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Authors: Azumi, Kaoru, and Yokosawa, Hideyoshi

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Characterization of Protease-Releasing Factors Isolated from Hemocytes of the Solitary Ascidian, *Halocynthia roretzi*

Kaoru Azumi* and Hideyoshi Yokosawa

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060, Japan

ABSTRACT—We have previously demonstrated that hemocytes of the solitary ascidian *Halocynthia roretzi* respond to several stimuli, such as calcium ionophore, lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate (PMA), and to release metalloproteases. Here, we show that *H. roretzi* hemocytes contained two substances, named protease-releasing factors A and B, which induced the release of metalloproteases from the hemocytes. Factor A was isolated from the acid-ethanol extract of hemocytes by gel filtration, while factor B was isolated from the hypotonic extract of hemocytes by cation exchange chromatography. The former factor was a heat-labile, large molecule and the latter was a heat-stable, small molecule. We found that these factors existed only in some types of hemocytes.

INTRODUCTION

In invertebrates, cellular and humoral reactions are two essential elements of the defense mechanisms of animals. Ascidians occupy a phylogenetical position between vertebrates and invertebrates. We have been investigating the defense mechanisms of a solitary ascidian, *Halocynthia roretzi* (Azumi and Yokosawa, 1996a). Hemocytes play a very important role in biological defense in *H. roretzi* (Azumi and Yokosawa, 1996a) as is the case in other ascidians (Wright, 1981), both of which lack immunoglobulins. In *H. roretzi*, hemocytes contain some humoral defense factors such as two antimicrobial substances (Azumi *et al.*, 1990) and LPS-binding protein (bacterial agglutinin) (Azumi *et al.*, 1991a). Also, *H. roretzi* hemocytes show several cellular defense reactions including phagocytosis against foreign materials (Ohtake *et al.*, 1994), aggregation at the site of the injured tunic (Takahashi *et al.*, 1994), release of metalloproteases in response to stimuli (Azumi *et al.*, 1991b), and release of phenoloxidase in a self- and nonself-recognition reaction (Akita and Hoshi, 1995).

In the course of our studies, we found that several types of hemocytes in *H. roretzi* hemolymph can be functionally distinguished on the basis of cellular reactions or abilities to produce defense-related substances (Azumi *et al.*, 1993). Recently, it has been reported that cytokine-like molecules exist in hemocytes or hemolymph of several invertebrates including ascidians and that they regulate the proliferation and cellular reactions of hemocytes (Beck and Habicht, 1996). In connection with this, we also found that *H. roretzi* plasma contained several growth factors that promoted the proliferation of mammalian cells (Iizuka *et al.*, 1997). In addition, *H. roretzi* plasma contains some factors that promote aggregation and phagocytosis by the hemocytes (Azumi and Yokosawa, 1996a). Taken together, it can be inferred that *H. roretzi* hemocytes or plasma contain some factors that act on the hemocytes and regulate their cellular reactions.

Here, we focused on the release of metalloproteases from *H. roretzi* hemocytes as one of the stimuli-induced cellular reactions and found two protease-releasing factors with different properties in *H. roretzi* hemocytes.

MATERIALS AND METHODS

Materials

Succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) and chymostatin were obtained from the Peptide Institute (Japan). Calcium ionophore (ionomycin) and lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 were purchased from Calbiochem Corp. (USA) and Difco Lab. (USA), respectively. β1-3 glucan was obtained from Wako Pure Chemical Ind. (Japan). Bestatin and leupeptin were provided by Dr. W. Tanaka of Nippon Kayaku Co. (Japan). Phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP) were purchased from Sigma Chemical Co. (USA). DEAE-Sepharose, CM-cellulose and Bio-Gel P-10 were obtained from Pharmacia Fine Chemicals AB (Sweden), Whatman Ltd (UK) and Bio-Rad Laboratories (USA), respectively. Bovine serum albumin (BSA, Fraction V) was obtained from Armour Pharmaceutical Co. (USA).

Collection of hemocytes

Solitary ascidians, *H. roretzi*, Type C (Numakunai and Hoshino, 1974), were harvested in Mutsu Bay, Japan. The hemolymph was collected from individual animals by cutting the tunic matrix without injuring internal organs. Hemocytes were collected by centrifugation (1,000 × g, 7 min) and were frozen at -20°C until use. Hemolymph from different individuals must not be mixed before centrifugation to

* Corresponding author: Tel. +81-11-706-3917; FAX. +81-11-706-4900.

avoid lysis of hemocytes due to contact reaction.

Extraction of protease-releasing factors from hemocytes

The extraction procedure was performed at 4°C as follows:

Factor A: Frozen hemocytes of *H. roretzi* (12 g wet weight) were thawed and homogenized with 50 ml of acid-ethanol solution (73% ethanol-0.18 M HCl) containing protease inhibitors (0.2 mM DFP, 0.1 mM leupeptin, 0.1 mM cymostatin and 0.4 mM PMSF) using a Teflon homogenizer (1,300 rpm, 5 strokes), and the solution was stirred overnight at 4°C. The homogenate was centrifuged (9,000 × g, 15 min), and the resulting supernatant was collected. The precipitate was extracted again by adding 50 ml of the above acid-ethanol solution and stirring for 4 hr at 4°C, and the precipitate was discarded after centrifugation. The supernatants (total 94 ml) obtained by the two extraction procedures were mixed, and the pH of the solution was adjusted to 5.2 with 1.1 ml of 2 M ammonium acetate. To the solution, 200 ml of cold (-20°C) ethanol followed by 400 ml of ether were added, and the mixture was stirred for 65 hr at -20°C. After centrifugation (9,000 × g, 15 min, -10°C), the resulting precipitate was dissolved in 40 ml of 1 M acetic acid and used as the crude extract (acid-ethanol extract) of protease-releasing factors.

Factor B: Frozen hemocytes (22 g wet weight) were thawed and homogenized with 88 ml of 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ and protease inhibitors (0.2 mM DFP, 0.1 mM leupeptin, and 0.1 mM cymostatin) using a Teflon homogenizer (1,300 rpm, 10 strokes), and the solution was stirred overnight at 4°C. The homogenate was centrifuged (9,000 × g, 15 min) and the resulting supernatant was used as the crude extract (hypotonic extract).

Preparation of hemocyte suspension

Hemolymph of *H. roretzi* was collected from individual animals into 50 ml of 0.56 M NaCl that contained 2.7 mM EDTA (pH 5.2) (EDTA solution) to prevent aggregation of hemocytes. EDTA solution was then added to give a final volume equal to twice that of the original hemolymph. After centrifugation (800 × g, 10 min) of this 2-fold diluted hemolymph, the resulting pellets (hemocytes) obtained from individual animals were gently washed with 5 ml of Ca^{2+}-, Mg^{2+}-free Herbst's artificial sea water (F-HASW; 450 mM NaCl, 9.4 mM KCl, 32 mM Na_2SO_4, 3.2 mM $NaHCO_3$, pH 7.6) and suspended in 50 ml of F-HASW.

Measurement of protease release from hemocytes

The release of protease from *H. roretzi* hemocytes in response to various stimuli was measured using the hemocyte suspension as described previously (Azumi *et al.*, 1991b, 1993). Briefly, to 500 µl of the hemocyte suspension, 6.3 µl of 0.4 M $CaCl_2$ were added to give a final concentration of 5 mM, and then the respective stimulant was immediately added: 25 µl of a solution of 1 mg/ml of factor A or B in F-HASW (its protein concentration was estimated by measuring the absorbance at 280 nm and assuming that $A^{1\%}_{280}$ =10.0), 25 µl of 1.26 mg/ml LPS or β1-3 glucan in F-HASW, or 5 µl of 50 µM ionomycin in methanol. After each mixture had been incubated for various times at 20°C, hemocytes were removed by centrifugation (8,000 × g, 5 min), and each resulting supernatant was subjected to assay for protease activity as described below.

Assay for protease activity

Protease activity was measured at 25°C in 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ and 0.2 mM bestatin using succinyl-Leu-Leu-Val-Tyr-MCA (20 µM) as a substrate. The appearance of fluorescence due to 7-amino-4-methylcoumarin (AMC) was monitored with excitation at 380 nm and emission at 460 nm.

Preparation of the acid extract of fractionated hemocytes

Hemocytes of *H. roretzi* were fractionated by discontinuous BSA density gradient centrifugation as described previously (Azumi *et al.*, 1993). Briefly, hemocytes obtained from one animal were washed with F-HASW that contained 1 mM EDTA (EF-HASW), suspended in 2 ml of EF-HASW, and layered over a discontinuous BSA gradient that consisted of 35%, 26%, 23%, 18% and 10% BSA in EF-HASW. The gradient was centrifuged at 8,000 × g for 30 min at 4°C in an SW 40 Ti rotor in a Beckman L8-55 ultracentrifuge. Hemocytes (groups L1-L4) present at each interface between layers and those (L5) in the pellet were collected, and each population of hemocytes was gently washed twice with EF-HASW. To each group (L1-L5) of hemocytes, 500 µl of 20 mM HCl was added, and each hemocyte suspension was frozen at -20°C until use. After thawing, each hemocyte suspension was incubated for 30 min on ice and then centrifuged (8,000 × g, 10 min). The supernatant was neutralized by adding one-tenth volume of 0.25 M HEPES, pH 8.0, and was used as the acid extract of fractionated hemocytes.

RESULTS

Isolation of two protease-releasing factors from ascidian hemocytes

Factor A: The acid-ethanol extract of *H. roretzi* hemocytes was dialyzed against 0.86 M acetic acid overnight using a dialysis tube (molecular weight cut, 10,000). After insoluble materials were removed by centrifugation, 8 ml of the resulting supernatant was applied to a Bio-Gel P-10 column (1.5 × 45 cm) previously equilibrated with 0.86 M acetic acid (Fig. 1). Two protein peak fractions, a high-molecular-weight PI fraction and a low-molecular-weight PII fraction, were detected and subjected to assay for protease-releasing activity. Activity was detected only in the PI fraction, named factor A.

Factor B: The hypotonic extract of *H. roretzi* hemocytes was dialyzed overnight against 50 mM sodium formate, pH 3.0, using a dialysis tube (molecular weight cut, 10,000). After insoluble materials were removed by centrifugation, the resulting supernatant (87 ml) was applied to a CM-cellulose column (50 ml volume) previously equilibrated with 50 mM sodium formate, pH 3.0. After washing with the equilibrating buffer, elution was carried out successively with 0.2 M NaCl,

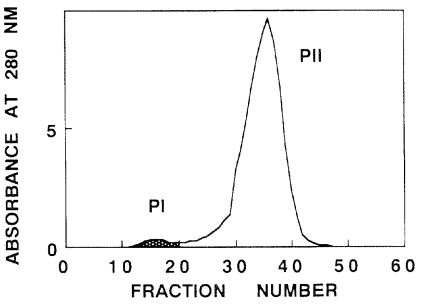


Fig. 1. Bio-Gel P-10 gel filtration of the acid-ethanol extract of *H. roretzi* hemocytes. The acid-ethanol extract of hemocytes was applied to a Bio-Gel P-10 column (1.5 × 45 cm) previously equilibrated with 0.86 M acetic acid. The eluate was collected in 2-ml fractions at a flow rate of 20 ml/hr. The high-molecular weight fraction (PI, fraction number 11 to 20) and the low-molecular weight fraction (PII, fraction number 21 to 47) were pooled separately and dialyzed against 1% acetic acid overnight using a dialysis tube (molecular weight cut, 500). After freeze-drying, the PI or PII fraction was dissolved in F-HASW and its protease-releasing activity was measured. Activity was detected only in the PI fraction, which was named factor A.

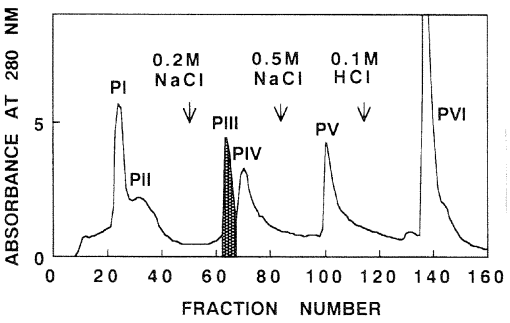


Fig. 2. CM-cellulose chromatography of the hypotonic extract of *H. roretzi* hemocytes. The hypotonic extract of hemocytes was applied to a CM-cellulose column (50 ml volume) previously equilibrated with 50 mM sodium formate, pH 3.0. After washing with the equilibrating buffer, elution was carried out successively with 0.2 M NaCl and 0.5 M NaCl, both of which had been dissolved in the same buffer, and finally with 0.1 M HCl. Fractions at six protein peaks, designated as PI (fraction number 21 to 28), PII (29 to 40), PIII (62 to 67), PIV (68 to 88), PV (98 to 109) and PVI (134-148), were dialyzed against 1% acetic acid overnight using a dialysis tube (molecular weight cut, 500). After freeze-drying, each fraction was dissolved in F-HASW and its protease-releasing activity was measured. Activity was detected only in the PIII fraction, which was named factor B.

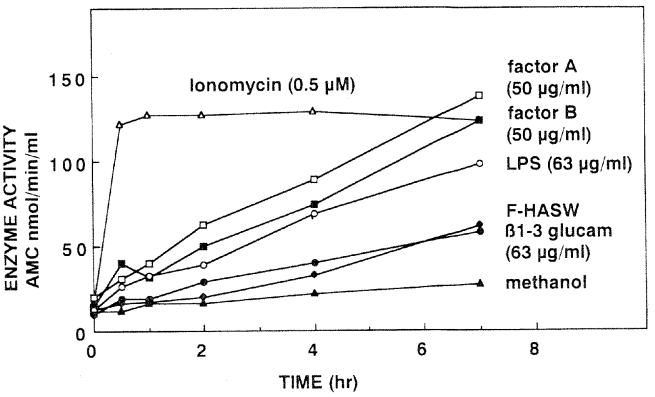


Fig. 3. Time course of the release of succinyl-Leu-Leu-Val-Tyr-MCA hydrolyzing activity from *H. roretzi* hemocytes. Various stimuli, factor A in F-HASW (50 μg/ml) (□), factor B in F-HASW (50 μg/ml) (■), LPS in F-HASW (63 μg/ml) (○), β1-3 glucan in F-HASW (63 μg/ml) (●), and ionomycin in methanol (0.5 μM) (△), were added to 500 μl of *H. roretzi* hemocyte suspension containing 5 mM $CaCl_2$. F-HASW (◆) and methanol (▲) were used as controls. After each mixture had been incubated for indicated times at 20°C, hemocytes were removed by centrifugation (8,000 × g, 5 min), and proteolytic activity in the resultant supernatant was measured with succinyl-Leu-Leu-Val-Tyr-MCA as the substrate. Each point represents the mean value calculated from the data of duplicate measurements.

0.5 M NaCl, and 0.1 M HCl (Fig. 2). Six protein fractions (PI, PII, PIII, PIV, PV and PVI) were subjected to assay for protease-releasing activity. Activity was detected only in the PIII fraction, named factor B.

Both factor A and factor B (50 μg/ml) can induce the release of protease from *H. roretzi* hemocytes in a time-dependent manner similar to that of LPS (Fig. 3). The extents of abilities of the two factors were similar to that of ionomycin, although both time-dependencies were different.

Characterization of factor A and factor B

Molecular sizes and susceptibilities toward heat (95°C, 15 min), acid (0.1 M HCl, 25°C, 1 hr) and alkali (0.1 M NaOH, 25°C, 1 hr) of protease-releasing factors A and B were examined. Factor A was a large molecule (molecular weight of >10,000) and was acid- and alkali-stable but heat-labile, while factor B was a small molecule (molecular weight of <10,000) and was alkali- and heat-stable but moderately acid-stable (remaining activity was 54%). Thus, we concluded that *H. roretzi* hemocytes contain at least two different factors that act on the hemocytes themselves and induce the release of protease from them.

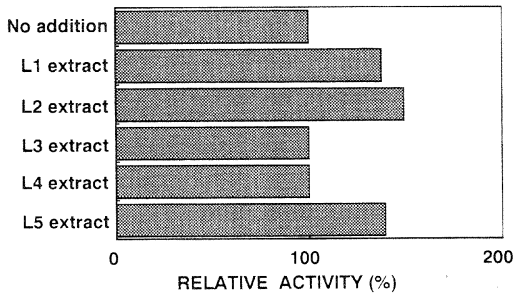


Fig. 4. Protease-releasing activity present in *H. roretzi* hemocytes of group L1 to L5. The neutralized acid extract of the hemocyte group of L1, L2, L3, L4 or L5 in a volume of 20 μl was added to 400 μl of the hemocyte suspension containing 5 mM $CaCl_2$. After each mixture had been incubated for 3 hr at 20°C, hemocytes were removed by centrifugation (8,000 × g, 5 min), and protease activity in the resultant supernatant was measured. The protease activity of the supernatant of each hemocyte group in the absence of the extract was defined as 100%. Each relative activity represents the mean value calculated from the data of duplicate measurements.

Identification of hemocyte types containing protease-releasing factors

The protease-releasing activity in each acid extract of fractionated hemocytes (L1, L2, L3, L4, and L5) was measured to identify the hemocyte types that contain protease-releasing factors. Activity was detected in the extracts of L1, L2 and L5, but not in those of L3 and L4 (Fig. 4). The extracts of L3 and L4 showed protease activity, but an increase in protease activity was undetectable by the addition of L3 or L4 extract to the hemocytes. On the other hand, protease activity could not be detected in the extracts of L1, L2 and L5, although these extracts showed protease-releasing activity. These results indicate that some acid-stable protease-releasing factors such as factor A or factor B are present in the hemocytes of L1, L2 and L5.

DISCUSSION

In the beginning of this study, we tried to find protease-releasing activity in the crude extract of *H. roretzi* hemocytes,

but activity could not be detected because the hemocyte extract had a high level of protease activity. Next, we measured the activity in protein fractions separated by several different chromatographic procedures using some hemocyte extracts or concentrated plasma protein preparations of *H. roretzi*: a) Pass-through fraction and two protein peak fractions, which had been obtained by gel filtration on Bio-Gel P-10 at 0.86 M acetic acid of 75% saturated ammonium sulfate precipitates of the plasma, showed little activity. b) Pass through fraction, and 0.2 M NaCl- and 0.5 M NaCl-eluted fractions, which had been obtained by DEAE-Sepharose chromatography at pH 8.0 of 75% saturated ammonium sulfate precipitates of the plasma, showed little activity. c) One of two protein peak fractions, which had been obtained by gel filtration on Bio-Gel P-10 at 0.86 M acetic acid of the acid-ethanol extract of the hemocytes, showed protease-releasing activity (see Fig. 1, factor A). d) Two 0.2 M NaCl- and 0.1 M HCl-eluted fractions, which had been obtained by CM-cellulose chromatography at pH 6.0 of the acid-ethanol extract of the hemocytes, showed little activity. e) One of six protein peak fractions, which had been obtained by CM-cellulose chromatography at pH 3.0 of the hypotonic extract of the hemocytes, showed protease-releasing activity (see Fig. 2, factor B). In conclusion, we succeeded in isolating two protease-releasing factors, A and B, from two different hemocyte extracts. However, we were not able to find activity in any fractions from *H. roretzi* plasma.

Both factors A and B induced the release of protease from *H. roretzi* hemocytes in a similar time-dependent manner to that of LPS (Fig. 3). Even at a one-tenth concentration both factors showed releasing activity (data not shown). Inhibitor spectra and substrate specificities of the proteases released from the hemocytes by treatments with factor A and B are the same as those previously reported for metalloproteases (Azumi *et al.*, 1993; Azumi and Yokosawa, 1996b) (data not shown). These results strongly suggest that factor A and factor B act on *H. roretzi* hemocytes to induce the release of the metalloproteases from them.

Although the procedure of isolation of factor A is essentially similar to that of mammalian transforming growth factors (TGF) (Roberts *et al.*, 1980), the properties of factor A and TGF are different: Factor A is heat-labile, whereas TGF is moderately heat-stable (Roberts *et al.*, 1980). Also it has been reported that a platelet-derived growth factor (PDGF) with a molecular weight of about 30,000 is heat-stable and has binding ability to the CM-cellulose column (Heldin *et al.*, 1981). Factor B is heat-stable and can adsorb to the CM-cellulose column, but its molecular weight is less than 10,000. Further study is needed to obtain structural information of these isolated factors.

We have already established a method for separating *H. roretzi* hemocytes into five groups by BSA density gradient centrifugation, and structural and functional information for each group of hemocytes has been reported previously (Azumi *et al.*, 1993; see Fig. 5). The hemocytes in group L1 contain mainly one type of small hemocyte, which has low-density granules. This hemocyte group, which releases proteases in response to LPS, calcium ionophore and PMA, contains the protease-releasing factors. Group L2 consists of two different types of hemocytes whose cellular granularities are different. This group does not release proteases in response to stimuli but contains the releasing factors. Both L3 and L4 groups contain a common cell type of small size and low density. In addition, L4 contains another type of large-sized cell. The hemocytes of L3 and L4 release proteases in response to calcium ionophore and PMA but do not contain the releasing factors. Group L5, which consists of only one type of hemocyte that has vacuoles of high density and occupies more than half of the hemocyte population in *H. roretzi* hemolymph, does not release proteases in response to stimuli but contains the releasing factors. The protease-releasing factors, found in L1, L2 and L5 groups, are thought to be released from these hemocytes in response to yet-unidentified stimuli and act on the hemocytes of L1, L3 or L4 to release metalloproteases (Fig. 5). The mechanisms for the release of these factors require further investigation. In addition, characterization and structural elucidation of these factors and of the isolated factors A and B, as well as analysis on the interaction between the factors and separated hemocytes, is needed for an understanding of the mechanisms for hemocyte-hemocyte communication.

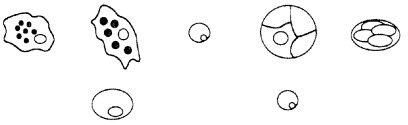
	Group				
	L1	L2	L3	L4	L5
Shape					
Release of protease	yes	no	yes	yes	no
Presence of protease releasing factor	yes	yes	no	no	yes

Fig. 5. Shape of, release of protease from and presence of protease-releasing factor in *H. roretzi* hemocyte group of L1, L2, L3, L4 or L5.

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