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Isolation and characterization of antigens present in the protective ES-thiol fraction of *Ostertagia ostertagi* and their evaluation as vaccine candidate

Proefschrift ter verkrijging van de graad van Doctor in de Diergeneeskundige Wetenschappen aan de Faculteit Diergeneeskunde
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Thank you...

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Yves

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2-D	two dimensional
3-D	three dimensional
4-D	four dimensional
AO	acridine orange
ASP	activation-associated secreted protein
BLAST	basic local alignment search tool
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CP	cysteine protease
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
DTT	dithiothreitol
E-64	L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane
ELISA	enzyme-linked immunosorbent assay
EPG	eggs per gram faeces
ES	excretory-secretory
ES-thiol	thiol binding excretory-secretory antigen
EST	expressed sequence tag
FEC	faecal egg counts
GFP	green fluorescent protein
GI	gastrointestinal
GST	glutathione-S-transferase
H-gal-GP	*Haemonchus* galactose-containing glycoprotein complex
HRPO	horse raddish peroxidase
HS	horse serum
Ig	immunoglobulin
kDa	kilo Dalton
L1	first stage larva
L2	second stage larva
L3	third stage larva
L3ex	third stage larva without sheath
L4	fourth stage larva
LC	liquid chromatography
ML	macrocyclic lactone
MS	mass spectrometry
MSP	major sperm protein
MEP	metalloprotease
mRNA	messenger ribonucleic acid
MW	molecular weight
OD	optical density
Oo-gal-GP	*Ostertagia* galactose-containing glycoprotein complex
OPA	*Ostertagia* polyprotein allergen
P	probability
PDB	Protein Data Bank
pI	isoelectric point

PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.05% Tween20
PCR	polymerase chain reaction
PGE	parasitic gastroenteritis
PNGase F	peptide-N-glycosidase
PRI	propidium iodide
PVDF	polyvinylidene difluoride
rpl-13a	ribosomal protein L13a
RACE	rapid amplification of complementary DNA ends
RFU	relative fluorescence unit
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSP	sperm-specific protein family class P
TBB	beta-tubulin
TCTP	translationally controlled tumor protein
TSBP	thiol sepharose binding protein
WT	wild-type
Z-Phe-Arg-AMC	carbobenzoxy-phenylalanyl-arginyl 7-amino-4-methyl coumarin

Introduction

0.1 Introduction

Gastrointestinal (GI) parasitic nematodes infect livestock worldwide. Their negative effect on animal growth and the costs related to anthelmintic treatment are responsible for major economic losses in animal production every year. While infection can result in clinical disease - i.e. parasitic gastroenteritis (PGE) - most losses can be attributed to subclinical infections. For grazing cattle in temperate climates, the most common and most pathogenic GI nematode is *Ostertagia ostertagi*.

0.2 *Ostertagia ostertagi*

O. ostertagi is a parasitic cattle nematode belonging to the superfamily of Strongyloidea and the family of Strongylidae (de Ley and Blaxter, 2004). The organism has a direct life-cycle consisting of a free-living phase on pasture and a parasitic phase in the host (Figure 0.1). *Ostertagia* eggs are passed on in the faeces and the first stage larva (L1) hatches in the faecal pat. After a first moult, the second stage larva (L2) emerges. While maintaining its loosely fitting cuticle (sheath), the L2 develops into an infectious third stage larva (L3). Under optimal conditions of temperature and humidity, development from egg to L3 occurs within 2 weeks. Infection is realized by the ingestion of grass contaminated with L3s. Ingested L3s exsheath in the rumen and penetrate the gastric glands of the abomasum within 6 hours. Next, they develop to fourth stage larvae (L4s) and migrate to the abomasal lumen to establish themselves as adults. The pre-patent period is approximately 21 days, although under certain conditions larvae can become arrested in their development at the early L4 stage for a period of up to 6 months.

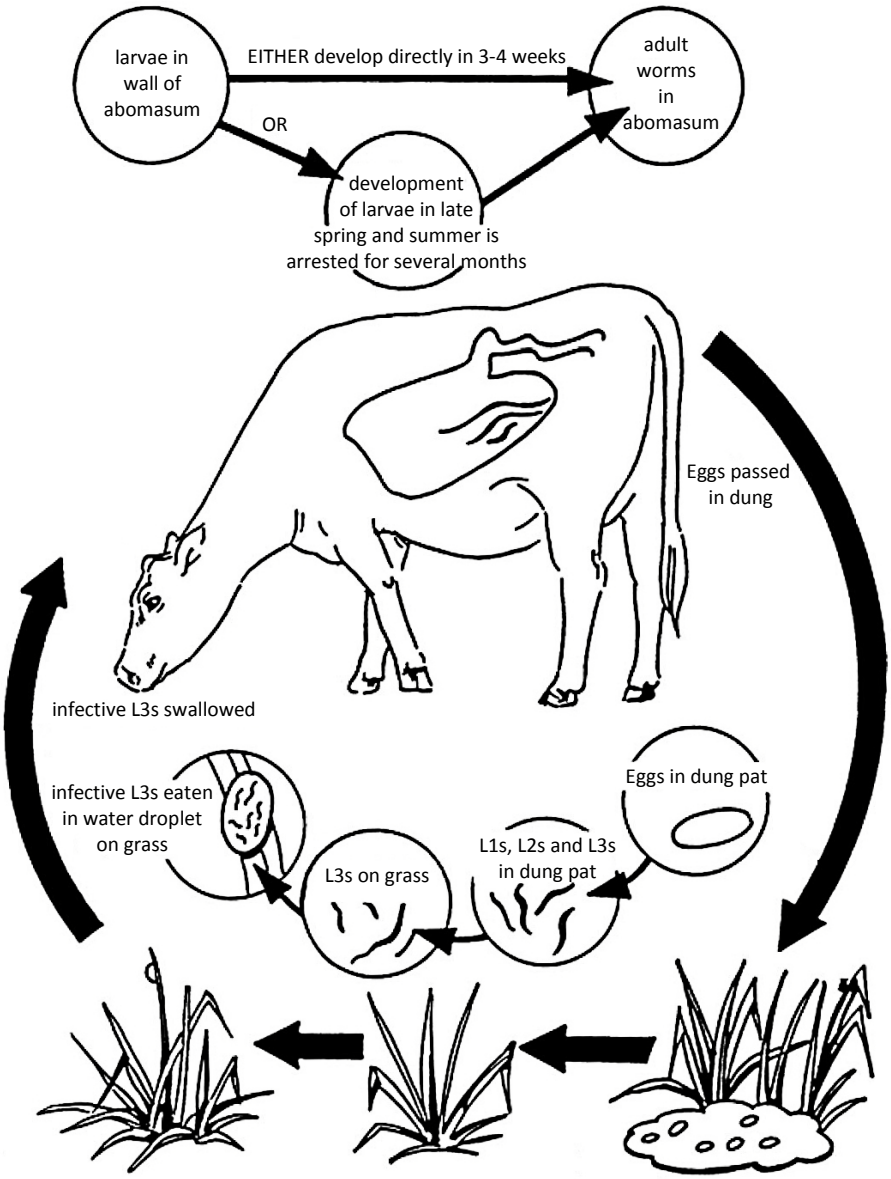

Figure 0.1 Scheme showing the life-cycle of *O. ostertagi* (Petalia™ & Petsite.com Ltd© - http://petalia.com.au)

0.3 Epidemiology

Nematode infections during spring are largely derived from over-wintered larvae present on pasture, but in some cases may partially consist of over-wintered adults or worms maturing from inhibited larvae. Infective larvae are ingested by animals that are turned out in the beginning of the new grazing season (May-June) (Figure 0.2). After 3 weeks, eggs are shed and develop into L3s. At this time of year, egg hatching is rather slow but becomes faster toward mid-summer as the temperature rises. Therefore, the majority of eggs deposited in April, May and June will reach the infective stage from mid July onwards, during the so-

called mid-summer rise. Timing of this rise is variable and weather-dependent (Hilderson *et al.*, 1987). Wet summers result in high pasture contamination from mid-July onwards, leading to PGE (Anderson *et al.*, 1965). When the summer is dry, larvae will accumulate in faecal pats and there will be little to no release. However, these larvae will emerge when wet weather returns, resulting in high pasture contamination in autumn. While autumn progresses an increasing proportion of ingested L3s will only evolve to the L4 stage and then go into arrested development. As a result, calves can harbor thousands of these "early" L4s but few developing forms or adults. This can lead to PGE in the following grazing season when the inhibited larvae start to mature (Anderson *et al.*, 1965). However, the latter type of ostertagiosis rarely occurs.

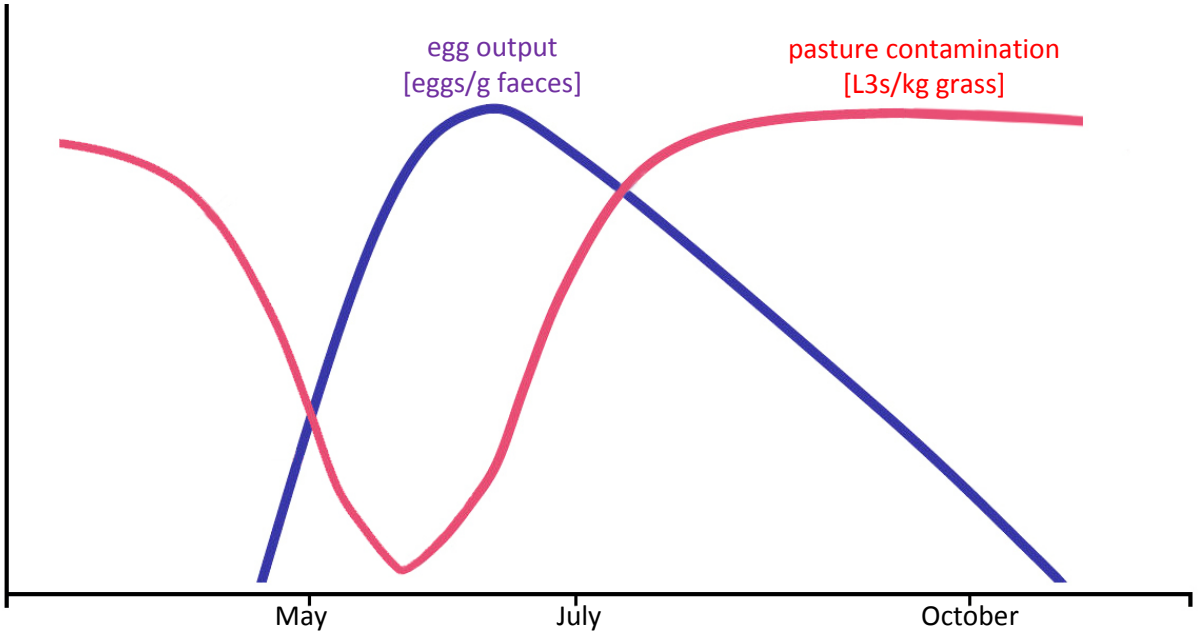

Figure 0.2 Diagram showing the egg output of cattle (eggs per gram faeces = EPG) versus pasture contamination of *Ostertagia* (L3s/kg grass) throughout the year

0.4 Pathogenesis and pathology of ostertagiosis

Abomasal nematodes are able to induce structural, biochemical, hormonal, nutritional and immunological changes in the host. When infective larvae penetrate the gastric glands these glands become dilated and enlarged (Figure 0.3a). The growing larvae cause the parasitized mucosal glands to become distended and the common cell lining of the abomasum - i.e. zymogenic cells, mucous cells and parietal cells - to be replaced by undifferentiated

epithelium (Figure 0.3b) (Murray and Jennings, 1970). As a result, the pH of the abomasum increases and pepsinogen is no longer converted into pepsin (McKellar, 1993). A decrease in peptic digestion and bacteriostatic activity can be seen. When emerging from the glands the adult parasites disrupt the junctions between the epithelial and the endothelial cells, causing an increase in plasma pepsinogen levels and loss of plasma proteins (McKellar, 1993). These changes in cell structure and coherence can result in clinical signs like inappetence, diarrhea, dull hair coat and weight loss, i.e. ostertagiosis.

(a) (b)

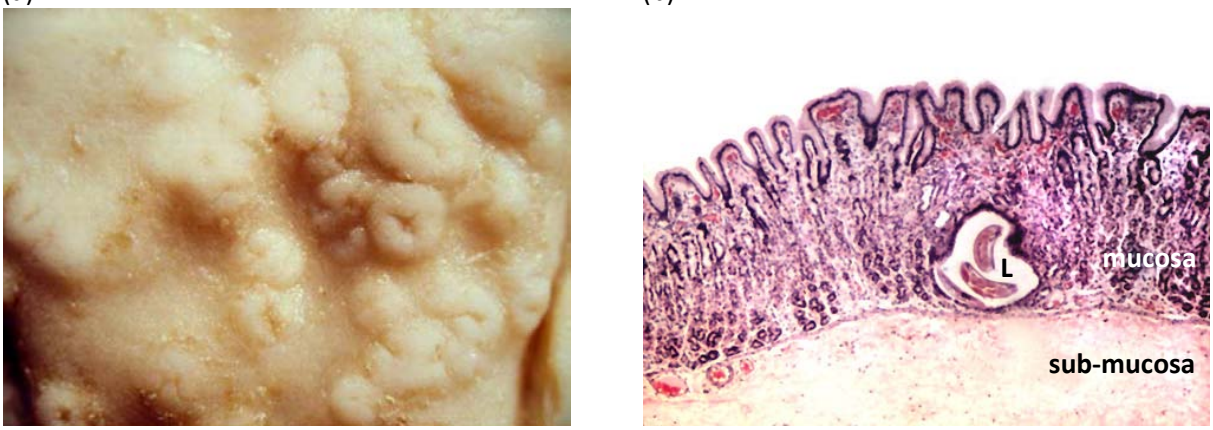

Figure 0.3 (a) Dilated and enlarged gastric glands afer infection with L3s. (b) Transversal section of an infected gastric gland containing a developing larva (L).

0.5 Economic importance

GI nematodes are regarded as a major source of production losses in cattle farming (Kloosterman *et al.*, 1992; Vercruysse and Claerebout, 2001). Young, first grazing season calves are the most susceptible to infection and suffer severe losses in terms of animal production as a result of a reduced growth performance and the costs related to anthelmintic treatment. Clinical PGE is typically seen in this age class when no appropriate preventive measures have been taken and causes a general loss of condition which eventually leads to death (Hilderson *et al.*, 1987). However, the development of anthelmintic drugs and their strategic use have reduced parasitism to a subclinical phenomenon. Substantial reductions in weight gain have been reported in untreated, first grazing season calves with subclinical infections (Shaw *et al.*, 1998). Subclinical infections can also be associated with decreased levels of milk production in adult cows. Anthelmintic treatment of *Ostertagia* infected cows or heifers increases milk production by an average of 0.35 kg milk

per day (Sanchez *et al.*, 2004). Additionally, carcass quality is affected by GI nematodes with reduced carcass weight, killing out percentage and related carcass measurements (Entrocasso *et al.*, 1986). Since there are approximately 89 million cattle in the EU (Dias *et al.*, 2008) and 100 million in the United States (Economic Research Service Service, 2006) it is understandable that it is of great economic importance to control *Ostertagia* infections in the best possible way.

0.6 Current control

At the moment, control of PGE in Europe is almost exclusively based on preventive treatment with synthetic chemotherapeutic drugs, i.e. anthelmintics. There are three major classes of drugs used to control nematodes in livestock: benzimidazoles (e.g. albendazole sulphoxide), nicotinic agonists (e.g. levamisole) and macrocyclic lactones (MLs) (e.g. ivermectin). Most frequently used are MLs since they have the highest efficacy, a broad spectrum of activity and persistent activity (Vercruysse and Rew, 2002). Animals are treated during the early part of the first grazing season to prevent recycling of the infection acquired from the over-wintered larvae on pasture. In addition, the recent availability of generic MLs and the lack of withdrawal time for milk (eprinomectin and moxidectin pour-on) led to an increased use of these anthelmintics in second grazing season animals and adult dairy cows.

Unfortunately, the intensive use of anthelmintics has its downsides. First, there are the financial repercussions for farmers as a result of recurrent treatments. Second, the development of natural immunity against GI nematodes can be negatively influenced by anthelmintics (Claerebout, 2002). Third, there is an increasing consumer demand for “cleaner and greener” agricultural products leaving a minimum amount of drug residues in food products and the environment (Joint FAO/WHO Expert Committee on Food Additives, 2002). Finally, the development of anthelmintic resistance in cattle nematodes might pose a problem in the future. Recent reports point out the increasing incidence of resistance to benzimidazoles, imidothiazoles and MLs in Argentina (reviewed by Anziani *et al.*, 2004), the United Kingdom (reviewed by Coles, 2005) and New Zealand (reviewed by Pomroy, 2006). For *O. ostertagi*, the first suspected cases of ivermectin resistance have been reported in New Zealand (Mason and McKay, 2006; Waghorn *et al.*, 2006) and Argentina (Suarez and Cristel, 2007). A recently discovered new class of anthelmintics, i.e. the amino-acetonitrile derivates, may provide a temporary solution for the current resistance problem (Kaminsky *et al.*, 2008). Nevertheless, these results have encouraged researchers to investigate alternative control methods for ostertagiosis.

0.7 Alternative control

There are several non-chemical alternative strategies for controlling nematode parasites in ruminant livestock.

One possibility is to optimize grazing management by introducing pasture resting, late turn-out, mowing, reduction of the livestock density, stock rotation onto clean pasture and interchange of grazing between different species e.g. sheep and cattle (reviewed by Waller, 2006). However, this approach requires a firm understanding of the epidemiology of infection and is not adapted to the local availability of grass.

A second strategy would be to develop cattle lines or breeds with an enhanced disease resistance. Examples include resistance of *Bos indicus* to cattle ticks (Donald, 1994) and trypanotolerance of the West African *Bos taurus* breeds (Murray *et al.*, 1991). Previous studies have shown predisposition to heavy *O. ostertagi* infections to be genetic in origin and that genetic variability provides feasible means to control GI parasite infection without anthelmintics (Gasbarre *et al.*, 1990; Kloosterman *et al.*, 1992; Sonstegard and Gasbarre, 2001). Unfortunately, no appropriate marker is available for determining the difference between resistant and resilient animals. Also, resistance to GI nematodes is likely to come at the expense of high rates of growth, reproduction or milk production. For instance, a negative correlation has been described between cow fertility and resistance to worm infection (Mackinnon *et al.*, 1990).

A third proposal focuses on the use of natural compounds. The predaceous microfungi *Duddingtonia flagrans* have been studied for their ability to reduce nematode parasitism. In short, these fungi produce high numbers of thick-walled resting spores - i.e. chlamydospores - which are shed with the parasite eggs in the fresh faeces where they germinate, grow and prey on parasite larvae as a food source hereby reducing pasture contamination (Waller and Thamsborg, 2004). The advantages of these fungi are that they survive passage through the bovine GI tract and grow rapidly in fresh dung. Although their potential has been demonstrated in several trials with sheep (Chandrawathani *et al.*, 2003; Flores-Crespo *et al.*, 2003; Waller *et al.*, 2004) and cattle (Dimander *et al.*, 2003) practical delivery systems still need to be developed. In addition, several plant products have been tested for their anti-parasitic potential. Good examples are plant tannins (Athanasiadou *et al.*, 2001; Marley *et al.*, 2003; Waller and Thamsborg, 2004) and cysteine proteases (Tagboto and Townson, 2001; Stepek *et al.*, 2004). While the results obtained so far look promising, this area of research is still in its infancy.

A final alternative for controlling parasitic nematode infection would be vaccination, which will be described in detail in the next chapter.

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Chapter 1

Designing vaccines against parasitic nematodes

1.1 Introduction

Vaccination has been shown to be an effective tool against various infectious agents. Outstanding successes have been achieved in the control of bacterial, viral and - to a lesser extent - protozoan infections. Furthermore, vaccines are potentially safer, cheaper and more efficacious as prophylactics than drugs.

Unfortunately, only limited progress has been made in designing vaccines against parasitic helminths. Successes include highly effective recombinant vaccines against the cestodes *Taenia ovis* in sheep, *Taenia saginata* in cattle, *Taenia solium* in pigs and *Echinococcus granulosus* in livestock animals (reviewed by Lightowlers, 2006) and a commercial vaccine against the bovine nematode *Dictyocaulus viviparus* (Dictol^{TM}, Fort Dodge).

Decades of study have shown that vaccine development against parasitic helminths is far from straightforward. In contrast with bacteria, viruses and protozoa, helminths are multicellular organisms which go through complex developmental changes within their mammalian hosts. They can occur in a variety of tissues, though many of them are specialized to single tissues like muscle, veins, brain or intestine. Also, these parasites are capable of modulating the host immune system, hereby protecting themselves and their offspring (Meeusen, 1996; Maizels *et al.*, 2004). Their relatively long life span in the host is direct evidence of this capacity. In general, helminth parasites favor strong Th2 responses in the host while inducing factors that prevent immunity from eliminating the parasite itself. Immunomodulating factors include cytokine homologs, protease inhibitors and nematode-specific glycan structures. Unfortunately, the exact mechanism of immunomodulation is not fully understood yet.

At the moment, little is known about the immune response that is responsible for protection as a result of vaccination. However, the success of a vaccine does depend on its capacity to activate the host immune system which can be monitored by the induction of antigen-specific antibodies.

The following section will present the progress made in the development of nematode vaccines. This overview will be limited to vaccination trials in which successful induction of a protective immune response was observed.

1.2 Vaccination by targeting the entire parasite

Initial attempts at creating nematode vaccines were based on using the entire parasite as a vaccine, either by infection with inactivated or attenuated parasites or by immunization with whole organism extract.

Nearly 50 years ago, this approach resulted in the commercialization of a vaccine against the cattle lungworm *Dictyocaulus viviparus* which consists of X-irradiated third stage larvae (L3s) and resulted in a reduction in worm burden of 90-99% (Jarrett *et al.*, 1958a, 1958b). In accordance, this approach was tested in several other host-parasite systems. In sheep, a significant reduction in worm burden was demonstrated for the lungworm *Dictyocaulus filaria* (Jovanovic *et al.*, 1965; Sharma *et al.,* 1988). In dogs, vaccination with irradiated larvae of the hookworm *Ancylostoma caninum* resulted in a 90% reduction in worm burden (Vinayak *et al.*, 1981). Similarly, golden hamsters immunized with treated *Ancylostoma ceylanicum* larvae demonstrated a 95-99% reduction in worm burden (Menon and Bhopale, 1985). For the rat parasite *Strongyloides ratti*, immunizing the host with microwave-irradiated larvae resulted in a significant reduction in worm burden and worm size as well as egg production (Conder and Williams, 1983). In pigs, radiation-attenuated eggs of *Ascaris suum* were capable of inducing an 83-94% reduction in lung worm burden (Tromba, 1978; Urban and Tromba, 1982, 1984). Immunization with irradiated larvae was also successful for the sheep parasites *Trichostrongylus colubriformis* with a 53-98% reduction in faecal egg counts (FEC) and a 97% reduction in worm burden (Gregg *et al.*, 1978) and *Haemonchus contortus* with a 98% reduction in FEC and a 97% reduction in worm burden (Jarret *et al.*, 1959; 1961; Smith and Angus, 1980). However, in the latter case high levels of protection could not be induced in younger animals (Urquhart *et al.*, 1966a, 1966b; Smith and Angus, 1980). Since lambs are most at risk for nematode infection, this vaccine was not commercially viable.

Additionally, somatic extract obtained from the entire parasite has been shown to induce protection against several infections. For *A. caninum*, dogs vaccinated with whole worm extract demonstrated a 45-64% reduction in worm burden (Vinayak *et al.*, 1981). Crude larval extract from *Trichinella spiralis* was capable of protecting mice against infection (Goven and de Buysscher, 1978) and reducing worm burdens in pigs with 78-88% (Marti *et al.*, 1987). Adult crude extract from the pig nematode *A. suum* was able to protect mice against infection, reducing worm burdens with 79-89% (Lukes, 1992). Furthermore, a somatic extract fraction of *H. contortus* containing low molecular weight (MW) antigens induced a 99% reduction in FEC and a 97% reduction in worm burden in sheep (Schallig and Van Leeuwen, 1997). In calves, vaccination with larval or adult worm homogenates of

Oesophagostomum radiatum induced protective immunity, resulting in a 29-100% reduction in FEC and a 12-99% reduction in worm burdens (Herlich *et al.*, 1973; Keith and Bremner, 1973; East *et al.*, 1988, 1989; Gasbarre and Canals, 1989). In contrast, attempts to induce protection against *Ostertagia ostertagi* using irradiated larvae (Anderson *et al.*, 1967; Bürger and Pfeiffer, 1969) or larval or somatic extract were unsuccessful (Williams *et al.*, 1974; Herlich and Douvres, 1979; Hilderson *et al.*, 1995).

Parasite	Host	Antigen	Reduction in worm burden (%)	Reduction in FEC (%)	Reference
A. caninum	dog	adult extract	45-64	N.D.	133
		X-irradiated larvae	90	N.D.	133
A. ceylanicum	hamster	X-irradiated larvae	95-99	N.D.	79
A. suum	pig	adult extract	79-89	N.D.	71
		X-irradiated eggs	83-94	N.D.	123, 127
D. filaria	sheep	X-irradiated larvae	(significant)	N.D.	55, 103
D. viviparus	cow	X-irradiated larvae	90-99	N.D.	49, 50
H. contortus	sheep	somatic extract	97	99	100
		X-irradiated larvae	97	98	51, 52, 108
O. radiatum	cow	adult extract	12-99	29-100	22, 23, 28, 42, 57
S. ratti	rat	μ-irradiated larvae	(significant)	(significant)	16
T. spiralis	mouse	larval extract	(significant)	N.D.	35
	pig	larval extract	78-88	N.D.	75
T. colubriformis	sheep	X-irradiated larvae	97	53-98	36

Table 1.1 Overview of successful vaccination trials against parasitic nematodes based on attenuated parasites or whole worm extract.

1.3 Vaccination by targeting specific parasite components

The limited success of the previously described approach can be attributed to several factors. First of all, protective antigens are usually relatively scarce within the complex mixture of parasite antigens. Second, immunomodulatory molecules present in parasite extracts could suppress or divert a protective immune response (Meeusen, 1996; Maizels *et al.*, 2004). Finally, it is not always possible to obtain a commercially stable formulation of vaccine material. Therefore, when designing parasite vaccines it is necessary to study potential sites of attack at the molecular level.

1.3.1 Internal antigens

In order to target the parasite's metabolism, a vaccine should be aimed against an internal component. In this case, the antigens are specifically expressed within the parasite and therefore not recognized by the host during infection. While there is no direct contact between the host and this type of antigens, blood feeding parasites will be able to take up host immunoglobulins (Igs) induced by vaccination followed by exposure of internal antigens. Although this approach has the advantage of subjecting the parasite antigens to limited selection pressure, natural infection will not boost the host immune response. Hence, multiple immunizations are necessary to maintain protection.

1.3.1.1 Gut antigens

The first studies aimed at targeting internal parasite antigens were conducted in the blood-feeding sheep nematode *Haemonchus contortus* (Munn and Greenwood, 1984). Initially, Munn *et al.* discovered a helical polymeric structure on the gut surface of *H. contortus*, termed **contortin**. Vaccinating young lambs with this gut structure resulted in protection against challenge infection (Munn *et al.*, 1987).

A second protective protein of *H. contortus* was an integral membrane glycoprotein of 110 kilo Dalton (kDa) derived from intestinal microvilli (Smith and Munn, 1990). Sheep immunized with this antigen, called **H11**, showed a reduction in FEC and worm burden of 90% and 75% respectively (reviewed by Newton and Munn, 1999). In addition, H11 protects young lambs (Tavernor *et al.*, 1992) and is effective against anthelmintic-resistant worms and in a range of breeds (Newton and Munn, 1999). Furthermore, the development of natural acquired immunity is not hampered by vaccination (Smith and Smith, 1993) and protection persists for about 23 weeks after vaccination (Andrews *et al.*, 1997). A recent field trial with native H11 showed a significant reduction in worm egg counts in vaccinated lambs and a greatly diminished pasture contamination, although the protective immunity induced by each vaccine lasted only 7 weeks and antigen titers were quite variable between different lambs (Lejambre *et al.*, 2008). Unfortunately, recombinant H11 expressed in *Escherichia coli* and baculovirus induced little to no protection in sheep (Knox and Smith, 2001; Knox *et al.*, 2003).

A third group of gut surface antigens was isolated from *H. contortus* extract by affinity chromatography using a lectin column i.e. the *Haemonchus* galactose-containing glycoprotein complex (**H-gal-GP**) (Smith *et al.*, 1994). Vaccinating sheep with this complex resulted in a 67-95% reduction in FEC and 61-67% reduction in worm burden (reviewed by Newton and Munn, 1999). Recently, the protective capacity of the H-gal-GP fraction has also

been demonstrated in the field (Lejambre *et al.*, 2008). H-gal-GP is made up of proteins ranging from 31 to 230 kDa (Smith and Smith, 1996) and contains neutral metalloproteases (MEPs) (Redmond *et al.*, 1997; Smith *et al.*, 1999), cysteine proteases (Smith *et al.*, 1999) and aspartyl proteases (Longbottom *et al.*, 1997; Smith *et al.*, 2003a). Partial protection could be obtained when using fractions of H-gal-GP indicating that multiple components are responsible for protection (Smith and Smith, 1996). Two pepsin-like aspartyl proteases present in H-gal-GP - i.e. HcPEP1 and HcPEP2 - proved to significantly reduce egg counts by 48% and worm numbers by 36% (Smith *et al.*, 2003a). Moreover, a vaccination trial using a combination of four identified MEPs, i.e. MEP1-4, rendered a reduction in FEC of 45% and a reduction in worm burden of 50%. MEP3 was found to be the most protective antigen used on its own, inducing a 33% reduction in FEC (Smith *et al.*, 2003b). In contrast, *E. coli* recombinants of the trombospondin, galectin, cystatin and MEP components of H-gal-GP were not protective (Newton and Meeusen, 2003; Knox *et al.*, 2003; Smith *et al.*, 2003b).

A fourth type of gut antigens which have proven their protective capacity are the **cysteine proteases** (CPs). These enzymes are located at the surface of *H. contortus* intestinal cells and show homology with mammalian cathepsin B (Skuce *et al.*, 1999). Vaccination with membrane extracts enriched for CP activity by thiol sepharose affinity chromatography - i.e. thiol sepharose binding proteins (TSBPs) - induced protection in lambs reducing FEC by 47% and worm burden by 77% after a single challenge infection (Knox *et al.*, 1999). In a subsequent study the CPs of the TSBP fraction were further purified using an affinity column coated with recombinant *H. contortus* cystatin (Redmond and Knox, 2004) and anion exchange chromatography (Knox *et al.*, 2005). This purified fraction conferred protection comparable to that obtained using the entire TSBP fraction, indicating that CPs are responsible for inducing protection. Recently, a mixture of three cathepsin B-like CPs - i.e. hmcp1, hmcp4 and hmcp6 - have been recombinantly expressed in *E. coli* and have been shown to moderately protect lambs, reducing FEC by 27% and worm burden by 29% (Redmond and Knox, 2006).

Monoclonal antibodies have been used to identify and purify two other internal *H. contortus* antigens called **p46** and **p52** (Jasmer *et al.*, 1993). Both proteins are encoded by the same GA1 gene and are originally expressed as a polyprotein of 100 kDa, i.e. p100 (Jasmer *et al.*, 1996). A mixture of these antigens significantly reduced FEC in goats by 50% and worm counts by 60% (Jasmer *et al.*, 1993). In addition, the GA1 proteins show similarities with three peptides (p45, p49 and p53) which were isolated from the protective H11 fraction using ion-exchange chromatography (Smith *et al.*, 1993). Vaccination of lambs with these so-called P1 antigens rendered a 69% reduction in FEC and a 30% reduction in worm burden.

In contrast with the results obtained for the sheep parasite *H. contortus*, limited progress has been made in identifying protective internal antigens of other important parasitic nematodes.

For the canine hookworm *Ancylostoma caninum*, three protective **internal antigens involved in blood digestion** have been described. Ac-CP2, a cathepsin B-like protease identified in the gut and recombinantly expressed in yeast, induced partial protection in dogs, reducing fecundity and worm size (Loukas *et al.*, 2004). A baculo recombinant of Ac-APR1, a cathepsin D-like aspartic acid protease, rendered an 18% reduction in worm burden in dogs (Hotez *et al.*, 2002). A novel glutathione-S-transferase - i.e. Ac-GST1 - was found to be expressed in limited amounts in the intestine as well as the hypodermis and muscle tissue. This enzyme is believed to have a detoxifying role during hemoglobin digestion. Vaccinating dogs with an enzymatically active yeast recombinant of Ac-GST1 resulted in a 32% reduction in FEC and a 39% reduction in worm burden (Zhan *et al.*, 2005). While this reduction was not significant, hamsters injected with this recombinant demonstrated a significant reduction in worm burdens of 50-54% after challenge with the human hookworm *Necator americanus* (Xiao *et al.*, 2008).

When combined, *O. ostertagi* homologs of H11 (**Oo12**) and H-gal-GP (**Oo-gal-GP**) are capable of inducing limited protection with a 30-50% reduction in FEC (Smith *et al.*, 2000). In addition, these antigens efficiently cross-protected sheep against an *H. contortus* infection with an 81-97% reduction in FEC. This suggests that *Ostertagia* does not ingest sufficient amounts of host Igs compared to blood-feeding nematodes (Smith *et al.*, 2001). Correspondingly, Siefker and Richard (2000a) demonstrated that vaccinating calves with an intestinal homogenate of the bovine stomach worm *Haemonchus placei* rendered protection against homologous challenge infection significantly reducing the number of female worms. Although *H. placei* and *O. ostertagi* contain similar epitopes on these intestinal antigens (Siefker and Rickard, 1998), no protection was observed against heterologous challenge infection with *O. ostertagi* (Siefker and Rickard, 2000b). Also, a membrane-bound antigen fraction isolated from adult worms and enriched for CPs failed to protect calves against an *Ostertagia* infection (Geldhof *et al.*, 2002).

1.3.1.2 Tropomyosins

Tropomyosins are present in all eukaryotic cells and interact with actine filaments (Perry, 2001). These antigens play a central role in cell motility and muscle movement (Pittenger *et al.*, 1994). A series of experiments in sheep in Australia have demonstrated the protective capacity of L3 tropomyosin from *Haemonchus contortus* and *Trichostrongylus colubriformis*

inducing a 43-51% and 54% reduction in worm burden respectively (Cobon *et al.*, 1989; O'Donnel *et al.*, 1989). Furthermore, injecting BALB/c mice with the full-length tropomyosin complementary deoxyribonucleic acid (cDNA) of the filarial parasite *Onchocerca volvulus* resulted in a 20% reduction in worm burden (Harrison and Bianco, 2000) and immunization with a recombinant fusion protein of the Ov-tropomyosin coupled to maltose-binding protein protected mice against *Onchocerca lienalis* reducing the number of skin microfilariae by 48-62% (Taylor *et al.*, 1996). Finally, a recent study has demonstrated the immunogenicity of a 41 kDa tropomyosin homolog of the rodent filarial parasite *Acanthocheilonema viteae*. Vaccinating jirds with native tropomyosin, an *E. coli* recombinant of Av-tropomyosin and cDNA resulted in a reduction in worm burdens of 29-64%, 28-35% and 41-43% respectively (Hartman *et al*., 1997, 2006).

Parasite	Host	Antigen	Reduction in worm burden (%)	Reduction in FEC (%)	Reference
A. vitae	jird	r-Av-tropomyosin	28-35	N.D.	40
		tropomyosin	29-64	N.D.	40
		tropomyosin (cDNA)	41-43	N.D.	40
A. caninum	dog	r-Ac-APR1	18	N.D.	46
		r-Ac-CP2	(significant)	(significant)	70
		r-Ac-GST1	39	32	143
H. contortus	goat	p46 + p52	60	50	53
	sheep	contortin	(significant)	N.D.	83
		H11	75	90	3, 65, 85, 111, 120
		H-gal-GP	61-67	67-95	65, 85, 113
		HcPEP1 + HcPEP2	36	48	118
		MEP1-MEP4	50	45	119
		Oo-12 + Oo-gal-GP	N.D.	81-97	116
		P1	30	69	112
		r-Hc-hmcp1/4/6	29	27	96
		tropomyosin (L3)	43-51	N.D.	15
		TSBP fraction	43-77	47-54	58, 62
		TSBP fraction (CPs)	33-46	28-56	62, 95
H. placei	cow	intestinal extract	(significant)	(significant)	105
N. americanus	hamster	r-Ac-GST1	50-54	N.D.	137
O. lienalis	mouse	r-Ov-tropomyosin	48-62	N.D.	121
O. volvulus	mouse	tropomyosin (cDNA)	20	N.D.	37
O. ostertagi	cow	Oo-12 + Oo-gal-GP	N.D.	30-50	116
T. colubriformis	sheep	tropomyosin (L3)	54	N.D.	87

Table 1.2 Overview of successful vaccination trials against parasitic nematodes based on internal antigens.

1.3.2 External antigens

External antigens come in direct contact with the host immune system and hereby represent a major antigenic and functional challenge to the host. They can be present on the surface of the parasite, be released by specialized secretory glands or be by-products of parasite digestion. External antigens fulfill an important role during parasitism with regard to tissue penetration, digestion of host tissue for nutrition and evasion of the host immune response (Tort *et al.*, 1999; Dzik, 2006).

1.3.2.1 Surface antigens

The surface of parasitic nematodes is an important site of host-parasite interaction, making it an interesting target for a vaccine. On the outside the epidermis is covered by a cuticle which is a multilayered extracellular matrix mainly consisting of collagens. A lipid-rich membrane-like layer called the epicuticle covers the cuticle and is known to undergo biophysical changes upon interacting with the host (Proudfoot *et al.*, 1990). Finally, the glycocalyx is a carbohydrate-rich surface coat loosely associated with the epicuticle and implicated in immune evasion (Maizels *et al.*, 1993).

The first protective surface antigen to be identified in ruminant parasites was an *Haemonchus contortus* glycoprotein, called **Hc-sL3** (Ashman *et al.*, 1995). This antigen is specifically expressed by L3s and vaccination reduces FEC in sheep with 52-69% (Jacobs *et al.*, 1999).

Harrison *et al.* (2003a) described an immunodominant carbohydrate antigen of *Trichostrongylus colubriformis* (Tc35CarLA) to be present on the surface of L3s. This high MW complex is highly resistant to digestion with a range of proteases and possibly plays a role in protecting larvae during transit through the hostile environment of the host (Harrison *et al.*, 2003b). It is currently being investigated as a potential vaccine candidate.

For *Ancylostoma caninum*, an immunodominant surface antigen (**Ac16**) was identified in the epicuticle and the basal layer of the cuticle of the juvenile and adult stages (Fujiwara *et al.*, 2007). Dogs vaccinated with an *E. coli* recombinant of Ac16 showed a 64% reduction in FEC and a significant decrease in anemia caused by the parasite. Furthermore, Ac16 homologs of *Ascaris suum* (As14), *Brugia malayi* (Bm-SXP-1) and *Onchocerca volvulus* (Ov17 / P36991) have been shown to induce protection in model organisms like mice and gerbils (Wang *et al.*, 1997; Tsuji *et al.*, 2001; Lustigman *et al.*, 2003).

Prior studies based on the immunization of jirds with irradiated *B. malayi* larvae have demonstrated the immunogenicity of nematode **paramyosin** located at the parasite surface (Li *et al.*, 1991). Moreover, jirds immunized with an *E. coli* fusion protein of Bm-paramyosin

showed a 43% reduction in worm burden and a significant reduction in worm length (Li *et al.*, 1993). Recently, paramyosin was identified as an abundant surface antigen of *Trichinella spiralis* larvae (Yang *et al.*, 2008). BALB/c mice vaccinated with an *E. coli* recombinant of Ts-paramyosin showed a 36% reduction in muscle larvae burden.

A final group of surface antigens that have been related with protection are **cuticular globins**. These globins are suggested to be involved in oxygen transport from the host environment to the parasite's tissue (Blaxter, 1993). An 18 kDa globin-like antigen from *T. colubriformis* induced 60-84% protection in guinea pigs (Frenkel *et al.*, 1992). In correspondence, an *Ostertagia* globin was purified from *O. ostertagi* worms by liquid chromatography (LC) (de Graaf *et al.*, 1996). This antigen induced significant protection against an experimental infection in cattle, reducing FEC by 52% and worm burden by 28%. Unfortunately, no consistent protection was observed in cattle exposed to natural infections on pasture due to great variations in FEC and worm burden reductions (Claerebout *et al.*, 2005).

1.3.2.2 Excretory-secretory antigens

Initial experiments were focused on testing the protective capacity of whole excretory-secretory (ES) material of juvenile and adult worms. ES material is a collection of antigens which are actively or passively released by the parasite during host infection. These products can be obtained by cultivating parasites *in vitro*. However, the success of this approach was limited. Some degree of protection was observed for *Haemonchus contortus* in sheep (Schallig and Van Leeuwen, 1997) and *Ostertagia radiatum* in calves (Gasbarre and Douvres, 1987). Vaccination trials in rodents have also confirmed the protective capacity of whole ES material of *Ascaris suum* (Matoff and Tersijski, 1968), *Trichostrongylus colubriformis* (Rothwell and Love, 1974), *Nippostrongylus brasiliensis* (Poulain *et al.*, 1976) and *Trichinella spiralis* (Robinson *et al.*, 1994). In contrast, ES material from *O. ostertagi* larvae failed to protect calves against infection (Herlich and Douvres, 1979; Hilderson *et al.*, 1995).

In more recent years, intensive efforts have been made to define the composition of the ES material from different parasites (e.g. Britton *et al.*, 1993; Knox, 2000; Yatsuda *et al.*, 2003; Vercauteren *et al.*, 2003; Bakker *et al.*, 2004; Matthews *et al.*, 2004; Craig *et al.*, 2006). This allowed the selection of single ES proteins with potential protective capacity.

The first group of ES antigens that have been targeted for vaccination are the **activation-associated secreted proteins** (ASPs). Early studies demonstrated the importance of secreted larval antigens for inducing a protective immune response against the dog parasite *Ancylostoma caninum* (Otto, 1940). The two most abundantly secreted antigens were

identified as ASP1 and ASP2 making them interesting targets for vaccination (Hotez *et al.*, 1996). As a result, the protective capacity of both native and recombinant nematode ASPs has been studied in several vaccination trials against different parasitic helminths. The first ASP to be natively evaluated as a vaccine was an *H. contortus* homolog of Ac-ASP2 called Hc24. This 24 kDa antigen was identified in a low MW antigen fraction obtained from ES material of adult worms. Four independent vaccination trials in sheep showed a reduction of more than 70% in worm burden and more than 60% in mean FEC (Schallig and Van Leeuwen, 1997; Schallig *et al.*, 1997; Kooyman *et al.*, 2000; Vervelde *et al.*, 2002). A positive correlation was detected between vaccine effectiveness and age (Kooyman *et al.*, 2000). Nevertheless, this fraction also contained an immunogenic 15 kDa antigen and it remains unclear whether Hc24 on its own is capable of inducing protection. Vaccination trials based on different vaccine batches of *E. coli* recombinant Hc24 and 15kDa protein (rec15/24) rendered variable levels of protection indicating a problem with reproducibility (Vervelde *et al.*, 2002). Interestingly, IgG1 antibody levels against rec15/24 were significantly higher when protection was obtained. It is also of note that cross-reactivity between antibodies specific for native and recombinant proteins was low and three-month old lambs were not protected after immunization. For *O. ostertagi*, ASP1 and ASP2 were found to be the most abundant components of a protective thiol binding excretory-secretory antigen (ES-thiol) fraction which induced a 56-60% reduction in FEC (Geldhof *et al.*, 2002, 2003, 2004). In addition, vaccination trials against *A. caninum* have shown that immunizing mice with an *E. coli* recombinant form of *A. caninum* ASP1 reduced lung worm burdens with 51-79% and recombinant homologs of *Ancylostoma duodenale* and *Necator americanus* were able to induce limited cross-protection (Ghosh *et al.*, 1996; Ghosh and Hotez, 1999; Sen *et al.*, 2000). In this case, the degree of protection was correlated with the percentage of amino acid identity with Ac-ASP1 (Sen *et al.*, 2000). While the recombinantly expressed Ac-ASP2 did not elicit protection in mice, an insect cell expressed Ac-ASP2 was able to induce a significant reduction in FEC and worm burden in dogs, the parasite's natural host (Bethony *et al.*, 2005). Recently, a yeast recombinant of *N. americanus* ASP2 has been shown to protect hamsters against challenge infection resulting in a 30-46% reduction in worm burdens (Xiao *et al.*, 2008).

Parasites release a wide array of **enzymes**, such as serine, aspartic, metallo- (MEPs) and cysteine proteases (CPs). Vaccination trials with ES fractions enriched for CPs have also rendered protection against *H. contortus* in sheep with a 52% reduction in FEC and a 50% reduction in worm burden (Bakker *et al.*, 2004) and against *O. ostertagi* in cattle with a 56-60% reduction in FEC (Geldhof *et al.*, 2002, 2004). Also, an *E. coli* recombinant of *N.*

americanus CP2 was capable of reducing worm burden in hamsters with 29% (Xiao *et al.*, 2008). For *A. caninum*, an astacin-like MEP secreted by L3s was recombinantly expressed in *E. coli* and used to immunize dogs. The immunization induced a humoral antibody response that was associated with the exodus of adult *A. caninum* hook worms from their normal habitat into the small intestine (Hotez *et al.*, 2003).

Parasites have also been known to secrete a variety of **inhibitors** capable of inhibiting proteases of the host and the parasite itself (Dzik, 2006; Knox, 2007). An adult-specific secreted tissue inhibitor of MEPs (Ac-TMP) (Zhan *et al.*, 2002) and a factor Xa serine protease inhibitor anticoagulant (Ac-AP) (Capello *et al.*, 1993, 1995) have been identified in ES material of adult *A. caninum* worms. A vaccination trial in dogs using *E. coli* recombinants of Ac-TMP or Ac-AP resulted in a modest reduction in worm burden in the small intestine (Hotez *et al.*, 2002). In addition, a migratory shift of hookworms from the small intestine to the colon was observed in vaccinated animals. For *Ancylostoma ceylanicum*, a Kunitz type serine protease inhibitor (AceKI1) was identified in adult ES material (Milstone *et al.*, 2000). AceKI1 expressed in *E. coli* successfully inhibited serine proteases, pancreatic elastase, neutrophil elastase, chymotrypsin and trypsin. A vaccine study in hamsters revealed partial protection against parasite-induced growth delay, although no effect on anemia was observed (Chu *et al.*, 2004).

Several ES antigens have been described to have **immunomodulating capabilities** and can therefore be considered as valuable vaccine candidates. Galectins are a family of lectins that are present in vertebrates and invertebrates and are characterized by a specific affinity for β-galactoside sugar structures (Kasai and Hirabayashi, 1996). Their function is not fully understood, although galectins are believed to be important mediators of allergic inflammatory responses induced by helminth parasites (Young and Meeusen, 2004). So far, two galectins of *H. contortus* - i.e. Hco-gal-m and Hco-gal-f - have been recombinantly expressed and used to vaccinate 9-10 month old goats (Yanming *et al.*, 2007). Animals injected with Hco-gal-m/f and Hco-gal-f showed a 41-46% reduction in worm burden and a 37-48% reduction in FEC. In addition, a preliminary survey of in vitro ES material of *Teladorsagia circumcincta* fourth stage larvae (L4s) revealed the presence of two different galectins (Craig *et al.*, 2006). A second example of an ES antigen with immunomodulating capacities is calreticulin, which was originally identified as a major Ca^{2+}-binding protein present in the endoplasmatic reticulum (Michalak *et al.*, 1992). This well-conserved protein has been detected in humans, plants, insects and parasites, and is involved in protein-protein interactions, metal binding, ribonucleic acid (RNA) binding and autoantibody binding (reviewed by Nakhasi *et al.*, 1998). A study of the human hookworm *N. americanus* showed

calreticulin to be an important parasite allergen with an immunomodulatory function (Pritchard *et al.*, 1999). A subsequent vaccination trial in mice using an *E. coli* recombinant of Na-calreticulin reduced lung worm burdens with 43-49% (Winter *et al.*, 2005). A third example of a protective immunomodulating ES antigen is a neutrophil inhibitory factor (NIF) found in *A. ceylanicum*. This factor was expressed in yeast and used to vaccinate hamsters resulting in an 86% reduction in FEC (Ali *et al.*, 2001).

To conclude, additional ES antigens with **unknown function** have been shown to induce protection against nematode infection. Using size-exclusion chromatography, a protective low MW ES fraction was obtained for *H. contortus* which rendered a 70% reduction in FEC and a 60-80% reduction in worm burden (Schallig *et al.*, 1997). For *O. ostertagi*, a low MW fraction called *Ostertagia* polyprotein allergen (OPA) fraction was obtained from ES material of L3s. Vaccinating calves with this fraction resulted in a 60% reduction in FEC (Vercauteren *et al.*, 2004). For *T. colubriformis*, immunization with adult ES antigens of 11, 17 and 30 kDa gave up to 50% protection in guinea pigs (Savin *et al.*, 1990; Dopheide *et al.*, 1990, 1991). Moreover, an *E. coli* recombinant of the 17 kDa ES protein induced 8-39% reduction in FEC and 40-42% reduction worm burden in neonatal lambs (Emery *et al.*, 1999). A recombinant 11 kDa ES protein of *A. ceylanicum* (AceES2) significantly reduced anemia in challenged hamsters (Bungiro *et al.*, 2004). Finally, a 16kDa antigen commonly expressed in human and pig *Ascaris* infections was detected in parasite ES material (Tsuji *et al.*, 2003). Vaccinating mice with an *E. coli* recombinant of this As16 protein resulted in a 58% reduction in lung worm burden.

Parasite	Host	Antigen	Reduction in worm burden (%)	Reduction in FEC (%)	Reference
A. caninum	dog	r-Ac16	N.D.	64	26
		r-Ac-AP	(significant)	N.D.	46
		r-Ac-ASP2	(significant)	(significant)	6
		r-Ac-MTP1	N.D.	N.D.	47
		r-Ac-TMP	(significant)	N.D.	46
	mouse	r-Ac-ASP1	51-79	N.D.	33, 34, 102
		r-Ad-ASP1	28	N.D.	102
		r-Na-ASP1	62	N.D.	102
A. ceylanicum	hamster	r-AceES2	(not significant)	N.D.	9
		r-Ac-AceKI1	N.D.	N.D.	13
		r-NIF	(not significant)	86	1
A. suum	mouse	adult ES	(significant)	N.D.	76
		r-As14	64	N.D.	124
		r-As16	58	N.D.	125
B. malayi	jird	r-Bm-paramyosin	43	N.D.	67
		SXP-1	(significant)	N.D.	134
H. contortus	goat	r-Hco-gal-m/f	41-46	37-48	139
	sheep	adult ES	64	32	100
		Hc24 + 15kDa	>70	60-80	64, 100, 101, 132
		Hc-sL3	52-69	N.D.	48
		TSBP fraction	50	52	5
N. americanus	hamster	r-Na-ASP2	30-46	N.D.	137
		r-Na-CP2	29	N.D.	137
	mouse	r-Na-calreticulin	43-49	N.D.	136
N. brasiliensis	rat	adult ES	(significant)	N.D.	91
O. radiatum	cow	larval ES	23	N.D.	27
O. volvulus	mouse	r-Ov17	(significant)	N.D.	70
O. ostertagi	cow	ES-thiol	0-18	56-60	29, 31
		globin	28	52	14
		OPA	(not significant)	60	131
T. spiralis	mouse	adult ES	(significant)	N.D.	97
		r-Ts-paramyosin	36	N.D.	138
T. colubriformis	guinea pig	11 kDa ES protein	(significant)	N.D.	20
		17 kDa ES protein	(significant)	N.D.	19
		18 kDa globin	60-84	N.D.	25
		30 kDa glycoprotein	(significant)	N.D.	99
		adult ES	(significant)	N.D.	98
	sheep	r-17 kDa ES protein	8-39	40-42	24

Table 1.3 Overview of successful vaccination trials against parasitic nematodes based on external antigens.

1.4 Strategy for developing anti-nematode vaccines

Decades of study in the field of anti-nematode vaccines have rendered a collection of parasite antigens which are capable of adequately protecting the host organism against infection. Interestingly, successful vaccination trials described in this chapter are mainly based on native protein fractions. Unfortunately, these native vaccines have several important disadvantages when it comes to commercialization. First of all, purifying sufficient amounts of nematode proteins is often an expensive and/or time-consuming task. Second, antigen preparations can show significant batch differences. Third, it is not always possible to obtain a commercially stable formulation of the vaccine. Moreover, while these native fractions are enriched for a specific internal or external parasite antigen, they usually remain rather crude protein mixtures containing additional unidentified antigens. Hence, it is rather difficult to attribute the induced protection solely to a single antigen and as a result the exact mechanism of protection remains unknown.

Hence, vaccine development will depend on obtaining an immunologically active recombinant form of the parasite antigen. The advances made in recombinant technology have made it relatively easy to produce vaccine candidates in different expressions systems. As a result, antigens are no longer isolated and tested in their native form but rather selected based on antibody recognition, localization or presumed function, followed by injection as a recombinant protein. However, while some recombinant antigens described here were able to protect the host against infection, most published vaccination trials were unsuccessful (excluded from our overview, reviewed by Geldhof *et al.*, 2007). This could either be explained by the fact that these proteins are not genuine protective antigens or that the recombinant version of the protective antigen differs too much from its native counterpart. These structural differences can include incorrect protein folding and post translational modifications such as glycan structures.

From these results it is clear that a direct recombinant approach is not efficient when searching for protective antigens. An extensive study of the original native parasite antigens would therefore be more appropriate. However, the main bottleneck in this approach will be obtaining sufficient amounts of parasite material. While this is a major problem in the field of human parasitology, livestock nematodes are more accessible for large-scale collection. Hence, more effort should be put into obtaining highly pure parasite antigens followed by a thorough analysis of the vaccine candidate in its native form, hereby taking into account the immunogenic importance of protein epitopes, general protein conformation, possibility of polymerization and the presence of immunogenic glycan structures. For this reason, the

following study will focus on further purifying and characterizing the protective native *O. ostertagi* fraction called ES-thiol (Geldhof *et al.*, 2002, 2004).

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Objectives

Objectives

The overall objective of this study was to evaluate the antigens present in the protective ES-thiol fraction of the parasitic cattle nematode *Ostertagia ostertagi* as putative vaccine candidates.

The four sub-objectives were:

-to further purify the native ES-thiol fraction to determine which one of the two most promising vaccine candidates, i.e. activation-associated secreted proteins (ASPs) and cysteine proteases (CPs) are responsible for the induced protection

-to assess the importance of protein conformation and glycan structures of the native ASPs for inducing protection

-to screen for new antigens present in the protective ES-thiol fraction

-to further characterize one of the identified antigens, i.e. the translationally controlled tumor protein (TCTP) and evaluate its potential as a vaccine or drug target

Chapter 2

Vaccination against *Ostertagia ostertagi* with subfractions of the protective ES-thiol fraction

Based on: Meyvis, Y., Geldhof, P., Gevaert, K., Timmerman, E., Vercruysse, J., Claerebout, E. (2007). Vaccination against *Ostertagia ostertagi* with subfractions of the protective ES-thiol fraction. Vet Parasitol 149, 239-245.

2.1 Introduction

Previous vaccination trials have shown that immunizing calves with excretory-secretoy (ES) material from adult *Ostertagia ostertagi* worms affinity purified on a thiol-sepharose column (i.e. ES-thiol) results in a 56-60% reduction in cumulative faecal egg counts (FEC) during two months after a first infection with third stage larvae (L3s). This reduction was accompanied by a significant reduction in worm length (Geldhof *et al.*, 2002, 2004).

The most abundant proteins in the ES-thiol fraction have been identified as activation-associated secreted proteins (ASPs) (Geldhof *et al.*, 2003). ASPs belong to a group of evolutionary related secreted proteins called the SCP/tpx-1/Ag5/PR-1/Sc7 family and are present in different organisms such as plants, mammals and nematodes. The exact function of ASPs is still unclear, although it is believed that they play a role in parasite infection and transition to parasitism (Hawdon *et al.*, 1996, 1999; Bin *et al.*, 1999, 2003; Moser *et al.*, 2005). A recent study in *O. ostertagi* demonstrated some ASP genes to be male enriched which might imply a role in reproduction (Visser *et al.*, 2008). ASP molecules have previously shown their protective capacity in vaccination trials against *Ancylostoma caninum* (Ghosh *et al.*, 1996, Ghosh and Hotez, 1999; Sen *et al.*, 2000), *Ancylostoma ceylanicum* (Goud *et al.*, 2004; Mendez *et al.*, 2005), *Onchocerca volvulus* (MacDonald *et al.*, 2005) and *Haemonchus contortus* (Schallig *et al.*, 1997a, 1997b; Vervelde *et al.*, 2002). Two ASP molecules of *O. ostertagi* have been described in ES-thiol, i.e. Oo-ASP1 and Oo-ASP2, with a molecular weight (MW) of around 30 kDa (Geldhof *et al.*, 2003).

Besides the ASPs, ES-thiol contains multiple cysteine proteases (CPs) with cathepsin L-like activity (Geldhof *et al.*, 2002). The function of these enzymes is unknown, but they might enable the parasite to destroy host tissue for feeding and penetration, and to evade the host immune system (Tort *et al.*, 1999; Dzik, 2006). The protective capacity of CPs has already been demonstrated against *Fasciola hepatica* (Wijffels *et al.*, 1994; Dalton *et al.*, 1996) and *H. contortus* (Boisvenue *et al.*, 1992; Knox *et al.*, 1999, 2005; Redmond and Knox, 2004).

At the moment it is still unclear which of these ES-thiol components are essential for rendering protection against an *O. ostertagi* challenge infection, i.e. the ASPs, the CPs or other so far unidentified proteins present in ES-thiol. Therefore, the objective of this chapter is to further fractionate ES-thiol in its different components and to determine their protective capacity separately.

2.2 Materials and methods

2.2.1 Antigen collection and fractionation

The protective ES-thiol fraction was purified as described previously (Geldhof *et al.*, 2002, 2004). A naïve Holstein calf (3-4 months old) was infected with 200,000 *Ostertagia* L3s. At day 21 post-infection, the animal was sacrificed and adult worms were collected from the abomasum and maintained in RPMI medium (Gibco) supplemented with gentamycin (0.2 mg/ml), penicillin (1,000 U/ml), streptomycin (1 mg/ml), L-glutamine (10 mM) and amphotericine B (Fungizone, Bristol-Myers Squibb) (5 µg/ml) for 72h at 37°C and 5% CO_2. Parasite viability was confirmed after 24h and 48h on the basis of structural integrity and motility. Culture supernatants were collected and passed through a 0.22 µm filter followed by dialysis against PBS at 4°C and a final concentration step on a Centriprep Ultra-15 membrane (Millipore; 10,000 MWCO). This material is referred to as ES. Prior to affinity chromatography, ES was pre-incubated with a final concentration of 2.5 mM dithiothreitol (DTT) for 30 min at 37°C. An activated Thiol-Sepharose 4B column (Sigma) with 5 ml bed volume, was equilibrated in 10 mM Tris-HCl, 0.5 M NaCl, pH 7.4. Protein samples (10 mg/run) were applied to the Thiol-Sepharose 4B column at a flow rate of 5 ml/hour. Unbound material was eluted by washing the column with equilibration buffer (10 mM Tris-HCl, 0.5 M NaCl, pH 7.4) until the OD_{280} had returned to a steady baseline. Bound material was eluted with equilibration buffer containing 50 mM DTT at a flow rate of 0.5 ml/min. The peak fractions were pooled. Next, DTT was removed from the eluted proteins by passage through a Sephadex G-25 column (GE Healthcare) at 5 ml/min in 10 mM Tris-HCl, pH 7.4. The peak fractions were again pooled and protein content was determined using the BCA method (Pierce Chemical Co., Rockford, IL, USA). The obtained protein fraction is referred to as ES-thiol.

A Q-Sepharose column (Amersham, 1 ml bed volume) was equilibrated in 10 mM Tris-HCl, pH 7.4. ES-thiol was applied to the column (1 ml/min). Unbound proteins were collected and bound proteins were eluted by a stepwise increase in NaCl concentration (25-50-75-100-125-175-250-500-1000 mM). Aliquots of all samples were analyzed on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing/reducing conditions followed by silver staining. The presence of ASPs was demonstrated by running the samples on a 10% SDS-PAGE under denaturing/reducing conditions followed by blotting onto a polyvinylidene difluoride (PVDF) membrane (Immobilon®, Milipore). The blot was blocked for 2h in 10% horse serum (HS) in phosphate buffered saline with 0.05% Tween20 (PBST), probed overnight with rabbit anti-rASP serum obtained by immunization with an *Escherichia coli* recombinant of Oo-ASP-1 (diluted 1/200 in 5% HS / PBST / Visser *et al.*, 2008)

and incubated for 2h with conjugate (goat anti-rabbit horse raddish peroxidase (HRPO), Sigma; diluted 1/5,000 in 5% HS / PBST). ASPs were visualized by adding 0.05% 3,3 diaminobenzidine tetrachloride in phosphate buffered saline (PBS) containing 0.01% H_2O_2 (v/v). CPs were monitored by incubating the samples with synthetic carbobenzoxy-phenylalanyl-arginyl 7-amino-4-methyl coumarin (Z-Phe-Arg-AMC) as was done in a previously described cathepsin assay (Geldhof *et al.*, 2000). Finally, the material was pooled into 3 different subfractions, i.e. an ASP fraction, a CP fraction and a rest fraction, followed by a concentration step on an Amicon Ultra-15 membrane (Millipore; 10,000 MWCO). Protein concentrations were determined using the BCA method (Pierce Chemical Co., Rockford, IL, USA). Ten μg of ES-thiol and its subfractions was analyzed by Coomassie Blue staining under denaturing and reducing conditions. The presence of ASPs was verified using the same Western blot as described above. CP activity was detected by gelatin-substrate gel under non-reducing conditions at pH 5.0 in the presence of 5 mM DTT. (Geldhof *et al.*, 2000).

2.2.2 Vaccination trial

A vaccination trial was designed as previously described (Geldhof *et al.*, 2002, 2004). A Holstein cross-breed population of 35 female helminth-free calves (8 months of age) was randomly divided into 5 groups of 7 animals. All animals were immunized three times intramuscularly in the neck with a three-week interval. One group received 100 μg of ES-thiol per immunization in combination with 750 μg of QuilA adjuvant. One group received the same amount of QuilA with Tris-buffer instead of antigen (i. e. the negative control group). Three remaining groups were immunized with the ASP, the CP and the rest fraction respectively. The quantity of each subfraction injected was equivalent to the respective amount received by the group injected with ES-thiol. Serum was taken from each animal before the first immunization and one week after the second immunization. The animals were challenged with a trickle infection of 25,000 infectious L3s (1,000 L3s/day; 5 days/week) which started at the day of the third immunization. All calves were euthanized three weeks after the last infection. Parasitological parameters - i.e. FEC, worm counts, worm lengths, percentage of fourth stage larvae (L4s) - were analyzed as described in previous trials (Geldhof *et al.*, 2002, 2004).

2.2.3 Statistical analysis

Statistical analysis was performed as described previously (Geldhof *et al.*, 2002, 2004). Data are shown as geometric means (+range). Indicators of worm fitness (i.e. FEC, worm burden and length of adult worms) were expected to be lower in vaccinated animals. A one-tailed

Mann-Whitney U-test for pairwise comparison was used to determine significance between group means of vaccinated animals and the adjuvant control. Probability (P) values smaller than 0.05 were considered to indicate significant differences. Furthermore, a Kruskal-Wallis test was performed to demonstrate significant differences between group means of vaccinated animals. Based on the Bonferroni correction, P values smaller than 0.01 were considered statistically significant.

2.2.4 Antibody response

The antibody responses to the different antigen preparations were tested using pooled sera from the different groups collected one week after the second immunization. Ten μg of ES-thiol and its subfractions was separated on a 10% SDS-PAGE under non-denaturing conditions and blotted onto a PVDF membrane (Immobilon®, Milipore). The blot was blocked overnight in 10% HS (diluted in PBST), probed for 2 h with pooled bovine serum (diluted 1/500 in 5% HS / PBST), and incubated for 1h with conjugate (rabbit anti-bovine-HRPO, Sigma; diluted 1/5,000 in 5% HS / PBST). Recognized proteins were visualized by adding 0.05% 3,3'-diaminobenzidine tetrachloride in PBS containing 0.01% H_2O_2 (v/v).

2.3 Results

2.3.1 Antigen collection and fractionation

The protein composition of ES-thiol and its three subfractions on a Coomassie-stained gel is shown in Figure 2.1. The profile of ES-thiol was identical to previously published results (Geldhof *et al.*, 2002, 2004). The ASP fraction (eluted at 0-25-50 mM NaCl) contained only one visible band at 30 kDa. Two major protein bands were detected in the CP fraction (eluted at 250-500-1000 mM NaCl) at 28 and 45 kDa. The rest fraction (eluted at 100-125-175 mM NaCl) showed multiple protein bands over the entire length of the gel, including a more abundant band at 48 kDa.

The Western blot analysis with antibodies against ASPs is shown in Figure 2.2. The 30 kDa band seen in the ASP fraction was confirmed to be ASPs. No ASP proteins could be detected in the CP fraction. Minor recognition was seen in the rest fraction, possibly indicating trace amounts of ASP.

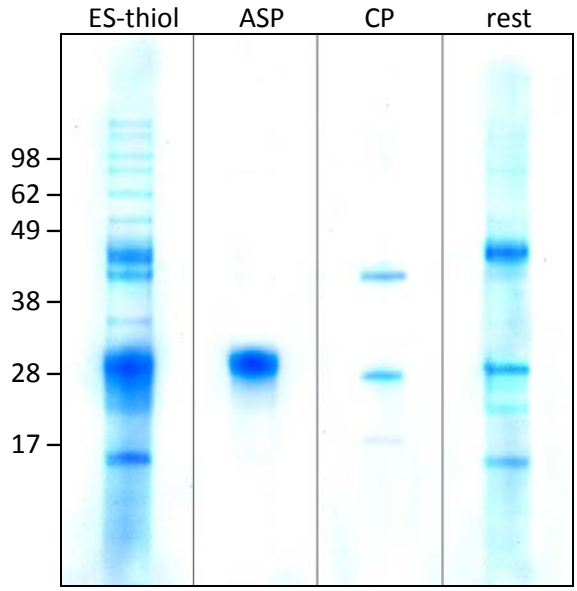

Figure 2.1 Comparison of the protein profiles of ES-thiol, the activation-associated secreted protein fraction (ASP), the cysteine protease fraction (CP) and the rest fraction (rest) on a 10% SDS-PAGE under denaturing/reducing conditions, visualized by Coomassie Blue staining. MWs of standards are presented in kDa.

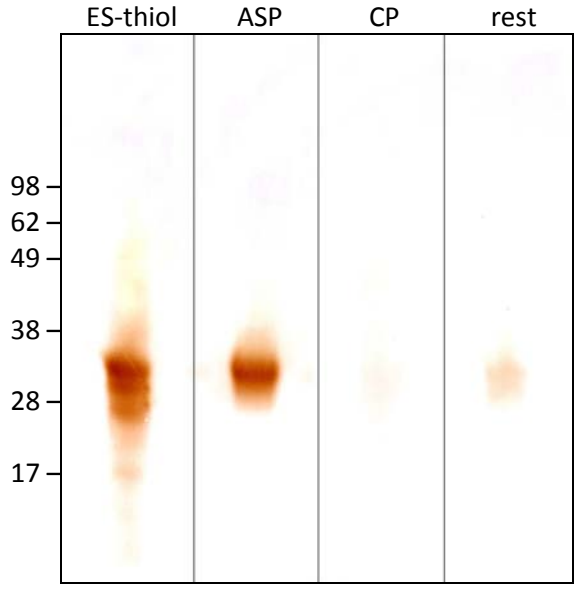

Figure 2.2 Detection of ASPs in ES-thiol, the activation-associated secreted protein fraction (ASP), the cysteine protease fraction (CP) and the rest fraction (rest) on a 10% SDS-PAGE under denaturing/reducing conditions using Western blotting and rabbit anti-rASP antibodies. MWs of standards are presented in kDa.

Figure 2.3 shows the CP activity in ES-thiol and the different subfractions as detected on a gelatine substrate gel before (-) and after (+) addition of L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64), a CP inhibitor. CP activity present in the ES-thiol fraction was highly enriched in the CP fraction. The ASP fraction did not contain any activity, while minor activity was seen in the rest fraction. Addition of an E-64 inhibitor completely abolished protease activity.

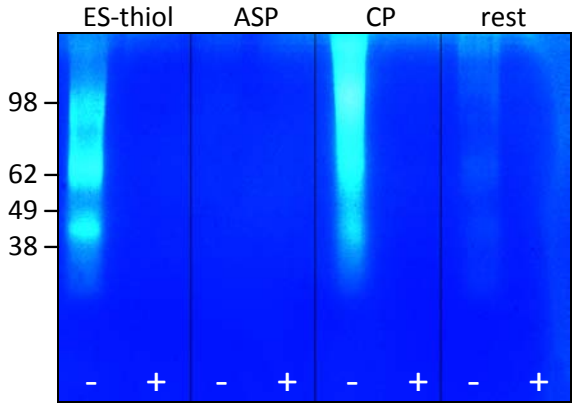

Figure 2.3 Visualization of cysteine protease activity in ES-thiol, the activation-associated secreted protein fraction (ASP), the cysteine protease fraction (CP) and the rest fraction (rest) on a non-reducing gelatin substrate gel incubated at pH 5.0 in the presence of 5 mM DTT (-) and the effect of adding the CP inhibitor E64 (+). MWs of standards are presented in kDa.

2.3.2 Vaccination trial

None of the animals showed adverse reactions to the immunizations and no clinical signs of ostertagiosis were observed. FEC during the vaccination trial are shown in Figure 2.4.

The geometric mean FEC of all vaccinated groups were lower than the control group throughout the experiment. The parasitological parameters are summarized in Table 2.1. Immunization with ES-thiol gave a statistically significant reduction in cumulative FEC of 62%. Groups injected with the ASP, CP and the rest fraction showed a reduction in cumulative FEC of 74%, 80% and 70% respectively. The percentage of L4s was lower than 2% in all groups and no difference could be seen between the different groups (data not shown). Animals immunized with ASPs and CPs respectively contained 47% and 28% fewer adult worms compared to the controls, although these reductions were not significant. No reduction in worm burden was observed in the ES-thiol group and the rest fraction group. Both female and male worms were significantly smaller in all treated groups with the exception of male

worms found in ES-thiol injected animals. No significant difference was seen between the parasitological parameters of the vaccinated groups.

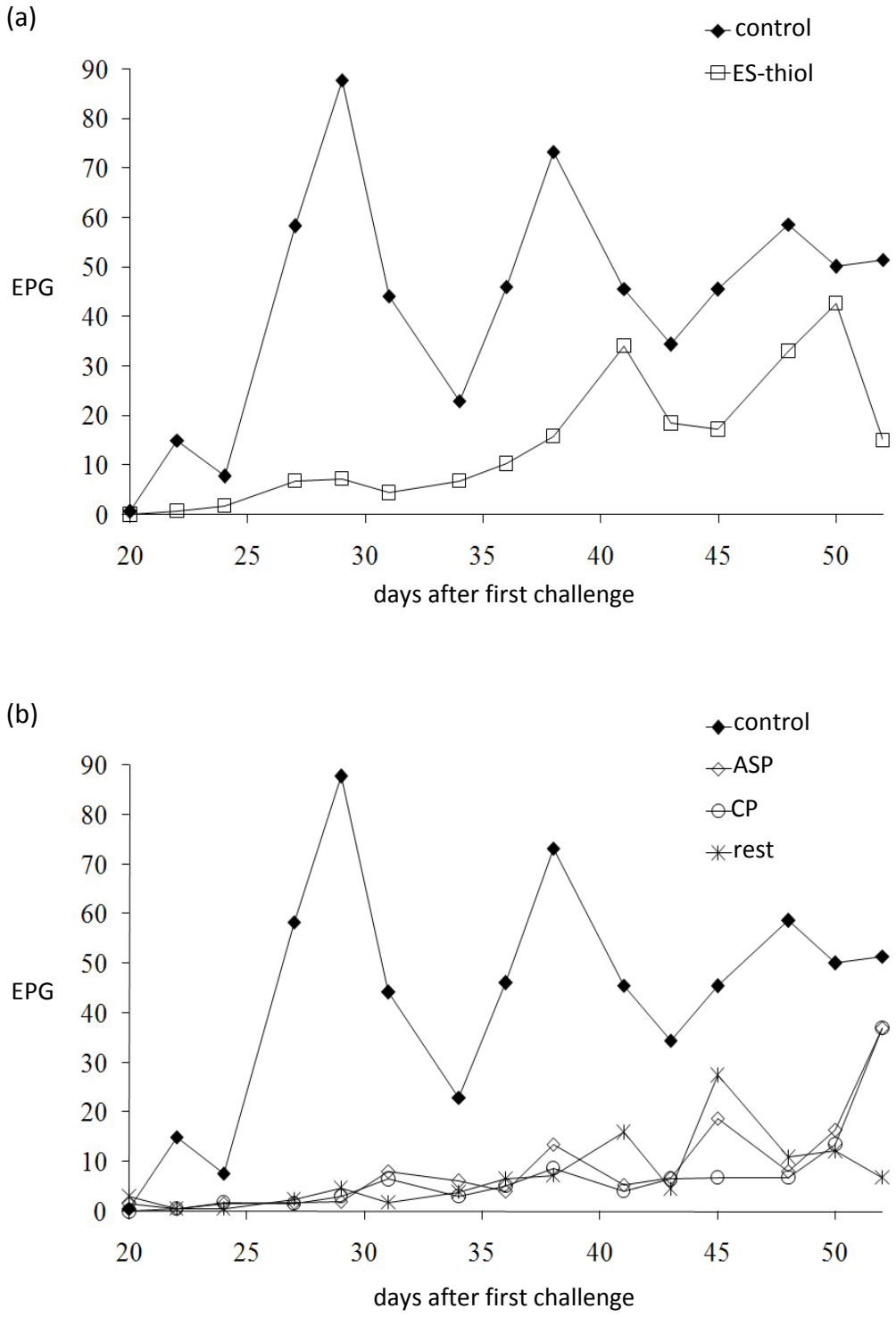

Figure 2.4 Geometric mean of FEC during the two-month period of the vaccination trial. (a) Animals vaccinated with QuilA versus ES-thiol; (b) Groups vaccinated with QuilA versus ASP, CP and rest fraction. (EPG = eggs per gram faeces).

Group	n	Cumul. FEC (EPG)	Reduction (%)	No. of worms	Worm length (mm)	
QuilA	7	2034 (650-4575)	---	2738 (550-8900)	F 9.29 M 7.41	(8.95-9.92) (6,97-7,92)
ES-thiol	7	768 * (63-2650)	62	2891 (1450-5050)	F 8.26 * M 6.65	(6.87-9.11) (5.26-7.71)
ASP	7	538 ** (125-1250)	74	1446 (400-5400)	F 8.22 ** M 6.91 *	(7.65-8.91) (6.15-7.57)
CP	7	407 ** (75-1363)	80	1797 (100-4500)	F 8.14 ** M 6.78 *	(7.33-9.03) (5.87-7.70)
rest	7	618 ** (238-2075)	70	2536 (450-6200)	F 8.24 * M 7.03 **	(7.93-8.58) (6.73-7.50)

Table 2.1 Parasitological parameters of the vaccination trial. Number of animals per group (n), Geometric mean cumulative FEC (eggs per gram faeces, EPG), total no. of worms (geometric mean + range) and worm lengths (geometric mean of 50 worms per animal + range). (F = female worms; M = male worms; significant differences between the control group (QuilA) and the treated groups are indicated with * = $P < 0.05$ and ** = $P < 0.01$).

2.3.3 Antibody response

Figure 2.5 shows antibody recognition of ES-thiol and its subfractions under non-reducing conditions by pooled sera from each group. Animals injected solely with QuilA did not recognize any ES-thiol proteins. The ES-thiol group recognized ES-thiol proteins over the entire length of the gel with one immunodominant band at 120 kDa. Multiple bands were recognized in the subfractions: two bands at 25 and 50 kDa in the ASP fraction, a band at 120 kDa in the CP and the rest fraction, and an additional 250 kDa band in the CP fraction.

Animals immunized with the ASP fraction showed one immunodominant band at 50 kDa and a minor band at 25 kDa in both the ES-thiol and the ASP fraction. These bands were cut from a non-reducing Coomassie-stained gel and both were identified as Oo-ASP1 by mass-spectrometry (results not shown). No proteins were recognized in the CP and rest fraction.

Animals injected with the CP fraction and the rest fraction showed a similar pattern. Two high MW bands around 120 kDa and 250 kDa were recognized in the CP fraction. The same 120 kDa band was detected in the rest fraction. Recognition of the 120 kDa band by the rest fraction group was stronger compared to the CP group. None of the animals recognized proteins from the ASP fraction.

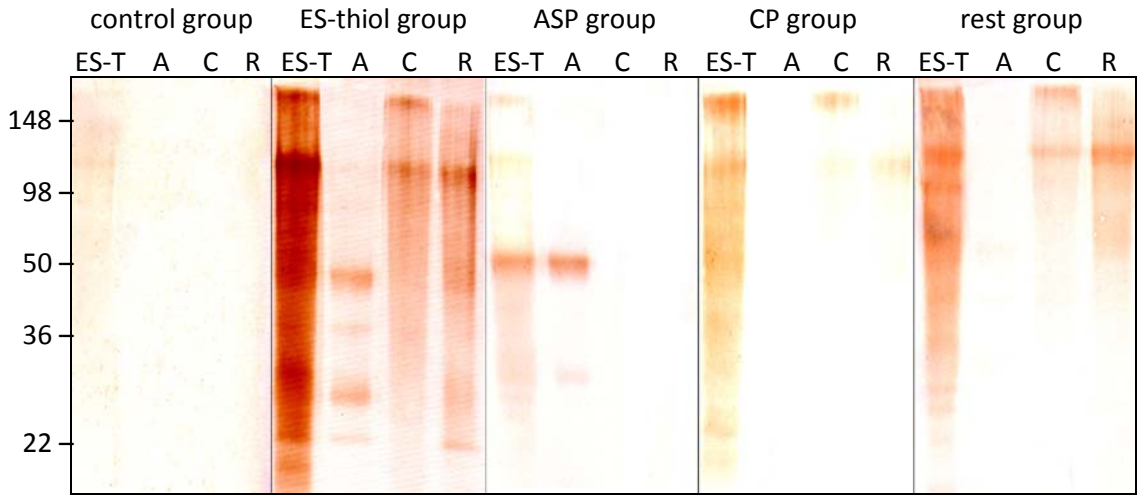

Figure 2.5 Detection of serum antibody responses to vaccinations using Western blotting. Sera from individual animals were taken 1 week after the second immunization and pooled for each group (QuilA, ES-thiol, ASP fraction, CP fraction and rest fraction). Lanes were loaded with equivalent amounts of ES-thiol (ES-T), ASP fraction (A), CP fraction (C) and rest fraction (R) in order to detect cross-reactivity. MWs of standards are presented in kDa.

2.4 Discussion

Published data on successful vaccination trials against *O. ostertagi* are still scarce. Smith *et al.* (2000) previously reported a 30-50% reduction in FEC by vaccination with gut membrane glycoproteins. More recently, Vercauteren *et al.* (2004) reported the protective capacity of the *Ostertagia* polyprotein allergen (OPA). Injection with this L3 ES antigen induced a reduction in FEC of 60% compared to control animals. Finally, the ES-thiol fraction was previously tested in two independent trials, with the main protective effect being a reduction in cumulative FEC of 56-60% (Geldhof *et al.*, 2002, 2004). In the present study injection with ES-thiol resulted in a 62% reduction in cumulative FEC demonstrating the protective capacity of ES-thiol for a third time. Although all animals received a similar dose of infective L3s, success of parasite establishment can vary significantly for each individual (Gasbarre *et al*., 2001). Calves can either be innately immune, acquired immune or immunologically non-responsive to an *Ostertagia* infection which would explain the large ranges in cumulative FEC values.

Analysis of the composition of ES-thiol previously indicated the presence of ASPs and CPs (Geldhof *et al.*, 2002; Geldhof *et al.*, 2003). Both classes of molecules are well known vaccine candidates, which have shown their protective capacity in different host-parasite systems.

Animals injected with the ASP fraction showed a 74 % reduction in cumulative FEC. Analysis of this fraction on gel and Western blot only demonstrated the presence of ASPs. As far as we know, this is the first time native ASPs from any parasite were tested in a vaccine trial in such a pure form. The only other native ASP tested as a vaccine candidate was the 24 kDa single-domain ASP2 homolog (Hc24) from the sheep parasite *Haemonchus contortus* (Schallig *et al.*, 1997a). Adult sheep injected with this protein fraction showed a 77% reduction in mean FEC and a 85% reduction in worm count compared to animals injected with only adjuvant. However, it is unclear if the Hc24 protein itself was actually responsible for the induced protection, since the protein fraction also contained an immunogenic 15 kDa antigen.

The CP fraction conferred an 80% reduction in cumulative FEC, which is the highest level of protection induced against *O. ostertagi* in cattle published to date. Three different bands of CP activity were seen on a gelatine substrate gel, suggesting the presence of at least three different CPs. Protection induced by purified native CPs has also been described in vaccination trials against *H. contortus* (Redmond and Knox, 2004; Knox *et al.*, 2005). Sheep injected with CPs obtained through anion exchange (MonoQ) or affinity chromatography with cystatin (a natural CP inhibitor) showed a reduction of 28-56% in FEC and a reduction of 33-46% in worm burdens. Integral membrane CPs from *O. ostertagi* did not induce protection (Geldhof *et al.*, 2002), although it is not clear whether these CPs are identical to those present in ES-thiol. Furthermore, it is important to note that the CP fraction used in this study also contained other proteins. Hence, it can not be concluded that the *O. ostertagi* CPs alone are responsible for protection. Further purification of this protein fraction is essential. One possibility would be to affinity purify these proteases according to the method described by Redmond and Knox (2004) using the parasite's cystatin as a ligand.

The rest fraction contained the remaining proteins present in ES-thiol and still remained a fairly complex mixture. Except for trace amounts of ASPs and CPs, the other components remain unknown. Protection rendered by this fraction could be attributed to ASPs, CPs or other so far unknown antigens. Further fractionation combined with a complete proteomic analysis will be essential in order to identify these unknown components and to test their protective capacities separately.

Surprisingly, all subfractions induced a similar level of protection with 70-80% reduction in cumulative FEC. This might suggest the presence of common protective antigens in the different subfractions. Although the subfractions were acquired from the anion exchange column under a wide salt gradient, one cannot exclude the possibility that trace amounts of some proteins are present in all fractions. Alternatively, multiple protective antigens could

be present in the different subfractions. For this reason, serum cross-recognition of the different groups was analyzed on Western blot. The animals injected with the ASP fraction did not show any cross-reactivity with other subfractions and only recognized the ASPs. This observation, combined with the protein profile of the ASP fraction, strongly suggests that the ASPs are the protective component of this subfraction. The analysis also indicated the presence of two cross-reacting HMW antigens in the CP and the rest fraction. Unfortunately, these proteins could not be visualized by Coomassie or silver staining. Hence, they could not be isolated for mass spectrometry (MS) analysis and their identity remains unknown. A possible explanation might be that these immunogenic antigens are in fact complex carbohydrate structures. Surprisingly, other proteins present in the CP and the rest fraction were not recognized by vaccinated animals which would suggest limited immunogenicity compared to these HMW antigens.

The results presented in this study strongly suggest that vaccination of calves with native *O. ostertagi* ASPs induces a protective immune response against a homologous challenge infection. This will enable us to compare the protective capacity of future recombinant ASPs to that of its native form. Recombinant ASP molecules have been evaluated for *Ancylostoma caninum* (Ghosh *et al.*, 1996; Ghosh and Hotez, 1999; Sen *et al.*, 2000), *Ancylostoma ceylanicum* (Goud *et al.*, 2004; Mendez *et al.*, 2005), *Onchocerca volvulus* (MacDonald *et al.*, 2005) and *H. contortus* (Vervelde *et al.*, 2002). These recombinants were produced in bacterial systems and the levels of protection varied between 21 and 79 % reduction in parasite burden. Although bacterial recombinants of parasite antigens have been used previously, expression of cysteine-rich antigens often results in the production of inclusion bodies, making it difficult to solubilize the antigen. Another problem might be the incorrect folding of the ASPs, disabling the formation of possible immunogenic epitopes and preventing proper recognition by the host. Switching to eukaryotic expression systems such as yeast, insect or mammalian cells might solve this problem. For example, Ac-ASP1 and Ac-ASP2 were expressed in *Pichia pastoris* to render soluble recombinant antigens at high yield and low cost (Goud *et al.*, 2004). Besides protein conformation, the glycan structures present on the ASPs may also be crucial for protection. Two putative N-glycosylation sites have been described in *O. ostertagi* ASP1 and ASP2 (Geldhof *et al.*, 2003). The glycan structures present on these sites might be important for the recognition by the host immune system or essential in the correct folding or the dimerization of the ASPs. In this trial, animals immunized with the native ASPs recognized two different forms: an immunodominant dimeric form at 50 kDa and a monomer at 25 kDa. The dimerization of ASP molecules might

result in the creation of additional epitopes, explaining its higher immunogenicity on Western blot.

In conclusion, we have obtained three subfractions of ES-thiol which all induced protection against *O. ostertagi*. Since the native ASPs were obtained in a highly pure form and animals injected with this fraction developed a specific immune response against these antigens without demonstrating cross-reactivity with antigens from the other subfractions, we will further investigate the ASPs for vaccine development. In order to help select an appropriate recombinant expression system for future production of *Ostertagia* ASPs, the next chapter will focus on the importance of protein conformation and glycosylations of ASPs with regard to their immunogenicity.

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Chapter 3

Glycosylation and conformation study of *Ostertagia ostertagi* activation-associated secreted proteins (ASPs)

Based on: Meyvis, Y., Callewaert, N., Gevaert, K., Timmerman, E., van Durme, J., Schymkowitz, J., Rousseau, F., Vercruysse, J., Claerebout, E., Geldhof, P. (2008). Hybrid N-glycans on the host protective activation-associated secreted proteins of *Ostertagia ostertagi* and their importance in immunogenicity. Mol Biochem Parasitol 161, 67-71.

3.1 Introduction

In the previous chapter, the ES-thiol fraction was further purified to obtain a subfraction highly enriched for activation-associated secreted proteins (ASPs). This fraction conferred a 74% reduction in faecal egg counts (FEC) when injected intramuscularly in combination with the adjuvant QuilA. This level of protection is sufficient to consider commercialization (Claerebout *et al.*, 2003). However, the further development of these antigens into a commercial vaccine requires their recombinant expression. Recently, the *Ostertagia ostertagi* ASP1 molecule has been recombinantly expressed using a baculovirus system and tested in a vaccination trial (Geldhof *et al*., 2008). However, immunized calves failed to recognize the native ASPs and no protection against a challenge infection was obtained. This lack of cross-reactivity may be due to structural differences between the recombinant ASP1 and its native version.

Sequence analysis of Oo-ASP1 and Oo-ASP2 has revealed the presence of two potential N-glycosylation sites (Geldhof *et al.*, 2003). The possible presence of glycan structures on the ASPs might have a dramatic effect on the immunogenicity of these molecules. The immunomodulatory importance of parasite glycans has already been described in other helminths such as *Schistosoma mansoni* (Okano *et al.*, 2001; Faveeuw *et al.*, 2003; Pearce *et al.*, 2004), *Trichinella spiralis* (Reason *et al.*, 1994), *Dictyocaulus viviparus* (Haslam *et al*., 2000; Kooyman *et al*., 2007) and *Brugia malayi* (Tawill *et al.*, 2004). In addition to glycosylations, the conformation of specific peptide epitopes can be essential to obtain protection against an invading parasite. The importance of the protein backbone for inducing protective immunity has already been demonstrated in vaccination trials against *Haemonchus contortus* (Smith and Smith, 1996; Munn *et al.*, 1997) and *D. viviparus* (Kooyman *et al.*, 2007).

In this chapter, the glycosylations present on the ASP antigens are characterized and the contribution of both glycosylations and protein conformation to antibody recognition by immunized animals will be evaluated.

3.2 Materials and methods

3.2.1 Antigen collection

ES-thiol was collected as described previously (Geldhof *et al.*, 2002). Ten μg was run on a denaturing/reducing 10% SDS-PAGE gel. Glycosylations were detected using the pro-Q Emerald 300 staining kit according to the manufacturer's protocol (Molecular Probes). Protein bands were made visible by subsequent Coomassie Blue staining.

3.2.2 Glycan analysis

Glycan analysis was done as described by Laroy *et al.* (2006). In short, N-glycans present on the ES-thiol antigens were removed by treatment with a recombinant peptide-N-glycosidase F (PNGase F) and fluorescently labelled with 8-amino-1,3,6-pyrenetrisulfonic acid. A subsequent linkage analysis was performed using a series of digestions of the labelled glycans with highly specific exoglycosidases (α-2,3/6/8-sialidase, β-1,4-galactosidase, β-hexosaminidase, α-1,2/3/4/6-fucosidase, α-1,3/4-fucosidase, α-1,2-mannosidase and α-1,2/3/6-mannosidase) followed by electrophoretic analysis on a DNA sequencer to determine the number of sugar residues which had been removed. Based on the resulting electropherograms the structure of the most abundant N-glycan was determined.

3.2.3 Deglycosylation/Denaturation/Reduction of ES-thiol

Aliquots of ES-thiol (20 μg/condition) were subjected to deglycosylation under native and denaturing/reducing conditions. Denatured and reduced ES-thiol was obtained by heating an aliquot at 95°C for 20 min in 0.5% sodium dodecyl sulphate (SDS) and 100 mM dithiothreitol (DTT), followed by the addition of 10% Triton X-100 to a final concentration of 1 %. All samples were diluted in 10 mM Tris-HCl buffer (pH 7.4) to identical volumes and 1 μl (= 100 U) of recombinant PNGase F was added. Deglycosylation was performed overnight at 37°C. Negative controls were diluted using the same buffers as treated samples and incubated without PNGase F. The ES-thiol samples (untreated, native treated with PNGase F and denatured and reduced treated with PNGase F) were subsequently separated on a 10% SDS-PAGE gel. Glycosylations and protein profiles were visualized using the pro-Q Emerald 300 staining kit (Molecular Probes) and subsequent Coomassie Blue staining.

3.2.4 Tandem MS

In order to determine which N-glycan was removed under native and denaturing/reducing conditions, the major ASP bands were cut from the gel and screened for the presence of peptides containing N-glycosylation sites by means of tandem mass spectrometry (MS). The gel pieces were washed with 100 μl of acetonitrile for 15 min and dried in a centrifugal vacuum concentrator. Seventy-five nanograms of sequencing-grade trypsin (Promega) in 10 μl of 50 mM NH_4HCO_3 (pH 8) was added to re-hydrate the protein bands. After 10 min, an additional 90 μl of 50 mM NH_4HCO_3 (pH 8) was added and digestion was carried out for 16 h at 37°C. Resulting peptide mixtures were dried, re-dissolved in 20 μl of 0.1% formic acid in 2/98 (v/v) acetonitrile (Baker)/water (liquid chromatography (LC)-MS grade water, Biosolve) and half of it was applied for automated nano-LC-MS/MS analysis on an Ultimate (Dionex,

Amsterdam, The Netherlands) in-line connected to an Esquire high capacity ion trap (Bruker Daltonics, Bremen, Germany). Peptides were first trapped on a trapping column (PepMap™ C18 column, 0.3 mm I.D. x 5 mm, Dionex) and after back-flushing, loaded on a 75 µm I.D. x 150 mm reverse-phase column (PepMap™ C18, Dionex). Peptides were eluted with a linear solvent gradient over 50 min going to 100% of 0.1% formic acid in acetonitrile/water (7/3, v/v). Using data-dependent acquisition, multiple charged ions with intensities above threshold 300,000 were selected for fragmentation. During MS/MS analysis, a MS/MS fragmentation amplitude of 0.7 V and a scan time of 40 ms were used. MS/MS spectra were converted to Mascot generic files (mgf) using the Automation Engine software (version 3.2, Bruker) and searched using the Mascot database search engine (http://www.matrixscience.com) against an *O. ostertagi* protein sequence database downloaded from the NCBInr protein database website (http://www.protein.sdu.dk/gpmaw/GPMAW/Databases/NCBInr/ncbinr.html). Mascot's parameter settings were as follows: enzyme: trypsin; variable modifications: acetyl (N-term), propionamide (C), deamidation (NQ), oxidation (M), pyro-glu (N-term Q), peptide mass and fragment mass tolerances: ± 0.5 Da, maximum number of missed cleavages: 1 and instrument type: electron spray ionization trap. An additional, parallel "no enzyme" search (i.e. without any restriction on the protease's specificity) was also performed. From both searches, only spectra that exceeded Mascot's threshold score (set at the 95% confidence level) and were ranked first were considered for identification.

3.2.5 3-D modeling of Oo-ASP1 and Oo-ASP2

In an attempt to model the three dimensional (3-D) structure of Oo-ASP1 and Oo-ASP2, a Basic Local Alignment Search Tool (BLAST) similarity search was conducted against the Protein Data Bank (PDB; http://www.wwpdb.org) to find homologous template sequences. Alternatively, the overall fold of Oo-ASP1 and Oo-ASP2 was predicted using the 3D-jury server (http://meta.bioinfo.pl) (Ginalski *et al.*, 2003). In short, the target sequence was threaded through the backbone structures of a collection of template proteins and a goodness of fit score was calculated for each sequence-structure alignment. According to the secondary structure profile, 3D-jury external modeling servers searched for structures in the PDB resembling most this particular structure prediction. The resulting alignments were used to build atomic models in MODELLER (Sali *et al.*, 1995) with the structure of *Necator americanus* ASP2 as a template (Asojo *et al.*, 2005). Although atomic model precision can never be reached using the fold recognition modeling methods described here, the full

models were used to localize the relative positions of the N-glycosylation sites on Oo-ASP1 and Oo-ASP2. Molecular graphics were created with YASARA (http://www.yasara.org).

3.2.6 ELISA

An enzyme-linked immunosorbent assay (ELISA) was set up to investigate antibody recognition of native ASPs by vaccinated animals and to determine whether different antibody subtypes recognize protein and/or glycan epitopes. Serum was obtained from cattle immunized with native ASPs (chapter 2). Blood from all animals (n=7) was collected before the first immunization (= pre-immune) and one week after the second immunization (=immune). Pre-immune and immune sera were pooled and stored at -20°C. ES-thiol samples (native untreated, denatured/reduced untreated, native treated with PNGase F and denatured/reduced treated with PNGase F) were diluted in carbonate coating buffer (0.1 M; pH 9.6) and coated in triplicate onto a 96-well plate (Nunc MaxisorpTM): 150 μl sample per well (5 μg/ml). Afterwards, the plate was incubated for 2h at 37°C with pooled serum from animals injected with native ASPs (200 μl/well; diluted 1/400 in 2% horse serum (HS) / PBST). Pre-immune serum was used as a negative control. Antibodies against bovine immunoglobulin (Ig) G1 (1/40; monoclonal, AbD Serotec), IgG2 (1/100; monoclonal, AbD Serotec), IgM (1/50; polyclonal conjugate, AbD Serotec) and IgA (1/25; polyclonal conjugate, AbD Serotec) were administered (200 μl/well; diluted in 2% BSA / PBST) for 2h at 37°C. Rabbit-anti-mouse horseradish-peroxidase (1/2,000; polyclonal conjugate, Sigma) was used as a conjugate for IgG1 and IgG2. Finally, O-phenylenediamine was added as a substrate (100 μl/well; 0.1% in citrate buffer, pH 5.0 / 0.01% H_2O_2) and the optical density (OD) was measured at 492 nm.

3.3 Results

3.3.1 Antigen collection

Purified ES-thiol was analyzed on a 10% SDS-PAGE. A Coomassie staining yielded one major protein band of 33 kDa (previously identified as Oo-ASP1 and Oo-ASP2) and additional minor bands between 45 and 92 kDa (Figure 3.1a). This corresponds with the results from previous ES-thiol purifications (Geldhof *et al.*, 2002, 2004; chapter 2). An Emerald 300 staining of the same gel revealed a highly intense band of 33 kDa, indicating that particularly the ASPs are glycosylated (Figure 3.1b).

3.3.2 Glycan analysis

The results of the exoglycosidase array sequencing of the released N-glycans are shown in Figure 3.2. Glycan structures are drawn according to the conventions of the Society for Glycobiology. The addition of sialidase and α-1,2-mannosidase did not affect the glycan profile, which allowed to conclude that no sialylated or high-mannose N-glycans are present. In contrast, treatment of the N-glycans with galactosidase, hexosaminidase and fucosidase resulted in distinct pattern changes. Careful interpretation of these data revealed several glycan structures (see ES-thiol panel). The majority of these glycans are of the paucimannosidic and hybrid type. We interpret the fucose residue on all fucosidase-sensitive glycan species to be linked to the protein-proximal GlcNAc residue, as degalactosylation and removal of outer branch GlcNAc residues is not hampered by the presence of the fucose residue. Treatment with α-1,3/4 fucosidase did not remove the residue, whereas treatment with α-1,2/3/4/6-fucosidase did. An α-1,2-linkage can not be formally excluded, but the α-1,6-linkage is the more common one in this position in eukaryotes and the fucosylated glycans run at the exact positions as standards with these structures in this method (not shown, method was calibrated with a large range of standards in our laboratory).

Based on these results it was concluded that more than 90% of all glycans were of the paucimannosidic type and of the hybrid type with a complex α-1,3-arm and an unprocessed α-1,6-arm (Figure 3.3). N-glycans contained an α-1,6-fucose core and differed in build-up at the terminal end of the α-1,3-arm (ending in galactose, N-acetyl glucosamine or mannose). A few percent of the N-glycans were complex biantennary.

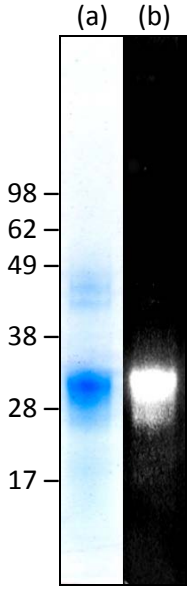

Figure 3.1 Analysis of ES-thiol on 10% SDS-PAGE visualized by Coomassie Blue (a) and Emerald 300 (b). Molecular weights (MWs) of standards are presented in kDa.

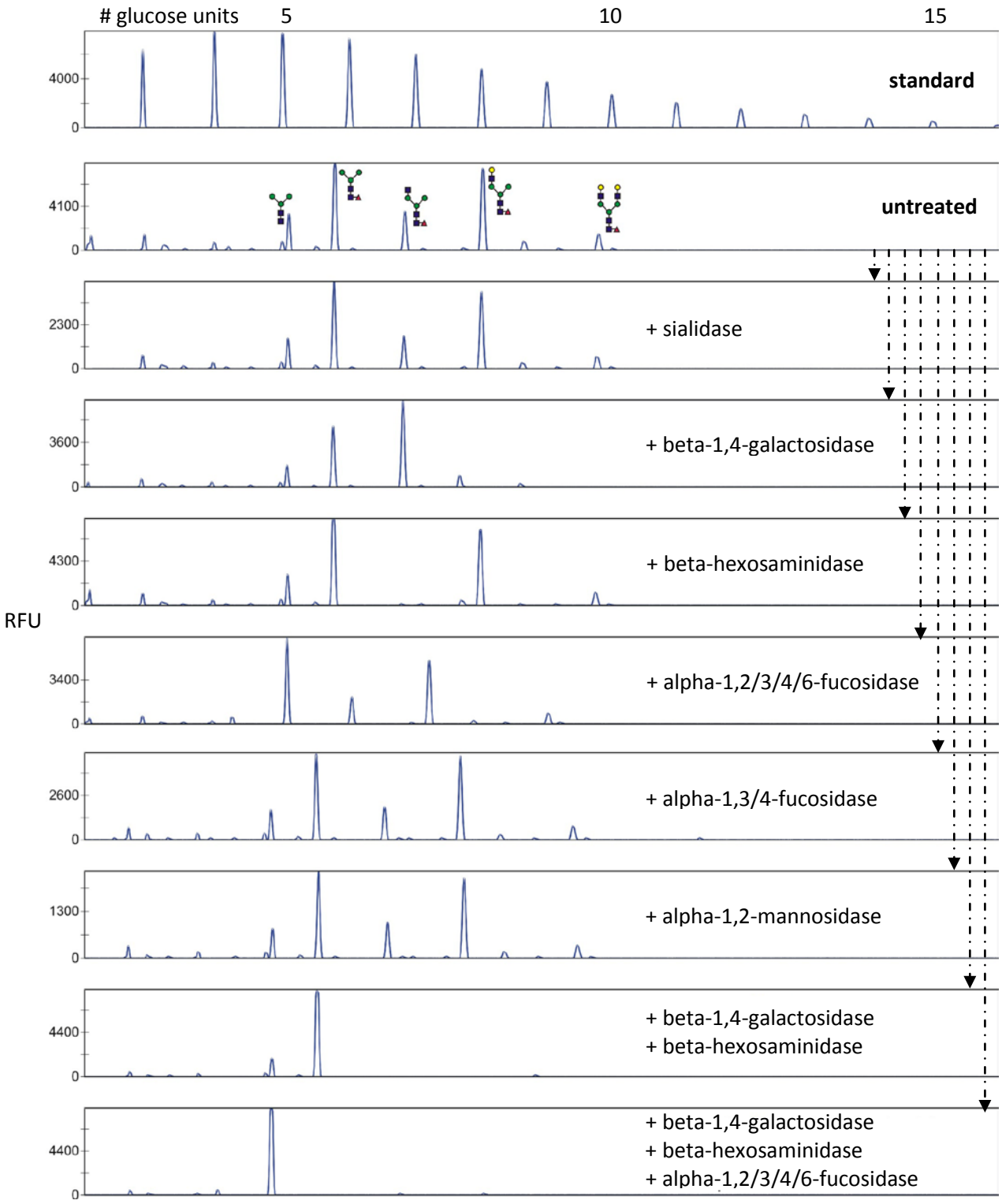

Figure 3.2 Exoglycosidase array sequencing of the N-glycans found on the proteins in the ES-thiol fraction. Spikes represent glycan structures with increasing size (from left to right). First panel = electrophoretic profile of malto-oligosaccharide reference standard. Second panel = profile of unprocessed N-glycans with schematic representation of different structures. Remaining panels = profiles after enzymatic treatment of N-glycans with specific exoglycosidases (indicated in panels). Shifts in the profile indicate the presence of sugar residues targeted by the respective enzymes. (RFU = relative fluorescence unit).

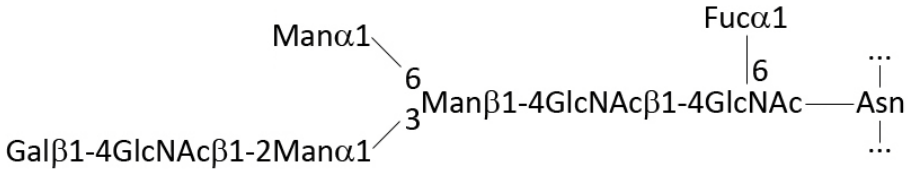

Figure 3.3 Schematic representation of the most common N-glycan found on *Ostertagia* ASP1 and ASP2.

3.3.3 Deglycosylation/Denaturation/Reduction of ES-thiol

Deglycosylation of native and denatured/reduced ES-thiol was monitored on a 10% SDS-PAGE. PNGase F treatment under native conditions yielded a shift of the 33 kDa band to 29 kDa (Figure 3.4a, lane 1 vs. lane 2). This shift was confirmed on Emerald staining together with a decrease in glycan intensity (Figure 3.4b, lane 1 vs. lane 2). Under denaturing and reducing conditions, deglycosylation resulted in a more pronounced shift from 33 kDa to 25 kDa (Figure 3.4a, lane 1 vs. lane 3) and no glycosylations were detected by Emerald staining (Figure 3.4b, lane 1 vs. 3).

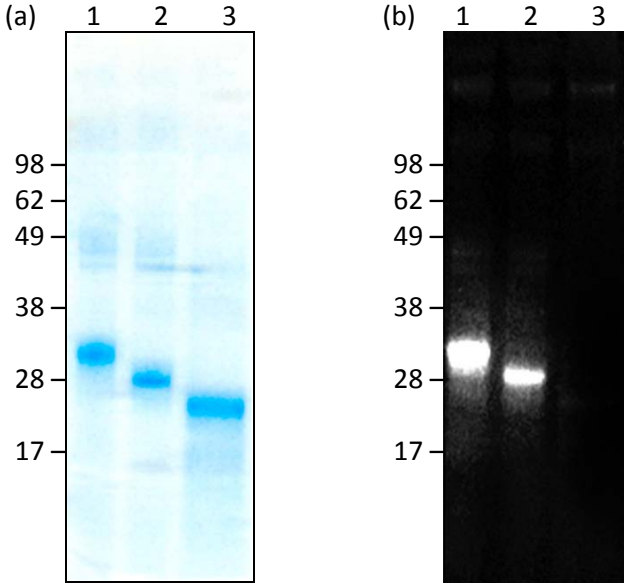

Figure 3.4 Analysis of ES-thiol on 10% SDS-PAGE before (lane 1) and after treatment with PNGase F under native (lane 2) and denaturing/reducing conditions (lane 3) visualized by Coomassie Blue (a) and Emerald 300 (b). MWs of standards are presented in kDa.

3.3.4 Tandem MS

The 33, 29 and 25 kDa bands (Figure 3.4a) were all identified as Oo-ASP1 and Oo-ASP2. An alignment of these ASP molecules is shown in Figure 3.5. No peptides containing a putative N-glycosylation site were detected in the spectrum of the 33 kDa band. For ASP1 the peptide RDIAGASPLLN*<Dam>*LTGAVQM<Mox>R was identified following trypsin digestion of the 29 and 25 kDa band, while the peptide EAGFCCPADLN*<Dam>*QTDEAR was only found in the digest of the 25 kDa band (N*<Dam>* indicates a deamidated asparagine - i.e. conversion to aspartic acid - due to PNGase F treatment). For ASP2 the peptide PLPIN*<Dam>*TSLAQNIAR was detected in the digest of the 25 kDa band. A peptide containing the second predicted N-glycosylation site was not detected in the 29 or 25 kDa band.

```
1    M Q A L I G I A A L Y L V L V T S N T E A G F C C   Oo-ASP1
1    M S A A V - V V A V L L A L F S - Y A E A G F C C   Oo-ASP2

26   P A D L N Q T D E A R X I F L D F H N Q V R R D I   Oo-ASP1
24   P N S L S Q S D S A R Q I F L D F H N D V R R N I   Oo-ASP2

51   A G A S P L L N L T G A V Q M R N V L G P A K N M   Oo-ASP1
49   A L G N G L I N W T V N A D A V - I L G P A Q N M   Oo-ASP2

76   Y R M D W D C N L E A K A K A M I W P C T T P L P   Oo-ASP1
73   Y K V D W D C N L E E V A A Q Q I A P C N D P L P   Oo-ASP2

101  I D T S I P Q N L A Q W L L F Q N S Q E X E V L T   Oo-ASP1
98   I N T S L A Q N I A R W L Y F K D S E E E T V L Q   Oo-ASP2

126  Q T P W S W V T A S L R N L Q P D T E A N I Y N W   Oo-ASP1
123  Q V S W Y W V S A S L G F M K G T K L D Q F A N Q   Oo-ASP2

151  Q I R P L S N I A N W Q N L K V G C A H K V C K F   Oo-ASP1
148  W A E P L A N I A N Y R N R K V G C A H K I C - -   Oo-ASP2

176  P T G T N M V V S C A Y G G E V L Q D N E V V W D   Oo-ASP1
171  P A Q Q N M V V S C V Y G S P K L A P N E V I W Q   Oo-ASP2

201  K G P T C M C N A Y P N S F C C N N L C D T I A A   Oo-ASP1
196  E G K A C V C D A R P D S F C C D N L C D T R D A   Oo-ASP2

226  A T L R N Q P C K S T                             Oo-ASP1
221  A S V R H Q C C A S P                             Oo-ASP2
```

Figure 3.5 Sequence alignment of *Ostertagia* ASP1 and ASP2. Peptide fragments identified by MS overspanning an N-glycosylation site are boxed. N-glycosylation sites are marked with an asterix.

3.3.5 3-D modeling of Oo-ASP1 and Oo-ASP2

According to the BLAST search *Necator americanus* ASP2 (PDB 1U53) was the best ranked template structure for Oo-ASP1 and Oo-ASP2. Because sequence identity was less than 30%, a pairwise sequence alignment was unreliable for conventional homology modeling (results not shown). Similarly, the 3D-jury external modeling servers presented Na-ASP2 to be the best structural template for Oo-ASP1 and Oo-ASP2. In contrast, these alignments were based on secondary structure conservation, not on pairwise sequence identity. Figure 3.6 shows the resulting structural alignment (superposition) of the Oo-ASP1 and Oo-ASP2 models. The overall fold was predicted to be a three layered alpha-beta-alpha sandwich. In both models, the two glycosylation sites appear on opposite sides of the structure's largest cavity. One glycosylation site seems to be structurally conserved: Asn58 in ASP1 and Asn56 in ASP2. Asn30 in ASP1 and Asn99 in ASP2 are located on a similar side of the structure but are not structurally conserved. Three putative disulphide bonds were found for ASP1 between Cys25-Cys82, Cys95-Cys173 and Cys168-Cys185, and for ASP2 between Cys23-Cys79, Cys92-Cys170 and Cys165-Cys180. These bonds were structurally conserved between ASP1 and ASP2.

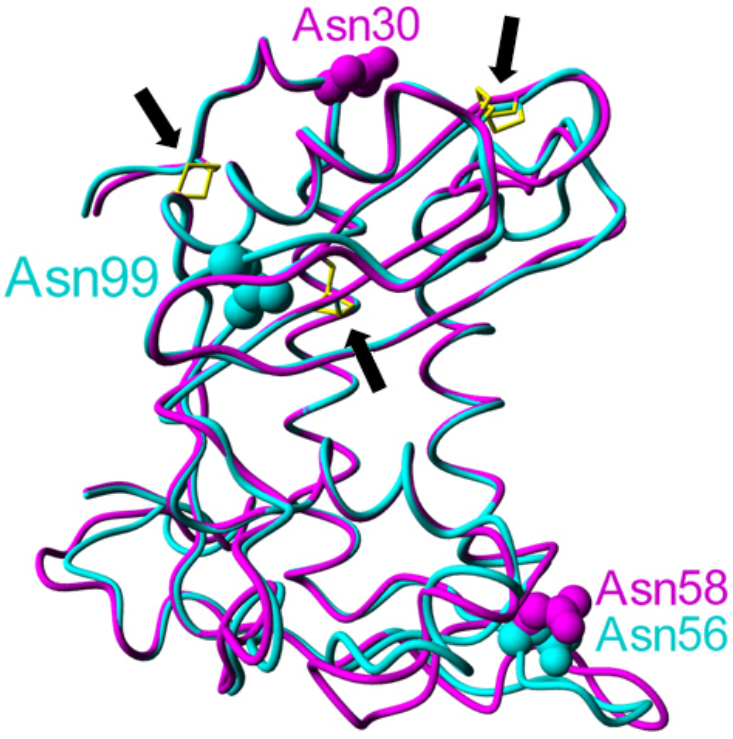

Figure 3.6 3-D modeling of *Ostertagia* ASP1 and ASP2. Both models are drawn in ribbon style representation. Oo-ASP1 is colored in magenta (dark), Oo-ASP2 in cyan (light). Putative disulphide bridges (yellow) are indicated by arrows. The N-glycosylated Asn residues are drawn in spherical representation.

3.3.6 ELISA

An ELISA was used to investigate antibody recognition by cattle immunized with native ASPs. Figure 3.7 shows the mean OD measurements for IgG1, IgG2, IgM and IgA. The removal of N-glycans by treatment with PNGase F did not yield a reduction in antibody recognition for any of the antibodies studied here. In contrast, denaturing and reducing the ASPs leaving the glycans intact reduced the OD values for IgG1, IgG2 and IgM with 89%, 84% and 71% respectively. OD values for IgA were relatively low and a similar effect could be observed, although these values did not differ significantly.

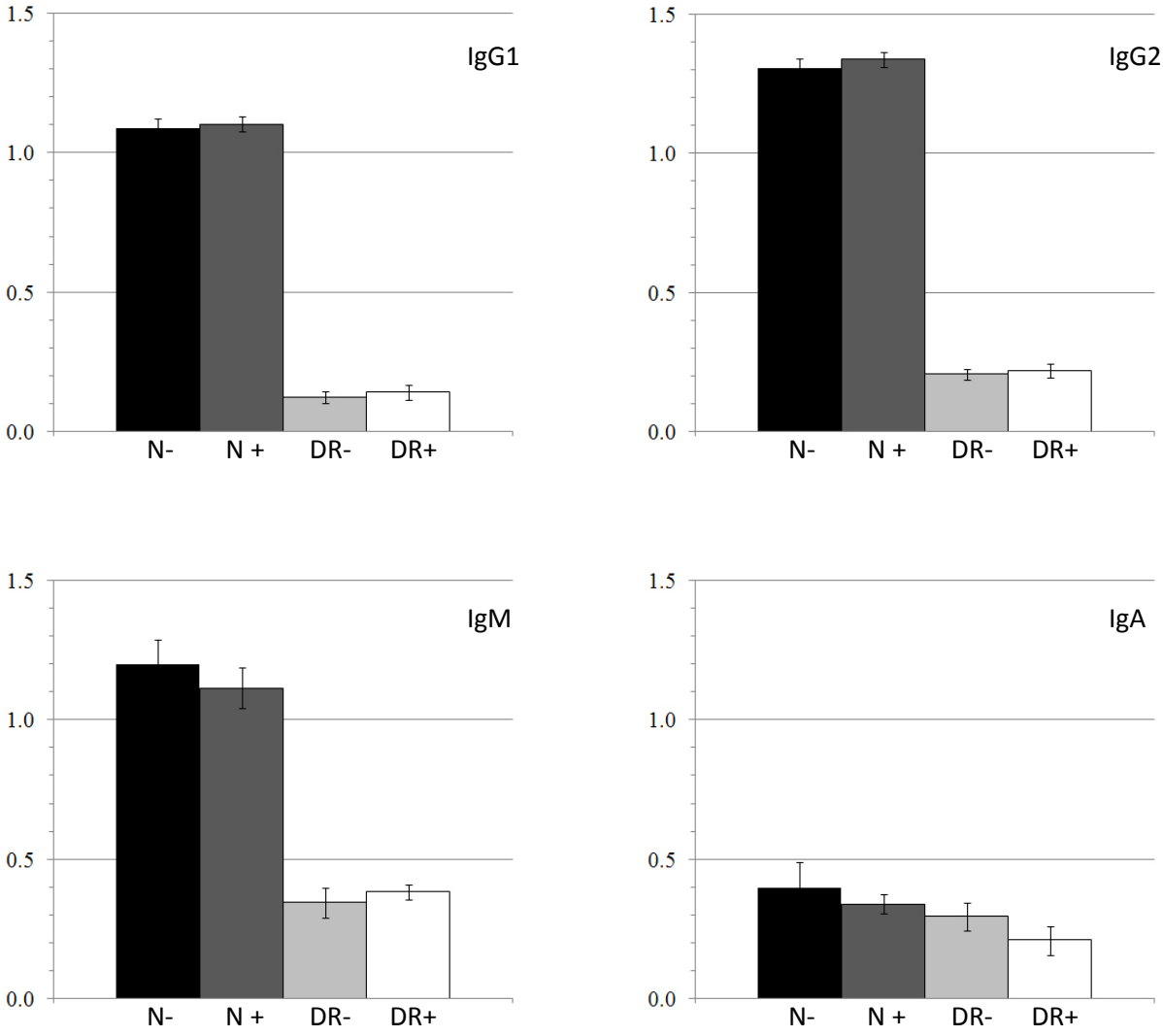

Figure 3.7 Effect of denaturation/reduction and deglycosylation of ES-thiol on recognition by IgG1, IgG2, IgM and IgA antibodies of animals immunized with native ASPs. Results are given as mean OD ± standard error. (N- = native ES-thiol, untreated; N+ = native ES-thiol, treated; DR- = denatured/reduced ES-thiol, untreated; DR+ = denatured/reduced ES-thiol, treated).

3.4 Discussion

In this chapter, we have investigated the presence and the nature of glycans present in the protective ES-thiol fraction of *O. ostertagi*. Glycan structures were identified specifically on the ASPs, which are the most abundant component of ES-thiol (Geldhof *et al.,* 2003).

Linkage analysis of the enzymatically removed glycan structures revealed mainly paucimannosidic and hybrid-type N-glycans containing an core α-1,6-fucose, a complex α-1,3-arm and an α-1,6-arm not processed by N-acetylglutaminyltransferase II. Hybrid N-glycans have already been detected in insects, mammals and the free-living nematode *Caenorhabditis elegans* (Cipollo *et al.*, 2002), but so far not in parasitic helminths.

Treatment with PNGase F under native conditions resulted in a 4 kDa shift in migration of the ASPs, along with a decrease in glycan intensity. This suggests the removal of one N-glycan which is readily available for the PNGase F enzyme to cut. Under denaturing/reducing conditions, an extra shift indicated the removal of a second N-glycan, leaving the ASPs fully deglycosylated. These results support the prediction of two N-glycosylation sites for ASP1 and ASP2 (Geldhof *et al.*, 2003). Potential N-glycosylation sites have also been detected in other ASP homologs. Two N-glycosylation sites have been found in the one-domain *Onchocerca volvulus* ASP1 (Tawe *et al.*, 2000) and the two-domain *Ancylostoma ceylanicum* ASP1 (Goud *et al.*, 2004). For the latter, both sites are located in the N-terminal part of the sequence, which shows the highest homology with Oo-ASP1 and Oo-ASP2 (Geldhof *et al.*, 2003). Also, single N-glycosylation sites have been detected in the single-domain *Ancylostoma caninum* ASP2 (Hawdon *et al.*, 1999) and *Haemonchus contortus* Hc24 antigen (Schallig *et al.*, 1997). However, none of the glycan structures present on these vaccine candidates have been studied in detail.

MS analysis of ASP1 demonstrated that the N-glycan on Asn58 was removed under native conditions, while the glycan on Asn30 was only removed after denaturing and reducing the protein. Removal of the N-glycan on Asn56 of ASP2 could not be confirmed by MS, however the N-glycan on Asn99 appeared to be removed under denaturing and reducing conditions. Interestingly, these “hidden” N-glycans of ASP1 and ASP2 occupy the same location in the 3-D model. Since Oo-ASPs are dimeric under native conditions (chapter 2), it is possible that the latter glycan structures are located close to the dimerization interface, making them initially unavailable for PNGase F to cut.

Our ELISA results suggest that the glycan structures on the ASPs are not immunogenic on their own. However, in order to be absolutely sure that these glycans are not important for inducing protection a vaccination trial can be performed using deglycosylated ASPs. Since a reduction in antibody recognition was observed for all subtypes after denaturing and

reducing the ASPs, the antibodies induced by vaccination with native ASPs seem to be directed against specific epitopes present in the protein backbone. A similar observation has been made in vaccination trials against *Dictyocaulus viviparus* where long-lasting protection was correlated with an antibody response against protein epitopes (Kooyman *et al.*, 2007). On the other hand, immunodominant N-glycans of *D. viviparus* only induced a short-lived immune response. *Ostertagia* ASP1 and ASP2 contain 13 respectively 14 cysteine residues (Geldhof *et al.*, 2003) and three putative intramolecular disulphide bonds were found which are likely to have an impact on protein conformation. Furthermore, ASP1 and ASP2 are dimeric under non-reducing conditions and only exist as monomers after reduction (chapter 2) suggesting the remaining cysteine residues are important for dimerization. In correspondance, a 3-D structural analysis of the ASP homolog of *Necator americanus* (Na-ASP2) showed the presence of a putative binding cavity that might be essential for dimerization and Na-ASP2 demonstrated structural and charge similarities with chemokines (Asojo *et al.*, 2005). These conformational characteristics are likely to be essential for the correct functioning of ASPs. Hence, they could be the key to proper antibody recognition and possibly crucial for inducing protection. From our ELISA results it is still unclear whether denaturing or reducing on its own is responsible for the drastic reduction in antibody recognition. Western blot analysis of non-reduced ASPs on a denatured SDS-PAGE still resulted in recognition by vaccinated animals (chapter 2) suggesting disulfide bridges are at least to some extent responsible for antibody recognition.

Numerous parasites confront their host with antigens equipped with exotic and immunogenic glycans, often as part of their immune evasion strategy (Hokke and Deelder, 2001; Nyame *et al.*, 2004). Despite the fact that ASP1 and ASP2 are the most abundant antigens released by adult *Ostertagia* parasites (Geldhof *et al.*, 2003), they seem to lack highly immunogenic sugar residues such as the α-1,3-fucose core described by van Die *et al.* (1999). In correspondence, the ELISA results indicated that N-glycan removal coincided with only a minor decrease in antibody recognition. The importance or role of the ASPs and their glycans for the parasite remains unclear. Interestingly, α-mannosidase-II knockout mice that solely express these types of hybrid N-glycans are known to develop an autoimmune disease resembling systemic lupus erythematosus (Green *et al.*, 2007). In this case, an increased infiltration and activation of macrophages in kidney tissue has been observed causing inflammation and tissue damage. However, it is still unclear whether the hybrid glycans discovered in *Ostertagia* have a role in the recruitment of immunocompetent cells in the gastrointestinal mucosa during an infection. Furthermore, while the immunogenicity of ASP glycans appears to be negligible, they might be important during protein synthesis inside the

adult worm. It is possible that the correct folding of ASPs along with the formation of essential epitopes depends on the glycosylation process in the Golgi apparatus.

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Chapter 4

Proteomic survey of the protective ES-thiol fraction of *Ostertagia ostertagi*

4.1 Introduction

Different screening techniques have been applied when looking for appropriate vaccine candidates in excretory-secretory (ES) material of *Ostertagia ostertagi*. First of all, a comprehensive substrate gel analysis has detected stage- and substrate-specific proteases including metallo-, serine and aspartyl proteases as well as enzymes with cathepsin L-like activity (Geldhof *et al.*, 2000). In addition, antibodies isolated from the abomasal mucus and the draining lymph nodes of infected cattle (de Maere *et al.*, 2002) and rabbit antibodies directed against parasite ES material (Vercauteren *et al.*, 2003) have been used to screen complementary DNA (cDNA) expression libraries of *O. ostertagi*. This approach led to the identification of several interesting ES antigens such as metalloproteases (MEPs), vitellogenin, an aspartyl protease inhibitor, a protein disulfide isomerase and an *Ostertagia* polyprotein allergen (OPA). Furthermore, the latter antigen has been demonstrated to successfully protect cattle against infection (Vercauteren *et al.*, 2004). While previous studies of the ES-thiol fraction have confirmed the presence of activation-associated secreted protein 1 (ASP1), ASP2 and several cysteine proteases (CPs) (Geldhof *et al.*, 2002, 2003), its composition remains largely unknown.

In chapter 2 vaccinating calves with any of the three subfractions of ES-thiol (i.e. the ASP, CP and rest fraction) resulted in significant protection against *Ostertagia* infection. Since animals injected with the CP and rest fraction did not recognize any antigens from the ASP fraction, other protective antigens besides ASPs are likely to be present within ES-thiol. In order to discover new potential vaccine candidates for *O. ostertagi*, a tandem mass spectrometry (MS) analysis of the subfractions of ES-thiol will be performed in this chapter.

4.2 Materials and methods

4.2.1 Antigen collection

The ES-thiol fraction and its subfractions were obtained as described in chapter 2 (section 2.2.1). Ten μg was run on a 10% SDS-PAGE under denaturing/reducing conditions. Protein bands were made visible by subsequent Coomassie Blue staining.

4.2.2 Tandem MS

A tandem MS analysis was performed on aliquots of the ASP, CP and rest fraction (100 μl/fraction) and protein bands visualized by Coomassie Blue staining. Samples were analyzed as described in chapter 2 (section 2.2.2). The resulting MS spectra were used to screen the protein database of *O. ostertagi* and the expressed sequence tag (EST) database of *O. ostertagi*, *Teladorsagia circumcincta* and *Haemonchus contortus*.

4.3 Results

4.3.1 Antigen collection

The peptide profile of ES-thiol and its three subfractions on a Coomassie-stained gel is shown in Figure 4.1.

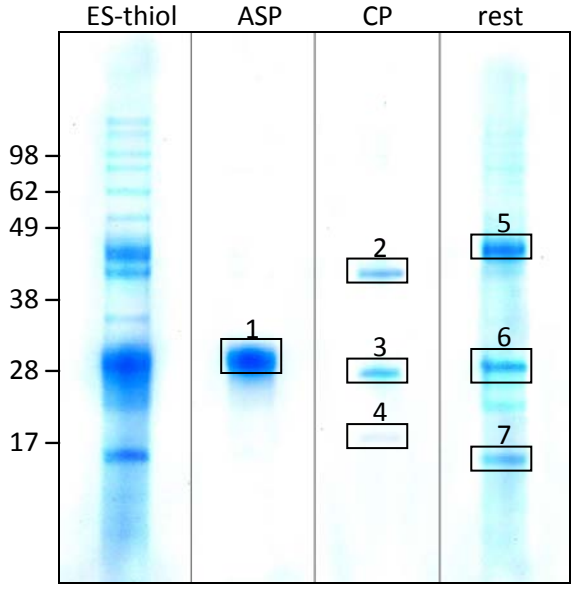

Figure 4.1 Protein profiles of ES-thiol, the activation-associated secreted protein fraction (ASP), the cysteine protease fraction (CP) and the rest fraction (rest) on a 10% SDS-PAGE under denaturing/reducing conditions, visualized by Coomassie Blue staining. Excized protein bands are boxed. Molecular weights (MWs) of standards are presented in kDa.

4.3.1 Tandem MS

Seven bands were labeled (Figure 4.1) and excised from the gel and analyzed together with an aliquot of the ASP, CP and rest fraction. The result of the analysis is summarized in Table 4.1. Only peptides giving significant database hits have been included. Hits with trypsin were discarded.

Fraction	Acc. no.	Identity/Homology	Organism	No. pept.	Band
ASP	CAD23183	ASP1	*O. ostertagi*	3	1
	CAD56659	ASP2	*O. ostertagi*	3	
	CAO00416	ASP3	*O. ostertagi*	1	
	CAN84553	AL1	*O. ostertagi*	2	
	CAD20463	globin-like ES protein F6	*O. ostertagi*	1	
	CAD20600	putative ES protein	*O. ostertagi*	1	
	CAD20601	putative ES-protein	*O. ostertagi*	1	
	BQ099827	profilin 1	*O. ostertagi*	1	
	BQ457734	malate dehydrogenase	*O. ostertagi*	1	
	CB037330	ancylostoma-secreted protein like protein	*T. circumcincta*	2	
	BM052082	ancylostoma-secreted protein like protein	*T. circumcincta*	1	
	CB036207	SSP (sperm-specific family class P protein)	*T. circumcincta*	1	
	CB036286	NEX1 annexin	*T. circumcincta*	1	
	BF662710	NEX1 annexin	*H. contortus*	2	
	CB015939	NEX1 annexin	*H. contortus*	2	
	CA956390	vitellogenin	*H. contortus*	1	
	BM138812	major sperm protein	*H. contortus*	1	
CP	CAD11605	MEP1	*O. ostertagi*	1	3
	CB038665	hypothetical protein CBG00622	*T. circumcincta*	2	2
	BG734323	C-type lectin	*T. circumcincta*	1	3 + 4
	BF059783	translationally-controlled tumor protein	*H. contortus*	1	
	AAM73859	putative serine/threonine protein kinase	*H. contortus*	2	3
	AAA29181	D-fructose-6-phosphate 1-phosphotransferase	*H. contortus*	2	3
	BM139416	asparagine synthase	*H. contortus*	2	4
	CA869788	unknown protein	*H. contortus*	2	4
rest	CAD23183	ASP1	*O. ostertagi*	2	7
	CAD56659	ASP2	*O. ostertagi*	1	
	AM747038	ASP3	*O. ostertagi*	1	7
	CAD20737	thioredoxin peroxidase	*O. ostertagi*	3	7
	AAC08344	40S ribosomal protein S28	*O. ostertagi*	1	6
	CAD12263	MEP2	*O. ostertagi*	2	6
	CB037817	MEP4	*T. circumcincta*	1	5

Table 4.1 Results of tandem MS analysis of the protective subfractions of ES-thiol. (Acc. no. = accession number; No. pept. = number of peptides with significant hits).

4.4 Discussion

In this chapter we have performed an MS analysis of the protective subfractions of ES-thiol. Although this approach has allowed us to obtain additional information about the composition of ES-thiol, some important remarks need to be made.

In chapter 2 we have seen that the three subfractions of ES-thiol induced a similar degree of protection in a vaccination trial. Western blot analysis also demonstrated cross-reactivity between the CP and the rest fraction. These results might indicate the presence of one or more common protective antigens. However, this could not be confirmed by MS analysis. One reason might be that there is no common antigen present at all and protection is indeed induced by different proteins. Another reason could be that one or more common antigens are present but can not be detected by MS either because of their limited amount or because their peptide sequence is not included in our search database. Although substrate gel analysis clearly indicated the presence of CPs in the CP fraction, no CPs were identified in our proteomic survey. Since the amount of CPs present in ES-thiol is limited, it is likely that MS is not able to detect these peptides. Because CPs could not be detected here, it is possible that also other antigens present in ES-thiol were not detected indicating the limitations of the MS approach for identifying proteins.

The ASP fraction which appeared to be highly pure based on Coomassie and Western blot analysis in chapter 2 was found to contain additional antigens. Since ASP1 and ASP2 are the most immunogenic components of the ASP fraction, it is tempting to attribute protection to these components.

An overview of the different types of antigens identified will be given in the following sections.

ASPs and ASP-like proteins

Two N-type single domain ASPs - i.e. ASP1 and ASP2 (Geldhof *et al.*, 2003) - were detected in the ASP fraction. This corresponds with the results from the anti-ASP Western blot obtained in chapter 2 (Figure 2.2). In addition, a C-type single domain ASP3 similar to *Ancylostoma caninum* ASP2 and *Haemonchus contortus* Hc24 (Visser *et al.*, 2008), an *Ostertagia* specific ASP-like protein named AL1 (Saverwyns *et al.*, 2008) and two ASP homologs from *Teladorsagia circumcincta* (cluster TDC00435 and TDC00460) were discovered.

Peptides from ASP1 and ASP3 were also found in a low MW band of the rest fraction (Figure 4.1; band 7), which could indicate the presence of degraded ASP molecules. Since these peptides were not recognized by immunized animals, it can be assumed that they are not capable of inducing protection on their own. This would also correspond with the enzyme-

linked immunosorbent assay (ELISA) results from chapter 3 where protein conformation was found to be essential for correct antibody recognition.

Although the potential of ASP as a vaccine candidate has been extensively described (section 1.3.2.2), its specific function remains unclear. While several papers have suggested a key role in parasitism (Hawdon *et al.*, 1996, 1999; Bin *et al.*, 1999, 2003; Moser *et al.*, 2005), a recent study in *O. ostertagi* has shown transcription of many ASP genes to be male enriched (Visser *et al.*, 2008) suggesting a function in reproduction. The fact that ASP1, ASP2 and several other ASP-like molecules were eluted in the same fraction from the anion exchange column suggests the presence of similar ionic groups, which could infer structural homology and function.

Vitellogenin, major sperm protein and sperm-specific protein

Additional antigens involved in reproduction were identified in the ASP fraction i.e. vitellogenin (cluster HCC00998), a major sperm protein (MSP, cluster HCC02230) and a sperm-specific protein family class P protein (SSP, cluster TDC00542).

Vitellogenin is an abundant component of egg yolk and is highly conserved among eukaryotic organisms (Chen *et al.*, 1997). It supplies the growing embryo with amino acids and was also detected in a previous immunoscreening of an *Ostertagia* cDNA database indicating its immunogenic character within the host (Vercauteren *et al.*, 2003).

MSP was originally discovered in *Caenorhabditis elegans* and makes up 15-20% of the total protein content of nematode sperm, while abscent in any other nematode cell type (Roberts, 2005). It is responsible for sperm motility (Roberts and Stewart, 2000) and is capable of triggering oocyte maturation and ovulation (Yamamoto *et al.*, 2006). In addition, MSP was found to be an immunogenic ES antigen of the bovine lungworm *Dictyocaulus viviparus* (Matthews *et al.*, 2004; Höglund *et al.*, 2008).

The SSP family is also known as the MSP-like family. SSPs are smaller than proteins of the MSP family and 17 SSPs have been identified in *C. elegans* (http://www.wormbase.org). Little is known about their function, although a moderate sequence homology and a high structural homology with MSPs suggests a similar function (Schormann *et al.*, 2004).

MEPs

Several MEPs were found to be present in the CP and the rest fraction. *Ostertagia* MEPs show homology with a family of zinc MEPs called astacins (Möhrlen *et al.*, 2003) which have been described in several parasitic nematodes such as *A. caninum* (Zhan *et al.*, 2002; Williamson *et al.*, 2006; Feng *et al.*, 2007), *Trichinella spiralis* (Lun *et al.*, 2003), *Strongyloides*

stercoralis (Gomez Gallego *et al.*, 2005) and *Onchocerca volvulus* (Borchert *et al.*, 2007). Furthermore, an astacin from *A. caninum* is considered an important vaccine candidate by the human hookworm vaccine initiative (Hotez *et al.*, 2003).

Ostertagia MEP1 and MEP2 have previously been detected in an immunoscreening using local antibodies of infected cattle (de Maere *et al.*, 2002). A truncated baculo-expressed MEP1 failed to protect calves against challenge infection with *O. ostertagi* (de Maere *et al.*, 2005). However, these animals did not recognize the native MET1 protein on Western blot indicating the lack of immunogenic epitopes in the recombinant MET1 fragment.

Profilin and annexin

An *Ostertagia* homolog of profilin (cluster OOC03037) and annexin (clusters TDC02079 and HCC01655) was detected in the ASP fraction.

Profilins are cytosolic proteins of 12-15 kDa which can be found in all eukaryotic cells (Radauer and Breiteneder, 2007). They are capable of binding actin and different regulatory proteins with prolin-rich regions and are key players in cell movement, cytokinesis and signaling. Several profilins found in flowering plants and pollens are known to be allergenic (Radauer and Breiteneder, 2007). Three different isoforms have been described in *C. elegans* (Polet *et al.*, 2006). Furthermore, several parasite profilins have been shown to modulate the host immune response and are capable of inducing interleukin 12 production in murine dendritic cells. This has been demonstrated for the protozoan organisms *Eimeria tenella*, *Plasmodium falciparum*, *Cryptosporidium parvum* and *Toxoplasma gondii* (Rosenberg *et al.*, 2005; Yarovinsky *et al.* 2005) and the trematode *Schistosoma japonicum* (Zhang *et al.*, 2008).

Annexins are cytosolic proteins capable of binding phospholipids and the cell membrane, and have been proposed to act as membrane-membrane or membrane-cytoskeleton linkers (Rescher and Gerke, 2004). While a secretion signal is missing, several annexins have been detected extracellularly. These annexins are able to bind to the cell surface and are believed to be involved in exocytosis (Creutz, 1992), plasminogen regulation (Kwon *et al.*, 2005), anti-coagulation (Rand, 2000) and anti-inflammation (Perretti and Gavins, 2003; Parente and Solito, 2004). Four annexins with different expression profiles and ligand-binding properties have been described in *C. elegans* (Nishioka *et al.*, 2007). Annexins have also been identified as an immunodominant component of the membrane of the intestinal protozoan parasite *Giardia lamblia* (Weiland *et al.*, 2003) and the tegument surface of *Schistosoma mansoni* (Braschi *et al.*, 2006) and *Taenia solium* metacestodes (Zhang *et al.*, 2007). A recent study has shown that *T. solium* metacestodes actively secrete annexin B1 which is capable of inducing apoptosis of eosinophils by binding their extracellular membrane (Yan *et al.*, 2008).

In addition, vaccinating pigs with an *Escherichia coli* recombinant of annexin B1 reduced the total number of cysticerci with 85% (Guo *et al.*, 2004).

Proteins with unknown function

A globin-like ES protein F6 and two putative ES proteins were found to be present in the ASP fraction. While the function of these antigens is unknown, their immunogenicity has already been demonstrated previously (Vercauteren *et al.*, 2003). In addition, three antigens with unknown function were detected in the CP fraction, i.e. a C-type lectin similar to *C. elegans* clec-87, an antigen similar to hypothetical protein CBG00622 from *Caenorhabditis briggsae* and an unknown protein from the EST database of *H. contortus* (cluster HCC04192). In order to evaluate these antigens as vaccine candidates, their full-length sequence should be determined followed by a characterization of the proteins they encode.

Cytosolic and metabolic proteins

Several proteins listed in the table are known to be present and function within the eukaryotic cell. These are thioredoxin peroxidase, malate dehydrogenase, serine/threonine protein kinase, phosphotransferase, asparagine synthase and ribosomal proteins. Since it is unlikely that these antigens are actively secreted by *Ostertagia* worms and are capable of inducing an immune response in the host, they will not be regarded as potential vaccine candidates for *O. ostertagi*. Their presence is probably a result of protein leakage from dying worms present in the culture medium during collection of ES material.

Translationally-controlled tumor protein

The translationally controlled tumor protein (TCTP) was detected in the CP fraction of ES-thiol (cluster HCC00192). TCTP is a highly conserved eukaryotic protein involved in various biological processes (reviewed by Bommer & Thiele, 2004). Interestingly, TCTP is a known secretion product of several parasitic organisms such as *P. falciparum* (MacDonald *et al.*, 2001), *S. mansoni* (Rao *et al.*, 2002), *Brugia malayi* and *Wuchereria bancrofti* (Gnanasekar *et al.*, 2002). Extracellular TCTP is able to promote local infiltration of eosinophils (reviewed by Bommer & Thiele, 2004) which is also observed in the abomasal mucosa of animals infected with *O. ostertagi*.

For these reasons, a detailed study of *Ostertagia* TCTP will be conducted in the following chapter and its potential as a vaccine target will be evaluated.

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Chapter 5

Study of the translationally controlled tumor protein (TCTP) in *Ostertagia ostertagi*

5.1 Introduction

In chapter 4, an *Ostertagia* homolog of the translationally controlled tumor protein (TCTP) was detected in the protective cysteine protease (CP) fraction of ES-thiol (peptide match with *Haemonchus contortus* cluster HCC00192). TCTP is a highly conserved eukaryotic protein which has been described in multiple organisms like protozoa, yeasts, plants and mammals (reviewed by Bommer & Thiele, 2004). It was originally discovered 25 years ago as an important transcription product of murine sarcoma and erythroleukaemia cells (Yenofsky *et al.*, 1982, 1983). Since then, TCTP has been shown to be involved in various biological processes such as cell growth, cell cycle progression, cell differentiation, malignant transformation and protection of cells against various stress conditions and apoptosis (reviewed by Bommer & Thiele, 2004). Studies in parasitic nematodes have suggested that intracellular expression of TCTP is linked with heat stress adaptation of *Trichinella spiralis* and *Trichinella pseudospiralis* (Mak *et al.*, 2001, 2007) and protection of *Brugia malayi* against oxidative stress (Gnanasekar and Ramasway, 2007). Although TCTP lacks a secretion signal (reviewed by Bommer & Thiele, 2004), several parasitic organisms such as *Plasmodium falciparum* (MacDonald *et al.*, 2001), *Schistosoma mansoni* (Rao *et al.*, 2002), *B. malayi* and *Wuchereria bancrofti* (Gnanasekar *et al.*, 2002) have been found to actively secrete TCTP during host infection. In this case, parasite TCTP is able to induce the release of histamine from basophils and promote local infiltration of eosinophils (reviewed by Bommer & Thiele, 2004). An accumulation of eosinophils has also been described in the abomasal mucosa of *Ostertagia ostertagi* infected cattle and the parasite appears to be directly responsible for this effect (reviewed by Claerebout and Vercruysse, 2000). Therefore, the aim of this chapter is to further characterize *O. ostertagi* TCTP and investigate its potential role in parasite survival and host-parasite interaction.

5.2 Materials and methods

5.2.1 Full-length sequence of *Ostertagia* TCTP

Degenerative forward and reverse TCTP primers were designed based on conserved sequences observed between *Caenorhabditis elegans* (NC_003279), *Teladorsagia circumcincta* (cluster TDC00659) and *Haemonchus contortus* (cluster HCC00192) and were used to pick up part of the *Oo-tctp* gene from a complementary DNA (cDNA) library. The full-length sequence of *Oo-tctp* was determined by 5' and 3' rapid amplication of cDNA ends (RACE) using the BD Marathon® cDNA amplification kit (BD Clontech). Following the manufacturer's protocol, the 5' and 3' RACE were conducted with the AP1 primer and the

degenerative primers Oo-TCTPrev1 (5'-CGRTATTCWATGATKGCSACTTGCCC-3') and Oo-TCTPfw1 (5'-CCGGGWGCCAAYCCTTCAGCCG-3') respectively. The amplified DNA sequences were resolved by the dideoxy chain terminator method (Big Dye™ terminator v3.1, Applied Biosystems) in a ABI3730xl genetic analyzer (Applied Biosystems) followed by sequence analysis in Lasergene (DNASTAR).

A Basic Local Alignment Search Tool (BLAST) search of the Oo-TCTP sequence was performed in the NCBI database (www.ncbi.nlm.nih.gov/BLAST) and the predicted amino acid sequence was aligned in Lasergene (DNASTAR) with TCTP homologs demonstrating the highest percentage of identity. An additional BLAST search was performed in the NEMBASE3 expressed sequence tag (EST) database (www.nematodes.org/nembase3). N-glycosylation sites were predicted with PROSITE (www.expasy.ch/tools/prosite) and signal peptides were identified with SignalP (www.cbs.dtu.dk/services/SignalP).

5.2.2 Recombinant expression and purification

The full-length Oo-TCTP sequence was cloned into the pGEM®-T-Easy vector (Promega) following polymerase chain reaction (PCR) using primers containing the restriction sites *EcoRI* and *XhoI* (Oo-TCTP-*EcoRI* 5'-GAATTCATGCTGATCTTCAAGGAC-3' and Oo-TCTP-*XhoI* 5'-CTCGAGAATCTTTTCAAAAATGA TGG-3'). The Oo-TCTP-pGEM®-T-Easy plasmid was purified (Qiagen Midi kit) and restricted with *EcoRI* and *XhoI* (Promega). The Oo-TCTP insert was gel-purified and unidirectionally ligated into the pET-21a(+) expression vector (Novagen) at the *EcoRI/XhoI* restriction site. The constructed plasmid was used to transform *Escherichia coli* Bl21-CodonPlus(DE3)-RIL competent cells (Stratagene) according to the manufacturer's protocol. A pre-culture of transformed *E. coli* cells (4 ml) was transferred to 200 ml 2xYT broth and grown in a shaker at 37°C (250 revolutions per minute (rpm)) to an OD of 0.6 (λ = 600 nm). Recombinant expression was induced by adding isopropyl-beta-D-thiogalactopyranoside (final concentration = 0.10 mM) followed by 2h incubation at 37°C (250 rpm). Cell lysis and protein solubilization were performed according to an established protocol (Frangioni and Neel, 1993).

The bacterial lysate was dialysed overnight at 4°C in T7 Tag binding buffer using a 10,000 MWCO Slide-A-Lyzer® dialysis cassette (Pierce) and the recombinant Oo-TCTP was purified using a T7 Tag purification kit (Novagen) as recommended by the manufacturer. Elution steps were pooled, dialyzed in 10 mM Tris-HCl (pH 7.4) and concentrated on a Centriprep Ultra-15 membrane (Millipore; 10,000 MWCO). The protein concentration was determined using the BCA method (Pierce Chemical Co., Rockford, IL, USA).

5.2.3 Production of monospecific antibodies

A white laboratory rabbit received two subsequent subcutaneous immunizations with 50 respectively 25 μg recombinant Oo-TCTP with a two week interval in combination with 50 μg QuilA adjuvant (Superfos, Taastrup, Denmark). Serum was collected before the first immunization (i.e. pre-immune) and 2 weeks after the final immunization (i.e. immune).

In order to enrich for monospecific antibodies 25 μg of recombinant Oo-TCTP was separated on a preparative SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon®, Milipore). The area around Oo-TCTP was cut out and blocked for 1h in 10% horse serum (HS) in PBST and probed overnight at 4°C with immune rabbit serum (1/40 in 2% HS / PBST). The blot was washed repeatedly (3x 10 min at room temperature) and bound antibodies were eluted by 5 min incubation in 5 mM glycine, 0.5 M NaCl, pH 2.5. Eluted antibodies (i.e. anti-Oo-TCTP) were neutralized by adding 1M Tris pH 8.5 (100 μl / 5 ml elution buffer) and stabilized with HS (final concentration = 5 %).

5.2.4 Stage-specific transcription

Ribonucleic acid (RNA) was extracted from the different life stages of *O. ostertagi* and cDNA was obtained as described by Van Zeveren *et al.* (2007). A semi-quantitative PCR was performed according to Geldhof *et al.* (2006) in order to determine the stage-specific transcription of *Oo-tctp*. In short, RNA was extracted from different life stages of *O. ostertagi* (i.e. third stage larvae (L3s), L3s without sheath (L3ex), fourth stage larvae (L4s), adult male worms, adult female worms and eggs) using Total RNA Isolation Reagent (ABgene® Ltd) and dissolved in 10 μl DEPC treated water. A one-step reverse transcriptase (RT) PCR reaction (Invitrogen) was run according to the manufacturer's protocol using *Oo-tctp* specific primers Oo-TCTPfw2 (5'-GGAATCGACATCGTTCTCAACCAC AAGC-3') and Oo-TCTPrev2 (5'-TTAAATCTTTTCAAAAATGATGGCCTC-3'). Each reaction mixture (25 μl) contained 1 μl cDNA, 1 μM forward/reverse primer, 4 mM dNTPs, 1.5 mM $MgCl_2$ and 0.6 U Taq DNA polymerase in 1X PCR buffer (Invitrogen). PCR parameters were 94°C of denaturation for 30 seconds, 60°C of primer annealing for 30 s and 72°C of primer extension for 30 s (30 cycles). A final extension at 72°C was performed for 10 min after which the samples were stored at 4°C. In order to account for sample-to-sample variation of the RNA isolates the transcription profile of the reference gene ribosomal protein L13a (*Oo-rpl-13a*; accession number BQ457724; 5'-AGAACTCAGAGCCGTCGGTA-3' and 5'-CTGAGCTTC TTGGGGAACAA-3'; Van Zeveren *et al.*, 2007) was included. The PCR reactions were loaded onto a 2.0% agarose gel and amplified PCR products were visualized with ethidium bromide. Photographs were made under a UV light using the Quantity One 4.5.1 Chemidoc EQ™ Software System (Bio-Rad, CA, USA).

5.2.5 Stage-specific translation

Extract and excretory-secretory (ES) material from *Ostertagia* L3s, L4s and adult worms were obtained as described previously (Geldhof *et al.*, 2000). Ten μg extract and ES-material of each developmental stage was separated on a 10% SDS-PAGE under denaturing/reducing conditions and blotted onto a PVDF membrane (Immobilon®, Milipore). The blot was blocked for 2h in 10% HS in PBST, probed overnight with monospecific rabbit anti-Oo-TCTP antibodies (section 5.2.3) and incubated for 2h with conjugate (Goat anti-rabbit horse raddish peroxidase (HRPO), Sigma; diluted 1/5,000 in 5% HS / PBST). TCTP was visualized by adding 0.05% 3,3 diaminobenzidine tetrachloride in PBS containing 0.01% H_2O_2 (v/v). One μg purified recombinant Oo-TCTP was included as a positive control.

5.2.6 Immunolocalization in *O. ostertagi*

Adult female *Ostertagia* worms were embedded in Tissue-Tek (Sakura Finetek Europe B. V.) and frozen in liquid nitrogen. A Jung CM3000 cryotome (Leica Instruments GmbH) was used to make 10 μm-thick sections. Sections were mounted on 3-aminopropyltriethoxy silane (Sigma) and fixated in xylene and isopropanol for 10 min. Slides were successively submerged in 94% - 80% - 70% - 50% ethanol followed by heated incubation in Antigen Retrieval solution (Biogenex) according to the manufacturer's protocol. Sections were washed in PBS (3 times for 5 min), blocked in 20% goat serum (diluted in PBS) for 30 min at room temperature and probed with monospecific rabbit anti-Oo-TCTP antibodies for 2h at 37°C. Pre-immune rabbit serum was used as a negative control (section 5.2.3). Detection was done with Alexa Fluor® 594 goat anti-rabbit immunoglobulin (Ig) G (H + L) (Molecular Probes, diluted 1/4,000, 5% goat serum in PBS) by 1h incubation at 37°C. Red fluorescence was registered with a Leitz DMRB microscope and a DC-100 camera (Leica Instruments GmbH).

5.2.7 Antibody response of infected cattle

In order to determine whether Oo-TCTP is secreted during host infection antibody recognition by naturally infected cows after their first grazing season was evaluated by Western blot analysis. This blot was performed with extract of *Ostertagia* eggs in which Oo-TCTP is typically expressed.

*Ostertagia e*ggs were washed three times in PBS followed by 10 min centrifugation at 3,000 g (4°C). Eggs were subjected to a freeze-thaw cycle (-70°C) and the pellet was resuspended in an equal volume of PBS. While placed on ice, eggs were crushed using a Vibra-Cell™ VC375 sonicator (Sonics & Materials, Inc.; power = 375 W; frequency = 20 kHz; time = 60 s; pulser = ON; duty cycle = 50%; micro-tip limit = 4.5). The lysate was incubated on ice for several

minutes followed by an additional sonication step. Finally, precipitate was removed by centrifugation for 10 min at 16,000 g (4°C). The supernatans was transferred to a fresh tube and protein concentrations were determined using the BCA method (Pierce Chemical Co., Rockford, IL, USA).

Sixty µg *Ostertagia* egg extract was prepared for two dimensional (2-D) SDS-PAGE with the 2-D PAGE cleanup kit (GE Healthcare) according to the manufacturer's instructions. The protein pellet was resolved in 125 µl rehydration solution (8 M urea; 2% CHAPS; 2% IPB buffer; 0.02% bromophenol blue; 0.28% dithiothreitol (DTT)) and was loaded on an Immobiline DryStrip (7 cm, linear pH 3-10, GE Healthcare) by overnight incubation at room temperature. Isoelectric focusing was performed on a 2117 Multiphor II electrophoresis system (LKB) at 200 V for 1 min and 3500V for 155 min (Imax = 2 mA; Pmax = 5 W; T = 20°C). The strip was washed twice for 15 min in SDS equilibration buffer (75 mM Tris-HCl pH 8.8; 6 M urea; 29.3% glycerol; 2% SDS; 0.02% bromophenol blue) containing 0.1% DTT and 0.25% iodoacetamide respectively. The second dimension was carried out on a 12% Tris-Glycine SDS-PAGE (10 mA for 15 min and 30 mA for 90 min). The resulting protein profile was visualized by Coomassie Blue staining.

The 2-D SDS-PAGE protocol was repeated, the separated proteins were blotted onto a PVDF membrane (Immobilon®, Milipore) and blocked for 2h in 10% HS (diluted in PBST). The blot was probed overnight with monospecific anti-Oo-TCTP antibodies (section 5.2.3), and incubated for 2h with conjugate (Goat anti-rabbit-HRPO, Sigma; diluted 1/5,000 in 5% HS / PBST). The Western blot was repeated with pooled bovine serum obtained from cattle before and after their first grazing season (n = 7; pre-immune vs. immune, diluted 1/500 in 5% HS / PBST) followed by 2h incubation with conjugate (Rabbit anti-bovine-HRPO, Sigma; diluted 1/5,000 in 5% HS / PBST). Recognized proteins were visualized by adding 0.05% 3,3'-diaminobenzidine tetrachloride in PBS containing 0.01% H_2O_2 (v/v).

5.2.8 Detection in eggs from different worm species

In order to determine whether the expression of TCTP in eggs is conserved among nematodes and trematodes, protein extract was prepared from isolated eggs of *C. elegans*, *O. ostertagi*, *Teladorsagia circumcincta*, *Cooperia oncophora*, *Haemonchus contortus*, *Ascaris suum* and *Fasciola hepatica*. Egg extract was prepared as described in section 5.2.7. Ten µg egg extract was separated on a 10% SDS-PAGE under denaturing/reducing conditions followed by Coomassie Blue staining and Western blot analysis using monospecific rabbit anti-Oo-TCTP antibodies as described in section 5.2.5.

5.2.9 Immunolocalization in *C. elegans*

Adult wildtype *C. elegans* worms were subjected to a freeze-crack procedure as described by Duerr (2006). Localization of Ce-TCTP was realized using monospecific anti-Oo-TCTP antibodies (section 5.2.3) following the protocol described for the *Ostertagia* sections (section 5.2.6).

5.3 Results

5.3.1 Full-length sequence of *Ostertagia* TCTP

The full-length Oo-TCTP cDNA sequence consists of 543 base pairs (bp) and the predicted protein (181 amino acids) has a molecular weight (MW) of 20.7 kDa and an isoelectric point (pI) of 4.56. A BLAST search in the NCBI database demonstrated that Oo-TCTP showed the highest similarity with TCTP of *Caenorhabditis briggsiae* (CAP23314 - 76%), *Caenorhabditis elegans* (CAB02099 - 76%), *Brugia malayi* (EDP33421 - 76%) and *Wuchereria bancrofti* (AAK71499 - 75%). In addition, a BLAST search in the NEMBASE3 EST database of the Strongylida indicated that the cDNA sequence of Oo-TCTP is similar to Tc-TCTP (cluster TDC00659 - 94%) and Hc-TCTP (cluster HCC00192 - 89%). A protein alignment of Oo-TCTP with these sequences is shown in Figure 5.1. No N-glycosylation sites or secretion signal peptides were predicted to be present.

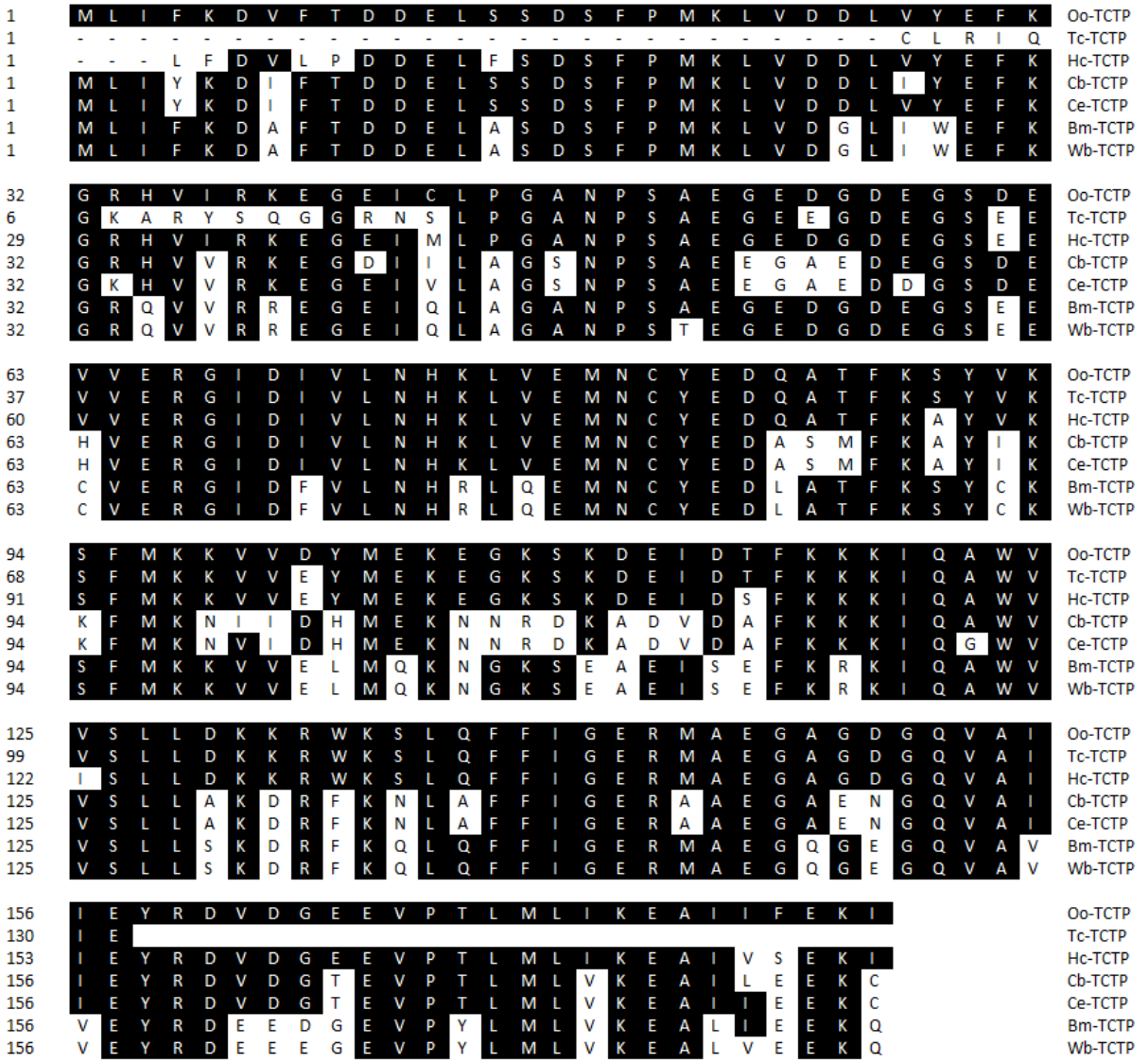

Figure 5.1 Alignment of the (predicted) amino acid sequences of TCTP from different nematodes. Database cluster/accession numbers are: Tc-TCTP *T. circumcincta (*TDC006592), Hc-TCTP *H. contortus* (HCC00192), Cb-TCTP *C. briggsiae* (CAP23314), Ce-TCTP *C. elegans* (CAB02099), Bm-TCTP *B. malayi* (EDP33421) and Wb-TCTP *W. bancrofti* (AAK71499). Amino acids matching those of Oo-TCTP are shaded in black.

5.3.2 Stage-specific transcription

The results of the semi-quantative PCR reaction are shown in Figure 5.2. Transcription of the reference gene *rpl-13a* (160 bp band) was stable throughout the different life stages. Limited levels of *Oo-tctp* transcripts (350 bp band) were detected in L3s both before and after exsheathing (lane 1 and 2) and a slight increase in transcription could be seen in L4s (lane 3).

In the adult stage Oo-TCTP was abundantly transcribed in female worms and eggs. No transcripts could be detected in adult males.

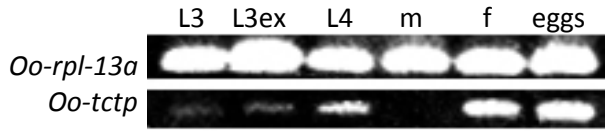

Figure 5.2 Stage-specific transcription pattern of the reference gene *Oo-rpl-13a* and *Oo-tctp* in *O. ostertagi* L3s, exsheathed L3s (L3ex), L4s, adult males (m), adult females (f) and eggs.

5.3.3 Stage-specific translation

Figure 5.3 shows the Western blot analysis which confirmed the result of the semi-quantative PCR. Limited amounts of Oo-TCTP were detected in L3s, L4s and adult males while Oo-TCTP was abundantly expressed in eggs. Small amounts of Oo-TCTP could be detected in ES material of adult worms.

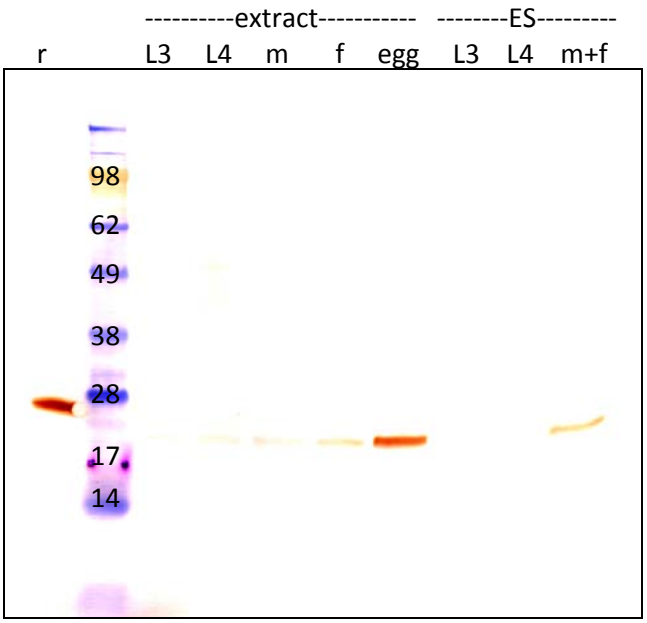

Figure 5.3 Detection of Oo-TCTP in extract and ES material of *O. ostertagi* L3s, L4s and adult male (m) and female worms (f) on a 10% SDS-PAGE under denaturing/reducing conditions using Western blotting and rabbit anti-Oo-TCTP antibodies. (r = *E. coli* recombinant of Oo-TCTP (positive control)). MWs of standards are presented in kDa.

5.3.4 Immunolocalization in *O. ostertagi*

Monospecific rabbit antibodies against Oo-TCTP were used to localize TCTP in sections of adult female *Ostertagia* worms (Figure 5.4). The negative control slides which were treated with pre-immune rabbit serum did not show any fluorescence. In contrast, sections incubated with monospecific antibodies demonstrated the presence of Oo-TCTP in the eggs.

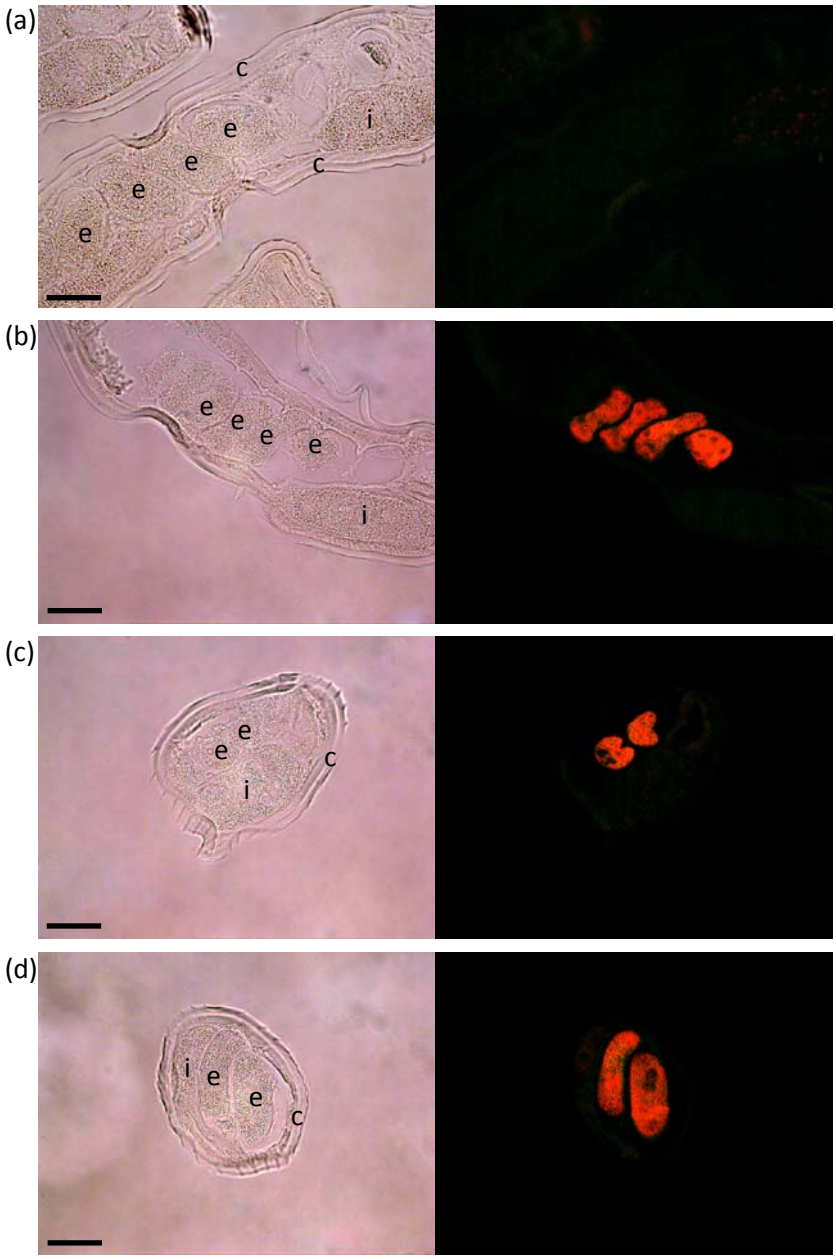

Figure 5.4 Detection of Oo-TCTP in sections of adult female *O. ostertagi* worms. (a) Negative control incubated with pre-immune rabbit serum. (b-d) Sections incubated with monospecific rabbit anti-Oo-TCTP. (e = egg; c = cuticle; i = intestine). Bar = 25μm.

5.3.5 Antibody response of infected cattle

Ostertagia egg extract was subjected to 2-D SDS-PAGE analysis followed by Coomassie staining (Figure 5.5a). Two abundant spots were detected between pI 6 and 7 with a MW of around 20 kDa. Multiple fainter spots were present between pI 4 and pI 9 with molecular masses varying from 5 to 60 kDa. Finally, a group of high MW proteins was detected with pI ranging from 4 to 6. Probing the 2-D SDS-PAGE Western blot with monospecific anti-Oo-TCTP antibodies rendered a spot at 20 kDa with pI 4.5 (Figure 5.5b). No proteins were recognized on the blot treated with serum obtained from pre-immune animals (Figure 5.5c). Incubation with serum from naturally infected animals resulted in recognition of numerous proteins above 40 kDa with pI 3 to 6. No signal was detected in the region of Oo-TCTP (Figure 5.5d).

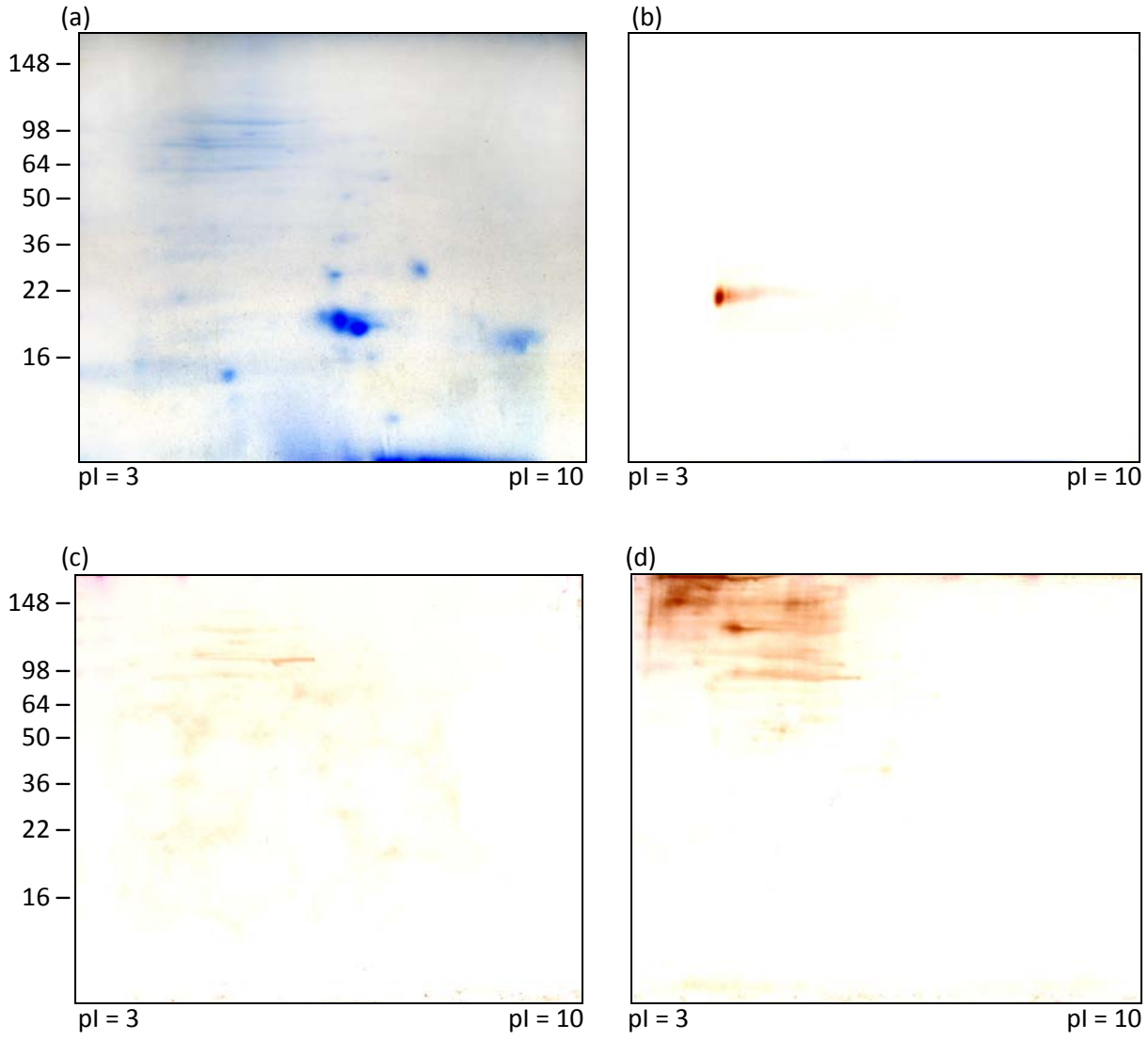

Figure 5.5 2-D SDS-PAGE analysis of *O. ostertagi* egg extract by (a) Coomassie Blue staining and (b) Western blotting using monospecific rabbit anti-Oo-TCTP and serum from (c) pre-immune vs. (d) immune cattle. MWs of standards are presented in kDa.

5.3.6 Detection in eggs from different worm species

The protein profile of egg extract obtained from different worms is shown in Figure 5.6a. Profiles for *C. elegans*, *O. ostertagi*, *Teladorsagia circumcincta*, *Cooperia oncophora*, *Haemonchus contortus* and *Ascaris suum* contain several high MW bands between 70 and 140 kDa which appear to be conserved. In contrast, the peptide profile for the trematode *F. hepatica* is composed of a different array of protein bands.

Western blot analysis indicated cross-reactivity of anti-Oo-TCTP antibodies with TCTP from other worm species and detected its presence in eggs from *C. elegans*, *O. ostertagi*, *T. circumcincta*, *C. oncophora* and *H. contortus* (Figure 5.6b). No bands were found in egg extracts from *A. suum* and *Fasciola hepatica*.

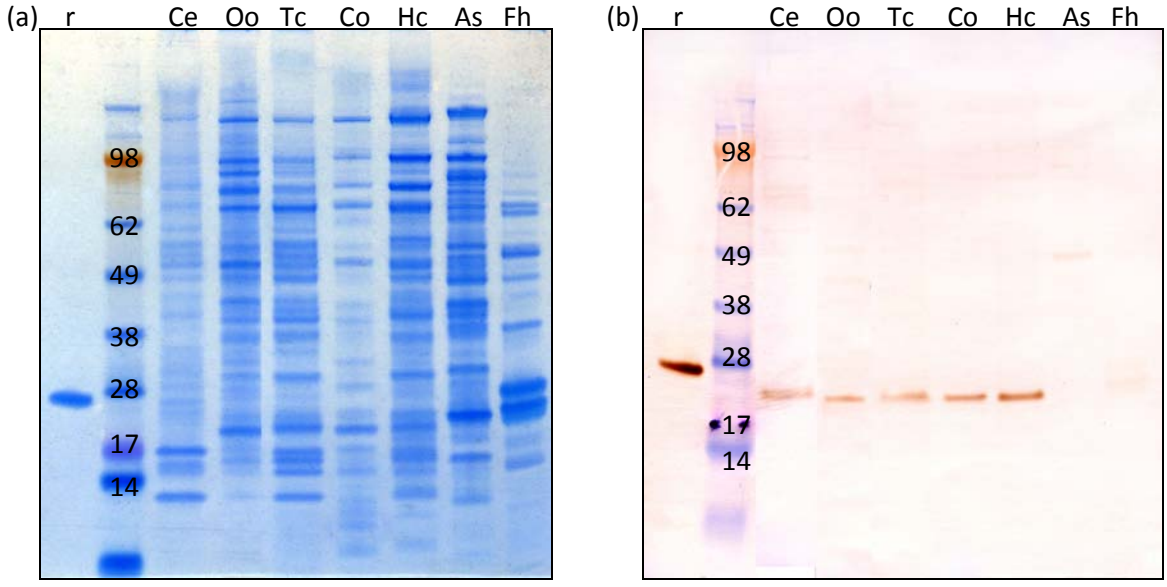

Figure 5.6 (a) Comparison of the protein profile of egg extract from *C. elegans* (Ce), *O. ostertagi* (Oo), *T. circumcincta* (Tc), *C. oncophora* (Co), *H. contortus* (Hc), *A. suum* (As) and *F. hepatica* (Fh) on a 10% SDS-PAGE under denaturing/reducing conditions, visualized by Coomassie Blue staining. (b) Detection of TCTP in egg extract by Western blotting using monospecific rabbit anti-Oo-TCTP. MWs of standards are presented in kDa. (r = *E. coli* recombinant of Oo-TCTP (positive control)). MWs of standards are presented in kDa.

5.3.7 Immunolocalization in *C. elegans*

Cross-reacting monospecific antibodies against Oo-TCTP were used to localize TCTP in freeze-crack preparations of adult hermaphrodite *C. elegans* worms. The negative control slides which were treated with pre-immune rabbit serum did not show any fluorescence

(Figure 5.7a). In correspondence with the immunolocalization results in *Ostertagia* worms, fluorescence was specifically detected in the eggs.

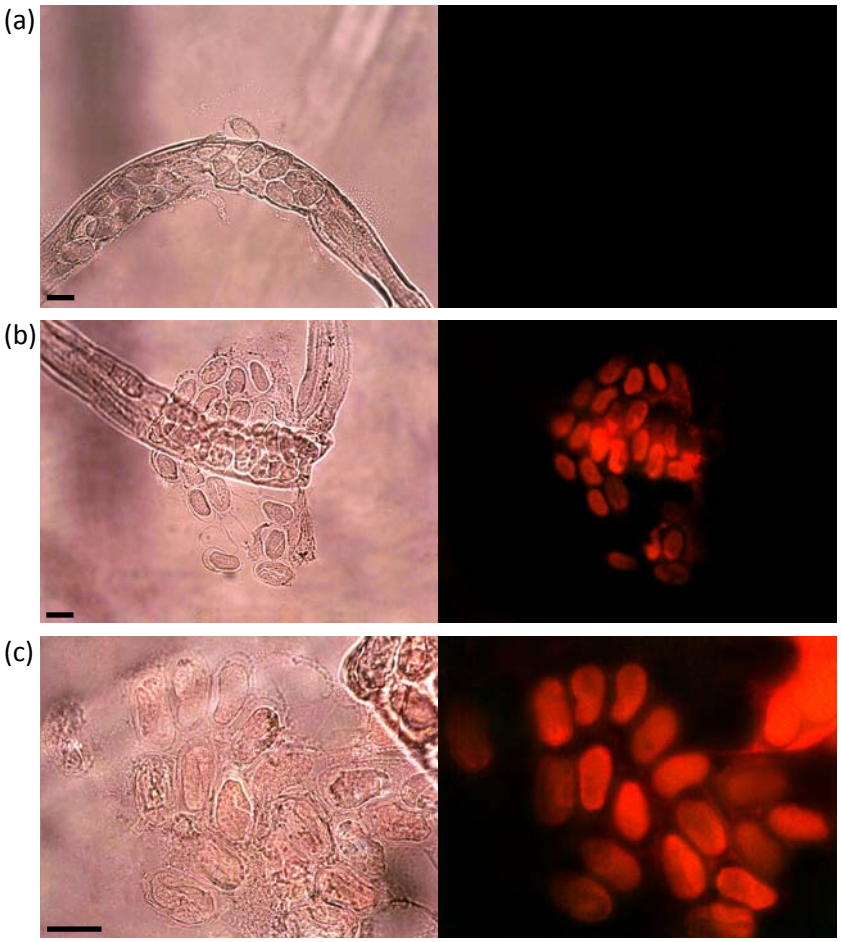

Figure 5.7 Detection of TCTP in freeze-crack preparations of adult *C. elegans* worms. (a) Negative control incubated with pre-immune rabbit serum. (b-c) Slides incubated with monospecific rabbit anti-Oo-TCTP. Bar = 25 μm.

5.4 Discussion

Based on the observations made for other parasite species where TCTP is highly secreted and capable of modulating the host immune response, we have studied the potential role of *Ostertagia* TCTP in affecting the immune response of cattle. Although Oo-TCTP was detected in ES material and the protective ES-thiol fraction of *O. ostertagia*, we have not found any evidence of active secretion by the parasite. Western blot analysis showed that Oo-TCTP was typically expressed in eggs, while only limited amounts were detected in ES material (Figure 5.3). Because adult *Ostertagia* worms tend to release their eggs in the RPMI medium during

ES collection (unpublished results) Oo-TCTP is likely to be a by-product due to leakage from or damage of eggs. Moreover, naturally infected cattle did not appear to develop an antibody response against *Ostertagia* TCTP (Figure 5.2), while multiple studies have acknowledged the immunogenicity of parasite TCTP (Walker *et al.*, 2000; Gnanasekar *et al.*, 2002; Roa *et al.*, 2002). This further suggests that the host does not come into contact with TCTP during infection.

When comparing the amino acid sequences of nematode TCTPs it is obvious that this antigen is highly conserved (Figure 5.1). Nevertheless, several amino acids differ between *Brugia* and *Wuchereria* TCTP and TCTP from other nematodes. These subtle differences might be a result of evolutionary adaptation and might explain why some parasite TCTPs are actively secreted during host infection while others are not. Furthermore, the immunomodulating characteristics of TCTP appear to be typical for parasites that - at one point during their life cycle - migrate through the host organism via the bloodstream or other tissues. The benefit for the parasite of being able to secrete an antigen that mimics an immunomodulator of the host will be more pronounced when released within the host organism as opposed to the 'external' environment of the gastrointestinal tract. This could explain why the secretion of TCTP has been optimized in Clade III nematodes (Blaxter *et al.*, 1998).

The transcription and translation profile of Oo-TCTP indicated limited expression in early developmental stages and adult male worms, while expression was upregulated in adult female worms, specifically in eggs. Correspondingly, Western blotting and immunolocalization in *O. ostertagi* detected the presence of TCTP in eggs. Previous studies have shown that TCTP is able to promote growth and proliferation in *Drosophila* (Hsu *et al.*, 2007), *Xenopus*, murine and human cells (Kubiak *et al.*, 2008). Moreover, an upregulation of TCTP has been described in maturing oocytes of mice (Vitale *et al.*, 2007) and in fertilized eggs and during early development of cephalochordates, a sister group of the vertebrates (Chen *et al.*, 2007). Furthermore, cloning studies involving bovine oocytes have demonstrated that TCTP has a beneficial effect on the potential of bovine somatic cell nuclei to develop into normal calves (Tani *et al.*, 2007). Hence, it is possible that Oo-TCTP fulfills a similar developmental or reproductive function in *Ostertagia*. Moreover, the results from the Western blot demonstrated that TCTP was also present in egg extract of *C. elegans*, *H. contortus* and *T. circumcincta* which could suggest a conserved function among the Clade V nematodes.

An interesting technique for studying the importance of *Ostertagia* TCTP would be RNA interference (RNAi). Unfortunately, previous studies in *Ostertagia* and other parasitic helminths have demonstrated the limited efficiency and specificity of this technique making

it unreliable to evaluate gene functions (Visser *et al.*, 2006; Geldhof *et al.*, 2007). However, the results obtained here show that *Ostertagia* TCTP displays significant homology with *C. elegans* TCTP both on sequence level and on protein localization. Hence, it is likely that TCTP has a similar function in these organisms. The advantages of the *C. elegans* model for studying the function and regulation of nematode genes have already been described extensively (reviewed by Britton and Murray, 2006). For these reasons, a comparative study of TCTP in *C. elegans* will be conducted in the next chapter.

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Chapter 6

Study of the translationally controlled tumor protein (TCTP) in *Caenorhabditis elegans*

Based on: Meyvis, Y., Houthoofd, W., Visser, A., Borgonie, G., Vercruysse, J., Claerebout, E., Geldhof, P. The translationally controlled tumor protein suppresses p53-dependent apoptosis in *Caenorhabditis elegans* germ cells during oocyte development. Dev Biol, submitted.

6.1 Introduction

In the previous chapter, we have reported that the translationally controlled tumor protein (TCTP) of *Ostertagia ostertagi* is not actively secreted during parasitism and does not appear to have a parasite-specific function. In contrast, TCTP was detected in eggs of several Clade V parasites and eggs from the model nematode *Caenorhabditis elegans*. So far, functional studies of TCTP have been conducted in human cell lines as well as murine and yeast models (reviewed by Bommer and Thiele, 2004; Chen *et al.*, 2007; Hsu *et al.*, 2007; Tani *et al.*, 2007; Vitale *et al.*, 2007; Kubiak *et al.*, 2008). However, the biological role of TCTP in nematodes remains unknown. Several large-scale RNA interference (RNAi) studies in *C. elegans* suggest that Ce-TCTP (referred to as Ce-TCT-1; accession number NP_492767) is involved in growth and reproduction (Fraser *et al.*, 2000; Simmer *et al.*, 2003; Rual *et al.*, 2004; Balklava *et al.*, 2007; Ceron *et al.*, 2007).

In this chapter, we will further investigate the function of TCT-1 in *C. elegans* in order to get a better understanding of its importance in Clade V nematodes. In addition, we will evaluate the possible role of TCTP in the process of programmed cell death or apoptosis as has been described in yeast and mammals (Li *et al.*, 2001; Tuyder *et al.*, 2002; Zhang *et al.*, 2002; Graidist *et al.*, 2004; Liu *et al.*, 2005; Yang *et al.*, 2005; Rinnerthaler *et al.*, 2006; Chen *et al.*, 2007; Susini *et al.*, 2008).

Previous research focused on apoptosis in *C. elegans* has shown that 131 out of 1090 somatic cells as well as 50% of the developing germ cells are destined to undergo apoptosis (Sulston and Horvitz, 1977; Sulston *et al.*, 1983; Gumienny *et al.*, 1999). Genetic and biochemical studies of these cells have allowed researchers to identify the key players in the apoptotic pathway (reviewed by Blum *et al.*, 2008). The core pathway consists of a canonical arrangement of three cell death genes - i.e. *ced-9*, *ced-4* and *ced-3* - which are under control of several pro- and anti-apoptotic proteins (Figure 6.1).

In short, the Bcl-2 like protein CED-9 is responsible for preventing apoptosis by retaining CED-4 (apoptosis protease activating factor-1) in the outer mitochondrial membrane. In the classical pathway - typically active in somatic cells - internal and external signals are able to induce transcription of the *egl-1* gene. When EGL-1 binds to CED-9, it causes a conformational change of CED-9 hereby inhibiting the interaction between CED-9 and CED-4. This allows for CED-4 to undergo oligomerization and promote activation of the CED-3 caspase leading to cell death. In addition, apoptosis can be mediated through alternative non-canonical pathways which have been observed in germ cells, cephalic companion neurons, the tail-spike cell and the linker cell (reviewed by Blum *et al.*, 2008). Although these

pathways are not yet fully understood, some of the regulators involved have been identified e.g. *lin-35/Rb*, *egl-38*, *pax-2*, *dpl-1*, *pal-1* and *ceh-30*.

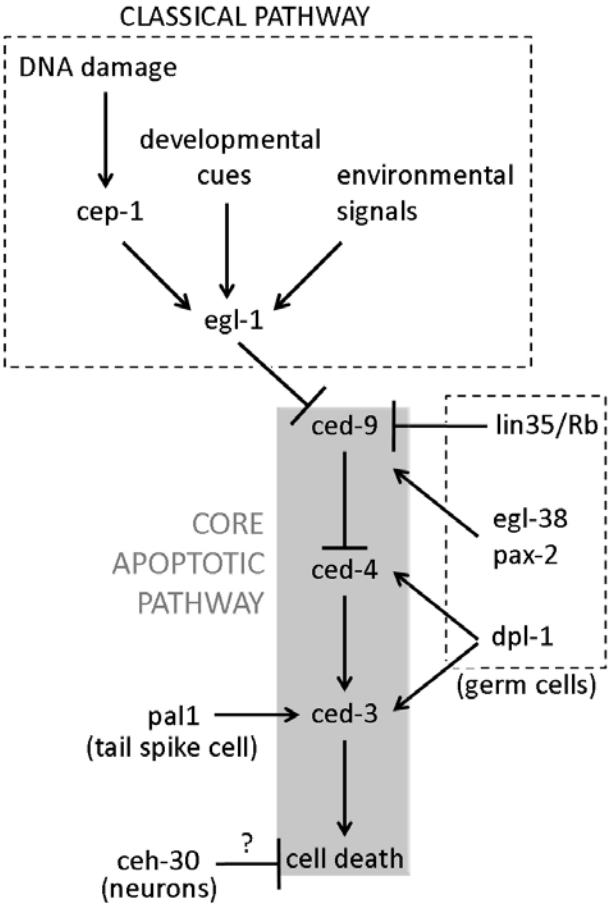

Figure 6.1 Apoptotic pathway in *C. elegans*.

6.2 Materials and methods

6.2.1 Stage-specific transcription

A semi-quantitative reverse transcriptase (RT) PCR was performed according to Geldhof *et al.* (2006) in order to determine the stage-specific transcription of *Ce-tct-1*. In short, RNA was extracted from first stage (L1), second stage (L2), third stage (L3) and fourth stage (L4) *C. elegans* larvae and adult worms using Total RNA Isolation Reagent (ABgene® Ltd) and dissolved in 10 µl DEPC treated water. A one-step RT-PCR reaction (Invitrogen) was run according to the manufacturer's protocol using 2.5 µl template RNA and *Ce-tct-1* specific primers (5'-ATGCTGATCTACAAGGATATTTTCAC-3' and 5'-GCAGTTCATCTCAACGAGCTT-3')

with 33 cycles of amplification (annealing temperature = 57°C). In order to account for sample-to-sample variation of the RNA isolates the transcription profile of the reference gene beta-tubulin 1 (*Ce-tbb-1*; accession number NM066966; 5'-AGGCCAACAATGGCAA ATAC-3' and 5'-AGTTCTGCTCCCTCGGTGTA-3') was included. The PCR reactions were loaded onto a 2.0% agarose gel and amplified PCR products were visualized with ethidium bromide.

6.2.2 GFP reporter constructs

Four different TCT-1 - green fluorescent protein (GFP) constructs were designed using the PCR-fusion based approach described by Hobert (2002) (Figure 6.2). A *C. elegans* fosmid clone containing the *Ce-tct-1* genomic sequence (WRM067bB09) provided by Professor Donald Moerman and Jaryn Perkins (Moerman Laboratory, Vancouver, Canada) served as a DNA template. A transcription amplicon was obtained using primers against the 1100 bp upstream region of the *Ce-tct-1* gene (5'-ACGTTGGATCCAAGCAAATGCAAGAGATTC GTC-3' and 5'-CAAGCAAATGCAAGAGATTCGTC-3'). Three translation amplicons were created including an upstream promoter region of 1100 bp (5'-ACGTTGGATCCAAGCAAATGCAAGAGA TTCGTC-3'), 544 bp (5'-TTCGCCAAGTTTACGATGATGGAACCC-3') and 241 bp (5'-CTCCAGCCG CCAAGAAGGAAGAGCC) together with the full-length *Ce-tct-1* gene and a 21 bp tag (5'-AGTCGACCTGCAGGCATG CAAGCTGCACTTCTCCTCGATGATGGC-3') complementary to the standard Fire Lab GFP-polylinker region (http://www.addgene.org/Fire_Lab). The GFP amplicon was obtained from the Fire Lab vector pPD95.75 using forward primer 5'-AGCTTGCATGCCTGCAGGTCG-3' and reverse primer 5'-AAGGGCCCGTACGGCCGACTAGTAGG-3'. A subsequent nested PCR was performed for fusing the 4 PCR products to the GFP tag (Hobert, 2002). The resulting GFP-constructs were gel purified and dissolved in ultra-pure water to a final concentration of around 500 ng/μl.

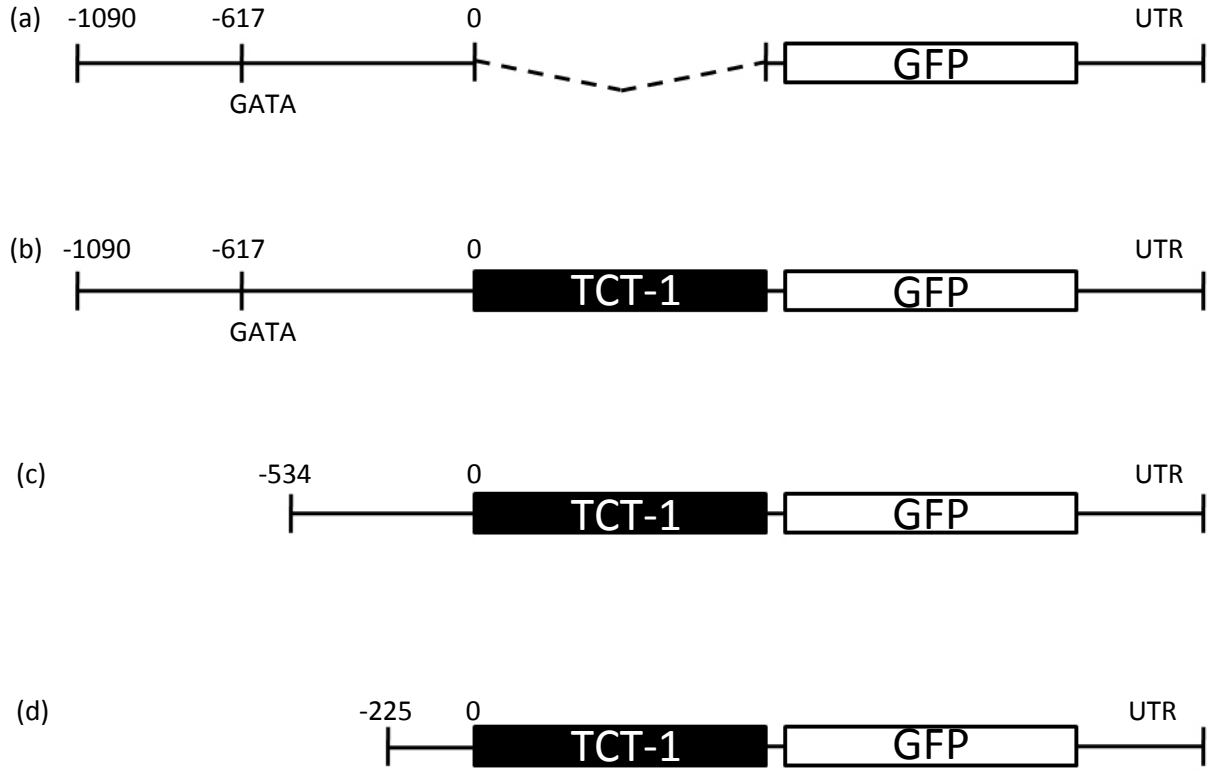

Figure 6.2 Schematic representation of GFP reporter constructs. (a) Transcription construct containing 1090 bp upstream promoter region. (b) Translation constructs containing the *Ce-tct-1* gene and 1090 bp promoter region, (c) 534 bp promoter region and (d) 225 bp promoter region. The location of the GATA DNA motif present in the promoter region is indicated. (UTR = untranslated region).

6.2.3 Transgenic animals

Wild-type (WT) N2 Bristol *C. elegans* worms were injected with GFP constructs (50 ng/µl DNA) as previously described by Mello and Fire (1995). The dominant injection marker rol-6 (50 ng/µl DNA) was included as a positive control. Injected constructs form large extrachromosomal arrays which are passed on to the next generation with 10-90% efficiency. Transformed animals were selected based on the dominant rol-6 phenotype and stable transmission was examined from the F_3 generation by epifluorescence and confocal microscopy at 488 nm (Nikon Eclipse TE2000-S).

6.2.4 RNAi setup

An RNAi experiment was carried out on WT N2 Bristol *C. elegans* worms according to Geldhof *et al.* (2006). In short, total RNA from adult *C. elegans* worms was prepared using

Trizol and subsequently converted into complementary DNA (cDNA) according to the manufacturer's protocol (Invitrogen). A 240 bp fragment of the *Ce-tct-1* gene (F25H2.11) was PCR amplified using the specific forward (5'-ATGCTGATCTACAAGGATATT TTCAC-3') and reverse primer (5'-GCAGTT CATCTCAACGAGCTT-3'). The fragment was ligated into the L4440 RNAi vector (Fire Lab) and double-stranded RNA (dsRNA) was prepared using the T7 Ribomax Express RNAi system (Promega). L2 worms were soaked in a mixture of lipofectin and dsRNA (1 mg/ml) for 24h (25°C). Control worms were incubated with dsRNA prepared from *O. ostertagi asp-1* (accession number AJ310812). The larvae were transferred to NGM plates seeded with an OP50 *Escherichia coli* strain and allowed to grow until the adult stage (25°C). At this point (t = 0h) knock down of the *Ce-tct-1* gene was checked by RNA preparation and subsequent semi-quantitative RT-PCR as described in section 6.2.1.

In order to obtain additional information about the phenotype resulting from TCT-1 knock down and gain insight in the position of TCT-1 in the apoptotic pathway, this protocol was repeated using three mutant *C. elegans* strains, i.e. *cep-1(gk138)* (deficient in the apoptosis initializing CEP-1/p53 protein), *ced-9(gf)(n1950)* (gain-of-function mutation which completely blocks CEP-1/p53 dependent germ cell apoptosis) and *ced-3(n717)* (deficient in the apoptosis effector caspase CED-3).

6.2.5 Reproduction

RNAi-treated young adult WT worms (t = 0h; n = 15) were transferred to a NGM 12-well plate. After 16h (25°C) the number of eggs and larvae was counted for each worm (mean ± standard error). A non-parametric one-tailed Mann-Whitney U-test for pairwise comparison was used to determine whether a significant reduction in brood size could be observed in knock down worms (probability (P) < 0.01). This analysis was repeated for the young adults of the F_1 generation. Additionally, embryogenesis of 3 specimens of the F_1 generation was monitored using a four dimensional (4-D) Axionplan 2 microscope with Nomarski interference (Zeiss) in a time-lapse course. Every 30 seconds, a Z-stack of 30 images was recorded with a Hamamatsu Newvicon camera (C2400-07) at a constant temperature of 20°C. These images were analyzed in the Simi Biocell software (version 4.0, Simi Gmbh, D-85705 Unterschleissheim, Germany) (Schnabel *et al.*, 1997). The division pattern or cell lineage of the early embryonic development until 50-cells was reconstructed. Timing of divisions and 3-D positions of all the cells were compared to the embryogenesis of untreated WT *C. elegans*.

6.2.6 Morphology

The morphology of the reproductive tract of RNAi-treated WT, *cep-1*, *ced-9(gf)* and *ced-3* adult worms was investigated by propidium iodide (PRI) staining according to the manufacturer's protocol (Molecular Probes; t = 16h). For each group and condition around 50 worms were fixed in boiling formaldehyde (4%) solution followed by 2h incubation at room temperature. Worms were washed twice with 2XSSC buffer (0.30 M sodium chloride; 0.030 M sodium citrate; pH 7.0) and treated with RNase A (100 μg/ml) for 20 min (37°C). Afterwards, worms were washed again with 2XSSC buffer followed by incubation with PRI (Molecular Probes; 4 μg/ml) for 3 min. Stained worms were transferred to a glass slide treated with poly-L-lysine and investigated by confocal microscopy at 543 nm (Nikon Eclipse TE2000-S).

6.2.7 Detection of germ cell apoptosis

RNAi-treated WT, *cep-1*, *ced-9(gf)* and *ced-3* adults worms were screened for germ cell apoptosis using an acridine orange (AO) staining at different points in time (t = 0h / 5h / 10h / 16h / 24h) according to Lettre *et al.* (2004). In short, worms were stained for 1h in the dark by adding 500 μl M9 buffer with AO (Molecular Probes; 0.020 mg/ml) to seeded NGM plates followed by 1h destaining on seeded NGM plates lacking AO. Worms were immobilized on agar pads containing sodium azide (15 mM) and the number of apoptotic germ cells present in one gonadal arm was determined (n = 10) by confocal microscopy at 488 nm (Nikon Eclipse TE2000-S). A non-parametric one-tailed Mann-Whitney U-test for pairwise comparison was used to determine whether a significant increase in apoptotic germ cells could be observed in knock down worms ($P < 0.01$).

6.3 Results

6.3.1 Stage-specific transcription

A semi-quantitative RT-PCR was performed to determine the temporal transcription pattern of *Ce-tct-1* during development. The results are shown in Figure 6.3. Transcription of the control gene *Ce-tbb-1* was consistent throughout the different life stages of *C. elegans* (180 bp band). No *Ce-tct-1* transcripts could be detected in the L1, L2 and L3 stage. Transcription of *Ce-tct-1* appeared to be initiated in the L4 stage (240 bp band) while the highest level of transcription was found in the adult stage.

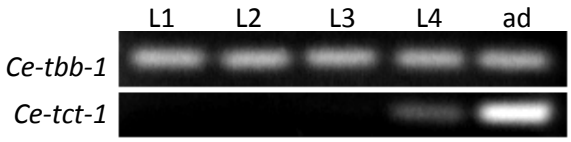

Figure 6.3 Stage-specific transcription pattern of the reference gene *Ce-tbb-1* and *Ce-tct-1* in L1, L2, L3 and L4 C*. elegans* larvae and adult worms (ad).

6.3.2 GFP reporter constructs

The spatial and temporal expression of TCT-1 was further examined by injecting WT worms with different GFP reporter constructs. A transcription construct was used to localize cells responsible for TCT-1 production. No fluorescence could be detected in the L1, L2 and L3 stage (results not shown). In contrast, transgenic worms displayed fluorescence in the L4 and adult stage. The intensity of the GFP signal was the highest in adult worms. A representative example is shown in Figure 6.4. Expression of TCT-1 was localized in the head and tail region of the worm in the vicinity of the gonad bends (Figure 6.4a), more specifically the first two cells forming the intestine (behind the terminal bulb of the pharynx) (Figure 6.4b) and the two final cells of the intestine (anterior of the anal depressor muscle) (Figure 6.4c). In addition, three translation constructs of TCT-1 were used in an attempt to determine its final destination after production. All constructs rendered identical results corresponding with the expression profile of the transcription construct (results not shown).

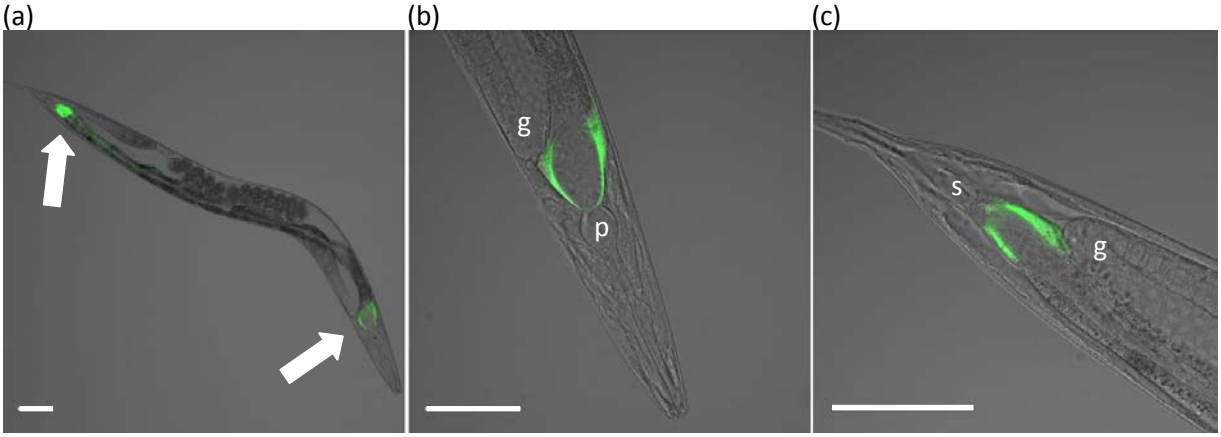

Figure 6.4 (a) Transgenic worm showing Ce-TCT-1::GFP fusion protein expression. GFP expression is observed in the L4 and adult stage. Fluorescence was detected in both ends of the worm (indicated by arrows). (b) Close-up of two intestinal cells producing TCT-1 near the pharynx (p). (c) Close-up of two intestinal cells producing TCT-1 before the anal sphincter (s). Production of TCT-1 occurred in the vicinity of the gonad bend (g). Bar = 60 μm.

6.3.3 Effect of RNAi on reproduction

In order to evaluate the function of *Ce-tct-1*, RNAi was performed to inhibit TCT-1 expression. Successful RNAi treatment was confirmed at the transcriptional level by subjecting adult worms to a semi-quantitative RT-PCR (Figure 6.5). All worms of the RNAi experiment showed consistent transcription of the control gene *Ce-tbb-1* (180 bp band). Transcription of *Ce-tct-1* in worms treated with *asp-1* control dsRNA was unaffected, while worms treated with *tct-1* dsRNA displayed a strong reduction in the number of *Ce-tct-1* transcripts (240 bp band).

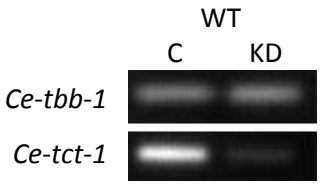

Figure 6.5 Effect of RNAi treatment on transcription pattern of the reference gene *Ce-tbb-1* and *Ce-tct-1* in WT *C. elegans* worms. (C = negative control group treated with *asp-1* dsRNA; KD = knock down group treated with *tct-1* dsRNA).

The brood size of young WT adult worms after RNAi was quantified in a time interval of 16h. The results are summarized in Table 6.1. After 16h, the *tct-1* treated worms of the F_0 generation had generated 90% less eggs and 83% less larvae compared to control worms. Similarly, the F_1 generation of *tct-1* treated worms produced 72% less eggs and 94% less larvae compared to control F_1 worms. Embryonic development of deposited eggs was investigated under a 4-D microscope in a time-lapse course. No difference in morphology or growth rate was observed between eggs from the control group and the knock down group. The eggs hatched at normal time point and produced fertile offspring (results not shown).

Group	n		F_0 generation (mean ± s.e.)	Reduction (%)		F_1 generation (mean ± s.e.)	Reduction (%)
C	15	e	57 ± 3.5	---	e	60 ± 4.8	---
		l	7.1 ± 0.9		l	6.2 ± 1.3	
KD	15	e	5.8 ± 1.4 *	90	e	17 ± 2.5 *	72
		l	1.2 ± 0.5 *	83	l	0.4 ± 0.2 *	94

Table 6.1 Effect of RNAi treatment on progeny size of WT *C. elegans* worms (t = 16h). (C = negative control group treated with *asp-1* dsRNA; KD = knock down group treated with *tct-1* dsRNA; e = number of eggs; l = number of larvae; s.e. = standard error; * = P < 0.01).

6.3.4 Effect of RNAi on morphology

In order to explain the reduced brood size observed in TCT-1 knock down worms, the morphology of the reproductive tract was monitored using a PRI staining. PRI is a DNA intercalating fluorescent agent which can be used to stain cell nuclei, hereby visualizing general worm morphology as well as germ cells, oocytes and spermatheca. The reproductive tract of *C. elegans* consists of two U-shaped gonads ending in a shared uterus (Figure 6.6a). In short, germ cells present in the ovaries undergo meiosis while migrating through the gonad bend. Germ cells differentiate into oocytes which mature in an assembly-line like manner. Mature oocytes are squeezed through the spermatheca and are fertilized. Eggs undergo a brief embryonic development, followed by deposition through the vulva around the 40-cell stage. An example of a PRI staining of WT worms (t = 16h) is shown in Figure 6.6b and 6.5c. While the total number of germ cells present in the distal part of the gonad did not appear to be affected by TCT-1 knock down, maturing oocytes in the proximal part showed structural differences compared to those of the controls, demonstrating an increased amount of cytoplasmic granules and disintegration of the plasma membrane (Figure 6.6b). The uterus of control worms contained multiple developing eggs with an increasing number of nuclei present in eggs near the vulva. In contrast, the uterus of TCT-1 knock down worms was filled with unicellular eggs containing one large ball-shaped nucleus (Figure 6.6c). In addition, some worms displayed larval hatching inside the uterus.

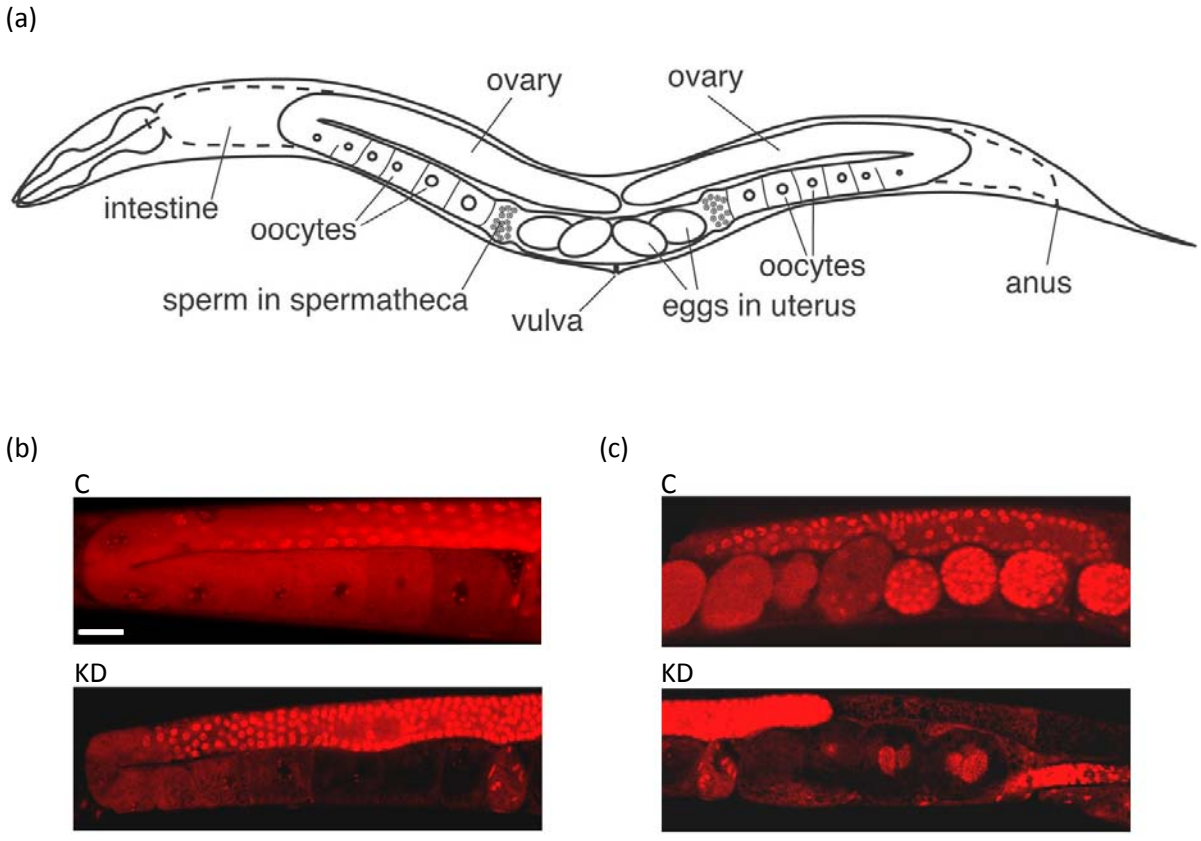

Figure 6.6 (a) Schematic representation of the reproductive tract of an adult hermaphrodite *C. elegans* worm (Zarkower, 2006). (b) Close-up of gonad bend (b) and uterus (c) of RNAi-treated WT *C. elegans* worms after fixation and PRI staining (t = 16h). (C = negative control group treated with *asp-1* dsRNA; KD = knock down group treated with *tct-1* dsRNA). Bar = 20 μm.

6.3.5 Effect of RNAi on germ cell apoptosis

To investigate whether the observed reduction in fecundity after RNAi is caused by apoptosis, an AO staining was performed of RNAi-treated WT worms (t = 0h, 5h, 10h, 16h and 24h). After digestion of stained bacteria, AO accumulates in apoptotic corpses of the germ cell line. From t = 10h, a significant increase in germ cell apoptosis could be observed in worms treated with *tct-1* dsRNA compared to control worms (Figure 6.7). The number of apoptotic bodies present in the gonads was counted and the results are summarized in Table 6.2 (p. 114). Worms treated with *tct-1* dsRNA showed an average of 3.3 apoptotic bodies per gonad vs. 0.4 in control worms.

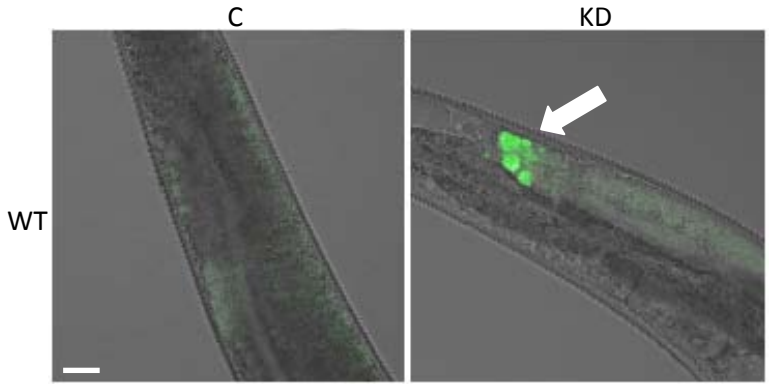

Figure 6.7 Pictures of the gonad bend of RNAi-treated WT *C. elegans* worms after AO staining. Apoptotic germ cells are seen as highly refractile green fluorescent discs (indicated by arrows). (C = control group treated with *asp-1* dsRNA; KD = knock down group treated with *tct-1* dsRNA). Bar = 10 μm.

6.3.6 Position of TCT-1 in the apoptotic pathway

In order to further investigate the position of TCT-1 in the apoptotic pathway, RNAi treatment was repeated using *cep-1*, *ced-9(gf)* and *ced-3* mutants. Successful RNAi treatment was confirmed at the transcriptional level by subjecting adult worms to a semi-quantitative RT-PCR (Figure 6.8). Again, all worms showed consistent transcription of the control gene *Ce-tbb-1* (180 bp band). Transcription of *Ce-tct-1* in worms treated with *asp-1* control dsRNA was unaffected, while worms treated with *tct-1* dsRNA displayed a clear reduction in the number of *Ce-tct-1* transcripts (240 bp band).

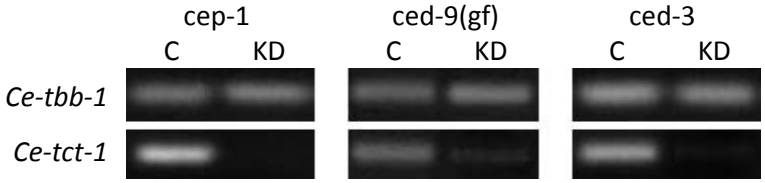

Figure 6.8 Effect of RNAi treatment on transcription pattern of the reference gene *Ce-tbb-1* and *Ce-tct-1* in *cep-1*, *ced-9(gf)* and *ced-3* mutant *C. elegans* worms. (C = negative control group treated with *asp-1* dsRNA; KD = knock down group treated with *tct-1* dsRNA).

TCT-1 knock down in *cep-1* mutants resulted in a similar phenotype as seen in WT *tct-1* treated worms (results not shown) and a significant increase in apoptosis from 0.0 to 3.4 (Table 6.2). *Tct-1* treated *ced-9(gf)* and *ced-3* mutants displayed normal oocyte maturation and egg development (results not shown) and had no apoptopic bodies in the gonad bend

(Table 6.2). Representative examples of an AO staining of *cep-1*, *ced-9(gf)* and *ced-3* gonads are shown in Figure 6.9.

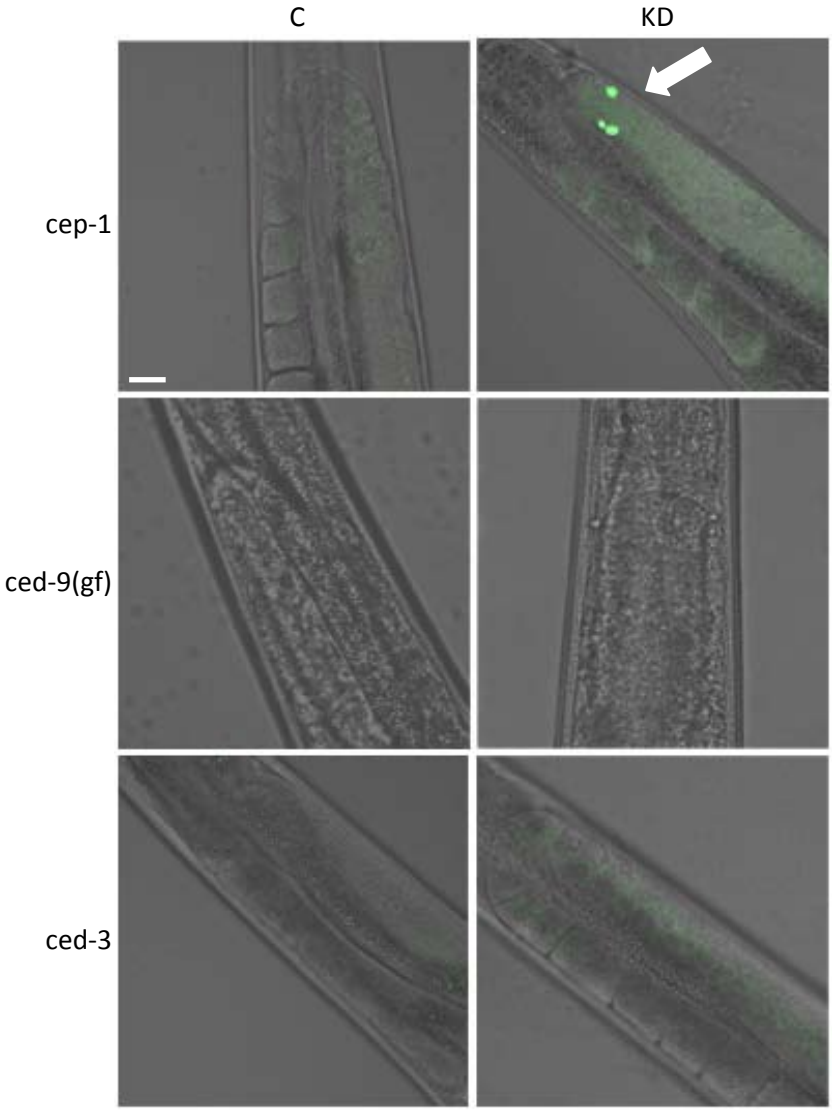

Figure 6.9 Pictures of the gonad bend of RNAi-treated *cep-1*, *ced-9(gf)* and *ced-3* mutant *C. elegans* worms after AO staining. Apoptotic germ cells are seen as highly refractile green fluorescent discs (indicated by arrows). (C = control group treated with *asp-1* dsRNA; KD = knock down group treated with *tct-1* dsRNA). Bar = 10 μm.

		# apoptotic bodies per gonad (mean ± s.e.)			
Group	**n**	**WT**	**cep-1**	**ced-9(gf)**	**ced-3**
C	10	0.4 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
KD	10	3.3 ± 0.7 *	3.1 ± 0.7 *	0.0 ± 0.0	0.0 ± 0.0

Table 6.2 Effect of RNAi treatment on the number of apoptotic bodies detected in the gonads after AO staining of WT, *cep-1*, *ced-9(gf)* and *ced-3* mutant *C. elegans* worms (t = 10h). (C = control group treated with *asp-1* dsRNA; KD = knock down group treated with *tct-1* dsRNA; s.e. = standard error; * = P < 0.01).

6.4 Discussion

In this chapter, we have demonstrated that *C. elegans* TCT-1 has a pivotal role in preventing germ cell apoptosis during oocyte differentiation and knock down of this gene has a major effect on worm reproduction.

Our RNAi experiment in WT *C. elegans* worms clearly demonstrated that TCT-1 knock down significantly increased apoptosis in germ cells present in the distal part of the gonad bend. This effect was maintained when treating worms deficient in the tumor suppressor protein p53 (CEP-1) indicating that TCT-1 acts downstream from *cep-1*. In contrast, germ cell apoptosis in *ced-9(gf)* and *ced-3* mutants was unaffected by TCT-1 knock down which suggests that TCT-1 operates within the p53-dependent apoptotic pathway between CEP-1 and CED-9 at the same level as EGL-1 (Figure 6.1). However, it still remains to be elucidated how TCT-1 is able to inhibit apoptosis in this step of the pathway. In mammals, TCTP directly interacts with anti-apoptopic members of the Bcl-2 family hereby preventing the activation of the pro-apoptopic protein Bax located in the mitochondrial membrane (Susini *et al.*, 2008). The apoptopic pathway in *C. elegans* does not involve such a Bax-like component, but depends on the direct interaction of CED-9 with CED-4 (reviewed by Domingos and Steller, 2007). Based on the role of TCTP in mammals, it is possible that also in *C. elegans* TCT-1 directly or indirectly interacts with CED-9 to stabilize the CED-9/CED-4 complex and prevents

uncontrolled germ cell apoptosis. The interaction between the Bcl-2 protein Bcl-xL and the mammalian TCTP is realized by the N-terminal BH3 domain in Bcl-xL and an N-terminal peptide sequence (amino acid 20-27) in TCTP which is conserved in all higher organisms (Yang *et al.*, 2005). However, these sequences are not conserved in either the Ce-TCT-1 or CED-9 (results not shown), so if the interaction postulated above occurs in *C. elegans*, it has to be mediated by different protein domains or an additional unknown component.

Previous large-scale RNAi screenings reported that knock down of TCT-1 resulted in a reduction in brood size, sterility and embryonic lethality (Simmer *et al.*, 2003; Rual *et al.*, 2004; Ceron *et al.*, 2007). These RNAi phenotypes are in line with the results from our study in which TCT-1 knock down dramatically affected the organism's reproductive capabilities reducing the number of eggs laid by the F_0 and F_1 generation with 90% and 72%. Furthermore, egg production was unaffected in RNAi-treated *ced-3* mutants indicating that the abnormal eggs observed in RNAi-treated WT worms and *cep-1* mutants are a direct result of increased germ cell apoptosis. Increased apoptosis would reduce the number of maturing oocytes and is likely to cause a reduction in egg production. In addition, the limited number of surviving oocytes would have to mature in the absence of so-called nursing oocytes which might explain the arrested development observed in eggs present in the uterus. A previous protein-protein interaction study in *C. elegans* based on the yeast-two-hybrid system suggested an interaction of TCT-1 with MAT-3 and DNC-2 (Li *et al.*, 2004). MAT-3 and DNC-2 play a key role in oogenesis respectively embryogenesis, hence it is possible that TCT-1 stabilizes these components during early development. However, since TCT-1 knock down in *ced-3* mutants did not result in abnormalities in oogenesis or embryogenesis it is not likely that this interaction is crucial.

Several RNAi reports also showed TCT-1 knock down to have a negative effect on growth rate (Fraser *et al.*, 2000; Rual *et al.*, 2004; Ceron *et al.*, 2007). However, no effect on growth was observed in any strain subjected to RNAi in this study. Moreover, development was unaffected in the progeny of RNAi-treated *ced-9(gf)* and *ced-3* mutants suggesting TCT-1 is not essential for promoting growth. This result stands in contrast with findings in *Drosophila*, *Xenopus* and murine and human cells where TCTP was found to have growth promoting potential in the first stages of development (reviewed by Bommer and Thiele, 2004; Chen *et al.*, 2007; Hsu *et al.*, 2007; Tani *et al.*, 2007; Vitale *et al.*, 2007; Kubiak *et al.*, 2008). From an evolutionary point of view, it is possible that the anti-apoptotic function of TCTP is conserved among eukaryotes, whereas evolutionary divergence could be responsible for loss of function in nematodes or gain of function in insects, amphibians or mammals such as

promoting growth, stimulating cell proliferation and inducing the release of histamine and certain cytokines (reviewed by Bommer and Thiele, 2004).

While knock down of Ce-TCT-1 was successful, a limited number of viable eggs were still produced by young adult hermaphrodites. These eggs were able to develop into adult worms without showing any abnormalities in growth. This might suggest that during maturation of the first oocytes transport of dsRNA is not yet fully operational, resulting in the presence of sufficient amounts of TCT-1 messenger RNA (mRNA) or protein for preventing apoptosis and allowing normal oocyte development. Expulsion of normal eggs might be hindered by the accumulation of abnormal eggs in the uterus, which would explain the presence of hatched larvae in some RNAi-treated worms.

GFP reporter constructs demonstrated that TCT-1 is produced by a limited number of intestinal cells, i.e. the first two cells after the pharynx and the last two cells before the anal sphincter. In correspondence, TCT-1 was previously identified in a mRNA screening targeting intestine-expressed *C. elegans* genes (Pauli *et al.*, 2006). Also, the promoter region of TCT-1 contains a GATA motif in cis orientation which is typical for intestinal genes. However, the two smallest translation constructs used in this study contained promoter regions that lacked this GATA motif. Nevertheless, the GFP expression pattern was not affected, which indicates the presence of additional promoter elements responsible for TCT-1 transcription and translation in these intestinal cells. In addition, a study of the expressed sequence tag (EST) database of the parasitic sheep nematode *Haemonchus contortus* showed that TCTP (HCC00192) was one of the most abundantly expressed genes with a substantial amount of ESTs (24 out of 59) originating from isolated parasite intestines (Geldhof *et al.*, 2005), suggesting a conserved expression pattern in nematodes.

The fact that TCT-1 fulfills a function in germ cells would require it to be transported from the intestine to the bend of the gonad. Interestingly, TCT-1 was produced in both ends of the adult worm in the vicinity of the gonad bend. Although TCTP is known to be transported and secreted outside the cell, the exact mechanism of transport is unknown. Because TCTP lacks a secretion signal in its sequence, a specialized transport system has been suggested (Bommer and Thiele, 2004). A genome-wide protein interaction search of *C. elegans* predicted TCT-1 to interact with the protein VHA-8 which is required for receptor-mediated endocytosis of yolk protein (Zhong and Sternberg, 2006). In this case, TCT-1 might be transported based on a similar mechanism as vitellogenin YP170 which is produced in the intestine and transported into developing oocytes (Grant and Hirsh, 1999) (Figure 6.10). Moreover, the presence of TCT-1 was detected in *C. elegans* eggs in chapter 5 using cross-reacting antibodies against Oo-TCTP (section 5.3.7). Unfortunately, the reporter constructs

used in this study failed to demonstrate TCT-1 transport outside the intestinal cells. Since TCT-1 is a rather small protein of 20 kDa the presence of a GFP tag of 27 kDa might prevent proper binding to a transport receptor, which would explain why no fluorescence was detected in developing oocytes and eggs.

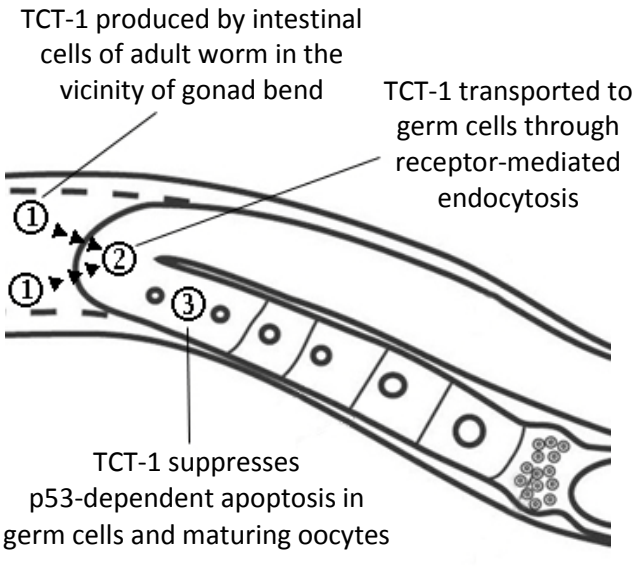

Figure 6.10 Suggested working mechanism of TCT-1 in *C. elegans.*

In chapter 5 we have demonstrated that Oo-TCTP is not an interesting vaccine candidate because it is present in *Ostertagia* eggs and is not accessible for the host immune system. However, Oo-TCTP might be an interesting drug target for interfering with parasite reproduction and hereby preventing pasture contamination. In vitro studies in *Plasmodium* have shown that the function of TCTP can be blocked by binding with artemisinin. Artemisinin has been successfully used as an anti-malarial drug (reviewed by Woodrow *et al.*, 2005) and its anti-schistosomal activity has been reported (reviewed by Utzinger *et al.*, 2007). Moreover, a *Plasmodium* strain with an increased expression of TCTP appeared to be more resistant to artemisinin treatment (Walker *et al.*, 2000). Dihydroartemisinin, the active metabolite of all artemisinin compounds, was also found to bind human TCTP which is considered to be a molecular target for many types of cancer (Fujita *et al.*, 2008). Therefore, the impact of artesunate - a water-soluble derivative of artemisinin - on *C. elegans* was investigated by soaking L2s and L3s for 24h in varying concentrations (0.1 - 0.5 - 1.0 mg/ml) and determining the progeny size after reaching the adult stage. However, no effect was

observed (results not shown). This lack of effect might be due to the limited intake of artesunate or its insufficient binding capacity with Ce-TCTP. Also, it is not certain whether binding to Ce-TCTP has phenotypic repercussions in this nematode. Since this is merely a preliminary result, the experimental setup to determine whether TCTP would be an appropriate drug target still requires further optimization such as testing several derivatives of artemisinin at different concentrations, increasing the soaking time or soaking L4s or adult worms.

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Chapter 7

General discussion and conclusions

The objective of this doctoral thesis was to identify protective antigens from the ES-thiol fraction of *Ostertagia ostertagi*, to evaluate their function and to determine the presence of immunogenic epitopes for further vaccine development. This chapter will discuss some interesting findings and future prospects as well as several important limitations we encountered in this project.

7.1 Identification of protective antigens

We have confirmed the protective capacity of ES-thiol in a vaccination trial for a third time and have subfractionated this protein mixture hereby separating the two most promising classes of vaccine candidates i.e. the activation-associated secreted proteins (ASPs) and the cysteine proteases (CPs). All subfractions rendered protection in cattle which indicates that they contain common protective antigens or that multiple protective antigens are present in ES-thiol.

7.1.1 ASP fraction

Although anion exchange chromatography rendered us one of the purest native ASP fractions that have been described to-date, proteomic analysis still indicated the presence of several antigens besides ASP1 and ASP2, such as ASP3, two unknown ASPs, ASP-like protein 1, a major sperm protein (MSP), a sperm-specific protein (SSP) and several excretory-secretory (ES) proteins. Hence, it still remains to be elucidated which of these antigens are responsible for inducing protection. Further purification steps based on size and hydrophobicity may provide a way to remove the remaining contaminants from ASP1 and ASP2 and confirm their protective capacity. Further separating ASP1 from ASP2 using standard chromatography methods will be difficult since they have the same size and a similar charge.

7.1.2 CP and rest fraction

Nearly all the CP activity present in ES-thiol was collected in one fraction. Protein profile analysis already demonstrated the presence of additional protein bands and this was confirmed by mass spectrometry (MS) which detected peptides from other proteins, among which the translationally controlled tumor protein (TCTP). Therefore, we are unable to attribute the induced protection solely to the CPs. In order to validate the protective potential of native CPs this antigen mixture requires further purification. One possible way of doing this would be by means of affinity chromatography. Previously, native CPs were successfully purified from the protective *Haemonchus contortus* thiol sepharose binding

protein (TSBP) fraction using a sepharose column coated with recombinant *Haemonchus* cystatin, the natural inhibitor of CPs (Redmond and Knox, 2004). The same cystatin was used in an attempt to purify the CPs present in *Ostertagia* ES-thiol, but no binding was observed (Geldhof *et al.*, unpublished results). Since cystatins from different nematode species display differences in amino acid sequence, it is possible that successful binding of *Ostertagia* CPs will only be realized when coating the column with recombinant *Ostertagia* cystatin.

Identifying the exact CPs present in the ES-thiol fraction turned out to be difficult. A previous screening of a complementary DNA (cDNA) database of *O. ostertagi* identified eight different CPs, six of which were expressed by adult worms (Geldhof *et al.*, unpublished results). Although small amounts of CPs suffice for detection on substrate gels or cathepsin L assays, MS analysis failed to detect any CPs. This can most likely be explained by the limited amount of CPs present in comparison to other 'contaminants' present in the CP fraction. If we are able to isolate the CPs as suggested, it may be possible to precipitate them and successfully perform tandem MS analysis.

The rest fraction remains the most complex protein mixture in our project, therefore it is tempting to avoid further study and focus our efforts on the ASP and the CP fraction. Interestingly, Western blot analysis demonstrated cross-reactivity between the rest fraction and the CP fraction. Since no common antigens could be identified in our MS analysis, further investigation is needed. Affinity chromatography using a sepharose column coated with antibodies from vaccinated animals may enable us to isolate common immunogenic antigens followed by identification using MS.

7.2 Anti-fecundity effect of vaccination

Vaccinating calves with ES-thiol or the subfractions resulted in a significant reduction in faecal egg counts (FEC). The same anti-fecundity effect has been observed in vaccination trials using a gut membrane protein fraction (Smith *et al.*, 2000) and the *Ostertagia* polyprotein allergen (OPA) fraction (Vercauteren *et al.*, 2004). Since these vaccines are obtained from different life-stages of *Ostertagia* and include worm extract as well as ES material, it is unlikely that all antigens are involved in parasite reproduction. In chapter 4, we identified several antigens in the ASP fraction that could have a function in reproduction e.g. vitellogenin, MSP and SSP. However, no reproduction-related antigens were found in the CP or the rest fraction. Therefore, it seems that interference with parasite fecundity is a secondary effect of vaccination. It is possible that the protective antigens in current vaccines are involved in the basic metabolism of the parasite or its defense against the host environment, and that targeting these components results in a general stress situation in

which the parasite chooses survival over egg production. Unfortunately, the function of most of these antigens is still unknown.

7.3 Functional analysis of antigens

In addition to isolating appropriate antigens for vaccine development, determining their function within the parasite or the host-parasite relationship will give us a better understanding of their importance and help us in the selection of parasite targets. However, functional analysis of parasite genes in *O. ostertagi* itself is hampered by several important drawbacks. First of all, the parasite can only be studied in its natural host which is less practical and implies more expensive and time-consuming protocols. Second, gene silencing techniques like RNA interference (RNAi) can not be used reliably in this parasite (Visser *et al.*, 2006; Geldhof *et al.*, 2007). Finally, attempts to develop an *in vitro* cell culture system for studying the function of *O. ostertagi* genes have not been successful so far (De Maere *et al.*, unpublished results).

The potential of the free-living nematode *Caenorhabditis elegans* as a model for functional analysis of nematode genes has been described extensively (reviewed by Britton and Murray, 2006). Parasitic nematodes and *C. elegans* display similarities in structure, development and reproduction. Comparative studies in *C. elegans* have characterized several conserved nematode genes such as a cathepsin L protease from *Haemonchus contortus* which is essential for embryonic development (Britton and Murray, 2002), the glutathione-S-transferase from *Onchocerca volvulus* which renders increased resistance to oxidative stress (Kampkötter *et al.*, 2003) and the metalloprotease *nas-37* from *H. contortus* which has a role in the moulting process (Davis *et al.*, 2004). In addition, *C. elegans* has been used for investigating the mechanisms of thiabendazole (Kwa *et al.*, 1995) and avermectin resistance in *H. contortus* (Yates *et al.*, 2003). In chapter 6, TCTP was found to have a reproductive function which appeared to be conserved between *O. ostertagi* and *C. elegans*. Although our results suggest that TCTP is not an appropriate vaccine candidate, it might still be an interesting drug target for disabling parasite reproduction.

Based on our results in chapter 6, *C. elegans* may prove to be a valid model system for determining the function of other ES-thiol antigens as well. However, since there are some important differences in basic biology and development between parasitic nematodes and the free-living nematode *C. elegans*, one has to be careful when extrapolating data from *C. elegans* to for example *O. ostertagi*. Parasite-specific genes are typically the result of a selection-based evolution and might therefore lack proper homologs in *C. elegans*. For example, a previous study of ASPs in *C. elegans* indicated limited homology with *Ostertagia*

ASPs with regard to protein sequence and spatial and temporal expression, hereby demonstrating that *C. elegans* is not an appropriate system for studying the function of *Ostertagia* ASP1 and ASP2 (Visser 2008). Furthermore, although genes can be conserved at the DNA or RNA level, it is possible that parasite antigens have acquired different or additional functions.

7.4 Importance of antigen epitopes

After purifying and identifying the proper antigen for vaccination, it is important to have a good understanding of the immunogenic epitopes that are present and essential for activating the host immune response. Two non-immunogenic N-glycan structures were discovered on each ASP molecule and protein conformation was found to be crucial for host recognition. Our results indicated that ASPs are typically dimeric under native conditions, so it is possible that homo- or hetero-dimerization is a prerequisite for proper recognition by host antibodies. The immunogenic importance of this dimeric structure could be evaluated in the future by denaturing and reducing the ASP fraction prior to vaccination. In addition, the enzyme-linked immunosorbent assay (ELISA) experiment used in chapter 3 can easily be adjusted to study new antigens for future research. Furthermore, with this test we are able to compare antibody recognition of native antigens with their recombinant version. These results will give us an idea which expression system is best fit for the production of the recombinant vaccine. Potential expression systems are *Escherichia coli* strains with optimized protein folding and the capacity of forming disulphide bonds (e.g. Origami™ and Rosetta-gami™ strains, Novagen), a baculovirus system or yeast strains of *Pichia pastoris* with modified glycosylation pathways that deliver soluble nematode antigens equipped with native glycan structures (Vervecken *et al.*, 2004; Li *et al.*, 2007). In addition, Murray *et al.* (2007) provided proof of principle that *C. elegans* can be used to produce nematode vaccine candidates.

Finally, instead of expressing complete worm antigens we can also attempt to design a vaccine that mimics the essential immunogenic epitopes. Identification of these so-called mimotopes can be achieved using phage-display technology (reviewed by Paschke, 2006). In short, a bacteriophage T7 library expressing random peptide sequences on phage coat proteins will be allowed to interact with immobilized antibodies from vaccinated animals. Phages displaying peptide sequences specifically recognized by these antibodies can subsequently be selected by repeated wash and elution steps (i.e. panning rounds) followed by sequencing of the selected peptides. This approach has already been applied for identifying mimotopes of the bluetongue virus (du Plessis *et al.*, 1994), the infectious bursal

disease virus (Wang *et al.*, 2005) and the spirochaete bacteria *Leptospira* (Tungtrakanpoung *et al.*, 2006). The serum from animals injected with the ASP fraction of *Ostertagia* can be used to screen for important ASP epitopes. This approach can also be repeated with serum from animals injected with the CP or the rest fraction as well as complete ES-thiol in order to define epitopes from the additional (unknown) protective antigens. A peptide-screening might also allow us to determine whether a common immunogenic epitope - and therefore a common antigen - is present in all subfractions. Once identified, these peptides can easily be expressed in *E. coli* and fused with carrier-proteins for ensuring stability and realizing proper epitope presentation to the host immune system.

7.5 Future prospects

The proteomics approach we applied for identifying new antigens in ES-thiol has previously been used for other parasitic nematodes such as *Trichinella spiralis* (Robinson and Connolly, 2005; Robinson *et al.*, 2007), *Trichinella pseudospiralis* (Robinson *et al.*, 2007), *Brugia malayi* (Hewitson *et al.*, 2008), *Haemonchus contortus* (Yatsuda *et al.*, 2003), *Dictyocaulus viviparus* (Matthews *et al.*, 2004), *Teladorsagia circumcincta* (Craig *et al.*, 2006) and *Trichostrongylus colubriformis* (Kiel *et al.*, 2007). However, an important bottleneck for *O. ostertagi* is the limited number of sequences that are available. At the moment, the NCBI and the NEMBASE database of *O. ostertagi* only consist of 148 proteins and 6,749 expressed sequence tags (ESTs) respectively. In order to increase the potential of our search tool, we have extended our database with a theoretical peptide library based on the EST sequences of *O. ostertagi* and the related parasites *T. circumcincta* and *H. contortus*. However, caution should be taken when interpreting positive peptide hits, especially when identification of EST homologs is based on one peptide sequence. In this case, the only way to be sure the identified protein is indeed present would be by detecting it in native material e.g. by means of antibodies aimed against its recombinant. Completion of the *O. ostertagi* transcriptome along with the transcriptome of other parasitic nematodes will be an important step forward in the identification of parasite antigens.

The use of native *O. ostertagi* antigens in vaccination trials was a major disadvantage in this study. Collecting parasite excretory-secretory (ES) material is an expensive and time-consuming procedure. Furthermore, due to the limited amount of protein that was available chromatography methods needed to be selected with careful consideration and limited optimization was possible. Therefore, future work should be focused on delivering active recombinant antigens of *Ostertagia* for further study.

Due to the high purity of the native ASP fraction obtained in this project and the specific immunogenicity of ASPs in protected animals, further development of an *Ostertagia* vaccine should be initially focused on delivering recombinant ASP1 and ASP2 that ressemble their native counterparts in terms of immunogenic epitopes. Once expression has been optimized, the produced ASPs can be subjected to a glycosylation and conformation study to confirm structural similarity with native ASPs. A subsequent vaccination trial will demonstrate their protective capacity.

The recombinant ASPs would also allow us to investigate the function of parasite ASPs in the host. These ASPs can be used to screen for interaction partners e.g. by using sections of the bovine abomasum and conducting interaction studies based on affinity chromatography or Biacore technology (GE Healthcare). Introducing a concentrated dose of ASPs at the level of the mucosa or by means of an air pouch model - as recently described for the hookworm *Necator americanus* (Mendez *et al.*, 2008) - might also give us an indication of the immunomodulatory effect of ASPs and help us optimize our vaccination strategy. If recombinantly expressed ASP1 and ASP2 fail to protect cattle, we could focus on characterizing and expressing other ASPs or other antigens that were found in ES-thiol. Finally, if any of these recombinant vaccines induce significant protection, their efficacy would still need to be confirmed in multiple field trials before commercialization is possible.

7.6 References

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Summary

Summary

Infections with parasitic gastrointestinal (GI) nematodes are a major economic constraint on livestock production worldwide. For grazing cattle in temperate climates, the most common and most pathogenic GI parasite is *Ostertagia ostertagi* which infects the abomasum. In Europe, control is almost exclusively based on the use of anthelmintic drugs. Unfortunately, this control strategy has several important disadvantages such as the high treatment costs, the interference with natural immunity, the presence of drug residues in consumer products and the environment, and the increasing threat of drug-resistant parasites. Therefore, alternative control methods are urgently needed. Vaccination is considered to be one of the most promising alternatives for combating nematode infections.

Chapter 1 gives an overview of the progress made in the development of anti-nematode vaccines. The major steps in developing a vaccine are (1) the identification and purification of protective worm antigens, (2) the large-scale production of these antigens, (3) the development of an efficient antigen delivery system for inducing an appropriate type of immune response and (4) the evaluation of the efficacy of the vaccine. So far, there is only one anti-nematode vaccine that is commercially available, namely the vaccine against the bovine lungworm *Dictyocaulus viviparus*. While decades of studies have revealed some interesting vaccine candidates for different parasites, delivering a commercial vaccine appears to be anything but straightforward. Native vaccines often are obtained by means of expensive and/or time-consuming procedures and their antigens are usually not fully characterized and not completely pure. In contrast, production of recombinant vaccines is relatively easy and cheap. Unfortunately, the majority of the recombinant antigens tested so far failed to induce protection against nematode infections.

Successful vaccination trials against *O. ostertagi* in cattle are scarce. At the moment, three native protein fractions have been isolated that are able to protect calves, i.e. a pooled gut membrane protein fraction containing a 120 kDa integral *Ostertagia* glycoprotein (Oo-12) and the *Ostertagia* galactose-containing glycoprotein complex (Oo-gal-GP), the *Ostertagia* polyprotein allergen (OPA) fraction and the thiol binding excretory-secretory antigen (ES-thiol) fraction. Vaccinating calves with the OPA or the ES-thiol fraction results in a 60% reduction in cumulative faecal egg counts (FEC) during a period of two months. This level of protection is sufficient to consider commercialization. However, the antigens responsible for inducing protection remain to be identified. Furthermore, recombinant *Ostertagia* vaccines tested so far - i.e. OPA, activation-associated secreted protein (ASP) 1, metalloprotease 1, a small heatshock protein and an aspartyl protease inhibitor - failed to protect cattle against infection.

The objective of this project was to isolate and characterize the protective antigens that are present in ES-thiol and evaluate their potential as vaccine candidate. In **chapter 2**, ES-thiol was further fractionated using Q-Sepharose anion exchange chromatography. The first fraction contained the abundantly present ASPs. The second fraction was highly enriched for cysteine proteases (CPs). All remaining elutions were pooled together to form the rest fraction. Calves (seven/group) were immunized three times intramuscularly with 100 μg of ES-thiol or equivalent amounts of the ASP, the CP or the rest fraction in combination with QuilA adjuvant. A negative control group only received QuilA. After the final immunization the animals were challenged with a trickle infection of 25,000 infectious third stage larvae (1,000 L3s/day; 5 days/week). During a two-month period the geometric mean cumulative FEC of the ES-thiol group was reduced by 62% compared to the QuilA control group (probability (P) < 0.05). Groups injected with the ASP, the CP and the rest fraction demonstrated a reduction in cumulative FEC of 74%, 80% and 70%, respectively (P < 0.01). Although no significant reductions in worm burdens were observed, adult male and female worms were significantly smaller in all vaccinated groups (P < 0.05), except for male worms from the ES-thiol group. Western blot analysis demonstrated that animals injected with the ASP fraction specifically recognized monomeric and dimeric ASP molecules without showing cross-reactivity with the other fractions. These results suggest that ASPs are able to induce protection against an *Ostertagia* infection. Animals injected with the CP and rest fraction showed cross-reactivity of several high molecular weight antigens.

In order to select an appropriate expression system for producing recombinant ASP1 and ASP2, we investigated whether glycans and/or structural epitopes present on native ASPs are key features in the induction of a protective immune response (**chapter 3**). We discovered that ASPs carry two hybrid N-glycans with a complex α-1,3-arm, an unprocessed α-1,6-arm and an α-1,6-fucose core. A theoretical 3-D model of ASP1 and ASP2 suggested the presence of a cavity which could be important for binding or dimerization. While removal of the glycan structures had little effect on antibody recognition by vaccinated animals, denaturing and reducing the proteins dramatically reduced recognition suggesting the importance of conformational protein backbone epitopes.

The protective capacity of the CP and the rest fraction indicates that additional protective antigens are present within ES-thiol. Therefore, a proteomic survey was undertaken to identify new potential vaccine candidates (**chapter 4**). A list of antigens was obtained, some of which had already proven their protective capacity against other parasites. Furthermore, several antigens in this screening appear to have a function in reproduction (e.g. vitellogenin, major sperm protein and sperm-specific protein). Since vaccination with ES-

thiol results in reduced fecundity, it is possible that these antigens play an important role in inducing protection. No CPs were picked up by mass spectrometry (MS), although this is most likely the result of the limited amount of CPs present in ES-thiol.

In **chapter 5**, one of the antigens identified in the proteomic analysis of the CP fraction was further studied for its vaccine potential i.e. the translationally controlled tumor protein (TCTP). While TCTP is actively secreted during host infection by several parasitic organisms such as *Schistosoma mansoni*, *Brugia malayi* and *Wuchereria bancrofti*, we found no evidence of active secretion by *Ostertagia* worms in cattle. Oo-TCTP did not appear to be recognized by naturally infected animals and also differed in several amino acids from secreted Bm- and Wb-TCTP. Oo-TCTP was found to be present in parasite eggs suggesting a function in development or reproduction. In addition, cross-reacting antibodies against Oo-TCTP detected TCTP in eggs from other Clade V nematodes such as *Teladorsagia circumcincta*, *Cooperia oncophora* and *Haemonchus contortus* as well as the nematode model organism *Caenorhabditis elegans*. In contrast, no TCTP could be found in eggs from the Clade III nematode *Ascaris suum* and the trematode *Fasciola hepatica*.

Because of the advantages of the *C. elegans* model for studying the function and regulation of nematode genes, a comparative study of TCTP was conducted in **chapter 6**. TCTP was typically expressed in adult hermaphrodite worms, more specifically in the anterior and posterior cells of the intestine near the gonad bend. Knocking down TCTP using RNA interference (RNAi) resulted in a reduction in egg production in the F_0 and F_1 generation of 90% and 72%. Further investigation showed that the uterus of RNAi-treated worms contained unicellular eggs with a large nucleus which were incapable of further embryonic development. Knocking down TCTP also resulted in an increase in p53-dependent germ cell apoptosis which coincided with degradation of developing oocytes. It was hypothesized that TCTP is transported from the intestine into the germ cells and developing oocytes where it fulfills an anti-apoptotic function. Targeting parasite TCTP may provide an alternative for controlling egg production in the host.

Chapter 7 presents a general discussion and future prospects. In this PhD project the protective ES-thiol fraction was subfractionated into an ASP, a CP and a rest fraction which all proved to be able to protect calves against infection with *O. ostertagi*. In addition, MS was used as a tool for the identification of protective antigens. Furthermore, an enzyme-linked immunosorbent assay (ELISA) experiment allowed the analysis of the importance of protein glycosylations and conformation with regard to antibody recognition. This ELISA setup will allow us to evaluate the immunogenicity of native antigens and their recombinants before

initiating a vaccination trial. Further research on defining the immunogenic epitopes present on native antigens in combination with modified recombinant expression systems to mimic these epitopes will allow a more efficient vaccine design in the future.

Samenvatting

Samenvatting

Wereldwijd hebben besmettingen met parasitaire gastro-intestinale nematoden een belangrijke economische impact op de veeteelt. Voor grazende runderen in gematigde klimaten is de lebmaagnematode *Ostertagia ostertagi* de meest voorkomende en meest pathogene parasiet. In Europa is bestrijding bijna uitsluitend gebaseerd op het gebruik van anthelmintica. Deze controlestrategie heeft echter een aantal belangrijke nadelen zoals de hoge behandelingskost, de negatieve invloed op de normale immuniteitsopbouw, de aanwezigheid van residuen in voedsel en omgeving en de dreigende resistentie tegen deze producten. Daarom is er nood aan alternatieve controlestrategieën. Vaccinatie wordt beschouwd als een veelbelovend alternatief om besmetting met nematoden te bestrijden.

Hoofdstuk 1 geeft een overzicht van de vooruitgang die geboekt werd op vlak van anti-nematodenvaccins. Belangrijk stappen binnen de ontwikkeling van deze vaccins zijn: (1) het identificeren en opzuiveren van protectieve wormantigenen, (2) het op grote schaal produceren van deze antigenen, (3) het ontwikkelen van een efficiënte toedieningswijze om een gepaste immuunrespons te induceren en (4) het evalueren van de doeltreffendheid van het vaccin. Tot nu toe is er slechts één anti-nematodenvaccin commercieel beschikbaar, namelijk het vaccin tegen de runderlongworm *Dictyocaulus viviparus*. Hoewel reeds enkele interessante vaccinkandidaten werden ontdekt voor verschillende andere parasieten, blijkt het ontwikkelen van een commercieel vaccin niet evident. Vaccins bestaande uit natief opgezuiverde antigenen worden vaak bekomen door middel van dure en/of tijdrovende protocols en de antigenen in kwestie zijn meestal niet compleet gekarakteriseerd en niet volledig zuiver. Het aanmaken van recombinante vaccins daarentegen is relatief eenvoudig en goedkoop. Niettemin is gebleken dat het merendeel van de geteste recombinante antigenen niet in staat is om bescherming tegen nematodenbesmetting te induceren.

Succesvolle vaccinatieproeven tegen *O. ostertagi* in runderen zijn zeldzaam. Momenteel zijn er drie natieve eiwitfracties beschikbaar die kalveren beschermen tegen infectie, namelijk een darmeiwitfractie bestaande uit een *Ostertagia* glycoproteine van 120 kDa (Oo-12) en het *Ostertagia* galactose bevattend glycoproteïnecomplex (Oo-gal-GP), de *Ostertagia* poly-proteïne allergeen (OPA) fractie en de thiol bindende excretie-secretie antigen (ES-thiol) fractie. Immunisatie van runderen met de OPA- en de ES-thiolfractie resulteert in een daling in cumulatieve ei-uitscheiding van 60% gedurende twee maanden. Deze bescherming is voldoende om commercialisatie te overwegen. Niettemin, de antigenen die in deze fracties verantwoordelijk zijn voor de bekomen bescherming moeten nog geïdentificeerd worden. Naast deze natief opgezuiverde eiwitfracties werden verschillende recombinante *Ostertagia* vaccins getest in kalveren - m.n. OPA, activatie-geassocieerd gesecreteerd proteïne (ASP) 1,

metallo-proteïnase 1, een hitteschok eiwit en een aspartyl proteïnase inhibitor - maar deze gaven geen bescherming tegen infectie.

De doelstelling van dit project was de opzuivering en karakterisering van protectieve antigenen aanwezig in ES-thiol en de evaluatie van hun mogelijkheden als vaccinkandidaat. In **hoofdstuk 2** werd ES-thiol verder gefractioneerd door middel van Q-Sepharose anionenuitwisselingschromatografie. De eerste fractie bevatte de abundant aanwezige ASPs. De tweede fractie was sterk aangerijkt voor cysteïne proteïnasen (CPs). De overige elutiestappen werden samengevoegd en vormden de restfractie. Kalveren (zeven/groep) werden driemaal intramusculair geïmmuniseerd met 100 μg ES-thiol of equivalente hoeveelheden van de ASP-, CP- of restfractie in combinatie met het adjuvans QuilA. Een negatieve controle-groep ontving alleen QuilA. Na de laatste immunisatie werden de dieren geïnfecteerd met 25.000 L3 larven (1.000 L3s/dag; 5 dagen/week). Over een periode van twee maanden was de gemiddelde cumulatieve ei-uitscheiding van de ES-thiol-groep 62% lager dan deze van de QuilA controlegroep (probabiliteit (P) < 0.05). Groepen geïmmuniseerd met de ASP-, CP- en restfractie vertoonden een daling in cumulatieve ei-uitscheiding van respectievelijk 74%, 80% en 70% (P < 0.01). Hoewel er geen significante daling in wormbesmetting werd vastgesteld, waren mannelijke en vrouwelijke wormen significant kleiner in alle gevaccineerde groepen (P < 0.05) met uitzondering van de mannelijke wormen in de ES-thiolgroep. Op Western blot werd aangetoond dat dieren die geïmmuniseerd waren met de ASP-fractie monomere en dimere ASP-moleculen herkenden zonder kruisreactiviteit te vertonen met de andere eiwitfracties. Deze resultaten suggereren dat ASPs in staat zijn om bescherming te induceren tegen een *Ostertagia*-infectie. Dieren geïmmuniseerd met de CP- en restfractie vertoonden kruisreactiviteit van enkele hoog-moleculaire antigenen.

Opdat een gepast expressiesysteem zou kunnen geselecteerd worden voor de productie van recombinante ASP1 en ASP2, werd nagegaan in welke mate suikerstructuren en/of structurele eiwitepitopen van belang zijn bij het induceren van een protectieve immuunrespons (**hoofdstuk 3**). We ontdekten dat ASPs voorzien zijn van twee hybride N-glycanen met een complexe α-1,3-arm, een eenvoudige α-1,6-arm en een α-1,6-fucose core. Een theoretisch 3-D model van ASP1 en ASP2 wees op de aanwezigheid van een ruimtelijke holte die van belang kan zijn voor binding of dimerisatie. Terwijl verwijdering van de suikerstructuren slechts een beperkt effect had op antilichaamherkenning door gevaccineerde dieren, nam deze herkenning sterk af na denaturatie en reductie van de eiwitten. Dit suggereert het belang van ruimtelijke eiwitepitopen bij immuunherkenning.

Het feit dat de CP- en restfractie eveneens bescherming induceerden bij kalveren wijst op de aanwezigheid van bijkomende protectieve antigenen in ES-thiol. Daarom werd een eiwitanalyse uitgevoerd om nieuwe potentiële vaccinkandidaten te identificeren (**hoofdstuk 4**). Een lijst van antigenen werd bekomen, waarvan enkele reeds hun vaccinpotentieel hebben bewezen tegen andere parasieten. Bovendien bleken verschillende antigenen uit deze analyse betokken te zijn bij de voortplanting, m.n. vitellogenine*, major sperm protein* en *sperm-specific protein*. Vermits vaccinatie met ES-thiol resulteert in een afname in eileg, is het mogelijk dat deze antigenen een belangrijke rol spelen bij het induceren van bescherming. Geen CPs werden opgepikt door middel van massaspectrometrie (MS). Dit is waarschijnlijk een gevolg van de beperkte hoeveelheid CPs die aanwezig is in ES-thiol.

In **hoofdstuk 5** werd één van de antigenen die geïdentificeerd werden in de CP-fractie verder bestudeerd voor zijn vaccinpotentieel, m.n. het translationeel-gecontroleerd tumor-proteïne (TCTP). Hoewel TCTP van *Schistosoma mansoni*, *Brugia malayi* en *Wuchereria bancrofti* actief gesecreteerd wordt tijdens infectie van de gastheer, hebben we geen bewijs gevonden van actieve secretie van TCTP door *Ostertagia*-wormen in runderen. Oo-TCTP werd niet herkend door natuurlijk geïnfecteerde dieren en verschilde in een aantal aminozuren van Bm- en Wb-TCTP. Oo-TCTP werd teruggevonden in parasieteieren wat een functie binnen ontwikkeling of voortplanting insinueert. Daarnaast werd TCTP gedetecteerd in eieren van andere nematoden uit Groep V zoals *Teladorsagia circumcincta*, *Cooperia oncophora* en *Haemonchus contortus* evenals het modelorganisme *Caenorhabditis elegans*. TCTP werd echter niet gevonden in de eieren van de nematode *Ascaris suum* (Groep III) en de trematode *Fasciola hepatica*.

Omwille van de voordelen van het *C. elegans* model voor het bestuderen van de functie en regulatie van parasietgenen, werd een vergelijkende studie van TCTP uitgevoerd in **hoofdstuk 6**. TCTP kwam tot expressie in volwassen hermafrodiete wormen, meer bepaald in de eerste en laatste darmcellen nabij de bocht van de gonaden. Het uitschakelen van TCTP door middel van RNA-interferentie (RNAi) resulteerde in een daling in eiproductie in de F_0- en F_1-generatie van respectievelijk 90% en 72%. Uit verder onderzoek bleek dat de uterus van RNAi-behandelde wormen ééncellige eieren met een grote kern bevatte die niet meer in staat waren om verder te ontwikkelen. Het uitschakelen van TCTP resulteerde eveneens in een toename in p53-afhankelijke apoptose in kiemcellen die gepaard ging met een afbraak van ontwikkelende oocyten. Er werd voorgesteld dat TCTP getransporteerd wordt van de darm naar kiemcellen en ontwikkelende oocyten, waar het een anti-apoptotische functie vervult. Parasitair TCTP zou bijgevolg een alternatief doelwit kunnen zijn om wormeiproductie in de gastheer tegen te gaan.

Hoofdstuk 7 omvat een algemene discussie en toekomstperspectieven. In dit doctoraatsproject werd de protectieve ES-thiolfractie gesubfractioneerd in een ASP-, een CP- en een restfractie die kalveren beschermden tegen infectie met *O. ostertagi*. Daarnaast werd gebruik gemaakt van MS om protectieve antigenen te identificeren en van een *enzyme-linked immunosorbent assay* (ELISA) experiment om het belang van suikerstructuren en eiwitconformatie voor antilichaamherkenning te onderzoeken. Deze ELISA zal ons toelaten om de immunogeniciteit van andere natieve antigenen en hun recombinant te bestuderen alvorens een vaccinatieproef te starten. Verder onderzoek voor het bepalen van immunogene epitopen in combinatie met aangepaste recombinante expressiesystemen zal een efficiëntere vaccinontwikkeling mogelijk maken in de toekomst.