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Characterization of Novel Metallo-Proteases Released from Ascidian Hemocytes by Treatment with Calcium Ionophore

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ABSTRACT—We have previously demonstrated that calcium ionophore induced the release of a novel metallo-protease from hemocytes of a solitary ascidian, *Halocynthia roretzi*. Here, we isolated the enzymes, PI and PII, from the culture media of *H. roretzi* hemocytes, which had been treated with calcium ionophore, A23187. The purification procedure included hydorophobic and anion-exchange chromatographies, and gel filtration. The molecular weights of the enzymes were estimated to be 11,000 by gel filtration, but the apparent sedimentation coefficients were 5.0 S, which suggests that the *H. roretzi* enzymes are of larger proteins with molecular weights of 80,000-90,000. The most susceptible substrate was succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide, and the optimum pH was 8.0, in either case of PI or PII. The activities of PI and PII enzymes were strongly inhibited by metal-chelating agents and propioxatin A, but not by phosphoramidon, a typical metallo-protease inhibitor. Zinc and calcium ions were found to be essential for the maximum expression of protease activity in both enzymes. Thus, the isolated enzymes are characterized as phosphoramidon-insensitive metallo-proteases, which are inhibited by propioxatin A. Extracellular roles of these enzymes were also discussed.

INTRODUCTION

Protease release in response to foreign invaders is involved in an event, which has an important role in host defense in invertebrates. For example, in horseshoe crabs, serine proteases are released from hemocytes in response to bacterial endotoxin (lipopolysaccharide, LPS) (Iwanaga, 1993): The released serine proteases make up the cascade system and trigger the coagulation in the hemolymph.

We have been studying the defense mechanisms of the solitary ascidian, *Halocynthia roretzi*, and have succeeded in isolating several defense factors, lectins (Yokosawa *et al.*, 1982; Harada-Azumi *et al.*, 1987) and trypsin inhibitors (Yokosawa *et al.*, 1985; Kumazaki *et al.*, 1990) from the hemolymph, and antibacterial and antiviral substances (Azumi *et al.*, 1990a, b) and LPS-binding protein (Azumi *et al.*, 1991a) from the hemocytes. We also analyzed the cellular reactions of *H. roretzi* hemocytes, such as hemocyte aggregation (Takahashi *et al.*, 1994), phagocytosis and a self and non-self recognition reaction. In the course of our study on the cellular response to stimuli, we found an enzyme release reaction of *H. roretzi* hemocytes in response to LPS (Azumi *et al.*, 1991b). The released enzyme hydrolyzed succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) and its activity was inhibited by EDTA and *o*-phenanthroline, but not by phosphoramidon and serine-protease inhibitors. Thus, the enzyme was characterized as a phosphoramidon-insensitive metallo-protease. Calcium ionophore and phorbol myristate acetate (PMA) also induced the release of the same protease from *H. roretzi* hemocytes (Azumi *et al.*, 1993). In addition, we showed that metallo-protease was released from specific populations of *H. roretzi* hemocytes (Azumi *et al.*, 1993). In connection with the stimulus-induced enzyme release, a phenoloxidase was released from hemocytes upon the self and non-self recognition reaction (Akita and Hoshi, 1995).

In the present communication, we describe the isolation and characterization of metallo-proteases from the culture media of *H. roretzi* hemocytes, which had been treated with calcium ionophore, A23187. The substrate specificities and inhibitor susceptibilities of the enzymes led us to propose that the enzymes are novel metallo-proteases, insensitive to phosphoramidon and sensitive to propioxatin A, an inhibitor of dipeptidyl-aminopeptidase.

MATERIALS AND METHODS

Materials

Peptidyl-MCAs, phosphoramidon, chymostatin, elastatinal, pepstatin A, diprotinin A and E-64 were obtained from the Peptide Institute (Japan). Diisopropylfluorophosphate, thiorphan, cytochrome c and calcium ionophore (A23187) were purchased from Sigma Chemical Co. (U.S.A.). *o*-Phenanthroline, *p*-chloromercuribenzoic acid and *N*-ethylmaleimide were obtained from Nacalai Tesque (Japan).

Aprotinin was from Seikagaku Kogyo Co. (Japan). Bestatin and leupeptin were provided by Dr. W. Tanaka of Nippon Kayaku Co. (Japan). Captopril and propioxatin A were generous gifts of Dr. A. Awaya of Mitsui Seiyaku Co. (Japan) and Dr. Y. Inaoka of Sankyo Co. (Japan), respectively. Brij 35 was from Pierce (U.S.A.). Sephadex G 50, phenyl-Sepharose, Mono Q, Superose 12 and blue dextran 2000 were from Pharmacia Fine Chemicals AB (Sweden). Chymotrypsinogen A was from Worthington Biochemical Corp. (U.S.A.). Immunogloublin G (IgG) and bovine serum albumin (BSA, Fraction V) were from ICN Pharmaceuticals Co. (U.S.A.) and Armour Pharmaceutical Co. (U.S.A.), respectively.

Preparation of hemocytes

Solitary ascidians, *H. roretzi*, Type C (Numakunai and Hoshino, 1973), were harvested in Mutsu Bay, Japan. The tunic matrix was extensively washed with seawater and cut without injuring the internal organs, and the hemolymph was collected from individual animals into 50 ml of 0.1% EDTA-0.56 M NaCl (EDTA solution) to prevent aggregation of hemocytes. EDTA solution was then added to yield a final volume equal to that of the hemolymph. After centrifugation (800 ×g, 10 min) of this 2-fold diluted hemolymph, the resulting pellet (hemocytes) obtained from individual animals was 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 was suspended in 50 ml of F-HASW.

Preparation of the crude enzyme

Fifty ml each of hemocyte suspension were prepared from each of four ascidians. Enzyme release was initiated by adding 25 μl of 10 mM calcium ionophore (A23187) in dimethyl sulfoxide (DMSO) to 50 ml of the hemocyte suspension (about 10^7 cells/ml) containing 2 mM $CaCl_2$. The suspension was gently mixed and then allowed to stand for 1 hr at 20°C. After hemocytes were removed by centrifugation (8000 ×g, 5 min), the resulting supernatants from four hemocyte suspensions were pooled and used as a crude enzyme solution.

Measurement of 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. Activities towards various peptidyl-MCAs were measured under the same conditions. The appearance of fluorescence due to 7-amino-4-methylcoumarin (AMC) was monitored with excitation at 380 nm and emission at 460 nm.

Determination of molecular weight

The enzyme solution obtained from the phenyl-Sepharose column in a volume of 3 ml was applied to a column (2 × 86 cm) of Sephadex G-50 previously equilibrated with 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$, 0.5 M NaCl and 0.005% Brij 35. Brij 35 was added as an effective stabilizer. The column was developed with the same buffer at a flow rate of 15 ml/hr. Effluents were collected in a volume of 2.7 ml and protease activity was assayed as described above. The molecular weight was estimated using blue dextran (void volume), chymotrypsinogen A (26 K), cytochrome *c* (12 K) and aprotinin (6.5 K) as standards.

Estimation of the sedimentation coefficient

The sedimentation velocity experiment was performed by the sucrose density-gradient centrifugation method. Sucrose was dissolved in 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ and 0.005% Brij 35 to give a concentration of 5%, 20% or 40%. Forty % solution in a volume of 0.25 ml was poured into a centrifuge tube and 4.5 ml of a linear gradient of sucrose solution from 5% to 20% was layered over 40% sucrose. Then 0.25 ml of the enzyme solution obtained from the phenyl-Sepharose column, which had been dialyzed against the above solution, was layered over a linear gradient of sucrose. The gradient was centrifuged at 300,000 ×g for 15 hr at 4°C in an SW 40 Ti rotor in a Beckman L8-55 ultracentrifuge. After centrifugation, the sucrose solution was fractionated (20 drops, 0.5 ml) and protease activity was measured as described above. The sedimentation coefficient was estimated using IgG (7 S), BSA (4.4 S) and chymotrypsinogen A (2.5 S) as standards.

Inhibition studies

The enzyme was preincubated at pH 8.0 and at 25°C for 20 min in the presence or absence of various concentrations of each inhibitor and the remaining activity was measured at pH 8.0 as described above.

Effects of various metal ions on EDTA-pretreated protease

The enzyme was incubated with 5 mM EDTA in 50 mM Tris-HCl, pH 8.0, containing 0.005% Brij 35 at 25°C for 30 min. The solution was then dialyzed against 1L of 50 mM Tris-HCl, pH 8.0, containing 0.005% Brij 35 at 4°C overnight and used as an EDTA-pretreated enzyme preparation. The enzyme preparation was again incubated with various metal ions in 50 mM Tris-HCl, pH 8.0, containing 0.005% Brij 35 at 25°C for 30 min and the recovered enzyme activity was assayed as described above.

RESULTS

Purification of metallo-proteases from the culture media

Metallo-proteases released from *H. roretzi* hemocytes in response to A23187 were purified from the culture media. The purification procedure was performed at 4°C.

Step 1. Phenyl-Sepharose column chromatography. The crude enzyme solution (183 ml) was applied to a column (1.7 ×12.5 cm) of phenyl-Sepharose previously equilibrated with 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$. The column was washed with the equilibration buffer and the effluent was collected in 10-ml fractions. Adsorbed materials were then eluted with 66% ethylene glycol in the same buffer and the

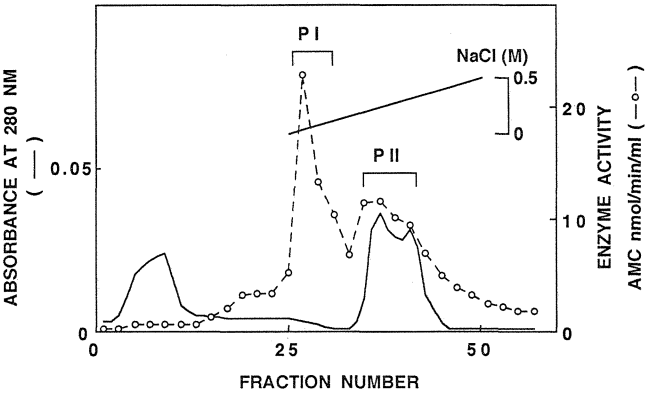

Fig. 1. Mono Q FPLC of metallo-proteases released from *H. roretzi* hemocytes. Active fractions obtained from the phenyl-Sepharose column were pooled, dialyzed against 1L of 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ overnight, and applied on a column (0.5 × 5 cm) of a Mono Q previously equilibrated with the same buffer. After washing the column with the same buffer, elution with a linear gradient of increasing NaCl concentrations (0-500 mM) was carried out. The effluent was collected in 1-ml fractions at a flow rate of 1 ml/min. The active fractions were separated into two peaks named PI and PII. Absorbance at 280 nm (——); succinyl-Leu-Leu-Val-Tyr-MCA-hydrolyzing activity (–o–).

effluent was collected in 5-ml fractions at a flow rate of 20 ml/hr. Succinyl-Ley-Leu-Val-Tyr-MCA-hydrolyzing activity was detected at the ethylene glycol-eluted fraction.

Step 2. Mono Q fast protein liquid chromatography (FPLC). Active fractions from the phenyl-Sepharose column were dialyzed against 1L of 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ overnight, and 3 ml each were applied on a column (0.5 × 5 cm) of Mono Q previously equilibrated with the same buffer using the FPLC system (Pharmacia, Sweden). After washing the column with the same buffer, elution with a linear gradient of increasing NaCl concentrations (0-500 mM) was carried out. The effluent was collected in 1-ml fractions at a flow rate of 1 ml/min. Enzyme activity was detected in two peaks named PI and PII (Fig. 1). As a result, the same chromatographies were repeated three times and PI and PII fractions were pooled respectively.

Step 3. The first Superose 12 gel filtration. Active fractions (2 ml) in PI or PII were applied to a Superose 12 column (1 × 30 cm) previously equilibrated with 50 mM Tris-HCl, pH 8.0, containing 1 mM $CaCl_2$, 0.5 M NaCl and 0.005% Brij 35. The effluent was collected in 0.5-ml fractions at a flow rate of 0.5 ml/min. Enzyme activity was detected at almost the same position in either case of PI or PII.

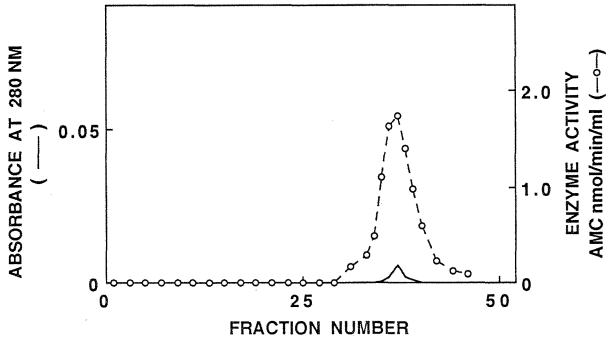

Fig. 2. The second Superose 12 gel filtration of metallo-protease PI. Active fractions (500 µl) of PI obtained from the first Superose 12 column were applied to the same column (1 × 30 cm) of Superose 12 previously equilibrated with 50 mM Tris-HCl, pH 8.0, containing 1 mM $CaCl_2$, 0.5 M NaCl and 0.005% Brij 35. The effluent was collected in 0.5-ml fractions at a flow rate of 0.5 ml/min. Absorbance at 280 nm (——); succinyl-Leu-Leu-Val-Tyr-MCA-hydrolyzing activity (–o–).

Step 4. The second Superose 12 gel filtration. Active fractions (500 µl) obtained from the first Superose 12 column were applied to the same column of Superose 12. All operations were performed under the same conditions as Step 3. The final steps of purification of PI and PII fractions gave single peaks of protease activity at the same positions, which were superimposable on the protein peaks in both cases (Fig. 2 in PI; data not shown in PII). Thus, the PI and PII enzymes were characterized as the molecules of the same size but different ionic properties.

Determination of molecular weight and sedimentation coefficient

The molecular weight of the enzyme obtained from the phenyl-Sepharose column was estimated to be 11,000 by gel filtration on Sephadex G 50. The purified PI and PII enzymes were eluted at the same positions by gel filtration. However, the sedimentation coefficient of the enzyme obtained from the phenyl-Sepharose column was determined to be 5.0 S (Fig. 3), which indicated that the "real" molecular weights of the enzymes were about 80,000-90,000.

Since the amounts of the purified enzymes were not adequate for the detection of silver-stained bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we could not ascertain the purities and the subunit structures of the enzyme preparations.

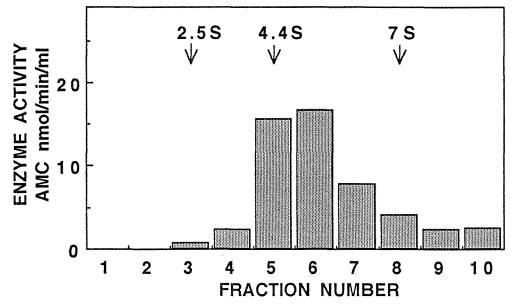

Fig. 3. The estimation of sedimentation velocity of *H. roretzi* metallo-protease. Forty % sucrose solution (0.25 ml) in 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ and 0.005% Brij 35 was poured into a centrifuge tube, and 4.5 ml of a linear gradient of 5-20% sucrose solution dissolved in the same buffer was layered over 40% sucrose. Then 0.25 ml of the enzyme solution obtained from the phenyl-Sepharose column was layered over a linear gradient of sucrose. The gradient was centrifuged at 300,000 ×g for 15 hr at 4°C in an SW 40 Ti rotor in a Beckman L8-55 ultracentrifuge. After centrifugation, the sucrose solution was fractionated (20 drops, 0.5 ml) and the protease activity was measured. The sedimentation coefficient was estimated using IgG (7 S), BSA (4.4 S) and chymotrypsinogen A (2.5 S) as standards.

pH optima of purified enzymes

Enzyme activities toward Succinyl-Leu-Leu-Val-Tyr-MCA of the purified enzymes PI and PII were measured at various pHs from 3.0 to 11.0 in buffers consisting of 0.1 M Tris, 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.1 M acetic acid, 0.1 M glycine, 10 mM $CaCl_2$, 0.005% Brij 35, 0.2 mM bestatin and appropriate amounts of NaOH or HCl to adjust the pH values. The pH optima were determined to be 8.0 in both cases.

Substrate specificities of purified enzymes

The substrate specificities of the purified enzymes PI and PII are shown in Table 1. Both of the enzymes had the same highly strict substrate specificities. Succinyl-Leu-Leu-Val-Tyr-MCA was most efficiently hydrolyzed by both enzymes. On the other hand, other substrates for chymotrypsin-like enzyme, together with those for trypsin-like and aminopeptidase-like enzymes, were hardly hydrolyzed. In addition, Succinyl-Arg-

Table 1. Substrate specificities of purified metallo-protease PI and PII

Substrate P6 P5 P4 P3 P2 P1 P1'	Activity (%) PI	PII
Suc-Leu-Leu-Val-Tyr-MCA	100[a]	100[a]
Ac-Ala-Ala-Tyr-MCA	4	8
Suc-Ala-Ala-Pro-Phe-MCA	2	4
Glt-Gly-Gly-Phe-MCA	0	0
Suc-Ala-Pro-Ala-MCA	1	1
Boc-Val-Pro-Arg-MCA	1	1
Boc-Val-Leu-Lys-MCA	10	13
Leu-MCA	0[b]	0[b]
Suc-Gly-Pro-Leu-Gly-Pro-MCA	1	1

[a]The activity toward succinyl-Leu-Leu-Val-Tyr-MCA was defined as 100%.
[b]The activity toward Leu-MCA was measured in the absence of bestatin.
Suc, succinyl; Ac, acetyl; Glt, glutaryl; Boc, *t*-butyloxycarbonyl

Table 2. Effects of various reagents on the activities of purified metallo-protease PI and PII

Inhibitors	Concentration (mM)	Inhibition (%) PI	PII
EDTA	1.0	94	92
o-Phenanthroline	0.5	93	93
	0.1	56	32
Propioxatin A	0.1	92	91
	0.01	75	72
Phosphoramidon	0.1	0	0
Thiorphan	0.01	3	8
Captopril	0.01	0	0
Diisopropylfluorophosphate	0.1	2	0
Leupeptin	0.1	27	34
Chymostatin	0.1	0	0
Elastatinal	0.1	0	0
N-Ethylmaleimide	0.1	0	0
p-Chloromercuribenzoic acid	0.1	0	0
E-64	0.1	0	0
Pepstatin A	0.1	9	0
Diprotin A	0.01	2	1

Pro-Phe-His-Leu-Leu-Val-Tyr-MCA (a substrate for renin) having the same structure as Succinyl-Leu-Leu-Val-Tyr-MCA and also Succinyl-Gly-Pro-Leu-Gly-Pro-MCA (a substrate for collagenase type I to III) were not hydrolyzed by the enzymes (data not shown).

Effects of various protease inhibitors on the activities of purified enzymes

Table 2 illustrates the effects of various protease inhibitors on the protease activities of purified enzymes PI and PII. Both enzymes had similar susceptibilities to various inhibitors. EDTA, *o*-phenanthroline and propioxatin A were strong inhibitors for both enzymes. However, other inhibitors for metallo-proteases, phosphoramidon, thiorphan and captopril, had little effect. Pepstatin, an aspartic protease inhibitor, and diprotin A, a dipeptidyl-aminopeptidase inhibitor, also had no effect. Leupeptin, an inhibitor for trypsin and cysteine protease, had a weak inhibitory effect (30% inhibition). Other trypsin and chymotrypsin inhibitors scarcely inhibited the activities. These results suggest that the purified proteases of PI and PII are characterized as phosphoramidon-insensitive metallo-proteases.

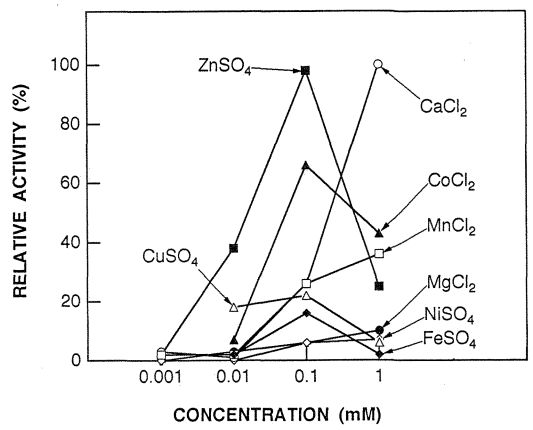

Fig. 4. Effects of various metal ions on the activity of EDTA-pretreated enzyme PI. The enzyme was incubated with 5 mM EDTA in 50 mM Tris-HCl, pH 8.0, containing 0.005% Brij 35 at 25°C for 30 min and was dialyzed against 1L of 50 mM Tris-HCl, pH 8.0, containing 0.005% Brij 35 at 4°C overnight. The EDTA-pretreated enzyme was then incubated with various metal ions in 50 mM Tris-HCl, pH 8.0, containing 0.005% Brij 35 at 25°C for 30 min. The enzyme reaction was initiated by adding succinyl-Leu-Leu-Val-Tyr-MCA and the recovery of enzyme activity was measured.

Effects of various metal ions on the activities of purified enzymes

The effects of various metal ions on the activity of the EDTA-pretreated protease PI are shown in Fig. 4. The protease activity was completely inhibited by EDTA. The activity was restored to full and almost 70% levels by the addition of 0.1 mM Zn^{2+} and 0.1 mM Co^{2+}, respectively. At higher concentrations of these metal ions, the activities were decreased. In the presence of 1 mM Ca^{2+}, the activity also recovered to full activity, but other metal ions including Cu^{2+}, Mg^{2+}, Ni^{2+} and Fe^{2+} had little effect (about 40% of the activity recovered in the presence of 1 mM Mn^{2+}). Exactly the same result was obtained in the case of protease PII (data not shown). These results also indicated that the proteases of PI and PII belong to the metallo-protease family which requires divalent metal ions for the expression of full activity.

DISCUSSION

We purified metallo-proteases from the culture media of *H. roretzi* hemocytes, which had been treated with calcium ionophore, A23187. We already found that Brij 35 has a stabilizing effect on protease activity. Enzyme activity was also stable in a 66% ethylene glycol-containing buffer. In the second purification step of Mono Q FPLC, however, the enzyme was unable to adsorb to the Mono Q column in the presence of

0.005% Brij 35. Therefore, dialysis and subsequent Mono Q FPLC were carried out in the absence of Brij 35, which resulted in the loss (about 30%) of protease activity. Gel filtration on Superose 12 was performed in the presence of 0.005% Brij 35, but the protease activity was reduced after the second gel filtration. We thought that Brij 35 had little stabilizing effect in the final step probably because of a low concentration of enzymes, but we cannot find other stabilizer in this case. Instability of the final preparations of enzymes made further purification of them very difficult. The protease activity was detected at two peaks (PI and PII) in Mono Q FPLC. The results of gel filtration, substrate specificities and inhibitor spectra suggest that the PI and PII proteases have almost the same properties, except for ionic properties.

The molecular weights of the proteases were estimated to be 11,000 by gel filtration on Sephadex G 50 and 6,500 by Superose 12 gel filtration. However, in the sucrose density-gradient centrifugation experiment, protease activity was detected at the fraction between BSA (4.4 S, MW, 66K) and IgG (7 S, MW, 160 K) and the apparent sedimentation coefficients of metallo-proteases were estimated to be 5.0 S. These results suggest that the enzymes of PI and PII may interact with the gel filtration matrix and that the apparent molecular weights were underestimated. From the S value, the molecular weights of the metallo-proteases were estimated to be 80,000-90,000.

The purified enzymes show endopeptidase activity toward succinyl-Leu-Leu-Val-Tyr-MCA to produce AMC as a product in the presence of an aminopeptidase inhibitor, bestatin. We preliminarily examined whether other sites than the site between Tyr and MCA bond could be cleaved by the proteases. After hydrolysis of various substrates with the enzymes in the absence of bestatin and subsequent inactivation of the enzymes by heat treatment, we checked whether further generation of AMC from the hydrolysis products could be detected by the addition of excess aminopeptidase. However, we couldn't detect an additional generation of AMC by aminopeptidase treatment. From these results, we concluded that the enzymes hydrolyzed only the Tyr-MCA bond.

Propioxatin A is a specific inhibitor of enkepharinase B (Inaoka *et al.*, 1986). However, propioxatin A strongly inhibited the *H. roretzi* metallo-proteases. Enkepharinase B is a dipeptidyl-aminopeptidase and is thought to be incapable of hydrolyzing succinyl-Leu-Leu-Val-Tyr-MCA. Another inhibitor of dipeptidyl-aminopeptidase, diprotin A, didn't inhibit the *H. roretzi* enzyme. These results suggest that the *H. roretzi* metallo-endopeptidases have active sites common to that of a specific dipeptidyl-aminopeptidase. To the best of our knowledge, this is the first report on metallo-proteases sensitive to propioxatin A.

We measured the activities of *H. roretzi* metallo-endopeptidases toward various bioactive peptides, but could not find any hydrolyzing activity toward the peptides. Recently, it has been reported that membrane-bound metallo-proteases can cleave extracellular domains of cell-surface proteins such as major histocompatibility complex (MHC) class I heavy chains (Demaria *et al.*, 1994) and natural killer cell-associated FCγRIII (Harrison *et al.*, 1991) to release their soluble forms. On the other hand, metallo-proteases have been reported to be secreted from cells. Human neutrophils stimulated with PMA released a high-molecular-weight metallo-protease (92 K), which is capable of degrading gelatin and type V collagen (Hibbs *et al.*, 1985). Another high-molecular-weight metallo-protease (88 K), which degraded gelatin, elastin and type IV and V collagen, was isolated from culture media of pig polymorphonuclear leucocytes (Murphy *et al.*, 1989). In marine invertebrates, the structure of a sea urchin embryo hatching enzyme (52 K), which is secreted from the eggs, has been reported; this enzyme belongs to the metallo-protease family (Lepage and Gache, 1989, 1990). In our preliminary study, *H. roretzi* metallo-proteases can degrade casein, but not gelatin, and collagen type IV and V. From the facts that *H. roretzi* metallo-proteases were released from *H. roretzi* hemocytes in response to various stimuli such as LPS, calcium ionophore, PMA, thrombin and concanavalin A (Azumi *et al.*, 1991b) and that proteases hydrolyzed proteins such as casein, we propose that the metallo-endopeptidases released from *H. roretzi* hemocytes may function extracellularly in producing bioactive peptides from proteins present in *H. roretzi* hemolymph.

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