Aspartic Proteinase in *Dugesia tigrina* (Girard) Planaria

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A proteolytic activity was identified in *Dugesia tigrina* planaria using the chromogenic substrate Phe–Ala–Ala–Phe (4-NO_2)–Phe–Val–Leu–O_4MP. The activity of the enzyme increased four times during the regeneration and presented a maximum at 120 hr being higher in tail than head regenerating segments. The protease that displays this activity was purified from worms by a single step on pepstatin-agarose followed by gel-filtration high performance liquid chromatography. The purification resulted in a 34-fold increase in specific activity and the final yield was 10%. The active *D. tigrina* hydrolase appears to be a dimeric protein composed of identical subunits with 34 kDa associated by disulphide bridges similar to vertebrate cathepsin D. By SDS-PAGE several bands were detected but upon gel filtration HPLC one proteolytically active component, termed Asp-68, was detected and isolated. The maximal activity was observed in a range between pH 3.5–5.0 and the enzyme became inactivated at a pH value above 7.2. The purified enzyme was not inhibited by inhibitors from serine (aprotinin, TPCK, PMSF and TLCK), metallo (EDTA) and cysteine proteinase (E-64) classes. In contrast, inhibitors such as pepstatin, EPNP, and 4-β-PMA efficiently inhibited the activity of the 68-kDa protease.

Introduction

Regenerating planarians are in a special physiological state which molecular basis is poorly understood. The morphological aspects of its regenerative process are well known and it appears that the formation of new tissues consists of two distinct stages involving the programming of non-differentiated blastema cells followed by differentiation of cells which become part of missing structures (Spiegelman and Duddley, 1973; Hori, 1992). However, localized degradation of the extracellular matrix and tissues are necessary for cells to migrate, and may comprise many proteolytic enzymes. This fact is supported by the evidence that: (a) an increasing cell afflux containing high lysosomes number (Coward *et al.*, 1974) which migrate to the tissue repairing, and (b) the digestion of exogenous material occurs intracellularly in planaria.

Despite the great amount of information available about morphological alteration (Slack, 1987) and some biochemical metabolic changes (Nishimura *et al.*, 1988; Nery da Matta *et al.*, 1992; 1993; 1994) during the regeneration, few data exist upon the role played by hydrolases during this complex biological process (Malczewska *et al.*, 1980; Landsperger *et al.*, 1981; Giovanni De Simone *et al.*, 1994).

Aspartic proteinases constitute a relatively small group of homologous proteolytic enzymes involved in a wide range of cellular functions, including removal of signal peptides from nascent poly-

Abbreviations: AP, aspartic proteinase; BSA, bovine serum albumin; E-64, L-*trans*-epoxysuccinyleucylamido-(4-guanidino) butane; EDTA, ethylenediaminetetraacetic acid; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; Hb, haemoglobin; HEPES, [N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid)]; HPLC, high performance liquid chromatography; Mes, [2-(N-morpholine-ethane-sulphonic acid) monohydrate]; NOG, *n*-octylglycoside; 4-β-PMA, 4-phorbol myristate; PBS, phosphate buffer pH 7.3 containing 150 mM NaCl; PMSF, phenylmethysulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TLCK, *N*-tosyl-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

peptides, enzyme activation, protein degradation, and cellular reorganization. Notwithstanding, they have received much interest because several play significant roles in human diseases. These enzymes have been found to be widely distributed in fungi, higher plants and mammalian cells. They are active at low or neutral pH and are characterized by having two aspartic residues in their catalytic sites. Most of them have molecular mass of about 40 kDa and have homologous sequences varying between 323 and 340 amino acid residues in length. These hydrolases arise to be synthesized in the form of precursors (Kay, 1985) and are found in the mature state predominantly in two chains form (Tang and Lin, 1994). As there is no information about the proteolytic maturation mechanism of the vacuolar enzymes of planaria and of the extensive protein degradation that takes place during the regeneration, it was of interest to purify proteinases of this animal in order to investigate its role in the course of the regenerative process. In this work, we describe the purification and partial characterization of the aspartic proteinase from *Dugesia tigrina* planaria. The data will contribute to the identification of important proteinases involved in the turn over of proteins of this animal and could help to understand some of the metabolic changes associated with the regenerative process.

Materials and Methods

Materials

Pepstatin A-agarose, N-octylglycoside, MES, TPCK, TLCK, SBTI, 4-PMA, aprotinine, gelatine, haemoglobin, Triton X-100, SDS, E-64 and Phe–Ala–Ala–Phe (4-NO_2)–Phe–Val–Leu–O_4MP were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Coomassie brilliant blue, molecular weight standard proteins and PVDF membrane were from Bio-Rad (Richmond, CA, U.S.A.). P10 and HV filters were obtained from Amicon Corp. (U.S.A.) and Millipore Corp. (MI, U.S.A.), respectively. The Shimpack Diol-150 HPLC column was from Wako Chemicals (U.S.A.), the electrophoresis reagents were from Serva (Heidelberg, Germany) and all other chemicals were from Merck (Darmstadt, Germany).

Planaria culture

Planarians were kept at room temperature (20–25 °C) in plastic trays containing spring water (purity) and fed once a week with fresh chicken liver. Before experiments, the animals were fasted for one week. For regeneration studies, worms were cut transversely into sections by means of an incision behind the auricles. Cephalic and caudal sections were collected in two separate fresh water trays at room temperature. Samples were collected every 24 h until completion of the regeneration process (192 hr).

Extract and pepstatin-agarose chromatography

After washing, regenerating planarians segments were disrupted by homogenizing in a potter homogenizer using distillate water and freeze-thawed four times in PBS. After dialysed against 0.1 M acetate buffer, pH 3.5, containing 0.1 M NaCl, the homogenates which derived from intact planarian were centrifuged at 105,000×*g* for 30 min at 4 °C and the supernatants applied immediately on a pepstatin-A agarose column (10 × 1 cm, I.D.), previously equilibrated in the same buffer. After washing with acetate buffer (10 bed volumes) the bound proteins were eluted with 0.1 M Tris-HCl buffer, pH 8.6, containing 1.0 M NaCl, pooled and concentrated using P10 microconcentrators.

Gel filtration high performance liquid chromatography and determination of molecular mass

After concentration, the pooled pepstatin-fractionated proteins were filtered (0.22 μm, HV nylon filters) and injected in a Shinpack Diol-150 (50 cm × 7.9 mm, I.D.) HPLC column previously equilibrated in 50 mM phosphate buffer pH 7.2. The proteins were fractionated on an automatic HPLC system (Shimadzu, 6A model), using the same equilibrating buffer, at a flow rate of 1 ml min^{-1}, during 28 min at 25 °C. Fractions were collected manually, pooled and concentrated using centrifugal ultrafiltration microconcentrators and stored at −20 °C for further studies. For molecular mass characterization the column was calibrated in the same buffer with the following markers: apoferritin (M_r 440,000), β-galactosidase (M_r 105,000),

bovine serum albumin (M_r 68,000), ovalbumin (M_r 45,000), and carbonic anhydrase (M_r 29,000).

Effect of pH

The effect of pH on the enzymatic activity was assayed under standard conditions and with a combination of following buffers: 0.1M sodium citrate (pH 2.5–3.0), sodium acetate (pH 3.5–5.5), Mes (pH 5.0–7.0), sodium phosphate (pH 6.0–7.5), HEPES (7.5–8.5) and Tris-HCl (7.5–9.5).

Enzymatic assays and protein estimation

Activity on haemoglobin was determined as described (Barret and Heath, 1977). The reaction mixture which contained 200 µl of 1% (w/v) Hb solution (dialyzed against 0.1 M sodium acetate buffer, pH 3.5) and 50 µl of enzyme solution, was incubated at 37 °C for 30 min. The reaction was stopped by adding 250 µl of 10% (w/v) ice-cold trichloroacetic acid (TCA) solution. The mixture was allowed to stand for 20 min and centrifuged, and the liberated peptides in the supernatant were read at 280 nm in a Hitachi spectrophotometer (model U 2000). This photometric absorbance assay is linear in the range of 0.01 to 1.0 AU. One unit (U) of specific activity was defined as the change in absorbance at 280 nm·min^{-1}·mg protein^{-1} at pH 3.5 and 37 °C. In all experiments control without enzyme was performed. Protein was estimated using the Lowry's method (Lowry *et al.*, 1951).

Polyacrylamide gel electrophoresis and detection of proteinase activity

The SDS-polyacrylamide gel electrophoresis was performed using 12.5% polyacrylamide gels in Laemmli buffers (Laemmli, 1970) under reducing and non-reducing conditions. The gels were silver (Bio-Rad staining kit) or coomassie blue R-250 stained. BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and trypsinogen (24 kDa) were used as standards for characterization of molecular mass. Stained protein bands were analysed (560 nm) and quantified with an analytical imaging instrument (Biomax, Millipore, MI, U.S.A.)

Inhibition studies

Inhibition studies were carried out a 100 µl reaction volume of 100 mM sodium acetate buffer, pH 3.5, containing 1 M NaCl, 10 µg enzyme (10 µl) and 10 µM – 1 mM of inhibitors (Table II). Each mixture was incubated for 15 min at room temperature (25 °C). Following incubation, 30 µl of 0.05 mM Phe–Ala–Ala–Phe (4-NO_2)–Phe–Val–Leu–O_4MP (in acetate buffer, pH 3.5) was added, and the absorbance change recorded at 300 nm, after 30 min of incubation at 25 °C. Control solutions, lacking inhibitors, were run simultaneously. Inhibition was expressed as per cent of the appropriate control activity.

Results

Characterization of proteolytic activity during the regeneration

The total activity of aspartic proteinase (AP) as measured using Hb as substrate was elevated at 120 h of both regenerating head and tail segments. Quantitative estimation of AP typically revealed approximately a 4-fold increase in hydrolytic activity during 96–168 h of tail regenerating (Fig. 1).

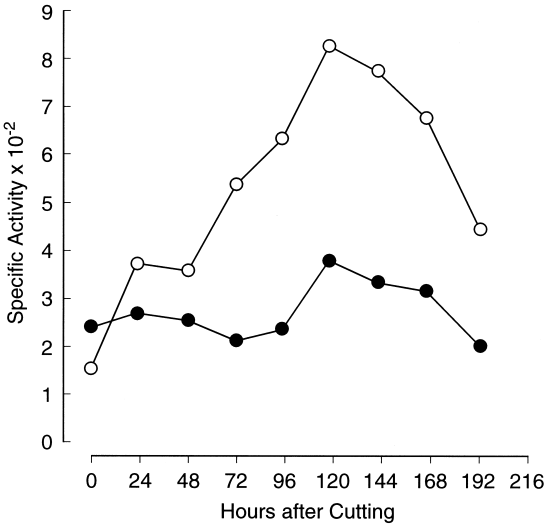

Fig. 1. Profiles of specific activity level of aspartic proteinase during the regeneration of the tail (○—○) and head (●—●) of *D. tigrina* planaria using haemoglobin as substrate. The enzymatic activity was expressed in OD 280 nm·min^{-1}·mg protein^{-1}. The results represent the mean of three experiments.

Purification of AP from D. tigrina

The purification of *D. tigrina* aspartic proteinase was monitored with Hb as substrate. The crude extract (3 g) of packed worms was chromatographed on a pepstatin-agarose column and about 120 μg of partially purified enzyme was obtained. A SDS-PAGE of pooled material is shown in Fig. 2A. Three minor bands with M_r 65 kDa, 60 kDa, 34 kDa and three major bands with M_r 21 kDa, 19 kDa and 16 kDa (Fig. 2A, lanes b and c) were seen by Coomassie blue staining. This electrophoretic behaviour was however influenced by reduction, the non-reduced enzyme (Fig. 2B) had a higher electrophoretic mobility (M_r 121 kDa, 34 kDa, 21–16 kDa) than the fully reduced samples, suggesting that the enzyme may exist as a homodimer connected by disulphide-bond(s) (Table I).

In order to purify and determine the M_r of the proteinase in their native state, HPLC gel-filtration was performed. Three major density peaks with retention time of 10.05 (125 kDa), 12.84 (68 kDa) and 20–21 min (20 kDa) were detected but only one of them (68 kDa) coincided with the proteinase activity peak (Fig. 3). This peak contained 1–2% of the absorbing material (Table I). At this point there was only a slight increase in specific activity during gel filtration (data not shown), thus indicating that the proteinase preparation was homogeneous. The proteinase was purified 36-fold with a yield of 10%.

Table I. Relative amounts of proteins determined in purified *D. tigrina* preparation by densitometric scanning of SDS-PAGE and HPLC.

Procedure	Molecular weight [kDa]	Relative % of proteinase forms
HPLC	125	34.2 ± 3.60
	68	1.5 ± 0.08
	≤20	64.2 ± 1.65
Gel scanning	65–60	24 ± 12
	31–24	18 ± 7
	≤24	58 ± 11

Both results represent the mean ± SD of three independent experiments.

Optimum pH and inhibition studies

The purified planaria proteinase (68 kDa) was maximally active at pH 3.5–4.0 with a significant reduction in activity noted at pH 3.0 or 5.0 (Fig. 4).

As shown in Table II, this enzyme was strongly inhibited by typical AP inhibitors, such as peps-

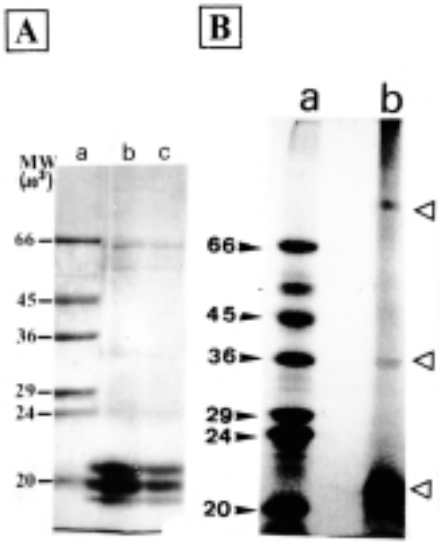

Fig. 2. SDS-PAGE (12.5%) under reducing (A) and non reducing (B) conditions of pooled peak from pepstatin-agarose column followed by staining with Coomassie blue-R 250. Lanes: a, standard molecular weight; b and c, purified proteinase (30 and 10 μg respectively). In the lane Bb about 45 μg of protein was applied.

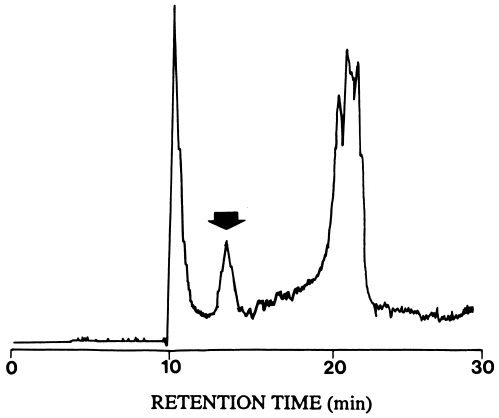

Fig. 3. Gel filtration chromatography analysis of the pepstatin-agarose purified proteinase using a Shinpack Diol-150 HPLC column (50 cm × 7.9 mm I.D.). About 100 μg of protein were analysed and elution performed using 10 mM phosphate buffer (pH 7.3) at a flow-rate of 1 ml·min^{-1}. Absorbance was measured at 280 nm using AUFS = 4. Protein peak with Hb-hydrolysing activity (retention time of 12.84 min) is indicated. This peak corresponds to a 1.6–1.8% of the total fractioned protein.

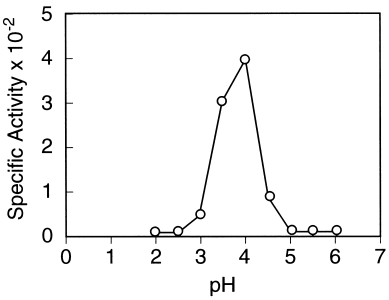

Fig. 4. Determination of the optimum pH of the aspartic proteinase activity on Hb. The enzyme was diluted in 100 mM of following buffers: -sodium citrate (pH 2.5–3.0), sodium acetate (pH 3.5–5.5), Mes (pH 5.0–7.0), -sodium phosphate (pH 6.0–7.5), HEPES (pH 7.5–8.5), and Tris-HCl (pH 7.5–9.5).

tatin (52%), EPNP (79%), and 4β-PMA (83%). Serine (TLCK, TPCK, PMSF, aprotinin and SBTI), metallo (EDTA) and cysteine (E-64) proteinase inhibitors did not show any effect. Thus, the inhibition studies indicate that the enzyme is a member of the pepsin family.

Stability and self digestion

Storage of the cell extracts at 4 °C for 1–2 days after disintegration of the cells resulted in an almost complete loss (>90%) of the proteinase activity.

Table II. Effect of inhibitors on the *D. tigrina* aspartic proteinase activity. The results are the means of two independent experiments (±5%) using Phe–Ala–Ala–Ala–Phe–(4-NO_2)–Phe–Val–Leu–O_4MP as substrate.

Reagents	Concentration	Residual activity [%]
Control	–	100
Aprotinin	100 μM	100
TLCK	100 μM	100
TPCK	100 μM	100
PMSF	1 mM	100
SBTI	50 μM	99
EDTA	10 mM	100
E-64	10 μM	90
pepstatin	10 μM	48
EPNP	10 μM	21
4β-PMA	10 μM	17

Abreviations: EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; 4-β-PMA, 4 phorbol myristate; PMSF, phenylmethysulfonyl fluoride; SBTI, soybean trypsin inhibitor; TLCK, *N*-tosyl-lysyne chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

In order to investigate if the 125 kDa protein may generate the active enzyme, a sample was maintained at pH 4.0 and pH 7.2 for 2 h and analysed by HPLC (Fig. 5). A graduate decreasing of the high molecular peak (125 kDa protein) with simultaneous increasing of low molecular weight peaks was observed only when the sample was incubated at pH 4.0. This suggests that the 125-kDa protein is a precursor form of the enzyme and that it is activated at low pH inducing self-digestion.

Discussion

Proteolytic enzymes play important roles in the life cycles of all uni- and multicellular organisms. The present results of the regeneration experiments show that the activity of AP increases approximately 4 times during the reabsorption phase and decreases to normal level after completion of the regeneration (Fig. 1). In order to establish the role of this enzyme in the regenerative process of planarians, it was necessary to obtain the enzyme in an apparent homogeneous state. The interpretation of the SDS-PAGE results for pepstatin-agarose isolated AP was initially rather difficult due to the presence of different forms of the enzyme. However, using the gel filtration HPLC step it was possible to identify the active form of the proteinase. Thus the mature form of the enzyme appears

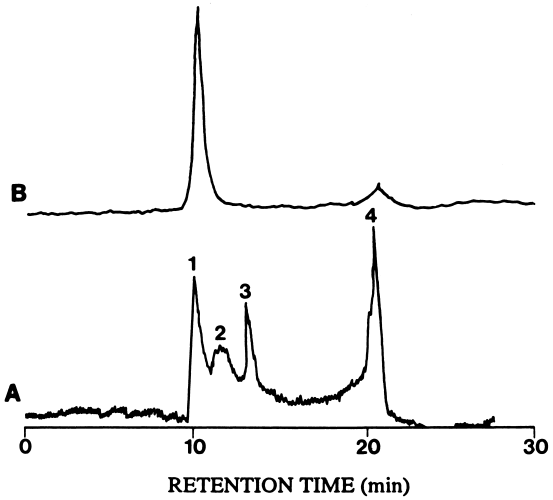

Fig. 5. HPLC of purified 125 kDa protein incubated for 2 h at pH 3.5 (A) and pH 7.0 (B). The chromatographic conditions were the same as in Fig. 3.

to be a dimeric protein composed by identical subunits of 34 kDa associated by disulphide bridges similar to the major acidic proteinases.

The AP enzymes of vertebrate/fungal origin consist of 323–340 amino acid residues and these have molecular mass of 40 kDa with exception of cathepsin E (Rawings and Barret, 1994; Hill and Phylip, 1997). An extra cys residue located at position-6 is responsible for dimerization of two identical 40 kDa subunits to give rise to the 80 kDa form of the native enzyme. By contrast, the retroviral members of the family including the proteases from HIV-1 and HIV-2 contain only 100 residues and form active enzymes by non-covalent dimerization (Kay and Dunn, 1990). An interesting phenomenon observed was the apparently spontaneous activation of the high molecular weight form of the AP enzyme at low pH. This was demonstrated in the present paper following its substantial alteration of the chromatographic behaviour and yield of the enzyme at pH 4.0 (Fig. 5) but appears not to be restricted to planarian enzyme but extensive to most AP studied so far (Hara *et al*., 1993). Several studies on biosynthesis of cathepsin D have revealed that this protein is synthesized as a prepro-enzyme which is activated, most likely in lysosomes, to a single-chain cathepsin and/or two-chains enzyme (Hasilik and Neufeuld, 1980; Hasilik *et al*., 1982). The fact the incubation of the 125 kDa form of the enzyme at low pH induced its self transformation in to several intermediary polypeptides indicates that it may be activated and that it may contain the pre-pro region of the enzyme (Nishimura *et al*., 1988; Tang and Lin, 1994).

Although some cysteine proteinases express activity under acidic conditions and some aspartic proteinases are active at near neutral pHs, the low pH optimum for the proteinase suggests that it belongs to the aspartic class of proteinase. This conclusion is supported by the finding that the 68 kDa proteinase activity was not inhibited by a range of serine, metallo, and cysteine inhibitors but was susceptible to the microbial proteinase inhibitor pepstatin and the active site-directed affinity label EPNP (Kay, 1985).

The activity at around pH 4.0, which corresponds to the physiological pH within the lysosomes, also suggests that the present enzyme exists and functions in these organelles. However, the activity detected during the major phase of tissue reabsorption likewise indicates that the enzyme is involved in the tissue remodeling phase. This fact is reinforced by the finding that the activity of cathepsins is major in tissue subjacent to the regenerating blastema. Consequently, their probable contribution is in a phase subsequent to the regeneration providing sufficient amino acid pool for protein synthesis. The other important physiological phenomenon, which may be played by this enzyme is in the maintenance of the dynamic equilibrium of intracellular proteins, contributing to protein degradation while other proteins are being resynthesized. Whatever the origin and possible function of the aspartic proteinase may be, it must fulfil an important role in the regenerating process of planarian.

In summary, the cathepsin D like enzyme from *D. tigrina* planaria was purified and partially characterized. The finding that a high enzymatic level is correlated with the phase of differentiation (81–160 h), suggested that it may play (a) an active role in the degradation of the extracellular matrix, promoting the enhance of new migration cells and constitutions of new tissues, or (b) a general physiological function in post-translational modification of proteins as demonstrated in several other systems (Nishimura and Kato, 1988; Nishimura *et al.,* 1989; 1990; Wiederander and Kirschke, 1989). Further studies are in progress to define the natural substrate(s) of this enzyme.

Acknowledgements

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