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ScienceDirect
International Journal for Parasitology 37 (2007) 1117–1125
www.elsevier.com/locate/ijpara

Expression and purification of an active cysteine protease of *Haemonchus contortus* using *Caenorhabditis elegans*

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Received 22 January 2007; received in revised form 27 February 2007; accepted 28 February 2007

Abstract

Many proteolytic enzymes of parasitic nematodes have been identified as possible targets of control. Testing these as vaccine or drug targets is often difficult due to the problems of expressing proteases in a correctly folded, active form in standard expression systems. In an effort to overcome these difficulties we have tested *Caenorhabditis elegans* as an expression system for a *Haemonchus contortus* cathepsin L cysteine protease, Hc-CPL-1. Recombinant Hc-CPL-1 with a polyhistidine tag added to the C-terminal was expressed in an active and glycosylated form in *C. elegans*. Optimal expression was obtained expressing *Hc-cpl-1* under control of the promoter of the homologous *C. elegans cpl-1* gene. The recombinant protein was purified from liquid cultures by nickel chelation chromatography in sufficient amounts for vaccination studies to be carried out. This study provides proof of principle that active, post-translationally modified parasitic nematode proteases can be expressed in *C. elegans* and this approach can be extended for expression of known protective antigens.

Keywords: Nematode; Parasite; *Caenorhabditis elegans*; Protein expression; Protease; Enzyme activity; Glycosylation

1. Introduction

The increasing problem of resistance to all classes of anthelmintics currently available highlights the need for novel control methods for parasitic nematodes of veterinary importance (Kaplan, 2004; Sargison et al., 2005). The absence of any new drugs nearing commercial development has led to greater interest in vaccine development, as well as alternative strategies including pasture management, nutrition and selective host breeding (Stear et al., 2007). A significant amount of research has been carried out on identification and testing of potential control targets, but translation of this to recombinant vaccine production remains a major hurdle. A number of highly effective antigens have been identified in protection trials against the major abomasal pathogens *Haemonchus contortus* and *Teladorsagia circumcincta* in sheep (Knox et al., 2003) and *Ostertagia ostertagi* in cattle (Claerebout et al., 2003; Vercauteren et al., 2004). However, despite the success of native proteins in inducing protective immunity, recombinant forms expressed in bacteria, yeast or insect cell expression systems are far less effective (Newton and Meeusen, 2003; Vercauteren et al., 2004). A few successes have been reported, such as the ability of a *Pichia pastoris*-expressed aspartyl protease of the dog hookworm *Ancylostoma caninum* (Ac-APR-1) (Loukas et al., 2005) and bacterially expressed *H. contortus* cysteine proteases (Redmond and Knox, 2006) to induce protection, although the latter were not as effective as the native proteins. The identified protective *H. contortus* gut antigens all have protease activity and are all glycosylated (Knox et al., 2003). Enzyme activity, indicative of appropriate folding, may

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0020-7519/$30.00
doi:10.1016/j.ijpara.2007.02.012

be required to induce appropriate protective immune responses, a suggestion supported by vaccine trials with dissociated or denatured antigen preparations, treatments which dramatically reduce the protection observed (Munn et al., 1997). In addition, a considerable proportion (>60%) of the antibody generated by vaccination with gut antigens of *Haemonchus* is to glycan moities (Geldhof et al., 2005; Knox, unpublished data). It is speculated that the limited success to date with recombinant forms, particularly of the H11 protective gut antigen, may be due to incorrect conformation and/or glycosylation differences between native and standard recombinant proteins (Newton and Meeusen, 2003).

A system which expresses parasitic nematode proteins in a form resembling the native protein, including correct folding and appropriate post-translational modifications, would, therefore, be advantageous to vaccine development. The similarities in basic biology and genome sequence between the free-living nematode *Caenorhabditis elegans* and parasitic nematodes, particularly other Clade V nematodes such as *H. contortus*, suggest *C. elegans* may be a suitable alternative expression system. Several studies have previously demonstrated expression of parasitic nematode genes in *C. elegans*. Kwa et al. (1995) showed that a β-tubulin gene, *tub-1*, from *H. contortus* could confer thiabendazole (TBZ) sensitivity when expressed in a *C. elegans* TBZ-resistant mutant. More recently, Pillai et al. (2005) reported expression in *C. elegans* of a cystatin gene from the filarial parasite *Acanthocheilonema viteae*. Although his-tagged *A. vitea* recombinant cystatin could be detected in transgenic *C. elegans* extracts by Western blot, the protein could not be purified by nickel chromatography, perhaps due to inaccessibility of the tag (Pillai et al., 2005).

In a previous study, we demonstrated rescue of a *C. elegans* cathepsin L protease mutant with the orthologous cathepsin L gene (*Hc-cpl-1*) from *H. contortus* (Britton and Murray, 2002). This demonstrated, firstly, correct folding and activation of the enzyme in the free-living nematode and, second, functional conservation of the parasite and *C. elegans* CPL-1 (Ce-CPL-1) proteases. In *C. elegans*, CPL-1 is required for correct processing of yolk proteins within developing embryos and loss of Ce-CPL-1 activity leads to embryonic lethality (Hashmi et al., 2002; Britton and Murray, 2004). *Ce-cpl-1* is expressed in the gut and the protein transported to the gonad via the pseudocoelomic fluid (Britton and Murray, 2004). Cathepsin L activity has also been implicated in egg development in the liver fluke *Fasciola hepatica*, in addition to its role in feeding. Vaccination of sheep and cattle with native *F. hepatica* cathepsin L protease reduced worm burden, egg output and egg hatching (Dalton et al., 2003). In this study, we examine whether *C. elegans* is a suitable system for bulk expression of Hc-CPL-1 and examine enzymatic activity and post-translational modification of the expressed protease. We also test the recombinant protein in protection studies in sheep to determine any effect on embryonic development.

2. Materials and methods

2.1. Generation of histidine tagged protein expression constructs

2.1.1. Ce-cpl-1 promoter construct

An *Hc-cpl-1* construct was previously generated in the pGEM-T vector (Promega) containing 1.76 kb of *Ce-cpl-1* upstream sequence, 512 bp of *Ce-cpl-1* 3′ untranslated region (UTR) and *Hc-cpl-1* coding sequence (Britton and Murray, 2002). To generate a polyhistidine tagged Hc-CPL-1 expression construct (*Hc-cpl-1-His*), the *Hc-cpl-1* coding sequence was excised from the above construct with *Xba*I and *Sal*I and a new *Hc-cpl-1* insert, encoding a 10× histidine tag at the C-terminal, was ligated into the *Xba*I/*Sal*I digested vector. The new insert was generated by PCR amplification of a previously isolated *Hc-cpl-1* cDNA clone using primers *Hc-cpl-1*-hF1 (5′CAA*ACT AGT*ATGCTACGTCTGTTGTCGTTGGCGC3′; underline indicates start codon; italics indicate *Spe*I restriction site to ligate into the *Xba*I restriction site of the vector) and *Hc-cpl-1*-hR1 (5′AGC*GTCGAC*CCTAGTGGTGGT GGTGGTGGTGGTGGTGGTGGTGAACGAGCGGG TAGCTGGCC3′; underline indicates complement of the stop codon; italics indicate *Sal*I restriction site).

2.1.2. GATA-Ce-cpl-1 promoter construct

A GATA enhancer-construct was generated containing six copies of a GATA enhancer element from the *C. elegans* cathepsin B protease *cpr-1*. The GATA concatamer was excised from clone pC1562 (Britton et al., 1998) by digestion with *Sph*I and cloned into the *Sph*I site present at the 5′ end of the *Ce-cpl-1* promoter in the rescue construct described above.

2.1.3. let-858 promoter construct

For cloning into *C. elegans let-858* promoter vector pPD118.25 (kindly provided by Andy Fire), His-tagged *Hc-cpl-1* was PCR amplified using primers *Hc-cpl-1*-LF1 (5′CAA*GCTAGC*ATGCTACGTCTGTTGTCGTTGGCG C3′; underline indicates start codon; italics indicate *Nhe*I restriction site) and *Hc-cpl-1*-LR1 (5′AGC*GCTAGC*CCTA GTGGTGGTGGTGGTGGTGGTGGTGGTGAAC GAGCGGGTAGCTGGCC3′; underline indicates complement of stop codon; italics indicate *Nhe*I restriction site). The PCR product was cloned initially into TOPO vector pCR2.1 (Invitrogen), excised from pCR2.1 with *Nhe*I and cloned into *Nhe*I digested, CIP (calf intestinal phosphatase) treated pPD118.25 vector. Automated DNA sequencing was carried out to confirm the identity of all cloned fragments (MWG Biotech).

2.2. Testing rescue activity of C. elegans-expressed Hc-CPL-1 his-tagged protein

Plasmid DNA for each of the *Hc-cpl-1-His* constructs was prepared using the Qiagen protocol and injected (final

concentration 25 μg/ml), together with *rol-6* plasmid pRF4 (final concentration 100 μg/ml; Kramer et al., 1990), into the gonad of *C. elegans* adult hermaphrodites. Transformation was carried out in *Ce-cpl-1* deletion strain VC322 (allele *ok360*) previously rescued with the *Ce-cpl-1* wild-type gene together with *dpy-7:green fluorescent protein (GFP)* as a marker gene (Britton and Murray, 2002). The rescued strain was used as *Ce-cpl-1* (*ok360*) homozygous mutants produce 95–100% dead embryos and cannot, therefore, be maintained. Progeny of the injected worms which showed a roller phenotype and loss of *dpy-7:GFP* were maintained and their progeny scored for embryonic survival. Whole worm PCR using gene-specific primers was carried out to confirm that roller progeny contained the *Hc-cpl-1-His* construct in a *Ce-cpl-1* (*ok360*) homozygous mutant background (Britton and Murray, 2002).

2.3. Caenorhabditis elegans culture and protein purification

Caenorhabditis elegans worms transformed with the *Hc-cpl-1-His* and *rol-6* constructs were grown on 30 9-cm normal growth medium (NGM) agar plates seeded with *Eschericia coli* OP50 (Sulston and Hodgkin, 1988) at 20 °C for 4 days, at which time the worms were adult stage. To prevent starvation, worms were fed with additional OP50 bacteria 24 h before washing off. Worms were washed from the plates using M9 buffer (Sulston and Hodgkin, 1988), left to settle on ice for 10 min and washed a further three times to remove OP50 bacteria. For liquid culture, *Hc-cpl-1-His*-transformed worms were grown initially on five 9-cm NGM agar plates for 5 days, at which time the OP50 bacteria had cleared from the plates. Worms were washed from the plates in M9 buffer and added to a 3 l flask containing 500 ml S medium (Sulston and Hodgkin, 1988) supplemented with 28 ml of packed OP50 culture. Worms were grown at 20 °C, 200 rpm for 5 days, with 56 ml OP50 added on day 2 to prevent starvation. Worms were separated from OP50 bacteria and worm debris by centrifugation at 1730*g* on a 60% (w/v) sucrose gradient. Worms were collected from the upper layer, washed three times in 0.1 M NaCl and once in M9 buffer, by allowing them to settle on ice. Worms from the agar plates or liquid culture were homogenised in M9 on ice using a standard glass homogeniser. The worm extract was centrifuged at 16,060*g* for 15 min and the supernatant collected. This was applied to a 2 ml column packed with ProBond Nickel Chelating Resin (Invitrogen) previously charged with Ni^{2+} ions, according to the manufacturer's instructions. The worm supernatant was left to bind to the column using gentle agitation for 1 h at room temperature. The column was then left to settle under gravity and the unbound supernatant collected. The column was washed four times in native wash buffer according to the manufacturer's instructions and the bound material eluted with 8 ml native elution buffer containing 400 mM imidazole. Initial Western blot studies indicated efficient binding and elution of the Hc-CPL-1-His protein from the column. The eluted sample was concentrated using Centricon 10 devices according the manufacturer's instructions (Amicon) and dialysed against PBS. Protein concentration of the concentrated sample was estimated using the Coomassie Protein Assay Reagent (Pierce).

2.4. SDS–PAGE and Western blotting

Adult total worm extracts were prepared by washing hermaphrodites in M9 buffer and heating to 100 °C for 15 min in lysis buffer (50 mM Tris pH 7.5, 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), 1 mM EDTA, 10 μM L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E64, Sigma)) containing 5% SDS and 5% β-mercaptoethanol. For comparison of protein expression levels equal numbers of age-matched worms were picked into M9 buffer and lysed as above. Before loading onto gels, samples were boiled for 10 min in an equal volume of loading buffer (lysis buffer containing 10% glycerol and 0.1% Bromophenol blue). Proteins were separated on 10% SDS–polyacrylamide gels using the Bio-Rad mini-system and blotted into polyvinylidene fluoride (PVDF) membrane. Western blotting was carried out by standard procedure using an antibody to the mature region of Ce-CPL-1 (Britton and Murray, 2004; 1:200 dilution) and an anti-His tag antibody (Invitrogen; 1:500 dilution). Alkaline phosphatase conjugated secondary antibodies (Molecular Probes) were used routinely at 1:3000 dilution. Substrate gel analysis was carried out essentially as described by Geldhof et al. (2000), using 0.1% final concentration of gelatin (Heussen and Dowdle, 1980).

2.5. Enzyme activity assays

The synthetic substrates carbobenzoxy-arginyl-arginyl 7-amino-4-methyl coumarin (Z-Arg-Arg-AMC) and carbobenzoxy-phenylalanyl-arginyl 7-amino-4-methyl coumarin (Z-Phe-Arg-AMC) (Sigma) were used to measure cathepsin L and B activities. Z-Arg-Arg-AMC is selective for cathepsin B activity, while both cathepsin L and B show activity with Z-Phe-Arg-AMC substrate (Hasnain et al., 1993). Stock solutions of the substrates were prepared in methanol at a concentration of 200 μM. The assay mixture contained 10 μl of the purified recombinant cathepsin L (approximately 5 μg protein), 5 μl 0.1 M dithiothreitol (DTT), 5 μl AMC substrate (final concentration 9 μM) and 90 μl of buffer (pH 3 and 4: 0.1 M acetate buffer, pH 5 and 6: 0.1 M phosphate buffer, pH 7: 0.1 M Tris buffer). For inhibition assays, the cysteine protease inhibitor E64 was added to the reaction mixture at a final concentration of 100 μM. The release of the fluorescent group, AMC, was monitored with a fluorescent spectrophotometer (Perkin-Elmer, UK) (excitation wavelength: 370 nm, emission wavelength: 450 nm) during 300 s. Results were calculated as the change in fluorescence over the 300 s period.

2.6. Analysis of glycosylation

Samples of approximately 1 μg of protein, eluted from the Nickel column, were incubated at room temperature for 1 h with various concentrations (0–500 U) of PNGase F in digest buffer according to the manufacturer's instructions (New England Biolabs). Ten microlitres of SDS–PAGE loading buffer was added to the samples, which were then boiled for 10 min and loaded onto a 10% SDS polyacrylamide mini-gel. Western blotting was carried out as described above.

To examine binding to Concanavalin A lectin (Con A), 400 μl (approximately 200 μg) purified protein in running buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 0.1 mM $MnCl_2$, 0.5% (v/v) Triton X-100) was applied to a 1 ml column of packed Con A agarose (Sigma) and incubated for 1 h with agitation. The column was left to settle under gravity and unbound protein collected following three washes with running buffer. A bound sample was eluted with 0.1 M methylmannopyranoside, followed by 0.1 M HCl. The HCl eluted fractions were collected into 2 M Tris to neutralise the acid. All fractions were concentrated using Centricon devices, dialysed against PBS and Western blotting carried out with anti-Ce-CPL-1 antibody as described above.

2.7. Protection trial with C. elegans-expressed Hc-CPL-1

Five-month-old worm-free Suffolk lambs were allocated into groups of seven, balanced for sex and weight. One group received three immunisations (each 70 μg *C. elegans* expressed cathepsin L protein) at 3 weekly intervals and were challenged with 5000 infective L3 *H. contortus* 1 week after the final immunisation. Five milligram Quil A was used as adjuvant and the final volume was adjusted to 2 ml with PBS. This was administered i.m. as two 1 ml doses into each hind leg, with control animals receiving adjuvant alone. Blood was sampled at weekly intervals and faecal egg counts (FEC) monitored three times weekly from day 16 after challenge until necropsy at days 34–36 when worm numbers were counted, as previously described (Smith and Smith, 1993). All procedures were carried out in accordance with the Animal (Scientific Procedures) Act and approved by the Moredun Ethical Committee.

2.8. ELISA

Microtitre plates were coated overnight at 4 °C with 2 μg/ml *C. elegans*-expressed Hc-CPL-1-His protein diluted in 50 mM bicarbonate buffer, pH 9.6. Total IgG titres for individual animals were measured as described previously (Redmond and Knox, 2004) and values expressed as the dilution factor required to obtain the mean of the group pre-immune serum values at a dilution of 1:20.

2.9. Immunolocalisation

Haemonchus contortus adult worms (33 days old) were embedded in paraffin wax and transverse sections cut and probed with pooled pre-immune or cathepsin L immune sera from vaccinated animals at a 1:100 dilution as described previously (Redmond et al., 2004). Fluorescein isothiocyanate (FITC)-labelled rabbit anti-sheep IgG was used at a dilution of 1:200. Sections were viewed under a fluorescence microscope (Olympus BX50) and images captured using a digital camera (Olympus DP70) at 200× magnification.

3. Results

3.1. Rescue of C. elegans cpl-1 mutant with his-tagged Hc-cpl-1

We previously reported rescue of *Ce-cpl-1* RNA interference (RNAi) or genetic mutants with the homologous *Hc-cpl-1* gene (Britton and Murray, 2002). In the present study, we aimed to determine whether active protease could be expressed in *C. elegans* and purified in sufficient quantities for the protease to be tested in protection studies. An *Hc-cpl-1* construct encoding a 10× histidine tag at the C-terminus of the protein (Hc-CPL-1-His) was generated and expressed in *Ce-cpl-1* genetic mutant (*ok360*), using the *rol-6* gene as a marker of transformation. The histidine tagged construct gave 95% rescue of the *Ce-cpl-1* mutant phenotype, identical to that observed with the non-histidine tagged construct (Britton and Murray, 2002). Addition of the C-terminal tag, therefore, had no effect on expression or activity of the Hc-CPL-1 protease.

As *Ce-cpl-1* is required maternally for embryonic development (Britton and Murray, 2004), it was necessary to inject *Ce-cpl-1* mutant adult hermaphrodites which had previously been rescued with the *Ce-cpl-1* wild-type gene, together with *dpy-7:GPF* as a marker gene, to allow development of viable embryos. All three roller lines obtained following microinjection of the *Hc-cpl-1-His* construct with *rol-6* as a marker gene had lost the original *Ce-cpl-1/dpy-7::GFP* array and carried only the *Hc-cpl-1/rol-6* array, as confirmed by PCR with species-specific *cpl-1* PCR primers. In addition to transformation of the *Ce-cpl-1* mutant strain, the *C. elegans* wild-type N2 Bristol strain was also transformed with the *Hc-cpl-1-His* construct. Similar numbers of transformants were obtained and identical levels of protein expression were observed, with both strains using total worm extracts on Western blots probed anti-his tag or anti-Ce-CPL-1 antibodies (Fig. 1A). No reactivity was observed with extracts from non-rescued *Ce-cpl-1* (*ok360*) homozygous mutant worms, indicating specific recognition of the expressed *H. contortus* CPL-1 protease (data not shown).

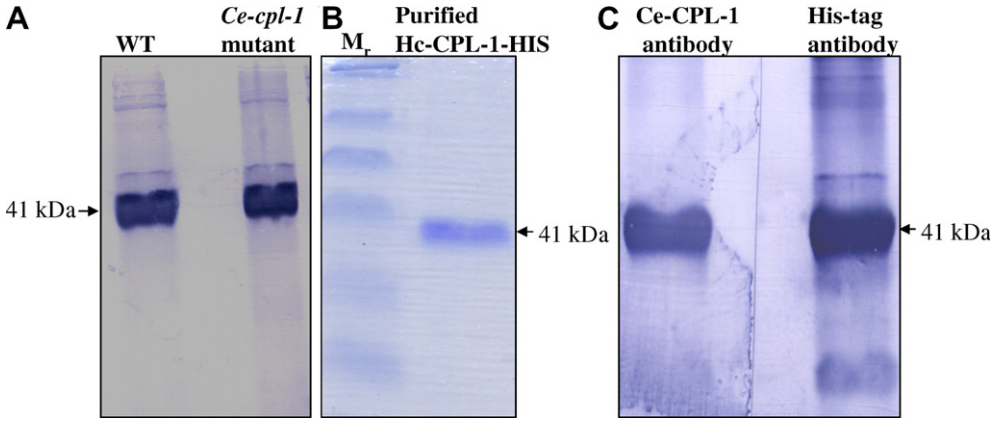

Fig. 1. Detection of *Caenorhabditis elegans*-expressed *Haemonchus contortus* CPL-1 protease. (A) Comparison of Hc-CPL-1-His protein in total adult worm extracts of *C. elegans* N2 wild-type (WT) and *cpl-1* (*ok360*) mutant worms transformed with *Hc-cpl-1-His* expression construct (75 adults per lane). The Western blot was probed with anti-His (C-terminal) antibody. (B) Coomassie staining of concentrated eluate following Nickel affinity chromatography of the soluble fraction of *Hc-cpl-1* transgenic *C. elegans*. (C) Western blot of affinity column eluate probed with antibodies to the mature region of Ce-CPL-1 and to the histidine tag. M_r indicates molecular mass marker proteins.

3.2. Purification of Hc-CPL-1-His and yield from solid and liquid cultures

Addition of the histidine tag allowed purification of *C. elegans*-expressed Hc-CPL-1 by nickel chelation chromatography. In initial studies, worms were cultured on 30 9-cm NGM plates and yielded approximately 200 μg purified Hc-CPL-1 from approximately 1 ml of packed worms. To increase the amount of purified protein obtained, liquid cultures (500 ml) were used and yielded approximately 2 mg purified cathepsin L protein from around 12 ml packed worms. Solid and liquid cultures of transgenic *C. elegans* were, therefore, equally efficient in producing Hc-CPL-1. The volume of liquid culture and all subsequent cleaning, homogenisation and purification procedures were easily manageable.

Analysis of protein eluted from the nickel column by SDS–PAGE and Coomassie staining showed the presence of a major band of approximately 41 kDa with very little contamination with other proteins (Fig. 1B). Western blotting with anti-His tag and anti-Ce-CPL-1 antibodies (Britton and Murray, 2004) confirmed that the major 41 kDa protein staining with Coomasssie was Hc-CPL-1-His (Fig. 1C).

3.3. Caenorhabditis elegans-expressed Hc-CPL-1-His is enzymatically active and glycosylated

Rescue of the *Ce-cpl-1* mutant phenotype with *Hc-cpl-1-His* indicated that the expressed protease was active and capable of carrying out the same enzymatic activity as Ce-CPL-1. Presence of enzymatic activity was also confirmed by gelatin substrate SDS–PAGE of the purified protein. A zone of activity at approximately 41 kDa was observed which overlayed the Coomassie stained band in Fig. 1B (not shown). To characterise the activity, purified Hc-CPL-1-His was incubated with the standard fluorogenic cysteine protease substrates Z-Phe-Arg-AMC and Z-Arg-Arg-AMC. The protein showed activity against Z-Phe-Arg-AMC, but not with the cathepsin B substrate Z-Arg-Arg-AMC. Optimum activity was detected at pH 5, indicating that Hc-CPL-1-His has typical cathepsin L activity (Fig. 2A). This activity was enhanced in the presence of the reducing agent DTT and inhibited by E64, indicating it is a typical cysteine protease (Fig. 2B).

Ce-CPL-1 has one potential N-linked glycosylation site which is conserved in Hc-CPL-1 and other parasitic nematode CPL-1 proteases (Britton and Murray, 2002). To examine glycosylation of Hc-CPL-1 expressed in *C. elegans*, purified Hc-CPL-1-His was incubated with various concentrations of PNGase F to remove N-linked glycans. This treatment led to a reduction of 2–3 kDa in the size of the Hc-CPL-1 protein, with no apparent intermediate sizes, indicating the presence of an N-linked glycan

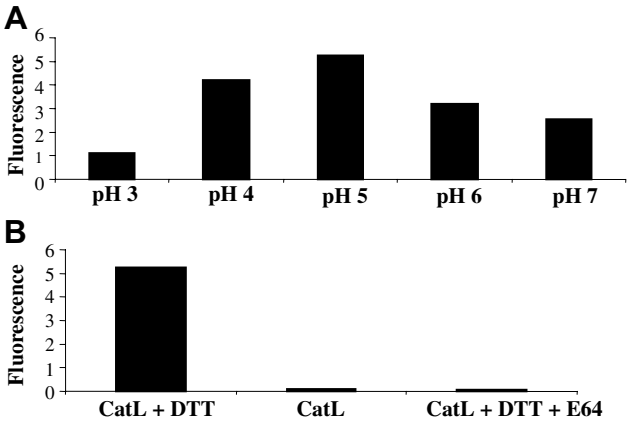

Fig. 2. Enzymatic activity of *Caenorhabditis elegans*-expressed Hc-CPL-1-His with Z-Phe-Arg-AMC substrate. Activity was measured at various pH values in the presence of dithiothreitol (DTT) (A) and at pH 5 in the presence or absence of DTT and E64 inhibitor (B). Results are shown as the change in fluorescence over a period of 300 s.

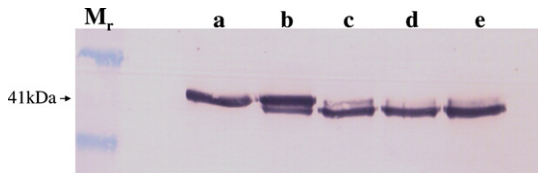

Fig. 3. PNGase F deglycosylation of Hc-CPL-1-His. Affinity purified protein was incubated with different units (U) of PNGase F (a, 0 U; b, 0.5 U; c, 5 U; d, 50 U; e, 500 U) for 1 h and analysed by Western blotting with anti-His antibody.

(Fig. 3). Although detailed analysis of native Hc-CPL-1 has not been carried out, this protease was identified as a minor protein present in an *H. contortus* fraction eluted from a Con A affinity column (Geldhof and Knox, unpublished data). To examine lectin binding of purified Hc-CPL-1-His, the protein was applied to a Con A-agarose column. Western blotting with anti-Ce-CPL-1 antibody of fractions eluted with methylmannopyranoside and HCl demonstrated the presence of the Hc-CPL-1 protein (not shown).

3.4. Expression of *Hc-cpl-1-His* under control of different *C. elegans* promoter and enhancer regions

The yield of protein from liquid cultures of transgenic *C. elegans* was sufficient for biochemical analysis and vaccine trials. To examine whether it was possible to increase expression of *Hc-cpl-1-His*, a GATA concatamer was inserted upstream of the 1.76 kb *Ce-cpl-1* promoter region used in the original construct. The GATA enhancer was derived from *C. elegans cpr-1* cathepsin B gene promoter and was previously shown to enhance expression of a *lacZ* reporter gene in gut and hypodermal cells (Britton et al., 1998). Hc-CPL-1-His expressed under control of the GATA enhancer-containing promoter was able to efficiently rescue the *Ce-cpl-1* mutant phenotype and Western blot analysis showed expression of Hc-CPL-1-His protein. However, the protein expression level with the GATA enhancer-construct was slightly lower than that with the *Ce-cpl-1* promoter alone (not shown).

Expression of Hc-CPL-His under control of the *C. elegans let-858* promoter was also examined. *Caenorhabditis elegans let-858* is expressed in all cells throughout development and has been used previously to direct expression of *C. elegans* and non-*C. elegans* genes (Paschinger et al., 2004; Pillai et al., 2005). Although we obtained *C. elegans* transgenic lines carrying the *Hc-cpl-1-His* construct under control of the *let-858* promoter, as indicated by PCR analysis, this construct did not rescue the *Ce-cpl-1* mutant phenotype and no protein could be detected by Western blot analysis with anti-Ce-CPL-1 or anti-His tag antibodies.

3.5. Protection trial and antibody response to vaccination with *Hc-CPL-1-His*

A protection trial was carried out with *C. elegans*-expressed Hc-CPL-1 to examine any effects, particularly on egg development. The FEC, adult worm burden and packed cell volume (PCV) at necropsy for the cathepsin L vaccinated and adjuvant control groups are shown in Table 1. No reduction in FEC and worm burden was observed in the vaccinated group and no difference in PCV values was measured. FEC from day 16 post-challenge to necropsy on day 33 showed no significant difference between the vaccinated and adjuvant control groups (data not shown). There was also no reduction in numbers of larvae hatching from eggs of Hc-CPL-1 vaccinated animals. Despite this lack of protection against challenge, high titres of total IgG to Hc-CPL-1-His were measured in ELISA in the cathepsin L vaccinated group. The antibody response in the adjuvant control group was negligible (Table 2).

3.6. Immunolocalisation on adult *H. contortus* sections

Transverse sections of *H. contortus* adult worms were probed with pooled pre-immune serum or pooled anti-cathepsin L serum collected at necropsy from vaccinated animals. Strong staining was observed around developing

Table 1
Faecal egg counts (FEC), adult worm burden and packed cell volume (PCV) at necropsy

Group	Adjuvant control			Cathepsin L vaccinated		
Animal number	Mean FEC	Adult worms	PCV values	Mean FEC	Adult worms	PCV values
A	1928	2150	28	1634	2750	26
B	2564	2600	22	1547	3550	27
C	1195	4650	30	1271	3350	26
D	1014	3050	28	1472	6150	28
E	943	4800	32	1756	5950	26
F	1271	2650	27	1853	3750	30
G	1383	1650	31	2052	2600	28
Mean	1471	3079	28	1655	4014	27
SEM	219	456	1.25	98	548	0.565
% protection	n/a	n/a	n/a	−12.5	−30.4	−3.7

Mean FEC represent the cumulative means for each animal from day 16 to end of trial. Adult worms are total worm burden at necropsy estimated from actual count in a 2% sample. PCV values are haematocrit value.

Table 2
Anti-Hc-CPL-1 IgG antibody titres

Group Animal number	Adjuvant control Total IgG titre	Cathepsin L vaccinated Total IgG titre
A	83	77 778
B	250	34 292
C	211	54 344
D	504	108 755
E	96	115 308
F	112	33 920
G	59	103 345
Mean	188	75 392
SEM	59	13 224

IgG values are expressed as the dilution factor required to obtain the mean of the group pre-immune serum values at a 1:20 dilution.

eggs within the uterus and on the uterine wall with the antiserum from the cathepsin L vaccinated group (Fig. 4A). No staining was observed in any other tissues, including the gut. Probing with pre-immune serum resulted in no staining of any tissues (Fig. 4B).

4. Discussion

The reported failure of recombinant forms of identified protective parasite antigens to induce protective immunity is thought to be due to inappropriate folding and/or glycosylation. This led us to examine the suitability of *C. elegans* as an alternative expression system, focussing on the *H. contortus* cathepsin L cysteine protease Hc-CPL-1. Several studies have previously shown that *C. elegans* can be used as a surrogate system for expression and analysis of parasitic nematode proteins (Kwa et al., 1995; Redmond et al., 2001; Britton and Murray, 2002; Kampkötter et al., 2003; Pillai et al., 2005). This study is the first to show that recombinant protein can be expressed and purified from extracts of transgenic *C. elegans* in sufficient amounts for protection studies to be carried out. Moreover, we show that the expressed protease is enzymatically active and glycosylated. PNGAse F digestion demonstrated the presence of one N-linked glycan on *C. elegans*-expressed Hc-CPL-1, consistent with glycosylation predicted from sequence analysis of nematode CPL-1 proteins identified to date (Britton and Murray, 2002) and shown experimentally to be the only *N*-glycosylation site on Ce-CPL-1 (Fan et al., 2005). Although similarity in glycan structures in native and *C. elegans*-expressed proteins will require detailed mass spectrometric analysis, this study provides proof of principle that biologically active and post-translationally modified parasitic nematode protein can be expressed in *C. elegans* in sufficient amounts for biochemical and immunisation studies.

Although this study focussed on Hc-CPL-1, it can potentially be used to test expression, activity and modification of any parasitic nematode protein. Of particular interest will be previously identified protective gut antigens of *H. contortus*, such as the aminopeptidase H11 and the cathepsin B proteases, known to be glycosylated (Knox et al., 2003). Protection trials with bacterially expressed *H. contortus* cathepsin B proteases resulted in moderate but significant decreases in worm burden and egg output (Redmond and Knox, 2006) and it will be important to test whether this can be enhanced with *C. elegans*-expressed forms. H11 is the most effective vaccine candidate identified (Smith et al., 1993) and detailed glycan analysis of H11 shows the presence of an unusual, highly fucosylated structure (Haslam et al., 1996) which may enhance immunogenicity. It is speculated that the inability of recombinant H11 expressed in baculovirus to induce protection may be due to inappropriate glycosylation (Newton and Meeusen, 2003). Interestingly, initial studies suggest that nematode fucosylated glycans may be involved in inducing Th2 responses in mice (Tawill et al., 2004). Analysis of the *C. elegans* glycome is progressing (Haslam and Dell, 2003; Hanneman et al., 2006) together with identification and functional analysis of many of the enzymes involved in glycosylation (Schachter, 2004). The *C. elegans* system and the availability of glycosylation mutants will allow more detailed examination of parasite glycans and their possible role(s) and effects on the host immune response (Dell et al., 1999) than is possible using standard expression systems.

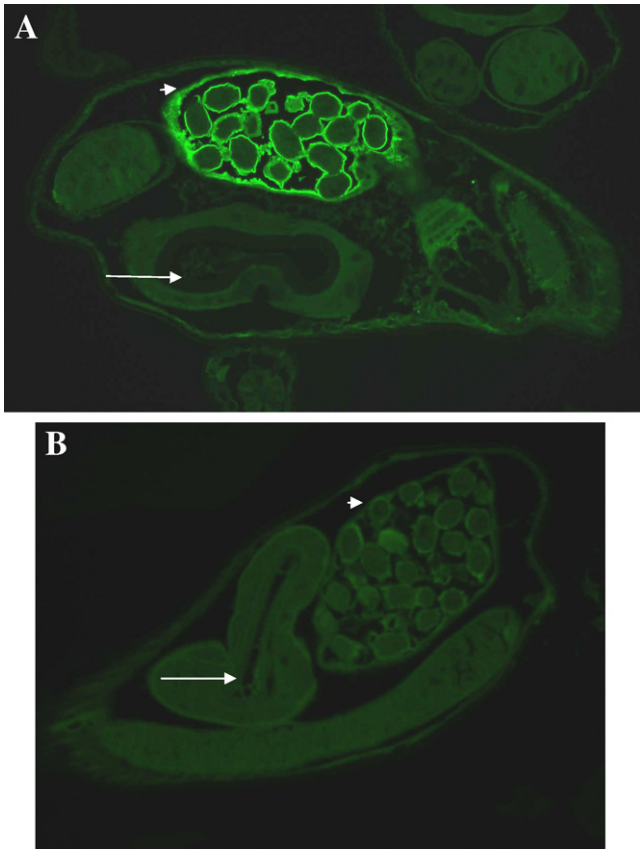

Fig. 4. Immunolocalisation of pooled antisera from the cathepsin L vaccinated group (A) and pre-immune serum from the same group (B) on adult *Haemonchus contortus* transverse sections. The uterus containing developing eggs is indicated by an arrowhead and the microvillar surface of the gut is indicated by an arrow. This image is typical of many sections incubated with these antisera. The diameter of sections is approximately 300 μm.

The levels of expression of Hc-CPL-1 in N2 wild-type worms and *Ce-cpl-1* mutant worms were identical indicating that expression of both the *C. elegans* and parasite proteases is not detrimental to worm development. This suggests that availability of a *C. elegans* genetic mutant is not a pre-requisite for expression of a related parasite gene. Hc-CPL-1 was expressed under the control of the *Ce-cpl-1* promoter, which produced sufficient protein for analysis and immunisation trials. Addition of GATA motifs, which previously enhanced expression from the *Ce-cpr-1* cathepsin B promoter (Britton et al., 1998) or use of the ubiquitously active *Ce-let-858* promoter (http://www.wormbase.org), resulted in reduced expression compared with the *Ce-cpl-1* promoter alone. This suggests that strong over-expression or inappropriate temporal and/or spatial expression may be detrimental and may lead to low level expression or silencing of introduced transgenes. Our findings suggest that, where possible, use of the promoter region of a related *C. elegans* gene is likely to lead to optimal expression levels of introduced parasite genes. Where this is not possible, use of a tissue-specific *C. elegans* promoter, such as *cpr-5* for gut-specific expression (Redmond et al., 2001), may be better than ubiquitous expression.

In the present study, the *Hc-cpl-1* gene was maintained in transformed *C. elegans* as an extrachromosomal array, along with the *rol-6* marker gene, rather than being integrated into the genome. In *C. elegans*, extrachromosomal arrays are lost at each generation and the *Hc-cpl-1* construct was maintained in around 70–80% of the population. This did not present a problem as the expression studies were carried out in *Ce-cpl-1* (*ok360*) mutant strain which produces inviable progeny and non-transformed worms were not maintained in the population. Integration of low-copy arrays can be readily achieved by irradiation of transformants, by introducing DNA by biolistic bombardment or by standard microinjection with transgene DNA together with a single stranded DNA oligo (Praitis et al., 2001; Evans, 2006). As well as obtaining 100% transformants, integration may lead to more stable expression and reduce any silencing effects of multi-copy arrays, if this occurs.

Ce-CPL-1 is essential during embryonic development for correct processing of yolk proteins (Britton and Murray, 2004) and rescue of the *C. elegans* embryonic lethal phenotype with *Hc-cpl-1* suggested conservation of function. Native Hc-CPL-1 has not been tested in protection trials due to the difficulty of obtaining sufficient quantities from parasite extracts. We examined whether vaccination of sheep with *C. elegans*-expressed Hc-CPL-1 provided any protection against challenge infection. No effects were observed on embryonic development, egg hatching nor on adult worms. Animals vaccinated with the recombinant protein produced significant antibody (IgG) titres to the recombinant protease and anti-vaccine antiserum bound strongly to developing eggs within the uterus in worm sections. However, no binding of antibody was detected in worms recovered at necropsy from immunised animals. This suggests that Hc-CPL-1 within the gonad in intact worms is inaccessible to antibody, at least in sufficient amounts to interfere with function. This is in contrast to gut antigens of *H. contortus* which, although considered hidden, are bound by circulating antibodies following vaccination with native protein (Knox et al., 2003). An alternative explanation for lack of protection is that additional proteases may compensate for immune-mediated inactivation of Hc-CPL-1. *C. elegans* expresses at least four cathepsin L-like enzymes (F41E6.6, R09F10.3, R07E3.1 and *cpl-1*; http://www.wormbase.org) and sequences related to these can be identified in *H. contortus* expressed sequence tag (EST) and genome sequence data (currently estimated at 95% genome coverage; http://www.sanger.ac.uk/Projects/H_contortus/). The lethal phenotype of *Ce-cpl-1* mutants indicates compensation does not occur in this nematode but we cannot rule this out for *H. contortus*.

We previously showed that Ce-CPL-1 is transported from the gut to the uterus as a pro-enzyme (Britton and Murray, 2004). In the present study, the Hc-CPL-1 protease purified from *C. elegans* adult worm extracts, and used in vaccination, was predominantly in the pro-form rather than the mature enzyme. This, most likely, explains the lower amount of activity measured with Z-Phe-Arg-AMC than may be expected. However, the rescue studies clearly demonstrated that the pro-enzyme was correctly folded and could be efficiently activated, indicating no inherent problem with the *C. elegans*-expressed protease. It seems unlikely that use of the Hc-CPL-1 pro-enzyme led to ineffective protection. Previous studies used the cathepsin L pro-enzyme of *F. hepatica* to induce protection: the mature protease was not used due to degradation problems (Dalton, personal communication). Although Hc-CPL-1 seems not to be an effective target of immune-mediated control of *H. contortus*, we have established that active, glycosylated parasite enzyme can be expressed and purified from transgenic *C. elegans* in sufficient amounts for biochemical analysis, vaccine testing or in vitro screening of inhibitors (Choe et al., 2005). This system can be extended to other parasite proteins and will be particularly important for expression of complex glycan epitopes and examination of their influence on the immune response to parasitic nematodes.

Acknowledgements

We are grateful to the Clinical and Parasitology Divisions at Moredun for their invaluable assistance in carrying out the vaccine trial. This work was funded by BBSRC and SEERAD (Scottish Executive Environment and Rural Affairs Department). P.G. is the recipient of a Marie Curie Intra-European Fellowship (No. 500859).

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