immune responses of hosts are variable (Jones and Ogilvie, 1972). In contrast, the variability in the binding affinity of pooled immune sera to the IVR products of individual worm populations (Fig. 6.9) may indicate variation in the structure of antibody binding epitopes between parasite populations.

Comparison of enzymes from somatic extracts of 'normal' and 'adapted' parasites, pooled from a number of hosts, revealed marked differences, in agreement with previous studies on AChE (Edwards, Burt and Ogilvie, 1971) and SOD (Knox and Jones, 1992), in similar parasitic populations. Proteolytic activity was analysed for the first time here, and 'adapted' worms were found to have dramatically elevated levels of these enzymes compared to 'normal' worms (Fig. 6.10e), although unique isoenzyme expression in 'adapted' parasites compared to 'normals' was not noted.

Non-specific esterase and AChE profiles were similar to those recorded previously (Edwards, Burt and Ogilvie, 1971), with adapted parasites exhibiting an intense staining band (band A) compared to 'normals'. In addition, the elevated SOD activity noted here in 'adapted' worms was in agreement with a previous study (Knox and Jones, 1992). Edwards, Burt and Ogilvie, (1971) also detected differences in the

isoenzyme profile of acid phosphatases between the normals and adapted, but this enzyme activity was not detected by gel analysis in either extract in the present study.

These results on the effect individual hosts have on enzyme expression, the effect of a specific immune response on a secondary parasite infection, and the results discussed in Chapters 4 and 5, indicate that enzyme expression in GI nematodes is modulated by the intra-host environment.

Earlier work by Jones and Ogilvie (1972), in which passive immunisation of rats with antiserum reduced establishment of challenge infection worms, showed that worms that were able to establish an infection had increased levels of AChE compared to 'normals' although not to the same level as 'adapted' parasites. These results indicate that AChE is an important enzyme for the adaptation process and supports the belief that AChE is important for parasite maintenance within the host, functioning as a 'biochemical holdfast, inhibiting local gut peristalsis (Lee, 1970), reducing mucus secretion, (Phillipp, 1983) and being involved in the control of cellular immune responses (Prichard, 1993b). Attempts at trying to determine when the adaptation process begins by examining the AChE profiles of earlier parasitic stages have found that the L3 stage has little activity and the L4 AChE profile resembles that of the adult parasite (Ogilvie, 1972). It was therefore concluded that the adaptation process probably occurs sometime between 24 and 96 hours after reinfection and probably begins during the L3/L4 moult in the lungs (Ogilvie, 1972).

It would be interesting to compare the enzyme profiles of the L3 and L4 stages of the parasite to investigate whether the heterogeniety of enzyme expression is found in all parasitic stages or whether it is a phenomena only of the adult stages. How marked parasite enzyme adaptation under natural conditions is also unknown, perhaps repeating some of these experiments using trickle infection regimes would mimic a more natural infection. It has been established that adapted worms express different antigens from 'normal' worms (Ogilvie, 1972; Jenkins and Phillipson, 1972 a and b). It has also been proposed that nematodes can vary their antigenic structure (Ogilvie, 1972; Maizels *et al,* 1993) and the variation in the proteinase and SOD profile of somatic extracts and proteinase and non-specific esterase profiles of *N. brasiliensis* IVR products from different rats would substantiate this argument. Maizels, Meghji and Ogilvie (1983) attempted to look at the surface antigens of different stages of *N.*

brasiliensis and detected great variation in the number and the molecular size of L3 surface antigens which may indicate that the parasite is able to express a number of different antigens by varying expression of the genes encoding surface antigens in a similar way to trypanosomes varying their surface glycoproteins. It may be that parasite enzyme expression is varied in a similar way.

Genetic variation in parasite populations can be detected by direct methods such as restriction fragment length polymorphism analysis or by analysis of gene products, usually by a range of electrophoretic techniques. The analysis of enzyme electrophoretic mobility had been used to compare parasite strains (e.g. Gasnier, Cabaret and Suarez, 1993; Eschevarria, Gennari and Tait, 1992). Profound differences in the esterase profiles of BZ-resistant and BZ-susceptible strains of *H. contortus* were noted (Sutherland, Lee and Lewis, 1988) but an analysis of isoenzyme expression in *H. contortus* resistant or susceptible to ivermectin revealed no profound differences. It is possible a mutation in one gene to enhance parasite survival may result in modified expression in related or unrelated gene families. Studies to compare alloenzyme variation in worms from wild and laboratory reared populations of *T. colubriformis* indicated only a small variation in genetic differences between the two parasite populations, however there were larger differences in alloenzyme expression between the two populations (N'zobadila, Cabaret and Gasnier, 1993). Similarly, alloenzyme analysis has been used to address the problem of species identification and evolutionary origin when it is difficult to make morphological comparisons (Vainola, Valtonen and Gibson, 1994; Gasnier, Cabaret and Suarez, 1993). It has however, been noted that differences in alloenzymes do not always indicate genetic differences as certain protein loci are prone to post translational modifications which alter electrophoretic mobility despite the lack of underlying nucleotide changes (reviewed Nadler, 1990).

In conclusion it appears that parasitic nematodes such as *N. brasiliensis* and *H. contortus* may be able to regulate their gene expression, and that some enzymes, most notably that of proteinase, SOD and AChE may be under the control of multigene families. The use of restriction fragment length polymorphism (RFLP) techniques, followed by southern blotting and subsequent hybridisation of specific DNA probes for the enzyme of interest, would provide information as to whether enzyme expression is under the control of multigene families. Additionally, restriction enzyme digestion of

the DNA from different parasite populations, followed by sequencing or DNA probing would indicate whether there was genetic polymorphism of the enzyme in question. Reverse transcriptase (RT)-PCR could also be used to quantify enzyme expression within a parasite population. This could then be used to determine whether differences observed in isoenzymes between parasite populations is due to modified gene expression. Some of these methods were used in the following Chapter to investigate SOD expression in populations of adult *N. brasiliensis*.

CHAPTER SEVEN

MOLECULAR ANALYSIS OF SUPEROXIDE DISMUTASE IN *NIPPOSTRONGYLUS BRASILIENSIS*

7.1 INTRODUCTION

The work described in Chapters four to six provided further evidence to support the hypothesis that parasite enzyme expression was responsive to the intra-host environment. The degree to which isoenzyme expression varied depended on the enzyme being studied and was most apparent in proteinase, esterase, acetylcholinesterase (AChE) and superoxide dismutase (SOD) expression. All of these enzymes which have been strongly implicated in parasite survival within the host.

While enzyme isoenzyme analyses demonstrated clear differences in secretion/expression between parasite populations of the same species, the functional significance of these differences could not be addressed by this approach. For example, individual isoenzymes may be antigenically quite distinct and/or these isoenzymes may have differing substrate specificities or affinities which may be advantageous for parasite survival. Alternatively, parasite enzyme expression may be relatively invariant within individual parasites and the differences noted here may simply reflect selection of parasites with the ability to survive in a particular host, this selection being due to the intra-host environment which may be quite variable between different individual hosts.

The objective of the work described here was to examine enzyme expression at the molecular level. SOD was chosen as the target enzyme because i) variable isoenzyme expression was clearly indicated in the work described in the previous chapter and previously (Knox and Jones, 1992, Knox *et al*, 1992); ii) host generated free radicals have been implicated in *N. brasiliensis* expulsion thus implying the relevance of the host/SOD interaction for immunity (Smith and Bryant, 1989a and b); iii) longevity of parasite survival has been correlated with the level of parasite antioxidant enzyme expression (Smith and Bryant, 1986; Batra *et al*, 1993) and, iv) SOD gene probes to the ovine GI nematode *H. contortus* were already available at Moredun (courtesy of Dr S. Liddell). In addition nucleic acid and protein data base searches indicated that SOD sequences were highly conserved between nematode species.

7.1.1 Rationale for Analysis of SOD Isoenzyme Expression in *N. brasiliensis* by RT-PCR

As small differences in amino acid composition between individual isoenzymes may be difficult to detect and quantitate, reverse transcription PCR (RT-PCR), using primers based on conserved regions of SOD genes were used to analyse specific gene expression. RT-PCR is now used widely for isolating specific genes and for semi-quantitative determination of specific gene expression by providing a measurement of the amount of mRNA encoding the protein of interest at a given point in time (Abbot, Poiesz, Byrne, Kwok, Sninsky and Ehrlich, 1988; Kain, Brown, Lenar, Ballon and Webster, 1993). In theory, specificity is provided by the choice of sequence specific primers and high stringency PCR conditions. This method of measuring mRNA transcripts also has advantages over other methods in that i) fewer parasites are required than used in conventional protein chemistry techniques or Northern blotting and ii) RT-PCR can be used to detect differences between individual parasites.

One potential difficulty with semi-quantitative RT-PCR is how to validate comparisons between analyses performed on different worm populations at different times. The detection of an RT-PCR product and the amount generated will be influenced by i) the amount of target transcript synthesised in the reverse transcription reaction which, in turn, is influenced by the efficiency of RNA extraction ii) the day to day variation in the efficiency of the individual components of a RT-PCR and product detection. To allow for these factors, RT-PCR reactions can be calibrated by performing parallel control reactions where the selected target is preferably, abundant and expressed by all the parasite stages of interest. For the work described in this chapter, tubulin was selected as a constitutively expressed protein for the control RT-PCR as it is known to be present in all parasite stages, it is not excreted/secreted and, therefore, no great differences between the levels of tubulin expression in different 7 d.p.i. parasite populations were expected.

Tubulins are the principle structural components of mitotic and meiotic spindles and are involved in intracellular transport, maintenance of various cell surface functions, overall cell shape and the internal cytoplasmic architecture (reviewed Cleveland and Sullivan, 1985; Sullivan, 1988). It consists of a 50 kDa heterodimer

made up of α and β proteins which self associate to form a cylindrical wall of hollow microtubule filaments (Sullivan, 1988). The β-tubulins are encoded by multigene families and, although the sequences of different members of the gene families are highly conserved, the sequences tend to be divergent towards the carboxy termini. (Cleveland and Sullivan, 1985). As a result of this variation, vertebrate β-tubulins have been grouped into 6 isotypes and, although differential functions among isotypes have been noted, they are not common (Geary *et al*, 1992).

7.1.2 Establishment of RT-PCR Assay for the Detection of *N. brasiliensis* SOD

To develop a RT-PCR assay to enable SOD expression in *N. brasiliensis* to be characterised in more detail, it was necessary to first isolate a DNA fragment encoding SOD. Briefly, SOD encoding complementary DNAs (cDNAs) were isolated using a sense oligonucleotide primer directed to the spliced leader 1 (SL1) sequence in combination with a SOD gene-specific antisense primer. SL1 consists of 22-nucleotides in the 5'-untranslated region of the majority (>70%) of nematode mRNA sequences (reviewed Bonen, 1993) and is thought to be required for correct removal of intron sequences, by a trans-splicing reaction, during gene transcription (Nilsen, 1989). The gene-specific primer used here (designated SOD7, Table 7.1) was directed towards arginine 146, a region of the SOD sequence near the 3' end which is highly conserved in all known Cu/Zn SOD sequences including nematode parasites such as *H. contortus* (Dr S. Liddell, personal communication). The RT-PCR reactions were carried out at moderate stringency (40°C) using total RNA isolated from adult *N. brasiliensis* as template after conversion to a RNA/DNA hybrid by reverse transcription. The identity of the PCR products were sought, in the first instance, by Southern blotting and probing with a defined SOD DNA fragment from the nematode *H. contortus* (courtesy of Dr S Liddell) and confirmed by cloning into a plasmid vector and DNA sequencing. After this *N. brasiliensis* SOD gene-specific sense and anti-sense primers were designed to develop the RT-PCR.

The amount of amplified product was determined using densitometry by reflectance (BioRad Model GS-670 image analysis system) to avoid the use radioisotopes (Abbot, Poiesz, Byrne, Kwok, Sninsky and Ehrich, 1988)

7.2 RESULTS

7.2.1 Extraction of mRNA from Adult *N. brasiliensis*

Adult *N. brasiliensis* mRNA (Section 2.5.3) was extracted using the Quick Prep Micro mRNA purification kit as described in Section 2.10.1.

Ribosomal RNAs and unbound mRNA were washed from the oligo (dT) cellulose using high salt buffer (Fig. 7.1, Panel A, Lanes 1 to 5). Further ribosomal RNA was eluted along with transfer RNAs using low salt buffer (Fig. 7.1, Panel A, Lane 6). Final washing with pre-warmed elution buffer yielded abundant mRNA, evident as a smear, over a broad size range (Fig. 7.1, panel B, Lanes 1 and 2).

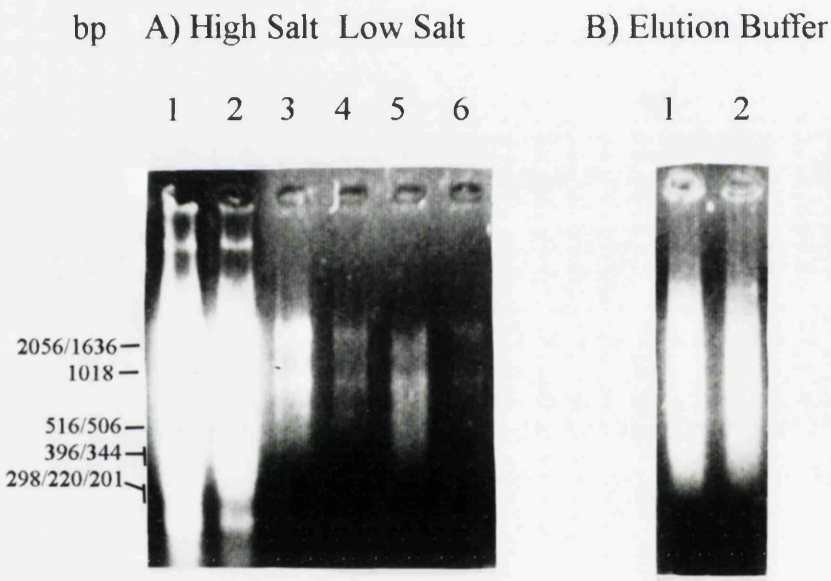
The amount of mRNA extracted was determined spectrophotometrically at 260 nm and 280 nm to quantitate the amount recovered (Section 2.10.2). Approximately five μg mRNA was recovered from 0.1g parasites. After extraction, the mRNA was fractionated in a 1.0% agarose gel and visualised by ethidium bromide staining (Section 2.10.7) and silver staining (Section 2.10.3).

Figure 7.1 mRNA Isolation from Adult *N. brasiliensis.*

RNA in aliquots (1/5th total volume) of the eluates during the mRNA isolation procedure was precipitated with ethanol and then re-dissolved in 10 µl TE buffer prior to analysis in a 1.0% agarose gel. Eluates analysed were high salt (Panel A, lanes 1 to 5), low salt (Panel 1, Lane 6) and elution buffer (Panel B, Lanes 1,2).

Figure 7.1

Adult *N. brasiliensis* mRNA



7.2.2 Isolation of Putative *N. brasiliensis* SOD Encoding cDNAs

PCR amplifications: First strand cDNA for use as PCR template DNA was synthesised as described in Section 2.10.5. Amplification of SOD encoding sequences was then attempted using a sense primer directed to the spliced leader sequence (SL1) and a sequence specific primer, SOD7 (Table 7.1), using 10 ng cDNA and medium stringency conditions (95°C for 1 min, 45°C for 3 min, 72°C for 3 min for 30 cycles, Section 2.10.6). The expected product size was calculated to be approximately 500 base pairs (bp). The PCR products yielded were analysed using 1% agarose gel electrophoresis (Fig. 7.2).

The PCR reaction yielded two prominent bands around the expected size range of 500 bp (Fig. 7.2, Lanes 1, 2 and 3 each of which represent different PCR reactions).

Table 7.1

Oligonucleotides Used for the Isolation of SOD Encoding cDNA from *N. brasiliensis*

Primer	Sequence	Primer length and type
SL 1	5' GGTTTAACCACCCAAGTTTGAG 3'	Sense primer 22 nts
SOD7	5' ACCGCAGCGAGGCGAGCGCAGCTTAACCAGT 3' *T G N A G A R L A C* region	Antisense primer 31 nts

Note: SL1 - sense primer found in the 5' untranslated region of approximately 70% of mature nematode mRNA transcripts. (See Fig. 7.5)

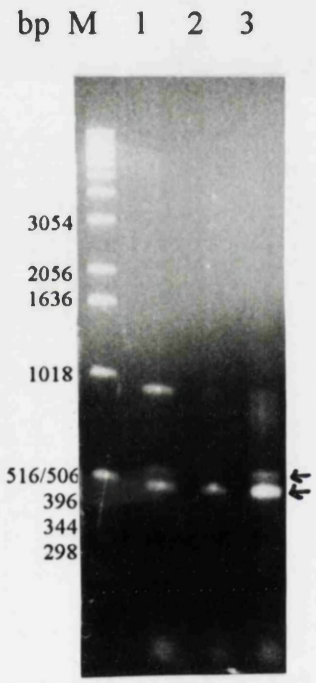
SOD7 - antisense primer to arganine 147, a highly conserved region in all known Cu/Zn SOD sequences (See Fig. 7.5 and Fig. 7.6)

Figure 7.2 PCR Amplification of Putative *N. brasiliensis* SOD Encoding cDNAs

The amplification products generated by three separate PCR amplifications with different amounts of first strand cDNA from adult *N. brasiliensis* (Fig. 7.2, Lanes 1, 2 and 3;.5 ng, 10 ng and 20 ng) using the SL1 sense primer and an antisense oligonucleotide primer directed to a highly conserved region of the SOD molecule (SOD7). The products (←) were fractionated in a 1.0% agarose gel and products at 500 and 450 bp were evident as expected.

Figure 7.2

RT-PCR of Putative *N. brasiliensis* SOD Encoding cDNA



Southern blot analysis: The identity of these 500 bp DNA fragments was confirmed by Southern blot transfer of the gel products (Fig. 7.2) to a nylon membrane (Hybond, Section 2.10.8) and subsequent probing with a digoxigenin labelled *H. contortus* SOD probe (in collaboration with Dr. S. Liddell) using moderate stringency annealing and washing conditions (Section 2.10.10, 16 h 42°C, and washed twice for 15 mins in 2X SSC and 1X SSC at 42°C). The SOD probe annealed to both the 450 bp and 500 bp DNA fragments from *N. brasiliensis* indicating that both encoded SODs (not shown). From hereon, the upper band is designated NBSOD1 and the lower NBSOD2. The band evident at 1000 bp (Fig. 7.2, lane 1) and the smear in Fig. 7.2 lane 3, were artifactual and did not hybridise to the SOD probe and were not evident in subsequent PCR amplifications.

Sub-cloning: The 450 and 500 bp PCR products were purified from 1% agarose gels using GENECLEAN™ and ligated into the plasmid vector pCR II as described in Section 2.10.12. The ligated DNA was then transformed into competent *E. coli* cells (Invitrogen, Section 2.10.13). Using aseptic techniques, ten (NBSOD2) and one (NBSOD1) putative recombinant (white) colonies were isolated and grown up overnight in 10 ml LB broth (Section 2.10.14).

Restriction digestion and hybridisation analysis of putative recombinant pCR II isolates: The putative recombinant plasmids generated by cloning SOD band 1 or 2 into pCR II were characterised by restriction enzyme analysis and Southern blotting followed by hybridisation with the defined *H. contortus* cDNA fragment. Plasmid DNA was isolated (Section 2.10.14) and digested with *Eco* R1 to excise the insert DNA fragment. The digestion products were analysed on 1% agarose gels (Fig. 7.3) and by hybridisation with the *H. contortus* SOD probe (Fig. 7.4).

Eco R1 digestion of purified plasmid preparations putatively containing the NBSOD2 insert clearly yielded products of the expected size approximately 500 bp, in 9 out of the 10 selected colonies (Fig. 7.3a, Lanes 1 to 7, 9 and 10) while the single putative NBSOD1 recombinant plasmid also give a fragment of approximately 500 bp after *Eco R1* digestion (Fig. 7.3b, lane 1).

The DNA fragment excised by *Eco RI* digestion hybridised to the *H. contortus* SOD DNA probe in every case except for Fig. 7.4a, lane 8, indicating that the cloning process had been successful.

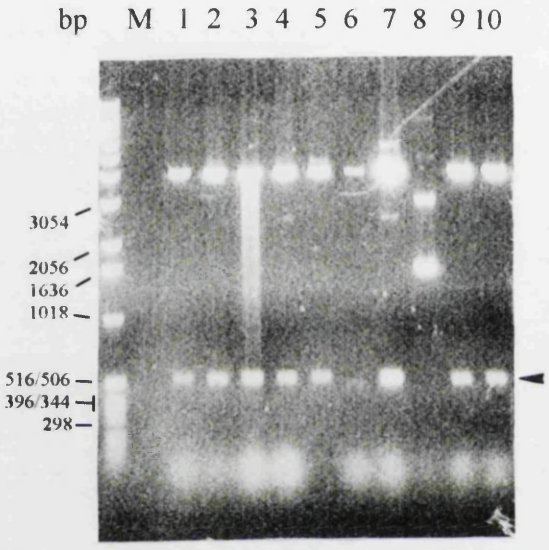
Figure 7.3 Restriction Enzyme Digestion of Putative Recombinant pCR II Plasmids Containing *N. brasiliensis* SOD Encoding cDNAs

Eco R1 digest products were fractionated in 1.0% agarose gels and the products of 10 recombinant plasmids carrying NBSOD2 (Fig. 7.3 A, lanes 1-10) and one carrying NBSOD1 (Fig. 7.3 B, lane 1) are shown.

Figure 7.3

Restriction Enzyme Excision of Putative SOD Encoding cDNAs from Plasmid pCR II

a) *Eco* RI Digestion of SOD Band 2



b) *Eco* RI Digestion of SOD Band 1

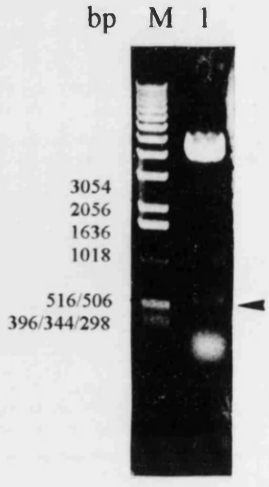


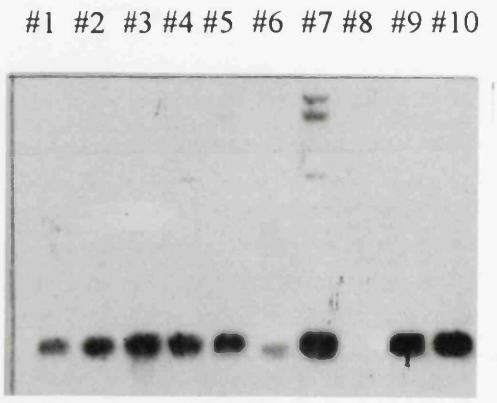
Figure 7.4 Southern Blot Analysis of Putative SOD Carrying Recombinant pCR II Plasmids

Plasmid DNA was digested with *Eco R1* and the digest products fractionated, blotted and probed with a defined *H. contortus* SOD probe as described in Section 2.10.8. Recombinant plasmids carrying NBSOD band 2 (Fig. 7.4a, lanes 1- 7 and 9 and 10), and NBSOD band 1 (Fig. 7.4b, lane 1) are shown.

Figure7.4

Southern Blot of *Eco* R1 Digests of pCR II Probed with *H. contortus* SOD

a) pCR II with SOD Band 2



b) pCR II with SOD Band 1

7.2.3 **Sequence Analysis of Putative *N. brasiliensis* SOD Inserts in pCR II**

N. brasiliensis NBSOD1, plasmid 1, and NBSOD2, plasmids 3, 4 and 7 were sequenced as described in Section 2.10.16 using the Pharmacia T7 Sequencing kit.

The nucleotide sequence of NBSOD1, plasmid 1 was 542 bp in length including the two primers while NBSOD2: 3, 4 and 7 were 490, 461 and 494 bp in length respectively including the primers SL1 and SOD7 (Fig. 7.5). These sequences were then compared to known helminth SOD gene sequences by data base searching using Genbank (Fig. 7.6) and shared 47 to 83 % homology at the amino acid level with cytosolic SOD sequences from *Brugia pahangi* (X76284) clones 1, 3, 4 and 7; 82, 83, 81 and 50 % respectively, *Schistosoma mansoni* (Q01137; Da Silva, Le Presle, Capron and Pierce, 1992), clones 1, 3, 4 and 7; 67, 67, 72 and 47 % respectively and *Onchocerca volvulus* (P24706; Henkle, Liebau, Mueller, Bergmann and Walter, 1991) clones 1, 3, 4 and 7; 82, 83, 82 and 49 % respectively. All clones were sequenced once.

Figure 7.5 Comparison of the Four *N. brasiliensis* SOD Clones

Nucleotide alignment of the four *N. brasiliensis* SOD clones *N. brasiliensis* NBSOD1 clone 1 (nippo1), NBSOD2 clone 3 (nippo3), NBSOD2 clone 4 (nippo4) and NBSOD2 clone 7 (nippo7). The sequence reads from 5' to 3' and includes the SL1 and SOD7 oligonucleotides as marked. The *N. brasiliensis* SOD specific primers are also marked as shown NSOD 1 and NSOD 3 (NSOD 3 is the reverse complement of the coding sequence). Unknown nucleotides are marked X and lower case nucleotides indicate discrepancies between sequence analyses. ↓ = Start Codon

Figure 7.5

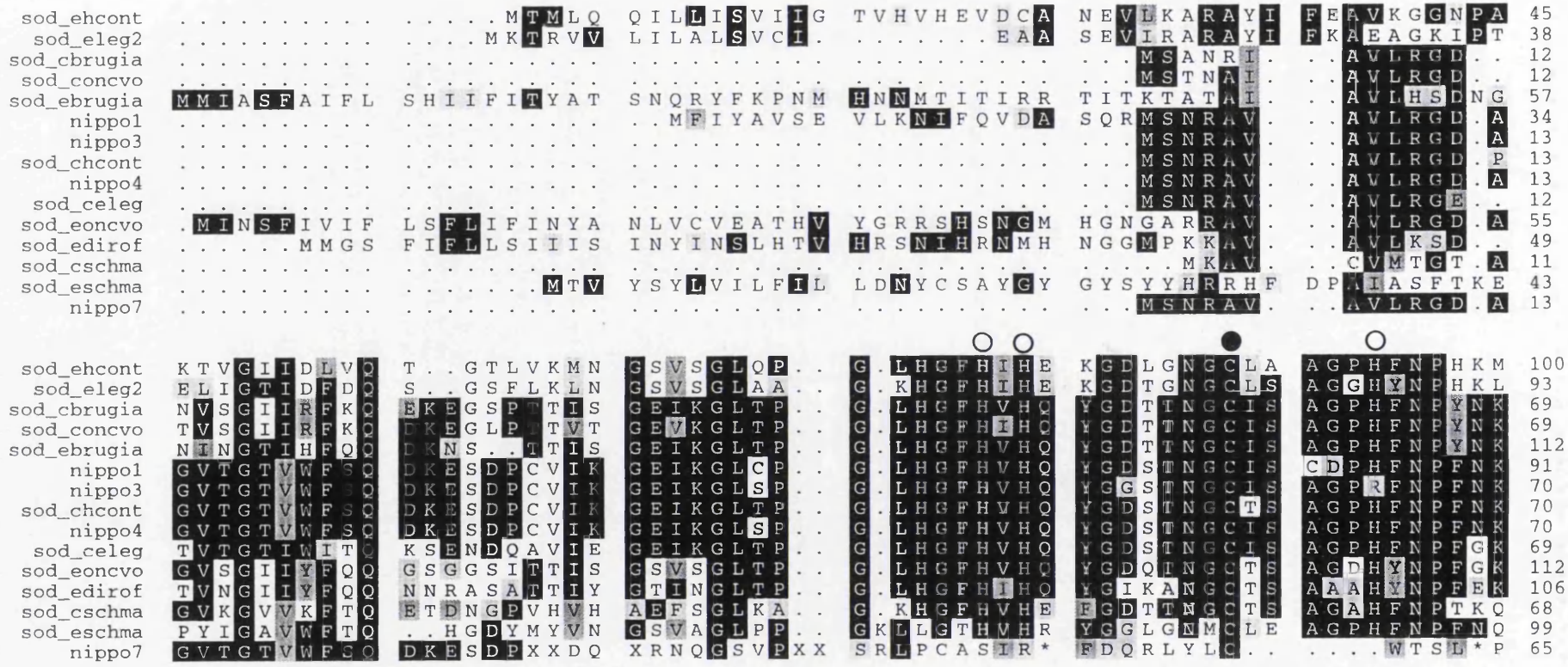
Sequence Comparisons of the Four *N. brasiliensis* SOD clones Showing the Positions of the *N. brasiliensis* SOD derived Primers

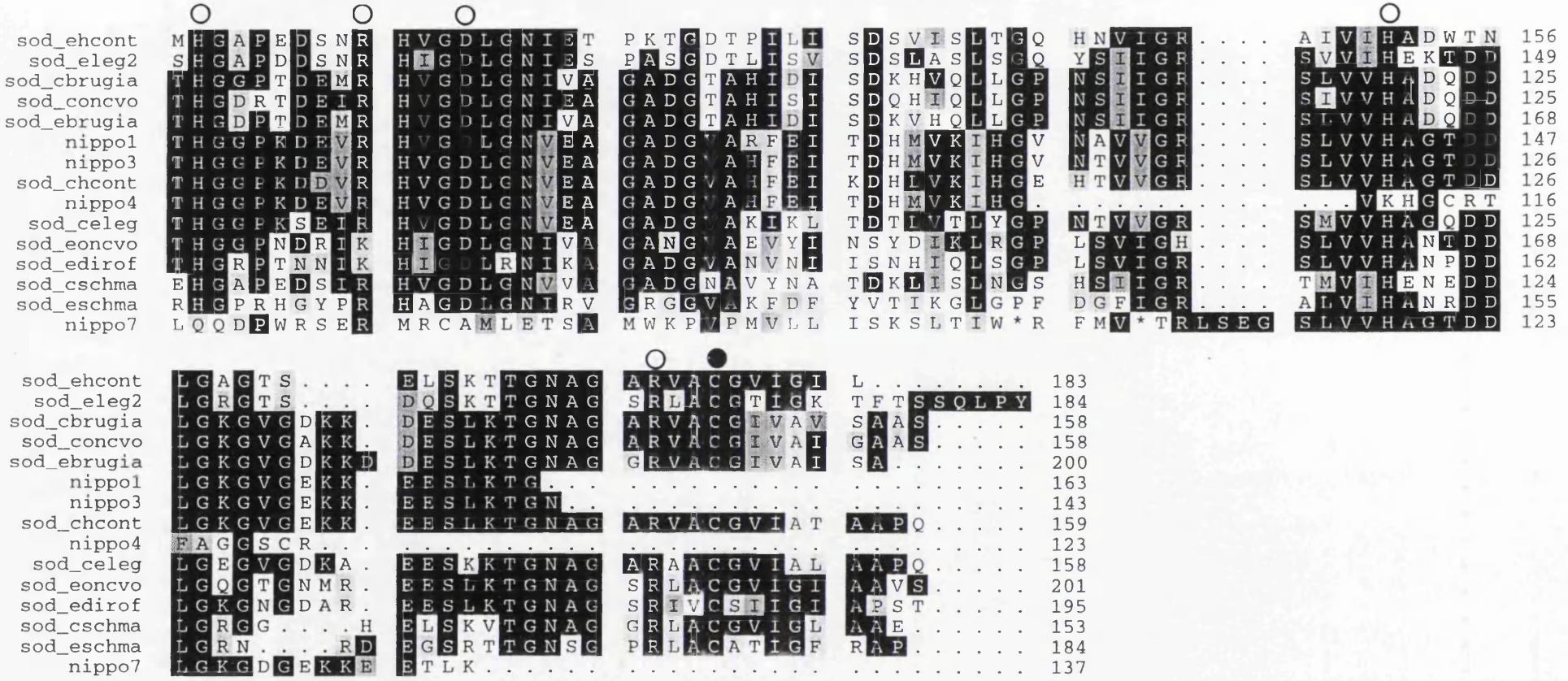
```
        1          SL1                                          50
nippo1  GGTTTAATTA CCCAAGTTTG AGTGCCGGAA TGTTCATCTA TGCGGTGTCG
nippo4  .......... .......... .......... .......... ..........
nippo7  .......... .......... .......... .......... ..........
nippo3  .......... .......... .......... .......... ..........

        51                                                   100
nippo1  GAGGTGCTGA AGAACATATT TCAGGTGGAC GCGTCACAAA GAATGAGCAA
nippo4  ..GGTTTAAT TACCCAAGTT TGAGGTGGAC GCGTCACAAA GAATGAGCAA
nippo7  .......... .SL1...... ....GTGGAC GCGTCACAAA GAATGAGCAA
nippo3  ..GGTTTAAT TACCCAAGTT TGAGGTCGAC GCGTCACAAG GAATGAGCAA

        101                                                  150
nippo1  CCGAGCAGTG GCCGTGTTGA GAGGTGATGC CGGAGTTACT GGGACGGTGT
nippo4  CCGAGCAGTG GCCGTGTTGA GAGGTGATGC CGGAGTTACT GGGACGGTGT
nippo7  CCGAGCAGTG GCCGTGTTGA GAGGTGATGC CGGAGTTACT GGGACGGTGT
nippo3  CCGTGCTGTG GCCGTGTTGA GAGGAGATGC CGGAGTTACC gggacgGtgt

        151                                                  200
nippo1  GGTTCAGTCA GGACAAGGAA TCGGACCC.G TGTgtGATCA AGGGCGAAAT
nippo4  GGTTCAGTCA GGACAAGGAA TCGGACCC.G TGTGTGATCA AGGGCGAAAT
nippo7  GGTTCAGTCA GGACAAGGAA TCGGACCC.G TXTXTGATCA AXXXCGAAAT
nippo3  GGTTCAGTCA GGACAAAGAA TCGGACCC.G TGTGTAATCA AGGGCGAAAT

        201                                                  250
nippo1  CAAGGGTCTG TGCCCTGGTC TTCACGGCTT CCATGTGCAT CAATACGGTG
nippo4  CAAGGGTCTG TCCCCTGGTC TTCACGGCTT CCATGTGCAT CAATACGGTG
nippo7  CAAGGGTCTG TCCCCXXXXC TTCACGGCTT CCATGTGCAT CAATACGGTG
nippo3  CAAAGGTCTG TCTCCTGGTC TTCACGGTTT CCATGTGCAT CAATACGGTG

        251                        NSOD,                     300
nippo1  ATTCGACCAA CGGCTGTATC TCTTGCGACC cTCACTTTAA CCCCTTCAAC
nippo4  ATTCGACCAA CGGCTGTATC TCTGCTGGAC CTCACTTTAA CCCCTTCAAC
nippo7  ATTCGACCAA CGGCTGTATC TCTGCTGGAC CTCACTTTAA CCCCTTCAAC
nippo3  gTTCGACCAA CGGCTGTATC TCTGCTGGAC CTCgCTTTAA CCCCTTCAAC

        301                                                  350
nippo1  AAGACCCATG GAGGTCCG.A AGGATGAGGT GCGCCATGTT GGAGACCTCG
nippo4  AAGACCCATG GAGGTCCG.A AGGATGAGGT GCGCCATGTT GGAGACCTCG
nippo7  AAGACCCATG GAGGTCCGAA agGATGAGGT GCGCCATGTT GGAGACCTCG
nippo3  AAGACCCATG GAGGTCCG.A AGGATGAGGT GCGCCATGTT GGAGATCTCG

        351                                                  400
nippo1  GCAATGTGGA AGCCGGTGCC GATGGTGTTG CTCGTTTCGA AATCACTGAC
nippo4  GCAATGTGGA AGCCGGTGCC GATGGTGTTG CTCATTTCGA AATCACTGAC
nippo7  GCAATGTGGA AGCCGGTGCC GATGGTGTTG CTCATTTCGA AATCACTGAC
nippo3  GAAACGTTGA AGCTGGAGCC GATGGTGTTG CTCATTTCGA GATCACTGAC

         401                                                 450
nippo1  CATATGGTAA AGATTCATGG TGTG.AACGC GGTTGTCGGA CGTTCGCTGG
nippo4  CATATGGTAA AGATTCATGG TGTGAAACAC GGTTGTCGGA CGTTCGCTGG
nippo7  CATATGGTAA AGATTCATGG TGTGAACACG GTTGTCGGAA GGTTCGCTGG
nippo3  CATATGGTGA AGATTCACGG TGTG.AACAC GGTTGTCGGA CGTTCGCTGG
```

```
        451                                                    500
nippo1  TGGTTCATGC CGGAACTGAC GACCTTGGCA AAGGAGTCGG TGAGAAGAAG
nippo4  TGGTTCATGC CGGAACTGAC GACCTTGGCA AAGGAGTCGG TGAGAAGAAG
nippo7  TGGTTCATGC CGGAACTGAC GACCTTGGCA AAGGAGACGG TGAGAAGAAG
nippo3  TGGTCCATGC CGGGACTGAT GACCTTGGCA AAGGAGTCGG TGAGAAGAAG

        501                                SOD7          545
nippo1  GAGGAGTCGC TGAAGACTGG TAAGCTGCGC TCGCCTCGCT GCGGT
nippo4  GAGGAGTCGC TGAAG..... .......... .......... .....
nippo7  GAGGAGACGC TGAAG..... .......... .......... .....
nippo3  GAGGAATCGC TGAAGACTGG TAACGTGCGC TCGCCTCGCT GCGGT
```

Figure 7.6 **Amino Acid Alignment of *N. brasiliensis* SOD with Known Nematode SOD Sequences**

The SOD sequences shown are as follows: sod_ehcont - EC SOD *H. contortus;* sod_eleg2 - *C. elegans* SOD gene 2, putatively extracellular (P34461); sod_cbrugia - cytosolic SOD *B. pahangi* (X76284)*;* sod_concvo - cytosolic SOD *O. volvulus* (P24706)*;* sod_ebrugia - EC SOD *B. pahangi;* sod_chcont - cytosolic SOD *H. contortus;* sod_celeg - cytosolic SOD *C. elegans* (X77020)*;* sod_eoncvo - EC SOD *O. volvulus* (X57105)*;* sod_edirof - EC SOD *Dirofilaria immitis* (V14994)*;* sod_cschma - cytosolic SOD *S. mansoni* (Q01137)*;* sod_eschma - EC SOD *S. mansoni* (P16026)*;* nippo1, nippo3, nippo4 SOD1 clone 1, SOD2 clones 3 and 4 respectively. Identical residues are shaded ■ , conserved residues are shaded ▓ and ▒ . Residues involved in catalysis and metal ion binding are marked ○. Cysteine residues used in intrachain di-sulphide bonding are marked ●.


sod_ehcont
sod_eleg2
sod_cbrugia
sod_concvo
sod_ebrugia
nippo1
nippo3
sod_chcont
nippo4
sod_celeg
sod_eoncvo
sod_edirof
sod_cschma
sod_eschma
nippo7


sod_ehcont MHGAPEDSNRHVGDLGNIETPKTGDTPILISDSVISLTGQHNVIGR....AIVIHADWTN 156
sod_eleg2 SHGAPDDSNRHIGDLGNIESPASGDTLISVSDSLASLSGQYSIIGR....SVVIHEKTDD 149
sod_cbrugia THGGPTDEMRHVGDLGNIVAGADGTAHIDISDKHVQLLGPNSIIGR....SLVVHADQDD 125
sod_concvo THGDRTDEIRHVGDLGNIEAGADGTAHISISDQHIQLLGPNSIIGR....SIVVHADQDD 125
sod_ebrugia THGDPTDEMRHVGDLGNIVAGADGTAHIDISDKVHQLLGPNSIIGR....SLVVHADQDD 168
nippo1 THGGPKDEVRHVGDLGNVEAGADGVARFEITDHMVKIHGVNAVVGR....SLVVHAGTDD 147
nippo3 THGGPKDEVRHVGDLGNVEAGADGVAHFEITDHMVKIHGVNTVVGR....SLVVHAGTDD 126
sod_chcont THGGPKDDVRHVGDLGNVEAGADGVAHFEIKDHLVKIHGEHTVVGR....SLVVHAGTDD 126
nippo4 THGGPKDEVRHVGDLGNVEAGADGVAHFEITDHMVKIHG.......... ....VKHGCRT 116
sod_celeg THGGPKSEIRHVGDLGNVEAGADGVAKIKLTDTLVTLYGPNTVVGR....SMVVHAGQDD 125
sod_eoncvo THGGPNDRIKHIGDLGNIVAGANGVAEVYINSYDIKLRGPLSVIGH....SLVVHANTDD 168
sod_edirof THGRPTNNIKHIGDLRNIKAGADGVANVNIISNHIQLSGPLSVIGR....SLVVHANPDD 162
sod_cschma EHGAPEDSIRHVGDLGNVVAGADGNAVYNATDKLISLNGSHSIIGR....TMVIHENEDD 124
sod_eschma RHGPRHGYPRHAGDLGNIRVGRGGVAKFDFYVTIKGLGPFDGFIGR....ALVIHANRDD 155
nippo7 LQQDPWRSERMRCAMLETSAMWKPVPMVLLISKSLTIW*RFMV*TRLSEGSLVVHAGTDD 123
sod_ehcont LGAGTS....ELSKTTGNAGARVACGVIGIL.......183
sod_eleg2 LGRGTS....DQSKTTGNAGSRLACGTIGKTFTSSQLPY 184
sod_cbrugia LGKGVGDKK.DESLKTGNAGARVACGIVAVSAAS..... 158
sod_concvo LGKGVGAKK.DESLKTGNAGARVACGIVAIGAAS..... 158
sod_ebrugia LGKGVGDKKDDESLKTGNAGGRVACGIVAISA..... 200
nippo1 LGKGVGEKK.EESLKTG.... 163
nippo3 LGKGVGEKK.EESLKTGN... 143
sod_chcont LGKGVGEKK.EESLKTGNAGARVACGVIATAAPQ..... 159
nippo4 FAGGSCR.... 123
sod_celeg LGEGVGDKA.EESKKTGNAGARAACGVIALAAPQ..... 158
sod_eoncvo LGQGTGNMR.EESLKTGNAGSRLACGVIGIAAVS..... 201
sod_edirof LGKGNGDAR.EESLKTGNAGSRIVCSIIGIAPST..... 195
sod_cschma LGRGG....HELSKVTGNAGGRLACGVIGLAAE..... 153
sod_eschma LGRN....RDEGSRTTGNSGPRLACATIGFRAP..... 184
nippo7 LGKGDGEKKEETLK..... 137

7.2.4 Development of a RT-PCR Assay to Detect *N. brasiliensis* mRNA Transcripts

Initial attempts to develop a SOD-specific RT-PCR assay were performed using the oligonucleotide primers NSOD 1 (sense) and NSOD 3 (antisense) directed to regions of complete sequence homology in the four sequence variants isolated at this point (Underlined Marked in Fig. 7.5, Table 7.2). First strand target cDNA was prepared as described (Section 2.5) and PCR amplification was allowed to proceed for 25 cycles with an annealing temperature of 40°C and the time intervals are listed in Section 2.10.6.

A product of the expected size, 250 bp (Fig. 7.5) was visible after fractionation of the reaction products in a 1% agarose gel (Fig. 7.7a). After agarose gel electrophoresis, the products were blot transferred to a nylon membrane and hybridised with a digoxigenin (DIG) labelled *N. brasiliensis* SOD probe prepared from the *N. brasiliensis* NBSOD1 pCR II construct (Section 2.10.9). The probe hybridised strongly to the 250 bp RT-PCR product confirming its identity (Fig. 7.7b). The PCR products were also visualised on silver stained 7.5% polyacrylamide gels (Fig. 7.7c), the latter procedure providing a more sensitive and accurate measurement of the size of small DNA fragments.

Table 7.2

Specific Oligonucleotides for *N. brasiliensis* SOD

Primer	Sequence	Primer length and type
NSOD1	5' CTGTATCTCTGCTGGACCTCACTT 3' *C I S A G P H F* region	Sense primer 248-272 24 nts
NSOD3	5' CCTCCTTCTTCTCTCCGACTCC 3' *G V G E K K E* region	Antisense primer 470-491 22 nts

Note: NSOD 1 - *N. brasiliensis* SOD specific sense SOD primer directed toward nucleotides 264-287 (Fig. 7.5)

NSOD 3 - *N. brasiliensis* SOD specific antisense primer directed towards nucleotides 483-504 (Fig. 7.5)

Figure 7.7 RT-PCR Detection of *N. brasiliensis* SOD Encoding mRNA Transcripts

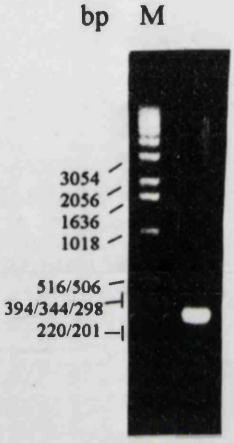
The PCR produces generated with primers NSOD 1 and NSOD 3 (final concentration 0.2 mM) using RT-PCR template from adult *N. brasiliensis* mRNA were visualised in 1% agarose (Fig. 7.7a) or 7.5% polyacrylamide (Fig. 7.7c) gels.

The PCR products were transferred to nylon and hybridised with a specific *N. brasiliensis* SOD probe (containing all four isolated and sequenced clones) labelled with DIG (Fig. 7.7b).

Figure 7.7

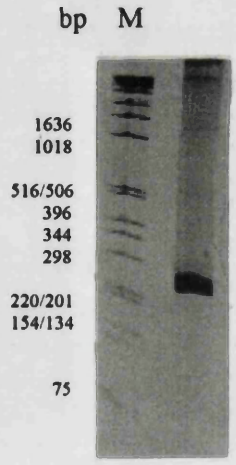
RT-PCR Amplifications Using Specific *N. brasiliensis* SOD Primers

a) PCR Using Primers NSOD1 and NSOD3



b) Southern Blot

c) Silver Gel



7.2.5 **The Quantitative Relationship between Target DNA and RT-PCR Product**

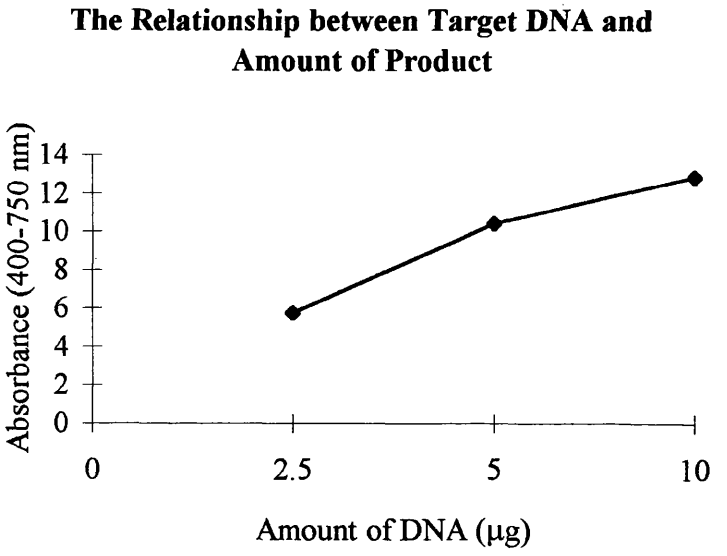
SOD RT-PCR reactions were set up with approximately 2.5, 5.0, 7.5 and 10.0 ng of target first strand cDNA using components as described in Section 2.10.6. Primer annealing was performed at 40°C for 25 cycles. The reaction products (5 µl of each) were visualised by silver staining after separation in 7.5% polyacrylamide gels. The amount of product visualised in relation to background stain was then quantified using densitometric scanning in reflectance mode.

It was observed that the amount of PCR product increased as the amount of target cDNA was increased from 2.5 ng to 10 ng (Fig. 7.8, Lanes 1 to 4; Graph 7.1a). Beyond this, the approximately linear relationship between product and target broke down (not shown).

When different volumes of the same RT-PCR reaction, representing 10 down to 2.5 µg product, were fractionated by gel electrophoresis and silver stained (Fig. 7.8, Lanes 5 to 7; Graph 7.1b), a linear relationship between amount of product and staining intensity was obtained (Graph 7.1b) and the quantitative staining broke down at 10 µg (Fig. 7.8, compare Lanes 7 and 6 with Lane 5). Finally, the consistency of the signal obtained in separate reactions was tested by executing 3 separate PCR reactions with 1.0 ng target DNA and visualising the products (Fig. 7.8, lanes 8 to 10) using the procedures described. The strength of the amplification product appeared to be the same in each lane and this was confirmed by densitometric analysis (Fig. 7.8, lanes 8,9 and 10, absorbance = 7.65, 7.74 and 7.84 respectively). These assays were carried out on three separate occasions and the same linear relationship was obtained each time.

Graph 7.1

a)


The Relationship between Target DNA and Amount of Product
Absorbance (400-750 nm)
14
12
10
8
6
4
2
0
0
2.5
5
10
Amount of DNA (μg)

b)

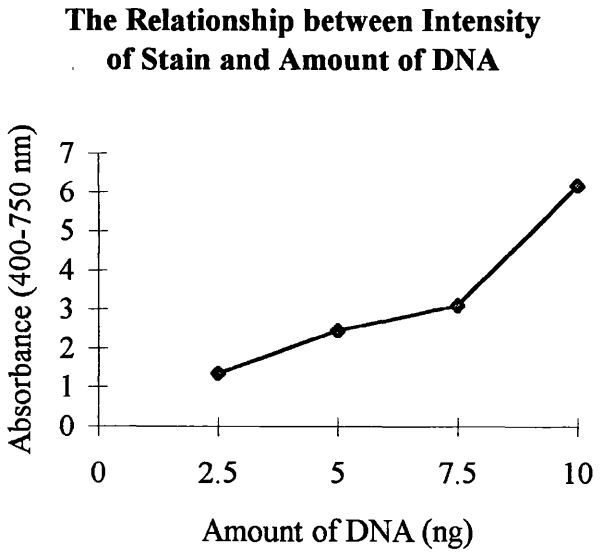

The Relationship between Intensity of Stain and Amount of DNA
Absorbance (400-750 nm)
7
6
5
4
3
2
1
0
0
2.5
5
7.5
10
Amount of DNA (ng)

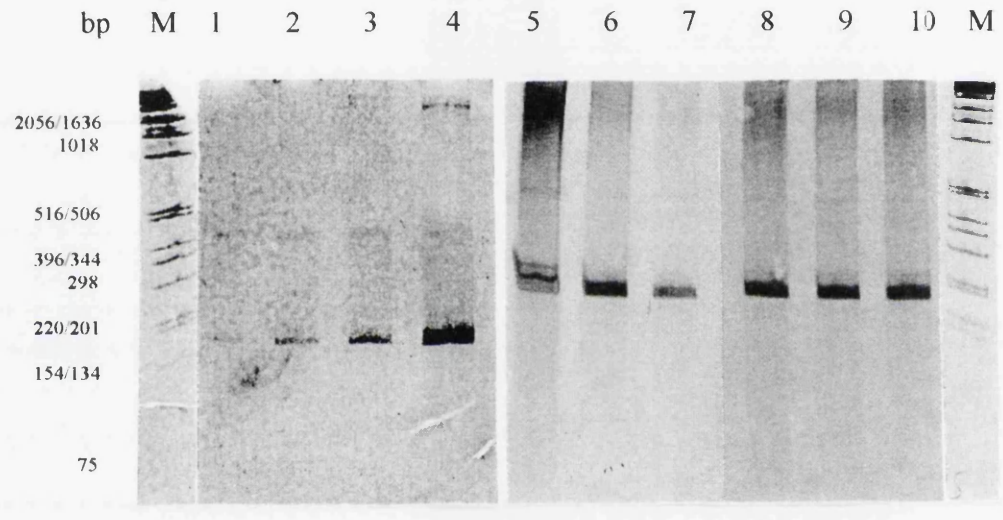
Figure 7.8 Silver Gel of SOD PCR's with Varying Amounts of RT-DNA from *N. brasiliensis*

RT-PCR amplifications with primers NSOD 1 and NSOD 3 at 40°C for 25 cycles, PCR products were then visualised by separation through 7.5% polyacrylamide gels followed by silver staining.

RT-PCR reactions with 2.5 ng (lane 1), 5 ng (lane 2), 7.5 ng (lane 3) and 10 ng (lane 4) target first strand cDNA in initial each reaction. Lanes 5-10 show the loading of varying amounts of RT-PCR product followed by silver staining for quantification (lane 5,10 μg; lane 6, 5 μg; and lane 7, 2.5 μg; Lanes 8-10, 5 μg each).

Figure 7.8

Silver Gel of SOD RT-PCRs for Quantification



7.2.6 **Quantification of SOD Expression in Different Parasite Populations**

First-strand cDNA was prepared as described in Section 2.10.5 from 200 7 d.p.i parasites and 1 µl used in the PCR amplifications.

'Normal' parasites: Adult *N. brasiliensis* were retrieved from the intestines of five male and five female rats 7 d.p.i (3,000 L3) and approximately 200 worms were snap frozen in liquid nitrogen while the remainder were homogenised in PBS for determination of enzyme activity. SOD expression in each population was assessed by measuring total homogenate SOD enzyme activity and by mRNA transcript analysis using RT-PCR. Table 7.3 shows total SOD enzyme activity levels in worm homogenates. The RT-PCR products are shown in Fig. 7.9. Initially, the products were run on 1% agarose gels (Fig. 7.9a) followed by transfer to a nylon membrane and hybridisation to a DIG labelled *N. brasiliensis* SOD DNA probe (Section 2.10.9) containing the four SOD plasmids. The outcome of densitometric scanning of these products visualised by Silver staining (Fig. 7.9c) is also shown in Table 7.3.

Total homogenate SOD activity was quite variable when worm populations from individual rats were compared, ranging from 8.6 to 28.0 U/mg protein. The mean homogenate SOD activity in parasites harvested from five female rats (14.9 U/mg protein) tended to be higher than that in parasites from the five male rats (12.3 U/mg protein) but the difference was not statistically significant.

The homogenate SOD isoenzyme profiles from these parasite populations were variable as recorded in Chapter 6, although there were no marked differences in the levels of SOD mRNA transcripts as judged by agarose gel electrophoresis with ethidium bromide staining of RT-PCR products (Fig. 7.9a) or when the products were probed with the NSOD pCR II plasmid probe after Southern transfer (Fig. 7.9b).

In addition three rats were given an initial infection of 500, 1000 or 2000. These worms were harvested and analysed by RT-PCR as previously described. The size of the infection administered to the host did not apparently affect the amount of SOD transcript detected by RT-PCR, it is however difficult to draw any conclusion as no RT-PCR product was obtained with the 1000 l3 sample (Fig. 7.10). Total

homogenate SOD activity however, tended to increase with increasing parasite dose (500, 1000 and 2000, Table 7.4).

'Normal' and 'adapted' N. brasiliensis compared: 'Adapted' parasites were harvested from rats following a challenge infection and were pooled for comparison with the worms harvested from individual rats. In accord with data presented in Chapter 6, homogenate SOD activity in 'adapted' parasites tended to be higher compared to the 'normal' worms (Table 7.2A).

These differences were not reflected when RT-PCR products from 'normal' and 'adapted' parasites were compared (Fig. 7.9 a, b and c; Lane A) and may reflect variable gel loading. Southern blotting results however, suggested that similar levels of SOD transcript was present in the adapted population compared to the ten normal populations (Fig. 7.9b, Lane A compared to Lanes 1 to 10), however as optimal conditions for the development of the blot were not sought this result may just reflect saturation of the system.

Table 7.3

Quantification of RT-PCR Products from Individual Worm Populations and 'Adapted' *N. brasiliensis*

	Sample	Absorbance	SOD Activity U/mg Protein
A)	**SOD PCR's**		**Mean n = 2**
1	1 F	5.12	16.1
2	2 F	2.41	8.6
3	3 F	4.74	13
4	4 F	3.65	10.5
5	5 F	10.14	27.95
6	1 M	6.41	17.3
7	2 M	6.26	16
8	3 M	2.94	9.5
9	4 M	3.61	9.9
10	5 M	3.16	8.9
A	Adapted	2.84	28.1

Note: 1 - 5 F = Adult *N. brasiliensis* harvested from 5 individual female rats; 1 - 5 M = Adult *N. brasiliensis* harvested from 5 individual male rats; Adapted = Pooled adult *N. brasiliensis* harvested from rats following a challenge infection.

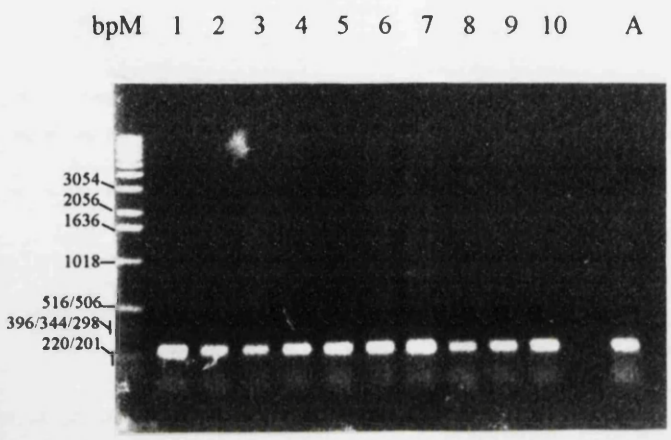
Figure 7.9 RT-PCR as a Method for Quantification of SOD Expression in *N. brasiliensis* using NSOD 1 and NSOD 3 Primers

RT-PCR products (5 μl) amplified from 1 μl single stranded cDNA template derived originally from 200 adult *N. brasiliensis* harvested from female rats 1 - 5 (Fig. 7.9a, b and c, lanes 1 - 5 respectively), male rats 1 - 5 (Fig. 7.9a, b and c, lanes 6 - 10 respectively) and 'adapted' parasites (Fig 7.9a, b and c, lane A) were separated on a 1 % agarose gel and stained with ethidium bromide (Fig. 7.9a). These products were then transferred to a nylon membrane and hybridised to a *N. brasiliensis* SOD specific DNA probe (Fig. 7.9b). The RT-PCR products (4 μl) were also visualised on a 7.5 % silver stained polyacrylamide gel (Fig. 7.9c) and scanned using densitometry for analysis.

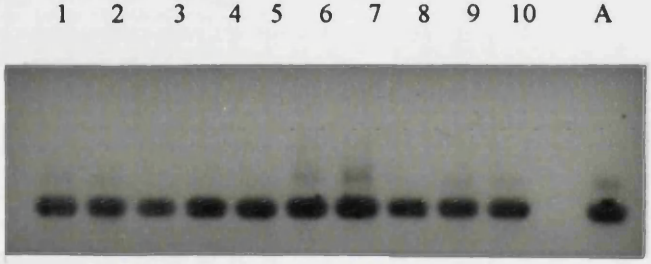
Figure 7.9

a) RT-PCR as a Method for Quantification of SOD Expression in *N. brasiliensis*

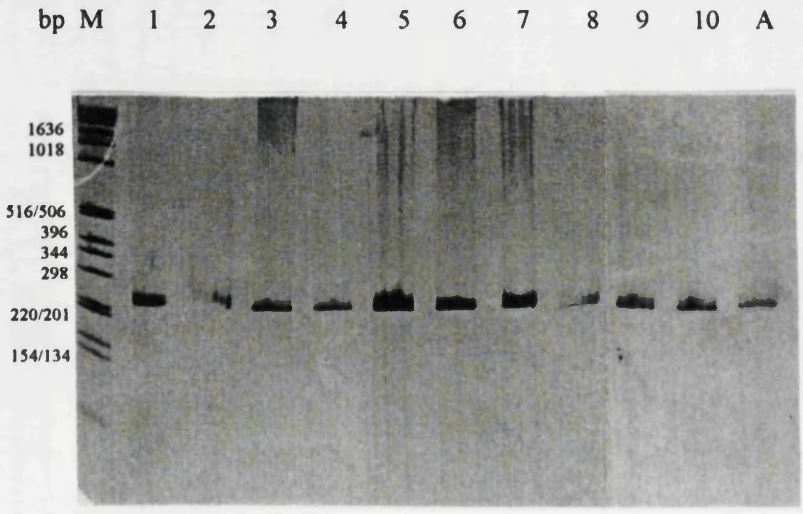
Individual Rats



b) Southern Blot



c) Silver Gel



Free radical exposure during* in vitro *culture: Adult *N. brasiliensis* were cultured *in vitro* as described in Section 2.4.2. To the culture media, various "cocktails" of free radical generating systems, scavengers or substrates were added as indicated Table 7.4.

Total homogenate SOD activity was elevated in parasites exposed to 10 mU xanthine oxidase (XO) in the presence of excess xanthine (X) when compared to those exposed to lower amounts (5, 2.5, 1.25 mU) of XO and the same X concentration. This trend was also evident when RT-PCR products were quantified by densitometry (Table 7.4) from a silver stained polyacrylamide gel of the RT-PCR products (Fig. 7.10c). In the treatments where it could be anticipated that superoxide radical exposure was minimised (XO/X/SOD; SOD alone and RPMI alone; Table 7.4), homogenate SOD activity and mRNA transcript levels, as measured by RT-PCR and densitometry, were consistently low. However, it is worthy of note that exposure to xanthine (0.2 mM X, Table 7.4), elevated SOD expression as judged by either method. Relatively high levels of SOD transcript were detected by RT-PCR in worms exposed to xanthine oxidase alone (2.5 mU XO, Table 7.2B) but this was not reflected in total homogenate SOD activity.

Table 7.4

Quantification by Densitometry of RT-PCR Products From Adult *N. brasiliensis* Exposed to Various Levels of Free Radical during *In Vitro* Culture

	Sample	Absorbance	SOD Activity U/mg protein
1	500	1.74	7.7
2	1000	0.00	8.2
3	2000	1.69	15.9
4	10 mU XO/X	6.05	15.8
5	5 mU XO/X	2.57	8.5
6	2.5 mU XO/X	0.00	6.5
7	1.25 mU XO/X	2.13	6.8
8	2.5 mU XO/X/SOD	1.15	7.3
9	0.2 mM X alone	9.27	25.3
10	2.5 mU XO alone	4.48	7.5
11	20 U SOD alone	1.94	8.1
12	RPMI 1640 alone	0.52	14.2

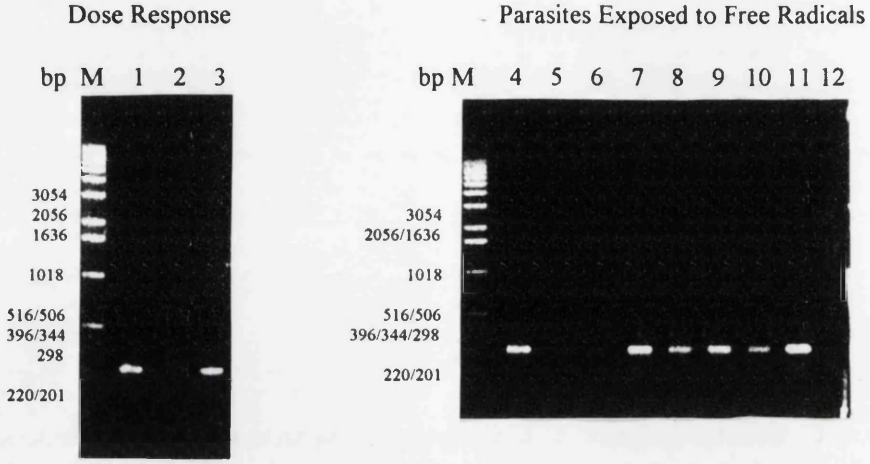
Note: Samples 1 -3, parasites harvested from individual rats exposed to an initial infections of 500, 1000 and 2000 L3 and frozen immediately upon harvest.
Samples 4 - 12, all parasites cultured in 1 ml RPMI 1640 (100 worms/ml) to which was added free radical generating compounds at the concentrations shown in the table; XO - xanthine oxidase; X - xanthine (2 mM); SOD - superoxide dismutase (20 U)

Figure 7.10 SOD Transcripts Obtained by RT-PCR on Adult *N. brasiliensis* Harvested from Rats with Varying Levels of Infection and from Parasites Cultured Under Different Experimental Conditions

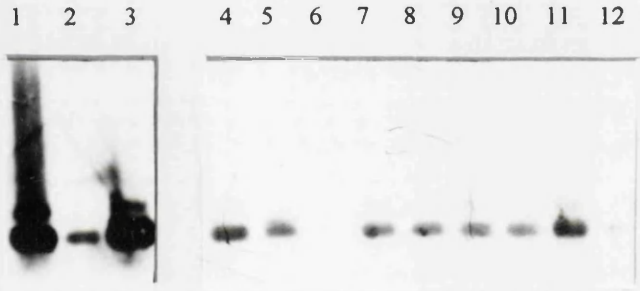
RT-PCR products were amplified using primers NSOD 1 and NSOD 3 from 1 µl single stranded cDNA template derived originally from 200 adult *N. brasiliensis* harvested from rats exposed to an initial infection of 500, 1000 and 2000 L3 (Fig. 7.10a, b and c, lanes 1 - 3 respectively) and from pooled parasites exposed to various levels of free radicals during *in vitro* culture (100/ ml; Fig. 7. 10a, b and c, lanes 4 - 12). Parasites were cultured under the following conditions; lane 4 - 0.2 mM xanthine (X) plus 10 mU xanthine oxidase (XO); lane 5 - 5 mU XO; lane 6 - 2.5 mU XO; lane 7 - 1.25 mU XO; lane 8 - 2.5 mU XO, 0.2 mM X and 20U superoxide dismutase (SOD); lane 9 - 0.2 mM X alone; lane 10 - 2.5 mU of XO alone; lane 11 - 20U of SOD alone; lane 12 - RPMI 1640 alone. The products from the RT-PCR amplifications (5 µl) were separated on a 1 % agarose gel and stained with ethidium bromide (Fig. 7.10a). These products were then transferred to a nylon membrane and hybridised to a *N. brasiliensis* SOD specific DNA probe (Fig. 7.10b) at moderate stringency (42°C, washed twice in 2X SSC for 15 min, and twice in 1X SSC for 15 min). The RT-PCR products (4 µl) were then visualised on a 7.5 % silver stained polyacrylamide gel (Fig. 7.10c) and scanned using densitometry for analysis.

Figure 7.10

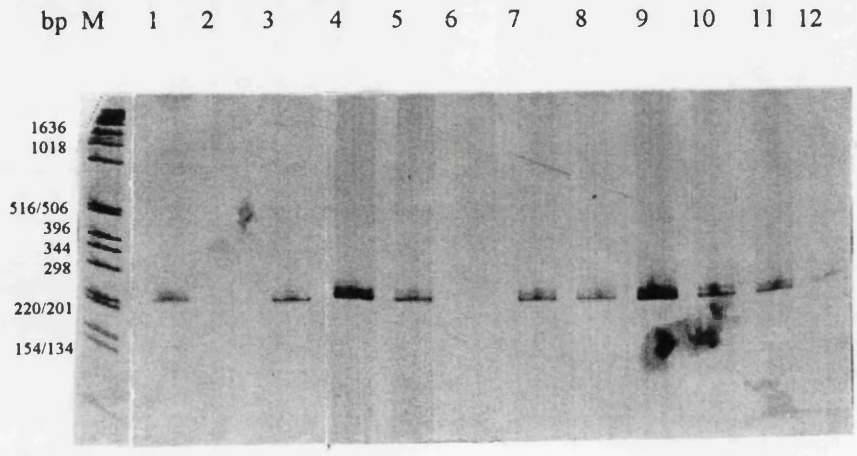
a) RT-PCR as a Method for Quantification of SOD Expression in *N. brasiliensis*



b) Southern Blot



c) Silver Gel

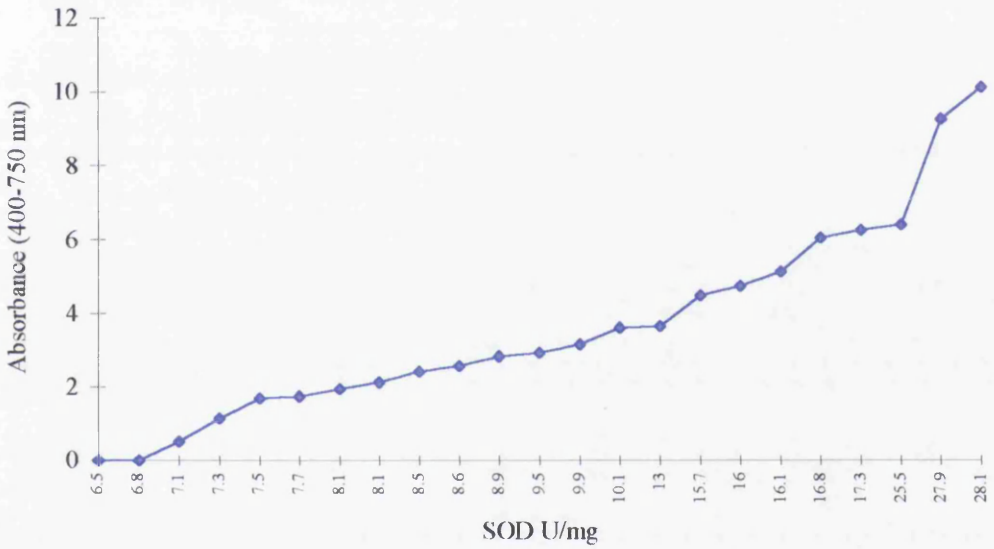


7.2.7 **The Relationship between Total Homogenate SOD Activity and RT-PCR Product**

The amount of RT-PCR product (Absorbance, Tables 7.3 and 7.4) were plotted (Graph 7.2) against SOD activity (U/mg) for all the samplings listed in Tables 7.3 and 7.4.

The relationship was approached linearity with a highly significant correlation coefficient of 0.77, n = 25 (Spearman Rank).

Graph 7.2 **The Relationship between SOD Activity and RT-PCR Product**



This graph shows the linear relationship between total SOD activity in homogenates of adult *N. brasiliensis* expressed as U/mg protein plotted against the amount of mRNA transcript as determined by RT-PCR amplification and densitometric quantification (blanked against gel background).

7.2.8 **Isolation of a *N. brasiliensis* Tubulin-Encoding cDNA and the Use of the mRNA from which it is Derived as a Constitutively Expressed RT-PCR Control**

First strand cDNA was prepared from mRNA as described earlier (Sections 2.10.4 and 5) and used as template for a series of PCR reactions to amplify *N. brasiliensis* β-tubulin cDNA fragments using oligonucleotide primers used previously to amplify *H.*

contortus β-tubulin sequences (Kwa, Jetty and Roos, 1994). In the first instance, amplifications were performed using βT30 (sense primer) and βT5 (antisense primer, Table 7.5; Kwa *et al*, 1994). The expected product size was approximately 125 bp (Fig. 7.11, lane 1), however no product was obtained with adult *N. brasiliensis* cDNA (Fig. 7.11, Lane 2).

Amplifications of *H. contortus* and *N. brasiliensis* cDNA using the SL1 primer defined above and βT5 (Table 7.5) gave a doublet at approximately 750 bp (Fig. 7.11, Lane 3), of the expected size with *H. contortus* cDNA but a range of products from 100 to 800 bp with *N. brasiliensis* cDNA (Fig. 7.11, Lane 4). This indicated that this primer pairing lacked specificity for use as an RT-PCR control in *N. brasiliensis* reactions. Therefore, a new sense primer, designated H.c βT, directed to a highly conserved region as judged by sequence comparisons of tubulin encoding genes from several parasite species, (Geary *et al*, 1992) was synthesised (Table 7.5). Using this sense primer and the antisense primer, βT5, a single product of the expected size of approximately 100 bp, was obtained with *N. brasiliensis* (Fig. 7.11, Lane 5).

To design oligonucleotide primers with specificity for *N. brasiliensis* tubulin, the 800 bp product of the SL1/βT5 amplification (Figure 7.11, lane 4) was cloned into the plasmid vector pCR II. The 5' and 3' nucleotide sequences of the insert were determined (Fig. 7.12) and compared with the *H. contortus* β-tubulin gene, gru-1 (Geary *et al*, 1992). Two primers, Ntub1 (sense primer) and Ntub2 (antisense primer), specific for *N. brasiliensis* tubulin were synthesised, however, amplification with these primers was unsuccessful (not shown). Subsequently, the primers H.c βT and βT5 were used in the RT-PCR reactions.

When the primers H.c βT and βT5 were used in RT-PCR reactions using aliquots of the same RT-cDNA used for the SOD determinations described above, variable results were obtained and a product of the correct size (100 bp) was only observed very occasionally (Figure 7.13; Lanes 2, 3 and 7) but was not detected in the majority of the reactions (Figure 7.13; Lanes 1, 4-6 and 8-22). The product did however hybridise to a *N. brasiliensis* β-tubulin DIG labelled DNA probe derived from the β-tubulin cloned from adult *N. brasiliensis* (Fig. 7.13b).

Table 7.5

Oligonucleotides Used for the Isolation of Tubulin Encoding cDNA from *N. brasiliensis*

Primer	Sequence	Primer length and type (nucleotides)
SL 1	5' GGTTTAACCACCCAAGTTTGAG 3'	Sense primer 22 nts
βT30	5' GAGAACACCGATGAAACAT 3'	Sense primer 19 nts
βT5	5' ACCAGACATTGTGACAGA 3'	Antisense primer 18 nts
H.c βT	5' GAAACRTTCTGTATTGAYACCGAA 3'	Sense primer 24 nts
Ntub 1	5' GCGCTGTTCTCGTTGATCTCG 3'	Sense primer 21 nts
Ntub 2	5' TTTGAGATGAGCAGCGTTCCA 3'	Antisense primer 21 nts

Note: **R** = A or G; **Y** = C or T

βT30 and βT5 primers designed by Kwa, Jetty and Roos, (1994) for the amplification of *H. contortus* β-tubulin.

H.c βT - designed to a highly conserved region of tubulin from several parasite species (Geary *et al*, 1992)

Ntub1 - *N. brasiliensis* β-tubulin specific sense primer

Ntub2 - *N. brasiliensis* β-tubulin specific antisense primer

Figure 7.11 PCR Amplifications from *N. brasiliensis* and *H. contortus* cDNA using Different β-tubulin Oligonucleotide Primers

Adult mRNA isolated from *N. brasiliensis* and *H. contortus* was subjected to RT-PCR using the primers shown. After 30 amplification cycles, the PCR products were analysed using 1% agarose gel electrophoresis and ethidium bromide staining.

Amplifications from *H. contortus* (Lanes 1 and 3) or *N. brasiliensis* (Lanes 2 and 4) cDNA are shown using either the βT30 and βT5 primer pairing (Lanes 1 and 2) or SL1 to βT5 (Lanes 3 and 4). Lane 5 shows the outcome of an amplification from *N. brasiliensis* cDNA using the newly designed primer H.c βT and βT5.

Figure 7.4

N. brasiliensis RT-PCR Amplifications Using *H. contortus* Tubulin Primers

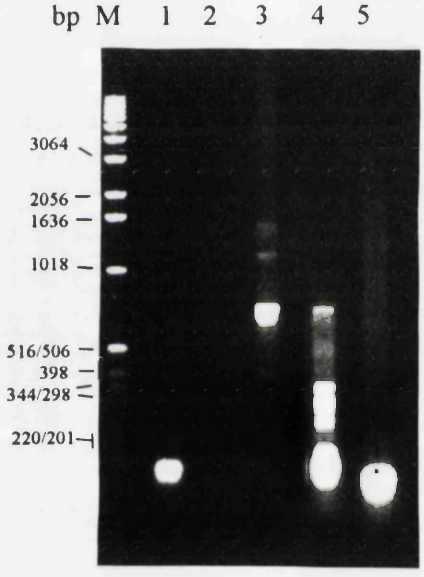


Figure 7.12 Nucleotide Alignment of *H. contortus* gru-1 gene with *N. brasiliensis* β-tubulin

Nucleotide comparison of *H. contortus* cDNAs encoding β-tubulin gene gru-1 (X67489; Geary *et al*, 1992) compared with incomplete *N. brasiliensis* β-tubulin fragments sequenced in the forward (upper segment, nucleotides 52 to 372) and reverse direction (lower segment, nucleotides 4 to 331). The upper sequence is of the *H. contortus* gru-1 gene and the lower is the putative adult *N. brasiliensis* β-tubulin. The sequences read from 5' to 3'. The specific *N. brasiliensis* tubulin primers are marked as shown Ntub1 and Ntub2 (primer 2 is the reverse complement of this sequence).

Figure 7.12

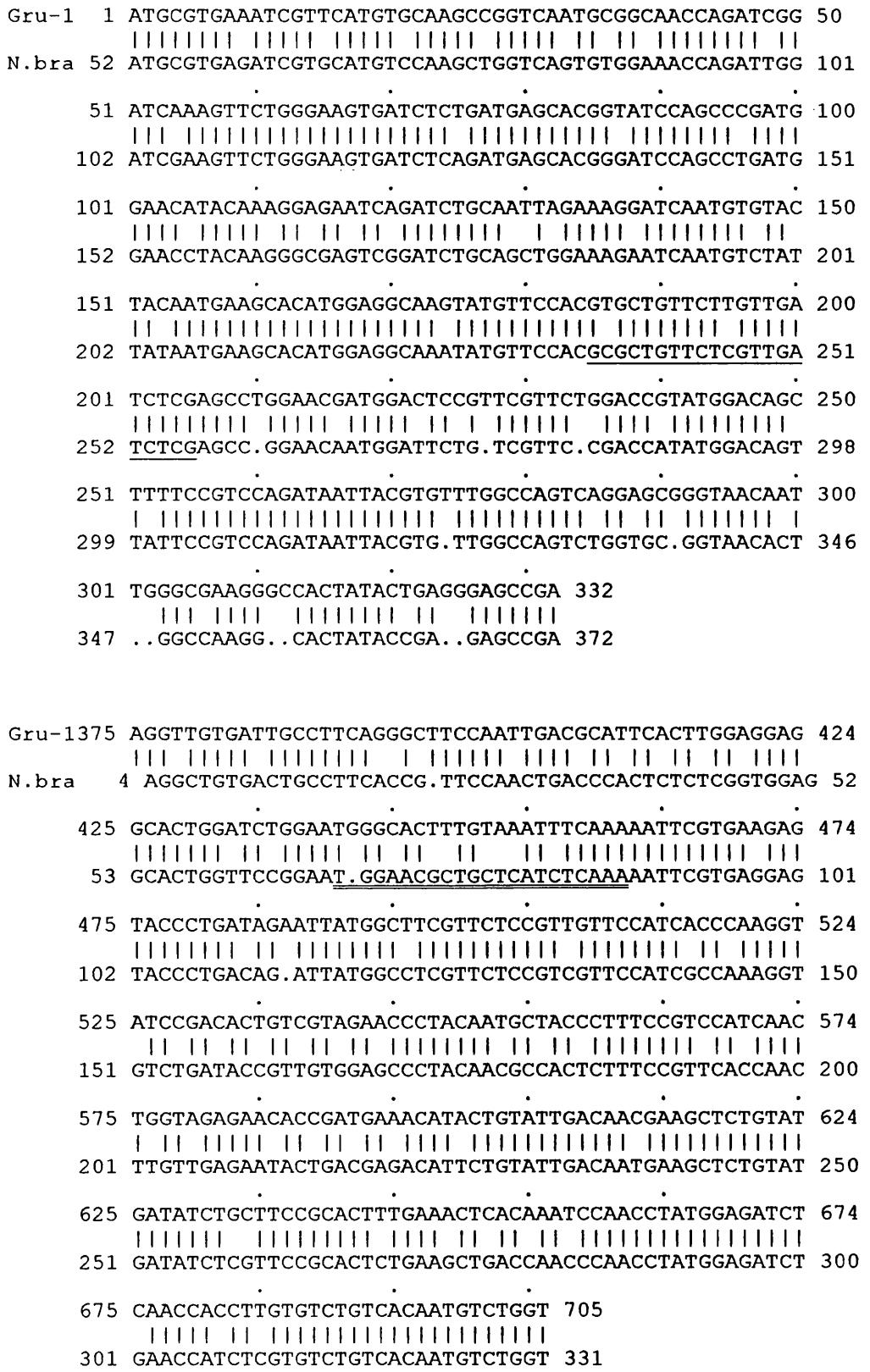

Gru-1 1 ATGCGTGAAATCGTTCATGTGCAAGCCGGTCAATGCGGCAACCAGATCGG 50
N.bra 52 ATGCGTGAGATCGTGCATGTCCAAGCTGGTCAGTGTGGAAACCAGATTGG 101
51 ATCAAAGTTCTGGGAAGTGATCTCTGATGAGCACGGTATCCAGCCCGATG 100
102 ATCGAAGTTCTGGGAAGTGATCTCAGATGAGCACGGGATCCAGCCTGATG 151
101 GAACATACAAAGGAGAATCAGATCTGCAATTAGAAAGGATCAATGTGTAC 150
152 GAACCTACAAGGGCGAGTCGGATCTGCAGCTGGAAAGAATCAATGTCTAT 201
151 TACAATGAAGCACATGGAGGCAAGTATGTTCCACGTGCTGTTCTTGTTGA 200
202 TATAATGAAGCACATGGAGGCAAATATGTTCCACGCGCTGTTCTCGTTGA 251
201 TCTCGAGCCTGGAACGATGGACTCCGTTCGTTCTGGACCGTATGGACAGC 250
252 TCTCGAGCC.GGAACAATGGATTCTG.TCGTTC.CGACCATATGGACAGT 298
251 TTTTCCGTCCAGATAATTACGTGTTTGGCCAGTCAGGAGCGGGTAACAAT 300
299 TATTCCGTCCAGATAATTACGTG.TTGGCCAGTCTGGTGC.GGTAACACT 346
301 TGGGCGAAGGGCCACTATACTGAGGGAGCCGA 332
347 ..GGCCAAGG..CACTATACCGA..GAGCCGA 372
Gru-1375 AGGTTGTGATTGCCTTCAGGGCTTCCAATTGACGCATTCACTTGGAGGAG 424
N.bra 4 AGGCTGTGACTGCCTTCACCG.TTCCAACTGACCCACTCTCTCGGTGGAG 52
425 GCACTGGATCTGGAATGGGCACTTTGTAAATTTCAAAAATTCGTGAAGAG 474
53 GCACTGGTTCCGGAAT.GGAACGCTGCTCATCTCAAAAATTCGTGAGGAG 101
475 TACCCTGATAGAATTATGGCTTCGTTCTCCGTTGTTCCATCACCCAAGGT 524
102 TACCCTGACAG.ATTATGGCCTCGTTCTCCGTCGTTCCATCGCCAAAGGT 150
525 ATCCGACACTGTCGTAGAACCCTACAATGCTACCCTTTCCGTCCATCAAC 574
151 GTCTGATACCGTTGTGGAGCCCTACAACGCCACTCTTTCCGTTCACCAAC 200
575 TGGTAGAGAACACCGATGAAACATACTGTATTGACAACGAAGCTCTGTAT 624
201 TTGTTGAGAATACTGACGAGACATTCTGTATTGACAATGAAGCTCTGTAT 250
625 GATATCTGCTTCCGCACTTTGAAACTCACAAATCCAACCTATGGAGATCT 674
251 GATATCTCGTTCCGCACTCTGAAGCTGACCAACCCAACCTATGGAGATCT 300
675 CAACCACCTTGTGTCTGTCACAATGTCTGGT 705
301 GAACCATCTCGTGTCTGTCACAATGTCTGGT 331

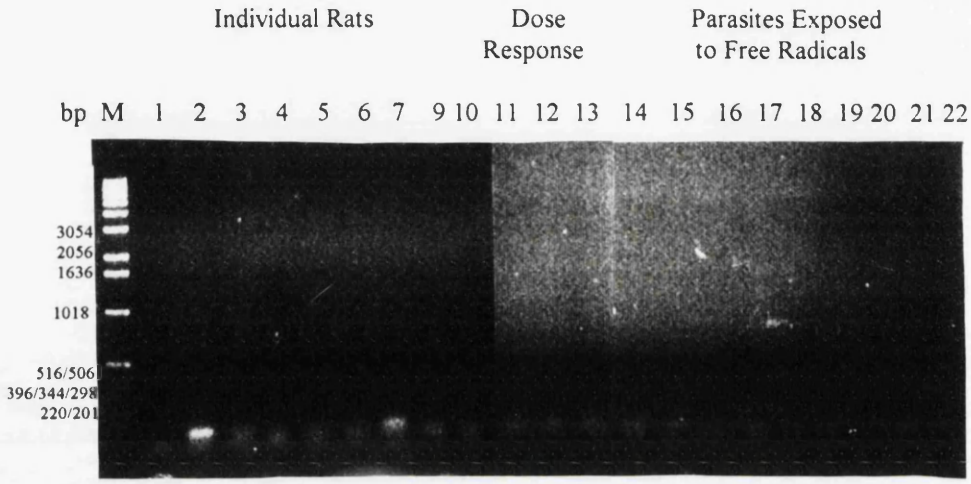
Figure 7.13 Tubulin Expression in *N. brasiliensis* using Primers H.cβT and βT5

RT-PCR products were amplified using primers NSOD 1 and NSOD 3 from 1 μl single stranded cDNA template derived originally from 200 adult *N. brasiliensis* harvested from female rats 1 - 5 (Fig. 7.13a, b and c, lanes 1 - 5 respectively), male rats 1, 2, 4 and 5 (Fig. 7.13a, b and c, lanes 6, 7, 9 and 10 respectively), rats exposed to an initial infection of 500, 1000 and 2000 L3 (Fig. 7.13a, b and c, lanes 11 - 13 respectively) and from pooled parasites exposed to various levels of free radicals during *in vitro* culture (100/ ml; Fig. 7. 13a, b and c, lanes 14 - 22). Parasites were cultured under the following conditions; lane 14 - 0.2 mM xanthine (X) plus 10 mU xanthine oxidase (XO); lane 15 - 5 mU XO; lane 16 - 2.5 mU XO; lane 17 - 1.25 mU XO; lane 18 - 2.5 mU XO, 0.2 mM X and 20U superoxide dismutase (SOD); lane 19 - 0.2 mM X alone; lane 20 - 2.5 mU of XO alone; lane 21 - 20U of SOD alone; lane 22 - RPMI 1640 alone.

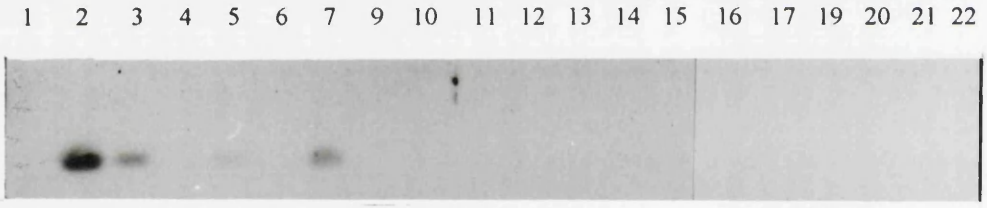
The products from the RT-PCR amplifications (5 μl) were separated on a 1 % agarose gel and stained with ethidium bromide (Fig. 7.13a). These products were then transferred to a nylon membrane and hybridised to a *N. brasiliensis* SOD specific DNA probe (Fig. 7.13b) at moderate stringency (42°C, washed twice in 2X SSC for 15 min, and twice in 1X SSC for 15 min). The RT-PCR products (4 μl) were then visualised on a 7.5 % silver stained polyacrylamide gel (Fig. 7.13c) and scanned using densitometry for analysis.

Figure 7.13

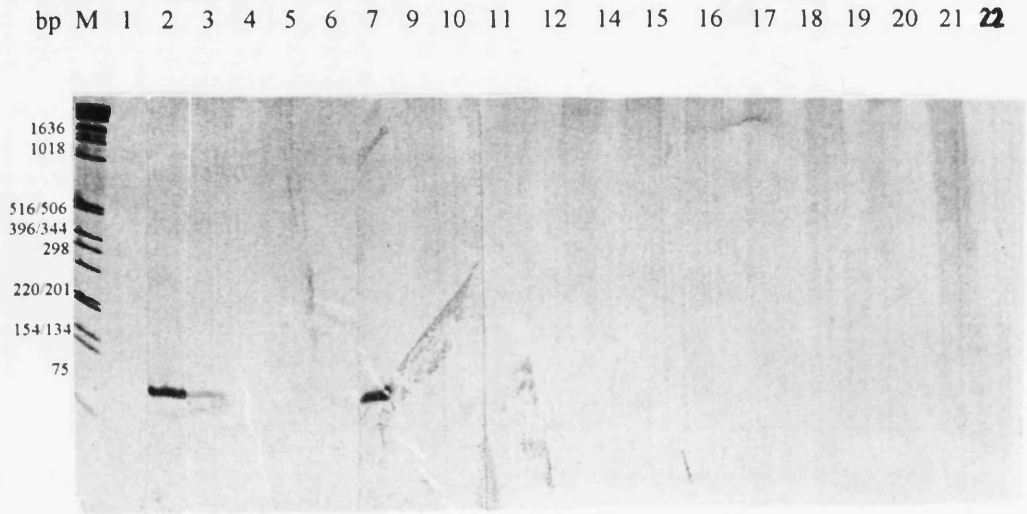
a) RT-PCR Amplifications of Tubulin in *N. brasiliensis* as Internal Controls for SOD Expression



b) Southern Blot



c) Silver Gel



7.3 DISCUSSION

In this chapter, SOD encoding cDNAs were isolated from adult *N. brasiliensis* using oligonucleotides derived from *H. contortus* and PCR amplification. The PCR products were cloned into a plasmid vector and sequenced. Comparison with database sequences confirmed that the cloned cDNA fragments encoded copper/zinc SOD sequences. This is the first time SOD encoding cDNA sequences have been described for *N. brasiliensis*. Using consensus nucleotide sequences identified by sequence alignments of the four distinct cDNAs isolated, species specific oligonucleotide primers were designed and synthesised and used to develop a RT-PCR assay for the detection of SOD mRNA transcripts in parasite populations. The quantitative data generated by this RT-PCR approach correlated to measurements of total enzyme activity on the same parasite samples (Tables 7.3 and 7.4). A similar approach was used to develop an RT-PCR assay for β-tubulin for use as a putative constitutively expressed control but this assay proved inconsistent.

SOD has been cloned and sequenced from the human parasitic helminths, *S. mansoni* (Hong, Lo Verde, Hammarskjold and Rokosh, 1992), *O. volvulus* (Henkle, Liebau, Muller, Bergmann and Walter, 1991) and *B. malayi* (Ou, Tang, McCrossan, Henkle-Duhrsen and Selkirk, 1995) and the important veterinary nematode *H. contortus* (Liddell, unpublished).

Amino acid alignment of the *N. brasiliensis* SOD clones 1, 3, 4 and 7 showed 67, 67, 72 and 42 % homology at the amino acid level with the cytosolic form of SOD from *S. mansoni* (Q01137; Da Silva *et al*, 1992), 82, 83, 82 and 49 % homology with *O. volvulus* cSOD (P24706; Henkle *et al*, 1991), 96, 97, 91 and 57 % homology with *H. contortus* cSOD and 82, 83, 81 and 50 % homology *B. pahangi* cSOD (X76284) respectively. SOD 7 had much lower homology to the other SOD sequences, possibly indicating that it encoded a distinct functional isoenzyme. However, the sequence data requires confirmation by repeat analysis which, unfortunately, due to lack of time I was not able to do. Amino acid alignment of the *N. brasiliensis* SOD sequences with those of other nematodes (Fig. 7.6) enabled identification of the histidine residues involved in catalysis, asparagine residues involved in metal ion binding and cysteine residues used in intrachain di-sulphide bonding in Cu/Zn SODs as identified by Henkle *et al*, (1991).

Good quality mRNA with a consistent yield between preparations was obtained as judged by the "quantity of target" dose response (Graph 7.1) and the overall correlation of RT-PCR product with total SOD activity (Graph 7.2). It is unlikely that this would have occurred if the mRNA yield had been variable and supports the results obtained during the RT-PCR assay (Fig. 7.9 and Fig. 7.10).

RT-PCR was chosen to determine SOD levels in the parasites because total SOD (activity per mg protein) determinations are relatively insensitive and the gel assay often yields inconsistent results as discussed in Chapter 5. RT-PCR is sensitive and potentially gene-specific and has a potential use in enabling identification of individual SOD isotypes by synthesis of primers designed to highly variant regions between SOD clones. This is especially relevant if an extracellular form of the enzyme is isolated. Stage-specific expression of individual SOD genes could also be examined with this procedure. This would not be possible with conventional Northern blotting methods because the coding sequences are so similar between the *N. brasiliensis* SOD variants that they would all recognise each other (Fig. 7.5).

The RT-PCR assay was developed by designing primers based on highly conserved regions in the four *N. brasiliensis* clones. RT-PCR using these primers amplified a single product of approximately 250 bp. These primers were designed so that they were unlikely to anneal to each other and were sufficiently distinct form other regions of the clones to prevent non-specific hybridisation. It was hoped that these primers should detect all SOD mRNA transcripts, though it should be kept in mind that increases in total SOD mRNA may not be reflected in an increase in total SOD activity as not all mRNA may be translated.

No marked differences in terms of product size were detected between the RT-PCR products of individual normal worm populations (Fig. 7.9), although earlier isoenzyme analysis (Chapter 6) indicated marked differences in electrophoretic mobility. This could be explained by the fact that the RT-PCR would detect all SODs. If it was possible to develop a more discriminatory RT-PCR assay as described above it may pick up different isoenzyme expression. Similarly, sequence analysis of SOD cDNAs derived from 'adapted' parasites may enable the identification of quite unique SOD encoding sequences not evident in 'normal' parasites i.e. SOD genes which are only transcribed in times of parasite stress.

Studies by Ou *et al* (1995) demonstrated that levels of mRNA for cytoplasmic SOD between different developmental stages of *B. malayi* was fairly equal however, by biochemical analyses, 10x more EC SOD was found in adults compared to microfilariae. These results could indicate that EC SOD levels are more variable than the cytosolic form and this would be in accord with a dynamic role, including the secretion of EC SOD, for use in detoxification of host generated free radicals. The use of SOD primers and molecular probes which do not distinguish between the two forms of the enzyme may mask variation of EC SOD if it is present in a lower quantity compared with the cytosolic form. It is therefore important to define differences in sequences between the different types of SOD.

Two forms of SOD, cytosolic and EC, identified by the presence of potential 'hydrophobic' signal sequences, have been recorded in *B. malayi* (Tang *et al*, 1994) and *H. contortus* (Liddell and Knox, 1995). Of the four *N. brasiliensis* SOD clones described *N. brasiliensis* NBSOD1 clone 1, contained an extra 50 bp at the 5' end of the sequence indicating that it may have encoded a signal peptide indicative of an extracellular function. Analysis however, by SIGCLEAVE utilising the von Heijne method (University of Wisconsin Genetics Package) failed to locate any signal sequence and identify a cleavage site in this region of the *N. brasiliensis* SOD 1 clone 1 and the reasons for this extension remain unclear. NBSOD1 #1 showed 80% homology at the nucleotide level to other nematode cSODs indicating that this clone probably represents a cytosolic form of the enzyme.

Total SOD activity was found to correlate, in a highly significant manner (linear correlation coefficient 0.77, n = 25), to the amount of RT-PCR product as visualised by PAGE and silver staining (Graph 7.2). This correlation tended to break down at low levels of SOD expression (< 6 U/mg) and at higher levels of enzyme activity (>25 U/mg) which may reflect the limits of the sensitivity of the assay or may have been due to less efficient mRNA extraction in these samples. In anticipation of the effects which inefficient mRNA extraction may have had on the assay, attempts were made to develop a parallel RT-PCR to quantify expression of a putative constitutively expressed protein, tubulin. The signal obtained from this PCR could be used as a correction factor for the SOD RT-PCR signal to allow for inconsistent mRNA yields. However attempts to develop a tubulin based assay were unsuccessful (Fig. 7.13a, b and c). A reason for

this may have been that the tubulin gene selected was inconsistently expressed and another tubulin gene may potentially have been better. Cyclophilin has also been used as an internal control to quantify cytokine gene expression (Zuo, Matsumura, Prehn, Marchevesky, Maltoff and Jordan, 1995) and glyceraldehyde phosphate deyhdrogenase has been used as an internal standard during quantitative RT-PCR of mRNA encoding angiotensin converting enzyme (Aschoff, Lazarus, Fanburg and Lanzillo, 1994). In an attempt to reduce the variability in the tubulin PCR amplifications which may have been due to variation between worm populations the annealing temperature of the PCR amplifications was reduced from 45°C to 32°C. Unfortunately there was still variation in PCR amplifications. These problems in amplification were not due to the RT-PCR technique as SOD could be amplified from all of these samples using specific primers. Variation in the success of the β-tubulin amplifications could be explained by low gene copy numbers (Abbott, Poisz, Byrne, Kwok, Sninsky and Ehrich, 1988) and it is known that *N. brasiliensis* tubulin is not as abundant as that of the rat host (Tang and Pritchard, 1988). If β-tubulin gene(s) were present in low copy numbers a damping effect on the PCR product could occur if a proportionally small amount of β-tubulin encoding DNA was present compared to non β-tubulin encoding DNA.

Myosin was also investigated for use as a control of SOD gene expression, however, a number of problems were encountered and due to the limited time left it was not a viable option to develop specific *N. brasiliensis* myosin primers, especially due to the lack of success with this route for tubulin.

Previous experiments carried out by Batra *et al*, (1993) in which adult *N. brasiliensis* were exposed to various free radical generating systems *in vitro* suggested that worm rejection involved a gradual decline in SOD and GSH levels of the parasite and an increase in sensitivity of the parasite to ROI within the rat intestine. Similarly *D. immitis* microfilariae were killed during *in vitro* culture by the addition of hydrogen peroxide and free radical generating systems (xanthine/xanthine oxidase), however no analysis was made of the parasites free radical scavenging enzymes (Rzepczyk and Bishop, 1984). In view of these results an attempt was made to culture the parasites under oxidative stress by incubating them with free radical generating substrates. The results obtained provided some evidence which linked increased levels of XO mediated superoxide anion production with an increase in parasite SOD expression (Fig. 7.10c,

lanes 4-6), however at a concentration of 1.25 mM a similar amount of product was detected as at 5 mM and at 2.5 mM XO no product was detected which may reflect a breakdown in the sensitivity of the assay at these levels or a variation in the efficiency of mRNA extraction in these samples. Maximal SOD expression was observed in the presence of 2.5 mM X alone (Fig 7.10c, lane 9) compared with incubation with SOD alone (Fig. 7.10c, lane 10) and little RT-PCR product was detected when the parasites were incubated in RPMI 1640 alone (Fig 7.10c, lane 12). There is evidence that nematode parasites contain xanthine oxidase and the addition of endogenous xanthine may lead to the production of superoxide anions within the live parasite and, as a result, cause dramatic changes in parasite SOD expression (D. P. Knox personal communication). The low amount of SOD in the SOD alone culture (Fig. 7.10, lane 10) may reflect a low level of enzyme being produced by the parasite while in culture (Table 7.4) because there is already an excess of the enzyme in the culture media which may be protecting the parasites from any endogenous oxidative damage. Similarly, the lack of product in the RPMI 1640 sample may indicate that the parasite was not making much mRNA at that time because it was not under oxidative attack. The low level of SOD expression recorded when the parasite was cultured in RPMI 1640 alone also agrees with the results in Chapter 6, where low levels of enzyme were detected in the IVR products. This could be indicative of specific expression of genes encoding cytosolic and putatively EC forms of the enzyme and elevations and reductions detected in the different groups may reflect which type of SOD gene is being expressed. If elevation in expression is due to one gene or gene type it may indicate the importance of a specific gene in parasite survival.

In summary, the work in this chapter focused on the isolation, cloning and sequencing of a *N. brasiliensis*-specific SOD gene. This work was followed by the development of a RT-PCR assay to measure *N. brasiliensis* SOD expression. The results from this assay indicated that RT-PCR can be used to analyse the dynamics of parasite SOD expression in response to the changing intra-host environment. This method of quantification of gene expression could also be implemented in order to analyse the expression of a variety of other parasite-derived proteins and would be useful for analysing the expression of parasite proteinases, which in this thesis (Chapters 4, 5 and 6), have also been shown to vary markedly in response to the intra-host

environment. It may also be possible to use this assay to discriminate between expression of individual genes which may encode a particular isoenzyme of interest.

CHAPTER EIGHT

GENERAL DISCUSSION

Gastrointestinal nematodes of ruminants cause considerable economic losses world wide by rendering animals unprofitable. At the moment the use of anthelmintics and good farm management practices are the main methods of helminth control, however resistance to anthelmintics has been detected world wide. In several parasite systems it has been shown that livestock do acquire immunity to GI nematodes infections and this has led to increasing research on the development of immunologically based control by vaccination, meanwhile new anthelmintics are continually being sought. However, a factor which is increasingly coming to light, as knowledge about the host/parasite relationship increases, is that a number of parasites can evade the host immune response (Maizels *et al*, 1993) and that, in an immunologically primed host, a population of worms is be able to establish and thrive (Ogilvie and Hockley, 1968). Parasite enzymes have been implicated in having important roles in parasite survival (Edwards, Burt and Ogilvie, 1971; Knox and Jones, 1992) and these components may vary genotypically and phenotypically within and between parasite populations. This issue was, in part, addressed here in the context of parasite enzyme expression.

In Chapter three, the proteinases released by the parasitic stages of *T. circumcincta* were identified. The results from these analyses demonstrated that these proteinases had optimal activities at alkaline pH and were mainly restricted to the metallo proteinase class. Stage-specific proteinase release was also demonstrated. These enzymes may play an important role in parasite maintenance and immune evasion and subsequent identification and isolation of enzymes or isoenzymes expressed by the parasite may play an essential role in the development of new anthelmintics or as antigens for use in vaccination.

In an attempt to establish if IVR proteinases from the adult stage of *T. circumcincta* were immunogenic in the infected host, antibody responses were sought by Western blotting and anti-enzyme antibody inhibition studies. From these results, it appeared that the IVR products were weakly immunogenic. Although incubation of IVR proteinases with immune plasma and gastric lymph did indicate some level of inhibition of proteinase activity, it was not possible to rule out the inhibitory effects of endogenous proteinase inhibitors in the sera and lymph. The weak antigenic nature of the parasite proteinases may indicate an adaptation of the parasite which enables it to survive in the host and may indicate that the parasite and host enzymes are structurally

very similar. In an attempt to demonstrate proteinase recognition by immune sera and purified IgG, enzyme-antibody complexes could be removed by precipitation using Protein G Sepharose prior to determination of total proteinase activity or visualisation using gelatin substrate gels (Todorova, Knox and Kennedy, 1995). If these methods were still not sensitive enough then purification of the proteinases by, for example, high performance liquid chromatography (Curley, O'Donovan, McNally, Mullally, O'Hara, Troy, O'Callaghan and Dalton, 1994; El Moudini, Rodier, Barrault, Ghazali and Jacquemin, 1995) followed by Western blotting, immunoprecipitation or the use of the purified enzyme in enzyme linked immuno-sorbent assays (ELISAs) may be a more successful pathways to pursue. More importantly, the effect of local immune responses should be appraised by examining the recognition of parasite enzymes by mucus and purified secretory IgA using the methods described above. The development of an ELISA to *Teladorsagia* antigens could also have a future use in serodiagnosis of *Teladorsagia* infections (Schallig, Hornok and Cornelissen, 1995).

The results discussed in Chapters four, five and six concentrated on seeking evidence for geographical strain divergence and changes in parasite enzyme expression induced by the intra-host environment which, to an extent, is influenced by elements of the host immune response. Differences in enzyme expression in the geographically distinct strains of *H. contortus* were most evident when comparing proteinase levels. These differences may have resulted from evolutionary adaptation by the parasites and this possibility could be investigated by isolation of genes encoding the enzymes of interest by, for example, the purification of the enzyme of interest and raising specific antibody to the enzyme for use in probing cDNA libraries, followed by the isolation and sequencing of genes of interest. Alternatively, these differences could arise by host mediated modulation of parasite enzyme expression. These effects may be mediated by the local physiological/biochemical environment or as a result of the elements comprising the early components of the host immune response. Infecting UK strains of sheep with the Australian parasite and vice versa would define the extent to which the observed effect was host mediated.

Proteinase and SOD isoenzymes expressed in parasites harvested from different individual rats (Chapter six) were quite variable despite the fact that all the rats received the same 'genetic pool' of larvae. Several parasite enzymes, including proteinases and

SOD, are thought to be encoded by multi-gene families (Pratt *et al*, 1992, Heussler and Dobbelaere, 1994; Liddell and Knox, 1995) which may explain why variable isoenzyme expression was detected in Chapter six. In addition, the SOD cDNA sequence variants from *N. brasiliensis* described in Chapter seven could be indicative of multi-gene family expression. The extent to which genetic polymorphism contributed to the variable SOD isoenzyme profiles detected here could be investigated further using techniques such as targeted PCR on individual worms with a single gene specific primer followed by southern blotting and hybridisation using a labelled SOD DNA probe. Individual gene products may confer antigenic and functional diversity and contribute to the ability of different parasite genotypes to survival in the host. This means that the variable profiles noted in Chapter six were not necessarily due to modulated gene expression. Differing 'isoenzyme' profiles could arise by switching on of 'new' i.e. dormant genes or as a result of the selection of different worm genotypes. These effects can only be investigated by quantifying gene expression by measuring mRNA transcript production as well as specific protein product levels. The use of RT-PCR with *N. brasiliensis* SOD as target (Chapter seven) was evaluated for this purpose.

It was noted that total SOD activity and SOD expression as measured by RT-PCR analysis varied in individual worm populations and these were elevated in the presence of increasing amounts of superoxide anions. Whether or not this was due to increased expression of specific SOD genes or of all SOD genes could not be deduced using the general SOD primers described here. Expression of cytosolic and extracellular SOD levels could be precisely examined by the development of transcript specific primers which were able to differentiate between the two types of SOD and between the different sequence transcripts. Higher levels of SOD have been detected in the L3 stage of *N. brasiliensis* compared with adults (Knox and Jones, 1992). indicating that *N. brasiliensis* SOD is developmentally regulated. This could be investigated further using the RT-PCR technique developed here. The same approach could be used to investigate proteinase expression within parasite populations which is often stage specific. An attempt was also made to standardise the amount of product obtained in the SOD amplifications using a constitutively expressed protein as target. The use of β-tubulin was assessed here. Highly variable amounts of amplified product were obtained from different worm populations despite consistent SOD product yields.

It is therefore unlikely that this β-tubulin is in fact constitutively expressed. Glycolytic enzymes provide a more reliable control as these enzymes generally demonstrate little genetic variation (Nadler, 1991). Actin (Van Hille, Lohri, Reuter & Herrmann, 1995) and cyclophorin (Zou *et al*, 1995) have also been used as standards for RT-PCR.

As mentioned previously, proteinases and antioxidant enzymes may play an important role in parasite survival. The results discussed in this study provide further evidence to support this theory. Some modulation of enzyme activity was demonstrated between all the parasite populations investigated in this study, but these differences cannot be specifically attributed to the parasite evading host immune responses, and the exact mechanisms require further definition. It is certainly pertinent to note the parasite's ability to express different forms of potentially important protective antigens when considering their future role for chemotherapuetic or immunological intervention.

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