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Chapter 4

PEPTIDASES IN PARASITIC NEMATODES: A REVIEW

David Malagón[1,2], Rocío Benítez[2], Martin Kašný[3] and Francisco Javier Adroher[2,*]

[1]Parasite Cell Biology Laboratory, Queensland Institute of Medical Research, Herston, Brisbane, Australia
[2]Departamento de Parasitología, Facultad de Farmacia, Universidad de Granada, Granada, Spain
[3]Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic

ABSTRACT

The nematodes are, after the insects, the group of organisms with the largest number of species identified. They include members of great medical, veterinary and agricultural significance, making this group one of the most important animal parasites. However there are many gaps in our knowledge of them. For example, there is still not a single nematode species for which we have detailed knowledge of feeding, digestion and nutritional requirements, showing that there are still many aspects to be learned about nutrition in nematodes [1]. Our understanding of the process of protein digestion, a very important function in the biology of any organism, is still poor since our knowledge is composed of fragmentary data for different groups of nematodes. It is believed that peptidases are essential during the development process and in the most critical moments of parasite-host interactions, and are thus directly involved with the growth and survival of the parasite. Their identification and characterization are important for basic understanding of the biology of the parasite, and their relevance to parasitic nematodes as virulence factors is clear. Consequently, peptidases are currently viewed as potential targets for vaccines, drugs and serodiagnosis. Despite this, in most cases, the precise physiological functions of peptidases in parasites are not known [2].

Peptidases comprise a large class of hydrolytic enzymes in parasitic nematodes, participating in nutrition through digestion of host proteins [3]. They also act in the

* E-mail address: fadroher@ugr.es.

moulting and resorption of the cuticle by processing and activating proenzymes or prohormones [4], degrading proteins that anchor the epidermis to the underlying cuticle (apolysis) [5], or by digesting the cuticle for resorption or facilitating its shedding (ecdysis) [6]. They are also active during embryonic development of the egg [7]. Peptidases are important in host-parasite relationships, being important virulence factors in some parasites [8]. The pathogenicity of several species of nematode has been significantly correlated with their peptidase activity. These include *Strongyloides stercoralis* [9], *Anisakis simplex* [10], *Onchocerca volvulus* [11], *Trichinella spiralis* [12], and *Ancylostoma caninum* [13].

All major types of peptidases have been described in nematodes. Aspartic peptidases have been described primarily in functions related to the digestion of nutrients. In invertebrates it is thought that, along with the cysteine peptidases, these have the same role as aspartic and serine peptidases in vertebrates [14]. In parasitic nematodes, the cysteine peptidases may be the class for which we have most information, since, owing to their great diversity, they cover virtually all functions in which peptidases are involved in parasitic nematodes. Cathepsins B and L are types of cysteine peptidases belonging to the papain family, and have been comprehensively studied in nematodes [15]. High variability has been found among the cathepsins B from different species of nematodes regarding optimum temperature and pH, and substrate affinity. It is thought that their main role is to digest nutrients and that the high interspecific variability observed is due to the nematode adapting to the ecological niche it occupies [16]. Cathepsins L also seem to be involved in the digestion of nutrients, as well as in processes of embryogenesis and moulting [2]. Many of these cathepsins L have counterparts in the free living nematode *Caenorhabditis elegans*, suggesting that they may be involved in conserved functions in different species of nematodes, but little is known about their precise functions [7]. The metallopeptidases are involved in the invasion of host tissues by the parasite, as they are able to degrade the extracellular matrix, and are also involved in the process of ecdysis and digestion of nutrients. The serine peptidases are also present in nematodes, and, along with the metallopeptidases, are believed to play the largest part in the invasion of host tissues by the parasites [10].

INTRODUCTION

The peptidases represent around 2% of the genes expressed in an organism [15]. The proteolytic enzymes of many parasites have been widely studied over the last two decades. In addition to their expected role in nutrition, at the expense of the host proteins, peptidases are considered essential during development and in the most critical moments of parasite-host interaction, and are thus directly involved in the growth and survival of the parasite. Their identification and characterization are important for our basic understanding of the biology of the parasite and it is easy to deduce their importance as virulence factors for parasitic nematodes. Consequently, peptidases are regarded as potential targets for vaccines, drugs and in serodiagnosis. Despite this, in most cases, the precise physiological functions of peptidases in these and other parasites are still unknown [2]. So, the main functions known for peptidases in parasitic nematodes are the following:

1) To facilitate penetration in, and through, the host tissues by digesting their proteins until the site where the parasite will establish itself is reached. A large part of the morbidity and mortality associated with infection is the result of the extensive

migration through the host by some parasites [17]. This migration normally involves an external digestive process in which the products are not usually assimilated. During the progression of the infective larva of parasitic nematodes of animals from the site of penetration – the skin or the digestive tract – to the establishment site, it is often possible to observe the secretion of proteolytic enzymes, as reported in geohelminths [18]. It has also been shown that the penetration of parasitic nematode larvae through the skin of mammals can sometimes be halted by the presence of peptidase inhibitors [9].

2) To protect the parasite from the attack of the host immune system, since they are able to degrade immunoglobulins bound to surface antigens [19]. In some cases peptidases are the main source of parasite antigens, favouring the immune response of the host. Many are especially immunogenic [20], making them extremely useful in serodiagnosis and vaccine development, although they also favour allergy and hypersensitivity processes in the host.
3) To take part in the moulting and resorption of the cuticle. This has been shown in certain species by the fact that when peptidase inhibitors are present the nematode cannot rid itself of the old cuticle during moulting [3]. Peptidases can participate in several ways during moulting: activating prohormones or proenzymes [4], degrading the proteins which anchor the cuticle to the underlying epidermis (apolysis) [5], or digesting the cuticle to resorb part of the proteins of which it is composed to form the new one or to facilitate shedding of the intact old cuticle (ecdysis) [6]. They may also take part in processing propeptides before they are incorporated as proteins into the new cuticle [21]. In nematodes, as opposed to arthropods, the animal grows and the cuticle, being elastic, stretches until it can no longer stretch further, at which point moulting occurs. In many cases the adult continues to grow, as does the cuticle, from the centre of the animal towards the extremes. In the epidermis of the adult stage of *Ascaris suum* enzymes have been found which participate in synthesizing the collagens of the cuticle, in spite of there being no further moults [22].
4) To be actively involved during the embryonic development of the egg, in the remodelling of the tissues during larval development and in the degradation of the nematode cuticle [7], and, finally, during development after eclosion [23].
5) As might be expected, to process and activate relevant peptides in many other metabolic processes, such as during the extension of pseudopods in nematode sperm [24]. This is a process in which peptidases participate by processing peptides involved in signal transduction pathways. Knowledge is currently increasing with regard to the vital processes involving peptidases, such as protein turnover (proteasomes) and development [25], and apoptosis [26], amongst others. This knowledge is generally being acquired from study of the free-living nematode *Caenorhabditis elegans*, and, to a lesser extent, from parasites [42].
6) Finally, to digest nutrients of proteic origin. This function is easily deduced since it is widely found in metazoans and is highly important. In nematodes, as in other animals, it takes place to facilitate the assimilation and utilization of the component units of the peptide molecules and may be internal or external [3]. However, despite its importance in nematode biology, protein digestion is still not well understood and our knowledge is comprised of fragmented data from different nematode groups.

The Peptidases and their Functions in Nematodes

Even when considering the complete phylum of nematodes there is no species for which all the details of feeding, digestion and nutritional requirements are known [1]. In any case, wide knowledge of feeding in one particular species, such as the model species *C. elegans*, could not be used to generalize for all groups due to the great variety of their feeding habits, related to the habitats they occupy. This is reflected in a series of anatomical characteristics and associated behaviours in both the digestive and absorptive processes. Sometimes in nematodes the occupation of a particular alimentary niche has phylogenetic implications, as in the ascarids. Depending on species, food generally consists of bacteria, algae, diatoms, protozoans, fungi, other nematodes, other invertebrates, vegetable matter, sap, haemolymph, blood, cell and fluids of animal tissues and gut contents. In some parasitic nematodes the larval stages may have completely different feeding habits to those of the adult, as in *Haemonchus contortus*, in which the larva develops free and exhibits survival stages until it is able to infect a host [1]. On the other hand, in mermitids, the larvae develop while parasitizing other invertebrates, usually insects, whereas the adults are free-living and feed on stored reserves [27]. The peptidases involved in feeding are found in the principal structures for this, these being the excretory/secretory system (E/S) and the digestive system (Figure 1).

The E/S system of nematodes may participate in feeding by secreting digestive enzymes. When this system was first described its function was thought to be to secrete manufactured substances and to excrete waste into the external medium. However, its main functions are currently thought to be secretion and osmoregulation, although functions can vary with species and developmental stage. Some evidence for this is provided by the observation that, in some nematodes, such as *Anisakis* sp., the enzymatic activity of structures associated with the E/S system is high and varied with many types of enzyme described (dehydrogenases, aminopeptidases, oxidoreductases, esterases and phosphorylases) [226]. It is also supported by ultrastructural studies of E/S tissue [28] and other evidence, such as the fact that excretion is mainly carried out in the intestine. However, excretion also takes place through the E/S system as it is known that CO_2 and different organic acids, amongst other products, are excreted through this system, which is of two basic types: glandular and tubular. The former is typical of free-living forms while the latter is more common in the parasitic forms, exhibiting a variety of designs but basically consisting of two lateral canals connected by a transverse canal. This system opens to the exterior via an excretory pore whose location is a taxonomic feature (Figure 1). This is the case of the genus *Hysterothylacium*, in which the excretory pore is located level with the nerve ring on the side of the front third of the parasite, unlike in the genus *Anisakis* (although both are anisakids) where it is found next to the mouth, through which peptidases can be released. Together with those from the oesophageal glands these can take part in invasion and migration by the infective third larval stage (L3) through the host tissues, as in the other ascaridoids. It has been shown that the secretions release antigenic substances, digestive enzymes and enzymes for moulting of the cuticle. In another anisakid, *Pseudoterranova decipiens*, a leucinoaminopeptidase is released during ecdysis of the final moult following stimulation of the neurosecretory cells of the E/S system [29]. In the trichostrongylids both cells of the E/S system and the oesophageal glands are involved in the release of moulting fluid by the larva during the second moult [30]. In *Angiostrongylus cantonensis* the main E/S antigens have been studied, revealing a significant quantity of peptidases and peptidase inhibitors (aldolase; CBR-PYP-1 protein; beta-amylase; heat shock

protein 70; proteosome subunit beta type-1; actin A3; peroxyredoxin; serine carboxy-peptidase; protein disulphide isomerase 1; fructose-bisphosphate aldolase 2; aspartyl protease inhibitor; lectin-5; hypothetical protein F01F1.12; cathepsin B-like cysteine proteinase 1; hemoglobinase-type cysteine proteinase; putative ferritin protein 2; and a hypothetical protein) [31].

The majority of nematodes have a complete digestive system (Figure 1), consisting of the buccal cavity and oesophagus, intestine and rectum. At the anterior end of the animal there are sensitive structures which are usually involved in the localization of food. This is sucked up by the oesophagus (circular, muscular and triradiate) and propelled up to and through the intestine by rapid contractions. The intestine is occluded by the pressure exerted on it by the hydrostatic skeleton of the pseudocele since it does not have associated musculature. When the muscles of the oesophagus are relaxed it occludes, thus preventing the food from returning towards the mouth. Among the oesophageal muscles there are several glands, usually three, one between each interradial zone (the dorsal being larger than the two ventrolateral), and which open into the oesophageal lumen. These glands generally secrete digestive substances such as proteolytic enzymes and amylases. In *Necator* spp. and *Ancylostoma* spp. the glandular secretions contain peptidases and hyaluronidases which degrade the tissue and capillaries engulfed by the parasite when it anchors itself with the buccal capsule to the intestine wall of the host [1], as well as anticoagulant proteins. The oesophagus can take several forms, depending on the order and species, and is thus an important taxonomic feature. In fact, some authors, such as Hsu [32], have related the level of development of the oesophageal glands of some nematodes to their feeding habits. Thus, in *Anisakis simplex*, the high level of development of the oesophageal glands has been related to the lesions which appear around the head of the parasite in the stomach wall of the cetacean definitive host [32-33]. It has also been observed in this species that proteolytic enzymes accumulate in granules within the oesophageal glands. It is thought that, due to the disposition of the glands, the secretions of the dorsal gland are involved in extracorporeal activities while those of the subventral glands are involved in intestinal digestion [34]. The oesophagus leads to the intestine, a simple tubular structure with an epithelium formed by a single layer of cells over the basal lamina, composed of collagens, with a brush border with numerous long folds supported by actin filaments in *Ascaris* sp. [35], increasing the surface area of the lumen by between 75 and 90 times [36]. These are very similar to the intestinal microvilli of mammals [37] and are covered by a glycocalyx. The front part of the intestine of *Ascaris* sp. is mainly for secretion and the rear part for absorption, with both apocrine and merocrine type secretions being described [36]. There are a large number of intestinal cells in the ascarids and transport processes take place in the intercellular spaces through the lateral membranes of the cells. In addition to absorbing nutrients, the epithelial cells probably excrete nitrogenated waste products, mainly ammonia. Excretion of this toxic substance is possible due to the availability of water. However, excretion is ureothelic in other nematodes which live in environments where water is a limiting factor [38]. Finally, the rectum and anus are situated at the end of the digestive tube. Some groups have rectal glands associated with the proctodeum, opening into the lumen of the rectum [1]. Waste products are expelled when the anus dilates, as there is a muscular sphincter, and hydrostatic pressure forces them out. Under experimental conditions, *Ascaris lumbricoides* (15-25 cm) renews the contents of its digestive tract in around 3 minutes while *C. elegans* (1 mm), which is much smaller, takes 45

seconds [39]. This gives an indication of the time required for digestion and/or absorption of nutrients.

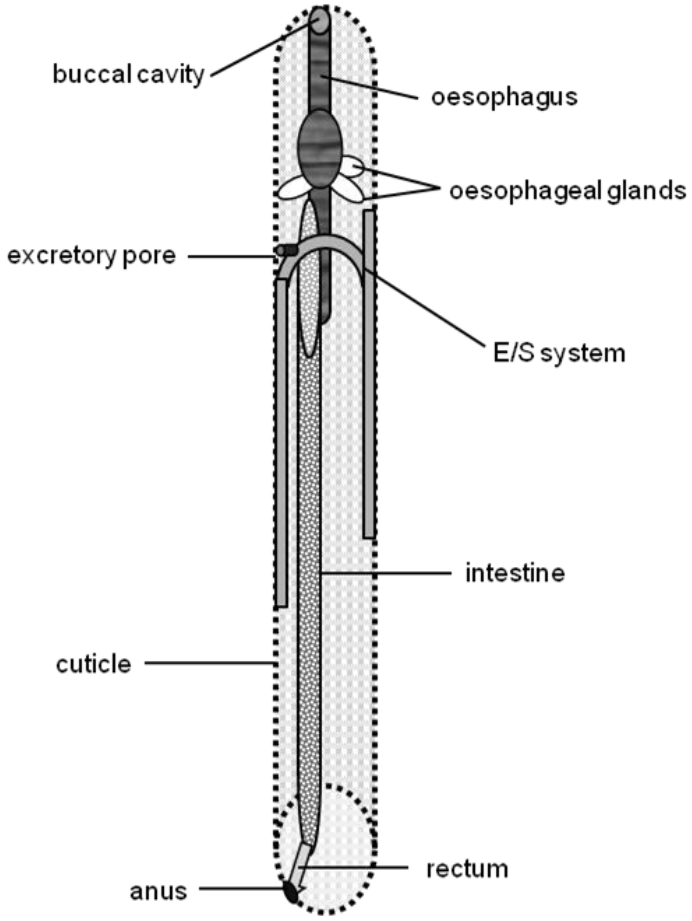

Figure 1. Diagram of digestive and Excretory/Secretory system of a nematode.

The digestive enzymes of nematodes comprise endopeptidases (of which the cysteine peptidases have been especially well studied in many organisms), exopeptidases, glycosidases, lipases, phospholipases and esterases. These may be secreted and released both externally and internally or are membrane proteins found on the luminal surface of the digestive tube. Van den Bossche and Borgers [37] reported that *A. suum* exhibited a low level of endocytosis in the intestinal epithelium suggesting that the acid hydrolases present in the intestinal microvilli might perform extracellular digestion of macromolecules in order to absorb the nutrients later. Several types of enzymes have been reported to be involved in the digestion of carbohydrates. In nematodes digestive enzymes have been described for all the principal nutrient groups. For example, in the case of the saccharides, the following were found in the intestine of *Ascaris* sp.: α-amylase, maltase, invertase, trehalase and isomaltase, but not lactase [36]. In *Nippostrongylus brasiliensis* and in other nematodes different glycosidases have been found and it is believed that these may be involved in the degradation of mucopolysaccharides. In parasitic nematodes of plants there are also cellulases, pectinases and chitinases [36]. Lipases have also been reported in nematodes. They have been found in different locations such as the intestine (*Ascaris* sp., *Leidynema* sp. and *Strongylus* sp.) and

the oesophagus (*Ancylostoma caninum*; in this hematophage they express themselves in the intestine where they combine with lipids, the saposins, which are believed to be active in the lysis of red blood cells [40]. As might be expected, the nutrient transport proteins are found in the luminal and basal surfaces of the digestive tube. In *Ascaris* sp., the absorption of hexoses in the intestine has been studied in detail [41]. The transport of cholesterol (which nematodes cannot synthesize) has been observed in the digestive tube of *A. lumbricoides* [36]. Although nutrient absorption takes place primarily in the intestine some nematodes use other structures as well. Nutrients may be absorbed through the cuticle (composed of modified keratinized cells), as in *Ascaridia galli* or *Ascaris* sp., and, especially, in certain parasites of insects, which lodge in the haemocoel, such as *Bradynema* sp. or *Mermis nigrescens*. The entomophile larva of *M. nigrescens* develops in the haemocoel of an orthopteran, absorbing glucose through the cuticle, mediated by a specific transport system [43] which is probably related to the ecological niche of this parasite. Furthermore, in some filarians absorption of glucose through the cuticle, together with leucine and adenine, has also been reported [36]. The great importance of peptidases for the assimilation of nutrients as well as in many other processes should be emphasized. They include a huge class of hydrolytic enzymes which are essential for all animals in the processes of digestion, development, cell dynamics, blood clotting, inflammation, scar tissue formation, processing of prohormones and many others.

In the nematodes, as in other organisms, the peptidases are distributed in different locations throughout the body of the animal, according to their function. For example, gelatinolytic activity in *A. simplex* [34] is mainly found in the intestinal lumen but is also present in the pseudocelom, the cells of the body wall and the muscles of the oesophagus. Accumulations of digested gelatin granules are found in the oesophageal glands. However, the excretory cells exhibit very little gelatinolytic activity, as occurs in *A. suum* with a specific aminopeptidase, which, furthermore, has been reported in the female reproductive tissues [44]. The glandular oesophagus of the filarians *Brugia pahangi* and *Brugia malayi* only appears in L3 with a granular distribution of cystein peptidases in which E/S products are released during the third moult [45]. Thus, as well as being spatial, the distribution of peptidases can also be temporal, depending on the developmental stage of the parasite. In electrophoresis of extracts of adult *Onchocerca volvulus* no gelatinolytic activity is observed, whereas it is present in larval extracts [46].

The main absorption route of amino acids is probably intestinal and it appears that some species are able to synthesize different amino acids from acetate. However, the majority of amino acids essential for mammals are probably also essential for nematodes. Thus, *C. elegans* and *Caenorhabdithis briggsae* require histidine, leucine, isoleucine, lysine, methionine (or its precursor homocysteine), phenylalanine, threonine, tryptophan, valine and arginine in their diet [1]. In the larva of *M. nigrescens* amino acid transport through the cuticle has been reported, and, in *Ascaris* sp., in the digestive tube, muscle and reproductive tissue. In the intestine of *Ascaris* sp. the assimilation of amino acids is a non-passive mediated process although it is not known whether this can occur against the gradient or whether it is sensitive to ions, as in the case of hexoses. However, in the muscle and reproductive tissue of *Ascaris* sp. the assimilation of a non-metabolizable amino acid, cycloleucine, through active transport in the reproductive tissue and diffusion in the muscle, has been observed. In the larva of *M. nigrescens* amino acid assimilation through the cuticle is a mediated process [47]. *A. simplex* ingests no food during L3 [48-49,224], demonstrated by Sommerville and Davey [50] on cultivating L3 of *A. simplex*, which had recently been extracted from the fish

Leionura atun. Here, they are found encapsulated in the viscera and mesenteries. During the first few days they did not feed orally so it is probable that a type of absorption through the cuticle took place. This has also been proposed for *Hysterothylacium aduncum* [51-52].

In some haematophages, such as *H. contortus* and the hookworms, numerous peptidases of different types have been identified (aspartic and metallopeptidases and a cathepsin B type cysteine peptidase) which are involved in haemoglobin digestion [53]. At the time of this study there was no direct evidence for haemoglobin digestion in the intestinal lumen. However, many peptidases expressed in the intestine and absent from E/S products have been described, implying that they act here. Furthermore, the optimum pH for many of these peptidases is lower than that of the host tissues, suggesting that they act in the acidic environment of the parasite intestine. Following many studies with cDNA of a large number of peptidases of all types, biochemical characterizations, expression of recombinant peptidases and localization of peptidase expression in the main haematophage nematodes (*Ancylostoma* spp., *Necator americanus* and *H. contortus*), peptidases have been found which are homologous to those found in haemoglobin digestion [54] by the trematode *Schistosoma mansoni* (one of the helminths which best shows this) [14,55] and by the protozoans *Plasmodium* spp. In *A. caninum* the haemoglobinolytic enzymatic cascade has been described in vitro [56]; and in the other haematophage nematodes, the known intestinal peptidases, homologous to those of *A. caninum, S. mansoni* y *Plasmodium* spp., have been assigned a position in the digestive cascade. However, in nematodes the process has only been described in *A. caninum*. This position is still hypothetical and provisional, equivalent to that occupied by its peptidase homologue in the processes already described. In this cascade the first to act would be the aspartic peptidases, generating the first peptides derived from the haemoglobin tetramer, followed by cysteine peptidases, metallopeptidases, and, finally, the exopeptidases, which would degrade the smallest peptides, giving rise to free amino acids and small oligopeptides. These can be absorbed through the intestinal epithelium [54]. It is clear that lysis of red blood cells could also occur in the intestine. Pores are created in the cell membranes [57] by the action of saposins, molecules with an affinity for lipids and thus able to adhere to the cell membrane and disrupt the cells [40]. These peptidases are accessible for the host immune system and are consequently being considered as vaccine targets against the illnesses caused by these nematodes, which have serious health and economic implications [58]. Some peptidases from these nematodes have already been analyzed. They exhibited different degrees of protection and were sometimes orthologous antigens shared by these haematophage nematodes [59]. One of these antigens, H-gal-GP, is highly protective against *H. contortus*. It is an intestinal cell membrane glycoprotein complex, and contains predominantly digestive peptidases. A study showed that adding protective antibodies from H-gal-GP immunized sheep to the H-gal-GP catalysed the haemoglobin digestion reaction, reducing the rate by 70–90%, while this reduction was only 30% when nonprotective IgG from sheep immunized with denatured H-gal-GP was added. These results support the theory that the mechanism of protection is by specific antibodies blocking the activity of the proteolytic enzymes, and thus, the ability of the parasite to feed [60].

In the free-living nematode *C. elegans* it appears that there is also a synergic process within the peptidases during the digestion of proteic nutrients. Thus, although the cysteinic cathepsins are able, in vitro, to digest some easily degradable proteins like casein by themselves, they require the prior collaboration of aspartic cathepsins to degrade other proteins. Cathepsin D (aspartic) degrades other proteins by itself, and, additionally, is able to

digest and inactivate itself when there is a lack of substrate. In effect, this is what takes place in vivo during fasting: after 4-8 hours without ingestion of food, levels of cathepsin D decrease first to 65%, while the cysteinic cathepsins do not decrease for at least 24 hours. The data show that, apart from the animal's age, its nutritional state is important for modulating lysosomal peptidase levels [61-63].

According to Delcroix et al. [14], the papain-type aspartic and cysteine peptidases assume the same role in invertebrate digestion as trypsins in that of vertebrates. In addition, this process occurs in an acidic environment. It was proposed that luminal or cellular acidic micro-environments may exist [64-65], a notion subsequently supported histochemically by the researchers [14]. This fits with proposals for other types of gastrointestinal parasites other than hookworms, the ascaridoids, in which it is thought that nutrient digestion is carried out through an enzymatic cascade similar to those described earlier at acidic pH. In *A. suum* the main intestinal activity is a result of an aminometallopeptidase. It has been suggested that in the gastrointestinal tract of the final host the metallopeptidases of the parasite complete the digestion initiated by the pepsin and other enzymes from the host [66]. This may also occur in another ascaridoid parasite, *H. aduncum*, in which it has been demonstrated that its aspartic activity is dependent on the pepsin of the culture medium (that mimics the pepsin from the host) being modulated by the level of exogenous pepsin (Figure 2). When pepsin activity is not in the milieu, the parasite tries to cover this deficiency with its own aspartic activity. Without exogenous predigestion by the pepsin in the medium, the larvae would require greater aspartate-peptidase activity [52]. Furthermore, it has been observed that infective L3 of *A. simplex* stimulate pepsin expression in the stomach of guinea pigs, an experimental host of this anisakid [223]. It should also be noted that pepsin has been shown to be necessary in the in vitro development and moulting of *A. simplex*. For optimum in vitro development of both these anisakids, besides pepsin, an acidic pH (pH 4.0) is necessary to facilitate its proteolytic activity [49,51]. The use of other peptidases such as papain or trypsin or other pH values has a negative effect on these nematodes [49,51,67-68]. It is thus possible that the dependence of *H. aduncum* on aspartic activity is related to the digestion of nutrients, and the decrease in its expression in culture is directly related to the presence of pepsin, as suggested by Rhoads et al. [66] for proteolytic activity in *A. suum.* This was mainly related to aspartate peptidases, but also metallopeptidases, and, to a lesser extent, cysteine peptidases. This activity seems to depend on the exogenous availability of nutrients of proteic origin, originating from previous digestion by the host [52]. The many ascaridoids which live in an acidic environment are able to withstand it due to their capacity for osmoregulation. *Ascaris suum* can maintain the concentration of the hydrochloric acid and the osmotic pressure in its haemolymph at levels lower than those in the intestinal content of the pig host [76]. It has long been known that these are factors that trigger the activation and development of infective larvae. So, in addition to pH, there are other stimuli in the environment that affect the parasite culture, such as temperature, CO_2 and environmental redox potential [50,69-71]. It has been found that the addition of hydrochloric acid during experimental infections with *A. suum* in neonate pigs inoculated with fertilized eggs resulted in more parasites in the lungs than in the controls, which were not given hydrochloric acid [72]. There is also another nematode, *A. cantonensis*, in which a combination of these two factors, pepsin and hydrochloric acid, has been found. Curiously, this is not a gastrointestinal parasite like the ascaridoids. This is a parasite whose final host is the rat, which is infected on ingesting the intermediate host (thought to be many species of snails). Then, the parasite reaches the brain through the host

tissues (the human is an accidental host in whom it can cause severe eosinophilic meningitis), and, finally, the adult migrates and settles in the pulmonary arteries. To facilitate isolation of infective larvae which are embedded in snail tissues, for experimental infection, specimens were prepared by digesting the infected snails with pepsin–HCl. It was shown that this method of collection contributed to more patent infections in rodents after inoculation of the larvae [73-74]. It was later found that during the pepsin preparation, transcripts of the infective larvae encode proteins that participate in a wide range of biological processes (among these, several peptidases). These findings suggest that treatment with pepsin–HCl not only digests the tissues of the snail host but also activates the infective larvae [75]. All these processes are likely to be carried out through the properties of the E/S system. Nevertheless, water and ion excretion are poorly understood in nematodes. The E/S systems seem to be involved in water excretion, but this has not yet been confirmed [76].

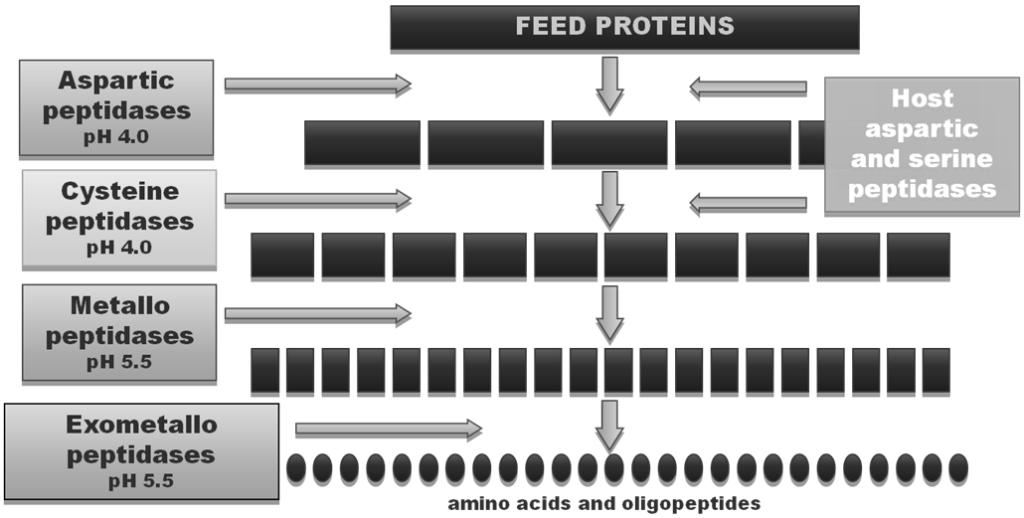

Figure 2**.** Proposed model for the digestion of feed proteins in the ascaridoid *Hysterothylacium aduncum* (gastrointestinal parasite of fishes). Based on Malagón et al. [52].

As mentioned previously, the peptidases have been considered potential targets for vaccines, drugs and serodiagnosis, and their importance is currently clear from the development of antiparasitic strategies initiated during the previous decade [77-79].This importance is largely due to their major role as virulence factors. In the nematodes in particular there are ample species which show a significant correlation between peptidase activity and pathogenicity. These include *Dirofilaria immitis* [80], *Nematospiroides dubius* [81], *Strongyloides stercoralis* [9], *A. simplex* [10], *N. americanus* [82-83], *Onchocerca* spp. [11,84], *Trichinella spiralis* [12], *Trichuris muris* [85], *Strongylus vulgaris* [219], *A. caninum* ([13] and *Teladorsagia* (*Ostertagia*) *circumcincta* [86]. A large part of the morbidity and mortality associated with infection is due to the extensive migration of the parasites through the host [17]. Migration of gastrointestinal parasites through the host tissues seems to be beneficial in that it favours more and faster growth than in those which do not migrate, leading to increased reproductive capacity of the parasite [87]. Balbuena et al. [88] found great variability in the location and routes followed by L3 of *H. aduncum* through the tissues of infected herring larvae and suggested that L3 migrate in search of an optimum environment

for their development within the host tissues and that this migration depends on their ontogenic development. The decision to penetrate and move through the host tissues depends on such factors as the condition of the host and/or the development of the parasite. This decision is also conditioned by the differences in susceptibility to the parasite of different host species and also within the same host species [for example, the host responding by isolating the parasite in a thick capsule may reduce its chances of developing [222]. Furthermore, the ability to detect small variations in the signals emitted by the host and to modulate their response accordingly will determine the extent of penetration and movement of the parasites within the host tissues [89]. Evidence for the secretion of hyaluronidase by both a parasitic nematode (*A. suum*) [90] and a nonparasitic nematode (*C. elegans*) [91] allowed Rhoads et al. [90] to suggest that this enzyme not only acts by facilitating the migration of the ascarids through the host tissue by its histolytic action but also that hyaluronic acid must be a requirement or signal for development to L4, since this molecule is involved in cell development. The presence of several hyaluronidases which can take part in tissue invasion has been described in *A. simplex, A. caninum* [92] and *A. suum* [90], with optimum pH of 4.0, 6.0 and 5.0-6.0, respectively (Table 1). *Ascaris* penetrates through the cecum and ascending colon [93]; *Ancylostoma*, through the skin, blood vessels and lungs; and *Anisakis* follows a cycle very similar to that of *H. aduncum*, often using the same intermediate and paratenic fish hosts, which in both cases are infected principally by ingestion of small crustaceans. It is possible that its optimum pH of 4.0 for its hyaluronidase indicates that it penetrates through the stomach (as proposed for *H. aduncum* due to an optimum pH for metallopeptidase type activity expressed only in the infective larva [52]), whereas optimum pH values for the other two hyaluronidases are higher since the other two parasites penetrate zones with higher pH. It is clear that the site where the parasite initiates host tissue invasion and subsequent migration through it is very important, the zone of action being related to the physicochemical properties of the employed peptidase. To a great extent, this is what determines the ecology and life cycle of the parasite. Anderson y Bartlett [94] reported that *Hysterothylacium haze* appeared to have lost the ability to return to the gastrointestinal tract of the host to develop to adulthood, as practised by other worms of the genus *Hysterothylacium*, (*H. haze* attains the adult stage in the visceral cavity of the host fish). This nematode is an extreme example of precocious development by some parasites in the host as a result of diverse evolutive acquisitions in their life cycles and that of their hosts. *A. suum* is a monoxenous parasite, and, when the eggs hatch in the host intestine, after their development in an external medium, the larvae do not immediately penetrate but rapidly travel along the narrow intestine and penetrate through the mucus of the cecum and the ascending colon, and, instead of going directly to the lungs, they accumulate in the liver for 48 hours [93]. They then migrate to the lungs, via the blood. Here, they moult to L4 and then return to the digestive tube to develop to adulthood. A possible explanation for this is that the ascarids may originally have had a heteroxenous life cycle based on the predator-prey relationships of their hosts thereby having at least two hosts, one intermediate and one definitive. It is thought that the migration undertaken by monoxenous ascarids through the host tissues is a simulation of the migration they used to undertake through their ancestral intermediate host, resulting from “memories” of the behavior of their ancestors in order to remain in the tissues of their intermediate host (the prey) [95]. Many parasitic nematodes produce an infective L3 to infect the host, comparable to the resting-stage dauer larva found in free-living nematodes. This is known as developmental arrest or hypobiosis and serves as a resting stage that allows many species of

nematode to withstand unfavourable conditions until a suitable host can be found [76]. It is clear that the invasion of host tissues has an important role in the development of parasitism, since the success of the parasite depends on it. Some species have attained a very wide distribution, such as the ascaridoid *H. aduncum*, which is regarded as one of the most abundant marine parasites [96-97]. This is also case of the insect parasite the *Steinernema carpocapsae*, which can parasitize a large number of different species of insects and has great potential for use in pest control. It has been demonstrated that the most virulent strains have more peptidases in their E/S products. But it seems that most of them come from *Xenorhabdus nematophila*, a bacterial species which lives in symbiosis with this insect parasitic nematode [98]. As a result, the peptidases have to evolve in this environment, as in the case of a metallopeptidase involved in invasion through the skin by the nematode *S. stercoralis*, which maintains its proteolytic activity after immunoprecipitation with IgG [99].

Table 1. Relationship between some hyaluronidase enzymes of nematodes and the tissues they invade

Nematode	Optimum pH for hyaluronidase	Place of invasion (pH)
Anisakis simplex	pH 4.0	Stomach (≈pH 2.0)
Ancylostoma caninum	pH 6.0	Skin (≈pH 5.5)
Ascaris suum	pH 5.0-6.0	Colon (≈pH 7.0)

In the plant-parasitic nematode, *Meloidogyne incognita*, the genome has recently been sequenced and the constitution of the degradome analyzed and compared with the peptidase genes of other nematodes, both free-living and parasites of animals [100]. The *M. incognita* degradome consists of at least 334 peptidases distributed in 43 families of the five known catalytic classes (i.e., 26 aspartic, 106 cysteine, 136 metallo, 52 serine and 14 threonine peptidases). A differential transcript level has been observed between eggs and infective juveniles and differences have also been found in the distribution of some peptidase families compared to those of five other nematodes (the plant-parasitic species *Meloidogyne hapla*, the model free-living species *C. elegans* and *C. briggsae,* the necromenic species *Pristionchus pacificus*, and the animal-parasitic species *B. malayi*). This could reflect specific aspects of the parasitic lifestyle of this organism; peptidases known to be used by other phytopathogenic microorganisms (bacteria), such as cysteine peptidases of the C48 sub-family, predicted to encode SUMO (small ubiquitin-like modifier) deconjugating enzymes and serine peptidases of the S16 family (Lon peptidases), were more abundant in *M. incognita* than *C. elegans*, which may reflect specific aspects of its interaction with host plant tissues. Together, these data reinforce the hypothesis that members of the nematode degradome may play a direct role in the host–parasite interaction. The comparative analysis of the *M. incognita* degradome with those from other nematodes has provided evidence that the global distribution of *M. incognita* peptidase classes closely resembles those of the other nematode genomes, which supports the current hypothesis that a core peptidase system is conserved throughout evolution [101-102]. The number of independent peptidase genes in parasitic species is very low and it is possible that genome compaction is an attribute of the parasitic lifestyle, as proposed for the root-knot nematode species *M. hapla*, considered the smallest metazoan genome yet completed [103],

and the human parasite *B. malayi* [104]. These studies are beginning to determine whether nematode genes have similar roles in pathogenicity.

Peptidase Classification and Nomenclature

The peptidases are hydrolases specifically acting on peptide bonds, with a single molecule able to break up to 1,000,000 bonds per second when a peptide bond would take centuries to break spontaneously [15]. The enzymes able to hydrolyze peptide bonds are termed peptidases. The classification of peptidases presents certain problems due to their being enzymes that generally exhibit degenerate substrate specificity. That is, the same enzyme is often able to hydrolyze peptide bonds in different amino acid residues (although generally belonging to the same group, defined by their chemical properties).

Table 2. Classification of peptidases by EC system

Sub-subclass	Peptidase type	Nº of entries
EC 3.4.11	Aminopeptidases	26
EC 3.4.13	Dipeptidases	12
EC 3.4.14	Dipeptidyl-peptidases and tripeptidyl-peptidases	9
EC 3.4.15	Peptidyl-dipeptidases	4
EC 3.4.16	Serine-type carboxypeptidases	4
EC 3.4.17	Metallocarboxypeptidases	20
EC 3.4.18	Cysteine-type carboxypeptidases	1
EC 3.4.19	Omega peptidases	9
EC 3.4.21	Serine endopeptidases	97
EC 3.4.22	Cysteine endopeptidases	58
EC 3.4.23	Aspartic endopeptidases	40
EC 3.4.24	Metalloendopeptidases	81
EC 3.4.25	Threonine endopeptidases	2
EC 3.4.99	Endopeptidases of unknown catalytic mechanism	0
	Total	363[a]

Notes: [a]Data from the section Enzyme Nomenclature (EC 3.4 (peptidases)) of the IUBMB website (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)) in 2011[107].

A widely used classification system is that proposed by the EC (Enzyme Commission), an internationally recognized organization whose current successor is the IUBMB (International Union of Biochemistry and Molecular Biology) (Table 2). This is a mechanistic system which considers the type of catalysis employed by the peptidase during the breaking of the peptide bond. It depends on the main functional group responsible for the catalysis, which, in turn, depends on the amino acid residues that form the active centre of the enzyme. For example, in the cysteine peptidases the characteristic triad of catalytic residues in the active centre is distinguished by having a cysteine residue in the catalytic site, giving the family its name. This has implications in the conditions required for this family to be active, the cysteine peptidases mainly being active between pH 4.0 and 7.0.

On the other hand, the peptidases are also classified according to their site of activity. Thus, according to the IUBMB, the peptidases are placed in group EC 3.4, which is divided into two groups, exopeptidases (EC 3.4.11-19) and endopeptidases (EC 3.4.21-25). The former hydrolyze the peptide bonds of those amino acid residues situated at one of the two extremes of the polypeptide chain, the amino or carboxyl terminal, with the aminopeptidases starting hydrolysis at the amino terminal and the carboxypeptidases at the carboxyl terminal. The endopeptidases attack the internal bonds of the chain, that is, they perform well towards the middle of long chains, far from the ends, and badly on the resulting oligopeptides. They have traditionally been referred to as proteases but this is often a simplification and the IUBMB recommends they be called peptidases. The term oligopeptidase is used for the endopeptidases which perform well on the oligopeptides, the smallest substrates proceeding from the action of proteases on a proteine [105] (Table 3). In the classification of a peptidase by catalytic type, a simple initial approximation for the classification of a new peptidase in terms of its mechanistic class is usually based on its response to class-specific inhibitors. Further data will still be necessary since definitive classification requires knowledge of the complete sequence of amino acids [106]. Serine endopeptidases, cysteine endopeptidases, aspartic endopeptidases and metalloendopeptidases are the main peptidases.

Two main types of catalysis have been identified in the peptidases (both with an acid-base mechanism): a covalent type, with a covalent enzyme-substrate complex due to a nucleophilic attack carried out by a group present in the enzyme (cysteine sulfhydryl in the cysteine peptidases and serine hydroxyl in the serine peptidases); and another without the covalent complex, where the nucleophile is water, through a mechanism involving at least one aspartyl residue (aspartic peptidases) or a metal ion (metallopeptidases) [109] (Table 4). Peptidases from all catalysis types have been described in parasitic organisms [8]. Hydrolysis of the peptide bond takes place through the collaboration of an unstable tetrahedral intermediate, produced by the active centre of the proteolytic enzyme, and the contiguous residues at either side of the catalytic site help to bind the substrate. The properties of these contiguous residues confer specificity for one sequence or another. Each position of the residues is given a name, following the widely used nomenclature system introduced in 1967 by Schechter and Berger [110] to describe the interaction of proteolytic enzymes with their substrates. This names different binding subsites (points of the enzyme which interact with the polypeptide substrate residues) of the peptidase. The amino acid residues of the substrate which are going to bind with the enzyme and are on the aminoterminal side of the peptide bond to be hydrolysed are numbered P1, P2, P3, successively, starting from the bond to be hydrolysed. The residues on the carboxyterminal side of the peptide bond to be hydrolysed in the substrate are numbered P'1, P'2, P'3, successively, in the same manner. Up to 6 residues can be numbered on each side of the bond. The subsites are residues found at the active centre of the peptidase and are numbered S1, S2, S3, S'1, S'2, S'3. Each subsite interacts with its corresponding residue in the substrate. These interactions may be electrical, hydrophobic, steric, etc. These are the peptidase residues which will define their specificity for a particular sequence in the substrate and for the bond to be hydrolysed:

In the Substrate: N -...- P3 – P2 – P1 $_{X}$ P'1 – P'2 – P'3 -...- C
In the Enzyme: N -...- S3 – S2 – S1 ¤ S'1 – S'2 – S'3 -...- C

Where "¤" represents the catalytic site residue and "X" represents the peptide bond to be hydrolysed. Both class-specific inhibitors and peptidase specific substrates are designed to interact specifically with the binding subsites of the active centre of the enzyme, emulating the natural polypeptide substrate residues (Figure 3).

Table 3. Basic classification of peptidases by the 'place of action' [108]

Endopeptidases	Cleave internal peptide alpha-bonds of polypeptide chain away from N-terminus or C-terminus.	
	Oligo-peptidases	Cleave shorter peptides and no proteins.[a]
Exopeptidases	Cleave peptide α-bonds adjacent to N-terminus or C-terminus of polypeptide chain.	
	Aminopeptidases	Cleave a single amino acid residue from N-terminus.
	Carboxypeptidases	Cleave a single amino acid residue from C-terminus.
	Dipeptidylpeptidases	Cleave a dipeptide from N-terminus.
	Tripeptidylpeptidases	Cleave a tripeptide from N-terminus.
	Peptidyldipeptidases	Cleave a dipeptide from C-terminus.
	Dipeptidases	Cleave dipeptides. Typically require both termini to be free.
Omegapeptidases	Cleave peptide α-bonds with no preference for N-terminus or C-terminus. They can also cleave isopeptide bonds.[b]	

Notes: Reprinted with permission from Elsevier, owner of the copyright [108]. [a] Peptide α-bonds are bonds where NH_2- or -COOH are directly attached to the α-carbon of the amino acid. [b] Isopeptide bonds are bonds where one or both of the NH_2- and -COOH groups are not directly attached to the α-carbon of the amino acid.

The problem with the EC classification system is that it groups together many enzymes which are very different from each other in terms of where and when they act, and, especially, in their evolutionary relationships. For this reason, Rawlings and Barrett [111], developed another classification system, MEROPS, in which taxonomic classification is performed (clan, family and unique peptidase) and the peptidases are grouped according to molecular structure, homology and function. It is based mainly on primary and tertiary structures of peptidases, and, according to some authors [108], is currently considered the most relevant approach to distinguish and group peptidases. "Merops" is the scientific name of a bird, the bee-eater (*Merops apiaster*), whose social behaviour inspired the authors to adopt the term "clan", as bee-eaters group their nests into families and clans. The inhabitants of each nest occupy a different part of the colony and have their own discrete area where the members hunt flying insects [112]. It has become clear that some proteolytic enzymes that depend upon the same residue for their catalytic activity belong to different families of proteins, sharing only a few general characteristics. Consequently, these families have been grouped into several "clans". A clan is defined as a group of families whose members have a common ancestor and that commonly have marked similarities, despite a lack of statistically significant correlation between their sequences (it mainly considers the catalytic centre and tertiary structure). Families of the same clan can be recognised because their peptidases have a

similar protein folding pattern; a family is a group of enzymes with significantly high homology in the sequence, more than half of its residues, and which has evolutionary relationships. A unique peptidase is generally a single peptidase or a set of proteins, all of which display a particular type of peptidase activity, and are closely related by sequence. Clans normally include a single catalytic type although some include two or more [113-116] (Table 5).

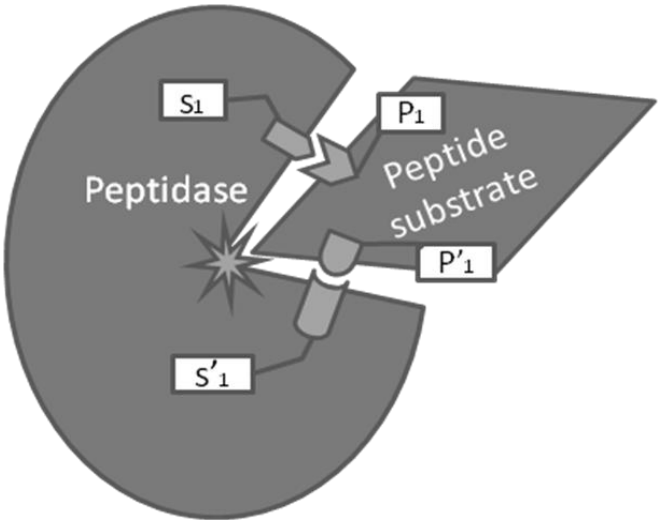

Figure 3. Diagram showing interaction between peptide substrate and the active site pocket of a peptidase.

1. Aspartic Peptidases

The aspartic peptidases are thought to mediate the peptide hydrolysis via general acid-base catalysis (without covalent complex formation). This often takes place through two aspartyl residues present in its active centre (Table 4). They are considered a highly conserved group compared to other peptidase classes, especially the cysteine and serine peptidases [54].

In *D. immitis* an aspartic peptidase is found mainly in the intestine, reproductive tissue and the developing eggs. It may thus be involved not only in nutrition but in gamete production and the development of the microfilarian [119].

In *A. cantonensis* a single aspartic peptidase is expressed after the stimuli of the digestion from the tissues of the intermediate host. It has been suggested that it may have a role in nutrition. This aspartic peptidase shows changes in the expression level of a putative aspartic peptidase in its development. The level increases from the infective larvae to the young adult, significantly decreases in male adults but increases in female adults. These findings could indicate differences in nutritional requirements at different developmental stages [75,120].

In extracts of hookworm *N. americanus* the digestion of haemoglobin and fibrinogen is inhibited by the aspartic peptidase inhibitor pepstatin A [83]. Two types of intestinal aspartic peptidases have been found in haematophage nematodes: those of cathepsin D type, which are lysosomal [83,121-123], and others more similar to mammalian pepsins [123]. This cathepsin

D has since been studied by Yang et al. [124] in *A. caninum* during embryonic development in the egg and the first stages of larval development, being found well distributed throughout all these stages, even in the infective larva and the E/S products. It has been suggested that it is involved in embryonic development, the same as other homologues in *C. elegans* [125], and that it may also play a part in host invasion and nutrient digestion, since, unlike the homologues of *C. elegans,* it is also found in L4 and the adult [124]. In *C. elegans* a cathepsin D (cathepsins are generally peptidases found in lysosomes and synthesized as inactive proenzymes) has been described which is thought to be involved in nutrient digestion and whose levels are modulated by the nutritional state of the nematode, decreasing during fasting [63].

Table 4. Basic classification of peptidases according to catalytic type[a] [108]

Serine Cysteine Threonine	S C T	The nucleophile attack during catalysis is facilitated by reactive group at amino acid side chain, a hydroxyl group (OH^-) of serine and TPs or a sulphydryl group (SH^-) of CPs.
		Catalytic triad of serine peptidases: Ser, Asp, His.
		Catalytic triad of CPs: Cys, His, Asn.
		Catalytic triad of TPs: Thr is conserved in active sites of all proteasomes [218]. All known threonine-type peptidases are N-terminal nucleophile peptidases [112].
Aspartic Metallo	A M	The nucleophile attack during catalysis is usually facilitated by an activated water molecule and followed by formation of tetrahedral intermediate.[b] The water molecule is bound by the side chains of aspartic residues of APs or by metal ions (e.g. one or two zinc ions, Zn^{2+}) of MPs.
		Catalytic dyad of APs: Asp and Asp.
		Catalytic zinc site of MPs is usually formed by His, Glu, Asp or Cys, which supply ligands for zinc.
Glutamic		The mechanisms of catalysis are similar to Aps, including an activated water molecule and tetrahedral intermediate. The water molecule is bound by the side chains of glutamic acid and glutamine residues.
		Catalytic dyad of APs: Gln and Glu.
Unknown		The peptidases of unknown catalytic type, that is, proteins where the sequence is known to belong to peptidases, but the mechanisms of catalysis are not determined [112].

Notes: Reprinted with permission from Elsevier, owner of the copyright [108]. [a] Catalytic type of peptidase is determined according to chemical mechanisms of catalysis related to the reactive group in the active site of peptidase. [b] Tetrahedral intermediate of AP catalysis is a formation that provides proton transfer from a water molecule to an aspartic acid dyad and another proton transfer from a dyad to a carbonyl oxygen of cleaved peptide bond [129]. A tetrahedral intermediate of MP is formed after attack of a zinc-bound water molecule on the carbonyl group of the cleaved peptide bond. This intermediate is further decomposed by transfer of glutamic acid proton to leaving the group [111]. AP, aspartic peptidase; CP, cysteine peptidase; MP, metallopeptidase; TP, threonine peptidase.

In *O. volvulus*, *A. caninum* and *C. elegans* a new family of aspartic peptidases was identified. This is closely related to the cathepsin D and E family, being more similar to cathepsins E. The latter are generally secreted and the adult female of *O. volvulus* is known to secrete specific aspartic peptidase inhibitors. It has thus been suggested that these peptidases are secreted by forming a complex with the inhibitor [126]. In *C. elegans* homogenate most activity is at an acidic pH and 90% is inhibited by the aspartic peptidase inhibitor pepstatin A, with evidence of up to 12 different aspartic peptidases being found [127].

In L4 and adults of *O. ostertagi* the presence of two aspartic peptidases which degrade mucins, haemoglobin and albumin has been reported, with one at pH 5.0 and the other at pH 3.0. The latter also degrades immunoglobulin G and would consequently be very useful for protecting the digestive tract of this nematode from attack by the immune system while it feeds on blood from the blood vessels irrigating the intestinal mucosa of the host [128].

In the insect parasitic nematode *S. carpocapsae* a secreted aspartic peptidase has been found which is a time dependent inducible gene, up-regulated in the gut of L3 and also expressed in the oesophagus. The expression of this peptidase can be induced in infective larvae with insect homogenate. Despite the high number of nematodes that are known to secrete aspartic peptidase, no clear function has been attributed to all of them, and the function of aspartic peptidase during the development stages remains unclear [98].

Table 5. Basic classification of peptidases according to molecular structure, homology and functions [108]

Clan[a]	Clan comprises a group of families probably sharing evolutionary ancestry, despite lack of statistically significant similarities in amino acid sequence. Distant relationship comes primarily from linear order of catalytic-site residues in polypeptide chains, and tertiary structure.[b] The name of each clan is formed from the letter for the catalytic type of peptidases (S, C, T, A, G, M or U, as for families) followed by second capital letter (e.g. CA).
Family	The family comprises peptidase members with evolutionary relationships based on primary structure similar to at least one another member of the family. Each family is named by a letter denoting the catalytic type (S, C, T, A, G, M or U) followed by number (e.g. C1).[c]
Unique peptidase	Unique peptidase is, in general, a single peptidase or a set of proteins all of which display a particular type of peptidase activity, and are closely related by sequence (e.g. C01.062).[d]

Notes: Reprinted with permission from Elsevier, owner of the copyright [108]. [a] The idea of using the terms 'family' and 'clan' for the groups of peptidases came from the ecological strategy of bee-eaters (*Merops apiaster*), which group their nests into families and clans. The inhabitants of each nest occupy a different part of the colony and have their own discrete area where the members hunt flying insects [112]. [b] Tertiary structure recognized by modelling is crucial for activity of many peptidases. For example, the polypeptide chain of papain forms two domains with a large cleft of the active site that blocks the pro-region part [117-118]. [c] Primary structure of peptidase determines statistically significant relationships in amino acid sequence with respect to a representative member, especially to its unit (peptidase unit is a part of the enzyme responsible for peptidase activity, [112]). [d] For example, cathepsin B-like peptidase, clan CA, family C1A, peptidase C01.062 (according to MEROPS classification).

2. Cysteine Peptidases

The cysteine peptidases usually contain a cysteine residue in their active centre, this being the principal residue of the typical catalytic triad (Cys/His/Asn) [114], which participates in covalent type catalysis. The sulfhydryl performs the nucleophilic attack, forming an intermediate covalent enzyme-substrate complex (Table 4).

Much is known about the cysteine peptidases in parasitic nematodes, since, due to their great diversity [16,130-132], they cover virtually all the functions in which peptides are involved in these animals. These enzymes are found in both larvae and adults and are thought to be involved in tissue penetration, nutrition and defense from the immune system of the host [77] as well as in moulting [66].

In *H. contortus* cysteine peptidases have been observed which produce extracorporeal degradation in vitro of tissues of an artificial extracellular matrix, presumably due to the E/S products, and which may help the parasite to reach and rupture the capillaries in the digestive tract mucosa in order to feed on the blood in vivo [3,133]. *Ostertagia ostertagi,* which feeds in the same way, exhibits cystein-like activity against mucins, suggesting that it may have a similar function [128].

In *A. suum* the cysteine peptidases have been related to the moulting process, as the presence of Z-Phe-Ala-FMK (cysteine peptidase inhibitor) prevents 77% of larvae from shedding the L3 cuticle, although their development continues [66]. In *B. pahangi* [4], *D. immitis* [134-135] and *O. volvulus* [136] they have also been related to moulting processes. In *Toxocara canis* a cathepsin Z has been related to moulting, although it expresses constitutively, suggesting that it must have another essential function [137]. It also expresses in *O. volvulus* and is again related to moulting [138]. In *C. elegans* it expresses at the same sites as in *O. volvulus* (in the epidermis of all the larval stages and in the oesophagus and gonads of the adult) and participates in moulting and other functions related to development such as spermatogenesis and embryonic development, suggesting it has a conserved function in nematodes [139]. In *N. americanus* a cysteine peptidase has been detected which is the most abundant in the fluids trapped between the L2 cuticle and that of the infective L3 before shedding of the old cuticle [140], suggesting it may also be involved in moulting.

In extracts of *H. contortus* [141] and in E/S products of *N. americanus* [83,221] cystein peptidases able to degrade haemoglobin, fibrinogen, collagen (only in *H. contortus*) and antibodies have been observed. In *H. contortus*, without considering the E/S products, the intestine has been shown to be the site where the highest concentration of cysteine peptidases is found [3].

Within the cysteine peptidase families there is one group which is widely distributed among the best studied parasites and one of the most important. This is the papain family, this also being the name of the clan to which they belong. The CA (papain type) clan is characterized by having the typical catalytic triad of cysteine peptidases in the active centre, consisting of three residues: cysteine, histidine and asparagine [15]. Analysis of the evolution, location and biological function of the cysteine peptidases belonging to this clan, in parasitic metazoa and protozoa, has increased understanding of the biochemistry and cellular function of this highly diverse family of enzymes, which take part in both intra- and extracellular processes [142]. For this reason, and given their diverse nature, they are regarded as attractive pharmacological targets, together with other cysteine peptidases, for the development of chemical agents to interfere with them. There are several reasons for this [15,143-145]:

- A sufficient number have different structural and biochemical properties from those of the host, including optimum pH and stability, alteration in peptide loops or domain extensions;
- The relative absence of redundancy with regard to the mammalian host. This means that the affinity spectrum for different substrates of the different cysteine peptidases in the hosts overlaps more than in the parasites;
- A higher concentration of cysteine peptidases in the intracellular compartments of the host than in the parasites. Cysteine peptidase activity of the host is generally more robust;
- Differentiated assimilation of inhibitors by parasites with regard to host, due in part to the great diversity in their cell location;
- The studies carried out to elucidate the mechanisms of resistance to cysteine peptidase inhibitors in parasite cultures show that they are independent of those of traditional antiparasitic agents.

Within the papain family there are two cathepsins which have been widely studied due to their presence in almost all organisms, cathepsins B and L. The term "cathepsin" was proposed by Willstätter and Bamann [146] to describe the peptidases which acted preferentially in an acidic medium and were later found in all cells and tissues and subsequently discovered to be of lysosomal origin. Thus, the term has traditionally referred to a lysosomal peptidase which can sometimes be secreted. They are widespread amongst the invertebrates and phyla of primitive protostome metazoa are known to express a wide range of cathepsins [77]. The papain family contains many cathepsins which are obviously cysteinic [147] and some of them are studied in nematodes [15]. They are organized in several multigenic families, some of which have been well characterized, such as those expressing in the intestinal tissues of the adults of *H. contortus* and *C. elegans.* This diversity reflects great functional specialization, the need for rapid protein digestion, or both, since all have a potential role in nutrient digestion [16,131-132,148].

In the cathepsins B of nematodes there is great variability in optimum pH and temperature, substrate affinity, etc. It is thought that their major role is nutrient digestion and that the high interspecific variability observed is due to the adaptation to its ecological niche undergone by each nematode [16]. Comparative phylogenetic analysis of some cathepsins B of both haematophagous and no-haematophagous helminths has been carried out. It has been shown that haematophagous worms' cathepsins B share some common motifs, known as hemoglobinase motifs, despite the fact that, phylogenetically, some of them may be more distant from each other, than from other cathepsins B belonging to the same group. For example cathepsins B of hookworms share this motif with those of schistosomes, whereas in nematodes such as *C. elegans* this does not occur. Interestingly, it appears in the intestinal nematode *A. suum*, but is not clearly understood since it is not known whether the cathepsin in question is expressed in all larval stages or during their migration in host tissues [149]. A cathepsin B with haemoglobinolytic activity from the intestine of *A. caninum* has been expressed in vitro, using a heterologous system. Optimal activity is achieved when it acts after a cathepsin D (aspartic peptidase) from the same parasite [56], and it is for this reason that both are placed in the enzymatic cascade that digests haemoglobin in this parasite. In *C. elegans* cathepsin B genes are expressed in the larval and adult digestive tube and also in the

intestine of *H. contortus* [148,150-151], being related to nutrient digestion [150]. Furthermore, the gene promoter of this cathepsin B of *H. contortus* has been inserted into *C. elegans* and it has been shown that the mechanisms controlling spatial distribution of this peptidase are conserved in parasitic and free-living nematodes [152]. It had previously been suggested that, in addition to the structural similarity, there was a functional homology between the cathepsins B of the nematodes, which could play an important part in their nutrition [148]. They are also thought to have a role in embryonic and larval development as it has been shown that a cathepsin B is expressed in the egg and the infective larva of *A. caninum* [153]. In *A. cantonensis* a cathepsin B has been characterized which shows high specific expression in the worm stages which cause dysfunction of the blood–brain barrier of hosts. This cathepsin *Ac*CB displayed different expression profiles in non-permissive host (mice) and permissive host (rats) derived larval stages and was involved in the maturation of dendritic cells, suggesting a potential role in the central nervous system invasion and immunoregulation during parasite–host interactions [154].

Cathepsins L are less abundant in terms of their level of expression [77,155]. They also seem to be mainly involved in nutrient digestion, embryogenesis and moulting [2,7,156], although they have also been implicated in tissue invasion and immune response evasion in parasitic nematodes [77]. Homologues of many of these cathepsins L have been found in *C. elegans*, suggesting that they may be involved in conserved functions in different species of nematodes, although little is known of their precise functions [7]. In *B. pahangi* and *B. malayi* [45] a series of cathepsins L were studied. When compared with their corresponding cathepsins in *O. volvulus* and *C. elegans* [7,136,157] it was concluded that they are also involved in remodelling of the cuticle and the egg capsule in filarians. In *O. volvulus* cathepsins L and Z (closely related) have been described. These participate directly in moulting of L3 [136], later confirmed by RNAi experiments [138]. In *C. elegans* a cathepsin L (*Ce*CL_1), essential for development and embryogenesis, has been studied in depth. On inhibiting its expression, development of up to 98% of the embryos was detained [2]. It has also been found in the egg capsule and the cuticle of *O. volvulus* (*Ov*CL_1), suggesting that it is conserved in nematodes. From this it can be deduced that this peptidase is of great importance and probably has an identical function in many nematodes. It is expressed during embryonic and postembryonic development in the intestine, epidermis, oesophagus, gonads and the outer part of the egg, near the capsule. It's produced during the intermoult period, approximately 4 hours before the moult, and then, after moulting, production decreases to basal levels. Consequently, it is thought to be involved in processing products used in the synthesis of the egg capsule or in its degradation, the same in the cuticle, and also for other functions. It has been suggested that *Ce*CL_1 degrades the deutoplasmic proteins to leave the necessary amino acids available for embryonic development and for the synthesis or maturation of the egg capsule, and, later, for remodelling of the larval cuticle during moulting. It may also act in the adult, during growth after the last moult, by degrading the old cuticle and processing the new or digesting the proteins of the materials used to anchor the cuticle. Finally, it may also act indirectly, processing and activating other enzymes and hormones involved in moulting [7]. In *H. aduncum* the evolution of activity of both cathepsin L and B has been measured during its life cycle in vitro. The former showed higher activity and an acidic optimum pH and the latter, lower activity and a basic-neutral optimum pH. Having considered these activity variations and the optimum pH values, it has been suggested that cathepsin L has a role in digestive processes while cathepsin B could be involved in cuticle renewal, among other

possible functions [158]. However, these data have been called into question since Rehman and Jasmer [16] reported that in *T. canis* and other nematodes such as hookworms there seemed to be modifications of the cathepsins L which caused them to show cathepsin B behaviour. This would suggest that both conventional substrates and inhibitors for this subclass of peptidases would offer only provisional information, at least in nematodes.

3. Metallopeptidases

Within the metallopeptidases the endopeptidases and exopeptidases are equally usual. The name is due to the presence of a coordinated metal ion in their active centre. This is frequently a divalent cation of zinc but can also be cobalt, manganese, nickel or copper, and is able to polarize the peptide bond before it breaks, through the nucleophilic attack of hydroxyl from the water molecule activated by the ion. A common feature is the presence of catalytic residues of glutamate, histidine, aspartate and lysine, which maintain the metal cations in their positions and are thus essential for catalysis (Table 4). They act as exopeptidases when they possess only one metal cation and can act as exo- or endopeptidases when two of them are involved. Metallopeptidases exhibit a relatively broad range of specificity to peptide substrates, usually defined by the substrate residues of positions P1 and P'1 [159-160].

The metallopeptidases intervene in the invasion of host tissues by the parasite as they are able to degrade the extracellular matrix [46]. In *S. stercoralis* it has been shown that a zinc-endometallopeptidase secreted by the infective larva is involved in penetrating the skin of the mammalian host. This metallopeptidase is extremely potent and attacks the principal components of the dermal extracellular matrix (elastine, collagens and glycoproteins). Penetration of the skin is inhibited by specific inhibitors for metallopeptidases [9]. Furthermore, this peptidase conserves its proteolytic activity after the action of the antibodies, indicating that immunogenicity is absent from its active centre. This would represent a great evolutionary advantage for infection of the host [99]. In *Strongyloides venezuelensis*, the infective larvae have a zinc-metallopeptidase activity, which has been assumed to play a major role in skin penetration [161]. This metallopeptidase activity seems to be a homologue of Ss40 of the previous activity of *S. stercoralis*. More recently, an astacin-like metallopeptidase transcript in infective larvae of *S. stercoralis*, referred to as "strongylastacin" [162], has been identified and which shows high homology with another transcript only expressed in the infective larva of *S. venezuelensis* [163]. For this reason it has been suggested that if the expression of this transcript is specific for the infective larva stage it perfectly matches the metalloprotease activity previously reported [161]. In *H. aduncum* the presence of a metallopeptidase has been reported, with a similar optimum pH to those found in the host stomach where the worm presumably initiates invasion of the stomach tissues. It is inhibited by 1,10-phenanthroline and dithiothreitol, and, during its development in vitro it only appears in the infective L3 stage, suggesting that it may be employed by the parasite to undertake penetration of the host tissues [52]. In *A. cantonensis* some findings suggest a crucial role of metallopeptidases in the penetration of the intestinal wall of the host. Several metallopeptidase transcripts have been identified, induced after a pepsin-HCl treatment, among the most abundantly expressed transcripts in the infective larvae only. It has been related with the previously reported secreted peptidases with collagenolytic and elastinolytic activities [75], that are inhibited by serine peptidase or metallopeptidase inhibitors [164]. In

the insect parasite *S. carpocapsae* an up-regulated astacin metallopeptidase has been reported during the parasitic stage, and strongly induced in vitro by insect tissues. This suggests that it plays a role in the parasitic process [165].

In *Trichuris suis* the presence of a secretory organ of a zinc-metallopeptidase has been reported and which has also been implicated in the degradation of host tissues during invasion [166]. In *H. contortus* a complex of several metallopeptidases associated with its intestinal microvilli, which, through immunization, offers moderate protection to sheep, has been described [227]. In addition to showing haemoglobinolytic activity, this complex may also be involved in the evasion of the immune system by degrading eotaxins, thus preventing the eosinophils from reaching the anchorage site of the parasite [167].

The first metalloendopeptidase activity described in nematodes was in the E/S products of *A. caninum* in 1983 [168]. Since it was able to degrade fibrinogen and plasminogen it was thought to prevent the host blood from coagulating. More recently, the cDNA of a metallopeptidase expressed in the intestinal lumen, *Ac*MEP$_1$, has been described and this is probably responsable for the activity mentioned previously [169]. In the same parasite there is another metallopeptidase, present both in the E/S products and extracts of larvae and adults, which also prevents coagulation by inhibiting fibrin precipitation and is also thought to be involved in tissue penetration due to its elastinolytic activity [168,170-171].

In *A. suum* an aminometallopeptidase released into the culture medium has been related to moulting as it reaches maximum concentration at the moment of passing from L3 to L4 [220]. The presence of amastatin (aminopeptidase inhibitor) prevents 34% of individuals from shedding the L3 cuticle. However, this does not halt development; the same aminopeptidase has later been found in the adult intestine, where it exhibits activity related with digestion [44]. In *B. pahangi* [4], *D. immitis* [134-135] and *O. volvulus* [136] aminopeptidases have also been detected in the moulting process. In the infective L3 of *H. contortus* there is a metalloendopeptidase which degrades an annular part of the L2 cuticle, so that it becomes detached, making way for the L3 cuticle [6].

In *H. aduncum* the expression of collagenase activity was studied during in vitro development and was observed to be of metallopeptidase type, principally inhibited by 1,10-phenanthroline. As L3 showed a different optimum pH to that of L4 and adult it was suggested that it has different functions in each developmental stage, with that of L3 possibly involved in migration of the parasite through the host tissues. Most activity was observed in the immature adult, after the final moult, suggesting that the collagenolytic activity may be involved in remodelling of the cuticle and in sexual maturity and digestion, as collagenolytic activity was shown to be significantly greater when no pepsin was added to the culture medium [172].

4. Serine Peptidases

This important group accounts for almost one third of all peptidases [108]. They are synthesized as inactive precursors (zymogens) and always consist of three domains: catalytic, substrate binding and zymogen activation. In order to be totally active they must be processed through the elimination of the zymogen domain (N-terminal extension) [173-174]. In their active centre the serine peptidases have an active serine residue, which, together with others of aspartate and histidine, form the catalytic triad His/Asp/Ser, which participates in covalent

type catalysis. The hydroxyl group of the serine carries out the nucleophilic attack, giving rise to the intermediate covalent enzyme-substrate complex (Table 4).

The serine peptidases are also present in nematodes, and, together with other enzymes such as metallopeptidases, have an important role in invasion of host tissues by the parasite [9-10,175-176]. They have been identified in different parasitic nematodes such as *N. americanus* [140], filarians *D. immitis* [80] and *O. volvulus* [84], ascarids *A. suum* [177] and *T. canis* [178], anisakids *A. simplex* and *P. decipiens* [10,34], and *T. spiralis* [20]. An example of how serine peptidases are likely candidates for the degradation of host tissues was reported by Morris and Sakanari [17], who related an extracellular serine peptidase, present in the pathogenic bacteria *Dichelobacter nodosus*, which destroys host tissues (by degrading elastin, keratin and collagen), with a serine peptidase from the E/S products of the *A. simplex* larva in in vitro culture with similar activity. In the E/S products of the infective L3 of *N. americanus* serine peptidase activity able to degrade immunoglobulins G, A and M has been detected [140] which may help the parasite during entry by offering protection from the antibodies generated by the immune response of the host. When the hosts are insects, they can defend themselves from pathogens by melanotic encapsulation, a humoral immune response related to the production of reactive oxygen species, which can kill microorganisms or parasites. It has been shown that melanization in insects can be inhibited by a secreted serine peptidase of the insect parasitic nematode *S. carpocapsae*, presumably by disrupting the enzyme cascade of melanisation, inhibiting the phenoloxidase activity (a key enzyme for this process) [179]. In addition, there is experimental evidence that they can alter host haemocytes and their actin filaments [180]. Finally, a very clear and interesting example of how the serine peptidases participate in the invasion of host tissues by a parasite has been shown in this insect parasitic nematode. A secreted chymotrypsin-like serine peptidase is expressed only during the parasitic stages in the insect haemocoel, especially in the infective larvae (pre-invasive nematodes inside the mid-gut). This peptidase caused histolysis in the insect mid-gut, and in vitro assays demonstrated that it can digest extracellular proteins and induce apoptosis in cultured insect cells, thus suggesting that this peptidase is involved in the pathogenesis [181]. Another secreted serine peptidase of this nematode is involved in the parasitic process since is not expressed in the arrested stage. However, this gene was overexpressed in recovered nematodes, particularly those in the gut lumen preparing to invade the insect hemocoelium (infective larvae). In vitro assays showed that the expression started after stimulation with insect tissues, especially peritrophic membrane and hemolymph (peritrophic membrane lines the host gut lumen and the hemolymph represents the first lines of defense) [182].

In *H. aduncum* the expression of proteolytic activity during in vitro development has been studied. The serine peptidases did not appear until after moulting to the adult stage. This may well be related to the attainment of sexual maturity, appearing during the sexual maturation of the parasite [52,172]. Serine peptidases in the filarian *O. volvulus* have been related to moulting, embryogenesis and spermatogenesis [183]. *D. immitis* has an alternative splicing of furin (a subtilisin-like peptidase, which are serine peptidases), generating sex-specific isoforms. These sex-specific isoforms suggest that this peptidase processes targets involved in sex-determination and/or reproduction. The patterns of alternative splicing found in *D. immitis* are not conserved within the furin genes of *O. volvulus* and *B. malayi*. The alternative splicing observed within nematode furin genes probably represents a method for increasing diversity without increasing gene number. The subtilisin-like peptidase (subtilases)

family is a group of serine peptidases involved in multiple processes such as the construction and maintenance of the cuticle, neural signalling and nematode development. At least two of these subfamilies of subtilases, kexin and pyrolysin, are present in nematodes. By cleaving or activating other proteins they play an essential part in the nematode biology. This makes these peptidases an attractive target for drug development. For example, cuticular collagen genes from *H. cortortus, A. suum* and *B. pahangi* have the conserved domain structure and kexin cleavage motifs found in *C. elegans* cuticular collagen genes, suggesting that this mechanism of collagen maturation is conserved among nematode species [184].

5. Peptidase Inhibitors in Parasitic Nematodes

Peptidase inhibitors have been studied in many groups of organisms including parasitic nematodes. For example, in *S. carpocapsae* it has been shown that the gene of a secreted cystatin is upregulated after induction by insect hemolymph and is thought to have a role among the parasitism genes expressed in *S. carpocapsae* throughout the parasitic cycle ([185]. In nematodes they would be expected to have a vital role in modulating proteolytic enzyme activity in, amongst others, development and parasite-host relationships. However, although not as widely studied as peptidases, their role is only clear in two cases:

- Protection of the digestive tract: in hookworms one of their functions seems to be to act as an anticoagulant, inhibiting several serine peptidases involved in host blood coagulation [186-188]. Consequently, some are promising pharmacological anticoagulants [189]. Aspartic peptidase inhibitors have been found in other groups of gastrointestinal parasites such as ascarids [190-191], and, more recently, trypsin-, chymotrypsin- and elastase-type serine peptidases have been found in *Ascaris* spp. [192-196] and *A. simplex* [17,225], where they are believed to be involved in protecting the parasite against host digestive enzymes and any other enzymes encountered while migrating through host tissues. In *Ancylostoma duodenale* an inhibitor acting on these enzymes has been characterized and shown to express itself in the oesophagus, intestine, and cuticle surface of the adult worms, suggesting that its role is to protect the exposed parts of the parasite from the host digestive environment [197]. Carboxypeptidase inhibitors which may have the same role have also been purified [198-199]. Some of these inhibitors are important allergens. Specifically, the aspartyl peptidase inhibitors from some parasitic nematodes (*A. caninum, Trichostrongylus colubriformis* and *O. volvulus*) are highly immunogenic, and have been proposed as potential vaccine antigens [200-202]. It has been reported that a single dose of the crude latex of *Carica papaya* provided comparable anthelmintic efficacy to the currently available synthetic anthelmintics, with respect to reductions in the egg output and worm burden, in pigs infected with the roundworm *A. suum* [203] and mice infected with *Heligmosomoides polygyrus*, one of the most common laboratory models for routine screening of potential drug candidates [204]. The mechanism of action of this phenomenon is related to the action of cysteine peptidases (such as papain) found in papaya latex [205], as shown by inhibiting the effect through addition of the cysteine peptidase inhibitor l-*trans*-epoxysuccinyl-leucylamido-4-guanidino-butane (E-64) [206]. In another ascaridoid,

A. simplex, as above, porcine pepsin has been shown to favor its development in in vitro culture [49] and resists it well, like *H. aduncum* [51], while papain and bovine trypsin had a negative effect on the former worm [67].

- Protection against host immune system: in the intestinal parasite *T. suis*, the inhibitors may be involved in a defense mechanism to escape the peptidase-mediated immune response of the mastocytes associated with the intestinal mucosa of the host [207]. In the filarian *B. malayi* it has been shown that certain inhibitors it produces are able to inhibit T-cell proliferation by preventing the processing of the antigen for its presentation by the antigen-presenting cells of the major class II histo-compatibility complex (MHC II) [208]. *N. brasiliensis* also modulates the immune response at the antigen processing level in the antigen-presenting cell [209]. In *A. cantonensis* certain cystatins are thought to be involved in host immunoregulation. It is surprising that in a non-permissive host, like the mouse, antibodies are generated against the cystatin of this nematode during an infection whereas in a permissive host, like the rat, no antibodies are generated [210]. For a non-permissive accidental host, human, a cystatin described in L3 of *A. simplex* is an important allergen which is expressed in the secretory gland and the basal layer of the cuticle [211]. In general, the cystatins in immune cells interfere with antigen processing and presentation, phagocytosis, expression of cytokines and nitric oxide, thus modifying the immune response. Consequently, it has been suggested that the cystatin-type molecules secreted from parasites down-modulate the host immune response [212]. In fact, it has been noted that in human and animal models helminth infections are concomitant with a reduction in the prevalence of inflammatory diseases and others related to hypersensitivity reactions. As a result, there is currently much interest in finding molecules from these parasites with antiallergic and/or anti-inflammatory properties [213-214].

Of course, peptidase inhibitors also take part in other regulatory processes, such as in the filarian *O. volvulus*, where they may have a regulatory role during moulting, embryogenesis and spermatogenesis [183].

Hosts have also developed peptidase inhibitors as a way of controlling nematode infections. For example, some plants such as the potato react to an infestation of the nematode *Globodera rostochiensis* by producing peptidase inhibitors [215]. Different plants have been created experimentally from transgenic crops (tomato and banana) in which there is heterologous expression of cystatin genes. These plants show, to a greater or lesser extent, resistance phenotypes against infestation by plant-parasitic nematodes (*M. incognita, Radopholus similis*) classified as serious pests [216-217]. The importance of the cysteine peptidases, the target of these cystatins, in nematodal development, is clear.

CONCLUSION

The nematodes are, after insects, the group of organisms with the largest number of species identified, indicating their great potential for adaptation and diversification. It includes members of medical, veterinary and agronomic significance, making this group one

of the most important among animal and plant parasites. Peptidases, as in others organisms, play a major role in the biology of nematodes, but a great amount of the processes in which they are involved are not well known. Most effort is invested in investigation of tissue and cuticle renewal during development, host-tissue invasion, protein digestion, and, interestingly, in the role of parasitic peptidase inhibitors. Fortunately, unlike other groups of parasitic helminths, the nematodes include a species used as a model organism of great importance*, C. elegans,* enabling us to extract much relevant information by means of comparative studies, especially those related to development, processing of peptides and protein renewal by the proteasome, and, to a lesser extent, digestion. Although invertebrates appear to share several general features, digestion obviously depends largely on the ecological niche occupied by the nematode as well as the food source. However, such important processes and characteristics of parasitism as invasion and migration through host tissues or the host immune system's interaction with peptidases and inhibitors from both plants and animal parasites need the development of research adapted to each group of parasites or at least to groups that share a niche. This would serve as a reference for study in other closely related species.

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