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Isolation and characterization of a protease from the marine sponge *Spheciospongia vesparia*

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A protein that showed activity against proteic (casein and hide powder azure) and synthetic (BAEE and HLPA) substrates was isolated from the marine sponge *Spheciospongia vesparia* The protease was purified from an aqueous extract by ammonium sulfate precipitation, gel filtration, hydrophobic and HPLC-anion exchange chromatographies. The purified protease showed a single band in SDS-PAGE minigels and had a molecular weight of 29,600, but when submitted to isoelectric focusing it showed 2 bands with isoelectric points of 4.56 and 4.43. Its catalytic action was inhibited by EDTA and 1,10-phenanthroline, so it seemed to be a metalloprotease.

Metalloprotease; Sponge; *Spheciospongia vesparia*

1. INTRODUCTION

Studies on marine sponges have focused mainly on small molecular weight compounds with a great variety of chemical structures and pharmacological properties. Proteins, enzymes and polypeptides have received little attention although they may be of greater importance. This is partly due to difficulties in obtaining pure substances. The most studied proteins have been the hemagglutinins [1–3] and aggregation factors [4,5]. Also, some toxic proteins are known. A lethal protein, named suberitine, isolated from the marine sponge *Suberites domuncula*, showed neurotoxic, ATPase and hemolytic properties [6]. In 1989 Hiratsuka [7] isolated a peptide (M_r 6,300) which was found to be a potent inhibitor for the K^+, Ca^{2+} and Mg^{2+}-ATPases of myosin, and actomyosin. Sova et al. [8] found a high-molecular-weight inhibitor of β-1,3 glucanases. This was a glycoprotein (M_r ~100,000) with a carbohydrate content of 40%

Agrell [9] reported in extracts of *Halicondria panicea*, amilolytic, lipolytic and proteolytic activities, the last one having pH optima at 4.3–6.8. More recently, an arginine kinase was isolated from several species (*Grantia compresa, Halichondria panicea*, etc.) and a creatine kinase from *Tethya aurantium* [10]. These enzymes are thought to be part of the energy metabolism of these organisms.

In the present paper we report the isolation and characterization of a protease from *Spheciospongia vesparia*, as well as information on its specificity.

Abbreviations: BAEE, α-*N*-benzoyl-L-arginine ethyl ester; BAPNA, α-*N*-benzoyl-D-arginine *p*-nitroanilide; BCA, bicinchoninic acid; HLPA, hippuryl-L-phenylalanine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid; EDTA ethylenediaminetetraacetic acid; PMA, phenyl mercuric acetate; PMSF, phenylmethylsulphonyl fluoride; HPLC, high performance liquid chromatography.

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2. EXPERIMENTAL

2.1. *Materials*

Ultrogel AcA-54, Octyl-Sepharose CL-4B, PhastGels for IEF, PAGE, PAGE-SDS, Low Molecular Weight Calibration Kit, Low pI Calibration Kit (pH 4–6.5) and Broad pI Calibration Kit (pH 3–10) were purchased from Pharmacia LKB Biotechnology Inc. Hide Powder Azure, casein, BAEE, HLPA, BAPNA, EDTA and 1,10-phenanthroline were from Sigma Chemical Co. The BCA and Micro BCA reagent concentrates were from Pierce. Other reagents were of biochemical research grade.

2.2. *Sponge extract*

Several specimens of *Spheciospongia vesparia*, collected in Puerto Morelos, Quintana Roo (−1 to −10 m), were transported frozen to the laboratory and stored at −25°C until used. A sample (500g wet weight) was cut into small pieces and washed thoroughly with tap water first, and then with distilled water. The following steps were performed at 4°C. The washed material was homogenized in a high speed blender with 300 ml of Tris buffer (0.05 M Tris-HCl pH 7.0, containing 0.03 M NaCl), then centrifuged for 50 min at 31,000×*g*. The precipitate was discarded and a two-step precipitation with ammonium sulfate was performed using the supernatant adjusted to pH 6.0. The firtst one (291 g/l; 50% saturation) was stored at 4°C for 12 h. After centrifugation (31,000×*g*, 45 min) the precipitate was discarded. The supernatant containing the activity was brought to 80% saturation. After 12 h the precipitate was collected centrifuging 45 min at 31,000×*g*, dissolved in water and dialyzed against the Tris buffer.

2.3. *Gel filtration chromatography*

The solution obtained in the previous step (35 ml) was loaded onto an Ultrogel AcA54 column (5×72 cm) equilibrated with the Tris

buffer. Fractions showing proteolytic activity were pooled and rechromatographed under the same conditions.

2.4. *Hydrophobic chromatography*

The active fractions were dialized against the elution buffer (0.05 M Tris-HCl, 0.3 M $(NH_4)_2SO_4$, pH 7.0) and applied to an Octyl Sepharose CL-4B column (1.6×43 cm). Unretained material was washed off with the starting buffer. The column was then washed with water and fractions containing activity were pooled and dialyzed against 0.05 M sodium phosphate buffer, pH 5.95.

2.5. *HPLC anion exchange chromatography*

The last preparation was then loaded onto a TSK-DEAE-5PW column (0.75×7.5 cm), equilibrated with 0.05 M sodium phosphate buffer, pH 5.95. Protein samples were eluted with a linear gradient of 0–1.0 M NaCl (0.03 M/min) in the same buffer.

2.6. *Activity measurements*

The protease activity was followed by two methods, the one proposed by Rinderknecht et al. [11] and by a casein assay similar to that previously described [12], but using 90 min at 37°C. One unit of activity corresponded to a change of 1.0 absorbance (A) U/min under the conditions described. The specific activity is the activity in U/mg of enzyme. Besides, activity over gelatin was assayed with the purified protease according to Pardo et al. [13].

2.7. *Effect of pH on hide powder azure hydrolysis*

To find the optimum pH for this protease, a curve of activity versus pH was obtained at 37°C. Fifty mM sodium citrate buffer was used in the pH range 3.0–6.0, 0.05 M Tris-HCl in the pH range 7.0–8.0, and 0.05 M glycine-NaOH buffer in the pH range 9–10.

2.8. *Enzyme assays*

In order to determine the substrate specificity of the sponge protease, several synthetic substrates were assayed. Initial rates of hydrolysis were determined using a Cary I spectrophotometer equipped with a thermostated cell compartment. Hydrolysis of BAEE was based on the method of Whitaker and Bender [14]. Activity was calculated on the basis of the increase in absorbance at 253 nm. Hydrolysis of the *p*-nitrophenyl ester of *N*-carbobenzoxy lysine was based on the method of Silverstein [15]. The increase in absorbance was measured at 340 nm. For HLPA we used the method described by Folk and Schirmer [16]. Activity was determined as the increase in absorbance at 254 nm. For BAPNA hydrolysis, an assay method described by Fink et al. [17] was used.

2.9. *Inhibition studies*

Inhibition of the proteolytic (hide powder azure) and esterolytic (BAEE) activities of the sponge protease was determined by preincubation of the enzyme (30 min at 37°C) with each of the following inhibitors (0.1 mM): phenyl mercuric acetate, phenyl methyl sulphonyl fluoride, EDTA and 1,10-phenanthroline.

2.10. *Protein determinations*

The concentration of the protease in samples was determined using the Pierce BCA protein assay reagent and bovine serum albumin as a standard [18].

2.11. *SDS-PAGE*

Electrophoresis was performed using a Phast System (Pharmacia-LKB) and protocols from that company. Homogeneus (12.5% polyacrylamide) and gradient (8–25% polyacrylamide) gels (0.45×43×50 mm) were used together with native and SDS buffer strips. Band positions were determined by means of a LKB 2202 UltroScan Laser Densitometer.

2.12. *Isoelectric focusing*

Phast Gel IEF 3–9 slabs (0.35×43×50 mm), were run in a Phast System using the protocols from the company.

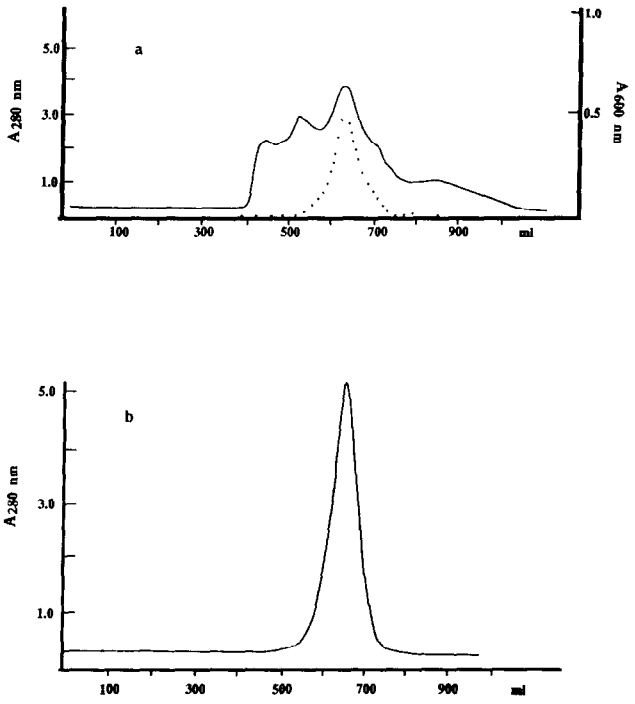

Fig. 1. Gel filtration chromatography of the redissolved ammonium sulfate precipitate (80% saturation) from the sponge extract. (a) The sample was applied to an Ultrogel AcA-54 column and eluted with 0.05 M Tris-HCl, 0.3 M NaCl pH 7.0 buffer. Solid and dotted lines represent absorbance at 280 nm and the activity against hide powder azure ($A_{600\ nm}$), respectively. (b) Rechromatography, under the same conditions, of the fractions containing the activity.

3. RESULTS

3.1. *Purification*

Fig. 1a shows the elution profile obtained after gel filtration chromatography. The proteolytic activity was found essentially in the main peak. Rechromatography of this fraction under the same conditions showed only one component (Fig. 1b) with specific activity of 0.14 U/mg. This active fraction was then applied to an Octyl-Sepharose CL-4B column and only two peaks were observed. The hydrophobic fraction eluted from the column with water contained the activity (0.94 U/mg). This fraction was further purified by means of HPLC on a TSK-DEAE-5PW column. The elution pattern revealed the presence of at least 6 components, but only one of them contained the proteolytic activity. This fraction was rechromatographed twice (Fig. 2), modifying slightly the NaCl gradient (0.02 M/min), and as observed in Fig. 2b, only one sharp symmetric peak was obtained. Table I summarizes the purification results; the most effective step in this procedure was the separation on the DEAE-TSK column, as is clearly demonstrated by the increase in the purification factor.

3.2. *Gel electrophoresis and isoelectric focusing*

The protease was tested for homogeneity by PAGE

where only one protein band was present. It also gave one single band by SDS electrophoresis (not shown), but when submitted to isoelectric focusing (pH gradient 3–9) gave 2 well-defined bands (Fig. 3). To determine their isoelectric points Phast Gels (pH 4.5–6) were used, obtaining the values of 4.56 and 4.43, making up 61% and 39%, respectively.

3.3. *Effect of pH on hide powder azure hydrolysis*

The optimum value for the hydrolysis of hide powder azure was obtained at pH 7.0.

3.4. *Substrate specificity and inhibitors*

The value of specific activity for the sponge protease against casein (2.51 U/mg) was of the order of those reported for other proteolytic enzymes like papain (2.14 U/mg) [19] and kallikrein (0.65 U/mg) [20]. This sponge enzyme did not show activivity towards gelatin. Concerning synthetic substrates, the sponge protease hydrolyzed in decreasing order BAEE, HLPA; and did not act upon BAPNA nor Nα-CBZ-Lysine-*p*-nitrophenyl ester. To compare the esterolytic activity of this protease with other proteolytic enzymes, the K_m value for BAEE was also determined, being 1.10 mM, similar to the value reported for cathepsin B (1.34 mM) [21] and much lower than that reported for papain (23 mM) [19] or asclepain (17.7 mM) [22].

On the other hand, typical inhibitors of metalloproteases, such as 1,10-phenantroline and EDTA, inhibited the sponge protease, while cysteine protease inhibitors, like PCA, or serine protease inhibitors, like PMSF, were ineffective.

3.5. *Molecular weight*

The molecular weight of the purified sponge protease, determined by SDS electrophoresis in polyacrylamide gels, was 29,600.

4. DISCUSSION

It has been suggested that proteolytic enzymes play important roles in various aspects of animal development. On the other hand, information on the biochemistry of marine sponges is at this moment unknown so, in the present study, we isolated and characterized a protease from the marine sponge *Spheciospongia vesparia*. This enzyme seems to be present as two forms of slightly different pI, but with the same molecular mass (29,600 Da). The sponge protease showed proteolytic activity on substrates as casein and hide powder azure; it was also capable to hydrolyze ester and amide bonds. Proteolytic and esterolytic activities of the enzyme were both inhibited by EDTA or 1,10-phenantroline, suggesting that a metal atom is essential for its activity.

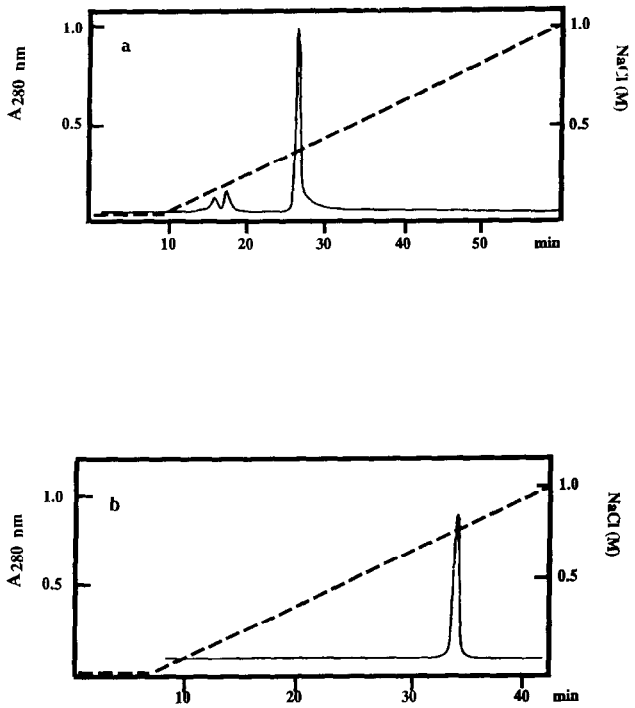

Fig. 2. Purification of the sponge protease by HPLC. The active material obtained from the hydrophobic chromatography was applied to a DEAE-TSK column, equilibrated with 0.5 M sodium phosphate buffer, pH 5.9, and eluted with a linear gradient. (a) First rechromatography of the active fraction, eluted with a linear NaCl gradient (0.03 M/min). (b) Second rechromatography with a slightly modified NaCl gradient (0.02 M/min)

Table 1

Purification of *Spheciospongia vesparia* protease

Purification step	Total volume (ml)	Total protein (mg/ml)	Specific activity (units/mg)[a]	Recovery %	Purification (fold)
1. Crude extract	540	2.14	0.016	100	1
2. Ultrogel AcA 54	60	0.316	0.14	22	9.0
3. Octyl Sepharose CL-4B	22	0.109	0.94	12.4	59.0
4. HPLC applied to column TSK DEAE 5PW	1.0	0.208	2.51	2.10	157

[a]One unit was defined as the amount of enzyme that caused an absorbance increase of 1.0 per min at 280 nm Activities were determined over casein.

1 2 3 4 5 6 7 8

Fig. 3. IEF on the PhastGel System of the sponge protease (Fig. 2b). Lane 2 and 4, 2.13 μg; lane 3, 1.32 μg; lane 5, 0.66 μg.

Also, the protease is rather unspecific, as it is the case for other metalloproteases like thermolysin [23], and carboxypeptidase A [24]. On the other hand, this protease did not act on gelatin, suggesting that it is not involved in the process of collagen catabolism in these organisms.

Further investigations on the metal content and the conformation of these enzymes are in progress.

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