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Proteolytic activity in *Hysterothylacium aduncum* (Nematoda: Anisakidae), a fish gastrointestinal parasite of worldwide distribution

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ABSTRACT

Proteases have a significant role in the life cycle of parasites and the pathogen–host relationship, being regarded as important virulence factors. In the parasitic nematode *Hysterothylacium aduncum* proteolytic activity was measured during in vitro development from third larval stage (L3) to mature adult, using DQ red casein as a fluorogenic substrate. Proteolytic activity was detected in all the developmental stages studied and at all pH values within the range employed (2.0–7.5). The assay with specific inhibitors permitted the determination of metalloprotease activity, and, to a lesser extent, that of aspartate- and cysteine-protease. Serine-protease activity was the lowest of those studied. In L3 recently collected from the host fish (L3-0 h), the greatest activity was found at an optimum pH of 4.0 and was mainly inhibited by 1,10-phenathroline (metalloprotease inhibitor). This metalloprotease activity in L3-0 h (infective stage) may be related to the invasion of the host tissues by this larva. In the other developmental stages, the greatest protease activity was found at pH 5.5, although at pH 4.0 a lower activity peak was detected. On the other hand, our data show that the proteolytic activity of the nematode varies according to the presence of pepsin (an aspartic-protease) in the culture medium. Thus, at pH 4.0, activity was greater in the absence of pepsin, with increasing aspartic-protease activity. Together with the detection of aspartic-, cysteine- and metallo-protease (enzymes involved in digestion in invertebrates) in all the developmental stages of the parasite taking place in the digestive tract of the host fish, this allows us to suggest that the pepsin in the culture medium mimics the predigestion conditions in the habitat of the worm within the host and that the activity detected may have, amongst others, a digestive function.

1. Introduction

Hysterothylacium aduncum is an anisakid nematode parasite of fish and crustaceans with a complex life cycle (Køie, 1993). It is present in marine ecosystems worldwide and has also been reported in fresh water.

Although its pathogenic effect on adult fish is still under discussion, González & Carvajal (1994) suggested that fish parasitized by this nematode are more susceptible to secondary infections affecting their life expectancy and commercial value. A serious pathogenic effect of this anisakid has been described in both larval and juvenile fish (Bristow, 1990; Balbuena et al., 2000), which may lead to economic problems, particularly in the field of aquaculture, due to the worldwide distribution of the parasite. Accidental ingestion of this parasite by man may cause digestive problems (Yagi et al., 1996) and hypersensitivity (Valero et al., 2003).

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In our laboratory we have developed a culture medium for *H. aduncum* from L3 collected from the host fish to egg-laying adult. In order to achieve complete development of the nematode, the medium must contain pepsin. This may be involved in the degradation of proteins in the medium, thus facilitating the assimilation of nutrients of protein origin by the parasite (Iglesias et al., 2002; Adroher et al., 2004).

Proteases have a wide variety of roles in living organisms. In parasitic nematodes they seem to be involved in a range of vital processes, including digestion/nutrition, moulting and larval development (Gamble et al., 1989; Hong et al., 1993; McKerrow, 1994; Hashmi et al., 2002). Furthermore, they also seem to play an important part in the pathogen–host relationship, acting as virulence factors (Cazzulo, 2003), as occurs in *Anisakis simplex* (Sakanari and McKerrow, 1990), *Onchocerca volvulus* (Lustigman, 1993) or *Ancylostoma caninum* (Hawdon et al., 1995).

In previous studies we have described the presence of cysteine proteases (cathepsin L- and B-like) in *H. aduncum* and also that of serine- and metallo-proteases with collagenolytic activity, which may be involved in nutrient digestion and tissue remodeling, taking place during moulting and tissue migration (Malagón et al., 2010a,b).

The aim of the present study was to perform a general characterization of proteolytic activity and to observe its variation during in vitro development of *H. aduncum* from L3 recently collected from the paratenic/intermediate host fish to mature adult stage.

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 on the fishmarket of Granada (Southern Spain). The worms over 8 mm in length found free in the host body cavity were processed as previously described (Malagón et al., 2010a). The larvae were then identified according to morphological features (Yoshinaga et al., 1987; Petter and Maillard, 1988). Next, a number of larvae were frozen (−80 °C) for enzyme determination (L3 from fish). The rest of the larvae were axenized and individually placed in culture at 15 °C, in a modified RPMI-1640 medium with pepsin and foetal bovine serum added (Iglesias et al., 2002).

2.2. Preparation of the extract and protein determination

The following samples were taken, washed during 20 min in a 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 (Adult-J, 6 worms per ml of extract), and mature adult after 42 days of cultivation (Adult-M, 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 followed by 10 bursts/ml of ultrasonic disintegration (on ice), each of 1 s duration at 15 μm amplitude. The extract was then centrifuged at 18,000 × *g* for 15 min at 4 °C and the supernatant was assayed for protein concentration using the Bradford method (Bradford, 1976) and stored at −80 °C.

2.3. Enzyme determination

Enzyme activity was monitored at 37 °C for 100 min and measured every 2 min, in black microtiter plates, by hydrolysis of the fluorogenic substrate DQ red casein (an internally quenched casein, heavily labelled with the pH-insensitive bodipy TR-X dye, from Molecular Probes-Invitrogen, to a final concentration 5 μg/ml). To monitor the effect of pH on the activity of the parasite extracts (6 μg protein), the assays were performed at final volume of 0.2 ml in the following buffers with pH increments of 0.5: 50 mM glycine–HCl (pH 2.0–4.0); 50 mM sodium acetate: 50 mM acetic acid (pH 4.5–5.5); 50 mM phosphate buffer (pH 6.0–6.5); 50 mM Tris–HCl (pH 7.0–7.5). The same buffers were also prepared with the addition of 6.5 mM dithiothreitol (DTT), in order to determine whether this thioreductor agent affected proteolytic activity. Cleavage of fluorescent conjugate was detected using a fluorimeter with λ excitation at 584 nm and λ emission at 620 nm. Enzyme activity was expressed as a variation (Δ) of the fluorescence relative units (FRU) min^{-1} mg^{-1} protein.

2.4. Inhibition assays

To assess the contribution of the different protease classes to hydrolysis of the DQ red casein, protease inhibitors were assayed by modifications of previously described methods (García-Carreño, 1992; Beynon and Salvesen, 2001; Salvesen and Nagase, 2001). Inhibitors were tested at a final concentration of 0.02 mM (pepstatin A), 2 mM (1,10-phenanthroline), 0.05 mM [L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)-butane or E64] and 1 mM [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride or AEBSF]. Control assays were with an aspartic protease [pepsin (EC: 3.4.23.1) for pepstatin A], a cysteine protease [papain (EC: 3.4.22.2) for E64], a serine protease [α-chymotrypsine (EC: 3.4.21.1) for AEBSF] and a metalloprotease [thermolysin (EC: 3.4.24.27) for 1,10-phenanthroline]. The parasite extracts were preincubated during 45 min with the inhibitors prior to adding substrate. A solvent (0.5% methanol, used for pepstatin A and 1,10-phenanthroline) control was also performed. Reactions were allowed to proceed and the protease activity was determined as described above. Inhibition assays were carried out at pH 3.0 (pepstatin A), pH 4.0 (pepstatin A, E64 and 1,10-phenanthroline), pH 5.5 (pepstatin A, E64, 1,10-phenanthroline and AEBSF) and at pH 7.0 (E64, 1,10-phenanthroline and AEBSF). The effect of inhibitors was expressed as percentage of inhibition (%*I*), determined as: %*I* = 100 − [(mean ΔFRU min^{-1} mg^{-1} protein in presence of

Table 1
Full-factorial ANOVA table of all data obtained to pH 4.0, using enzymatic activity as dependent variable and worm developmental stage (Stage), pepsin (absence/presence in the culture medium) and dithiothreitol (DTT, presence/absence in the assay mixture) as independent variables.

Source of variation	Sum of squares	df	Mean square	*F*-value	*p*-Value
Intersection	0.046	1	0.046	0.623	0.436
Stage	1.405	4	0.351	4.757	0.004
Pepsin	0.068	1	0.068	0.917	0.345
DTT	0.095	1	0.095	1.281	0.266
Stage-pepsin	1.470	3	0.490	6.635	0.001
Stage-DTT	0.555	4	0.139	1.879	0.137
Pepsin-DTT	2.17×10^{-6}	1	2.17×10^{-6}	0.000	0.996
Stage-pepsin-DTT	0.011	3	0.004	0.050	0.985
Error	2.510	34	0.074		
Total	6.131	52			

the inhibitor/mean ΔFRU min^{-1} mg^{-1} protein in absence of the inhibitor) $\times$ 100].

2.5. Statistical method

The experiments were repeated two- to five-times in duplicate. The data are expressed as mean ± standard error (SE). The data were processed using SPSS software (version 14.0 for Windows). Values were tested for significance by ANOVA (Tables 1 and 2); when ANOVA was not applicable, the data were tested for the Kruskal–Wallis (K–W) non parametric test to "k" independent samples. After ANOVA, when the data were statistically significant, planned *post hoc* comparisons by pairs were performed (with Bonferroni correction) using Student's *t*-test or Tamhane's T2-test (for unequal variances); and Mann–Whitney's *U* test for the Kruskal–Wallis test. The significance level was $p < 0.05$.

3. Results and discussion

Fig. 1 shows the activity of somatic extract from *H. aduncum* against DQ red casein with regard to pH and developmental stage. Activity was detected in all the stages assayed and across the pH range studied (2.0–7.5). No significant differences were observed in any stage of the nematode on comparing the effect of the DDT on its proteolytic activity (with/without DTT; 1-way ANOVA or K–W test, $p > 0.05$ in all cases).

Activity tended to be concentrated mainly on the pH interval 3.5–7.0, being greatest at pH 5.5 except in the case of L3-0 h at pH 4.0.

The proteolytic activity assay in the presence of DTT should reveal the possible presence of cysteine-proteases. In general, our results (Fig. 1) do not show great variations in proteolytic activity, except for that of L3-0 h at pH 4.0, where there was notable inhibition. This may be due to the ability of the sulphydryl groups to form a complex with the metal ion of some proteases, thus inactivating them (Dunn, 2001). As shown in Fig. 2, at pH 4.0 in L3-0 h most of the activity (70%) was due to metalloproteases. This pronounced sensitivity to DTT does not occur in other larval developmental stages, where there was hardly any metalloprotease activity at pH 4.0 (Fig. 2a and b).

In the life cycle of *H. aduncum*, L3 must pass through the digestive mucosa of the host fish in order to establish themselves in the visceral cavity. Furthermore, Andersen (1993) and Iglesias et al. (2002) suggested that this L3, once it had attained the necessary level of development, could return to the digestive tract of the fish and complete its development to adult stage here. We believe that the metalloprotease activity detected in L3-0 h may be involved in the passage of the larva through the digestive mucosa of the fish. This is especially likely if we consider that several metalloproteases involved in tissue invasion processes in the host have been detected in other nematodes such as *Strongyloides stercoralis*, *S. venezuelensis*, *Trichuris suis*, and even in another anisakid, *A. simplex* (McKerrow et al., 1990; Sakanari and McKerrow, 1990; Hill et al., 1993; Maruyama et al., 2006). In the latter and in other nematodes, hyaluronidases involved in tissue penetration have been detected, with acidic optimum pH values (between 4.0 and 6.0) coinciding in many cases with the pH in the zone of penetration (Hotez et al., 1994; Rhoads et al., 2001), as occurs in *H. aduncum* (Fig. 1). In any case, the capacity for penetration of this parasite must be considerable since it infects a wide variety of host species (Adroher et al., 1996;

Table 2
Full-factorial ANOVA table of all data obtained to pH 5.5, using enzymatic activity as dependent variable and worm developmental stage (Stage), pepsin (absence/presence in the culture medium) and dithiothreitol (DTT, presence/absence in the assay mixture) as independent variables.

Source of variation	Sum of squares	df	Mean square	*F*-value	*p*-Value
Intersection	12.685	1	12.685	203.312	0.000
Stage	1.283	4	0.321	5.139	0.002
Pepsin	0.058	1	0.058	0.936	0.340
DTT	0.046	1	0.046	0.745	0.394
Stage-pepsin	0.088	3	0.029	0.472	0.704
Stage-DTT	0.018	4	0.005	0.073	0.990
Pepsin-DTT	0.003	1	0.003	0.045	0.834
Stage-pepsin-DTT	0.14	3	0.005	0.076	0.972
Error	2.121	34	0.062		
Total	19.376	52			

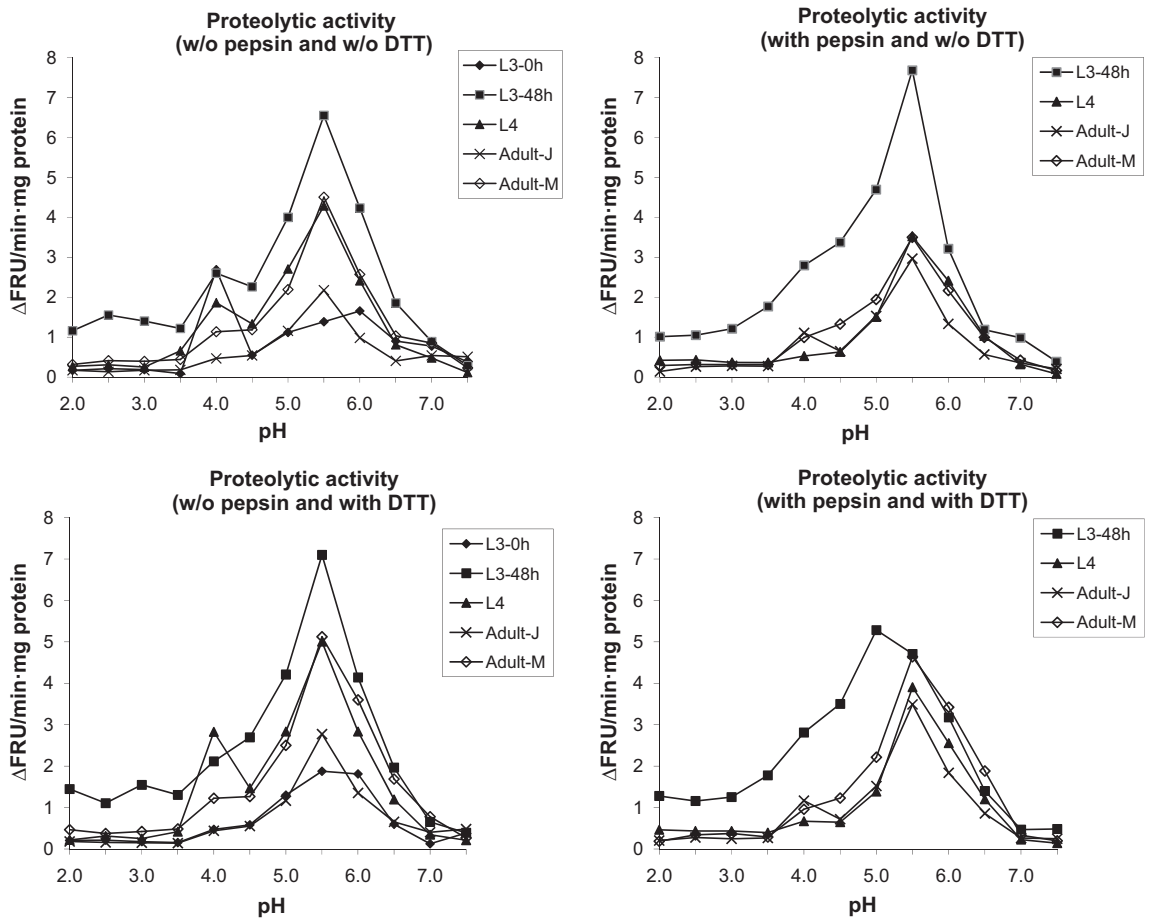

Fig. 1. pH profile of DQ red casein cleaving activity in *Hysterothylacium aduncum*, with or without dithiothreitol (DTT) in the assay, during its in vitro development. The parasites were cultured with or without pepsin as indicated in Section 2. Each point is the mean of two to three experiments in duplicate. The SE of the data was between ±0.01 and ±5.46.

Rello et al., 2008, 2009; and others) and is regarded as one of the most abundant marine parasites (Balbuena et al., 1998; Klimpel and Rückert, 2005). In many fish host, the L3 is encapsulated rather than free in the visceral cavity. Larsen (1980) suggested that the L3 perforates the capsule with his tooth and kept open by histolytic enzymes secreted by the larva, in order to obtain nutrients from outside the capsule. If this happens, perhaps the described metalloprotease could also be involved in this process.

The proteolytic activity measured at pH 5.5 was minimal in L3-0 h (3-way ANOVA, $F = 5.139$, df = 4, $p = 0.002$ (see Table 1); and one-tail Student's t-test in all cases $p < 0.02$) (Figs. 1 and 3), being mainly due to metalloproteases, as occurred at pH 4.0 (Fig. 2). Digestion in acid compartments appears to be widespread in invertebrates (Delcroix et al., 2006). However, the low activity of the proteases normally responsible for digestion at acidic pH, such as aspartic proteases, suggests that, in this developmental stage, a different mechanism is employed for the assimilation of nutrients of protein origin. Transcuticular nutrition has been recorded in some nematodes (see Thompson and Geary, 1995). This situation is in conformity with a possible acquisition of nutrients through the cuticle in this stage, as occurs in *A. simplex* (Iglesias et al., 1997), where L3 are unable to ingest food orally until moulting to L4 is complete (Yasuraoka et al., 1967; Sommerville and Davey, 1976). In fact, the proteolytic activity profiles of L3 and L4 are different (Dziekońska-Rynko et al., 2003).

The L3 of *H. aduncum* recommence their development, moulting to L4 and finally to adult, in the gastrointestinal tract of carnivorous fish, such as the gadoids (Punt, 1941; Berland, 1961), or under the in vitro culture conditions described by Iglesias et al., 2002, in which the larvae have access to large quantities of nutrients of protein origin. For this reason, the larvae of subsequent developmental stages, which can ingest nutrients orally, can begin to exhibit greater proteolytic activity.

Since L3-48 h seems unable to take food orally and is able of moulting to L4 in the absence of nutrients (Iglesias et al., 2002), their high proteolytic activity (Fig. 1)—greater than in any of the other developmental stages, except Adult-M, at least at pH 5.5 (3-way ANOVA, $F = 5.139$, df = 4, $p = 0.002$ (see Table 1); and one-tail Student t-test, $p \leq 0.05$)—is most likely due to the imminent moulting to L4, which usually takes place in vitro after 72 h culture, induced by the culture conditions (Iglesias et al., 2002). In

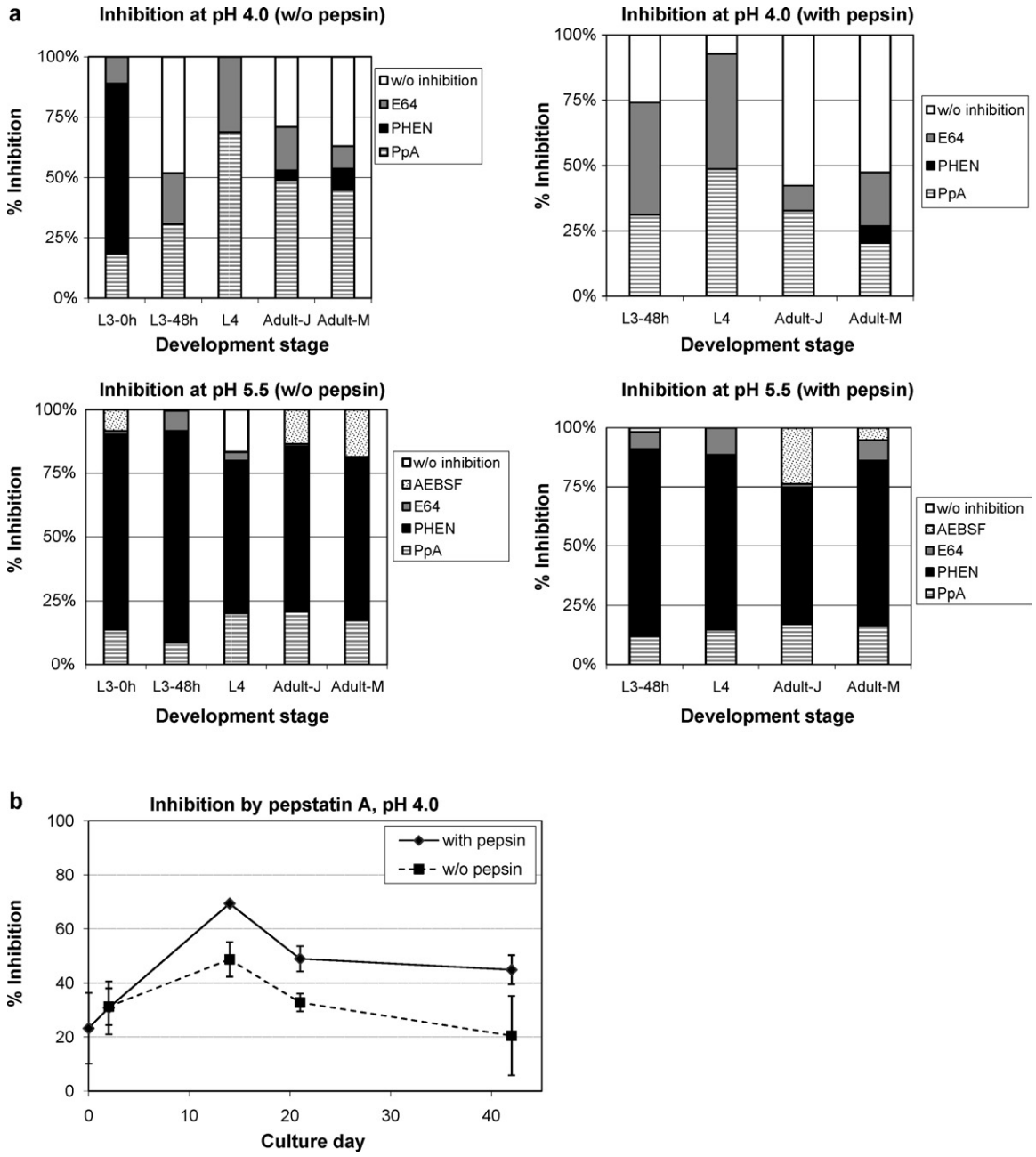

Fig. 2. (a) Inhibition percentage of optimal DQ red casein cleaving activity (pH 4.0 and 5.5 in L3-0 h, L3-48 h, L4, immature (Adult-J) and mature adults (Adult-M)) by pepstatin A, 1,10-phenanthroline (PHEN), AEBSF and E64 in *Hysterothylacium aduncum*, during its in vitro development, cultured with or without pepsin. Each inhibition percentage is the mean of two to five experiments in duplicate. (b) Inhibition percentage of DQ red casein cleaving activity at pH 4.0 by pepstatin A in *H. aduncum*, during its in vitro development, cultured with or without pepsin. Each point is the mean of two to five experiments in duplicate ± SE.

a similar nematode, *Ascaris suum*, it has been related to an aminometallopeptidase released into the culture medium by the moulting process, since its maximum concentration is found at the moment of passing from L3 to L4 (Rhoads et al., 1997). It is also found in the intestine of the adult, where it exhibits a high level of activity related to digestion (Rhoads and Fetterer, 1998), as may occur in *H. aduncum*. Aminopeptidases have also been detected in the moulting processes of several filarians such as *Brugia pahangi* (Hong et al., 1993), *Dirofilaria immitis* (Richer et al., 1992) and *Onchocerca volvulus* (Lustigman et al., 1996). Other metalloproteases have been detected in the moulting of *Haemonchus contortus* (Gamble et al., 1989).

We have shown a significant statistical interaction between the variables pepsin and developmental stage, at least at pH 4.0 (3-way ANOVA, $F = 6.635$, df = 3, $p = 0.001$;

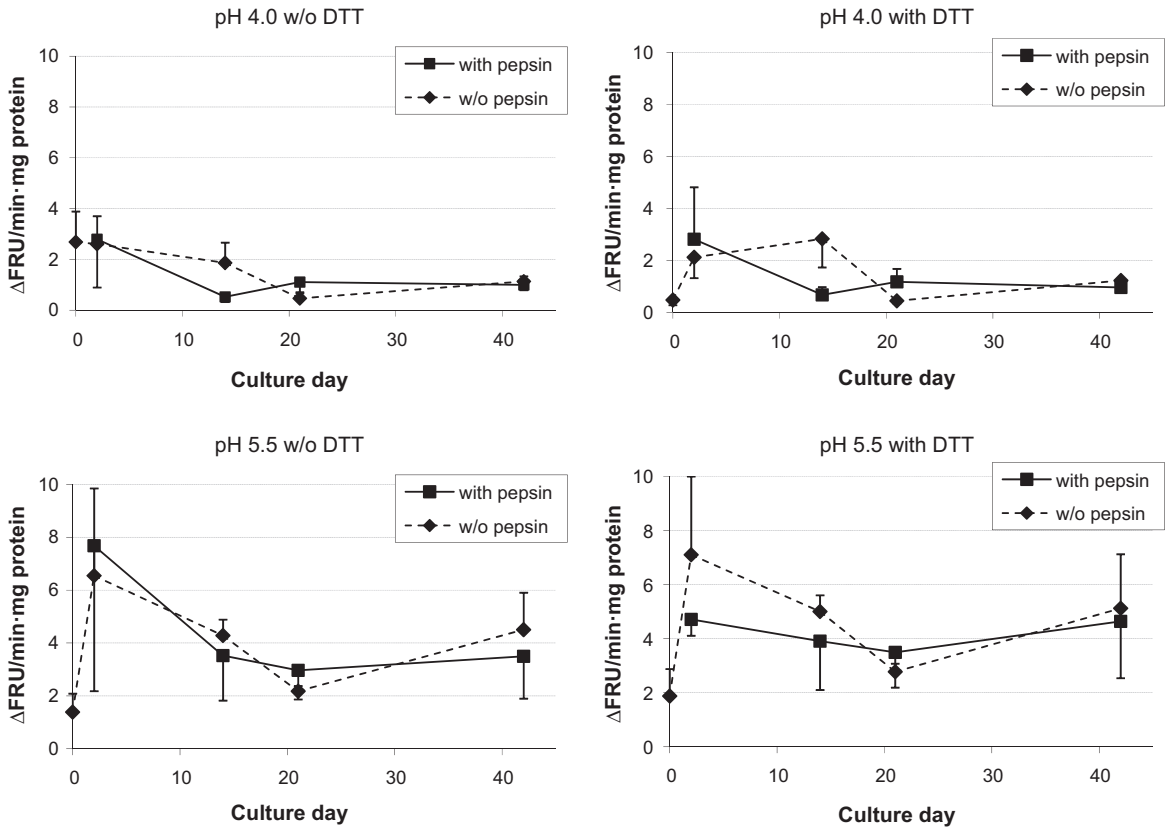

Fig. 3. Variation of DQ red casein cleaving activity *Hysterothylacium aduncum* at pH 4.0, and 5.5, with or without dithiothreitol in the assay, during its in vitro development. The parasites were cultured with or without pepsin as indicated in Section 2. Each point is the mean of two to three experiments in duplicate ± SE.

see Table 2). In the L4 stage there was a notable decrease in proteolytic activity at the two optimum pH values assayed, especially at pH 4.0 in the larvae cultivated in a medium with pepsin (Fig. 3). At this pH, our results show greater proteolytic activity when L4 arises in the absence of exogenous pepsin, opposite to L4 arisen with pepsin in the culture medium (one-tailed Student's *t*-test, $p < 0.003$). Not only did this activity double but the proportion of the activity due to aspartic proteases increased from 50 to 70% (Figs. 2 and 3). Iglesias et al. (2002) showed that moulting from L4 to adult was significantly facilitated by the presence of exogenous pepsin. Consequently, we believe that L4 require high assimilation of nutrients, at least those of protein origin, to be able to moult to adulthood. Without exogenous predigestion by the pepsin in the medium, the larvae would require greater aspartate-protease activity, which they themselves over-express. Under natural conditions, the pepsin added to the culture medium is substituted for that found in the digestive tract of the final host fish, where the larvae develop to adulthood. This increases the availability of nutrients for the development of the parasite. 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 (Dziekońska-Rynko et al., 1997a). 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 (Iglesias et al., 2001, 2002). The use of other proteases such as papain or trypsin or of other pH values has a negative effect on these nematodes (Dziekońska-Rynko et al., 1997b; Iglesias et al., 1997, 2001, 2002).

According to Delcroix et al. (2006), the papain-type aspartic and cysteine proteases assume the same role in invertebrate digestion as trypsins in that of vertebrates. Furthermore, the majority of the aspartic proteases described in nematodes are related to digestive processes (Hawdon et al., 1989; Brown et al., 1995; Geldhof et al., 2000; Williamson et al., 2003a,b). 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. (1998) for proteolytic activity in *A. suum*, a parasite from the same superfamily as *H. aduncum*.

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 metalloproteases of the parasite complete the digestion initiated by the pepsin and other enzymes from the host (Rhoads et al.,

1998). This may also occur in another gastrointestinal parasite, *H. aduncum*, since high metalloprotease activity at an optimum pH 5.5, representing between 60% and 90% of total proteolytic activity at this pH, is expressed in all developmental stages of the parasite (Fig. 2).

Serine protease activity, both at pH 5.5 (Fig. 2) and 7.0 (results not shown), did not appear until after moulting to the adult stage. In the filarian *O. volvulus* it has been related to moulting, embryogenesis and spermatogenesis (Ford et al., 2005). Thus, in *H. aduncum*, it may well be related to the attainment of sexual maturity, appearing during the sexual maturation of the parasite.

In *H. aduncum* cysteine proteases do not appear to account for much of the total protease activity. Most of their activity was measured at pH 4.0, accounting for between 15 and 40% of the total activity, while less than 10% was detected at pH 5.5 (Fig. 2). Both cases showed the same distribution profile throughout the different developmental stages, activity being lowest in the adults. At pH 7.0 activity, albeit very low (results not shown), was only found in adults, suggesting these activities may have different functions. In nematodes, many functions for cysteine proteases have been described (Britton et al., 1999; Hashmi et al., 2002), which, bearing in mind the optimum pH and the developmental stage in which the activity is expressed, leads us to believe that the acidic pH cysteine proteases may participate in digestion and the neutral pH in sexual maturation, as proposed by Malagón et al. (2010a).

In conclusion, metalloprotease-like activity has been shown to occur in the infective L3 of *H. aduncum* with an optimum pH of 4.0 and may be involved in invasion of the host. Activity which was probably digestive was also observed. This was mainly related to aspartate proteases, but also metalloproteases, and, to a lesser extent, cysteine proteases. This activity seems to depend on the exogenous availability of nutrients of protein origin, originating from previous digestion by the host or from the presence of pepsin in the culture medium. Other serine protease-like activities may be related to the sexual maturation of the nematode. Further work is required in order to thoroughly characterize the proteases present in *H. aduncum*.

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