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## The effects of plant cysteine proteinases on the nematode cuticle

Victor S Njom ${ }^{1,2,}$ Tim Winks ${ }^{1,3}$, Oumu Diallo ${ }^{1,3}$, Ann Lowe ${ }^{4}$, Jerzy Behnke ${ }^{4}$, Mark J Dickman

Running Title: Nematode cuticle breakdown by plant cysteine proteinases


#### Abstract

Background: Plant-derived cysteine proteinases of the papain family (CPs) attack nematodes by digesting the cuticle leading to rupture and death of the worm. The nematode cuticle is composed of collagens and cuticlins but the specific molecular target(s) for the proteinases have yet to be identified.

Methods: This study followed the course of nematode cuticle disruption using immunohistochemistry, scanning electron microscopy and proteomics, using a free-living nematode, Caenorhabditis elegans and the murine GI nematode Heligmosomoides bakeri (H. polygyrus) as target organisms.

Results: Immunohistochemistry indicated that DPY-7 collagen is a target for CPs on the cuticle of C. elegans. The time-course of loss of DPY-7 from the cuticle allowed us to use it to visualise the process of cuticle disruption. There was a marked difference in the time-course of damage to the cuticles of the two species of nematode, with $H$. bakeri being more rapidly hydrolysed. In general, the CPs' mode of attack on the nematode cuticle was by degrading the structural proteins, leading to loss of integrity of the cuticle, and finally death of the nematode. Proteomic analysis failed conclusively to identify structural targets for CPs, but preliminary data suggested that COL-87 and CUT-19 may be important targets for the CPs, the digestion of which may contribute to cuticle disruption and death of the worm. Cuticle globin was also identified as a cuticular target. The presence of more than one target protein may slow the development of resistance against this new class of anthelmintic.


Conclusions: Scanning electron microscopy and immunohistochemistry allowed the process of disruption of the cuticle to be followed with time. Cuticle collagens and cuticlins are molecular targets for plant cysteine proteinases. However, the presence of tyrosine crosslinks in nematode cuticle proteins seriously impeded protein identification by proteomic analyses.

Multiple cuticle targets exist, probably making resistance to this new anthelmintic slow to develop.


Keywords: C. elegans, H. bakeri, papain, papaya latex, cuticle, anthelmintic, proteomics, imaging, immunohistochemistry

## Background

Parasitic nematodes cause enormous public health, agricultural and economic problems worldwide, as pathogens of humans [1, 2], livestock [3] and crops [4]. In humans, treatment of gastro-intestinal (GI) nematode/soil transmitted helminth (STH) infections is usually with one or a combination of two or all three common classes of synthetic anthelmintics: benzimidazoles, nicotinic acetylcholine agonists and macrocyclic lactones [5], whose modes of action range from binding to microtubules and blockage of metabolic pathways to effects on neuromuscular transmission [6]. The intensive use of drugs and the dependence of treatment of nematode infection on only a few drugs with similar modes of action have put pressure on the drug candidates with resulting loss of potency due to development of resistance by target nematodes [7-9]. Nematode resistance to anthelmintics is a crisis in certain livestock industries particularly in small ruminant animals, where triple resistant nematodes have been reported [10]. Though the greatest problem is in treatment of ruminants, there are signs that resistance is also developing in human populations [7, 8, 11, 12].

Nematodes are protected from their environment by their cuticle, which also confers shape and integrity to the worms. The cuticle is made of two important structural protein types; collagens and cuticlins, encoded by about 160 and at least 8 genes, respectively in C. elegans. These structural proteins are strengthened by the presence of disulphide and tyrosine-based crosslinks [13-15]. The parasitic nematode species that inhabit the gastrointestinal tract produce proteinase inhibitors of serine proteinases and pepsin, and hence are able to avoid being digested and can survive in the gut lumen [16-18]. However, in the GI tract they are not exposed to high concentrations of cysteine proteinases (CPs) and therefore do not experience selective pressure to develop inhibitors to this class of proteinase in order to survive in the gut. Their protective cuticle may therefore be sensitive to digestion by this class of proteinases. Tropical countries have relied to some extent on plant extracts for the treatment of nematode infections [19], including extracts that contain CPs [20-22].

Many plant CPs are in the papain family (sub family C1A in the phylogenetic classification in the Merops database - http://merops.sanger.ac.uk/ ) [23]. They attack the nematode cuticle, weakening its structure sufficiently to allow the internal high hydrostatic pressure in the pseudocoelomic cavity to rupture the cuticle, resulting in evisceration and death of the worm. This mode of action appears to be the same both in vitro and in vivo [24-27]. Free-living and plant parasitic nematodes undergo the same fate as animal GI nematodes [28-30]

To accomplish growth, the cuticle is shed five times in the life of a nematode in a process known as moulting or ecdysis [15]. This involves the digestion of the old cuticle by cysteine and metallo-proteinases [15, 31]. It is possible that the anthelmintic action of plant CPs may therefore mimic the process of removal of the old unwanted cuticle during moulting.

For CPs to be accepted as an anthelmintic for livestock or for human use, we need to understand more about the mode of action, safety and toxicity. We have therefore investigated cuticle
disruption by CPs of a well annotated free living nematode, C. elegans, using an immunohistochemical approach, then described the time-dependent process of cuticle digestion of C. elegans and a murine GI nematode, Heligmosomoides bakeri, using scanning electron microscopy and immunohistochemistry. We also undertook a proteomic approach in an attempt to identify the molecular targets for CPs. The presence of multiple targets for CPs in the cuticle is highly likely to decrease the chance of future resistance developing against the drug.

## Materials and Methods

## C. elegans culture

The C. elegans genome contains two cystatins, the functions of which include the inhibition of papain-like CPs [29]. The following C. elegans strains were used in this study: Bristol N 2 wild type (WT), the cystatin gene null mutant RB1207 cpi-2(ok1256) [29] and cuticle collagen gene mutant dpy-7(qm63) [32]. We used a slight modification of the protocol described by Stiernagle, T. in www.wormbook.org [13]. The C. elegans strains were cultured on plates of nematode growth medium (NGM) agar spread with an Escherichia coli (OP50) lawn. Worms were washed from each plate with approximately 10 ml of ice-cold M9 buffer into 50 ml sterile centrifuge tubes. The worms were settled on ice for 15 min and the supernatant containing food bacteria removed with a Pasteur pipette leaving the worm suspension. Twenty ml of $60 \%(\mathrm{w} / \mathrm{v})$ sucrose were added to the tube and mixed by inversion, then centrifuged at $121 \times \mathrm{g}$ for 2 min . Ten ml of this suspension containing the worms were aspirated into a new tube and washed twice with ice cold M9 by centrifuging at $121 \times g$ for 2 min. The agar debris and bacterial sediments at the bottom of the tube were discarded. Worms were aliquoted in volumes of 1 ml ( $\sim 4500$ worms) and stored at $-20^{\circ} \mathrm{C}$ until use. To obtain a synchronised population we used a modification of the protocol described by Stiernagle, T. in www.wormbook.org [13], and adult
worms were washed off the plates with K medium (prepared as $53 \mathrm{mM} \mathrm{NaCl}, 32 \mathrm{mM} \mathrm{KCl}$ ). The worm suspension was passed through a $5 \mu \mathrm{~m}$ microplate sieve to remove any L1 and L2 larval stages. The resulting suspension was centrifuged at $755 \times g$ for 30 min . The supernatant was removed from the tube without disturbing the worms and replaced with egg isolation bleach ( $1 \%$ sodium hypochlorite and $0.5 \% \mathrm{KOH}$ ). The tubes were shaken for 7 min to disrupt the worms and release their eggs, then the tube was centrifuged for 3 min at $755 \mathrm{x} g$. The supernatant was replaced with fresh K medium and the process was repeated 3 times to remove any trace of the bleach solution. The tube was shaken on a rotary shaker overnight to allow L1 to hatch. The contents of the tube were then allowed to settle and the supernatant was removed leaving 2 ml in the 50 ml tube, which was transferred to several NGM agar plates with the aid of a pipette and incubated at $15^{\circ} \mathrm{C}$ for $24,39,55,74$ or 95 hrs to obtain L2, L2-L3, L3-L4, L4 and adult worms, respectively. All experiments on C. elegans described in this paper were undertaken using worms harvested after 95 hrs .

## Heligmosomoides bakeri culture

We used the method described by [33]. Briefly, oral gavage with a blunt-ended needle was used to infect 7 week-old BKW mice with L3 of H. bakeri, (Home Office Licence 40/3138) [34]. The mice were housed and maintained at the University of Nottingham, BioSupport Unit. Mice were provided with water and food ad libitum. At least two weeks post-infection the mice were sacrificed by asphyxiation with $\mathrm{CO}_{2}$, and dissected. The intestine was carefully removed and placed inside a 15 cm dia. Petri dish containing pre-warmed $\left(37^{\circ} \mathrm{C}\right)$ Hanks’ balanced salt solution (HBSS). To quicken the emergence of the adult worms from the mouse intestinal lumen the intestine was carefully slit open longitudinally and incubated in HBSS or suspended in gauze in HBSS in a 50 ml beaker kept in a $37^{\circ} \mathrm{C}$ water bath. Worms collecting in the bottom of the beaker were tipped into a Petri dish and with the aid of a stereomicroscope adult worms that had migrated out of the gut lumen were pipetted or picked up with the aid
of fine forceps and transferred into another Petri dish containing HBSS. Worms were later separated into males and females and aliquots were stored in 2 ml mini-fuge tubes at $-20^{\circ} \mathrm{C}$.

## Preparation of worm cuticles

We used a modification of the method described by [35]. An aliquot of either C. elegans strains or $H$. bakeri (containing $\sim 4500$ C. elegans or $\sim 120$ H bakeri adult worms) in a 1.5 ml mini-fuge tube was thawed and 1 ml of $\mathrm{H}_{2} \mathrm{O}$ was added, and vortexed to mix. The mini-fuge tube was centrifuged and the water was decanted. Following phosphate buffered saline (PBS) washes, 1 ml of $1 \%(\mathrm{w} / \mathrm{v})$ sodium dodecyl sulphate (SDS) in 0.125 M Tris- HCl pH 6.8 , was added to the pellet, boiled for 5 min , incubated at ambient temperature for 1 h and centrifuged at $121 \mathrm{x} g$ for 5 min , and the supernatant was taken off. The procedure was repeated for $H$. bakeri but not for C. elegans, because C. elegans fragmented and lost their intact morphology. After the SDS wash, the worm pellet was washed again in PBS and centrifuged at $121 \mathrm{x} g$ and the last supernatant was taken off. The prepared worm cuticles were finally washed in $\mathrm{H}_{2} \mathrm{O}$ and stored in PBS at $-20^{\circ} \mathrm{C}$ until they were used. $\beta$-mercaptoethanol [35] was excluded in this procedure because it fragmented the cuticles leading to loss of their intact cylindrical form.

## Preparation of CPs

The two preparations of CPs used in this study were purified papain from papaya latex, purchased from Sigma-Aldrich UK (product No. P3125, 2x crystallised aqueous suspension) and papaya latex supernatant (PLS), prepared as described [26]. PLS contains a mixture of four papaya CPs; chymopapain, glycyl endopeptidase, caricain and papain (in order of abundance) [36]. On the day of use the enzyme preparations were titrated for the molar concentration of active enzyme, using the irreversible CP inactivator L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E64) (Sigma-Aldrich product number E3132) [37, 38] The active enzyme concentration was diluted with water to give a $4 \mu \mathrm{M}$ stock.

## Immunohistochemistry

The C. elegans collagen gene dpy-7 knock-out affects body shape (dumpy) [39, 40]. The DPY-7 cuticle collagen is predicted to have a carboxyl-terminal domain of 40 residues that is not shared with other C. elegans cuticle collagens [39]. The DPY-7-5a monoclonal antibody recognises specifically this "C"-terminal region of DPY-7 [39]. Using this antibody, we predicted that the presence or absence of a signal detection from cuticles with or without digestion by CP will indicate whether DPY-7 collagen is degraded or not by the CP . Additionally, DPY-7 immunohistochemistry can be used to monitor changes in cuticle structure during digestion of the cuticle components by a CP. For these experiments we used wild-type (WT) C. elegans, and dpy-7 null strain MQ375. We used two slightly different methodologies; the first used a mini-fuge tube and the second was performed in 24-well plates. We used the mini-fuge tube method because we suspected disturbance and possible breaking of worms during centrifugation, whereas in well plates there was minimal or no disturbance of worms.

In the tube experiments, aliquots of washed WT or mutant dpy-7(qm63) C. elegans were thawed and rinsed with water by centrifugation at $121 \mathrm{x} g$ for 2 min . The worms were partially reduced and made permeable with $1 \%$ dithiothreitol (DTT) or not [41], and were then incubated with $1 \mu \mathrm{M}$ papain or PLS, or papain or PLS +1 mM E64, at time-points of 5,10 , 15 and 30 min at $37^{\circ} \mathrm{C}$. Enzyme activity was then stopped with 1 mM E64. The worms were washed with Tris buffered saline ( pH 7.0 ) with Tween-20 (TBST), by centrifugation at 121 x $g$ for 4 min . The washing was repeated 3 more times to remove any trace of CP and nonspecific binding sites were blocked for 4 hrs with $750 \mu \mathrm{l}$ of $5 \%$ skimmed milk in TBST. The worms were probed with 1 ml of a 1:200 dilution of DPY-7 antibody for 4 hrs or overnight followed by 1 ml of a 1:500 dilution of goat anti-mouse $\operatorname{IgG}$ secondary antibody Alexa fluor 488 conjugate (ThermoFisher Scientific UK), in the dark for 2 hrs and from here samples
were protected from light by wrapping in aluminium foil. The worm samples were centrifuged at $121 \mathrm{x} g$ for 2 min . The washing was repeated twice. After washing, $10 \mu \mathrm{l}$ of worm suspension were pipetted onto a grease-free slide and mixed with mounting medium for fluorescence (Vectashield H-1200) and protected with a coverslip.

In the 24 -well plate method, all the conditions were the same as in the tube method except that the worms were not washed by centrifugation, but manually by pipetting the reagent with minimal disturbance to the worms which were not made permeable with $1 \%$ DTT. It is important to note that in all cases the CP activity was totally eliminated by washing the samples in 1 mM E64, followed by 3 washes in TBST for 4 min before application of antibody, eliminating the possibility of hydrolysis of the antibody by CP [42]. The worms were imaged with a DMI4000B (Leica) inverted widefield fluorescence microscope and the images were stored electronically.

## Scanning electron microscopy (SEM)

Whole nematodes were used for this experiment. Approximately 30 C . elegans or 10 H . bakeri were added into each of four 1.5 ml mini-fuge tubes. The worms were incubated with $1 \mu \mathrm{M}$ (final concentration) of CP , or $\mathrm{CP}+\mathrm{E} 64$ at a temperature of $37^{\circ} \mathrm{C}$ for 10,15 , and 30 min . At each time point, activity of CPs was stopped with $50 \mu \mathrm{l}$ of 1 mM E64. The samples were then diluted with PBS and centrifuged at $121 \times g$ for 2 min and the supernatant was removed. This washing step was repeated three times to remove any trace of CP. The samples were fixed in $2.5 \%$ glutaraldehyde in 0.1 M phosphate buffer pH 6.8 for 1 h , before being washed for 20 min three times in PBS then fixed and stained with $1 \%$ osmium tetroxide in 0.1 M phosphate buffer pH 6.8 for 1 hr at ambient temperature. The samples were washed three times in water, and dehydrated by sequentially placing in $30 \%, 50 \%, 70 \%, 90 \%$ and $100 \%$ ethanol. The specimens were then dried using a Polaron E3000 critical point dryer. The dried samples were mounted onto aluminium stubs using carbon discs. The stubs were gold
sputter coated (approximately 10nm thick) using a Polaron E5100 SEM coating unit. All specimens were viewed and photographed using a JEOL JSM 840 scanning electron microscope at 23 kV , and the images were stored electronically.

## Digestion of worm cuticles with CPs for proteomic analyses

Either an aliquot ( $\sim 4500$ C. elegans or 120 H. bakeri) of prepared worm cuticles or whole worms was incubated in $1 \mu \mathrm{M}$ papain (final active concentration) or PLS (both activated with 4 mM L-cysteine), or papain or PLS +1 mM E64 as the control, at $37^{\circ} \mathrm{C}$ for 10,15 and 30 minutes. Twenty five $\mu 1$ of the supernatant was collected at each time point and mixed with $20 \mu \mathrm{l}$ of 1 mM E64 to stop further CP activity.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of CP digested worm supernatant

The supernatant was mixed at a ratio of 1:1with 2 x sample buffer [4\% SDS, $20 \%$ glycerol, $10 \%$ DTT, $0.004 \%$ bromophenol blue and 0.125 M Tris- HCl pH 6.5 ] and boiled for 5 min . Twenty $\mu 1$ of the boiled sample was loaded onto a $12 \%$ or $15 \%$ polyacrylamide 12 -well precast Mini Protean gel (Bio-Rad). Following electrophoresis at 120V, the gel was removed and fixed for 30 min in 5 ml of $7 \%(\mathrm{v} / \mathrm{v})$ glacial acetic acid in $40 \%(\mathrm{v} / \mathrm{v})$ methanol. Later the gel was stained with 150 ml of $0.25 \%$ (w/v) colloidal Coomassie brilliant blue G concentrate in $50 \%$ methanol, $10 \%$ acetic acid, $40 \%$ water, for at least 4 hrs . After staining the gel was rinsed with $10 \%$ acetic acid in $40 \%$ methanol, for 1 min . Rinsing was repeated, and the gel was de-stained overnight in $25 \%$ methanol on a shaker at ambient temperature. The next day the gel was washed, scanned using a Bio-Rad gel imager (Gel Doc XR +SYSTEM) and the gel was recovered and fixed in $1 \%$ formic acid.

## Peptide extraction and mass spectroscopy (LC/MS/MS)

In-gel tryptic digestion was a slight modification of the method by [43][44] (see attached Supplementary Material).

## Results

## Effects of CPs on the DPY-7 cuticle collagen of C. elegans

Figure 1 illustrates the immunochemical staining of the cuticles of C. elegans after incubation with $1 \mu \mathrm{M}$ papain. The DPY-7 collagen locates to parallel circumferential thread-like bands within the cuticle [32]. When WT C. elegans cuticles were incubated for 5 mins in $1 \mu \mathrm{M}$ papain plus the CP inhibitor E64, a wild type pattern of localisation of DPY-7 was observed (Figure 1A) where the DPY-7 circumferential thread-like bands are intact and appear the same as for untreated specimens [32]. Staining of the C. elegans mutant strain dpy-7(qm63) was performed as a negative control; this strain lacks the DPY-7 collagen and hence has no staining for DPY-7 (Figure 1B). When WT C. elegans cuticles were incubated with $1 \mu \mathrm{M}$ papain for 5 mins, frequent areas of structural disruption of the circumferential band structures were observed (arrowed red in Figures 1C, D and E). The areas of major disruption appeared to be relatively regularly spaced (between 4 to $6 \mu \mathrm{~m}$ ), although many parts of the cuticles had also lost some circumferential bands. Both the alae and the entire cuticle components totally disappeared after 10 min of incubation. This suggests that DPY-7 is a target protein for papain. Figure 2 shows representative images following the immunochemical staining of the cuticles of WT C. elegans after worms were incubated with $1 \mu \mathrm{M}$ papain or papain +E 64 in a 24 -well plate without prior reduction in $1 \%$ DTT. The advantage of the plate method was that the worms were not disturbed by centrifugation, which allowed us to monitor progressively the activity of CP on the worms. The images presented here were of treated worms lying at the bottom of the wells, which were imaged without transferring to microscope slides. Worms incubated in papain for 5 min (Figure 2B) were disrupted in the same regular pattern (red arrows) as was seen in worms prepared using the tube method (Figure 1). This is in contrast with worms incubated in $1 \mu \mathrm{M}$ papain + E64 (Figure 2A). In Figure 2C after 30 min in $1 \mu \mathrm{M}$ papain the DPY-7 staining had mostly disappeared, and what was left had very
little resolution (arrowed red). Some of the DPY-7 fluorescence remained until the cuticle was almost totally disrupted, indicating that this collagen species, or other proteins that are linked to it and holding it within the cuticle, may be a late target(s) for the CPs.

## Effects of CPs on C. elegans or H. bakeri visualised using scanning electron microscopy (SEM)

In order to throw more light on the means by which CPs cause disruption of nematode cuticles, we went on to investigate by SEM the changes that occurred to adult $H$. bakeri cuticles, as well as C. elegans cuticles, including those of a knockout of a CP inhibitor (cpi2) [30]. Whole WT, or mutant cpi-2(ok1256) strains of C. elegans or H. bakeri were incubated with CP or $\mathrm{CP}+$ E64 at time points of 10,15 and 30 min , then fixed and prepared for SEM. Figure 3 consists of electron micrographs of WT C. elegans incubated with $1 \mu \mathrm{M}$ papain with or without a molar excess of E64. The WT C. elegans incubated with papain + E64 appeared to be intact (Figure 3A), with the alae of the worm (arrowed yellow) running longitudinally along the worm's body. This is in contrast with the worms incubated in $1 \mu \mathrm{M}$ papain (B-D) where the cuticles have varying degrees of damage. At 10 min of incubation in papain, the cuticle surfaces of the WT worms were wrinkled and disrupted (Figure 3B). The disruption was apparently extensive at 15 min of incubation in papain (Figure 3C) whereas at 30 min , it appears that the worm has been split open longitudinally (Figure 3D). The electron micrographs of cpi-2 (ok1256) mutant C. elegans incubated in $1 \mu \mathrm{M}$ papain or papain + E64 are shown in Figures 4 and 5. Figure 4 illustrates the entire worms at low power, with the alae arrowed yellow in Figure 4A. Figure 4B illustrates a worm after 5 min in papain, with wrinkling of the cuticle. After 15 min , extensive wrinkling and blistering of the cuticle can be seen (Figure 4C). At 30 min the cuticle has been split along the alae, with the cuticle on either side either folded over on itself, or missing completely (Figure 4D). At higher magnification, the cpi-2(ok1256) mutant C. elegans were damaged by papain (Figure 5 B-D)
when contrasted to worms incubated in papain+E64, where the cuticle, including the alae (yellow arrow), appears to be intact, even after 30 min incubation (Figure 5A). At 10 min of incubation, worms incubated in papain showed tears longitudinally along the alae (arrowed red, Figure 5B). The papain-induced tearing may have caused the cuticles to detach from the rest of the body by 15 min in what appear to be sheets of cuticle (arrowed red in Figure 5C). A ribbon-like structure (arrowed yellow) appears to be the alae still intact on the opposite side of the worm, with the entire inner contents of the nematode having been lost by 30 min of incubation with papain (Figure 5D).

Compared to C. elegans, when incubated with $1 \mu \mathrm{M}$ papain $H$. bakeri showed greater susceptibility to damage by CP at all the incubation times (Figure 6). At 10 min of incubation the worms already appeared totally digested with only a fragment of the gut being anatomically discernible (Figure 6B). The worms were totally digested at 15 and 30 min of incubation with only the insoluble precipitates left after incubation (Figure 6C and D). The damage to H. bakeri was caused by CP action as worms incubated in papain + E64 were not affected but retained their intact status (Figure 6A).

Target proteins for CPs on intact $\boldsymbol{H}$. bakeri or prepared $\boldsymbol{H}$. bakeri cuticles, or whole $\boldsymbol{C}$. elegans

Nematode cuticles are substrates for CPs, and some cleaved products of hydrolysis are likely to be soluble. We analysed soluble products from prepared cuticles or whole H. bakeri incubated in CP or CP + E64, with SDS-PAGE. Bands that were unique in the papain digests or in the papain + E64 incubations were selected for in gel trypsin digestion in conjunction with mass spectrometry analysis to identify the corresponding proteins (Supplementary Tables 1 and 2). Cuticle globin (gi/8569651), required for respiration by the nematode [45, 46] was one of the proteins identified using papain (Supplementary Table 1). In addition, a single peptide identified the structural protein CUT-19. However, as only a single peptide
was identified, further validation is required. Cuticlins are major and important structural components of the nematode cuticle and their hydrolysis is expected to lead to loss of integrity of the entire cuticle structure, weakening it sufficiently to enable its rupture through the high hydrostatic pressure within the pseudoceolomic cavity.
C. elegans or washed C. elegans cuticles were incubated with papain or papain + E64 (Supplementary Tables 3 and 4). Similarly, whole C. elegans or washed C. elegans cuticles were also incubated with PLS and PLS plus E64 (Supplementary Table 5). Following SDSPAGE the unique bands were selected for in gel trypsin digestion in conjunction with mass spectrometry analysis to identify the corresponding proteins. Of particular interest was the identification of an important cuticle structural protein, COL-87. However, as the protein was identified by a single peptide, further validation is required

## Discussion

In view of the threat of nematode resistance, our attention is on the development of drugs with multiple modes of action i.e. with more than one target molecule and reduced likelihood of development of resistance. Focus has been on CPs and earlier reports of their effectiveness as anthelmintics [26-28, 47]. Although CPs attack and destroy nematode cuticles, the molecular target(s) and possible sites of activity on the structural proteins that constitute the cuticle have not been investigated.

For an anthelmintic based on CPs to be used on a large scale at an economic price, it is likely that a preparation such as PLS will be employed. This is a mixture of four closely related CPs, one of which is papain [36]. In order to simplify the interpretation of our data, particularly those using the proteomic approach, we decided to employ purified papain in our experiments alongside PLS. The effect of papain on the nematode cuticle is superficially similar to that of PLS, but it is unlikely that the two preparations will have identical effects.

DPY-7 collagen is a target for CPs on the cuticle of C. elegans. Our study found that the loss of DPY-7 immunoreactivity in C. elegans is time-dependent and that hydrolysis of DPY-7 or its disappearance by other means probably began before 5 min at a $1 \mu \mathrm{M}$ concentration of CPs, whereas total loss of DPY-7 immunoreactivity appeared to take place when the worms were incubated longer in papain for up to 30 min . The time course of the loss of DPY-7 immunoreactivity on C. elegans was slow enough to allow us to use it to visualise anatomical disruption of the entire nematode cuticle by CP . The nematode cuticle is a multi-layered structure with about $80 \%$ of its protein as collagen [40]. DPY-7, DPY-2, DPY-3, DPY-8 and DPY-10 are obligate partners and are necessary in the formation of the thin thread-like structures needed for the genesis and maintenance of the annular furrows of C. elegans cuticles [39]. Therefore, loss of DPY-7 immunoreactivity by CP would suggest the destruction of the framework of the cuticle leading to loss of cuticular structure. This could be due to the hydrolysis by CP of any one or more of these components, or of others that have not yet been identified, resulting in collapse of the whole architecture of the cuticle, possibly seen as wrinkling on the surface as is usually associated with CP attack on the cuticles of parasitic nematodes [28]. As the time of incubation was increased, more of the DPY-7 and probably the other cuticle collagen proteins were hydrolysed, making the cuticle weaker, the physical result of which is the loss of integrity of the cuticle as seen with SEM and loss of DPY-7 immunoreactivity observed in immunohistochemical imaging of C. elegans incubated in CP . The disruption and digestion of the $H$. bakeri or C. elegans cuticles by CPs was a timedependent but quite rapid process, producing severe damage to the cuticles. This suggests that there are many targets for CPs on nematode cuticles, most of which remained unidentified.

SEM demonstrated that dead $H$. bakeri are more susceptible to CP attack than dead C. elegans. An earlier report [30] indicated that the dose of CP that kills a parasitic nematode was unable to cause the death of wild type C. elegans. C. elegans possess CP inhibitors, presumably to
protect against exogenous CPs in their external environments containing bacteria, fungi and decaying plant material [30]. With H. bakeri, Stepek et al [48] observed cuticular damage after 15 min of incubating living $H$. bakeri in $200 \mu \mathrm{M}$ papain, a 100 -fold higher concentration than was used in this study. The difference in the amount of CP needed to cause cuticular damage to living and dead $H$. bakeri might be related to the presence or absence of cystatin secretions. H. bakeri cystatin(s) is involved in immune-regulation [17] and is presumed to be a secreted protein, so could influence CP activity if the animal is alive. The influence of cystatins in dead worms might be lessened by the inability to release cystatins from a store elsewhere in the worm and mobilised to the cuticles as may occur in the living nematode, as seems to be the case in live C. elegans [30]. As components of secretory products of parasitic nematodes, cystatins may be deposited in the cuticles [17, 48, 49]. In the situation where there are cystatins within the cuticles, our cuticle preparation would most likely have removed any cystatins, making the cuticles more susceptible to the action of CPs.

A cuticle related protein, extracellular cuticle globin, was identified from $H$. bakeri samples incubated with CPs. Its absence in the cuticles incubated in papain + E64 indicates that it was released by papain. This extracellular cuticle globin has high-affinity oxygen binding and is required by the parasitic nematodes to obtain oxygen in their near anaerobic environment within the host gut [50]. Disruption of cuticle globin by papain would disengage the mechanism through which the worm obtains oxygen from its host. We therefore conclude that CPs are able to disrupt the mechanism of oxygen uptake from the host, another potential killing method.

Nematode cuticle structural proteins are held together by covalent tyrosine cross-links [51]. The failure to identify many structural proteins is likely to be due to the inability of MS software to identify peptides containing tyrosine cross-links. The presence of the cross-links as well as the likelihood that many of the cross-links are formed between different cuticular collagen and cuticlin gene products will make the resulting structure impossible for the
software to recognise. The only peptides that could be recognised would be those that do not contain tyrosine cross-links and are the product of a single gene. In C. elegans about 160 and at least 8 functionally defined genes encode for cuticle collagens and cuticlins respectively [40, 52], which are all likely to be substrates for the formation of tyrosine-based crosslinks. [15, 53]

We identified a single peptide from each of two cuticle structural proteins, COL-87 and CUT19, in digests of H. bakeri and C. elegans, which may suggest that, along with DPY-7, these structural proteins may be cuticular targets for CPs. No examples of structural cuticle components were found in any of our control samples where the action of the CPs was blocked by the irreversible CP inactivator E64. Other proteomic analyses of nematodes have failed to identify significant numbers of peptides from cuticle proteins [42, 46], presumably for the reasons outlined above. For these reasons, we consider COL-87 and CUT-19, along with DPY7, to be possible CP targets in the cuticle.

The pattern of activity of CPs on nematodes is evidently novel and involves the targeting of a number of different gene products, making resistance of nematodes to anthelmintics derived from CPs difficult to achieve. We therefore suggest that CPs are good candidates for an anthelmintic with a completely novel mode of action from those attributed to other anthelmintics, and that development of resistance against CPs by nematodes will be slow as it will probably require simultaneous mutations of a number of different genes encoding collagens, cuticlins, and possibly other essential components of the nematode cuticle.

## Abbreviations:

CP: Cysteine proteinase,

DTT: Dithiothrietol

E64: L-trans-epoxysuccinyl-leucylamido 4-guanidino butane

HBSS: Hank's balanced salt solution

LC/MS/MS: Liquid chromatography-tandem mass spectrometry

PBS: Phosphate-buffered isotonic saline

PLS: Papaya latex supernatant

SEM: Scanning electron microscopy

SDS-PAGE: Sodium dodecyl sulphate- polyacryalamide gel electrophoresis
TBST: Tris buffered saline with Tween-20
WT: Wild type

## DECLARATIONS

Ethics approval and consent to participate. Mice were maintained and treated as covered by Home Office Licence 40/3138. No human material was used in the course of this work.

Consent for publication. All authors consented to publication

Competing interests. There are no competing interests

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Author contributions. David J Buttle, Victor S Njom, Mark Dickman, Ian Duce and Iain Johnstone were involved in the design of various parts of this study. Victor Njom carried out the experiments. Tim Winks and Oumu Diallo cultured and maintained C. elegans and aided with the proteomic and antibody experiments, Ian Duce performed SEM, Ann Lowe and Jerzy

Behnke maintained and infected mice with H. bakeri, Victor S Njom and David J Buttle wrote the manuscript.

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## References

1. de Silva NR: Impact of mass chemotherapy on the morbidity due to soil-transmitted nematodes. Acta Trop 2003, 86:197-214.
2. Gyapong M, Nartey A, Oti E, Page S: The social and economic impact of neglected tropical diseases in sub-Saharan Africa. In: Neglected Tropical Diseases - Sub-Saharan Africa. Edited by Gyapong J, Boatin B. Cham: Springer International Publishing; 2016: 349365.
3. Roeber F, Jex AR, Gasser RB: Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance - an Australian perspective. Parasit Vectors 2013, 6:153.
4. Nicol JM, Turner SJ, Coyne DL, Den Nijs L, Hockland S, Maafi ZT: Current nematode threats to world agriculture. In: Genomics and Molecular Genetics of Plant-nematode Interactions: Springer; 2011: 21-43.
5. Albonico M, Rinaldi L, Sciascia S, Morgoglione ME, Piemonte M, Maurelli MP, Musella V, Utzinger J, Ali SM, Ame SM et al: Comparison of three copromicroscopic methods to assess albendazole efficacy against soil-transmitted helminth infections in school-aged children on Pemba Island. Trans R Soc Trop Med Hyg 2013, 107:493-501.
6. Martin RJ: Modes of action of anthelmintic drugs. Vet J 1997, 154:11-34.
7. Geerts $S$, Gryseels B: Drug resistance in human helminths: current situation and lessons from livestock. Clin Microbiol Rev 2000, 13:202-222.
8. Geerts S, Gryseels B: Anthelmintic resistance in human helminths: a review. Trop Med Int Health 2001, 6:915-921.
9. Shalaby HA: Anthelmintics Resistance; How to Overcome it? Iranian J Parasitol 2013, 8:18-32.
10. Geurden T, Hoste H, Jacquiet P, Traversa D, Sotiraki S, di Regalbono AF, Tzanidakis N, Kostopoulou D, Gaillac C, Privat S et al: Anthelmintic resistance and multidrug resistance in sheep gastrointestinal nematodes in France, Greece and Italy. Vet Parasitol 2014, 201:59-66.
11. Kaplan RM: Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol 2004, 20:477-481.
12. Vercruysse J, Levecke B, Prichard R: Human soil-transmitted helminths: implications of mass drug administration. Curr Opin Infect Dis 2012, 25:703-708.
13. Fetterer RH, Rhoads ML, Urban Jr JF: Synthesis of tyrosine-derived cross-links in Ascaris suum cuticular proteins. J Parasitol 1993, 79:160-166.
14. Edens WA, Sharling L, Cheng G, Shapira R, Kinkade JM, Lee T, Edens HA, Tang X, Sullards C, Flaherty DB et al: Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multi-domain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox. J Cell Biol 2001, 154:879-891.
15. Page AP, Stepek G, Winter AD, Pertab D: Enzymology of the nematode cuticle: A potential drug target? Int J Parasitol: Drugs Drug Res 2014, 4:133-141.
16. Hawley JH, Martzen MR, Peanasky RJ: Proteinase inhibitors in Ascarida. Parasitol Today 1994, 10:308-313.
17. Hewitson JP, Grainger JR, Maizels RM: Helminth immune-regulation: The role of parasite secreted proteins in modulating host immunity. Mol Biochem Parasitol 2009, 167:1-11.
18. Molehin AJ, Gobert GN, McManus DP: Serine protease inhibitors of parasitic helminths. Parasitology 2012, 139:681-695.
19. Waller PJ, Bernes G, Thamsborg SM, Sukura A, Richter SH, Ingebrigtsen K, Höglund: Plants as de-worming agents of livestock in the Nordic Countries: historical perspective, popular beliefs and prospects for the future. Acta Vet Scand 2001, 42:31-44.
20. Berger J, Asenjo CP: Anthelmintic Activity of Fresh Pineapple Juice. Science 1939, 90:299-300.
21. Berger J, Asenjo CP: Anthelmintic activity of crystalline papain. Science 1940, 91:387 - 388.
22. Satrija F, Nansen P, Bjorn H, Murtini S, He S: Effect of papaya latex against Ascaris suum in naturally infected pigs. J Helminthol 1994, 68:343-346.
23. Rawlings ND, Barrett AJ, Finn R: Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 2016, 44: D343-D350.
24. Stepek G, Buttle DJ, Duce IR, Behnke JM: Human gastrointestinal nematode infections: are new control methods required? Int $J$ Exp Pathol 2006, 87:325-341.
25. Behnke JM, Buttle DJ, Stepek G, Lowe A, Duce IR: Developing novel anthelmintics from plant cysteine proteinases. Parasit Vectors 2008, 1:29.
26. Buttle DJ, Behnke JM, Bartley Y, Elsheikha HM, Bartley DJ, Garnett MC, Donnan AA, Jackson F, Lowe A, Duce IR: Oral dosing with papaya latex is an effective anthelmintic treatment for sheep infected with Haemonchus contortus. Parasit Vectors 2011, 4:36.
27. Levecke B, Buttle DJ, Behnke JM, Duce IR, Vercruysse J: Cysteine proteinases from papaya (Carica papaya) in the treatment of experimental Trichuris suis infection in pigs: two randomized controlled trials. Parasit Vectors 2014, 7:255.
28. Stepek G, Lowe A, Buttle DJ, Duce IR, Behnke JM: The anthelmintic efficacy of plantderived cysteine proteinases against the rodent gastrointestinal nematode, Heligmosomoides polygyrus, in vivo. Parasitology 2007, 134:1409-1419.
29. Stepek G, Curtis RH, Kerry BR, Shewry PR, Clark SJ, Lowe A, Duce IR, Buttle DJ, Behnke JM: Nematicidal effects of cysteine proteinases against sedentary plant parasitic nematodes. Parasitology 2007, 134:1831-1183.
30. Phiri AM, De Pomerai D, Buttle DJ, Behnke JM: Developing a rapid throughput screen for detection of nematicidal activity of plant cysteine proteinases: the role of Caenorhabditis elegans cystatins. Parasitology 2014, 141:164-180.
31. Guiliano DB, Hong X, McKerrow JH, Blaxter ML, Oksov Y, Liu J, Ghedin E, Lustigman S: A gene family of cathepsin L-like proteases of filarial nematodes are associated with larval molting and cuticle and eggshell remodeling. Mol Biochem Parasitol 2004, 136:227242.
32. Thein MC, McCormack G, Winter AD, Johnstone IL, Shoemaker CB, Page AP: Caenorhabditis elegans exoskeleton collagen COL-19: An adult-specific marker for collagen modification and assembly, and the analysis of organismal morphology. Developmental Dynamics 2003, 226:523-539.
33. Luoga W, Mansur F, Stepek G, Lowe A, Duce IR, Buttle DJ, Behnke JM: Host genetic influences on the anthelmintic efficacy of papaya-derived cysteine proteinases in mice. Parasitology 2015, 142:989-998.
34. Behnke JM, Harris PD: Heligmosomoides bakeri: a new name for an old worm? Trends Parasitol 2010, 26:524-529.
35. Cox GN, Kusch M, Edgar RS: Cuticle of Caenorhabditis elegans: its isolation and partial characterization. J Cell Biol 1981, 90:7-17.
36. Buttle DJ, Dando PM, Coe PF, Sharp SL, Shepherd ST, Barrett AJ: The preparation of fully active chymopapain free of contaminating proteinases. Biol Chem Hoppe-Seyler 1990, 371:1083-1088.
37. Zucker S, Buttle DJ, Nicklin MJ, Barrett AJ: The proteolytic activities of chymopapain, papain, and papaya proteinase III. Biochim Biophys Acta 1985, 828:196-204.
38. Luoga W, Mansur F, Buttle DJ, Duce IR, Garnett MC, Behnke JM: The anthelmintic efficacy of papaya latex in a rodent-nematode model is not dependent on fasting before treatment. J Helminthol 2012, 86:311-316.
39. McMahon L, Muriel JM, Roberts B, Quinn M, Johnstone IL: Two Sets of Interacting Collagens Form Functionally Distinct Substructures within a Caenorhabditis elegans Extracellular Matrix. Mol Biol Cell 2003, 14:1366-1378.
40. Page AP, Johnstone IL: The cuticle. WormBook 2007, 19:1-15.
41. Thein MC, Winter AD, Stepek G, McCormack G, Stapleton G, Johnstone IL, Page AP: Combined Extracellular Matrix Cross-linking Activity of the Peroxidase MLT-7 and the Dual Oxidase BLI-547 3 Is Critical for Post-Embryonic Viability in Caenorhabditis elegans. J Biol Chem 2009, 284:17549-17563.
42. Andrew SM, Titus JA: Purification of immunoglobulin G. Curr Protoc Immunol 2001, 2.
43. Van Steendam K, De Ceuleneer M, Dhaenens M, Van Hoofstat D, Deforce D: Mass spectrometry-based proteomics as a tool to identify biological matrices in forensic science. Int J Leg Med 2013, 127:287-298.
44. Wang T, Van Steendam K, Dhaenens M, Vlaminck J, Deforce D, Jex AR, Gasser RB, Geldhof P: Proteomic analysis of the excretory-secretory products from larval stages of Ascaris suum reveals high abundance of glycosyl hydrolases. PLoS Neg Trop Dis 2013, 7: e2467.
45. Barrett J, Brophy PM: Ascaris haemoglobin: new tricks for an old protein. Parasitol Today 2000, 16:90-91.
46. Blaxter ML: Nemoglobins: divergent nematode globins. Parasitol Today 1993, 9:353 360.
47. Stepek G, Lowe A, Buttle DJ, Duce IR, Behnke JM: In vitro and in vivo anthelmintic efficacy of plant cysteine proteinases against the rodent gastrointestinal nematode, Trichuris muris. Parasitology 2006, 132:681-689.
48. Stepek G, Buttle DJ, Duce IR, Lowe, A, Behnke JM: Assessment of the anthelmintic effect of natural plant cysteine proteinases against the gastrointestinal nematode, Heligmosomoides polygyrus, in vitro. Parasitology 2005, 130: 203-211.
49. Hewitson JP, Harcus Y, Murray J, van Agtmaal M, Filbey KJ, Grainger JR, Bridgett S, Blaxter ML, Ashton PD, Ashford DA et al: Proteomic analysis of secretory products from the model gastrointestinal nematode Heligmosomoides polygyrus reveals dominance of Venom Allergen-Like (VAL) proteins. J Proteom 2011, 74:1573-1594.
50. Daub J, Loukas A, Pritchard DI, Blaxter M: A survey of genes expressed in adults of the human hookworm, Necator americanus. Parasitology 2000, 120:171-184.
51. The cuticle [http://www.wormbook.org]
52. Cox GN, Kusch M, Denevi K, Edgar RS: Temporal regulation of cuticle synthesis during development of Caenorhabditis elegans. Dev Biol 1981, 84:277-285.
53. Fetterer RH, Hill DE, Urban JF, Jr.: The cuticular biology in developmental stages of Ascaris suum. Acta Trop 1990, 47:289-295.

## FIGURE LEGENDS

Figure. 1: Immunohistochemical investigation of the activities of papain on prepared cuticles of WT (Bristol N2) C. elegans. The worm cuticles were partially reduced with $1 \%$

DTT to increase permeability using the tube method then probed with the DPY-7 antibody. A: Mutant dpy-7(qm63) C. elegans used as the negative control. B and C: Disruption of the WT cuticle after 5 minutes of incubation in $1 \mu \mathrm{M}$ papain. D: A worm incubated in $1 \mu \mathrm{M}$ papain inactivated with E64 prior to incubation. E: A magnified image showing the regular pattern of disruption (arrowed red) and progressive disappearance of DPY-7 at 5 min of incubation with $1 \mu \mathrm{M}$ papain, indicating the sequence of events leading to the collapse of the cuticle structure. $B a r=25 \mu \mathrm{~m}$.

Figure 2: Immunolocalisation of DPY-7 in WT C. elegans (Bristol N2) cuticles incubated with papain or papain + E64 in a 24 -well plate. Whole intact worms were incubated with 1 $\mu \mathrm{M}$ papain or papain + E64 without prior reduction in $1 \%$ DTT. A: A worm incubated with papain + E64 for 30 min . B: Disruption of the C. elegans (Bristol N2 WT) cuticle (arrowed red), after 5 min incubation. C: Total disappearance of the collagen stripes and loss of immunoreactivity (yellow brackets) after incubation for $30 \mathrm{~min} . \mathrm{Bar}=25 \mu \mathrm{~m}$.

Figure 3: SEM of WT (Bristol N2) C. elegans after incubation in $1 \boldsymbol{\mu}$ M papain or papain + E64 at time points of $\mathbf{1 0}, \mathbf{1 5}$ and 30 mins. A: The worms were apparently intact and unaffected when incubated with papain +E 64 for 30 min , with the alae clearly visible (arrowed yellow). B: After 10 min of incubation in papain, the cuticle appeared to be split longitudinally, which became more extensive at 15 min of incubation (C). By 30 min , the cuticle was split longitudinally, with a section of the cuticle totally destroyed or curled in on itself $(\mathbf{D})$. Bar $=$ $25 \mu \mathrm{~m}$.

Figure 4: SEM images of cpi-2 (RB1207, ok1256) C. elegans incubated in papain or papain + E64. A: cpi-2(RB1207, ok1256) C. elegans worms incubated in papain + E64 for 30 min retained their intact status with the alae (arrowed yellow) visibly running longitudinally along the worm. B: At 10 min of incubation in papain cpi-2(RB1207, ok1256) C. elegans showed
wrinkling of the cuticles. C: By 15 min extensive blistering of the cuticles was apparent. At 30 $\min$ most of the cuticle was lost or split along the alae exposing the internal cavity $(\mathbf{D})$. Bar $=$ $50 \mu \mathrm{~m}$.

Figure 5: SEM images of cpi-2(RB1207, ok1256) C. elegans incubated in papain or papain + E64. A: Worms incubated for 30 min in papain + E64 clearly showed the delicate intact structure of the cuticle, with the alae being clearly visible (yellow arrow). B: By contrast, after 10 min of incubation, worms incubated in papain showed tearing or were split longitudinally along a line very close to the alae (arrowed red). C: At 15 min , the papain-induced cuticle disruption probably caused the cuticles to detach from the rest of the body in what appeared to be sheets of cuticle (arrowed red). D: After 30 min of incubation the cuticle was often split longitudinally, in some cases with the alae on the opposite side of the worm still intact (yellow arrow). $\mathrm{Bar}=10 \mu \mathrm{~m}$.

Figure 6: SEM of $\boldsymbol{H}$. bakeri incubated in papain or papain + E64. H.bakeri incubated in papain + E64 for 30 min were intact and appeared undamaged (A) whereas worms incubated in $1 \mu \mathrm{M}$ papain for 10 min or longer showed rapid and extensive digestion and were no longer recognisable (B 10, C 15 min ). After 30 min of incubation, all that remained were sheets of insoluble material (D). Bar $=25 \mu \mathrm{~m}$.

## SUPPLEMENTARY MATERIAL

## Peptide extraction and mass spectrometry (LC/MS/MS)

Briefly described; gels were washed twice in water. Bands of interest were cut out in 1 mm pieces and were de-stained with in 200 mM ammonium bicarbonate in $40 \%$ acetonitrile at $37^{\circ} \mathrm{C}$ for 30 min . The excised gel pieces were dehydrated and rehydrated with acetonitrile and 50 mM ammonium bicarbonate in $50 \%$ acetonitrile in water by incubating at $37^{\circ} \mathrm{C}$ for 15 min respectively. The supernatant was taken off and the samples were vacuum-dried for 30 min . The samples were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide for 30 min in the dark. Then the gel pieces
were washed with 50 mM ammonium bicarbonate for 15 min at $37^{\circ} \mathrm{C}$ and dried in a vacuum concentrator for 30 min at $37^{\circ} \mathrm{C}$ and thereafter digested with 200 ng of trypsin (per band) at $37^{\circ} \mathrm{C}$ overnight. Peptides were extracted in acetonitrile and dried on a vacuum concentrator. On the day of analysis, the dried peptides were re-suspended in $5 \%$ formic acid and $6 \mu \mathrm{l}$ was injected into the reverse-phase column of coupled LC/MS for protein analysis.

Protein identities were obtained by MS analysis with the aid of an AmaZon ETD (Bruker Daltonics) through an online nano liquid chromatography system (Ultimate 3000 RSLC, Dionex). Online acquisition of mass spectra was done using "Profile MS" with automatic dependent MS/MS scans. MS signals for each band or sample were converted to mascot generic files (mgf) using the software Bruker DataAnalysis 4.2. MS converted mgfs were used to query standard databases (NCBInr or Swiss-Prot) or a merger of the two standard databases with an in-house database made up of FASTA files of the C. elegans genome downloaded from WormBase. Queries were submitted using Mascot Server v.2.2, 01 (Matrix Science). The eukaryote and restricted C. elegans search taxonomy were used for $H$. bakeri (because it has no full genome annotation) and restricted search taxonomy - C. elegans for $C$ elegans. The following parameters were used: peptide mass tolerance $= \pm 1.2 \mathrm{Da}$, fragment MS/MS tolerance $= \pm 0.6 \mathrm{Da}$, peptide charge $=2+/ 3+$. Electrospray ionisation trap $($ ESI-TRAP $)$ was selected as Instrument. Tryptic enzyme specificity with up to two missed cleavages was applied to all searches. Carbamidomethylation of cysteine and oxidised methionine were selected for fixed and variable modifications respectively for the tryptic digests. Mascot calculated peptide ion score cut-off of $\geq 20$ was used to filter and protein identification was based on a minimum of two unique peptides. Peptide matches above homology or identity threshold were selected (for significance threshold, $\mathrm{P}<0.05$ ).

Table 1 Summary of proteins identified with LC/MS/MS from H. bakeri digested with $1 \mu M$

| Protein code | Protein name/species | Mascot score |
| :---: | :---: | :---: |
| gi\|345499008 | Myoglobin-1 [Heligmosomoides bakeri] | 429 |
| gi\|6626 | Actin [Caenorhabditis elegans] | 399 |
| gi\|283480611 | ADP/ATP translocase [Haemonchus contortus] | 169 |
| gi\|1707910 | RecName: Full=Myoglobin; AltName: Full=Globin, body wall isoform [Heligmosomoides bakeri] | 169 |
| gi\|560138732 | Protein synthesis factor and Translation elongation factor EFTu EF1A domain containing protein [Haemonchus contortus] | 164 |
| gi\|283480611 | ADP/ATP translocase [Haemonchus contortus] | 164 |
| gi\|560133126 | Mitochondrial substrate solute carrier domain containing protein [Haemonchus contortus] | 144 |
| gi\|560138732 | Protein synthesis factor and Translation elongation factor EFTu EF1A domain containing protein [Haemonchus contortus] | 144 |
| gi\|215981768 | Elongation factor 1 alpha [Melitaea interrupta] | 136 |
| gi\|51311 | Unnamed protein product [Mus musculus] | 132 |
| gi\|560133126 | Mitochondrial substrate solute carrier domain containing protein [Haemonchus contortus] | 132 |
| gi\|205360870 | Histone cluster 1, H4d [Xenopus laevis] | 121 |
| gi\|162280611 | Actin variant 1 [Dictyocaulus viviparus] | 121 |
| gi\|215981768 | Elongation factor 1 alpha [Melitaea interrupta] | 121 |
| gi\|51311 | Unnamed protein product [Mus musculus] | 121 |
| gi\|195042120 | GH12093 [Drosophila grimshawi] | 119 |
| gi\|465968844 | Histone H4 [Chelonia mydas] | 119 |
| gi\|194739625 | Epididymis luminal protein 176 [Homo sapiens] | 119 |
| gi\|205360870 | Histone cluster 1, H4d [Xenopus laevis] | 119 |
| gi\|162280611 | Actin variant 1 [Dictyocaulus viviparus] | 119 |
| gi\|511915211 | PREDICTED: histone H4-like [Mustela putorius furo] | 118 |
| gi\|223582 | Histone H4 [Mus musculus] | 115 |
| gi\|560131185 | Lipid transport protein and Vitellinogen and von Willebrand factor domain containing protein [Haemonchus contortus] | 112 |
| gi\|597854071 | Hypothetical protein Y032_0093g2638 [Ancylostoma ceylanicum] | 110 |
| gi\|58378714 | AGAP006782-PA [Anopheles gambiae str. PEST] | 109 |
| gi\|533205512 | Collagen alpha-1 (I) chain-like Chinchilla lanigera | 108 |
| gi\|342210213 | Histone H4, partial [Nemertean sp. 1 SA-2011] | 105 |
| gi\|225710442 | Actin, muscle [Caligus rogercresseyi] | 105 |
| gi\|560121263 | Globin domain containing protein [Haemonchus contortus] | 104 |
| gi\|48527433 | Actine [Elaeis guineensis] | 103 |
| gi\|83699695 | Myosin heavy chain [Haemonchus contortus] | 102 |


| gi/8569651 | Cuticle globin - [Syngamus trachea] | 98 |
| :---: | :---: | :---: |
| gi\|403271599 | PREDICTED: ADP/ATP translocase 2-like isoform 1 [Saimiri boliviensis boliviensis] | 92 |
| gi\|5702223 | Type 1 actin, partial [Pleurochrysis carterae] | 91 |
| gi\|136429 | RecName: Full=Trypsin; Flags: Precursor [Mus musculus] | 90 |
| gi\|83699695 | Myosin heavy chain [Haemonchus contortus] | 90 |
| gi\|560117494 | ATPase domain containing protein [Haemonchus contortus] | 88 |
| gi\|568287539 | von Willebrand factor type D domain protein [Necator americanus] | 88 |
| gi\|597890337 | Hypothetical protein Y032_0703g1670 [Ancylostoma ceylanicum] | 85 |
| gi\|507684739 | PREDICTED: keratin, type II cytoskeletal 6B [Echinops telfairi] | 84 |
| gi\|296434222 | alpha tubulin [Saccoglossus kowalevskii] | 83 |
| gi\|21667223 | alpha-tubulin 2 [Strongylocentrotus droebachiensis] | 83 |
| gi\|403492612 | alpha tubulin, partial [Adineta vaga] | 83 |
| gi\|326935547 | PREDICTED: keratin, type II cytoskeletal 5-like [Meleagris gallopavo] | 82 |
| gi\|281323596 | histone H4 [Stylocellus sp. Borneo 13] | 82 |
| gi\|560138732 | Protein synthesis factor and Translation elongation factor EFTu EF1A domain containing protein [Haemonchus contortus] | 81 |
| gi\|6225602 | RecName: Full=32 kDa beta-galactoside-binding lectin; AltName: Full=Galectin-1 (Haemonchus contortus) | 79 |
| gi\|281323596 | histone H4 [Stylocellus sp. Borneo 13] | 78 |
| gi\|403492580 | alpha tubulin, partial [Adineta ricciae] | 78 |
| gi\|38453896 | translation elongation factor 1 alpha [Nematostella vectensis] | 78 |
| gi\|597854071 | Hypothetical protein Y032_0093g2638 [Ancylostoma ceylanicum] | 78 |
| gi\|8926583 | beta-actin [Aspergillus terreus] | 76 |
| gi\|224016155 | elongation factor-1 alpha [Polygonia zephyrus] | 73 |
| gi\|568294029 | lipoprotein amino terminal region [Necator americanus] | 73 |
| gi\|18152531 | Thioredoxin peroxidase [Ostertagia ostertagi] | 73 |
| gi\|2182027 | mitochondrial processing peptidase [Teladorsagia circumcincta] | 72 |
| gi\|560132238 | Peptidase M16 domain containing protein [Haemonchus contortus] | 72 |
| gi\|498125699 | transketolase [Ruegeria conchae] | 72 |
| gi\|568292924 | Myosin, essential light chain family protein [Necator americanus] | 72 |
| gi\|187234917 | elongation factor-1 alpha, partial [Deidamia inscripta] | 72 |
| gi\|498125699 | transketolase [Ruegeria conchae] | 72 |
| gi\|560121268 | Globin domain containing protein [Haemonchus contortus] | 71 |
| gi\|546744680 | ATP synthase subunit alpha [Succinatimonas sp. CAG:777] | 69 |
| gi\|4107495 | translation elongation factor 1-alpha [Euplotes aediculatus] | 68 |
| gi\|402867694 | PREDICTED: keratin, type II cytoskeletal 8-like [Papio anubis] | 68 |
| gi\|4107495 | translation elongation factor 1-alpha [Euplotes aediculatus] | 68 |


| gi\|597838583 | hypothetical protein Y032_0251g186 [Ancylostoma <br> ceylanicum] | 66 |
| :--- | :--- | :--- |
| gi\|568287539 | Von Willebrand factor type D domain protein [Necator <br> americanus] | 65 |
| gi\|17569137 | Protein PDI-2, isoform [Caenorhabditis elegans] | 60 |

Table 2 Summary of proteins identified with LC/MS/MS from H. bakeri incubated in $1 \mu M$

## PLS (Search taxonomy $=$ C. elegans)

| Protein code | Protein name / species | Mascot score |
| :---: | :---: | :---: |
| gi\|17509391 | Protein UNC-15, isoform a | 159 |
| P0DM41 | Actin-1 | 108 |
| gi\|6628 | actin | 108 |
| P10567 | Paramyosin unc-15 | 90 |
| ACT1_CAEEL | Actin-1 | 76 |
| Q9XWA6 | Protein-tyrosine-phosphatase CELE_Y113G7C. 1 | 67 |
| gil 156400 | myosin heavy chain | 66 |
| Q9Y0V2 | Mitochondrial import inner membrane translocase subunit Tim10B tin-9.2 | 50 |
| gi\|25148479 | Protein MATH-41 | 46 |
| Q9U296 | Malic enzyme men-1 | 46 |
| O76640 | T08E11.4 math-41 | 45 |
| A9D0C3 | T02H6.1a CELE_T02H6.1 | 42 |
| T10B_CAEEL | Mitochondrial import inner membrane translocase subunit Tim10B tin-9.2 | 40 |
| gi\|71999370 | Protein CATP-7, isoform a | 38 |
| Q9N323 | Y59H11AR.2a catp-7 | 38 |
| SAHH_CAEEL | Adenosylhomocysteinase ahcy-1 | 37 |
| Q9Y0V2 | Mitochondrial import inner membrane translocase subunit Tim10B tin-9.2 | 37 |
| C13A7_CAEEL | Putative cytochrome P450 CYP13A7 cyp-13A7 | 36 |
| Q7YTS8 | C31H5.7 | 35 |
| Q9XWA6 | Protein-tyrosine-phosphatase CELE_Y113G7C. 1 | 34 |
| G5EBY3 | F20G4.3 GN=nmy-2 | 33 |
| O16297 | DNA helicase mcm-7 | 33 |
| gi\|17533825 | BTB and MATH domain containing | 30 |
| gi\|72000134 | Protein SRJ-23, isoform a | 30 |
| C7IVS4 | DNA polymerase OS CELE_Y47D3A. 29 | 30 |
| gi\|71985287 | Inactive angiotensin-converting enzyme-related protein | 29 |
| Q10010 | Uncharacterized protein T19C3.4 | 29 |
| O45615 | H12I19.2 srz | 29 |
| U4PBT1 | Y38F2AR.5a tfte | 29 |
| H2KYS3-2 | Isoform b of Cytochrome P450 daf-9 | 28 |
| gi\|25145561 | Bloom syndrome protein homolog | 26 |
| Q95XK8 | Y54F10BM. 11 fbxa-66 | 26 |
| C0Z3L2 | H38K22.5d gly-6 | 26 |
| P90901 | Intermediate filament protein ifa-1 | 26 |
| Q9XV94 | F16H6.7 | 25 |
| O17934 | Nuclear hormone receptor family member nhr-199 | 25 |
| GSLG1_CAEEL | Golgi apparatus protein 1 homolog | 21 |
| CUT19_CAEEL | Cuticlin-like protein 19 cutl-19 | 20 |

Table 3 Summary of proteins identified with LC/MS/MS from whole C. elegans incubated
with $1 \mu$ M papain. (Search taxonomy $=$ C. elegans)

| Protein code | protein name/species | Mascot score |
| :---: | :---: | :---: |
| gi\|156400 | Myosin heavy chain | 2791 |
| gi\|71991728 | ANT-1.1, isoform a | 1852 |
| gi\|71994099 | ACT-4, isoform c | 1839 |
| gi\|17568987 | ACT-4, isoform b | 1628 |
| gi\|735952 | ADP/ATP translocase | 1619 |
| gi\|17541180 | ANT-1.3 | 1276 |
| gi\|17552884 | EEF-1A. 1 | 878 |
| gi\|71997271 | LEC-1, isoform b | 778 |
| gi\|25153023 | LEC-1, isoform a | 778 |
| gi\|17541790 | R05G6.7 | 768 |
| gi\|71991083 | VIT-6, isoform a | 672 |
| gi\|32566139 | MYO-3 | 659 |
| gi\|25150292 | MYO-2 | 635 |
| gi\|17551718 | ACT-5 | 627 |
| gi\|133901794 | F01G4.6, isoform a | 601 |
| gi\|17554342 | NEX-1 | 596 |
| gi\|295767 | myosin heavy chain 2 | 561 |
| gi\|17541098 | HSP-1 | 485 |
| gi\|17534101 | F45D11.14 | 467 |
| gi\|17570201 | VIT-5 | 439 |
| gi\|17570199 | VIT-4 | 439 |
| gi\|32566409 | F46H5.3, isoform b | 438 |
| gi\|6920 | put. vitellogenin | 427 |
| gi\|17534333 | RPL-5 | 424 |
| gi\|6786 | myosin 1 | 415 |
| gi\|17508449 | MYO-1 | 415 |
| gi\|71990071 | LEC-2, isoform a | 399 |
| gi\|71990079 | LEC-2, isoform b | 399 |
| gi\|17509481 | ANT-1.2 | 397 |
| gi\|29428264 | RecName: Full=Vitellogenin-3; Flags: Precursor | 388 |
| gi\|17544026 | Y69A2AR.18, isoform a | 380 |
| gi\|604515 | Na,K-ATPase alpha subunit | 337 |
| gi\|17507559 | RPL-7 | 332 |
| gi\|25144756 | ATP-2 | 318 |
| gi\|32565886 | UNC-22, isoform a | 310 |
| gi\|392901026 | UNC-22, isoform d | 310 |
| gi\|17509869 | PHB-1 | 303 |


| gi\|17543386 | RPS-4 | 301 |
| :---: | :---: | :---: |
| gi\|156352 | heat shock protein 70A | 297 |
| gi\|17554770 | RPS-3 | 290 |
| gi\|392886622 | EEF-2, isoform a | 289 |
| gi\|552062 | actin, partial | 287 |
| gi\|17570195 | VIT-2, isoform a | 260 |
| gi\|71983779 | DIM-1, isoform a | 259 |
| gi\|71988506 | SCA-1, isoform b | 258 |
| gi\|17506425 | AHCY-1 | 254 |
| gi\|6924 | vitellogenin | 247 |
| gi\|17510479 | RPL-1, isoform a | 235 |
| gi\|71998537 | UNC-52, isoform e | 216 |
| gi\|289722 | basement membrane proteoglycan | 216 |
| gi\|17568359 | FTT-2, isoform a | 216 |
| gi\|17541600 | PAR-5 | 208 |
| gi\|829165 | cytoplasmic intermediate filament protein | 207 |
| gi\|453232784 | F40F4.6 | 200 |
| gi\|17554192 | LEC-4 | 199 |
| gi\|17542014 | RPS-8 | 175 |
| gi\|71983645 | BEN-1 | 156 |
| gi\|17534771 | HSP-4 | 143 |
| gi\|156346 | BiP, heat shock protein 3 | 143 |
| gi\|17534703 | FGT-1, isoform a | 143 |
| gi\|17570193 | VIT-1 | 137 |
| gi\|17506815 | RLA-0 | 134 |
| gi\|17507981 | HSP-70 | 130 |
| gi\|17534013 | F44E5.4 | 130 |
| gi\|392886736 | C41G7.9, isoform a | 127 |
| gi\|6744 | gpd-2 gene product | 123 |
| gi\|71987720 | LET-805, isoform b | 122 |
| gi\|17549915 | TBB-4 | 119 |
| gi\|17533087 | TSN-1 | 119 |
| gi\|1036784 | triosephosphate isomerase | 118 |
| gi\|71983985 | ALDO-2, isoform a | 117 |
| gi\|71983990 | ALDO-2, isoform b | 117 |
| gi\|552071 | myosin II | 109 |
| gi\|133906874 | F53A2.7 | 107 |


| gi\|17557310 | RPL-2 | 106 |
| :---: | :---: | :---: |
| gi\|17553700 | RPS-1 | 105 |
| gi\|17564550 | T22F3.3, isoform a | 103 |
| gi\|17561652 | MYO-5 | 101 |
| gi\|71988063 | H28O16.1, isoform a | 101 |
| gi\|17551082 | SAMS-1 | 100 |
| gi\|17538494 | SAMS-3, isoform a | 100 |
| gi\|32565909 | SAMS-3, isoform d | 100 |
| gi\|25145633 | SAMS-5, isoform a | 100 |
| gi\|584868 | RecName: Full=Collagen alpha-2(IV) chain; AltName: Full=Lethal protein 2; Flags: Precursor | 95 |
| gi\|392900056 | IDH-1, isoform b | 91 |
| gi\|17564950 | LEC-10 | 91 |
| gi\|17531535 | CDC-48.1 | 90 |
| gi\|71989645 | PCK-2, isoform a | 90 |
| gi\|17569053 | MEC-7 | 88 |
| gi\|17532375 | CDC-48.2 | 86 |
| gi\|17538698 | VHA-8 | 85 |
| gi\|7506104 | hypothetical protein M6.1 | 84 |
| gi\|17554310 | MDH-2 | 84 |
| gi\|241065 | alpha-actinin=actin-binding protein Peptide Partial, 910 aa | 82 |
| gi\|17553678 | UCR-1 | 81 |
| gi\|32564821 | K02E7.6 | 80 |
| gi\|17543174 | VHA-3 | 80 |
| gi\|17559068 | CPR-4 | 80 |
| gi\|25147133 | GOT-2.2, isoform a | 79 |
| gi\|17543600 | Y54G2A. 18 | 78 |
| gi\|32565833 | RPL-7A, isoform c | 75 |
| gi\|17561568 | F57F4.4 | 74 |
| gi\|17554768 | RPS-0 | 68 |
| gi\|17567355 | F28B4.3 | 66 |
| gi\|17557712 | ATP-5 | 59 |
| gi\|71982026 | TBA-2 | 58 |
| gi\|1405416 | alpha-1 tubulin | 58 |
| gi\|17565854 | VHA-13 | 58 |
| gi\|72000666 | T21H3.1, isoform a | 57 |
| gi\|32564411 | PCK-1, isoform d | 53 |
| gi\|17560798 | AAGR-3, isoform b | 53 |
| gi\|17555174 | CTS-1 | 51 |
| gi\|17542012 | RPS-2 | 51 |
| gi\|17508493 | PAS-5 | 49 |
| gi\|17534029 | TBA-4 | 49 |
| gi\|17555336 | TBA-7 | 49 |


| gi\|17506225 | CYC-1 | 49 |
| :---: | :---: | :---: |
| gi\|17563244 | RPS-27 | 49 |
| gi\|17549909 | ASP-4 | 48 |
| gi\|17533883 | F41C3.5 | 48 |
| gi\|17541222 | RACK-1 | 48 |
| gi\|17553758 | CRI-3 | 46 |
| gi\|2282574 | flavoprotein subunit of complex II | 45 |
| gi\|17550100 | SDHA-1 | 45 |
| gi\|17570047 | NEP-22 | 45 |
| gi\|17506981 | AARS-2 | 44 |
| gi\|17536967 | COPB-1 | 44 |
| gi\|71981411 | UNC-44, isoform f | 44 |
| gi\|71981393 | UNC-44, isoform b | 44 |
| gi\|71981389 | UNC-44, isoform a | 44 |
| gi\|17559162 | DAF-21 | 44 |
| gi\|1703238 | RecName: Full=Fructose-bisphosphate aldolase 1; <br> AltName: Full=Aldolase CE-1; Short=CE1 | 43 |
| gi\|17508501 | PBS-7 | 43 |
| gi\|72000923 | TTN-1, isoform g | 43 |
| gi\|6643 | G-protein | 43 |
| gi\|71997204 | VAB-10, isoform b | 42 |
| gi\|27801760 | VAB-10B protein | 42 |
| gi\|27801756 | VAB-10A protein | 42 |
| gi\|498329 | uses second of two potential start sites | 42 |
| gi\|7497374 | hypothetical protein C44B7.10 | 42 |
| gi\|71989991 | F52C6.3 | 42 |
| gi\|25148479 | MATH-41 | 41 |
| gi\|193205005 | PHB-2 | 40 |
| gi\|193211092 | T25C12.3 | 40 |
| gi\|17531783 | ART-1 | 39 |
| gi\|5834894 | NADH dehydrogenase subunit 5 | 38 |
| gi\|71999370 | CATP-7, isoform a | 37 |
| gi\|312738 | Cytoplasmic intermediate filament (IF) protein | 36 |
| gi\|17531383 | B0495.7 | 36 |
| gi\|17570205 | UCR-2.1, isoform a | 36 |
| gi\|17508687 | RPS-6, isoform a | 36 |
| gi\|17532641 | CYN-4 | 36 |
| gi\|17554946 | ECH-6 | 35 |
| gi\|17531429 | AQP-2, isoform a | 35 |
| gi\|17542706 | VHA-5 | 34 |
| gi\|17505833 | SDHA-2 | 34 |
| gi\|17569137 | PDI-2, isoform a | 34 |
| gi\|17570191 | VHA-12 | 34 |


| gi\|7509723 | COL-87 | 34 |
| :--- | :--- | :--- |
| gi\|7509723 | hypothetical protein Y39G8C.b | 34 |
| gi\|7506668 | hypothetical protein R12C12.7 | 34 |
| gi\|86565532 | F29B9.12 | 34 |
| gi\|17562024 | HSP-6 | 33 |
| gi\|17540338 | ELO-6 | 33 |
| gi\|392900718 | ENPL-1, isoform a | 33 |
| gi\|71995207 | GRD-13 | 33 |
| gi\|392895266 | LET-767, isoform b | 32 |
| gi\|392887757 | CLEC-115 | 32 |
| gi\|17509265 | T26E3.7 | 32 |
| gi\|71989076 | PAM-1, isoform b | 32 |
| gi\|17536425 | T23G7.3 | 31 |
| gi\|115534168 | R02F2.2 | 31 |
| gi\|115533004 | Y38H8A.2, isoform a | 31 |
| gi\|71998965 | PAS-7 | 30 |
| gi\|71984538 | RPL-3, isoform a | 30 |
| gi\|17567343 | PCCA-1 | 30 |
| gi\|17506191 | IMB-3 | 29 |
| gi\|32564395 | CPG-2 | 29 |
| gi\|32563753 | CUL-4 | 27 |
| gi\|25146366 | DLST-1 | 27 |
| gi\|133901658 | NSF-1, isoform a | 27 |
| gi\|1584496 | chemosensory receptor | 27 |
| gi\|17508669 | RPL-4 | 27 |
| gi\|17539652 | TKT-1 | 27 |
| gi\|17544676 | GDH-1 | 26 |
| gi\|17560088 | DLAT-1 | 26 |
| gi\|17536635 | VHA-6 | 26 |
| gi\|7507925 | hypothetical protein T18H9.2 | 26 |
| gi\|17506835 | MRPL-54 | 25 |
| gi\|17559824 | EEF-1G, isoform a | 25 |
| gi\|71988919 | VHA-17 | 25 |
| gi\|17510085 | Y47H10A.4 | 25 |
|  |  |  |

Table 4 Summary of proteins released by papain from washed C elegans identified by
LC/MS/MS. (Search taxonomy $=$ C. elegans)

| Protein code | Protein name/species | Mascot score |
| :---: | :---: | :---: |
| gi\|17570199 | VIT-4 | 1530 |
| gi\|17570201 | VIT-5 | 1501 |
| gi\|808359103 | VIT-3 | 1460 |
| gi\|17570195 | VIT-2, isoform a | 1246 |
| gi\|156400 | myosin heavy chain | 1145 |
| gi\|17570193 | VIT-1 | 858 |
| gi\|17541098 | HSP-1 | 335 |
| gi\|25150292 | MYO-2 | 310 |
| gi\|735952 | ADP/ATP translocase | 286 |
| gi\|17509869 | PHB-1 | 131 |
| gi\|829165 | cytoplasmic intermediate filament protein | 121 |
| gi\|6786 | myosin 1 | 108 |
| gi\|17555172 | CHC-1 | 97 |
| gi\|17507981 | HSP-70 | 87 |
| gi\|6626 | actin | 86 |
| gi\|17568987 | ACT-4, isoform b | 86 |
| gi\|17565854 | VHA-13 | 83 |
| gi\|17534771 | HSP-4, isoform a | 80 |
| gi\|156346 | BiP, heat shock protein 3 | 80 |
| gi\|32566139 | MYO-3 | 76 |
| gi\|735952 | ADP/ATP translocase | 71 |
| gi\|71989645 | PCK-2, isoform a | 63 |
| gi\|17534333 | RPL-5 | 62 |
| gi\|17541790 | VDAC-1 | 59 |
| gi\|312738 | Cytoplasmic intermediate filament (IF) protein | 54 |
| gi\|17561568 | F57F4.4 | 44 |
| gi\|71999370 | CATP-7, isoform a | 43 |
| gi\|2282574 | flavoprotein subunit of complex II | 43 |
| gi\|25148479 | MATH-41 | 40 |
| gi\|17554084 | VMS-1 | 34 |
| gi\|17505833 | SDHA-2 | 34 |
| gi\|17562024 | HSP-6 | 33 |
| gi\|71999370 | CATP-7, isoform a | 33 |
| gi\|17561652 | MYO-5 | 32 |
| gi\|392920913 | PYC-1, isoform a | 29 |
| gi\|212645067 | TAF-1 | 29 |
| gi\|25144707 | GLR-1 | 28 |
| gi\|7504305 | hypothetical protein F55E10.3 | 28 |
| gi\|25143302 | SOP-3, isoform a | 27 |
| gi\|604515 | Na ,K-ATPase alpha subunit | 27 |
| gi\|17555492 | PCK-1, isoform a | 27 |
| gi\|17539652 | TKT-1 | 26 |

