Invited Review

Enzymology of the nematode cuticle: A potential drug target?

Antony P. Page*, Gillian Stepek, Alan D. Winter, David Pertab

Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G61 1QH, UK

ARTICLE INFO

Article history:
Received 7 April 2014
Received in revised form 14 May 2014
Accepted 15 May 2014
Available online 6 June 2014

Keywords:
Nematode
Cuticle
Collagen
Moulting
Ecdysis
Protease

C. elegans

ABSTRACT

All nematodes possess an external structure known as the cuticle, which is crucial for their development and survival. This structure is composed primarily of collagen, which is secreted from the underlying hypodermal cells. Extensive studies using the free-living nematode Caenorhabditis elegans demonstrate that formation of the cuticle requires the activity of an extensive range of enzymes. Enzymes are required both pre-secretion, for synthesis of component proteins such as collagen, and post-secretion, for removal of the previous developmental stage cuticle, in a process known as moulting or exsheathment. The excretion/secretion products of numerous parasitic nematodes contain metallo-, serine and cysteine proteases, and these proteases are conserved across the nematode phylum and many are involved in the moulting/exsheathment process. This review highlights the enzymes required for cuticle formation, with a focus on the post-secretion moulting events. Where orthologues of the C. elegans enzymes have been identified in parasitic nematodes these may represent novel candidate targets for future drug/vaccine development.

© 2014 Published by Elsevier Ltd. on behalf of Australian Society for Parasitology Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. The cuticle

All nematodes are encased in an exoskeleton (known as the cuticle), a structure key to the success and diversity of nematode species. This complex extracellular matrix covers the outermost layer of cells and is required for body shape, movement, and functions as the primary interface with the environment (Fig. 1A). The cuticle structure and its biogenesis have been most extensively studied in the free-living model nematode Caenorhabditis elegans (Singh and Sulston, 1978; Page and Winter, 2003; Page and Johnstone, 2007). As a new cuticle is generated for each developmental stage, it is synthesised five times during the nematode lifecycle, with synthesis of the first cuticle beginning during late embryogenesis. Progression through development requires that the cuticle from the previous stage is shed and replaced with the new cuticle in a process known as moulting. The importance of the cuticle in maintaining body shape has been illustrated by genetic analysis in C. elegans. Strains carrying mutations in either the structural components of the cuticle, or the enzymes required for cuticle formation, result in dumpy morphological defects when mutated; these include phenotypes known in C. elegans nomenclature as dumpy (or Dpy, which are short and fat), roller (or Rol, helically twisted), long (or Lon), squat (or Sqt, short and twisted) and blistered (or Blt, fluid-filled blistering of the cuticle) (Page and Johnstone, 2007). Similar morphological phenotypes are found in mutants where the enzymes required for collagen and cuticle synthesis are defective (Page and Winter, 2003; Page and Johnstone, 2007). In addition to collagen, an unusual highly cross-linked class of insoluble protein called cuticlin is present in the nematode cuticle (Sapio et al., 2005), with C. elegans cuticlin mutants displaying dumpy morphological defects in specific developmental stages (Muriel et al., 2003; Sapio et al., 2005).

Collagen biogenesis is a complex, multi-step process with modifications that occur both intra- and extra-cellularly and requires the function of numerous enzymes (Fig. 1B). Some of the key enzymes involved in this pathway in C. elegans will be discussed in detail and their relevance to important human and animal parasitic nematodes will be highlighted.

http://dx.doi.org/10.1016/j.ijpdr.2014.05.003
2211-3207/© 2014 Published by Elsevier Ltd. on behalf of Australian Society for Parasitology Inc.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).
2. Enzymatic modifications occurring within the endoplasmic reticulum

2.1. Hydroxylation of proline residues

The first step in collagen synthesis involves the co-translational hydroxylation of Y position proline residues to 4-hydroxyproline in the Gly-X-Y domain, a modification that is required to stabilise the final assembled collagen triple helix. This reaction is catalysed by collagen prolyl 4-hydroxylase (C-P4H), which in multi-cellular animals are oligomeric enzymes consisting of α subunits, which contain the active site residues, and β subunits, formed by protein disulphide isomerase (PDI). Our lab has studied C-P4H extensively in the free-living nematode C. elegans where they are developmentally essential. C. elegans lacking a single C-P4H α subunit (DPY-18) are viable but show abnormal body morphology, cuticle structure, collagen localisation, and reduced levels of cuticular 4-hydroxyproline (Winter and Page, 2000), while combined loss of both α subunits (DPY-18 and PHY-2) results in embryonic lethality (Winter and Page, 2000; Winter et al., 2007b). Oligomeric C-P4Hs in all species examined contain only one type of β subunit PDI, which is present in all forms of the complex. Therefore, in C. elegans single loss of the β subunit (PDI-2) results in phenotypes equivalent to combined disruption of both α subunits (Winter and Page, 2000; Winter et al., 2007b) (Fig. 2). Recombinant C. elegans C-P4H are effectively inhibited using co-substrate analogues and in vivo these compounds replicate the phenotypes found by genetic disruption in C. elegans (Myllyharju et al., 2002). In vitro and in vivo work demonstrated that the C. elegans C-P4H complexes were formed from combinations of subunits that are unique to nematodes (Myllyharju et al., 2002). Similar analysis of a close relative of C. elegans, Caenorhabditis briggsae, revealed further nematode-specific C-P4H complexes that differ significantly from their vertebrate counterparts (Winter et al., 2007b).

Using a novel RNAi approach, our lab demonstrated that C-P4H is also developmentally essential in the human infective filarial nematode Brugia malayi (Winter et al., 2013). Analysis of the B. malayi genome (Ghedin et al., 2007) identified modification of all potential C-P4H subunits. Simultaneous RNAi of both α subunits (Bma-PHY-1 and -2) in cultured B. malayi adult females resulted in a highly penetrant body morphology defect in the released microfilariae (L1s) (Winter et al., 2013) (Fig. 2). This effect was replicated following RNAi of the single β subunit (Bma-PDI-2) (Winter et al., 2013). These results are supported by previous studies which showed that 4-hydroxyproline levels in B. malayi cuticle collagens were similar to those of C. elegans (Cox et al., 1981; Petralanda and Piessens, 1991) and that moulting of cultured B. malayi L3s was dependent on addition of ascorbate, a co-factor essential for C-P4H activity (Rajan et al., 2003). While the B. malayi RNAi results clearly mirrored the C. elegans genetic analysis, biochemical approaches revealed unusual aspects of the parasitic nematode proteins. In contrast to α subunits from every other multi-cellular species examined, recombinant Bma-PHY-1 and -2 proteins did not require PDI for solubility (Winter et al., 2003). Also, co-expression of all three B. malayi C-P4H components (Bma-PHY-1, -2 and Bma-PDI-2) in all combinations failed to produce highly active enzyme. In addition, the B. malayi proteins did not complement a C. elegans C-P4H mutant. Importantly, lack of complementation using B. malayi proteins is not a general phenomenon, as Bma-PDI-2 can complement a C. elegans pdi-2 mutant. This also confirmed that Bma-PDI-2 was a bona fide C-P4H β subunit. Further investigation into the B. malayi proteins revealed that parasite-derived, but not recombinant, B. malayi material contained a non-reducible covalent bond linking the subunits. This indicated that B. malayi C-P4H activity was dependent on this modification and its absence in recombinant proteins accounted for lack of activity/function in the assays employed. Enzymes from additional parasitic nematode species will need to be examined to determine if the B. malayi enzymes are unique in this respect. However, a covalent crosslink is not found between C-P4H subunits from any other species examined to date, including the extensively studied vertebrate enzymes. It is possible that the major differences in enzyme assembly between nematodes and vertebrate enzymes may be exploitable in the development of nematode-specific C-P4H inhibitors.

2.2. Procollagen registration: disulphide bond formation

The next important step in collagen biogenesis is the registration and trimerisation of the collagen chains. Registration involves the correct association of the monomers and is catalysed by PDI through disulphide bond formation of highly conserved C-terminal cysteine clusters in the cuticle collagens. This event may be key to the selection and assembly of the correct partners to form homo- or indeed hetero-trimers. In addition to the role in proline hydroxylation, it has been established that PDI-2 is also involved in this oxidative registration step in C. elegans (Winter et al., 2007b). Mutations in pdi-2 cause severe cuticle defects and adult sterility, whereas the combined mutations of pdi-1, pdi-2 and pdi-3 cause embryonic lethality and it was concluded that PDI-2 performs two essential functions during morphogenesis: one that is C-P4H dependent and the other contributing to disulphide bond formation. The importance of disulphide bonds and the oxidative/reductive state of the cuticle has also been found to be critical during the moulting process (Stenvall et al., 2011) and will be addressed further under the moulting section.
The reader is referred to the web version of this article.

MLT-7. (For interpretation of the references to colour in this figure legend, the
Fig. 1B. The cuticle collagen biogenesis pathway in C. elegans. Steps within the
endoplasmic reticulum (ER) (blue) include, proline hydroxylation by DPY-18,
disulphide bond formation by PDI, and proline isomerisation, by proline isomerases
(PIPlases). Export from the ER requires a functional COPII-pathway (sec-23). All
cuticle collagens are predicted to be N-terminally processed by the subtilisin-like
(PPIases). Export from the ER coincides with the N- and C-terminal processing events.
The removal of the globular non-Gly-X-Y domain results in protein
insolubility and precedes the final multimerisation and cross-
linking events. A number of key enzymes play important roles in
the post-ER maturation of collagens, and may therefore represent
novel drug targets.

3. Modifications that occur outside the endoplasmic reticulum

The transit of collagen from the ER to through the secretory
pathway coincides with the N- and C-terminal processing events.
The removal of the globular non-Gly-X-Y domain results in protein
insolubility and precedes the final multimerisation and cross-
linking events. A number of key enzymes play important roles in
the post-ER maturation of collagens, and may therefore represent
novel drug targets.

3.1. Collagen N-terminal processing

All C. elegans cuticle collagens possess a highly conserved N-terminal Kex2, furin-type cleavage site (RX/K/R/R), which has been
eperimentally defined as a requirement for processing the cuticle
collagens ROL-6, SQT-1 (Yang and Kramer, 1994, 1999) and DPY-5
(Thacker et al., 2006). The serine protease BLI-4 is required for
post-embryonic viability in C. elegans (Peters et al., 1991; Thacker
et al., 1995) and was originally defined by a viable partial loss-
of-function mutant with a blistered adult cuticle. Similarly, RNAi
depletion of BLI-4 reveals another cuticle-related phenotype as
moulting defects were observed (Kamath et al., 2003). These
findings suggested that BLI-4 is the enzyme that processes the
N-terminal pro-region of the cuticle collagens and this is supported
by its hypodermal expression pattern (Thacker et al., 1995) and by
the genetic suppression of the bli-4 phenotype by mutations in the
cuticle collagen gene dpy-5 (Thacker et al., 2006). Orthologues of
BLI-4 are present in many parasitic nematode genomes including
Onchocerca volvulus (Poole et al., 2003). Thus, BLI-4 is an essential
enzyme during embryogenesis, and is required for formation of the
cuticle in C. elegans, most likely due to processing the N-terminal
pro-region of cuticle collagens, and is likely to perform this role
in a range of parasitic nematode species.

A targeted RNAi screen for disruption of cuticle collagen
localisation performed in our lab, identified BLI-5 (Page et al.,
2006). Mutation of bli-5 resulted in a blistered cuticle, while over-
expression produced a severe moulting phenotype (Page et al.,
2006). Protein annotation suggested BLI-5 was a protease inhibi-
tor; however biochemical characterisation of C. elegans BLI-5
revealed that it activated, rather than inhibited, serine protease
activity (Stepek et al., 2010b). BLI-5 orthologues from the parasitic
nematodes Haemonchus contortus and B. malayi were both able to
complement the C. elegans bli-5 mutant and both also activated
serine protease activity in vitro (Stepek et al., 2010b). The biochemical
characterisation and phenotypic similarity between bli-5 and -4
mutants suggests BLI-5 might regulate BLI-4 (Stepek et al.,
2010b). In support of this, both bli-5 and bli-4 were found to share
common temporal and spatial expression patterns and combined
bli-4/bli-5 RNAi produced a variable but synergistic effect (Stepek
et al., 2010b).

3.2. Collagen C-terminal processing

The C-terminal processing of vertebrate fibrillar collagens is an
essential step that is carried out by zinc metalloproteases of the
astacin BMP class (bone morphogenic protein) (Canty and Kadler,
2005). The nematode astacins (NAS) represent a large gene family
of 39 members in C. elegans that includes the subgroup V forms
that, in addition to a metalloprotease domain, have a unique
nematode-specific domain arrangement that includes an epider-

In vitro analysis of vertebrate type III collagen demonstrates
that trimer folding is rate-limited by the slow cis-trans isomerisa-
tion of proline and hydroxyproline residues and requires the
assistance of peptidyl prolyl cis-trans isomerase (PPIase) enzymes
(Bachinger, 1987). The PPIase enzyme superfamily contains the
cyclophilin (CYN) and FK506-binding protein (FKB) families,
members of which are defined as receptors for the structurally
unrelated immunosuppressive drugs cyclosporin A (CsA) and
FK506, respectively.

CsA (Page et al., 1995; Bell et al., 1996) and novel PPIase inhibi-
tors (Yang et al., 2007; Dunsmore et al., 2011) cause severe gut
and cuticle related phenotypes in C. elegans suggesting that PPIase
enzymes are required to fold the structural components, such as
the proline and hydroxyproline-rich collagens. To date, the exact
combination of CYN enzymes, from a pool of 18, that are involved
in this process have not been defined, as genetic analysis of cysns in
C. elegans revealed widespread redundancy (Bell et al., 1996).
However, combined loss of two ER-localised C. elegans FKBs, frb-4
and -5, caused a cold-sensitive lethal phenotype with associated
collagen and cuticle matrix defects (Winter et al., 2007a). It is
therefore envisaged that this rate-limiting folding event is ther-
urally catalysed under normal conditions and that only under
adverse cold stress conditions is there an absolute requirement
for these PPIase enzymes.
The final steps in collagen maturation and cuticle synthesis are the structural cross-linking events. The cuticle collagens and cuticulins are covalently cross-linked via di- and tri-tyrosine cross-links. These non-reducible cross-links impart the characteristic strength and integrity to the cuticle (Page, 2001) and are distinct from the hydroxylysine-derived cross-links commonly found in the vertebrate collagens (Myllyharju and Kivirikko, 2004). The major cuticle cross-linking enzyme is a NADPH dual oxidase enzyme called BLI-3 (Edens et al., 2001; Simmer et al., 2003) (Fig. 1B). This large enzyme has a membrane-bound peroxide generating domain and a pseudo-peroxidase domain (Edens et al., 2001). RNAi (Edens et al., 2001) or mutation (Simmer et al., 2003) of BLI-3 results in weakened cuticles that lack tyrosine cross-links, producing adult worms that are devoid of cuticle struts and exhibit dumpy and blister phenotypes. BLI-3 does however lack a highly conserved haem-binding motif that is essential for peroxidase activity, and through RNAi screens an additional Haem peroxidase called MLT-7 was subsequently identified (Thein et al., 2003, 2009). MLT-7 protein has haem-binding and metridin ShK toxin domains, with mutants arresting as larvae with moulting defects. Like bli-3 mutants, mlt-7 mutants have fragile, permeable cuticles that are devoid of tyrosine cross-links and combined bli-3/mlt-7 mutants arrest prematurely as severely misshapen L1s. BLI-3 and MLT-7 are therefore the key enzymes that cooperate to promote the oxidative cross-linking of the collagens with a model suggesting that BLI-3 produces the H2O2 that is used by MLT-7 to generate the tyrosine linkages (Thein et al., 2009). Within their peroxidase domains, the MLT-7 orthologues from H. contortus and B. malayi show 82% and 50% identity, respectively, with C. elegans MLT-7 (Thein et al., 2009). The plant nematode, Meloidogyne incognita, has a dual oxidase gene (Midux) that encodes a multifunctional enzyme involved in tyrosine cross-linking of the developing cuticle during normal cuticle biosynthesis (Charlton et al., 2010). RNAi knockdown of this gene resulted in a slower developmental rate and a reduction in plant infectivity (Bakhetia et al., 2005). This Duox enzyme has significant identity to BLI-3 in C. elegans.

4. The enzymology of the moulting process

All nematodes are encased in a protective cuticle but this resilient structure restricts growth and therefore must be successively shed and re-synthesised. All nematodes undergo four cuticle molts from the L1 hatchling to the mature adult. The moulting process replaces the cuticle from the previous stage with a new cuticle, and occurs in three general steps: (1) lethargus, in which the activity of the worm decreases; (2) apolysis, in which the old cuticle separates from the hypodermis; and (3) ecdysis, in which the old cuticle is completely shed and the worm emerges as the next stage with a new cuticle. In C. elegans, this moulting process is repeated every 10 h and lasts for approximately 2 h, with the final ecdysis stage occurring rapidly within a matter of minutes (Singh and Sulston, 1978).
The process of moulting is unique to the Ecdysozoa clade, which in addition to nematodes, also includes arthropods, and may therefore represent a more general target for a wider group of pest species. The endocrine trigger for the moulting process in nematodes remains to be identified but in arthropods, such as Drosophila, this pathway is well characterised and centres on the hormone ecdysone, the nuclear ecdysone receptor (ECR) and ultraspiracle (USP) (Tzertzinis et al., 2010). In C. elegans, an ecdysone hormone has not been identified but a functional ECR homologue has been characterised in the filarial nematode B. malayi (Tzertzinis et al., 2010). The moulting process in C. elegans does require cholesterol, an essential low-density lipoprotein receptor (LRP-1) (Yochem et al., 1999) and an essential sterol synthetising enzyme LET-767 that is localised to the nematode gut (Kuervers et al., 2003). The genome of C. elegans does, however, encode a large family of orphan nuclear hormone receptors, two of which, NHR-23 and NHR-25, are essential for proper moulting (Kostrouchova et al., 1998, 2001).

Characterisation of the components of the moulting pathway in C. elegans is helping to uncover many nematode-specific features that may prove fruitful in future nematode control strategies. A global RNAi screen in C. elegans uncovered 159 genes that, when disrupted, affect the moulting process to some degree (Frand et al., 2005); this list includes proteases, protease inhibitors, peroxidasises, matrix components, sterol sensing proteins, nucleic acid binding/interacting proteins, signalling proteins and a range of other novel proteins, many of which are exclusively found in members of the nematode phylum. The importance of a key moulting component, MLT-10, identified in this screen (Frand et al., 2005) has been confirmed following a cholesterol-sensitivity mutagenic mutation (Meli et al., 2010). MLT-10 is a member of a large family of novel nematode-specific proline-rich proteins and is essential for both the synthesis of the new and removal of the old cuticle (Meli et al., 2010).

The oxidative state of disulphide linkages in the nematode cuticle has recently been shown to be a key feature in the apolysis process. Characterisation of the sole selenocysteine containing protein in C. elegans, thioredoxin TRX-R-1, revealed that in combination with the glutathione reductase (GSR-1), both proteins are essential for the normal moulting process (Stenvall et al., 2011). It was noted that the cuticular disulphide linkages are actively reduced during apolysis, an effect that could be induced prematurely by incubating worms in strong reducing agents or glutathione (GSH). TRX-R-1 is expressed in the hypodermis and has an absolute requirement for the proper digestion of the anterior cap, an event that concludes the moulting process and allows the worms to escape from the old cuticle (Davis et al., 2004). This specific enzymatic function is analogous to exsheathment in H. contortus, where an anterior refractile ring forms in the L2 cuticle sheath prior to infection of the host by the L3 stage (Gamble et al., 1989, 1996). A specific function was recently established when recombinantly-expressed C. elegans NAS-37 and NAS-36 were shown to induce refractile ring formation in the H. contortus L2 cuticle sheath (Davis et al., 2004; Stepek et al., 2011). Both H. contortus and B. malayi have a NAS-36, but lack a NAS-37 orthologue. The B. malayi gene can complement C. elegans strains mutant in either nas-36 or nas-37, and a combined mutant of nas-36/nas-37, indicating that NAS-36 is the dominant molecule involved in cuticle ecdysis in parasitic nematodes. This result also confirmed that NAS-36 metalloprotease plays a functionally conserved role in phylogenetically divergent nematode species (Stepek et al., 2011).

4.2. Additional cuticle-associated zinc metalloproteases

Zinc metalloproteases, with a similar crucial involvement in the L2 to L3 moult and exsheathment of L3, are present in the excretion/secretion (ES) of the infective L3 of many additional parasitic nematodes, including Brugia pahangi (Hong et al., 1993), Dirofilaria immitis (Rieher et al., 1992), Ancylostoma caninum and Ancylostoma duodenale (Hotze et al., 1990; Hawdon et al., 1995), and the bovine filarial nematode, Setaria cervi (similar to Wuchereria bancrofti) (Pokharel et al., 2006). A similar zinc metalloprotease is also involved in the formation and degradation of the old and new cuticles during the moulting of the L3 to L4 of Ascaris suum (Rhoads et al., 1998) and the nematode of fish, Hysterophrylaum aduncum (Malagon et al., 2010b).

The third-stage larvae of many parasitic nematodes, including Trichuris suis (Hill et al., 1993), Trichinella spiralis (Jen et al., 2003) and the fish nematode, H. aduncum (Malagon et al., 2010a), secrete metalloproteases that aid in the penetration of the host tissues. The hookworms, A. caninum and Necator americanus, the filarial nematode, O. volvulus, and the gastrointestinal nematode, Strongyloides stercoralis, secrete astacin metalloproteases (Ac-MTP-1, Na-MTP-1, Ov-AST-1 (onchoastacin) and Ss40 (strongylastacin), respectively), that aid in host skin penetration by hookworm L3 (Culley et al., 2000; Williamson et al., 2006) and S. stercoralis L3 (Brindley et al., 1995) and migration through the host tissue by filarial microfilariae (Borchert et al., 2007) to initiate infection. These enzymes have collagenolytic activity to help aid

Mutations in nas-36 and nas-37 result in moulting defects where worms are incarcerated in the cuticle from the previous stage, which remains unshed due to a tight anterior ring of undigested cuticle (Davis et al., 2004; Suzuki et al., 2004) (Fig. 3). NAS-37 is expressed in the C. elegans hypodermis just prior to ecdysis. The protein accumulates in the anterior cuticle and aids the proper digestion of the anterior cap, an event that concludes the moulting process and allows the worms to escape from the old cuticle (Davis et al., 2004). This specific enzymatic function is analogous to exsheathment in H. contortus, where an anterior refractile ring forms in the L2 cuticle sheath prior to infection of the host by the L3 stage (Gamble et al., 1989, 1996). A specific function was recently established when recombinantly-expressed C. elegans NAS-37 and NAS-36 were shown to induce refractile ring formation in the H. contortus L2 cuticle sheath (Davis et al., 2004; Stepek et al., 2011). Both H. contortus and B. malayi have a NAS-36, but lack a NAS-37 orthologue. The B. malayi gene can complement C. elegans strains mutant in either nas-36 or nas-37, and a combined mutant of nas-36/nas-37, indicating that NAS-36 is the dominant molecule involved in cuticle ecdysis in parasitic nematodes. This result also confirmed that NAS-36 metalloprotease plays a functionally conserved role in phylogenetically divergent nematode species (Stepek et al., 2011).

4.1. Ecdysis enzymes

In addition to the collagen biogenesis and cuticle assembly pathways, numerous enzymes play key roles in the subsequent cuticle shedding and moulting processes. The astacin metalloproteases, as well as being involved in procollagen C-processing, are also involved in the ecdysis process in C. elegans, and recombinant forms of these C. elegans enzymes can specifically digest the cuticle and permit exsheathment of the parasitic nematode H. contortus (Davis et al., 2004; Stepek et al., 2011). NAS-36 and -37 represent the subgroup V astacins that play key roles in cuticle ecdysis in C. elegans (Novelli et al., 2004).

Fig. 3. Moulting defects due to mutations in C. elegans metalloprotease NAS-37. (A) Mid-body constriction due to unshed cuticle and incomplete ecdysis due to mutation in nas-37. (B) SEM image of mid-body cuticle constriction due to nas-37 mutation. Scale bars 100 μm.
the degradation of the collagen in the host skin so that the parasite can penetrate and infect the host and are thus potential targets for new vaccines or drugs (Zhan et al., 2002; Hotez et al., 2003; Williamson et al., 2006).

ACN-1 is an angiotensin-converting enzyme (ACE)-like protein from *C. elegans* that is essential for larval development and adult morphogenesis, in particular the ecdisis step in moultling. Expression of *acn-1* is regulated by the moultling-associated genes *nhr-23* and *nhr-25* (Brooks et al., 2003) however, no homologues have been found in parasitic nematodes.

5. Cuticle-associated cysteine and serine proteases and their inhibitors

Cysteine proteases in nematodes (as for serine proteases, metalloproteases and aspartic proteases) have potential roles in digestion of the old cuticle, degradation of cuticular proteins and activation of moultling enzymes by processing their proenzymes. *C. elegans* encodes a cathepsin-L cysteine protease (Ce-cpl-1) that is similar to the proteases in *O. volvulus*, *B. pahangi*, *H. contortus*, *Dictyocaulus viviparus*, *Toxoeca canis*, *A. caninum*, *A. suum*, and plant parasitic nematodes such as *Heterodera glycines* (Britton and Murray, 2002), and is believed to be involved in post-embryonic development through degradation of the eggshell and cuticular proteins. Expression of this gene is greatest ~4 h prior to each moult, indicating a possible role in moultling to degrade the old cuticle whilst processing the next cuticle. This cysteine protease is either involved directly or indirectly by regulating other enzymes involved in the moultling process (Hashmi et al., 2002). *B. malayi* adults express the cathepsin-like cysteine proteases *Bm-cpl-1, Bm-cpl-5* and *Bm-cpz-1* that all have potential roles in embryogenesis, larval moultling and eggshell and cuticle remodelling, and share similarities with the *C. elegans* homologues (Ford et al., 2009). The cathepsin L cysteine proteases, cpl-1, -4 and -5 are secreted from *O. volvulus* (Guiliano et al., 2004), are involved in the L3 to L4 moult (Lustigman et al., 1992, 1996) and share many characteristics with the CPLs of *B. malayi* (Guiliano et al., 2004), *D. immitis* (Richer et al., 1992, 1993) and *B. pahangi* (Guiliano et al., 2004).

The role of the cathepsin Z-like cysteine protease (Ce-cpz-1) in *C. elegans* development is multifunctional, with the mRNA expressed throughout the lifecycle, increasing just prior to each moult. RNAi and gene knock-out result in severe moultling defects, and larval arrest at the L2 to L3 moult, thus suggesting a possible role for this enzyme in the moultling pathway; e.g., degrading cuticular proteins before ecdisis, or controlling proteins with essential roles during moultling (Hashmi et al., 2006). It is similar to *Ov-cpz* from *O. volvulus* and *Tc-cpz* from *T. canis*, indicating that the CPZ-1 function is possible conserved in other nematodes (Hashmi et al., 2002). *N. americanus* express an L3 specific cysteine protease that is found in the exsheathing fluid and may therefore play a role in ecdisis (Kumar and Pritchard, 1992).

The cysteine proteases involved in the moultling pathway of *C. elegans* and parasitic nematodes, such as *H. contortus*, are developmentally regulated, although their exact role remains to be established. *C. elegans* CPI-2a is a cystatin-like inhibitor that is present in the cuticle of all post embryonic stages and may have an essential role during moulting (Miller and Sands, 1977). Cuticle damage and a decrease in nematode motility also occurred when *Heligmosomoides polygyrus*, *Trichuris muris*, *Protospirula muricola* and the plant parasitic nematodes, *M. incognita*, *Meloidogyne javanica* and *Globodera rostochiensis*, were incubated with plant cysteine proteases, such as papain and bromelain, in vitro. This cuticle damage and loss of activity led to nematode death. This digestion of the cuticle represents a novel potentially exploitable, mechanism of action. Cuticle damage occurred together with loss of worm motility. However, the cuticle proteins that are sensitive to this plant cysteine protease digestion remain unknown, although as only the post-infection stages are affected, this suggests that the susceptible cuticle components are in the parasitic stage cuticles, not the free living stage cuticles (Stepek et al., 2005; Stepek et al., 2006, 2007a,b). Digestion of the cuticle in vivo, just as in vitro, also occurred when *H. polygyrus*, *T. muris* and *P. muricola* infected mice were treated with the plant cysteine proteases, fruit bromelain and crude papaya latex (Stepek et al., 2006, 2007a,b). *H. polygyrus* adult worms were expelled rapidly within 4 h of treatment if they were damaged.
resulting in a decrease in faecal egg count by 87–97% and a decrease in worm burden by 92%, suggesting that cysteine proteases from plants may be a candidate for novel anthelmintics. However, this treatment has no effect against the development of mucusal-dwelling L3 and L4 stages, only the adults in the gut lumen (Stepek et al., 2007b). Proteases from nematophagous bacteria and fungi can penetrate the cuticle of plant parasitic nematodes, causing infection of the nematode (Tian et al., 2009). Serine proteases from Bacillus (Lian et al., 2007; Huang et al., 2009), Hirsutella rhossiliensis (Wang et al., 2009), Paecilomyces lilacinus (Yang et al., 2011) and Pezizomycotina (Li et al., 2010) degrade the nematode cuticle, causing death of the nematode.

Thus, serine proteases from bacteria and fungi, and cysteine proteases from plants degrade nematode cuticles; however, the cuticle substrates for these proteases are still, as yet, unknown, indicating a requirement for further studies to determine these cuticle substrates that are strong potential candidates for novel targets for the much-needed new drugs or vaccines.

7. Concluding remarks

Significant progress has been made regarding our understanding of the structure, assembly and turnover of the C. elegans nematode cuticle. Many of the key enzymes involved in this process have now been identified and described in several important parasitic nematode species (Table 1). It is becoming clear that enzymes involved in the biogenesis of this critical exoskeleton and more significantly, in the molting process, represent a chink in the armour of these resilient metazoa. With the advent of completed genomes and the development of crucial experimental tools such as RNA interference, research into the enzymology of the cuticle in the parasitic nematodes is entering a promising era that may uncover novel drug targets and their inhibitors.

Acknowledgements

This work is supported by the Biotechnology and Biological Sciences and Biochemical Research Council (UK). The authors have declared that no competing interests exist.

References

Bachinger, H.P., 1987. The influence of peptidyl-prolyl cis-trans isomerase on the in vitro folding of type III collagens. J. Biol. Chem. 262, 17144–17148.

Bakhetia, M., Charlton, W., Atkinson, H.J., McPherson, M.J., 2005. RNA interference of dual oxidase in the plant nematode Meloidogyne incognita. Mol. Plant. Microbe Interact. 18, 1099–1106.

Bell, A., Roberts, H.C., Chappell, L.H., 1996. The antiparasite effects of cyclosporin A: possible drug targets and clinical applications. Gen. Pharmacol. 27, 963–971.

Borchert, N., Becker-Pauly, C., Wagner, A., Fischer, P., Stocker, W., Brattig, N.W., 2007. Identification and characterization of onchoastacin, an astacin-like metalloproteinase from the filaria Onchocerca volvulus. Microbes Infect. 9, 498–506.

Brindley, P.J., Gam, A.A., McKerrow, J.H., Neva, F.A., 1995. 5α: the zinc endopeptidase secreted by infective larvae of Strongyloides stercoralis. Exp. Parasitol. 80, 1–7.

Britton, C., Murray, L., 2002. A cathepsin L protease essential for postembryonic development. Dev. Biol. 86, 456–470.

Britton, C., Murray, L., 2003. An essential role in molting and morphogenesis of Caenorhabditis elegans for ACN-1, a novel member of the angiotsin-converting enzyme family that lacks a metallopeptidase active site. J. Biol. Chem. 278, 52340–52346.

Carry, E.G., Kader, K.E., 2005. Protocollagen trafficking, processing and fibrillogenesis. J. Cell Sci. 118, 1341–1353.

Charlton, W.L., Harel, H.Y., Bakhetia, M., Hibbard, J.K., Atkinson, H.J., McPherson, M.J., 2010. Additive effects of plant expressed double-stranded RNAs on root-knot nematode development. Int. J. Parasitol. 40, 855–864.

Cox, G.N., Straprans, S., Edgar, R.S., 1981. The cuticle of Caenorhabditis elegans. II. Stage-specific changes in ultrastructure and protein composition during postembryonic development. Dev. Biol. 86, 456–470.

Culey, P., Brown, A., Conroy, D.M., Sabroe, I., Pritchard, D.J., Williams, T.J., 2000. Eotaxin is specifically cleaved by hookworm metalloproteases preventing its action in vitro and in vivo. J. Immunol. 165, 6447–6453.

Davis, M.W., Birnie, A.J., Chan, A.C., Page, A.P., Jorgensen, E.M., 2004. A conserved metalloprotease mediates ecdysis in Caenorhabditis elegans. Development 131, 6001–6008.

Drake, L.J., Bianco, A.E., Bundy, D.A., Ashall, F., 1994. Characterization of peptidyl-prolyl cis-trans isomerase of the adult Trichuris muris. Parasitology 109, 623–630.

Dunsmore, C.J., Malone, K.J., Bailey, K.R., Wear, M.A., Florance, H., Shirran, S., Barran, P.E., Page, A.P., Walkinshaw, M.D., Turner, N.J., 2011. Design and synthesis of conformationally constrained cyclophilin inhibitors showing a cyclosporin-A pheno type in C. elegans. Chembiochem. 12, 802–810.

Edens, W.A., Shadinger, L., Cheng, G., Shapira, R., Kinkade, J.M., Lee, T., Edens, H.A., Tang, X., Sullards, C., Flaherty, D.B., Benian, G.M., Lambeth, J.D., 2001. Tyrosine cross-linking of extracellular matrix is catalysed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox. J. Cell Biol. 154, 879–891.

Eschenlauer, S.C., Page, A.P., 2003. The Caenorhabditis elegans ER960 homolog protein disulfide isomerase-3 has disulfide isomerase and transglutaminase-like cross-linking activity and is involved in the maintenance of body morphology. J. Biol. Chem. 278, 4227–4237.
Lustigman, S., McKerrow, J.H., Shah, K., Lui, J., Huima, T., Hough, M., Brotman, B., Lun, H.M., Mak, C.H., Ko, R.C., 2003. Characterization and cloning of metalloproteases from third- and fourth-stage larvae of the ovine nematode 

Kuervers, L.M., Jones, C.L., O’Neil, N.J., Baillie, D.L., 2003. The sterol modifying enzyme CHR3: a zinc metalloprotease from culture fluids of adult parasites. Exp. Parasitol. 80, 205–211.

Kostrouchova, M., Krause, M., Kostrouch, Z., Rall, J.E., 1998. CHR3: a receptor CHR3 is a critical regulator of all four larval molts of the nematode Caenorhabditis elegans.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Page, A.P., McCormack, G., Birnie, A.J., 2006. Biosynthesis and enzymology of the Caenorhabditis elegans cuticle: identification and characterization of a novel serine protease inhibitor. Int. J. Parasitol. 36, 681–689.

Pokharel, D.R., Rai, R., Kumar, P., Chaturvedi, C.M., Rathaur, S., 2006. Tissue destruction in the fluke Haemonchus contortus caused by matrix metalloproteinases. Exp. Parasitol. 110, 383–390.

Page, A.P., 2001. The nematode cuticle: synthesis, modification and mutants. In: Kennedy, M.W., Harnett, (Eds.), Parasitic Nematodes. CABI, St Albans, UK, pp. 73–87.

Page, A.P., Johnstone, L.L. 2007. The cuticle. In: WormBook (Ed.), The C. elegans Research Community. Wormbook. http://dx.doi.org/10.1895/wormbook.1.138.1.

Page, A.P., Winter, A.D., 2003. Enzymes involved in the biogenesis of the nematode cuticle. Adv. Parasitol. 53, 309–359.

Page, A.P., Kumar, S., Carlow, C.K.S., 1995. Parasite cyclophilins and antiparasite drug targets. Trends Parasitol. 11, 97–102.

Page, A.P., McCormack, G., Birnie, A.J., 2006. Biosynthesis and enzymology of the Caenorhabditis elegans cuticle: identification and characterization of a novel serine protease inhibitor. Int. J. Parasitol. 36, 681–689.

Pokharel, D.R., Rai, R., Kumar, P., Chaturvedi, C.M., Rathaur, S., 2006. Tissue destruction in the fluke Haemonchus contortus caused by matrix metalloproteinases. Exp. Parasitol. 110, 383–390.

Pokharel, D.R., Rai, R., Kumar, P., Chaturvedi, C.M., Rathaur, S., 2006. Tissue destruction in the fluke Haemonchus contortus caused by matrix metalloproteinases. Exp. Parasitol. 110, 383–390.

Pokharel, D.R., Rai, R., Kumar, P., Chaturvedi, C.M., Rathaur, S., 2006. Tissue destruction in the fluke Haemonchus contortus caused by matrix metalloproteinases. Exp. Parasitol. 110, 383–390.

Pokharel, D.R., Rai, R., Kumar, P., Chaturvedi, C.M., Rathaur, S., 2006. Tissue destruction in the fluke Haemonchus contortus caused by matrix metalloproteinases. Exp. Parasitol. 110, 383–390.

Pokharel, D.R., Rai, R., Kumar, P., Chaturvedi, C.M., Rathaur, S., 2006. Tissue destruction in the fluke Haemonchus contortus caused by matrix metalloproteinases. Exp. Parasitol. 110, 383–390.

Pokharel, D.R., Rai, R., Kumar, P., Chaturvedi, C.M., Rathaur, S., 2006. Tissue destruction in the fluke Haemonchus contortus caused by matrix metalloproteinases. Exp. Parasitol. 110, 383–390.

Pokharel, D.R., Rai, R., Kumar, P., Chaturvedi, C.M., Rathaur, S., 2006. Tissue destruction in the fluke Haemonchus contortus caused by matrix metalloproteinases. Exp. Parasitol. 110, 383–390.
Stepek, G., Buttle, D.J., Duce, I.R., Lowe, A., Behnke, J.M., 2005. Assessment of the anthelmintic effect of natural plant cysteine proteinases against the gastrointestinal nematode, *Heligmosomoides polygyrus*, in vitro. Parasitology 130, 203–211.

Stepek, G., Lowe, A.E., Buttle, D.J., Duce, I.R., Behnke, J.M., 2006. *In vitro* and *in vivo* anthelmintic efficacy of plant cysteine proteinases against the rodent gastrointestinal nematode, *Trichuris muris*. Parasitology 132, 681–689.

Stepek, G., Lowe, A.E., Buttle, D.J., Duce, I.R., Behnke, J.M., 2007a. Anthelmintic action of plant cysteine proteinases against the rodent stomach nematode, *Protospirura muricola*, *in vitro* and *in vivo*. Parasitology 134, 103–112.

Stepek, G., Lowe, A.E., Buttle, D.J., Duce, I.R., Behnke, J.M., 2007b. The anthelmintic efficacy of plant-derived cysteine proteinases against the rodent gastrointestinal nematode, *Heligmosomoides polygyrus*, *in vivo*. Parasitology 134, 1409–1419.

Stepek, G., McCormack, G., Page, A.P., 2010a. Collagen processing and cuticle formation is catalysed by the astacin metalloprotease DPY-31 in free-living and parasitic nematodes. Int. J. Parasitol. 40, 533–542.

Stepek, G., McCormack, G., Page, A.P., 2010b. The kunitz domain protein BLU-5 plays a functionally conserved role in cuticle formation in a diverse range of nematodes. Mol. Biochem. Parasitol. 169, 1–11.

Stepek, G., McCormack, G., Birnie, AJ, Page, A.P., 2011. The astacin metalloprotease moulting enzyme NAS-36 is required for normal cuticle ecdisis in free-living and parasitic nematodes. Parasitology 138, 237–248.

Suzuki, M., Sagoh, N., Iwasaki, H., Inoue, H., Takahashi, K., 2004. Metalloproteases with EGF, CUB, and thrombospondin-1 domains function in molting of *Caenorhabditis elegans*. Biol. Chem. 385, 565–568.

Thacker, C., Peters, K., Srayko, M., Rose, A.M., 1995. The bli-4 locus of *Caenorhabditis elegans* encodes structurally distinct ken2/subtilisin-like endoproteases essential for early development and adult morphology. Genes Dev. 9, 950–971.

Thacker, C., Sheps, J.A., Rose, A.M., 2006. *Caenorhabditis elegans* dpy-5 is a cuticle procollagen processed by a proprotein convertase. Cell. Mol. Life Sci. 63, 1193–1204.

Thein, M.C., McCormack, G., Winter, A.D., Johnstone, I.L., Shoemaker, C.B., Page, A.P., 2003. The *Caenorhabditis elegans* exoskeleton collagen COL-19: an adult-specific marker for collagen modification, assembly and the analysis of organismal morphology. Dev. Dyn. 226, 523–539.

Thein, M.C., Winter, A.D., Stepek, G., McCormack, G., Stapleton, G., Johnstone, I.L., Page, A.P., 2009. Combined extracellular matrix cross-linking activity of the peroxidase MLT-7 and the dual oxidoase BLU-3 is critical for post-embryonic viability in *Caenorhabditis elegans*. J. Biol. Chem. 284, 17549–17563.

Tian, B., Huang, W., Huang, J., Jiang, X., Qin, L., 2009. Investigation of protease-mediated cuticle-degradation of nematodes by using an improved immunofluorescence-localization method. J. Invertebr. Pathol. 101, 143–146.

Tzertzinis, G., Igana, AL., Palli, S.R., Robinson-Rechavi, M., Gissendanner, C.R., Liu, C., Uhnasch, T.R., Maina, C.V., 2010. Molecular evidence for a functional ec Dysyme signaling system in *Brugia malayi*. PLoS Negl. Trop. Dis. 4, e625.

Wang, B., Liu, X., Wu, W., Liu, X., Li, S., 2008. Purification, characterization, and gene cloning of an alkaline serine protease from a highly virulent strain of the nematode-endoparasitic fungus *Hirsutella rhossiliensis*. Microbiol. Res. 164, 665–673.

Williamson, A.L., Lustigman, S., Oksov, Y., Deumic, V., Plieskatt, J., Mendez, S., Zhan, R., Bottazzi, M.E., Hotez, P.J., Loukas, A., 2006. *Ankylostoma caninum* MTP-1, an astacin-like metalloprotease secreted by infective hookworm larvae, is involved in tissue migration. Infect. Immun. 74, 961–967.

Winter, A.D., Page, A.P., 2000. Prolyl 4-hydroxylase is an essential procollagen-modifying enzyme required for exoskeleton formation and the maintenance of body shape in the nematode *Caenorhabditis elegans*. Mol. Cell. Biol. 20, 4084–4093.

Winter, A.D., Myllyharju, J., Page, A.P., 2003. A hypoderomally expressed prolyl 4-hydroxylase from the filarial nematode *Brugia malayi* is soluble and active in the absence of protein disulfide isomerase. J. Biol. Chem. 278, 2554–2562.

Winter, A.D., Eschenlauer, S.C.P., McCormack, G., Page, A.P., 2007a. Loss of secretory pathway PKS06-binding proteins results in cold-sensitive lethality and associate extracellular matrix defects in the nematode *Caenorhabditis elegans*, J. Biol. Chem. 282, 12813–12821.

Winter, A.D., Keshkalo, K., Kukkola, L., McCormack, G., Felix, M.A., Myllyharju, J., Page, A.P., 2007b. Differences in collagen prolyl 4-hydroxylase assembly between two *Caenorhabditis* nematode species despite high amino acid sequence identity of the enzyme subunits. Matrix Biol. 26, 382–395.

Winter, A.D., McCormack, G., Page, A.P., 2007c. Protein disulfide isomerase activity is essential for viability and extracellular matrix formation in the nematode *Caenorhabditis elegans*. Dev. Biol. 308, 449–461.

Winter, A.D., McCormack, G., Myllyharju, J., Page, A.P., 2013. Prolyl 4-hydroxylase activity is essential for development and cuticle formation in the human infective parasitic nematode *Brugia malayi*. J. Biol. Chem. 288, 1750–1761.

Yang, J., Kramer, J.M., 1994. In vitro mutagenesis of *Caenorhabditis elegans* cuticle collagens identifies a potential subtilisin-like protease cleavage site and demonstrates that carboxyl domain disulfide bonding is required for normal function but not assembly. Mol. Cell. Biol. 14, 2722–2730.

Yang, J., Kramer, J.M., 1999. Proteolytic processing of *Caenorhabditis elegans* SQT-1 cuticle collagen is inhibited in right roller mutants whereas cross-linking is inhibited in left roller mutants. J. Biol. Chem. 274, 32744–32749.

Yang, Y., Moir, E., Kontopidis, G., Taylor, P., Wear, M.A., Malone, K., Dunsmore, C.J., Page, A.P., Turner, N.J., Walkinshaw, M.D., 2007. Structure-based discovery of a family of synthetic cyclophillin inhibitors showing a cyclosporin-A phenotype in *Caenorhabditis elegans*. Biochem. Biophys. Res. Commun. 363, 1013–1019.

Yang, J., Zhao, X., Liang, L., Xia, Z., Lei, L., Niu, X., Zou, C., Zhang, K.Q., 2011. Overexpression of a cuticle-degrading protease *Ver112 increases the nematocidal activity of *Paelolomys lilacinus*. Appl. Microbiol. Biotechnol. 89, 1895–1903.

Yochem, J., Tuck, S., Greenwald, I., Han, M., 1999. A gp330/megalin-related protein is required in the major epidermis of *Caenorhabditis elegans* for completion of molting. Development 126, 597–606.

Zhan, B., Hotez, P.J., Wang, Y., Hawdon, J.M., 2006. A developmentally regulated metalloprotease secreted by host-stimulated *Ancylostoma caninum* third-stage infective larvae is a member of the astacin family of proteases. Mol. Biochem. Parasitol. 120, 291–296.