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Vaccination against the nematode *Haemonchus contortus* with a thiol-binding fraction from the excretory/secretory products (ES)

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Abstract

Fractionated excretory/secretory products (ES) of adult *Haemonchus contortus* were evaluated as protective antigens. The proteins were successively eluted from a Thiol Sepharose column using 25 mM cysteine followed by 25 mM Dl-dithiothreitol (DTT). Sheep were vaccinated three times and challenged with 5000 third stage infective larvae (L3) of *H. contortus*. Highest level of protection was found in sheep vaccinated with the DTT-eluted fraction in which egg output and worm burden were reduced by 52 and 50%, respectively, compared to the adjuvant control group. There was a positive correlation between fecundity (number of eggs per female) and the cumulative EPG or worm burden. Serum and mucus antibody levels of ES-specific immunoglobulins increased after immunizations and after challenge for IgG, IgA and IgE. The harvesting of *H. contortus* from animals clustered per group revealed the presence of cysteine protease activity in the ES of all groups but in addition to that, metalloprotease activity was also detected in the groups vaccinated with the DTT-eluted fraction, total ES and adjuvant only, in contrast to previous batches of ES (completely inhibited by E64) obtained from non vaccinated animals.

Keywords: *Haemonchus contortus*; Excretory/secretory products; Vaccination; Thiol-binding proteins

1. Introduction

Haemonchus contortus is a blood-sucking nematode that primarily infects the abomasum of sheep and goats worldwide. Infections with this parasite can cause, mainly in young animals, anaemia and weight loss that in some cases result in death. Anthelmintic resistance has been detected in several *H. contortus* strains [1,2] and concerns about the effects of the residues on the health of consumers have focussed attention on the development of a vaccine. Some antigens have been tested with considerable success including gut proteins such as microsomal aminopeptidase H11 [3], glycoprotein complex H-gal-GP [4] as well as larval antigens [5]. Total excretory/secretory products (ES), or partially ES components such as 15 and 24 kDa proteins (Hc15 and Hc24) have also been tested with considerable promise [6]. Most of these antigens are major constituents of protein fractions obtained by sub cellular fractionations and/or ion exchange chromatography. Analysis of the complexity of these enriched fractions mainly relied on rather insensitive evaluation of 1D gels [3–6]. Consequently, the contribution of many unidentified proteins to protection observed in vaccination experiments remains undetermined.

Protein fractionation by virtue of covalent thiol-binding properties is a highly specific method resulting in the complete removal of many individual proteins. Recently, *Ostertagia ostertagi* thiol-binding protein extracts from adult membrane extracts and ES of adult worms were tested in a vaccination trial in bovine [7]. The thiol-binding fraction from ES induced a reduction in faecal egg count (EPG) of 60%, whilst adult membrane extracts had no protective effect. In contrast, Triton X-100-soluble membrane-associated thiol-binding fractions of *H. contortus* protected sheep against a challenge infection with *H. contortus*, while water-soluble and Tween 20-soluble membrane associated fractions induced no protection [8]. Thiol-binding fractions of *H. contortus* have been shown to be enriched in cysteine protease activity [7,8]. These proteases have attracted interest because they may

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be critical for the life cycle or pathogenicity of many nematodes and trematodes. The ability to degrade host proteins like haemoglobin suggested a function of cysteine proteases in parasite nutrition [9–12]. Analysis of ESTs of *H. contortus* indicated the abundance of cysteine proteases, representing 17% of the sequences from a gut cDNA library [13] and almost 5% from a total worm cDNA library (http://nema.cap.ed.ac.uk/nematodeESTs/Haemonchus/Haemonchus.html). It is unknown which of the probably over 50 different expressed cysteine protease genes encode a product that is present in ES of *H. contortus*. Considering the high degree of cysteine activity in ES and their potential critical function, we decided to fractionate ES of *H. contortus* based on the catalytic properties of the cysteine proteases through a thiol sepharose column to investigate if the fractions would generate a substantial reduction in faecal egg counts and worm burden in sheep challenged with *H. contortus*.

2. Materials and methods

2.1. Preparation of ES

Adult *H. contortus* (Moredun strain) were harvested from the abomasum of donor sheep at 25–35 days post-infection. ES was obtained as described previously [14] with some modifications. Briefly, the worms were washed three times in PBS containing antibiotics (100 I.U./ml penicillin, 100 μg/ml streptomycin, 5 μg/ml gentamicin) and incubated at 37 °C (5% CO_2) in RPMI 1640 (Gibco, Paisley, UK) containing antibiotics (same concentrations as in PBS). The medium was changed after 4 h incubation and new medium added with 2% glucose for overnight incubation at 37 °C. The media from both time points were pooled, concentrated and desalted (10 mM Tris, pH 7.4) in 3 kDa filters (Centripep YM3, Millipore).

2.2. Chromatography on Thiol-Sepharose and analysis of the fractions

ES were applied to a Thiol-Sepharose column as previously described for membrane extract of *H. contortus* [8]. In addition to the elution with 25 mM of cysteine [8] (Sigma–Aldrich Co., USA), a second elution was performed with 25 mM Dl-dithiothreitol (DTT, ICN Biomedicals Inc., USA). A third elution with 100 mM DTT was introduced to check for remaining bound proteins to the column. The peptide profiles of the fractions (cysteine-eluted, DTT-eluted and unbound) was visualised using 2D-SDS–polyacrylamide gel electrophoresis (2D-SDS–PAGE). ES and the purified fractions were precipitated in a final concentration of 10% trichloroacetic acid (TCA, Merck, Germany) containing 10 mM DTT and resuspended in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS), 2% carrierampholyte mixture pH 3–10 non linear (NL) (IPG buffer, Amersham Biosciences, Germany) and 20 mM DTT. For isoelectric focusing (IEF) the protein samples (70 μg protein) were supplemented with protease inhibitors (Complete protease Inhibitor Cocktail, Roche, Germany) and loaded on 13 cm IPG strips (pH 3–10 NL). The protocols for IEF and second dimensional electrophoresis were the same as described previously [15]. The gels were silver stained, compared both manually and by Image Master 2D v4.01 software (Amersham Biosciences) in order to correlate spots to previously made identifications. Zymographic protease activities were monitored by 1D-gelatin-substrate analysis under non-reducing conditions [9]. After electrophoresis, the gels were washed three times with 2.5% Triton-X-100 (Serva, Germany) and incubated with 20 mM Tris/50 mM NaCl buffer pH 5.0 supplemented with 2 mM DTT. Inhibition experiments were performed with 100 μM L-transepoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E64, Roche), 0.4 mM ethylenediaminotetraacetic acid (EDTA), 1 μM Pepstatin (Roche), 2 mM 4-(2-aminoethyl)-benzenesulfonyl-flouride, hydrochloride (AEBSF, Pefabloc, Roche), in 20 mM Tris/50 mM NaCl pH 5,0, supplemented with 2 mM DTT. Samples were incubated with these inhibitors before and after electrophoresis for 30 min and 20 h, respectively at 37 °C. Gels were stained with Coomassie Blue (0.5% (w/v) in acetic acid:ethanol:water, 1:3.5:5.5, respectively).

2.3. Immunisation trial and parasitological procedures

Twenty-nine Zwart-Bles lambs were reared and kept indoors in nematode-free conditions and were 9–9.5 months old when the experiment started. Five groups, balanced for sex and weight, were made with six animals per vaccinated group and five animals in the control group. The lambs were vaccinated sub cutaneously at week 0, 3 and 6 with 15 μg of the cysteine-eluted fraction (group 1), 15 μg of the DTT-eluted fraction (group 2), 60 μg of the unbound fraction (group 3), 75 μg ES (group 4) per immunisation and PBS only (group 5). The doses were estimated based on the yields of the fractionation (described in results). The antigens or PBS were dissolved in aluminium hydroxide gel ($Al(OH)_3$) (Allhydrogel, Superfos Biosector, Denmark) and each animal received 1 mg adjuvant/injection. All sheep were orally challenged at week 8 with 5000 L3 *H. contortus* and slaughtered at week 12. The abomasum was opened immediately to harvest the contents and tissue biopsies. The empty abomasum was thoroughly washed with 0.9% saline solution to remove the adhering worms subsequently. The abomasum was soaked in 0.9% saline at 37 °C for 5 h. Worm burdens were measured according to Eysker and Kooyman [16] on 1:50 aliquots and parasites were sorted by sex. To determine fecundity, worms were dissolved in 1% bleachwater and eggs of 20 female worms were counted per sheep. Faecal samples were collected every week and after challenge three times a week and the EPG was measured according to the modified McMaster

method. Serum samples were collected weekly and stored at −20 °C.

2.4. Isolation of mucus Ig

The abomasal tissue (∼25 cm^2) collected at the time of slaughter was stored until use at −20 °C. Mucus isolation was performed according to Kanobana et al. [17]. The protein concentration was measured (Bradford assay) and the samples adjusted to a final concentration of 0.5 mg protein/ml for the performance of enzyme linked immunosorbent assay (ELISA).

2.5. Enzyme linked immunosorbent assay

ELISA plates (96 well, Greiner, Germany) were coated with 2 μg/ml ES diluted in 0.06 M carbonate buffer pH 9.6 and incubated for 18 h at room temperature. The plates were washed three times with milliQ containing 0.05% Tween-20 in an automatic washer (EL404, Biotek Instruments), blocked with 1% gelatine in PBS and incubated for 1 h at 37 °C. Plates were stored at −20 °C until use. After thawing and washing of the plates, serum (1:5 dilution for IgA/IgE, 1:20 for IgG-isotype ELISA) or mucus (1:10 for all isotypes) dilutions were added and incubated for 1 h at 37 °C. Plates were washed four times and incubated for 1 h at 37 °C with monoclonals anti-sheep IgG, anti-sheep IgA (Serotec) or anti-sheep IgE (IE7) [18] diluted 1:400 in PBS containing 0.1% gelatin and 0.05% Tween-20 (PBS-GT). After washing, the plates were incubated for 1 h at 37 °C with rabbit–anti-mouse immunoglobulin-coupled to alkaline phosphate (Dako) diluted 1:3000 in PBS-GT. The colorimetric reaction was developed with *p*-nitrophenyl phosphate disodiumsalt (PNPP, Pierce) for 30 min at room temperature, followed by 18 h incubation at 4 °C. Absorbance was measured at 405 nm. Each individual sample was tested in duplicate and results are presented as a percentage of the positive control serum (pool of hyperimmune sheep) that was present in duplicate on every plate. The hyperimmune sheep sera were obtained from five animals orally infected for several times and then challenged with a high dose of *H. contortus*. The mucus results were presented in OD values.

2.6. Lymphocyte proliferation assay (LPA)

Draining lymphnodes were pressed through a fine mesh to obtain the lymphocytes, which were washed twice with PBS containing heparin (10 I.E./ml) and adjusted to 2×10^6 cells/ml in RPMI 1640 containing 10% FCS, 500 I.U./ml penicillin, 0.5 mg/ml streptomycin and 2 mM L-glutamine. LPA was performed as previously described [14] with slight modifications. ES were used at a concentration of 10 μg/ml and concanavalin A (conA) at 5 μg/ml. Data are expressed as stimulation indices (SI) where SI = c.p.m. (experimental)/c.p.m.(medium control).

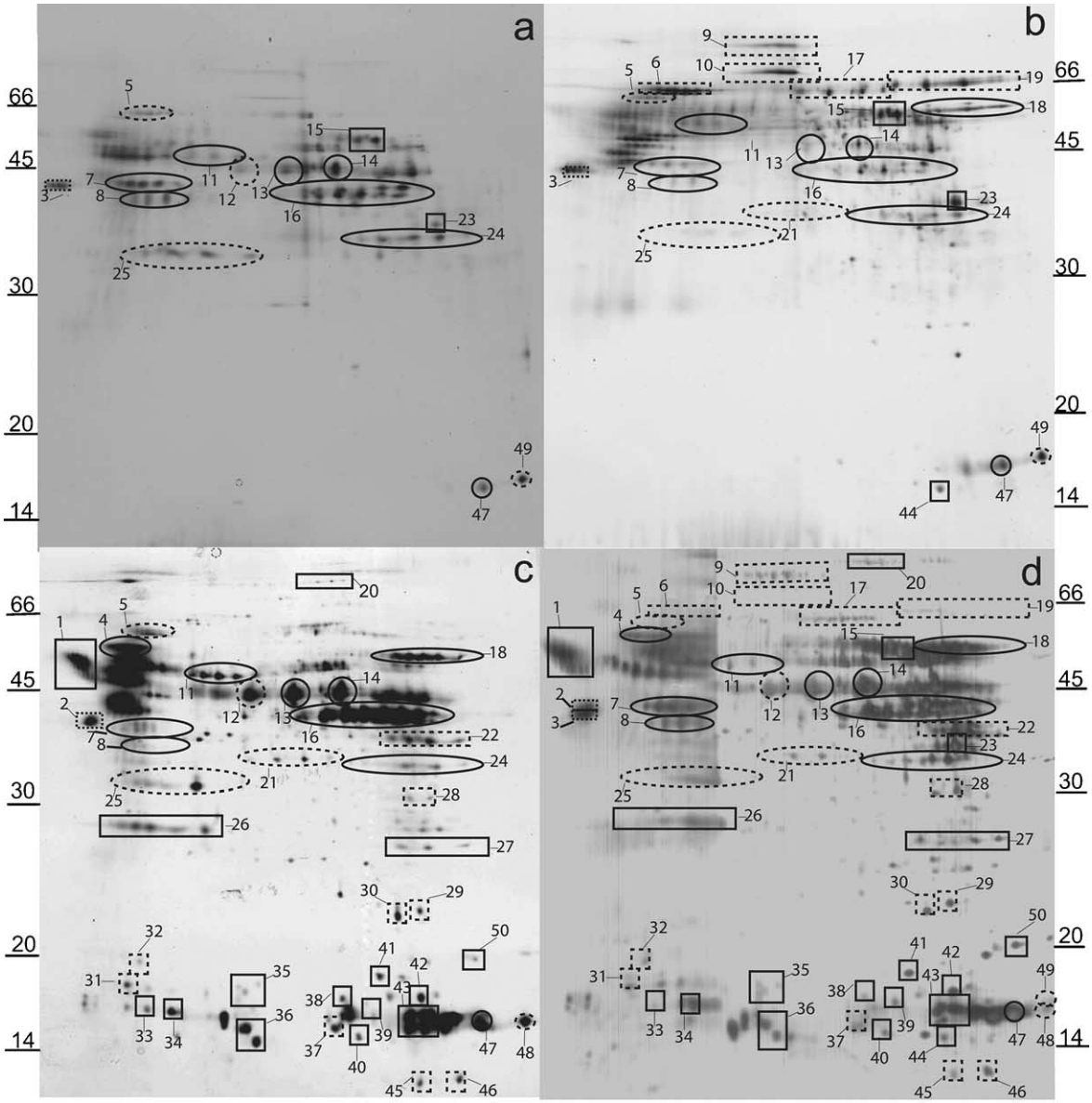
2.7. Statistical analysis

The data were statistically analysed through the non-parametric test of Kruskall–Wallis in the SPSS statistical package software (Chicago, IL, USA). Differences between the groups were considered significant at $P < 0.05$. Pairwise comparisons of the groups were calculated by post-hoc analysis as advised for Kruskall–Wallis. The Bonferroni correction was applied to avoid false positive associations generated by the multiple comparison. Correlations between immunoglobulin levels and parasitological parameters were analysed with the Spearman's rank correlation test with significance at $P < 0.01$.

3. Results

3.1. Fractionation of ES by Thiol-Sepharose chromatography

Thiol-binding protein fractions of ES and membrane proteins of parasitic nematodes have been shown to contain protective properties [7,8]. Theoretically all proteins containing reduced cysteine residues can bind covalently to thiol-sepharose. The reactivity and accessibility of the SH-groups is determined by their sequence context and location within the tertiary structure, which also determines the ease by which bound proteins can be eluted. In addition, proteins may stick non-covalently with high affinity to bound proteins although the binding and the washing were done in a high-ionic strength (0.5 M NaCl) to reduce such binding. Here, 21% of total ES was bound and eluted in two steps of increasing strength by successively 25 mM cysteine (10% of total ES) and 25 mM DTT (11% of total ES). The third elution with higher DTT concentration (100 mM) did not contain any protein as checked by analysing a concentrated sample on a silver stained gel (data not shown). This implicates maximally 10-fold enrichment but a more accurate estimate of the degree of purification of individual proteins could only be obtained by 2D-PAGE (Fig. 1), as no specific enzymatic assays or mono-specific antisera can be applied here for this purpose. Intensely stained spots (or groups of spots) exclusively located either in the eluted fractions or in the unbound fraction are indicated by square boxes whereas spots present in one of the eluted fractions and the unbound fraction are enclosed in ovals. By comparison with the recently established proteomic map of *H. contortus* ES [15], identities were assigned to numbered (groups of) spots and indicated in the legend of Fig. 1 and Table 1. Their abundance in each fraction was estimated from spot intensity and documented in Table 1. Most importantly, several groups of antigens with proposed protective properties were almost exclusively present in the unbound fraction. These include the Hc15 (spots 35, 36, 39, 40, 42, 43, see Fig. 1a–d) [6,19], Hc24 (groups 20, 25) [6,19], Hc40 (group 4) [20] and H11 proteins (group 50)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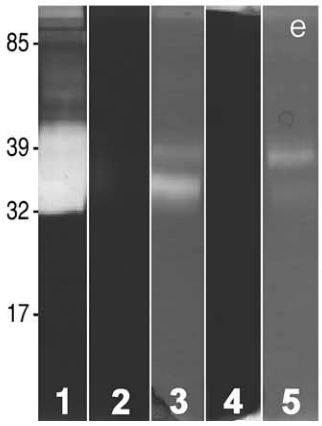
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[3]. The apical gut protein GA1 spots (group 16) [21] were predominantly found in the unbound fraction.

3.2. Determination of doses to be used for vaccination

Protease activity was monitored using 6 μg protein of total ES, the cysteine-eluted fraction and the unbound fraction (Fig. 1e, lanes 1, 3 and 5, respectively). The DTT-eluted fraction showed no activity up to 12 μg protein (Fig. 1e, lane 4). Protease activity was found to be maximal at pH 5.0 (data not shown) and activity of total ES was completely inhibited by E64, a cysteine protease inhibitor (Fig. 1e, lane 2). The total amount of protease activity present in the bound and unbound fractions seems less than in eluted ES. However, it must be noted that thiol-binding fraction and subsequent elution, especially under harsh conditions containing 25 mM DTT, could lead to permanent inactivation of proteases. Therefore, the amount of protease activity present in a fraction was not used as a parameter to determine the doses required for vaccination.

As the proteins present in the two eluted fractions became relatively more enriched than the unbound proteins (Fig. 1a–c) the doses of immunizations were adjusted based on the yields of fractionation for each fraction. The animals from the groups with the two thiol-binding fractions received 15 μg whereas the group with the unbound fraction received 60 μg.

3.3. Parasitological parameters

Although not statistically significant, there was a noteworthy trend for the DTT-eluted group displaying a lesser egg excretion and worm burden. The DTT-eluted group (Fig. 2) had a reduction of 52% compared to the adjuvant control group (Table 2), the animals immunised with the cysteine-eluted fraction had a 36% reduction in EPG, while groups 3 and 4, vaccinated with the unbound fraction and ES, respectively, showed 21 and 13% reduction in EPG. The worm counts (Table 2) followed the same tendency, with group 2 more protected than the other groups. In groups 1, 2, and 4 more female than male worms were found (Table 2).

The fecundity values (Fig. 3) revealed that the numbers of eggs per female found in animals vaccinated with the DTT- or cysteine-eluted fraction were significantly lower than the other groups ($P < 0.002$ with groups 3–5). There was a significant ($P < 0.01$) correlation between fecundity and cumulative EPG ($r = 0.56$) or worm burden ($r = 0.63$).

3.4. ELISA

The vaccinated animals had higher ES-specific immunoglobulin levels (Fig. 4a–c) compared to the adjuvant control group. Before challenge at day 56, ES-specific IgG, IgA and IgE increased significantly after each immunization compared to the adjuvant control (G5), as indicated in Fig. 1a–c. There was a peak 14 days after challenge for IgG and IgA and for IgE 1 week later. In general, the cysteine-eluted group (G1) had higher IgG levels than the other vaccinated groups (Fig. 4a, $P < 0.01$ at days 35, 56, 77) and total ES group (G4) was higher than all the other groups 7 days after challenge ($P < 0.002$, day 63). For IgE, G2 was the group with highest levels and differed significantly from G4 and G5 at days 7 and 49 ($P < 0.002$) and all the groups were higher than G4 and G5 after 3 and 4 weeks of challenge ($P < 0.002$). For IgA, only day 49 was significant and G4 did not differ from adjuvant group, whereas G1 and G2 were higher than all the other groups ($P < 0.002$).

The mean mucus antibody levels (Fig. 4d) showed local ES-specific IgA responses more intense than IgG and IgE responses. Animals vaccinated with the cysteine-eluted fraction had the highest IgA response against ES antigen, but the response did not differ significantly from the other groups.

3.5. Lymphocyte proliferation assay (LPA)

The ES-specific lymphocyte proliferation responses (Fig. 5) were more intense, although not significantly, for DTT-eluted group, compared to other groups. This was due to two animals (numbers 254 and 274) with low worm burdens (100 and 650, respectively) and high stimulation indices (SI of 367 and 90, respectively). As positive control, all animals showed a proliferative response against conA.

3.6. Protease profiles from the ES of the vaccinated groups

Zymograms of ES from previous batches (data not shown) and from five different batches of ES (Fig. 6a, lanes 1–5) showed that the protease activity of ES from *H. contortus* is completely inhibited by E64 (Fig. 6b, lanes 1–5). We wanted to investigate if the effect of vaccination would influence the protease activity of the worms retrieved from the different groups of animals. Therefore, worms were collected from all animals, pooled per group and subsequently ES were harvested and concentrated. The protease activities of these products were determined in 1D-gelatin-substrate

Fig. 1. 2D-SDS–PAGE and gelatine substrate gels of ES derived from parasites harvested of the different thiol-binding fractions of ES of *H. contortus* (12.5%). For the fractionation of ES a Thiol-Sepharose column was used. The proteins were eluted in two steps, firstly with 25 mM cysteine (a); secondly with 25 mM DTT (b); unbound fraction (c) and total ES (d). Rectangles indicate spots present either only in the unbound or in the bound fractions (cysteine and/or DTT-eluted) and ovals indicate (groups of) spots common to the eluted and unbound fractions. Solid lines represent identifications made previously by mass spectrometry [15] and dashed lines point out non-identified spots. Some spots in the fractions could not unambiguously be correlated to spots in the total ES and are therefore not boxed. (e) Protease activity of total ES (lane 1), total ES inhibited by E64 (lane 2), cysteine eluted fraction (lane 3), DTT-eluted fraction (lane 4) and unbound fraction (lane 5). Molecular weight markers on the left (kDa).

Table 1
Spots from Fig. 1a–d were marked, numbered and co-localised with the 2D pattern of ES of *H. contortus* identified previously by proteomic analysis [15]. In each fraction (cysteine eluted, DTT-eluted or unbound) the presence of each (group of) spot was estimated by crosses (maximum 6)

Spot(s)	Protein description (accession number)	Fraction		
		Cysteine eluted	DTT eluted	Unbound
1	Serpin[a] (*Bos taurus*, AAF23888)			+ + + + + +
2	Unknown			+ + + + + +
3	Unknown	+ + +	+ + +	
4	Hc40 (AAC03562, BF060098)			+ + + + + +
5	Unknown	+	+	+ + + +
6	Unknown		+ + + + + +	
7	Metallopeptidase mep1 (AAC31568)	+ + +	+ +	+
8	Metallopeptidase mep2 (AAC28740)	+ + +	+ +	+
9	Unknown		+ + + + + +	
10	Unknown		+ + + + + +	
11	Serine protease (BF422825)			+ + + + + +
12	Unknown	+		+ + + + +
13	Apical gut (GA1) (AAB01192)	+	+	+ + +
14	Apical gut (GA1) (AAB01192)	+ +	+	+ + +
15	Glutamate dehydrogenase	+ +	+ + +	
16	Apical gut (GA1) (AAB01192)	+ +	+	+ + +
17	Unknown		+ + + + + +	
18	Aldehyde dehydrogenase (BE228187)		+ +	+ + + +
19	Unknown		+ + + + + +	
20	H11-2, H11-4 (CAC39009, CAB57358)			+ + + + + +
21	Unknown		+	+ + + + +
22	Unknown			+ + + + + +
23	Aldolase (BF642891)	+ +	+ + + +	
24	Lactate/malate dehydrogenase[a] (*Ascaris suum* BM281165)	+ + +	+ +	+
25	Unknown	+ + +	+	+ +
26	Hc24 (AAC47714)			+ + + + + +
27	Hc24 (BF060283, BF059857)			+ + + + + +
28	Unknown			+ + + + + +
29	Unknown			+ + + + + +
30	Unknown			+ + + + + +
31	Unknown			+ + + + + +
32	Unknown			+ + + + + +
33	Transthyretin like domain (BM138829)			+ + + + + +
34	Hc15 (AAC47713)			+ + + + + +
35	Hc15 (AAC47713)			+ + + + + +
36	Hc15 (AAC47713)			+ + + + + +
37	Unknown			+ + + + + +
38	Hc15 (AAC47713)			+ + + + + +
39	Hc15 (AAC47713)			+ + + + + +
40	Hc15 (AAC47713)			+ + + + + +
41	Superoxide dismutase (Q27666)			+ + + + + +
42	Hc15 (AAC47713)			+ + + + + +
43	Hc15 (AAC47713)			+ + + + + +
44	Unknown		+ + + + + +	
45	Unknown			+ + + + + +
46	Unknown			+ + + + + +
47	Y105C5B.5 (BG734233)	+ +	+ +	+ + + +
48	Unknown			+ + + + + +
49	Unknown	+ + +	+ + +	
50	Cyclophilin 3 (BM138858)			+ + + + + +

[a] No sequence available from *H. contortus*, identification was made by similarity to other species [15].

gels. All five groups showed activity in a range from 32 to 200 kDa in the presence of DTT (Fig. 6c). Most of this activity was inhibited by E64. However, in contrast to a "normal" profile of previously batches of ES, in the groups vaccinated with the DTT-eluted fraction (group 2), ES (group 4), and the adjuvant control (group 5) a zone of proteolysis remained at around 45 kDa, being particularly prominent in the DTT-eluted and adjuvant control groups (Fig. 6d, lanes 2, 4, 5). This zone was completely inhibited by the addition of EDTA to the incubation buffer (data not shown). Based on the inhibition pattern we concluded that the 45 kDa band had a metalloprotease profile.

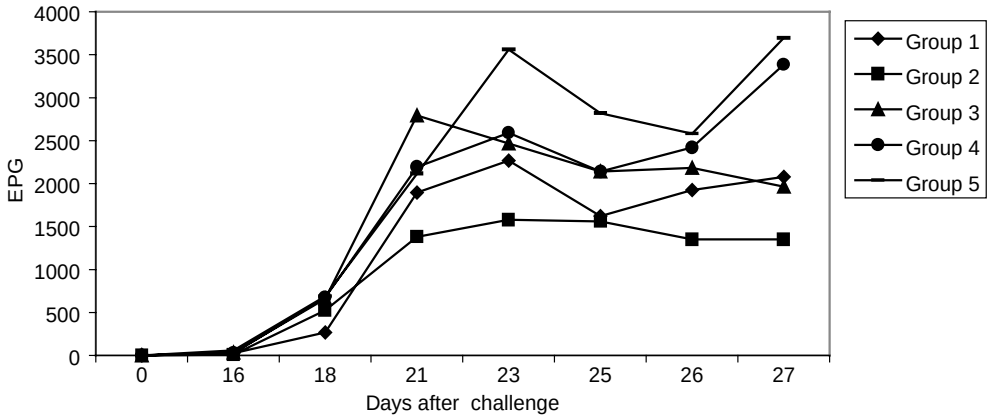

Fig. 2. Mean faecal egg counts of the animals vaccinated with cysteine-eluted fraction (group 1), with DTT-eluted fraction (group 2), with unbound fraction (group 3), with ES (group 4) and adjuvant only (group 5).

Table 2
Number of animals per group (*N*), cumulative faecal egg counts from day 0 till day 27 after challenge (mean ± S.E.), percentage of reduction in faecal egg counts (mean), total worm burden (mean ± S.E.), worm burden reduction (mean) and total number of male and female worms (mean) 27 days after challenge with 5000 L3 *H. contortus*

Group	*N*	Cumulative EPG	Reduction in EPG (%)	Worm burden	Reduction in worm burden (%)	Males	Females
1	6	9975 (4352)	35.7	1500 (527)	33.6	642	858
2	6	77521 (1568)	51.5	1124 (239)	50.2	416	708
3	6	112238 (4670)	21.2	1975 (651)	12.6	1025	950
4	6	113433 (4297)	13.4	1916 (503)	15.2	883	1033
5	5	115520 (5646)		2260 (523)		1190	1070

Animals were vaccinated in weeks 0, 3 and 6 with the cysteine-eluted fraction (group 1), DTT-eluted fraction (group 2), unbound fraction (group 3), ES (group 4) and adjuvant only (group 5).

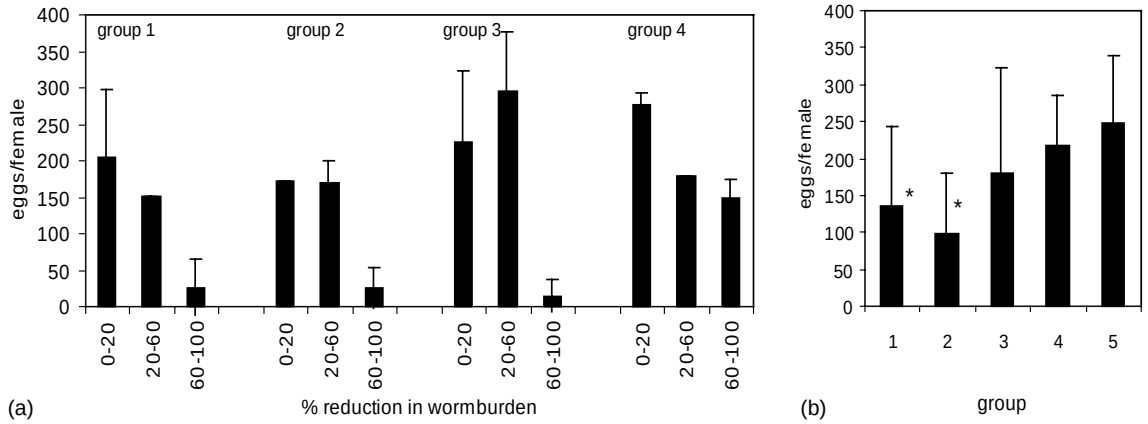

Fig. 3. Worm fecundity at 27 days post challenge. Animals were vaccinated with the cysteine-eluted fraction (group 1), DTT-eluted fraction (group 2), unbound fraction (group 3), ES (group 4) and adjuvant only (group 5). Number of eggs per female (mean ± S.E.). Within each group sheep were divided according to their level of protection (0–20%, 20–60%, 60–100%), i.e. percentage reduction in worm number (a). Number of eggs per female (mean ± S.E.) per group (b). The symbol (*) indicates significant difference between the indicated group and group 3–5 ($P < 0.002$).

4. Discussion

The results indicate that vaccination with the ES thiol-binding proteins of *H. contortus* induced partial protection in sheep after a challenge with 5000 L3 of *H. contortus*. The 2D protein profiles of the fractions eluted with either cysteine or DTT revealed a pattern with clear quantitative and qualitative differences (Fig. 1). Many spots could be assigned to proteins of known identity using the recently published proteome map of *H. contortus* ES [15]. The fractions used for vaccination documented on silver stained high-resolution 2D gels allows for a future re-evaluation of the current

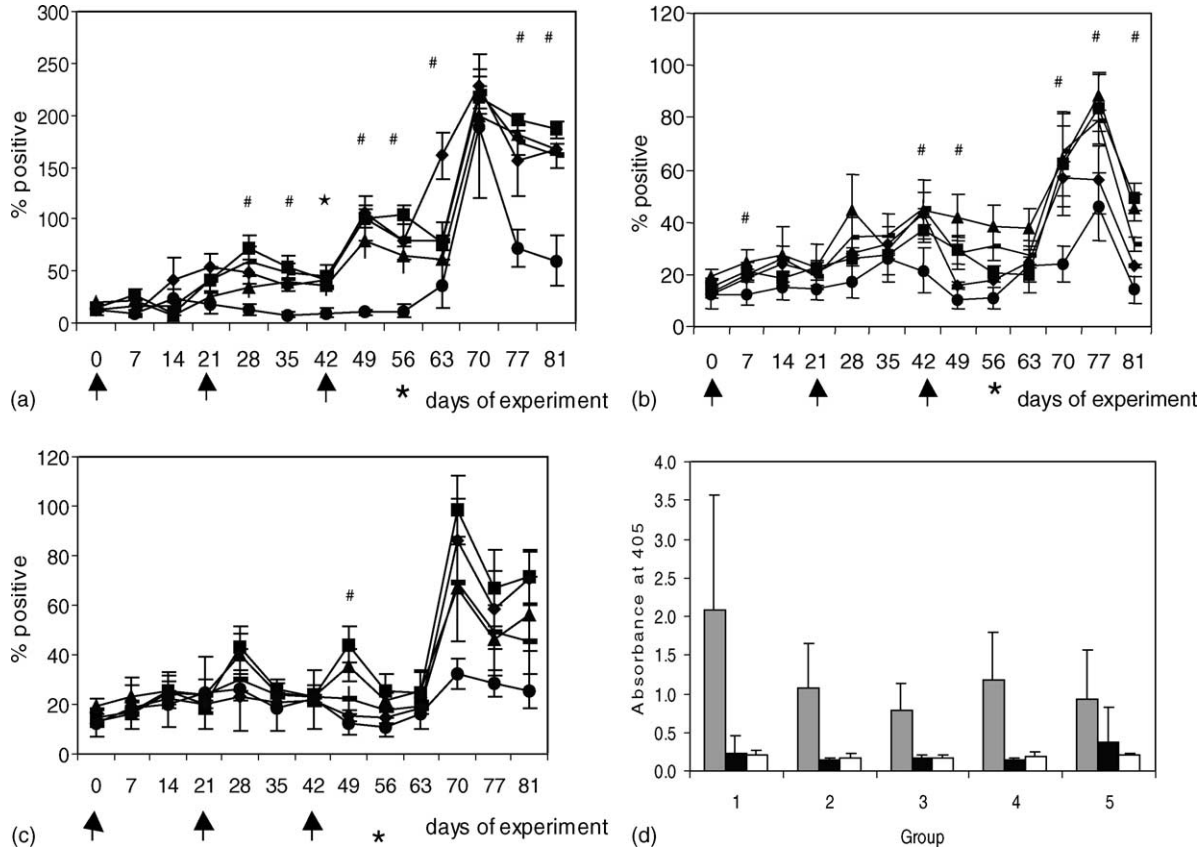

Fig. 4. ES-specific IgG (a); IgE (b); IgA (c) (mean ± S.D.) to *H. contortus*. The animals were vaccinated at week 0, 3 and 6 (arrow) and challenged at week 8 with 5000 L3 *H. contortus* (*). Results are presented as percentage absorbance of the positive control serum. The animals were vaccinated with the cysteine-eluted fraction (group 1 (■)), DTT-eluted fraction (group 2 (▲)), unbound fraction (group 3 (▬)), ES (group 4 (◆)) and adjuvant only (group 5 (●)). IgA (grey bar), IgG (black bar) and IgE (white bar) mucus antibody levels (mean ± S.D.) at 27 d.p.c. to *H. contortus* ES (d). The symbols (#) stands for $P < 0.05$ and (★) for $P < 0.06$ at Kruskal–Wallis.

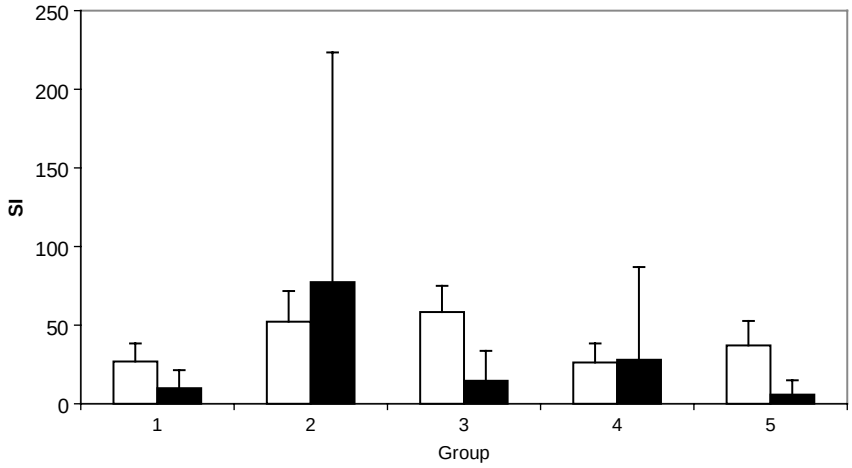

Fig. 5. Lymphocyte proliferation response (mean SI ± S.E.), 27 days post challenge, to conA (open bar) and *H. contortus* ES (black bar) in the different groups vaccinated with the cysteine-eluted fraction (group 1), DTT-eluted fraction (group 2), unbound fraction (group 3), ES (group 4) and adjuvant only (group 5).

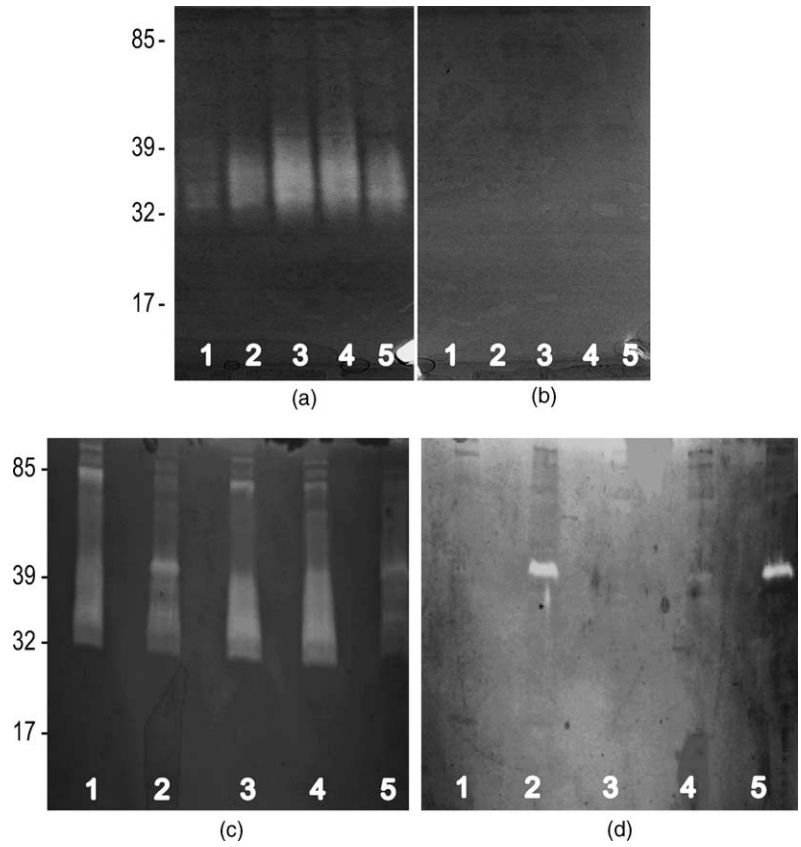

Fig. 6. Gelatine substrate gels of ES incubated before and after electrophoresis with DTT (a and c) or with E64 and DTT (b and d). Five different batches of ES harvested from donor sheep (5 μg per lane, a and b) and ES derived from parasites harvested from the five different vaccinated groups (10 μg/lane, c and d). Group 1 vaccinated with cysteine-eluted fraction, group 2 with DTT-eluted fraction, group 3 with unbound fraction, group 4 with ES and group 5 with adjuvant only. Molecular weight markers are indicated on the left in kilodalton.

experiment when the identities of additional spots become resolved. Standard used 1D gels clearly do not support this type of update and, moreover, underestimate the complexity of partially purified fractions. The DTT-eluted fraction conferred more protection (50%) than the cysteine-eluted fraction (34%) whereas the group vaccinated with total ES showed a reduction around 15% in EPG and worm burden, lower levels compared to a previous experiment, where reductions in the same parameters were 32 and 63%, respectively [14]. The present experiment used $Al(OH)_3$ as adjuvant, whilst diethyl dioctadecyl ammonium bromide (DDA) was used in the former [22]. DDA induces a Th-1 response while $Al(OH)_3$ induces a strong Th-2 response [23]. However, in another recent study in our department, sheep vaccinated with ES in $Al(OH)_3$ showed high percentage reductions in both EPG and worm burden (89 and 77%) compared to the adjuvant alone controls suggesting that the adjuvant is not the reason for the different protection levels found here and previously [22]. We recently demonstrated that the 2D profiles of different batches of ES show no major differences [15], although it cannot be excluded that also very small differences can have an effect on protective properties.

We deliberately decided for a lower dose based on the proportion of the thiol-binding proteins in total ES, thus the amount of antigen (15 μg) used in this experiment was lower when compared to other vaccination trials with *H. contortus*, in which between 50 and 200 μg was used [5,8,22,24,25]. Increasing the amount of a fractionated antigen administered could possibly increase the relevant immune response and, with it, the level of protection observed.

The number of eggs per female found in worms from the groups vaccinated with the DTT- or cysteine-eluted fractions was significantly lower compared to the other groups (Fig. 3). A positive relation was found between fecundity, EPG and worm burden. Vaccination of sheep and cattle with fractions enriched for cysteine protease also induced a significant decrease in worm fecundity of *Fasciola hepatica* [26] and *O. ostertagi* [7].

The vaccination schedule induced particularly marked increases in serum levels of antigen-specific IgG with lesser intensities in IgA and IgE levels (Fig. 4). In contrast, mucus antibody levels, a measure of the local immune response, were only elevated for IgA compared to the controls. No correlation was found between fecundity or protection and antibody levels in serum and mucus. Serum IgG antibody

levels have been correlated with protection using, for example, H11 as antigen [27,28]. Local parasite-specific IgA responses have been associated with reduced fecundity of *O. circumcincta* [29] in sheep and of *O. ostertagi* [7] in calves but the correlations in this last experiment were low ($r = -0.36$). Studies using purified larval surface antigens, cuticular collagen proteins or total *H. contortus* for immunisation had no clear relation between specific antibody levels and the protection status of the sheep [5,24,30,31].

We analysed the protease activity profiles of ES derived from worms retrieved from vaccinated animals after necropsy. Cysteine protease activity was detected in all groups, but metalloprotease activity was also present in the group vaccinated with the DTT-eluted fraction, the adjuvant control group and less clearly in ES-vaccinated group (Fig. 6c–d). Metalloprotease activity was absent from five different batches of ES that were previously tested, as shown by complete inhibition by E64 at pH 5.0, but also at pH 3.0 and 8.0 (data not shown).

The differing degrees of protection observed for the two thiol-binding fractions, cysteine eluted (50%) and the DTT-eluted (34%) could mean that the DTT fraction contains unique protective components or that a common component is more abundant in the DTT fraction. The absence of protease activity in the DTT-eluted fraction could be explained by inactivation, as the concentration of DTT (25 mM) used to elute the proteins may have irreversibly altered the protease structure. This is an inherent limitation correlating protection to enzymatic activity as measured for instance by zymograms as inactivated enzymes can still induce immune responses. To obtain more insight, we co-localised as many as spots as possible with the previous identified ones [15]. There are a few groups of spots left, with predicted molecular weights around 30–40 kD, that could possibly represent cysteine proteases (for example groups 3, 21, 24 and 25 and other unmarked spots below group 24, Fig. 1a and b). There are several unknown proteins predominantly and exclusively present in the DTT fraction, such as spots 6, 9, 10, 17, 19, 44 (Table 1), which represent interesting groups to be identified, as this was the fraction giving highest protection. Obviously further fractionation will remain necessary to identify proteins responsible for the conferred protection, as abundant spots do not necessarily correlate a higher degree of immunity. This is a problem often overlooked when relying exclusively in 1D gel analysis, since in other studies with gastrointestinal nematodes fractionated extracts were tested in vaccinations as a first-step identification of possible protective antigens [7,8,21,22,32,33,34].

In conclusion, we have demonstrated protective properties of ES thiol-binding proteins which may contain cysteine proteases, as confirmed previously for membrane protein thiol-binding proteins [8]. In addition, other groups of known and unknown proteins were shown to be present in these fractions that might contribute to the protective effects. Their precise documentation by 2D protein gel analysis will facilitate a better evaluation of future studies on differently or more extensively purified fractions and will hopefully lead to the selection of specific gene products to be tested in a recombinant vaccine.

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