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Running Title: Nematode cuticle breakdown by plant cysteine proteinases

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Abstract

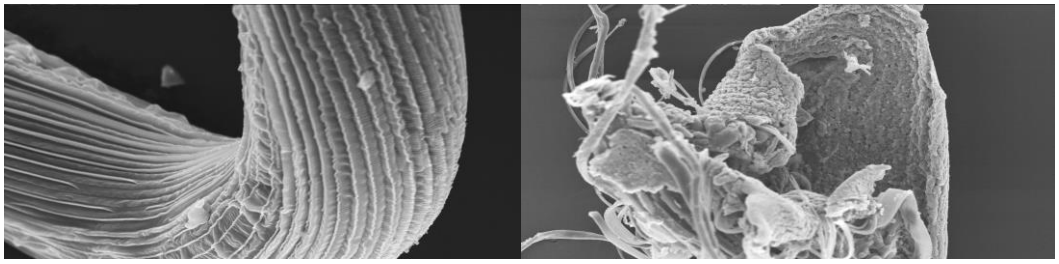
Background: Plant-derived cysteine proteinases of the papain family (CPs) attack nematodes by digesting the cuticle leading to rupture and death of the worm. The nematode cuticle is composed of collagens and cuticlins but the specific molecular target(s) for the proteinases have yet to be identified.

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 N2 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% (w/v) sucrose were added to the tube and mixed by inversion, then centrifuged at 121 x *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 x *g* for 2 min. The agar debris and bacterial sediments at the bottom of the tube were discarded. Worms were aliquoted in volumes of 1ml (~4500 worms) and stored at -20°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 mM NaCl, 32 mM KCl). The worm suspension was passed through a 5 µm microplate sieve to remove any L1 and L2 larval stages. The resulting suspension was centrifuged at 755 x *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% 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 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 15ºC 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 CO_2, and dissected. The intestine was carefully removed and placed inside a 15 cm dia. Petri dish containing pre-warmed (37˚C) 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 50ml beaker kept in a 37ºC 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˚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 ~4500 *C. elegans* or ~120 *H bakeri* adult worms) in a 1.5 ml mini-fuge tube was thawed and 1ml of H_2O 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% (w/v) sodium dodecyl sulphate (SDS) in 0.125M 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 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 x *g* and the last supernatant was taken off. The prepared worm cuticles were finally washed in H_2O and stored in PBS at -20^{o}C until they were used. β-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 4µM 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 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 µM papain or PLS, or papain or PLS + 1 mM E64, at time-points of 5, 10, 15 and 30 min at 37^{o}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 non-specific binding sites were blocked for 4 hrs with 750 µl 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 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 x *g* for 2 min. The washing was repeated twice. After washing, 10 µl 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 µM (final concentration) of CP, or CP + E64 at a temperature of 37ºC for 10, 15, and 30 min. At each time point, activity of CPs was stopped with 50 µl of 1 mM E64. The samples were then diluted with PBS and centrifuged at 121 x *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 23kV, and the images were stored electronically.

Digestion of worm cuticles with CPs for proteomic analyses

Either an aliquot (~4500 *C. elegans* or 120 *H. bakeri*) of prepared worm cuticles or whole worms was incubated in 1µM papain (final active concentration) or PLS (both activated with 4 mM L-cysteine), or papain or PLS + 1 mM E64 as the control, at 37ºC for 10, 15 and 30 minutes. Twenty five µl of the supernatant was collected at each time point and mixed with 20 µl 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 2x sample buffer [4% SDS, 20% glycerol, 10% DTT, 0.004% bromophenol blue and 0.125M Tris-HCl pH 6.5] and boiled for 5 min. Twenty µl 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% (v/v) glacial acetic acid in 40% (v/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 µM 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 µM 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 µM 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 µm), 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 µM papain or papain + E64 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 µM papain + E64 (Figure 2A). In Figure 2C after 30 min in 1µM 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 (*cpi-2*) [30]. Whole WT, or mutant *cpi-2(ok1256)* strains of *C. elegans* or *H. bakeri* were incubated with CP or 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 1µM 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 µM 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 µM 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 1µM 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 *H. bakeri* or prepared *H. bakeri* cuticles, or whole *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 SDS-PAGE 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 µM 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 time-dependent 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 μM 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 CUT-19, 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 DPY-7, 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: 349-365.

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 plant-derived 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**:227-242.

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.

536 38. Luoga W, Mansur F, Buttle DJ, Duce IR, Garnett MC, Behnke JM: **The anthelmintic**
537 **efficacy of papaya latex in a rodent-nematode model is not dependent on fasting before**
538 **treatment**. *J Helminthol* 2012, **86**:311-316.

539 39. McMahon L, Muriel JM, Roberts B, Quinn M, Johnstone IL: **Two Sets of Interacting**
540 **Collagens Form Functionally Distinct Substructures within a *Caenorhabditis elegans***
541 **Extracellular Matrix**. *Mol Biol Cell* 2003, **14**:1366-1378.

542 40. Page AP, Johnstone IL: **The cuticle**. *WormBook* 2007, **19**:1-15.

543 41. Thein MC, Winter AD, Stepek G, McCormack G, Stapleton G, Johnstone IL, Page AP:
544 **Combined Extracellular Matrix Cross-linking Activity of the Peroxidase MLT-7 and the**
545 **Dual Oxidase BLI**-547 **3 Is Critical for Post-Embryonic Viability in *Caenorhabditis***
546 ***elegans***. *J Biol Chem* 2009, **284**:17549-17563.

547 42. Andrew SM, Titus JA: **Purification of immunoglobulin G**. *Curr Protoc Immunol* 2001,
548 **2**.

549 43. Van Steendam K, De Ceuleneer M, Dhaenens M, Van Hoofstat D, Deforce D: **Mass**
550 **spectrometry-based proteomics as a tool to identify biological matrices in forensic science**.
551 *Int J Leg Med* 2013, **127**:287-298.

552 44. Wang T, Van Steendam K, Dhaenens M, Vlaminck J, Deforce D, Jex AR, Gasser RB,
553 Geldhof P: **Proteomic analysis of the excretory-secretory products from larval stages of**
554 ***Ascaris suum* reveals high abundance of glycosyl hydrolases**. *PLoS Neg Trop Dis* 2013, **7**:
555 e2467.

556 45. Barrett J, Brophy PM: ***Ascaris* haemoglobin: new tricks for an old protein**. *Parasitol*
557 *Today* 2000, **16**:90-91.

558 46. Blaxter ML: **Nemoglobins: divergent nematode globins**. *Parasitol Today* 1993, **9**:353 -
559 360.

560 47. Stepek G, Lowe A, Buttle DJ, Duce IR, Behnke JM: ***In vitro* and *in vivo* anthelmintic**
561 **efficacy of plant cysteine proteinases against the rodent gastrointestinal nematode,**
562 ***Trichuris muris***. *Parasitology* 2006, **132**:681 - 689.

563 48. Stepek G, Buttle DJ, Duce IR, Lowe, A, Behnke JM: **Assessment of the anthelmintic**
564 **effect of natural plant cysteine proteinases against the gastrointestinal**
565 **nematode, *Heligmosomoides polygyrus, in vitro***. *Parasitology* 2005, **130**: 203-211.

566 49. Hewitson JP, Harcus Y, Murray J, van Agtmaal M, Filbey KJ, Grainger JR, Bridgett S,
567 Blaxter ML, Ashton PD, Ashford DA *et al*: **Proteomic analysis of secretory products from**
568 **the model gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of**
569 **Venom Allergen-Like (VAL) proteins**. *J Proteom* 2011, **74**:1573-1594.

570 50. Daub J, Loukas A, Pritchard DI, Blaxter M: **A survey of genes expressed in adults of the**
571 human **hookworm, *Necator americanus***. *Parasitology* 2000, **120**:171-184.

572 51. *The cuticle* [http://www.wormbook.org]

573 52. Cox GN, Kusch M, Denevi K, Edgar RS: **Temporal regulation of cuticle synthesis**
574 **during development of *Caenorhabditis elegans***. *Dev Biol* 1981, **84**:277-285.

575 53. Fetterer RH, Hill DE, Urban JF, Jr.: **The cuticular biology in developmental stages of**
576 ***Ascaris suum***. *Acta Trop* 1990, **47**:289-295.

577

578 **FIGURE LEGENDS**

579 **Figure. 1: Immunohistochemical investigation of the activities of papain on prepared**
580 **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 µM papain. **D:** A worm incubated in 1 µM 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 1µM papain, indicating the sequence of events leading to the collapse of the cuticle structure. Bar = 25µm.

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 µM 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 min. Bar = 25 µm.

Figure 3: **SEM of WT (Bristol N2) *C. elegans* after incubation in 1 µM papain or papain + E64 at time points of 10, 15 and 30 mins. A:** The worms were apparently intact and unaffected when incubated with papain + E64 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 (**D**). Bar = 25 µm.

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 (**D**). Bar = 50 µm.

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). Bar = 10 µm.

Figure 6: SEM of *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 1µM 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 µm.

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^{o}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^{o}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^{o}C and dried in a vacuum concentrator for 30 min at 37^{o}C and thereafter digested with 200 ng of trypsin (per band) at 37^{o}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 µl 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 = ± 1.2 Da, fragment MS/MS tolerance = ± 0.6 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 ≥ 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, $P<0.05$).

653

654 ***Table 1 Summary of proteins identified with LC/MS/MS from H. bakeri digested with 1 µM***

655 ***papain (Search taxonomy = eukaryotic)***

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 ceylanicum]*	66
gi|568287539	Von Willebrand factor type D domain protein *[Necator americanus]*	65
gi|17569137	Protein PDI-2, isoform *[Caenorhabditis elegans]*	60

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657

658 ***Table 2 Summary of proteins identified with LC/MS/MS from H. bakeri incubated in 1 µM***

659 ***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
gi|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 tftc	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

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662 ***Table 3 Summary of proteins identified with LC/MS/MS from whole C. elegans incubated***

663 ***with 1 µM 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; 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

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666 ***Table 4 Summary of proteins released by papain from washed C elegans identified by***

667 ***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

gi|156400	myosin heavy chain	25

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