



Cathepsin B- and L-like cysteine protease activities during the in vitro development of *Hysterothylacium aduncum* (Nematoda: Anisakidae), a worldwide fish parasite

David Malagón^a, Manuel Díaz-López^b, Rocío Benítez^a, Francisco Javier Adroher^{a,*}

^a Departamento de Parasitología, Facultad de Farmacia, Universidad de Granada, Granada, Spain

^b Departamento de Biología Aplicada, Escuela Politécnica Superior, Universidad de Almería, Almería, Spain

ARTICLE INFO

Article history:

Received 3 July 2009

Received in revised form 9 November 2009

Accepted 13 November 2009

Available online 20 November 2009

Keywords:

Anisakidosis

Cathepsin

Cysteine protease

Fish parasite

Hysterothylacium aduncum

Nematode

Ontogeny

ABSTRACT

Proteinases play an important role as virulence factors both in the life-cycle of parasites and in the pathogen–host relationship. *Hysterothylacium aduncum* is a worldwide fish parasite nematode which has been associated with non-invasive anisakidosis and allergic responses to fish consumption in humans. Cysteine proteinases have been associated with allergy to plant pollens, detergents and dust mites. In this study the presence of two types of cysteine proteinases (cathepsin B and cathepsin L) during in vitro development of *H. aduncum* is investigated. Specific fluorescent substrates were used to determine cathepsin activities. The activity detected with substrate Z-FR-AMC was identified as cathepsin L (optimum pH = 5.5; range 3.5–6.5). Cathepsin B activity was only identified with Z-RR-AMC (optimum pH = 7.0–7.5; range 5.0–8.0). The start of cultivation led to increased activity of both cathepsins (1.8-fold for cathepsin B and 6.3-fold for cathepsin L). These activities varied according to the developmental stage. Cathepsin B activity decreased after M4, returning to its initial level. Cathepsin L activity also decreased after M4, but still maintained a high level (4–6 times the initial level) in adult stages. Having considered these activity variations and the optimum pH values, we suggest that cathepsin L has a role in digestive processes while cathepsin B could be involved in cuticle renewal, among other possible functions.

© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Hysterothylacium aduncum is an anisakid nematode parasite of marine and freshwater fish and crustaceans throughout the world [1,2]. Many of these fish are consumed by man, leading to the possibility of non-invasive anisakidosis [3] or allergic responses [4]. Some fish, such as the anchovy (*Engraulis encrasicolus*) or the sardine (*Sardina pilchardus*) act as intermediate/paratenic hosts [5,6] in which the parasite is found in the visceral cavity and/or in muscle tissue. Other larger teleost fish such as the hake (*Merluccius* spp.) or the cod (*Gadus morhua*) act as definitive hosts, where the worm is found in the intestine [7,8]. The presence of these parasites in fish may lead to the mortality of fish larvae [9]. Furthermore, the stress experienced by parasitised adult fish makes them more susceptible to other infections [10].

One of the conditions necessary for the successful in vitro development of the parasite is the presence of pepsin, which could degrade the proteins in the culture medium to facilitate their assimilation by the parasite and/or help in ecdysis [11,12]. Pathogenicity has been correlated with protease activity in several species of

nematode, such as another anisakid, *Anisakis simplex* [13]. Proteases are considered essential during development and the most critical moments of parasite–host interaction and are thus directly involved in the growth and survival of the parasite [14,15]. Thus, according to these authors, their identification and characterisation are vital to further our knowledge of the biology of these parasites and they may be considered as potential targets for vaccines, drugs and in serodiagnosis. They will also be highly important in forthcoming antiparasitic initiatives.

Cathepsin proteinases have been identified in many parasitic organisms, both in larval and adult stages, and are involved in roles as diverse as tissue and skin penetration, virulence, and immune evasion [16]. The most common and well-known cysteine proteases in nematodes are type B (EC 3.4.22.1) and L (EC 3.4.22.15) cathepsins included in family C1, *clan* CA of the papain superfamily [17,18]. The main role cathepsins B is believed to be the digestion of nutrients while their high interspecific variability observed is thought to be an adaptation by the nematode to the ecological niche it occupies [19]. On the other hand, it has been suggested that the cathepsins L of nematodes are not only involved in nutrient digestion but also in embryogeny, tissue invasion, avoidance of the immune response, and, above all, in moulting [20,21].

Although cysteine protease activity has been detected in an anisakid such as *A. simplex* [22], this has not been characterised. The aim of the present study is to verify cathepsin activity in *H. aduncum*

* Corresponding author. Departamento de Parasitología, Facultad de Farmacia, Campus de Cartuja, Universidad de Granada, 18071-Granada, Spain. Fax: +34 958 243862.

E-mail address: fadroher@ugr.es (F.J. Adroher).

and to observe its variation during the *in vitro* development of this worm from L3 recently collected from the intermediate/paratenic host fish to mature adult.

2. Materials and methods

2.1. Collection of L3 larvae and cultivation

The L3 of *H. aduncum* were collected from horse mackerel (*Trachurus trachurus*) purchased at the fish market in Granada (Southern Spain). The worms over 8 mm in length found free in the host body cavity, were collected and morphologically identified [2,8,23]. A number of larvae were frozen (-80°C) for enzyme determination (L3 0 h). The rest of the larvae were processed to the cultivation in a modified RPMI-1640 medium with pepsin and foetal bovine serum added, at 15°C , as previously indicated [11].

2.2. Preparation of the extract and protein determination

The following samples were taken, washed during 20 min in an 0.9% NaCl solution and frozen (-80°C) for enzyme determination: L3 from fish (L3 0 h, 30 larvae per ml of extract), L3 after 48 h of cultivation (L3 48 h, 25 larvae per ml of extract), L4 after 14 days of cultivation (L4, 12 larvae per ml of extract), immature adult after 21 days of cultivation (L5J, 6 worms per ml of extract), and mature adult 42 days of cultivation (L5, 3 worms per ml of extract). In order to determine whether the pepsin in the culture medium affected proteolytic activity a control was prepared using parasites cultured without pepsin for the last week prior to collection, except L3 48 h, which was collected after 48 h in culture without pepsin. Somatic extract was prepared by immersion of the larvae in de-ionized water, in the proportions mentioned above, followed by 10 bursts/ml of ultrasonic disintegration (on ice), each of 1 s duration at 15 micron amplitude. The extract was then centrifuged at $18000\times g$ for 15 min at 4°C and the supernatant was assayed for protein concentration using the Bradford method [24] and stored at -80°C .

2.3. Enzyme determination

The techniques previously described [25,26] were modified and used to determine enzyme activity. The parasite extracts ($6\ \mu\text{g}$ protein) were diluted to a final volume of 0.2 ml in the following buffers with pH increments of 0.5: 100 mM glycine-HCl (pH 2.0–3.5); 100 mM sodium acetate:100 mM acetic acid (pH 4.0–5.5) and 100 mM phosphate buffer (pH 6.0–8.0). Each buffer contained 1 mM dithiothreitol and one of the following fluorogenic peptide substrates at a final concentration of 0.1 mM: N- α -benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amido-4-methyl-coumarin (Z-FR-AMC) or N- α -benzyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methyl-coumarin (Z-RR-AMC). Before the fluorogenic substrates were used, they were dissolved in dimethylsulfoxide (DMSO) at 10 mM and stored at -80°C until use. The final DMSO concentration was 1% for all assays. Ionic strength was maintained constant at 0.25 M by addition of NaCl as necessary. Reactions were incubated at 37°C for 100 min and measured every 2 min. Release of 7-amino-4-methyl-coumarin (AMC) was detected using a fluorimeter with λ excitation at 355 nm and λ emission at 460 nm. To inhibition assays, 0.01 mM E64 [L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane], an irreversible cysteine protease inhibitor, was added to the reaction mixture. Enzyme activity units (U) were calculated as nanomoles of AMC released $\text{min}^{-1}\ \text{mg}^{-1}$ protein.

2.4. Statistical method

The data were processed using SPSS software (version 14.0 for Windows). Values were tested for significance by 1- or 2-way ANOVA, depending on variable number; when ANOVA was not applicable, the

data were tested for the Kruskal–Wallis (K–W) non-parametric test to “k” independent samples. When the data were statistically significant, *post hoc* multiple comparisons by pairs were performed using the Bonferroni test for the ANOVA; and Mann–Whitney’s U (U M–W) test with Bonferroni’s correction for the Kruskal–Wallis test. The significance level was $p < 0.05$.

3. Results and discussion

Fig. 1A shows the cathepsin B-like activity of the parasite, identified with the substrate Z-RR-AMC, as a function of pH and developmental stage of *H. aduncum*. Activity was detected in all the stages studied with pH range 5.0–8.0. No significant differences were found between the different development stages (K–W, $p = 0.512$), regardless of whether pepsin was used (K–W, $p = 0.465$). The addition of E64 to the reaction inhibited this cathepsin activity. This

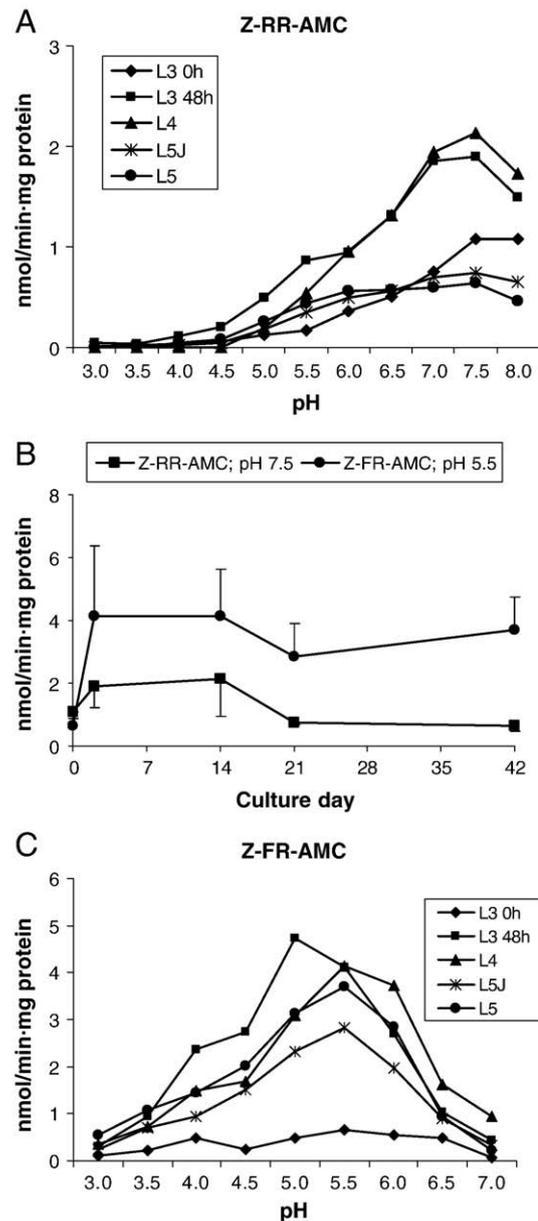


Fig. 1. Cathepsins in *Hysterothylacium aduncum* during its *in vitro* development. Each point is the mean of two to three experiments in triplicate. Panel A: pH profile of Z-RR-AMC cleaving activity. Panel B: Variation in cathepsin activities. Cleaving activity profile of Z-RR-AMC at pH 7.5 (■) and of Z-FR-AMC at pH 5.5 (●). Panel C: pH profile of Z-FR-AMC cleaving activity.

is the first time that this type of activity has been shown in this nematode. Cathepsin activity has generally been associated with lysosomes, which is why optimum activity usually takes place at around pH 5. But, in marked contrast to the vertebrate proteases, the parasite proteins are more active and remain stable at neutral or alkaline pH. This broad pH profile of the parasite cysteine protease is consistent with the numerous extralysosomal functions that have been characterised [18]. Our results show that the cathepsin B-like activity of *H. aduncum* is highest at a neutral pH (7.0–7.5). This leads us to suggest that this cathepsin does not act in the lysosome and is able to function extracellularly within the parasite or is secreted to the exterior. Furthermore, according to Barrett et al. [17], these data show that only the endopeptidase activity of cathepsin B is being measured.

Fig. 1B shows the cathepsin B-like activity at pH 7.5 during the different developmental stages of the nematode. It can be seen that L3, found in the body cavity of the intermediate/paratenic host fish, shows low activity which increases notably after 48 h in culture (L3 48 h and L4), and then decreases to levels similar or even lower than the initial in the adult stages studied. Although this cathepsin has often been associated with the intracellular digestion of nutrients due to its location in the lysosome, it may possibly perform other functions. Recently, several cathepsins B have been reported in hookworms, both in the digestive system (suggesting a possible role in the acquisition of nutrients) as in the excretion–secretion (ES) products [27,28]. Cysteine proteases from hookworm and filarial nematodes have been related to moulting of the L3 (see [18] for references; [29,30]). Thus, this cathepsin may take part in the remodelling of the cuticle from shortly before moulting. In fact, the greatest activity was during L3 48 h and L4 prior to moults M3 and M4, respectively.

Fig. 1C shows cysteine protease activity during the development of *H. aduncum*, as a function of pH with substrate Z-FR-AMC. All the larval stages studied showed an optimum pH of 5.5. Comparison of the groups cultured with and without pepsin (results not shown) showed no significant differences between them (K–W, $p=0.638$). In contrast, on comparing all the developmental stages, L3 0 h showed significantly lower activity (K–W, $p<0.001$) than the other stages (U–M–W, $p<0.001$ in all cases). The incorporation of a specific cysteine protease inhibitor (E64) inhibited this cathepsin activity. Fig. 1B shows the variation in enzymatic activity at pH 5.5 with Z-FR-AMC during the *in vitro* development of *H. aduncum*. Significant differences in activity can be observed (2-way ANOVA, $p<0.001$), L3 0 h showing the lowest activity of all ($p<0.005$). No significant differences were found (2-way ANOVA, $p=0.393$) between the parasites cultured with pepsin and those without (results not shown).

Substrate Z-RR-AMC is specific for cathepsin B, while Z-FR-AMC is recognised as an excellent substrate for cathepsin L, although also sensitive to cathepsins B. It is thus possible that the activity of two types of enzymes is being measured at the same time, although it is unlikely that both activities (B and L) show the same optimum pH (5.5). Furthermore, if cathepsin B were present it should show activity with substrate Z-RR-AMC at an optimum pH close to 5.5 and this did not occur. The optimum pH (5.5) is analogous to those reported in somatic extracts of other parasitic nematodes for the substrate Z-FR-AMC (activity range ca. 4.5–6.5) [25,31]. We obtained similar results in *H. aduncum*, detecting activity at pH between 3.5 and 6.5, approximately (Fig. 1C). However, the cathepsin B-like activity measured in the ES products of *Ancylostoma caninum* with this substrate was high, being detected between pH 5.0 and 9.0, showing almost no activity with substrate Z-RR-AMC [32]. The same occurred with the ES products of *Haemonchus contortus* [33,34]. Thus, although we cannot completely discount cathepsin B-like activity with substrate Z-FR-AMC in *H. aduncum*, it is to be supposed that, given our experimental conditions, we have measured almost exclusively cathepsin L-like activity.

Bearing in mind that digestion takes place in an acidic medium in invertebrates [35], cathepsin L-like activity with optimum pH of 5.5 could take part in the digestion of nutrients, either intracellularly in the lysosomes or extracellularly in the gut lumen of the nematode. The activity profile (Fig. 1B) throughout the *in vitro* development of *H. aduncum* may support this idea, since activity increases significantly after placing the worm in the culture; its development is re-initiated from the L3 which is found in the body cavity of its intermediate/paratenic host in a state of “hypometabolic dormancy”, as described in other nematodes [36,37]. This increase in cathepsin L-like activity remains more or less constant throughout the *in vitro* development of the parasite.

However, Loukas et al. [25] described a cathepsin L-like in extracts of infective larvae of *Toxocara canis* capable of cleaving both substrate Z-FR-AMC and Z-RR-AMC, although at different optimum pH values (5.0 and 6.5, respectively), with activity ratios of a similar magnitude to those obtained in this study with *H. aduncum* (results not shown). This cathepsin has not yet been described in other parasitic helminths.

It is believed that some cases of allergic response in humans, attributed to fish, are really due to the ingestion of the parasites they harbour. In fact, anisakids, particularly *A. simplex* s.l., are now considered to be responsible for a great number of cases of allergic responses. However, some authors have demonstrated that *A. simplex* and *H. aduncum* possess allergens in common and have suggested that the latter may also be responsible for many allergic responses following consumption of fish, as it is found in fish worldwide [4]. Cysteine proteases from dust mites, filarias, trematodes, and parasitic protozoa are also responsible for allergic responses in humans and animals (see references in [18]).

Cathepsins may be employed as vaccines. Thus, significant protection against *Fasciola hepatica* and *H. contortus* in animals has been documented [38,39]. Similar strategies may be effective against many parasitic organisms [18].

In conclusion, the somatic extracts of *H. aduncum* possess two different cathepsin proteinases which show different specific activity for each substrate used and are expressed differentially throughout ontogenic development. Cathepsin L-like showed optimum activity at pH 5.5 with Z-FR-AMC and could be involved in the digestion of nutrients. Cathepsin B-like showed optimum activity at pH 7.0–7.5 with Z-RR-AMC, and, given the variations it exhibits during ontogenic development, may take part in moulting. A more detailed characterisation of these cathepsins is required if we are to investigate their possible role as an allergen for man and their possible use in diagnosis or vaccines and in the development of inhibitors for use in chemotherapy to control infection in farmed fish.

Acknowledgments

This work has been funded by Spanish Grant ACU01-027 from the INIA and Research Group BIO-243 from Junta de Andalucía. David Malagón is a recipient of a predoctoral fellowship from Consejería de Agricultura y Pesca, Junta de Andalucía (Spain). The English translation was by Mr. Robert Abrahams, BSc.

References

- [1] Moravec F, Nagasawa K, Urawa S. Some fish nematodes from fresh water in Hokkaido, Japan. *Folia Parasitol* 1985;32:305–16.
- [2] Yoshinaga T, Ogawa K, Wakabayashi H. Experimental life cycle of *Hysterothylacium aduncum* (Nematoda: Anisakidae) in fresh water. *Fish Pathol* 1987;22:243–51.
- [3] Yagi K, Nagasawa K, Ishikura H, Nakagawa A, Sato N, Kikuchi K, Ishikura H. Female worm *Hysterothylacium aduncum* excreted from human: a case report. *Jpn J Parasitol* 1996;45:12–23.
- [4] Valero A, Terrados S, Díaz V, Reguera V, Lozano J. Determination of IgE in the serum of patients with allergic reactions to four species of fish-parasite anisakids. *J Invest Allergol Clin Immunol* 2003;13:94–8.
- [5] Rello FJ, Adroher FJ, Valero A. *Hysterothylacium aduncum*, the only anisakid parasite of sardines (*Sardina pilchardus*) from the southern and eastern coasts of Spain. *Parasitol Res* 2008;104:117–21.

- [6] Rello FJ, Valero A, Benítez R, Adroher FJ. The fishing area as a possible indicator of the infection by anisakids in anchovies (*Engraulis encrasicolus*) from southwestern Europe. *Int J Food Microbiol* 2009;129:277–81.
- [7] Andersen K. *Hysterothylacium aduncum* (Rudolphi, 1802) infection in cod from the Oslofjord: seasonal occurrence of third- and fourth-stage larvae as well as adult worms. *Parasitol Res* 1993;79:67–72.
- [8] Koje M. Aspects of the life cycle and morphology of *Hysterothylacium aduncum* (Rudolphi, 1802) (Nematoda, Ascaridoidea, Anisakidae). *Can J Zool* 1993;71:1289–96.
- [9] Balbuena JA, Karlsbakk E, Kvenseth AM, Saksvik M, Nylund A. Growth and migration of third-stage larvae of *Hysterothylacium aduncum* (Nematoda: Anisakidae) in larval herring *Clupea harengus*. *J Parasitol* 2000;86:1271–5.
- [10] González L, Carvajal J. Parásitos en los cultivos marinos de salmónidos en el sur de Chile. *Invest Pesq (Chile)* 1994;38:87–96.
- [11] Iglesias L, Valero A, Gálvez L, Benítez R, Adroher FJ. In vitro cultivation of *Hysterothylacium aduncum* (Nematoda: Anisakidae) from 3rd-stage larvae to egg-laying adults. *Parasitology* 2002;125:467–75.
- [12] Adroher FJ, Malagón D, Valero A, Benítez R. In vitro development of the fish parasite *Hysterothylacium aduncum* from the third larval stage recovered from a host to the third larval stage hatched from the egg. *Dis Aquat Org* 2004;58:41–5.
- [13] Sakanari JA, McKerrow JH. Identification of the secreted neutral proteases from *Anisakis simplex*. *J Parasitol* 1990;76:625–30.
- [14] Tort J, Brindley PJ, Knox DP, Wolfe KH, Dalton JP. Proteinases and associated genes of parasitic helminths. *Adv Parasitol* 1999;43:161–266.
- [15] Knox DP, Skuce PJ, Newlands GF, Redmond DL. Nematode gut peptidases, proteins and vaccination. In: Kennedy MW, Harnett W, editors. *Parasitic nematodes: molecular biology, biochemistry and immunology*. Wallingford-Oxon: CAB International; 2001. p. 247–68.
- [16] Dalton JP, Clough KA, Jones MK, Brindley PJ. Characterization of the cathepsin-like cysteine proteinases of *Schistosoma mansoni*. *Infect Immun* 1996;64:1328–34.
- [17] Barrett AJ, Rawlings ND, Woessner JF. *Handbook of proteolytic enzymes*. Academic Press: San Diego; 1998.
- [18] Sajid M, McKerrow JH. Cysteine proteases of parasitic organism. *Mol Biochem Parasitol* 2002;120:1–21.
- [19] Rehman A, Jasmer DP. Defined characteristics of cathepsin B-like proteins from nematodes: inferred functional diversity and phylogenetic relationships. *Mol Biochem Parasitol* 1999;102:297–310.
- [20] Hashmi S, Britton C, Liu J, Guiliano DB, Oksov Y, Lustigman S. Cathepsin L is essential for embryogenesis and development of *Caenorhabditis elegans*. *J Biol Chem* 2002;277:3477–86.
- [21] Britton C, Murray L. A cathepsin L protease essential for *Caenorhabditis elegans* embryogenesis is functionally conserved in parasitic nematodes. *Mol Biochem Parasitol* 2002;122:21–33.
- [22] Morris SR, Sakanari JA. Characterization of the serine protease and serine protease inhibitor from the tissue-penetrating nematode *Anisakis simplex*. *J Biol Chem* 1994;269:27650–6.
- [23] Petter AJ, Maillard C. Larves d'ascarides parasites de poissons en Méditerranée occidentale. *Bull Mus Natl Hist Nat 4e Sér* 1988;10(A):347–69.
- [24] Bradford MM. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- [25] Loukas A, Selzer PM, Maizels RM. Characterisation of Tc-clp-1, a cathepsin L-like cysteine protease from *Toxocara canis* infective larvae. *Mol Biochem Parasitol* 1998;92:275–89.
- [26] Yasuda Y, Kageyama T, Akamine A, Shibata M, Kominami E, Uchiyama Y, Yamamoto K. Characterization of new fluorogenic substrates for the rapid and sensitive assay of cathepsin E and cathepsin D. *J Biochem* 1999;125:1137–43.
- [27] Ranjit N, Zhan B, Stenzel DJ, Mulvenna J, Fujiwara R, Hotez PJ, Loukas A. A family of cathepsin B cysteine proteases expressed in the gut of the human hookworm, *Necator americanus*. *Mol Biochem Parasitol* 2008;160:90–9.
- [28] Mulvenna J, Hamilton B, Nagaraj SH, Smyth D, Loukas A, Gorman JJ. Proteomics analysis of the excretory/secretory component of the blood-feeding stage of the hookworm, *Ancylostoma caninum*. *Mol Cell Proteomics* 2009;8:109–21.
- [29] Guiliano DB, Hong X, McKerrow JH, Blaxter ML, Oksov Y, Liu J, Ghedin E, Lustigman S. A gene family of cathepsin L-like proteases of filarial nematodes are associated with larval molting and cuticle and eggshell remodeling. *Mol Biochem Parasitol* 2004;136:227–42.
- [30] Kumar S, Pritchard DI. The partial characterization of proteases present in the excretory/secretory products and exsheathing fluid of the infective (L3) larva of *Necator americanus*. *Int J Parasitol* 1992;22:563–72.
- [31] Kamata I, Yamada M, Uchikawa R, Matsuda S, Arizono N. Cysteine protease of the nematode *Nippostrongylus brasiliensis* preferentially evokes an IgE/IgG1 antibody response in rats. *Clin Exp Immunol* 1995;102:71–7.
- [32] Dowd AJ, Dalton JP, Loukas AC, Prociw P, Brindley PJ. Secretion of cysteine proteinase activity by the zoonotic hookworm *Ancylostoma caninum*. *Am J Trop Med Hyg* 1994;51:341–7.
- [33] Karanu FN, Rurangirwa FR, McGuire TC, Jasmer DP. *Haemonchus contortus*: identification of proteases with diverse characteristics in adult worm excretory-secretory products. *Exp Parasitol* 1993;77:362–71.
- [34] Rhoads ML, Fetterer RH. Developmentally regulated secretion of cathepsin L-like cysteine proteases by *Haemonchus contortus*. *J Parasitol* 1995;81:505–12.
- [35] Delcroix M, Sajid M, Caffrey CR, Lim KC, Dvorak J, Hsieh I, Bahgat M, Dissous C, McKerrow JH. A multienzyme network functions in intestinal protein digestion by a plathyhelminth parasite. *J Biol Chem* 2006;281:39316–29.
- [36] Petronijevic T, Rogers WP. Undissociated bases as the stimulus for development of the early parasitic stages of nematodes. *Int J Parasitol* 1987;17:911–5.
- [37] Dávila C, Malagón D, Valero A, Benítez R, Adroher FJ. *Anisakis simplex*: CO₂-fixing enzymes and development throughout the in vitro cultivation from third larval stage to adult. *Exp Parasitol* 2006;114:10–5.
- [38] Dalton JP, McGonicle S, Rolph TP, Andrews SJ. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. *Infect Immun* 1996;64:5066–74.
- [39] Skuce PJ, Redmond DL, Liddell S, Stewart EM, Newlands GF, Smith WD, Knox DP. Molecular cloning and characterization of gut-derived cysteine proteinases associated with a host protective extract from *Haemonchus contortus*. *Parasitology* 1999;119:405–12.