Localization of a 24-kDa collagenase in the *Gymnorhynchus gigas* plerocercoid

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Abstract

A 24-kDa collagenase was localized in the *Gymnorhynchus gigas* plerocercoid immunohistochemically by peroxidase complex staining using polyclonal antibodies from NMRI mouse sera immunized with purified enzyme. Immunoreactivity was determined at different parts of the body (scolex, vesicle and caudal region) and mainly localized in microtriches and parenchymal tissues of the scolex and vesicle. These results, along with the absence of the enzyme in the plerocercoid excretion–secretion products, suggest that the 24-kDa collagenase is produced by parenchymal cells in the anterior region and transported to the outer regions of the worm It is possible that the enzyme plays an important role in degrading parasite tissues during the moulting process.

Introduction

Gymnorhynchus gigas is a common fish parasite that invades muscle tissue in teleosts (intermediate host) and the gastrointestinal system of elasmobranchs (definitive host) (Campbell & Beveridge, 1994). To date, many of its biological aspects including its interrelationships with hosts are unknown. In a previous study (Vázquez-López *et al*., 1999) we determined the existence of multiple endo- and exo- proteases in parasite crude extracts. We have also described and characterized a 24-kDa protease with high activity against collagen under pH and temperature conditions found in the parasite's hosts. Moreover, this activity was inhibited by specific cysteine and metallo-protease inhibitors (principal protease types implicated in degradation of tissues). Based on these observations, we postulated that this enzyme might be implicated in penetration processes into the teleost muscle or the gastrointestinal system of elasmobranchs as well as in the moulting processes (Vázquez-López *et al*., 1999). However the localization of the enzyme and its possible mechanisms of secretion/excretion are not yet understood. The aim of the present study was to examine, by means of immunohistochemistry, the distribution of the 24-kDa collagenase in the different parts of the *G. gigas* plerocercoid body. We also examined its presence in excretion secretion (ES) products of the pleroceroid to study its possible role in the biology of the parasite.

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Materials and methods

Parasite

Gymnorhynchus gigas plerocercoids obtained from *Brama raii* musculature, naturally infected, were washed by agitation until the surrounding membrane was eliminated. The wash medium comprised: 10 mM phosphate-buffered saline (PBS, pH 7.2) supplemented with penicillin (1 mg ml^{-1}) and streptomycin sulphate (2 mg ml^{-1}) to avoid possible contamination.

Incubation of the plerocercoids and collection of the ES products

For the collection of the ES products, vital plerocercoids from eight worms were incubated in Petri plates with a culture medium at room temperature (22°C) for 96 h, under aseptic conditions. The culture medium comprised: 199 medium with Earl's salts and 2.20 g l^{-1} $NaHCO_3$ (ICN Flow; ICN Biomedicals, Barcelona, Spain), which was buffered with Hepes to pH 7.3 and supplemented with L-glutamine (0.1 mg ml^{-1}), penicillin (1 mg ml^{-1}), streptomycin sulphate (2 mg ml^{-1}) and 0.65% (wt vol^{-1}) glucose. The medium was changed every 12 h, filtered, pooled, dialysed against saline

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solution (0.9% NaCl) overnight at 4°C and concentrated in a Centricon-10 protein ultraconcentrator (AMICON, Beverly, Massachusetts) with a size exclusion of 10-kDa. The final protein concentration in the ES products sample, prepared as above was determined by the Lowry method (1951) and adjusted to a final concentration of 3 mg ml^{-1}. This ES product sample was stored at −80°C until use to determine the presence of the 24-kDa collagenase by enzyme assays or by an immunoblot procedure using a polyclonal specific anti-24-kDa collagenase.

Enzyme assays

Proteolytic activity in the plerocercoid ES products sample was determined by spectrophotometric assays as described Vázquez-López *et al*. (1999) using different pH and substrates. The substrates assayed were: azocoll, collagen (insoluble collagen from bovine Achilles tendon type V), elastin-orcein, keratin-azure, haemoglobin (from pig), plasma (from pig) and IgG (whole-molecule goat antihuman IgG) at 5 mg ml^{-1} (Sigma Chemical Co., St Louis, Missouri). The effect of pH on the proteolytic activity was tested at acid, neutral and basic pH using a citrate–citric acid buffer (pH 4) and phosphate buffered saline, PBS, (pH 7 and 8). Absorbance of supernatants was tested, following 24 h of incubation at 22°C.

Immunohistochemical localization

For the immunohistochemical localization of the 24-kDa collagenase, we employed peroxidase complex staining using polyclonal antibodies from NMRI mouse serum immunized with purified 24-kDa collagenase. The protease was purified as previously described (Vázquez-López *et al*., 1999).

Preparation of polyclonal specific antibodies to the 24-kDa collagenase

To prepare anti-24-kDa antibodies, NMRI mice were injected intraperitoneally three times, on days 0, +1 and +10, with 6.25 μg of the purified 24-kDa collagenase using 2′-2-dideoxiadenosin (DDA) as adjuvant (1 mg ml^{-1}). On day +21 the mice were bled using Nöller's technique and sera were separated using vacutainer tubes. Sera containing anti-24-kDa-antibodies were selected by enzyme-linked immunosorbent assay (ELISA), Western blot procedures and collagenase inhibition assays.

ELISA technique

The ELISA technique described by Armas-Serra *et al*. (1995a) was adopted. One hundred microlitres of purified 24-kDa protease (Vázquez-López *et al*., 1999) (5 μg ml^{-1} protein) in sodium carbonate buffer (0.1 M, pH 9.6) were coated on to wells of polystyrene plates (Costar, USA) at 37°C for 2 h. After coating and between all subsequent steps, the plates were washed four times in PBS, pH 7.4 containing 0.05% Tween-20 (PBSTw). As a blocking agent, 200 μl of 1% BSA in PBSTw was added and incubated for 1 h at 37°C. One hundred microlitres of diluted sera (1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640) (normal or anti-24-kDa) were incubated for 1 h at 37°C and after subsequent washing, 100 μl of antimouse IgG (whole molecule) peroxidase conjugate developed in sheep (Sigma Chemical Co., St Louis, Missouri) were reacted at dilutions 1:500, 1:1000 and 1:2000 for 1 h at 37°C. A colour reaction was developed by the addition of 100 μl of 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and H_2O_2 and stopped after 20 min by the addition of 50 μl 0.2 M fluorhydric acid (HF). Absorbance was read at 405 nm.

Immunoblot procedure

The immunoblot procedure described by Armas-Serra *et al*. (1995a) was followed. Plerocercoid crude extract, purified 24-kDa collagenase and ES products were electrophoresed in a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then equilibrated in Western blot buffer (25 mM TRIS, 192 mM glycine, 20% methanol, pH 8.3) for 10 min and protein bands were electroblotted onto nitrocellulose paper overnight at 40 mA and 4°C. The blots were blocked for 30 min with PBSTw and then for 1 h with PBSTw containg 5% (w/v) non-fat powdered milk. Confirmed anti 24-kDa collagenase sera by ELISA or normal sera were diluted 1:200 in PBSTw and reacted for 2 h at room temperature. After three rinses in PBSTw and incubation with antimouse IgG (whole molecule) peroxidase conjugate developed in sheep (Sigma) for 2 h at a dilution of 1:500 in PBSTw, blots were washed three times and developed using 3,3′-diaminobenzidine (Sigma). The colour reaction was stopped by washing the paper with distilled water.

Inhibition assays

The specificity of the antibodies against the 24-kDa protease was probed by collagenase inhibition assays, as described by Vázquez-López *et al*. (1999). Fifty microlitres of purified protease (0.0125 mg ml^{-1}) were incubated overnight with 5, 10 and 25 μl of confirmed anti 24-kDa collagenase sera (i.e. confirmed by ELISA and immunoblot) or normal sera and 200 μl of 100 mM citrate–citric acid buffer, pH 4, (optimal pH) at 22°C. The mixture was then incubated at 22°C for 10 min in the presence of collagen Achilles tendon type V (5 mg ml^{-1}) (Sigma), and the amount of hydrolysed substrate was determined using a ninhydrin assay as described by Armas-Serra *et al*. (1995b).

Immunolocalization

Sera containing the anti 24-kDa antibodies were selected and used in immunohistochemical localization assays following the procedure described by Pérez-Serrano *et al*. (1995). For these studies, the scolex, vesicle and caudal region of *G. gigas* plerocercoids (Casado *et al*., 1999) were separated and fixed with 3% paraformaldehyde in PBS (pH 7.2) for 3 h. After fixation, the three parts were processed for paraffin embedding. Sections of scolex, vesicle and caudal region were cut at 9 μm, deparaffinized then incubated with 3% H_2O_2 for 20 min to inhibit endogenous peroxidase activity. Sections were then incubated for 30 min in TRIS buffer with 5% (w/v) non-fat powdered milk at room temperature in a humidified chamber to block non-specific binding. Sections were then incubated without additional washing, at room temperature overnight at 4°C with sera containing

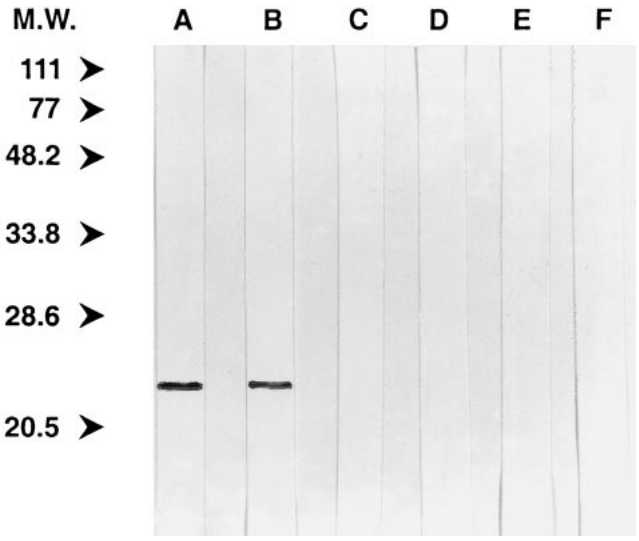

Fig. 1. Immunoblot analysis of the 24-kDa collagenase purified from the *Gymnorhynchus gigas* plerocercoid. Following SDS/PAGE of the crude extract (a, d), the purified 24-kDa protease (b, e) or ES products (c,f), blots were probed with anti-24-kDa collagenase sera (a,b,c) or control sera (d,e,f). Molecular weight markers are shown on the left.

anti 24-kDa antibody diluted in TRIS buffer at pH 7.6 (1:10 dilution). After three 5 min washes in TRIS buffer the sections were incubated for 90 min at 22°C in antimouse IgG (whole molecule) peroxidase conjugate developed in sheep (Sigma) at 1:500 dilution, then rinsed three times in TRIS buffer. Peroxidase activity was revealed with 0.06% 3,3′-diaminobenzidine (Sigma) in TRIS buffer with 0.01% H_2O_2 for 5 min. Sections were washed in distilled water, dehydrated in graded concentrations of ethanol and mounted in DPX (Sigma). Control sections were processed using a similar procedure, but the primary antibody was replaced with sera from untreated mice.

Chemicals

Each of the proteinase substrates, peroxidase conjugate and electrophoresis calibration kits were purchased from Sigma. Other chemicals were of analytical grade.

Results and Discussion

We previously prepared antibodies to the enzymatic anti 24-kDa collagenase and validated their specificity by ELISA, immunoblot and enzymatic assays inhibition assays. As shown in fig. 1, SDS-PAGE/immunoblots of crude extract or pure protease using sera from mice inoculated with the 24-kDa protease revealed a reaction to a single band at 24-kDa, while normal sera did not show any reaction. These results were corroborated by inhibition assays (fig. 2). The collagenolytic activity was abolished in the presence of sera anti 24-kDa protease in a concentration-dependent manner relative to controls with no added sera or normal sera, as occurs with other parasite proteases (Armas-Serra *et al.*, 1995a).

The 24-kDa protease in *G. gigas* plerocercoids was localized in each part of the body examined (scolex, vesicle and caudal region) using polyclonal antibodies from NMRI mouse sera immunized with pure enzyme (fig. 3b,c,d). Control sections did not show immunoreactivity (fig. 3a). These staining patterns in each of the plerocercoid regions suggest that 24-kDa collagenase is one of the principal collagenases of the *G. gigas* plerocercoid as previously hypothesized (Vázquez-López *et al.*, 1999). Interestingly, it appears from our micrographs that the caudal region posesses the lowest collagenase activity, whereas the scolex and vesicle were

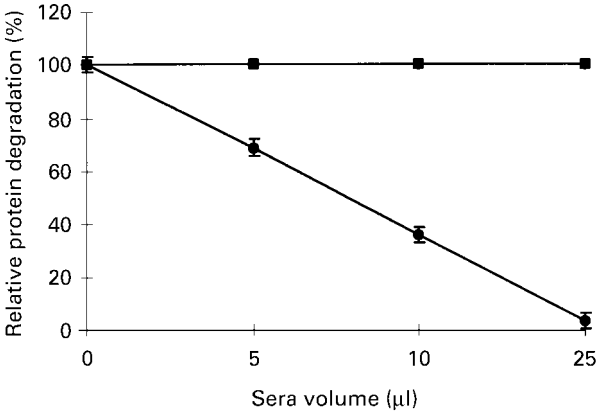

Fig. 2. Inhibitory effect of anti-24-kDa collagenase sera and normal sera on the proteinase activity of the purified 24-kDa collagenase as determined using collagen Achilles tendon type V as substrate. Relative protein degradation without sera (×), normal sera (■) and anti-24-kDa collagenase sera (●).

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darkly stained (fig. 3b,c,d). Similar results were obtained by other authors, as Kwa (1972a) for instance, demonstrated collagenolytic activity mainly in the scolex of *Spirometra erinacei* plerocercoids, suggesting the importance of this region in the synthesis of proteolytic enzymes in this cestode.

The distribution of the 24-kDa immunoreactivity in the different structures of the plerocercoid was not uniform. Our results demonstrate that the major immunoreactivity was localized in the outer regions of the scolex, vesicle and caudal regions of the tegument (fig. 3b,c,d) where microtriches are present (Casado *et al*., 1999). In the scolex and vesicle however, immunoreactivity was also very patent in parenchymal tissues (fig. 3b,c). Collectively, these data suggest that the 24-kDa collagenase is synthesized in gland cells within the parenchymal tissues as occurs in other plerocercoids (Osaki, 1990; Kim *et al*., 1992; Polzer & Conradt, 1994) and that in the *G. gigas* plerocercoid it seems to be principally present in the anterior region of the worm (scolex and vesicle) (N. Casado, personal communication). The enzyme is released from the worm following breakdown of the tegumental plasma membrane, as previously described for other cestodes (Kwa, 1972a; Ohman-James, 1973; Andersen, 1975; Kuperman & Davydov, 1982; Fukase *et al*., 1984, 1985; Kim *et al*., 1992; Polzer & Conradt, 1994). Following its release, the enzyme accumulates in the microtriche region, where it may play important biological roles. Kwa (1972a,b) and Kim *et al*., (1992) demonstrated a similar pattern of distribution for proteases in other cestodes, implicating these enzymes in two important biological mechanisms: (i) to facilitate the penetration process across host tissues by degradation of host tissue collagen; and (ii) to facilitate the moulting process. In

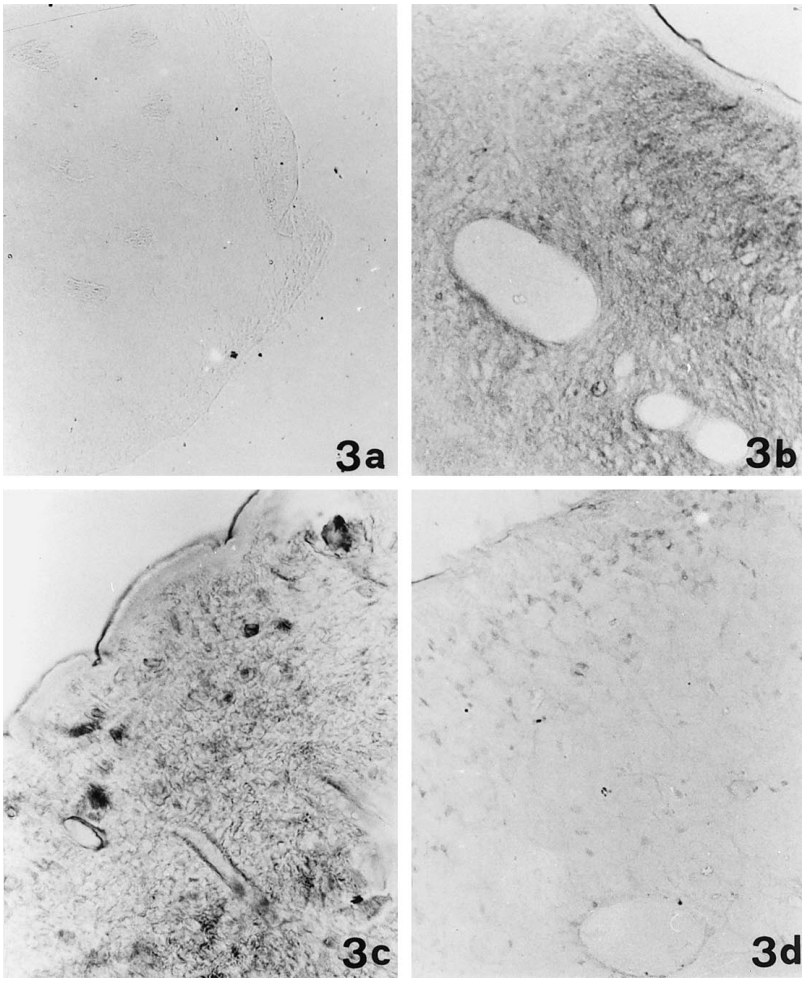

Fig. 3. Immunohistochemical localization of the 24-kDa collagenase in the *Gymnorhynchus gigas* plerocercoid by peroxidase complex staining using polyclonal antibodies from NMRI mouse sera immunized with purified enzyme: a) control section using a 1:10 diluted control sera as a primary antibody (×90), b) scolex region (×250), c) vesicle region (×150) and d) caudal region (×200).

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Table 1. Proteolytic activities of excretion-secretion products from *Gymnorhynchus gigas* plerocercoid to various substrates at acid, neutral and basic pH. Results are shown as absorbance ± S.E. of the supernatants after 24 h of incubation at 22°C.

	Substrates							
pH	Azocoll	Elastin-orcein	Keratin-azure	Haemoglobin	Plasma	IgG	Collagen	Fibrinogen
4	$0.008 \pm 1\ 10^{-2}$	$0.007 \pm 7\ 10^{-4}$	$0.083 \pm 21\ 10^{-4}$	$0.285 \pm 13\ 10^{-3}$	$0.0393 \pm 46\ 10^{-4}$	$0.0596 \pm 8\ 10^{-3}$	0	0
7	$0.663 \pm 8.9\ 10^{-2}$	$0.024 \pm 23\ 10^{-4}$	$0.016 \pm 17\ 10^{-4}$	$0.076 \pm 2\ 10^{-3}$	$0.0363 \pm 42\ 10^{-4}$	$0.0186 \pm 21\ 10^{-3}$	0	0
8	$0.257 \pm 4.2\ 10^{-2}$	$0.019 \pm 42\ 10^{-4}$	$0.0153 \pm 16\ 10^{-4}$	$0.092 \pm 1\ 10^{-3}$	$0.061 \pm 1\ 10^{-3}$	$0.0093 \pm 9\ 10^{-3}$	0	0

Note that these data indicate the activity against collagen is not released as an ES product.

order to clarify the biological role of the *G. gigas* 24-kDa collagenase, we analysed by immunoblot and enzymatic assays its presence in plerocercoid ES products. Results showed that the enzyme is not released as an ES product. So, SDS/PAGE immunoblots of ES products using normal sera or anti-24-kDa sera did not show a reaction (fig. 1) and by enzymatic assays no collagenolytic activity was detected (table 1) while each of the other substrates assayed were degraded under similar conditions as occurs in *G. gigas* plerocercoid crude extract (Vázquez-López *et al*., 1999).

These results, along with the localization of the 24-kDa collagenase in the anterior region of the plerocercoid of *G. gigas*, suggest that this larva stores the enzyme in the outer body region, principally the scolex and vesicle. This location suggests that the parasite uses the enzyme in the moulting process, degrading the collagen of the caudal region union, an early event required for successful infection of the elasmobranch host. Further investigations will be carried out to clarify additional biological aspects of this common but poorly characterized parasite.

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