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Original article

An AC-5 cathepsin B-like protease purified from *Haemonchus contortus* excretory secretory products shows protective antigen potential for lambs

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Abstract – The immunogenic properties of cysteine proteases obtained from excretory/secretory products (ES) of *Haemonchus contortus* were investigated with a fraction purified with a recombinant *H. contortus* cystatin affinity column. The enrichment of *H. contortus* ES for cysteine protease was confirmed with substrate SDS-PAGE gels since the cystatin-binding fraction activity was three times higher than total ES, despite representing only 3% of total ES. This activity was inhibited by a specific cysteine protease inhibitor (E64) and by recombinant cystatin. The one-dimensional profile of the cystatin-binding fraction displayed a single band with a molecular mass of 43 kDa. Mass spectrometry showed this to be AC-5, a cathepsin B-like cysteine protease which had not been identified in ES products of *H. contortus* before. The cystatin binding fraction was tested as an immunogen in lambs which were vaccinated three times (week 0, 2.5 and 5), challenged with 10 000 L3 *H. contortus* (week 6) before necropsy and compared to unvaccinated challenge controls and another group given total ES ($n = 10$ per group). The group vaccinated with cystatin-binding proteins showed 36% and 32% mean worm burden and eggs per gram of faeces (EPG) reductions, respectively, compared to the controls but total ES was almost without effect. After challenge the cystatin-binding proteins induced significantly higher local and systemic ES specific IgA and IgG responses.

***Haemonchus contortus* / excretory-secretory products / cysteine protease / vaccination / AC-5**

1. INTRODUCTION

Cysteine proteases have been identified in most helminth parasites as members of the papain-like clan, the largest subfamily among the cysteine protease class [13, 26]. Their presumed functions such as nutrition uptake, tissue penetration and evasion of host immune responses emphasize their importance as targets for helminth control [13, 23, 26, 33, 39].

Cysteine proteases of *Haemonchus contortus*, the most pathogenic gastrointestinal

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nematode parasite of small ruminants, have been the subject of extensive studies. *H. contortus* infects the abomasum of sheep, goats and other small ruminants and gains nourishment from the fact that it feeds on blood, often resulting in severe anaemia. Cysteine protease activity was detected in the microvillar intestinal tissue of the parasite gut and in its excreted/secreted products (ES) [10, 19, 22, 23]. The most predominant cysteine proteases are the cathepsin B-like proteases (CBL), which are encoded by a family of at least 22 genes [8]. Their abundant expression is apparent from the analysis of all 21 975 expressed sequenced tag (EST) of *H. contortus* present in GenBank. Approximately 4% of these appear to be derived from CBL genes. However, only a few of these proteases have so far been characterized in more detail. The cysteine proteases detected in ES products of *H. contortus* ranged in size from 32 up to 51 kDa [10, 23] whereas the predicted molecular weights of cysteine proteases encoded by clustered EST range from 30 to 45 kDa. Despite the high abundance in EST and the presence of a signal peptide, the cysteine proteases were not identified during the proteomic mapping of the most abundant secreted proteins of *H. contortus* [37]. The use of biotinylated inhibitors combined with a proteomic approach enabled the identification of nine different cysteine proteases present in ES (AC4, GCP7, HMCP1, HMCP1-like, HMCP2, HMCP-2-like, HMCP7, HMCP8, HMCP9) [38].

Specific cysteine protease inhibitors such as cystatin can provide an alternative method for enrichment of excreted cysteine proteases by affinity chromatography. Cystatins, members of the family 2 cysteine protease inhibitors, are natural, reversible, tight-binding cysteine protease inhibitors and represent important regulators of proteolytic processes [7, 30, 35].

In this report we enriched ES for cysteine proteases using recombinant *H. contortus* cystatin affinity chromatography, identified the proteins thus enriched and evaluated their protective effect against a challenge infection of *H. contortus* in a vaccination trial.

2. MATERIALS AND METHODS

2.1. Expression and purification of the recombinant *H. contortus* cystatin

H. contortus cystatin (GenBank^{TM} Accession No. AF035945) was expressed and purified as described by [18] with slight modifications. The soluble fraction was dialysed against 20 mM Tris-HCl, pH 7.4 and purified through sequential chromatography with ion exchange columns (Mono Q and Mono S, GE). The fractions were analysed by SDS-polyacrylamide gel electrophoresis (1D-SDS-PAGE).

2.2. Chromatography on sepharose-cystatin column and fractionation

Adult *H. contortus* (Moredun isolate) were harvested from the abomasum of donor sheep at 25 to 35 days post-infection. Total ES was obtained as described previously [2]. Two affinity columns were prepared: one with recombinant *H. contortus* cystatin and one with an unrelated *Escherichia coli* recombinant protein (*Cooperia punctata* Cp-ASP-1a, Accession No. gi 13625909). Freeze-dried CNBr-activated Sepharose 4B Fast Flow (2 mg, GE) was swollen in 50 mL 1 mM HCl and washed with 200 mL 1 mM HCl using a sintered glass filter. After washing, 50 mL coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3) was added and removed under vacuum. The column material was gently mixed with 4 mL of either purified recombinant cystatin or Cp-ASP-1a recombinant protein (1 mg/mL) in coupling buffer for 4 h at room temperature (RT) and washed with 50 mL coupling buffer, using sintered glass filter and under vacuum. Remaining active groups were blocked with 0.1 M Tris-HCl pH 8.0 for 2 h at RT. The column material was rinsed (0.1 M Tris, 0.5 M NaCl pH 8.0 (50 mL) followed by 0.1 M acetate buffer, 0.5 M NaCl pH 4.0 (50 mL; this cycle was repeated three times), resuspended in PBS and packed into a column (BioRad, Hercules, CA, USA). The columns were washed in equilibration buffer (50 mL 50 mM acetate buffer, 0.15 M NaCl pH 5.0) at a flow rate of 1 mL/min using the Econo System (Controller-model ES-1, Pump-model EP-1, UV monitor-model EM-1, Biorad). Before loading on the Cp-ASP-1a Sepharose 4B column (flow rate 0.2 mL/min), 5 mg total ES was dialysed against equilibration buffer for 18 h at 4 °C (Slide-a-Lyzer, Pierce, Rockford, IL, USA). The unbound fraction (20 mL), obtained after washing the column with equilibration solution, was loaded on the cystatin

Sepharose 4B column (flow rate 0.2 mL/min) and washed with 20 mL equilibration buffer. For elution 10 mM Tris-NaCl pH 7.4, 0.1% CHAPS, 6 M Urea (50 mL, flow rate 1 mL/min) was used. The fractions were dialysed against 10 mM Tris pH 7.4, 0.05% CHAPS for 18 h at 4 °C (Slide-a-Lyzer, Pierce), concentrated in 3 kDa filters (Centripep YM3, Millipore, Billerica, MA, USA) and the protein concentrations were measured.

2.3. SDS-PAGE analysis and cysteine protease activity of the cystatin binding fraction

The protein profiles of the cystatin-binding fractions were visualised by silver staining after SDS-PAGE. Protease activity was further characterized by electrophoresis under non-reducing conditions on gelatin substrate containing gels as described earlier [11]. Protease activity was defined using class-indicative inhibitors (from Roche, Indianapolis, IN, USA) namely the cysteinyl (L-transepoxysuccinyl-L-leucylamido- (4-guanidino)-butane; E64; 40 µM), metallo (ethylenediaminotetraacetic acid; EDTA, 400 µM), aspartyl (Pepstatin, 1.4 µM) and serine (4-(2-Aminoethyl)-benzenesulfonyl-flouride hydrochloride; AEBSF, 2 mM) protease inhibitors as well as 0.12 µg/mL recombinant cystatin or 0.43 µg/mL *B. bovis* recombinant protein (as control for *E. coli* proteins) in 20 mM Tris/ 50 mM NaCl pH 5.0, supplemented with 2 mM DTT).

2.4. Immunisation trial and parasitological procedures

Thirty Zwart-Bles lambs, 6–6.5 months of age and kept indoors since birth to exclude helminth infections, were randomly divided into three groups with 10 animals each. The doses for the immunization were chosen proportionally to the purification (3% bound to the cystatin column). Animals from group 1 received 2 µg of the cystatin-binding fraction whereas group 2 was immunised with 75 µg of total ES and group 3 was the adjuvant control group (PBS only). The animals were vaccinated subcutaneously, at weeks 0, 2.5 and 5 from the start of the experiment. The antigens or PBS were dissolved in aluminium hydroxide gel ($Al(OH)_3$; Allhydrogel, Superfos Biosector, Denmark) and each animal received 1.5 mg adjuvant/injection (1 mL/animal). At week 6 all animals were orally infected with 10 000 L3 *H. contortus* and killed at week 10. Faecal samples were collected weekly and after challenge three times a week and egg counting was performed according to the modified McMaster method. Worms were harvested and counted as described [5]. All animal procedures were in accordance with the Ethical Committee from the Faculty of Veterinary Medicine from Utrecht University.

2.5. Immunological parameters

2.5.1. Lymphocyte proliferation assay (LPA)

Animals were bled from the jugular vein in weeks 0, 6, 7 and 10 for isolation of lymphocytes [2] and LPA was done according to Schallig et al. [28] using 10 µg/mL ES and 5 µg/mL concanavalin A (conA) for lymphocyte stimulation. The results are presented as stimulation indices (SI) where SI = c.p.m. (experimental)/c.p.m. (medium control).

2.5.2. Mucus harvesting

Individual abomasal tissues (~50 cm^2) were collected at the time of slaughter for mucus isolation according to Kanobana et al. [9]. All mucus samples were diluted to a concentration of 1 mg/mL for the performance of the enzyme linked immunosorbent assay (ELISA).

2.5.3. ELISA

The ELISA were performed as described previously [2] with slight modifications. Briefly, ELISA plates coated with ES (2 µg/mL) were incubated with either serum or mucus, diluted at 1:20 for IgE and 1:100 for IgG in serum and 1:10 for all isotypes in mucus. The positive control serum consisted of a pool from 5 hyperimmune sheep which had been repeatedly infected with *H. contortus*. Each individual sample was tested in duplicate and the results are shown either as a percentage of the positive control serum that was present in duplicate on every plate or as OD values for the mucus samples.

2.6. Identification of cystatin-binding fraction by mass spectrometry

The material that bound to the cystatin column (see section 2.2.) was trypsinised and analysed using liquid chromatography MS/MS as described previously [37]. In summary, the bound fraction was delivered at 3 µL/min to a nano-LC system coupled to a Q-TOF (Micromass Ltd., Manchester, UK) and using a Famos autosampler (LCPackings, Amsterdam, The Netherlands) and trapped on an AquaTM C18RP column (Phenomenex, Torrance, CA; column dimensions 1 cm × 100 µm inner diameter). After flow splitting down to 150–200 ηL/min, peptides were transferred to the analytical column (PepMap; LC Packings, Amsterdam, The Netherlands; column dimensions 25 cm × 50 µm inner diameter) in a gradient of acetonitrile (1% per min). Fragmentation of eluting peptides was performed in a data-dependent mode, and mass spectra were acquired in a full-scan mode. The MS/MS derived data were searched against GenBank Protein and EST databases using MASCOT software with the following parameters: oxidation of methionine as a variable modification, peptide and fragment mass tolerances of 0.3 and 0.2 Da respectively with a maximum of one missed cleavage.

2.7. Statistical analysis

Statistical analyses were carried out using the SPSS statistical package software (Chicago, IL, USA) and data were analysed with the non-parametric Kruskal-Wallis test. Subsequent group pairwise comparisons were analysed through the Post Hoc test as advised for Kruskal-Wallis and the confidence level was set at $p < 0.05$ (two-tailed). The Bonferroni correction was employed to avoid possible false positive associations generated by multiple comparisons. Correlations between the immunoglobulin levels and parasitological parameters were tested using the Spearman rank correlation coefficient and considered significant at $p < 0.05$.

3. RESULTS

3.1. Purification of ES by cystatin affinity chromatography

Three percent of total ES protein bound to the recombinant cystatin column and the proteins were analysed by 1D-SDS-PAGE (Fig. 1) and gelatin-substrate gels (Fig. 2). Despite the bound protein fraction resolving as a single band around 43 kDa (Fig. 1, lane 3), this fraction displayed strong protease activity, over a wide molecular size range with a lower limit of 30 kDa (Fig. 2, lane 1). Further analyses were performed using different protease inhibitors. Recombinant cystatin and the specific cysteine protease inhibitor E64 (Fig. 2, lane 2 and 4, respectively) inhibited the activity of the cystatin binding fraction in contrast with AEBSF, a *C. punctata* recombinant protein or a mixture of EDTA, AEBSF and Pepstatin (Fig. 2, lane 3, 5 and 6).

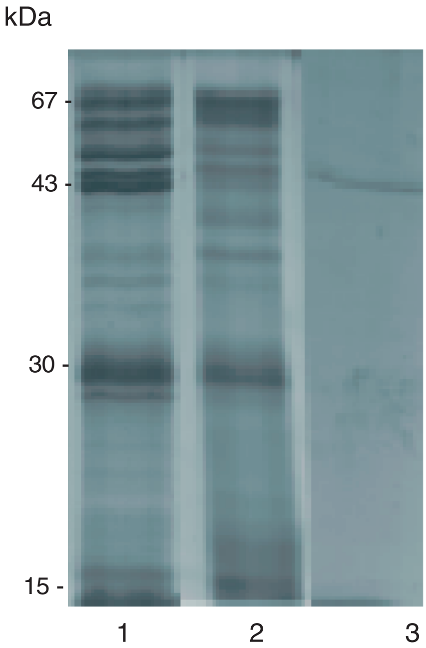


Figure 1. Silver-stained 1D SDS-PAGE gel (15%) analysis of ES products (5 µg, lane 1), 1st unbound fraction (1.25 µg, lane 2) and cystatin-binding fraction (0.5 µg, lane 3). Molecular markers are indicated on the left (in kDa).

Although a quantitative analysis of the specific activity of the partially purified *H. contortus* proteases was not feasible, a titration of ES and the cystatin-bound fraction by substrate-SDS-PAGE analysis indicated that protease activity was enriched at least four fold after purification (not shown).

3.2. Cystatin binding fraction analysis by mass spectrometry

The MS/MS spectra generated an identification of a sequence stretch composed of the 16 aminoacids, FFEYDGVVSGGPYLGK (See figure in Appendix), which was specifically identified to the cathepsin B-like protease AC-5 (AAA29176) and homologous EST

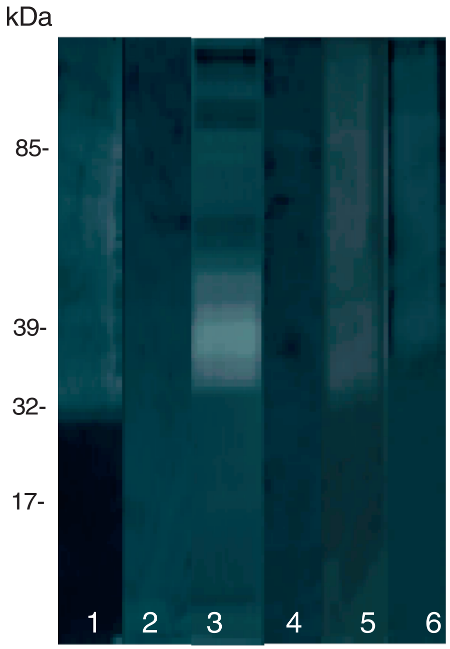


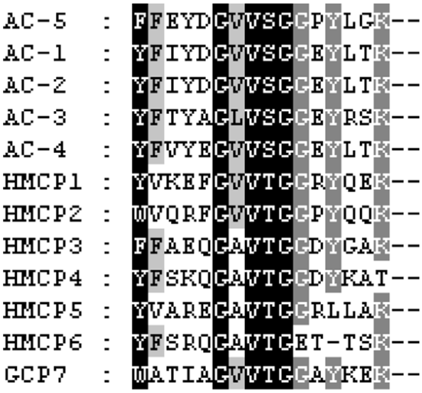
Figure 2. 1D-substrate analysis of 2 µg cystatin-binding fraction. The fractions were incubated before and after electrophoresis with only DTT (lane 1), DTT and recombinant cystatin (lane 2), DTT and AEBSF (lane 3), DTT and E64 (lane 4), DTT, EDTA, AEBSF and Pepstatin (lane 5) and unrelated recombinant (Cp-ASP-1a) (lane 6). Molecular markers are indicated in the middle (in kDa).

(like BG734242) This sequence corresponds to aminoacids 170 to 185 and is located after the active site, in a region of high divergence between cathepsin B-like proteases allowing unambiguous identification of AC-5 (Fig. 3). The predicted molecular weight of AC-5 with the pro-region and without signal peptide is 36.7 kDa and without the pro-region is 29 kDa while the identified band was 43 kDa.

3.3. Immunological parameters

3.3.1. LPA

Lymphocytes from all groups proliferated after stimulation with concanavalin A were used as the positive control (Fig. 4a) although no significant differences were evident between groups. With ES as mitogen, the response in both vaccinated groups, 1 (cystatin) and 2 (ES), was greatly enhanced after the third immunisation (Fig. 4b, open bar) compared to the adjuvant group 3 ($p < 0.05$). Group 2, stimulated with total ES, displayed the highest ES-specific proliferation response for all time points compared to the other groups ($p < 0.05$ at week 4 after challenge). The local lymphocytes (Fig. 4b, striped bar) were more reactive to ES antigens than peripheral lymphocytes (Fig. 4b, white, grey and checkered bars). A negative correlation was found between cumulative eggs per gram of faeces (EPG) and LPA at the lymphocytes isolated from local lymph nodes at 4 weeks after challenge ($p < 0.05$, $r = -0.046$).



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AC-5   : FFEYDCVVSCCPYLGK--
AC-1   : YFIYDCVVSCCEYLTK--
AC-2   : YFIYDCVVSCCEYLTK--
AC-3   : YFTYACLVSCCEYRSK--
AC-4   : YFVYECVVSCCEYLTK--
HMCP1  : YVKEFCVVTCCRYQEK--
HMCP2  : WVQRFCVVTCCPYQQK--
HMCP3  : FFAEQCAVTCCDYGAK--
HMCP4  : YFSKQCAVTCCDYKAT--
HMCP5  : YVARECAVTCCRLLAK--
HMCP6  : YFSRQCAVTCET-TSK--
GCP7   : WATIACVVTCCAYKEK--
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Figure 3. Sequence identification of the cathepsin B-like AC-5 from *H. contortus*. The aminoacid stretch identified from AC-5 by mass spectrometry aligned to CBL sequences from *H. contortus.* Positions having four or more identical residues are shaded. GenBank accession numbers AC-5-AAA29176, AC1-AAA29175, AC2-AAA29171, AC3-AAA29178, AC4-AAA29177, HMCP1-CAA93275, HMCP2-CAA93276, HMCP3-CAA 93277, HMCP4-CAA93278, HMCP5-CAA93279, HMCP6-CAB03627, GCP7-AAC05262.

3.3.2. ELISA

IgG anti-ES antibody concentrations increased markedly following immunisation with the

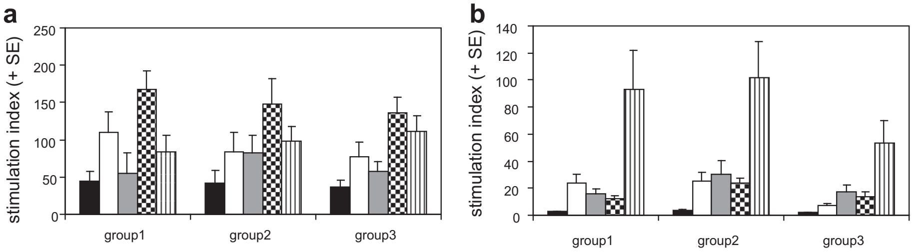

Figure 4. Lymphocyte proliferation responses (mean SI index + S.E.). Lymphocytes isolated from blood in week 0 (black bar), after the third immunization (white bar), 1 week after challenge (grey bar) and 4 weeks after challenge (chequered bar) and lymphocytes isolated from the lymph nodes 4 weeks after challenge (striped bar) with *H. contortus* were stimulated with con A (a) and total ES (b). Group 1 was vaccinated with cystatin-binding fraction, group 2 with total ES and group 3 was the adjuvant control group.

cystatin-binding fraction (group 1) and total ES (group 2) with levels continuing to increase post challenge until the end of the experiment (Fig. 5a). Similarly, IgE levels (Fig. 5b) were elevated two to three-fold in these groups up to the time of infection where control group 3 showed a marked IgE response 2 weeks post challenge. IgA levels showed little response to immunisation but rose rapidly following infection with the response in the cystatin-binding group 1 being two-fold and significantly higher than that in the total ES group 2 and the adjuvant control group 3 (Fig. 5c).

Local immunoglobulins measured in the mucus showed that all immunised animals had higher local IgA and IgG responses compared to the controls (Fig. 5d). IgE was undetectable in all the groups. Group 1, vaccinated with the cystatin-binding fraction, exhibited the highest IgA- and IgG-levels ($p < 0.05$). Among all the correlations tested for systemic and local immunoglobulins with the parasitological parameters, only three correlations were found. Negative correlations between local IgA and local IgE with worm burden ($r = -0.40$ and $r = -0.42$, respectively, $p < 0.05$) and local IgG with cumulative EPG ($r = -0.37$, $p < 0.05$).

3.4. Parasitological parameters

Mean EPG levels (Fig. 6) in all three groups increased until 25 days after challenge. From day 28 onwards a decrease in EPG was observed for group 1 (cystatin-bound fraction) in comparison to groups 2 (ES) and 3 (control) (Fig. 6). In general, animals of group 1 had lower mean EPG levels than the other groups ($p < 0.05$ at day 31).

Animals vaccinated with the cystatin-binding fraction (group 1) had means of 32% EPG and 36% worm burden reductions compared to the adjuvant control group (not significant). No effects were seen in Group 2, vaccinated with total ES (Tab. I) and changes in between groups in the sex ratio of the worms were not observed. There were positive correlations between EPG and worm burden ($r = 0.64$, $p < 0.01$), EPG and fecundity ($r = 0.42$, $p < 0.05$), worm burden and fecundity ($r = 0.40$, $p < 0.05$) and negative correlations between fecundity and protection ($r = -0.59$ for EPG and $r = -0.53$ for worm burden, $p < 0.01$).

4. DISCUSSION

Previous work showed that vaccination with adult ES antigens enriched for cysteine proteases using Thiol-Sepharose affinity purification, can induce a protective immune response in sheep against *H. contortus* [2]. Protection was indicated by reductions in egg output and worm burden by 52 and 50%, respectively, compared to the adjuvant control group. However, these

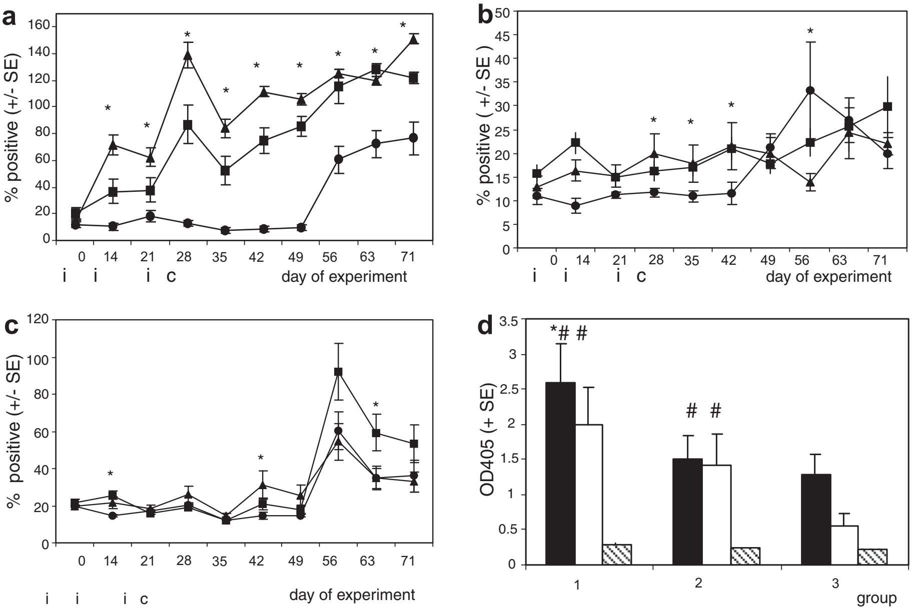

Figure 5. Serum IgG (a), IgE (b) and IgA (c) responses (mean ± S.E.) to *H. contortus* ES products. Animals were vaccinated in week 0, 2.5 and 5 (i) and challenged in week 6 (c) with 10 000 L_3 *H. contortus*. Group 1 (■) was vaccinated with cystatin-binding fraction, group 2 (▲) with total ES and group 3 (●) was the adjuvant control group. * Indicates significant difference between the groups ($p < 0.05$). (d) Mucus IgA- (black bar), IgG- (white bar) and IgE- (striped bar) mean antibody levels (± S.E.) to *H. contortus* ES 33 days after challenge infection. # indicates significant difference with group 3, * indicates significant difference with group 2.

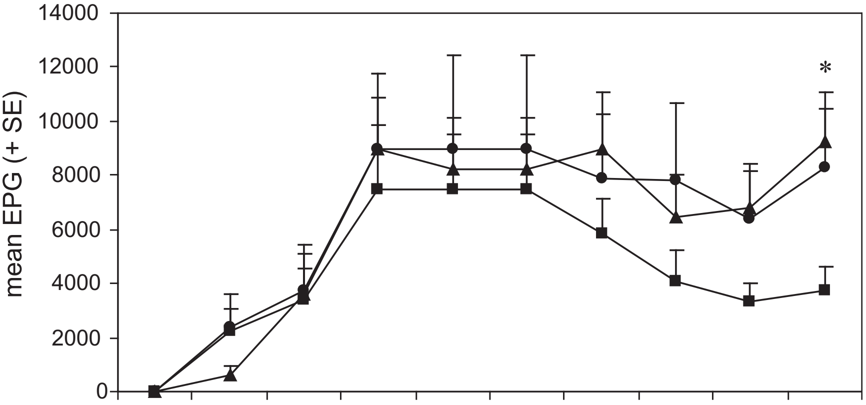

Figure 6. Mean faecal egg counts (± S.E.) of the animals vaccinated with the cystatin-binding fraction (group 1, ■), total ES (group 2, ▲) or adjuvant control group (group 3, ●) after challenge infection with 10 000 L_3 *H. contortus*.

Table I. Parasitological results per group. Groups (1, 2 or 3) with 10 animals per group, mean of cumulative EPG (S.E.), percentage reduction in EPG (mean), mean of worm burden (S.E.), percentage worm burden reduction (mean) and percentage of females. Animals were vaccinated in week 0, 2.5 and 5 with the cystatin bound fraction (group 1), total ES (group 2) or used as the adjuvant control group (group 3), challenged in week 6 with 10 000 L_3 *H. contortus* and slaughtered in week 10. The reductions are calculated based on the mean of group 3 (adjuvant control).

Group (n = 10)	Cumulative EPG	EPG reduction (%)	Worm burden	Worm burden reduction (%)	Females (%)
1	43300 (11728)	32	2935 (2353)	36	55
2	59435 (12438)	6	4750 (2358)	−3.6	57
3	63375 (20195)		4585 (2121)		58

protective fractions contained a number of proteins including several metalloproteases, aminopeptidases and an apical gut protein [12]. Here, we have purified the 43 kDa AC-5 cysteine protease from ES using a recombinant *H. contortus* cystatin affinity column. Lambs vaccinated with this AC-5 had mean reductions of 32% and 36% in cumulative egg output and worm burden, respectively, compared to the adjuvant control group (Tab. I). This level of protection is comparable to that of one of our previous studies [2] and with that observed in lambs vaccinated with *H. contortus* intestinal cysteine proteases purified from worm extracts using cystatin [20].

The AC-5 cysteine protease described here (Fig. 6) had not been identified in ES products of *H. contortus* before. Other secreted cysteine proteases such as AC-4 and GCP7 in ES [31] and HMCP-1, HMCP-1-like, HMCP-2 and HMCP-2-like, HMCP-7, HMCP-8 and HMCP-9 [35] that had been identified before by Mass Spectrometry were not detected in the present cystatin bound fraction. 2D gel analysis of this fraction resolved only a single row of spots (data not shown). Since only a single protease was identified by MS, this suggests that the 43 kDa peptide is a single protein with different pIs and not a mixture of other cysteine proteases with different pIs but of similar molecular weight.

A characteristic of type-2 cystatins is the conservation of the pentapeptide sequence, recognised as a target enzyme-binding site [1, 4]. Two rice cysteine protease inhibitors (OCI-OCII), with small differences in their target enzyme-binding site showed different degrees of affinity for *Meloidogyne hapla* cysteine proteases [1, 15]. Thus, variation in the pentapeptide sequence may determine differences in affinity for different proteases and may indicate that the previously identified [38] seven secreted cysteine proteases are absent from the *H. contortus* cystatin-1-bound fraction because of a low affinity for this inhibitor. Alternatively it cannot be excluded that many secreted CBL have a cystatin bound to their active site thus prohibiting their binding to the cystatin column.

The enrichment of *H. contortus* ES for cysteine protease activity was confirmed using substrate SDS-PAGE gels (Fig. 2) with activity in the cystatin-binding fraction being judged to be at least 4-fold higher than in whole ES (not shown). This analysis also showed that the protease resolved as several zones of apparently differing molecular size, an observation which, at first sight, conflicts with the above discussion. This is likely to reflect post-translational modifications such as glycosylation, with two potential asparagines in the AC-5 sequence predicted to be potentially N-glycosylated by NetNGlyc[1]. Moreover, substrate gels are run under non-reducing conditions.

AC-5 is described to be, among five CBL tested in two *H. contortus* isolates, the only homozygote and monoallelic CBL gene whereas the others were extremely polymorphic

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[1] http://www.cbs.dtu.dk/services/NetNGlyc/

[24]. Therefore, AC-5 may be a particularly good vaccine target.

Animals vaccinated with the cystatin-binding fraction and subsequently challenged with *H. contortus* showed significantly higher mucosal ES-specific IgA levels (Fig. 5) and abomasal lymphocyte proliferative responses (Fig. 4) compared to the control animals. This may indicate the importance of the local antibody response against a challenge infection with *H. contortus* and was in agreement with higher mucosal *Ostertagia* specific IgA levels in protected calves after vaccination with thiol-binding proteins derived from ES [6]. The cystatin-binding fraction also induced strong ES-specific systemic and local IgG responses. Previously, vaccination with thiol binding ES proteins of *H. contortus* resulted in significant higher systemic IgG_1 responses [2].

The total ES fraction did not confer any reduction in EPG or worms, in agreement with a recent report [2]. Variable results have been obtained with vaccination with ES in the past within our group [2, 27, 29, 34], due we suspect to variability between different ES batches (as observed by variable protein patterns observed on 1 and 2-dimensional protein gels) combined with the fact that some individual sheep animals fail to respond to vaccination.

There is now a consistent body of evidence suggesting that ES cysteine proteases may be appropriate targets for vaccine development against helminths of livestock. Calves vaccinated with a Thiol-Sepharose-enriched fraction of *Ostertagia ostertagi* ES had a 60% reduction in egg output [6]. In a further experiment, this immunogen was subfractionated through Q-Sepharose anion exchange chromatography and a group injected with a resultant cysteine protease enriched fraction had a reduction of 80% in cumulative faecal egg output compared to controls [16]. A similar thiol-enriched fraction was tested in goats, with 89% and 68% in egg and worm reduction, respectively [25]. These experiments above used Freunds or QuilA as adjuvants, in contrast to the aluminium hydroxide employed in ours. In addition, cysteine proteases from the regurgitant of mature worms induced 80% protection in sheep against *Fasciola hepatica*, as judged by egg output, [36] and secreted cathepsin Ls are lead vaccine candidates for *Fasciola* and schistosomiasis (reviewed in [14]) and hookworms in man (e.g. [3]). The identification of AC-5, here, as an ES immunogen from *H. contortus,* adds to this body of evidence and expands the previously identified set of immunogens from *H. contortus* such as H11, Hc40, H-gal-GP [17, 21, 32].

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REFERENCES

[1] Arai S., Matsumoto I., Emori Y., Abe K., Plant seed and their target enzymes of endogenous and exogenous origin, J. Agric. Food Chem. (2002) 50: 6612–6617.

[2] Bakker N., Vervelde L., Kanobana K., Knox D., Cornelissen A.W., de Vries E., Yatsuda A.P., Vaccination against the nematode *Haemonchus contortus* with a thiol-binding fraction induces different protease profiles in the excretory/secretory products (ES), Vaccine (2004) 22:618–628.

[3] Bethony J.M., Loukas A., Hotez P.J., Knox D.P., Vaccines against blood-feeding nematodes of humans and livestock, Parasitology (2006) 133:S63–S79.

[4] Bode W., Huber R., Structural basis of the endoprotease-protein inhibitor interaction, Biochim. Biophys. Acta (2000) 1477:241–252.

[5] Eysker M., Kooyman F.N.J., Notes on necropsy and herbage processing techniques for gastrointestinal nematodes of ruminants, Vet. Parasitol. (1993) 46: 205–213.

[6] Geldhof P., Claerbout E., Knox D., Vercauteren I., Looszova A., Vercruysse J., Vaccination of calves against *Ostertagia ostertagi* with cysteine protease enriched protein fractions, Parasite Immunol. (2002) 24:263–270.

[7] Hartmann S., Lucius R., Modulation of host immune responses by nematode cystatins, Int. J. Parasitol. (2003) 33:1291–1302.

[8] Jasmer D.P., Mitreva M.D., McCarter J.P., mRNA sequences for *Haemonchus contortus* intestinal cathepsin B-like cysteine proteases display an extreme in abundance and diversity compared with other adult mammalian parasitic nematodes, Mol. Biochem. Parasitol. (2004) 137:297–305.

[9] Kanobana K., Ploeger H.W., Vervelde L., Immune expulsion of the trichostrongylid Cooperia oncophora is associated with increased eosinophilia and mucosal IgA, Int. J. Parasitol. (2002) 32:1389–1398.

[10] Karanu F.N., Rurangirwa F., McGuire T.C., Jasmer D.P., *Haemonchus contortus*: Identification of proteases with diverse characteristics in adult worm excretory-secretory products, Exp. Parasitol. (1993) 77:362–371.

[11] Knox D.P., Redmond D.L., Jones D.G., Characterization of proteinases in extracts of adult *Haemonchus contortus* the ovine abomasal nematode, Parasitology (1993) 106:395–404.

[12] Knox D.P., Smith W.D., Vaccination against gastrointestinal nematode parasites of ruminants using gut-expressed antigens, Vet. Parasitol. (2001) 100: 21–32.

[13] Lecaille F., Kaleta J., Brömme D., Human and parasitic papain-like cysteine proteases: their role in physiology and pathology and recent developments in inhibitor design, Chem. Rev. (2002) 102:4459–4488.

[14] McManus D.P., Dalton J.P., Vaccines against the zoonotic trematodes *Schistosoma japonicum*, *Fasciola hepatica* and *Fasciola gigantica*, Parasitology (2006) 133:S43–S61.

[15] Michaud D., Cantin L., Bonadé-Bottino M., Jouanin L., Vrain T.C., Identification of stable plant cystatin/nematode protease complexes using mildly denaturing gelatin/polyacrylamide gel electrophoresis, Electrophoresis (1996) 17:1373–1379.

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

[17] Munn E.A., Smith T.S., Smith H., James F.M., Smith F.C., Andrews S.J., Vaccination against *Haemonchus contortus* with denatured forms of the protective antigen H11, Parasite Immunol. (1997) 19: 243–248.

[18] Newlands G.F.J., Skuce P.J., Knox D.P., Smith W.D., Cloning and expression of cystatin a potent cysteine protease inhibitor from the gut of *Haemonchus contortus*, Parasitology (2000) 122:371–378.

[19] Pratt D., Cox G.N., Milhausen M.J., Boisvenue R.J., A developmentally regulated cysteine protease gene family in *Haemonchus contortus*, Mol. Biochem. Parasitol. (1990) 43:181–191.

[20] Redmond D.L., Knox D.P., Protection studies in sheep using affinity-purified and recombinant cysteine proteinases of adult *Haemonchus contortus*, Vaccine (2004) 22:4252–4261.

[21] Rehman A., Jasmer D.P., A tissue specific approach for analysis of membrane and secreted protein antigens from *Haemonchus contortus* gut and its application to diverse nematode species, Mol. Biochem. Parasitol. (1998) 97:55–68.

[22] Rhoads M.L., Fetterer R.H., Biochemical and immunochemical characterisation of ^{125}I-labelled cuticle components of *Haemonchus contortus*, Mol. Biochem. Parasitol. (1990) 42:155–164.

[23] Rhoads M.L., Fetterer R.H., Developmentally regulated secretion of cathepsin L-like cysteine proteases by *Haemonchus contortus*, J. Parasitol. (1995) 81:505–512.

[24] Ruiz A., Molina J.M., Njue A., Prichard R.K., Genetic variability in cysteine protease genes of *Haemonchus contortus*, Parasitology (2004) 128:549–559.

[25] Ruiz A., Molina J.M., González J.F., Conde M.M., Martín S., Hernández Y.I., Immunoprotection in goats against *Haemonchus contortus* after immunization with cysteine protease enriched protein fractions, Vet. Res. (2004) 35:565–572.

[26] Sajid M., McKerrow J.H., Cysteine proteases of parasitic organisms, Mol. Biochem. Parasitol. (2002) 120:1–21.

[27] Schallig H.D.F.H., Van Leeuwen M.A.W., Cornelissen A.W.C.A., Protective immunity induced by vaccination with two *Haemonchus contortus* excretory secretory proteins in sheep, Parasite Immunol. (1997) 19:447–453.

[28] Schallig H.D., van Leeuwen M.A., Hendrikx W.M., Immune responses of Texel sheep to excretory/secretory products of adult *Haemonchus contortus*, Parasitology (1994) 108:351–357.

[29] Schallig H.D., Van Leeuwen M.A., Protective immunity to the blood-feeding nematode *Haemonchus contortus* induced by vaccination with parasite low molecular weight antigens, Parasitology (1997) 114:293–299.

[30] Schierack P., Lucius R., Sonnenburg B., Schilling K., Hartmann S., Parasite-specific immunomodulatory functions of filarial cystatin, Infect. Immun. (2003) 71:2422–2429.

[31] Shompole S., Jasmer D.P., Cathepsin B-like cysteine proteases confer intestinal cysteine protease activity in *Haemonchus contortus*, J. Biol. Chem. (2001) 276 (4):2928–2934.

[32] Smith W.D., Smith S.K., Murray J.M., Protection studies with integral membrane fractions of *Haemonchus contortus*, Parasite Immunol. (1994) 16:231–241.

[33] Tort J., Brindley P.J., Knox D., Wolfe K.H., Dalton J.P., Proteinases and associated genes of parasitic helminths, Adv. Parasitol. (1999) 43:161–266.

[34] Vervelde L., Van Leeuwen M.A., Kruidenier M., Kooyman F.N., Huntley J.F., Van Die I., Cornelissen A.W., Protection studies with recombinant excretory/secretory proteins of *Haemonchus contortus*, Parasite Immunol. (2002) 24 (4):189–201.

[35] Vray B., Hartmann S., Hoebeke J., Immunomodulatory properties of cystatins, Cell. Mol. Life Sci. (2002) 59:1503–1512.

[36] Wijffels G.L., Salvatore L., Dosen M., Waddington J., Wilson L., Thompson C., et al., Vaccination of sheep with purified cysteine proteases of *Fasciola hepatica* decreases worm fecundity, Exp. Parasitol. (1994) 78:132–148.

[37] Yatsuda A.P., Krijgsveld J., Cornelissen A.W.C.A., Heck A.J.R., de Vries E., Comprehensive analysis in the secreted proteins of the parasite *Haemonchus contortus* reveals extensive sequence variation and differential immune recognition, J. Biol. Chem. (2003) 278:16941–16951.

[38] Yatsuda A.P., Bakker N., Krijgsveld J., Knox D.P., Heck A.J., de Vries E., Identification of secreted cysteine proteases from the parasitic nematode *Haemonchus contortus* detected by biotinylated inhibitors, Infect. Immun. (2006) 74:1989–1993.

[39] Young C.J., McKeand J.B., Knox D.P., Proteases released *in vitro* by parasitic stages of *Teladorsagia circumcinda* an ovine abomasal parasite, Parasitology (1995) 110:465–471.

APPENDIX

Peptide sequence identified by MS/MS. Ions y and b detected are shown in the peptide sequence of the AC-5 cysteine protease identified.