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Identification and characterization of the proteolytic enzymes in the developmental stages of the eel-pathogenic nematode *Anguillicola crassus*

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Abstract. The proteolytic activities of homogenates prepared from the second larva (L2) and the third larva (L3) as well as the adult stage of the eel-pathogenic nematode *Anguillicola crassus* were examined using hemoglobin, azocoll, elastin-orcein, and keratin azure as substrates. Whole bodies of L2 larvae, the anterior third of the bodies of L3 larvae, and the anterior fifth of the bodies of adults were studied. Extracts of L2 contained a trypsin-like proteinase exhibiting a molecular weight of 38000 Da on gelatin-substrate gel electrophoresis. The proteinase showed a pH optimum at 8 and activity against azocoll and keratin. An apparent molecular weight of 25000 Da was determined for the trypsin-like proteinase of the L3. This enzyme possessed collagenolytic, keratinolytic and slight elastinolytic activity at an optimal pH of 8. Samples of adults contained an aspartyl proteinase with a molecular weight of 90000 Da. When hemoglobin was used as the substrate, the enzyme displayed optimal activity at pH 5. It was concluded that the proteinases of the larval stages are penetration enzymes, whereas that of the adult stage is a digestive enzyme.

Swim-bladder nematodes of the genus *Anguillicola* (Camallanata, Anguillicolidae) have been known to parasitize eels in different geographic regions of the world (Kuwahara et al. 1974; Moravec and Taraschewski 1988). Recently, the east Asian species *Anguillicola crassus* has been the focus of attention, since it invaded Europe (Neumann 1985) and quickly spread over the continent (Peters and Hartmann 1986; Taraschewski et al. 1987; Kennedy and Fitch 1990; Køie 1991; Szekely et al. 1991). Mass mortality as well as pathological alterations due to these nematodes have been reported from wild and cultured eels (Boon et al. 1990; Molnar et al. 1991; Sprengel and Lüchtenberg 1991). Levamisole is the drug of choice for the treatment of *A. crassus* (Taraschewski et al. 1988). However, only the adult worms residing in the lumen of the swim bladder are killed by this drug, whereas the larvae (L3, L4) localized inside the wall of the swim bladder are not affected (Hartmann 1989). Many ultrastructural features of the adult worms and of the larval stages have been documented (Taraschewski et al. 1988; Lamah et al. 1990; Taraschewski and Stemmer 1992). In addition, detailed knowledge about the life cycle of this parasite, which involves copepods as intermediate hosts and small fishes such as sticklebacks as paratenic hosts, has become available (Belpaire et al. 1989; De Charleroy et al. 1990; Kennedy and Fitch 1990). However, nothing is yet known about the mechanisms of penetration and of food uptake and digestion of this parasite. The aim of the present study was to identify and characterize the proteinases found in the developmental stages of *A. crassus* and to discuss these findings in terms of the descriptions on the biology and chemotherapy of this pathogenic parasite.

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Materials and methods

Parasites and hosts

Adults of *Anguillicola crassus* were obtained from eels caught in the river Weser. Male and female worms were dissected from the swim bladders and treated separately. Mature eggs from gravide females, i.e., L2 larvae enclosed by an egg sheath, were transferred to tap water at 22° C. As soon as the larvae had hatched, they were collected. L3 larvae of the parasite were dissected from experimentally infected copepods (*Megacyclops viridis*) beginning on the 12th day postinfection. The copepods had been allowed to feed on hatched L2 larvae. After they had ingested these larvae, they were kept at 22° C and fed on yeast.

Preparation of parasite crude extracts

For the preparation of crude extracts, whole bodies of L2 larvae, the anterior third of the bodies of L3 larvae, and the anterior fifth of the bodies of adults were homogenized in bidistilled water. After centrifugation at 12000 *g* for 10 min, the supernatant was either used immediately or stored at −80° C.

Enzyme assays

Proteolytic activity was determined at 25° C using the substrates azocasein (Serva), azocoll (Calbiochem), elastin-orcein, keratin azure, and hemoglobin (all purchased from Sigma). The effect of pH on the proteolytic activity against azocasein was tested over the pH range of 2–12 using 0.1 M citrate/0.2 M Na_2HPO_4 buffer (pH 2–8.5) and 0.1 M glycine/NaOH buffer (pH 8.5–12). The reaction mixtures comprised 20-µl aliquots of crude extracts or fractions, 20 µl buffer, and 20 µl azocasein solution (1.5%, w/v). Undigested protein was removed by acid precipitation, and the absorbance of the supernatant was measured with an Eppendorf photometer at 366 nm. For substrate specificity, the other protein substrates were used. Reaction mixtures contained 20-µl aliquots of crude extracts or fractions, 930 µl buffer, and 50 µl substrate (5 mg/ml). Incubations were carried out for 72 h. Undigested protein was removed by centrifugation, and the absorbance of the supernatants was measured at 280 (hemoglobin), 520 (azocoll), 550 (elastin-orcein), or 595 nm (keratin azure); 1 hydrolysis unit (HU) was defined as the amount of substrate required to give an absorbance of 1.0 per milligram of protein and hour of assay time. Soluble proteins were determined according to the method of Read and Northcote (1981) using bovine serum albumin as the standard.

Gelatin-substrate gel electrophoresis

For the identification of proteolytic activity in several stages of *A. crassus* and the determination of their apparent molecular weight under nonreduced conditions, crude extracts of the developmental stages were subjected to electrophoresis in a substrate gel; 10% polyacrylamide gels copolymerized with gelatin (0.1%, w/v) and a 4% nongelatin stacking gel were used. Samples were diluted 1:1 (v/v) in sample buffer [4% sodium dodecyl sulfate (SDS), 15% glycerol, 0.025 bromphenol blue in stacking buffer, 0.25 M TRIS-HCl (pH 6.8), and 0.4% SDS]. TRIS-glycerine (pH 8.3) running buffer was used, and the gel was run at 20 mA/gel. After electrophoresis, the gels were washed in 2% Triton X-100 for 1 h to remove SDS. The gels were then immersed in citrate buffer for 72 h at 25° C and stained in methanol/acetic acid/water (20:10:70, by vol.) containing 0.4% (w/v) Coomassie brilliant blue. Gels were destained with methanol/acetic acid/water (45:10:45, by vol.). Proteinases were detected as clear bands on a blue background. The electrophoretic migration of the proteinases was compared with that of standard proteins (Sigma).

At the end of electrophoresis, the gel was divided into 2.5-mm-wide slices by a gel cutter (fixed blades of high-grade steel). These slices were transferred into capped plastic centrifuge tubes (Eppendorf) filled with 0.5 ml bidistilled water. After the tubes had been shaken for 24 h at 4° C, the mixture of water and eluted enzymes was used at once or stored at −80° C.

Inhibitor studies

Inhibitors were used to determine the classes to which the proteinases belong. Samples of aliquots were incubated with phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), ethylenediaminetetraacetic acid (EDTA), *p*-chloromercuribenzoate (*p*-CMB), *N*-ethylmaleimide (NEM), and pepstatin for 20 min at room temperature. Incubation of samples and measurement of proteolytic activity were carried out as described above.

Results

The substrate gel electrophoresis of nonreduced samples revealed proteolytic activity in each developmental stage of *Anguillicola crassus* investigated. The apparent molecular weights of the proteolytic enzymes in extracts from L2 larvae, L3 larvae, and adults were 38000, 25000 and 90000 Da, respectively (Fig. 1). With azocasein as the substrate, the pH optimum of the enzymes from both larval stages was demonstrated as a single peak at pH 8. By contrast, a peak at pH 5 was observed for the proteinase of the adult (Fig. 2).

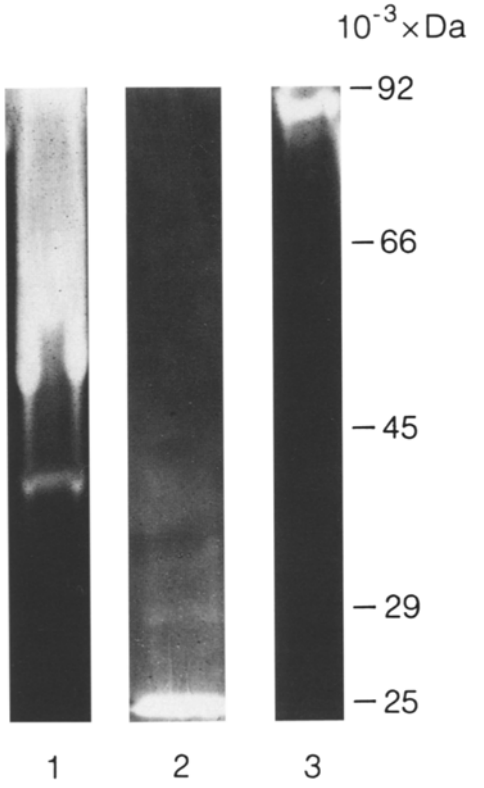

Fig. 1. Gelatin-substrate gel electrophoresis of crude extracts from several developmental stages of *Anguillicola crassus*. *Lane 1*, Larval stage L2; *lane 2*, larval stage L3; *lane 3*, adult stage. The *numbers* refer to molecular mass expressed in kilodaltons. Standard proteins are as follows: a, phosphorylase b (92.5 kDa); b, bovine serum albumin (66 kDa); c, ovalbumin (45 kDa); d, carbonic anhydrase (29 kDa); e, chymotrypsinogen (25 kDa). Hydrolysis has occurred during migration in the case of L2

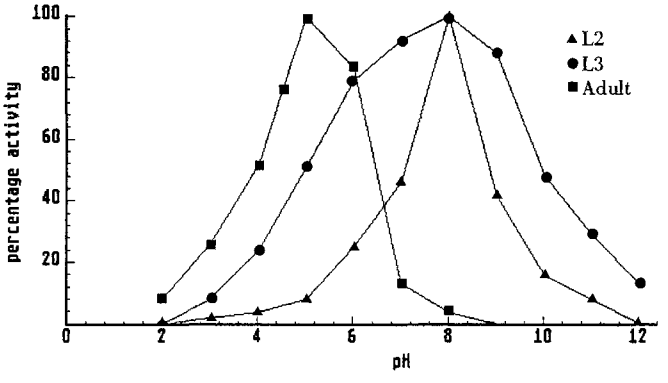

Fig. 2. Effect of pH on the hydrolysis of azocasein by the proteinases of the larval stages L2 and L3 and the adult of *Anguillicola crassus*

Table 1. Proteolytic activity in crude extracts of different developmental stages of *Anguillicola crassus*

Substrate	L2	L3	Adult
Azocoll (520 nm)	0.8±0.1	7.8±0.3	–
Elastin-orcein (550 nm)	–	0.3±0.1	–
Keratin azure (595 nm)	0.2±0.1	1.3±0.2	–
Hemoglobin (280 nm)	–	–	1.4±0.1

Results are expressed as the change in absorbance per milligram of protein and hour of assay time at 25° C. Data represent mean values ±SD for six experiments

Table 2. Inhibition of proteinase activity of different stages of *Anguillicola crassus* by specific proteinase inhibitors

Inhibitors	Concentration	Inhibition (%)		
		L2	L3	Adult
Serine proteinase:				
PMSF	10 mM	90	82	0
SBTI	125 µg/ml	83	75	0
Metalloproteinase:				
EDTA	10 mM	0	0	0
Cysteine proteinase:				
p-CMB	5 mM	0	0	0
NEM	10 mM	0	0	0
Aspartyl proteinase:				
Pepstatin	5 mM	0	0	73

PMSF, Phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; EDTA, ethylenediaminetetraacetic acid; *p*-CMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide

Table 1 summarizes the proteolytic activities of samples of the various stages of *A. crassus* that were detected using the protein substrates azocoll, elastin-orcein, keratin azure, and hemoglobin. Proteinase activity was higher in the L3 larvae than in the L2 larvae or the adult parasites. The proteinase of the L2 showed azocollytic and slight keratinolytic activity, whereas the proteinase of the L3 possessed azocollytic, keratinolytic, and slight elastinolytic activity. Both larval proteinases were incapable of degrading hemoglobin. In contrast, the proteinase of the adult showed activity against hemoglobin but was incapable of degrading the other protein substrates. The inhibitor profile assay revealed that approximately 85% of the proteinase activity of the L2 and 80% of the proteolytic activity of the L3 was inhibited by serine proteinase inhibitors (Table 2). The larval proteinases were not inhibited by either metalloproteinase, cysteine, or aspartyl proteinase inhibitors. By contrast, 73% of the proteolytic activity of the adult parasite was inhibited by the aspartyl proteinase inhibitor pepstatin, whereas no effect was observed for the other inhibitors. On the basis of these results, the proteolytic enzymes of the larval stages of *A. crassus* may be classified as trypsin-like proteinases, whereas the enzyme of the adult is most likely an aspartyl proteinase.

Discussion

The current report presents baseline data on the proteinase content of crude extracts prepared from the L2 and L3 larvae and the adult stage of the eel-pathogenic nematode *Anguillicola crassus*. We report that enzyme extracts of the investigated developmental stages of this parasite exhibit proteolytic activities with different pH optima, substrate specifities, and inhibition profiles, which indicates the stage specificity of the proteinases.

According to the results of the study, the proteolytic enzyme of the L2 larva of *A. crassus* can be considered a trypsin-like proteinase. This conclusion can be drawn from the observed alkaline pH optimum and the substrate and inhibitor specificities. It has been suggested that this enzyme is used for the penetration of the larval stage through the intestinal wall of the copepod rather than as a digestive proteinase, since the intestinal lumen of this larval stage is closed as shown by electron microscopy (Taraschewski and Stemmer 1992). The only available reference on proteinases in L2 larvae of a nematode is that of Knox and Kennedy (1988) for *Ascaris suum*; these authors have found proteolytic activities similar to those described in the present study for *A. crassus*.

In the L3 larvae of *A. crassus*, collagen was the major macromolecule degraded by its histolytic trypsin-like proteinase, which suggests that this larval enzyme is capable of degrading one major component of gastrointestinal tissues. Keratin, another constituent of tissues, was also hydrolyzed significantly. Elastin, which is only a minor component of these tissues, was barely degraded. The intestinal lumen of the L3 was open and contained remnants of tissue, but in contrast to the adult, the L3 larva does not possess a circumoral row of teeth (Taraschewski and Stemmer 1992). Similar to what has been postulated for the penetrating larvae of the fly *Hypoderma lineatum* (Lecroisey et al. 1983), the L3 of *A. crassus* probably reabsorbs the secreted proteinase together with the degradation products of the gastrointestinal tissue. The proteinase activity of the L3 larva of *Anisakis simplex* (Sakanari and McKerrow 1990) resembles that of the L3 of *A. crassus*. By contrast, elastase activity at neutral pH has been identified in L3 larvae of *Strongyloides stercoralis* (McKerrow et al. 1990).

Scanning electron micrographs of the mouth opening of adult *A. crassus*, showing circumoral teeth and ingested erythrocytes of the eel host (Taraschewski et al. 1987), suggest that the adult stage of this parasite does not need any histolytic proteinases to bore the swimbladder wall for ingestion of blood. Therefore, the aspartyl proteinase of adult *A. crassus* seems to function by degrading the hemoglobin of the ingested erythrocytes.

The alteration of the proteinases from a histolytic to a digestive character during the life cycle of *A. crassus* corresponds with the chemotherapeutic efficacy of levamisole. Only the adult stage provided with a hemoglobinolytic proteinase is affected by the drug (dissolved

in the blood of the eel), whereas the (non-blood-sucking) L3 stage, equipped only with a histolytic proteinase, is not affected (Hartmann 1989). The change observed in the proteinase class during the life cycle of *A. crassus* is similar to the differences seen in proteolytic activity between the larval (Knox and Kennedy 1988) and the adult (Rupova et al. 1984) stages of *Ascaris suum*. However, the appearance of an aspartyl proteinase with a preference for hemoglobin is somewhat surprising, since the adult stage of this nematode is not considered to suck blood. In summary, the present study suggests that the changes in the type of proteinase during the development of *A. crassus* are an adaptation of the parasite to alterations in its environmental conditions.

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