The Specificity of Sea Urchin Hatching Enzyme (Envelysin) Places It in the Mammalian Matrix Metalloproteinase Family†

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ABSTRACT: The sea urchin hatching enzyme (HEz) is a protease capable of dissolving the fertilization envelope that surrounds the embryo as a protective coat during early development. We have now purified a 37-kDa active enzyme from the supernatant fluid of hatched blastula medium of the species *Hemicentrotus pulcherrimus*. The purified enzyme was completely inhibited by α_2-macroglobulin and the chelating agents EDTA, EGTA, and 1,10-phenanthroline and was slightly inhibited by chymostatin and pepstatin, but was not inhibited by various other serine and thiol protease inhibitors. These results suggest that HEz is a metalloproteinase. Quantitative analyses of the products of HEz's action on various peptides revealed that the enzyme preferentially cleaved the peptide bonds on the amino side of bulky hydrophobic residues, -Leu, -Ile, and -Phe as well as -Tyr, in a similar but more limited fashion than thermolysin. Furthermore, although substance P was a good substrate of HEz, snake venom α-protease, and thermolysin, the analogue [Sar^9]substance P was a poor substrate for HEz. This analogue was a good substrate for thermolysin and α-protease, but the scissile bonds were altered from those of substance P. The failure of HEz and α-protease to cleave the P_1–P_1' bond that meets the primary specificity is ascribed to the presence of an imino acid residue (proline or sarcosine) or the absence of any amino acid at the P_2' or P_3' position, in contrast to the simple P_2' restriction of thermolysin. Since such a tendency to [Sar^9]substance P has been reported to be characteristic of stromelysin, one of the mammalian matrix metalloproteinases (MMPs), HEz and possibly α-protease are rationally classified as members of the MMP family (collagenase gene family), rather than of the bacterial metalloproteinase family. In this context we propose the name "envelysin" for the HEz of sea urchin, or of wider sources, if established. The HEz of *H. pulcherrimus* exhibited a unique species specificity; it could not dissolve the fertilization envelope of *Anthocidaris crassispina*, a species of a neighboring taxonomical family, but could dissolve those of more distantly related species, even if with less efficiency.

A sea urchin embryo is surrounded by the fertilization envelope (FE),[1] a protective coat made up of the slightly modified and elevated vitelline coat scaffold and several proteins and enzymes secreted from the cortical granules immediately after fertilization. By 10 min after fertilization it hardens by cross-linking relatively high molecular weight proteins via oxidative linking of tyrosine residues into dityrosine, trityrosine, and pulcherosine, the reaction being catalyzed by ovoperoxidase [Nomura et al. (1990a) and references therein]. When the embryo develops to midblastula stage in about 12 h, it secretes the protease, hatching enzyme (HEz; Ishida, 1936), to make an opening in the FE; it then swims out by ciliary motion. The FE "ghosts" are dissolved completely within a few hours.

For more than 50 years, definitive purification and characterization of HEz were not accomplished (Sugawara, 1943; Yasumasu, 1960, 1961; Barrett et al., 1971; Barrett & Edwards, 1976; Edwards et al., 1977; Takeuchi et al., 1979; Post et al., 1988). Satisfactory purification was attained by the use of Procion–agarose (Lepage & Gache, 1989), or by a formerly used method (urea treatment and DEAE-cellulose) coupled with reactive red 120–agarose chromatography (Roe & Lennarz, 1990). An M_r value of 51 000 has been reported for an active HEz which was autolyzed to an inactive form of M_r 30 000 (Lepage & Gache, 1989). In contrast, another HEz has an M_r of 57 000 as a secreted enzyme which is converted during purification to the second active form of M_r 33 000 (Roe & Lennarz, 1990).

Another important feature of a protease is its specificity at various levels. The primary specificity denotes the amino acid residue at either side of the scissile bond (P_1– or –P_1'), and the secondary specificity denotes the short range of the amino acid sequence on both sides of the scissile bond affecting catalytic efficiency [P_m-----P_1–P_1'-----P_n', according to the expression by Schechter and Berger (1967)]. These basic specificities of HEz, thus far, have not been determined. A previous report argued that HEz is specific to the carboxyl side of Glu- and Asp- residues (Barrett & Edwards, 1976). Others have claimed that HEz is chymotrypsin-like on the basis of experiments on a crude preparation of the enzyme using small substrates and inhibitors of chymotrypsin (Hoshi et al., 1979; Post et al., 1988). As to the protein–substrate

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[1] Abbreviations: FE, fertilization envelope; HEz, hatching enzyme; MMP, matrix metalloproteinase; ASW, artificial seawater; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Sar, sarcosine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, N-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; DMSO, dimethyl sulfoxide; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DNP, dinitrophenyl; PZ, [[(phenylazo)benzyl]oxy]carbonyl; Cbz, (benzyloxy)carbonyl.

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specificity, it has been reported that the HEz attacks a limited subset of high molecular mass (>100-kDa) proteins in FE (Roe & Lennarz, 1990), which is somewhat different from the result of Uher and Carroll (1987). For more precise elucidation of the mechanisms of hatching, i.e., FE dissolution by HEz, however, much more detailed studies are required.

In order to settle the debates and confusion with respect to the HEz's specificity, especially the primary and the secondary, we started the experiments to determine the sites and extents of the cleavage in various commercially available bioactive peptides by the *Hemicentrotus pulcherrimus* HEz. For this purpose we have purified the enzyme to an almost electrophoretically homogeneous level and retained the hatching activity at high salt concentration (0.5 M NaCl) as in seawater for the first time in the long history of the investigation of this Japanese sea urchin species. Thermolysin, a well-known bacterial metalloproteinase, and α-protease from rattlesnake venom were also used for comparison. The results on the cleavage of the peptides, especially substance P, its analogue [Sar^9]substance P, and Cbz-Gly-Pro-Leu-Gly-Pro, led to the conclusion that, with respect to secondary specificity, HEz and α-protease are more closely related to stromelysin (MMP-3) than to thermolysin and established the concept "P_2'/P_3' restriction" versus "P_2' restriction". After we had reached the above conclusion, we encountered a paper on cDNA cloning of the HEz of another species *Paracentrotus lividus* (Lepage & Gache, 1990) strongly supporting our conclusion. A major part of this work was presented at the 61st Annual Meeting of the Zoological Society of Japan in Niigata (Oct 1990; Nomura et al., 1990b).

Materials and Methods

Materials. The sea urchins *H. pulcherrimus*, *Anthocidaris crassispina*, *Clypeaster japonicus*, *Astriclypeus manni*, and *Peronella japonica* were collected intertidally near Noto Marine Laboratory on the Japan Sea coast (Toyama Bay), and *Glyptocidaris crenularis* urchins were collected from Aomori Bay by courtesy of the Asamushi Marine Biological Station. The protease inhibitors of *Streptomyces* origin listed in Table I, most of the peptides except otherwise noted, and thermolysin were purchased from Peptide Institute Inc. (Osaka). Chelating agents and most other reagents of analytical and HPLC grades were from Wako Pure Chemical Industries (Osaka) except as otherwise indicated. α_2-Macroglobulin was a product of Boehringer Mannheim (Germany). The rattlesnake venom α-protease was purchased from P-L Biochemicals Inc. (Milwaukee, WI). Trisma base, HEPES, CHAPS, 3-amino-1,2,4-triazole, [Sar^9]substance P, bovine insulin, dimethylated casein, laminarin, α-chymotrypsin (type VII), subtilisin (Carlsberg), glucose oxidase, and horseradish peroxidase were from Sigma (St. Louis, MO). Oxidized insulin B chain was prepared by the method of Griffin et al. (1966) and further purified by HPLC on a reverse-phase C8 column.

Fertilization and Embryo Culture. The collected and washed eggs were fertilized as previously described (Nomura et al., 1990a). Embryos were cultured in Millipore-filtered (0.45 μm) seawater at 10 °C until the start of hatching, when the medium was 10-fold concentrated by allowing the embryos to settle followed by aspiration of fluid. When more than 95% of the embryos had hatched, the supernatant from low-speed centrifugation was collected and further centrifuged at 100000g for 1 h. The final supernatant fluid was used as a crude HEz solution for further purification.

Purification of Hatching Enzyme. A purification method simplified by modification of that of Lepage & Gache (1989) was used. Solid NaCl was added to the above supernatant fluid to attain 1 M and loaded on a reactive red 120–agarose column (3 × 15 cm, Sigma) equilibrated with buffer A (10 mM HEPES, pH 8.2, containing 10 mM $CaCl_2$, 1 M NaCl, and 0.5% CHAPS) and eluted with a gradient of 0–60% ethylene glycol. Most of the hatching activity was eluted near the end of the gradient, and the active fractions were collected, dialyzed against buffer A, concentrated with Diaflo PM10 membrane (Amicon), and further purified on a Superose 12 HR column (1 × 30 cm, Pharmacia). The active fractions were collected, concentrated, and stored frozen. Electrophoresis was performed by the method of Laemmli (1970) with Coomassie Brilliant Blue staining.

Hatching Enzyme, Caseinolytic Activity, and Glucanase Assays. Hatching enzyme activity, i.e., the FE-dissolving activity, was assayed by a slightly modified microscopical method of Barrett and Edwards (1976), which was improved from that of Yasumasu (1960) by the use of ethanol-fixed 4–8-cell stage embryos. The enzyme samples to be tested were preincubated at 20 °C for 10 min in 10 mM HEPES (pH 8.2) containing 175 μg/mL penicillin G and 175 μg/mL streptomycin in artificial seawater (ASW) consisting of 454 mM NaCl, 9.7 mM KCl, 24.9 mM $MgCl_2$, 9.6 mM $CaCl_2$, 21.7 mM $MgSO_4$, and 4.4 mM $NaHCO_3$. The reaction was started by addition of the embryo suspension (200 embryos in 20 μL of the above buffer). The final volume of the reaction mixture was 70 μL. The hatching activity was expressed by the reciprocals of the time (h) for 50% of the embryos to be denuded. A unit of HEz activity was defined as the activity to denude 50% embryos in 1 h at 20 °C. Caseinolytic activity was assayed by the method of Barrett and Edwards (1976) using dimethylated casein, quantifying the newly generated amino groups with trinitrobenzenesulfonic acid by measuring the absorbance at 340 nm. Glucanase activity was assayed by the method of Talbot et al. (1982), using soluble laminarin as substrate and glucose oxidase and horseradish peroxidase as secondary enzymes.

Effects of Inhibitors. The effects of various inhibitors against the HEz's activity were examined both on the FE of ethanol-fixed embryos and on substance P. Most inhibitors were dissolved in H_2O, except for the water-insoluble inhibitors, chymostatin, pepstatin, 1,10-phenanthroline, PMSF, and TPCK, which were dissolved in DMSO. The final concentration of most inhibitors was 1 mM, except for 10 mM of the chelating agents. The serum high-M_r proteinase inhibitor α_2-macroglobulin was used at 1 mg/mL.

Hydrolyses of Peptides by Hatching Enzyme and Other Proteases. For hydrolysis by HEz, the peptides were dissolved to 1 mM in 10 mM HEPES (pH 8.2) in ASW. The reaction was started by adding 50 μL of enzyme solution in buffer A (40 μg/mL or 14.8 units/mL) to the 100 μL of enzyme solution, and the mixture was incubated at 20 °C for 3 or 12 h. The reaction was terminated by freezing at −80 °C followed by lyophilization. For hydrolyses by other enzymes, the reaction mixture was constructed with 50 μL of 0.2 M Tris-HCl (pH 7.5) containing 20 mM $CaCl_2$, 130 μL of a 1 mM peptide solution, 10 μL of H_2O, and 10 μL of 1 mg/mL enzyme solution. The times of incubation at 37 °C were 1 h for thermolysin, α-protease, and chymotrypsin and 5 h for subtilisin. The reaction was stopped in the same way as for HEz.

Isolation of Hydrolysis Products. The lyophilisate was dissolved in 200 μL of H_2O, and 150 μL was loaded on a reverse-phase C8 column (Senshupak, 8 × 250 mm) and eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at 40 °C. The absorbance was monitored at 225 nm at

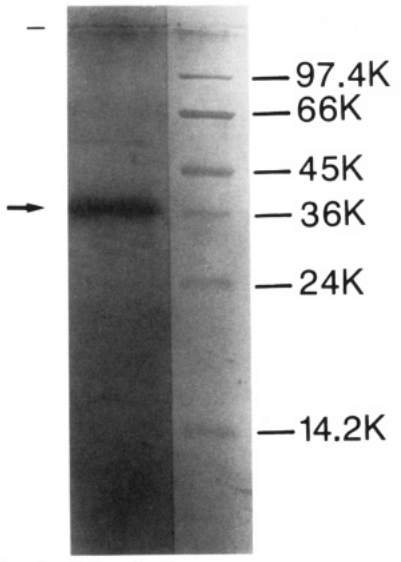

FIGURE 1: SDS–PAGE analysis of the purified *H. pulcherrimus* hatching enzyme. The acrylamide concentration of the separation gel was 15%, and protein bands were stained with Coomassie Brilliant Blue. The bar near the top of the gel indicates the interface of the stacking and separation gels. The arrowhead indicates the band of HEz of M_r 37 000.

the range of 0.16, on a Shimadzu LC-4A liquid chromatograph. An adequate gradient was chosen for each peptide to achieve the best resolution of the product fragments and the parent peptide, e.g., from 5 to 42% in 35 min for oxidized insulin B chain, and for most other peptides from 0 to 30–50% in 30–40 min. Each peak was collected with a Gilson CPR REF 45500 peak collector, with care not to lose the peaks of low absorbance due to the absence of aromatic amino acid residue(s). The peak fractions were lyophilized and used for the following analyses.

Amino Acid Analysis of Peptide Fragments. The lyophilisates were hydrolyzed with constant-boiling HCl (5.7 N) at 110 °C for 20 h, dried in a vacuum desiccator over NaOH pellets, and dissolved in 230 μL of 0.02 N HCl, and 100 μL was automatically loaded on a Hitachi 835-50 amino acid analyzer.

Identification and Quantification of Peptide Fragments. Since the amino acid sequences of the parent peptides were known, each fragment was easily identified, in most cases, by its amino acid composition. Quantification of each fragment was also achieved by the results of amino acid analysis. Consequently, the site of cleavage and its extent were determined by delineating the fragments and summing up the amounts of peptides generated from either the amino or carboxyl side of a certain peptide bond cleaved by the enzymes.

Species Specificity. Ethanol-fixed 4–8-cell stage embryos of five species of sea urchins in addition to *H. pulcherrimus* were prepared and used in the HEz assay by the method of Barrett and Edwards (1976). A partially purified HEz preparation prior to the final purification on Superose 12 HR chromatography was used in this experiment. At adequate time intervals the number of denuded embryos were counted and its percentage was calculated.

RESULTS

Purification of Hatching Enzyme. The SDS–PAGE analysis with Coomassie Blue staining of the finally purified HEz demonstrated that it is almost homogeneous, exhibiting a major protein band of 37 kDa with a couple of faint bands in the 57–65-kDa region (Figure 1). When the HEz was preincubated at 37 °C for 1 h, it exhibited no band of 37 kDa but a few bands in the 10–30-kDa region. When it was pretreated with 50% trichloroacetic acid, however, it exhibited the 37-kDa band without any change in the gel pattern. The finally purified enzyme preparation demonstrated both hatching and caseinolytic activities but no glucanase activity. From 10 L of hatching supernatant, 259 μg of purified enzyme was obtained with a specific activity of 1612 units/mg (149-fold increase over the supernatant). The recovery of activity at each step was as follows: first red agarose, 23%; second red agarose, 9%; and Superose 12HR, 5%. This low value is presumably due to autolytic degradation during the isolation procedure extending over many days.

Table I: Effects of Protease Inhibitors on Hatching Enzyme Activities[a]

inhibitor	rel act. on the FE of fixed *H. pulcherrimus* embryos: crude enzyme	rel act. on the FE of fixed *H. pulcherrimus* embryos: purified enzyme	rel act. on substance P: purified enzyme
control	100	100	100
1% DMSO control	100	96	100
TLCK	100	100	100
antipain	100	104	
leupeptin	100	104	100
elastatinal	100	100	
bestatin	100	100	
phosphoramidon	100	96	100
E-64	100	96	–
TPCK	100	96	85
chymostatin	8	90	23
pepstatin	55	87	83
PMSF	100	87	100
1,10-phenanthroline	0	0	7
α_2-macroglobulin	0	0	–
EDTA (10 mM)	0	0	0
EGTA (10 mM)	0	0	–

[a] The concentration of the inhibitors was 1 mM except for 10 mM of the chelating agents, 1,10-phenanthroline, EDTA, and EGTA, and 1 mg/mL of α_2-macroglobulin.

Effects of Inhibitors. Various inhibitors were tested for their effects on the hatching activity and substance P hydrolysis, as shown in Table I. The three chelating agents 1,10-phenanthroline, EDTA, and EGTA, as well as the serum high-molecular-weight protease inhibitor α_2-macroglobulin, completely inhibited the hatching activity of both crude and purified HEz. However, phosphoramidon, a *Streptomyces* inhibitor for metalloproteinases, was not inhibitory. Most microbial inhibitors of serine and thiol proteases and chloromethyl ketone type inhibitors were ineffective in inhibiting hatching activity of both crude and purified HEzs. PMSF, a serine protease inhibitor, was slightly effective on the purified enzyme while it was ineffective with the crude enzyme. The above results indicate that the HEz is a metalloproteinase and rule out the possibility that it is either a chymotrypsin-like serine protease or a type of calcium-activated thiol protease called calpain (EC 3.4.22.17). Somewhat puzzling was the observation that chymostatin, a *Streptomyces* inhibitor of chymotrypsin and papain, was highly inhibitory with crude HEz but only slighty effective with purified HEz. Pepstatin, an inhibitor for pepsin and other aspartic proteases, was moderately effective for both crude and purified enzymes. When substance P was used as a substrate, the inhibitory spectrum of the purified enzyme was slightly different from that of the hatching activity; the inhibition by chymostatin was more remarkable, although less than that on the crude enzyme's hatching activity. The results are suggestive of the presence of another metalloproteinase or proteinases with

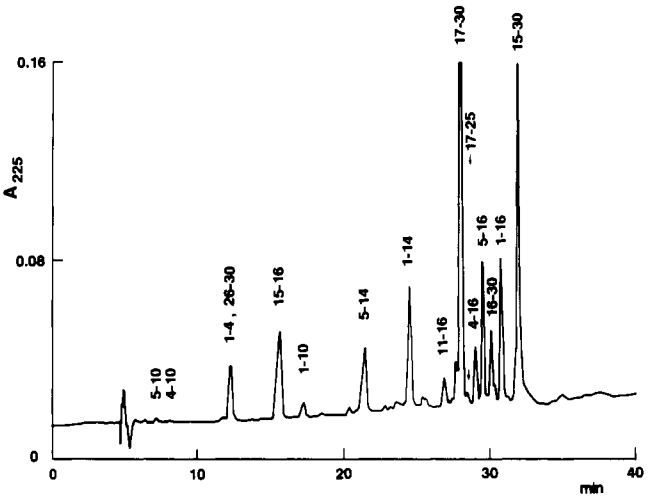

FIGURE 2: Reverse-phase HPLC chromatogram of the hatching enzyme hydrolysate (12 h) of oxidized bovine insulin B chain. Elution was carried out by a gradient of acetonitrile (5–42% in 35 min) in 0.1% trifluoroacetic acid. Each peak was identified as the peptide fragment, with the sequence as indicated above it as determined by amino acid analysis. Intact oxidized insulin B chain (1–30) was eluted at 34 min on a separate elution.

greater sensitivity than HEz to the inhibitors chymostatin and pepstatin. The results also indicate that the inhibition spectrum varies depending on the nature of substrate used. Since chymostatin and pepstatin are mostly constituted of hydrophobic residues and without Arg residues, the apparent inhibition is presumably via substrate competition or nonproductive binding rather than by the action of C-terminal aldehyde groups.

Hydrolysis of Oxidized Bovine Insulin B Chain. A typical chromatogram for the HEz hydrolysis of insulin B chain (12 h) is shown in Figure 2. Each peak was identified as indicated, by amino acid analysis and reference to the sequence of the whole chain (Figure 3). The following fragments with the sequence and percentage yield in parentheses were obtained: 5–10 (trace), 4–10 (trace), 1–4 (38%), 26–30 (8%), 15–16 (29%), 1–10 (6%), 5–14 (20%), 1–14 (16%), 11–16 (6%), 17–30 (65%), 4–16 (7%), 5–16 (16%), 16–30 (6%), 1–16 (13%), and 15–30 (17%). By longer incubation with more enzyme, a trace amount of the fragment 5–11 derived by the cleavage at the Leu^{11}-Val^{12} bond was detected in the HPLC chromatogram (data not shown).

The four enzymes tested exhibited different cleavage patterns as depicted in Figure 3. At 3 h, HEz preferentially cleaved the Tyr^{16}-Leu^{17} bond and, to a lesser extent, the Ala^{14}-Leu^{15} bond. After 12-h incubation the cleavage of these bonds did not increase substantially, but rather an unspecific cleavage at Gln^4-His^5 increased. Thermolysin preferentially cleaved the amino side of four -Leu residues, moderately at -Phe and -Tyr residues, and to a lesser extent at two -Val residues. A trace amount of the fragment 26–29 generated by the unspecific cleavage at Lys^{29}-Ala^{30} was detected. The cleavage pattern is the same as that reported by Morihara and Tsuzuki (1966) except that an additional cleavage at Phe^1-Val^2 was observed in the current work. It is also similar to that of α-protease which cleaves it at four -Leu residues and Gly^{23}-Phe^{24} (Pfleiderer & Krauss, 1965). The action of chymotrypsin for 1 h was the most specific, cleaving the Tyr^{16}-Leu^{17} bond completely and the carboxyl side of two Phe-residues to different extents. Subtilisin (5 h) cleaved the carboxyl side of Leu-, Phe-, Tyr-, Gln-, and Asn- and, furthermore, at acidic and basic residues. In this experiment HEz and chymotrypsin turned out to cleave most preferentially the same peptide bond, Tyr^{16}-Leu^{17}. It cannot be deduced, however, that both enzymes have the same specificity. Rather, the results indicate that HEz's primary specificity is close to that of thermolysin, although the cleavage sites are more limited depending on the amino acid sequence around the scissile bonds (secondary specificity). In order to verify the thermolysin-like specificity of HEz, a set of smaller peptides was used and the sites and extents of cleavage were determined as described below.

Hydrolysis of Small Peptides. The results on 14 peptides consisting of 8–13 amino acid residues are shown in Figure 4. Angiotensin I was cleaved by HEz only partially at Pro^7-Phe^8, while thermolysin cleaved almost completely at Arg^2-Val^3 and Pro^7-Phe^8, and partially at Tyr^4-Ile^5 and His^9-Leu^{10}. Similar cleavage patterns were obtained on bradykinin and dynorphin A (1–13); the scissile bonds by HEz

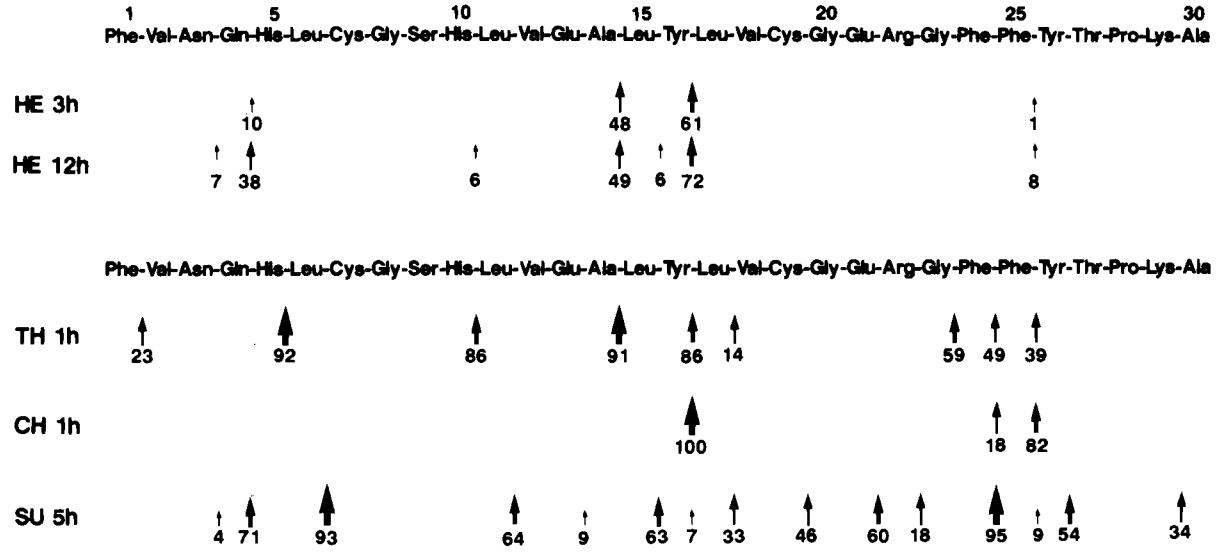

FIGURE 3: Sites and relative extents of cleavage in the oxidized insulin B chain by the action of hatching enzyme and other three proteases. The times of incubation in hours are shown after the enzyme names. The numbers accompanying the arrows indicate the relative extents of cleavage in percentages. Various sizes of arrowheads indicate four grades of relative extent of cleavage: less than 11%, 11–50%, 51–90%, and more than 90% in the order of increasing size. Abbreviations for enzymes are as follows: HE, hatching enzyme; TH, thermolysin; CH, chymotrypsin; SU, subtilisin (Carlsberg). Cys denotes cysteic acid.

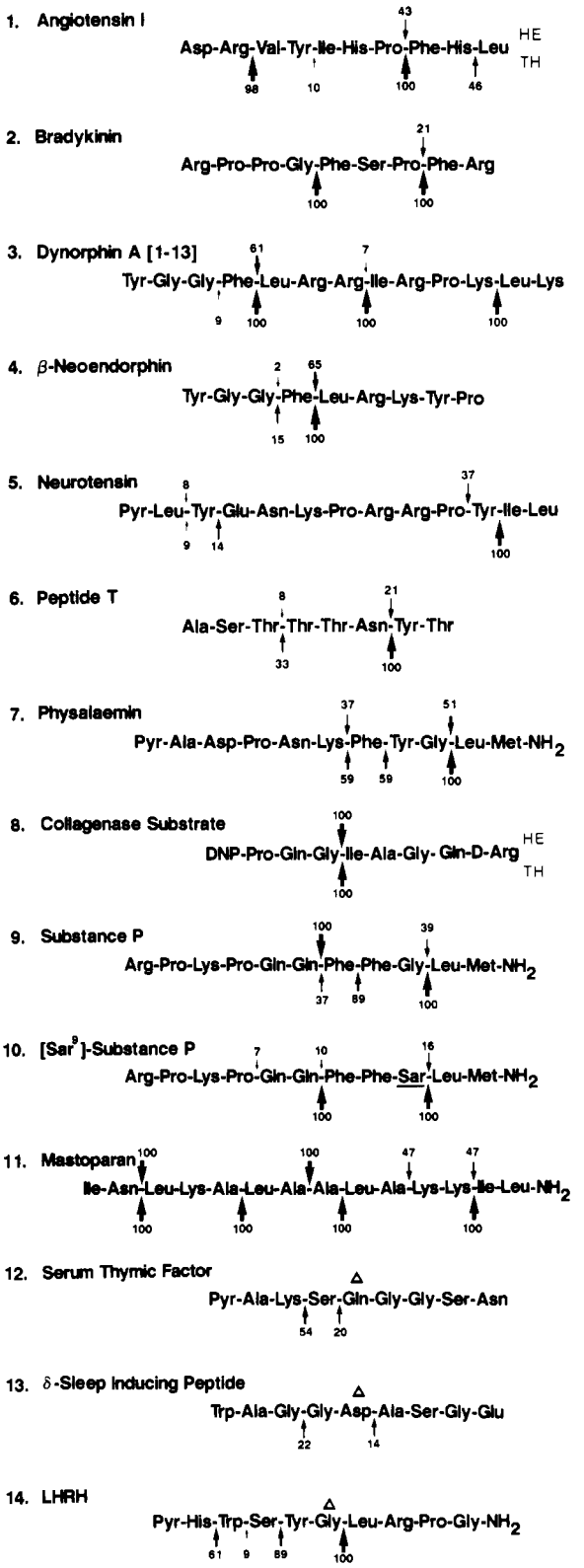

FIGURE 4: Sites and relative extents of cleavage of various peptides by hatching enzyme and thermolysin. The arrows above and below the sequences indicate cleavages by HEz and thermolysin, respectively. The usage of numbers and arrows is the same as in Figure 3. The triangles above peptides 12–14 indicate "no cleavage" by HEz.

action were more limited than thermolysin. Especially, it should be emphasized that the HEz cleavage at the hydrophobic residues (-P_1') accompanying Pro two residues downstream (P_3' position) was suppressed to null or to a very low extent: -Ile-His-Pro, -Phe-Ser-Pro, and -Ile-Arg-Pro, respectively, in the above three peptides. Both enzymes failed to cleave the Lys^7-Tyr^8 bond in β-neoendorphin because it was followed by a -Pro^9 residue (P_2' position). In the C-terminal region of neurotensin the two enzymes exhibited different action patterns; HEz cleaved Pro^{10}-Tyr^{11} partially while thermolysin cleaved Tyr^{11}-Ile^{12} completely. The amino-terminal region, Pyr-Leu-Tyr-Glu-, had low susceptibility to both enzymes. The lower extent and limited cleavage by HEz as compared with thermolysin were also shown on physalaemin.

In contrast to the above seven peptides with lower susceptibility to HEz than to thermolysin, there were some peptides with susceptibility to HEz as high as to thermolysin: synthetic substrate for vertebrate collagenase, substance P, and mastoparan. Collagenase substrate that mimics the sequence around the scissile bond Gly–Ile in native collagen (Masui et al., 1977) was completely cleaved by both enzymes at the same position.

Substance P was cleaved by HEz 100% at Gln^6-Phe^7 and 39% at Gly^9-Leu^{10}. In sharp contrast, thermolysin cleaved 100% at Gly^9-Leu^{10}, 89% at Phe^7-Phe^8, and 37% at Gln^6-Phe^7, so to say, in an opposite manner. The analogue [Sar^9]substance P, containing a sarcosine (*N*-methyl-Gly) residue at position 9, provided us the most interesting and valuable result. The cleavage of the bond Gln^6-Phe^7 that exhibited high susceptibility to HEz when in substance P was suppressed to only 10%, and the bond Gly^9-Leu^{10} was suppressed to 16%, with an additional and unexpected cleavage at Pro^4-Gln^5 of 7%. Thermolysin was as active with the analogue as with substance P, although with an altered cleavage pattern. It cleaved 100% at the Sar^9-Leu^{10} in the analogue in the same manner as in substance P, while the cleavage of Phe^7-Phe^8 was completely suppressed, and instead Gln^6-Phe^7 was cleaved 100%. Substance P was also cleaved by α-protease at Gln^6-Phe^7 (36%) and Gly^9-Leu^{10} (100%), but the [Sar^9] analogue was exclusively cleaved at Sar^9-Leu^{10} with only a trace of cleavage at Gln^6-Phe^7. The suppression of the HEz and α-protease cleavage at the Gln^6-Phe^7 bond in [Sar^9]substance P is similar to that first reported on with human fibroblast stromelysin (Teahan et al., 1989); it will be later discussed in detail.

Mastoparan was also a good substrate for both enzymes. Thermolysin cleaved the peptide at three -Leu and one -Ile positions completely into five distinctive peptide fragments, leaving intact the Ile-Leu bond in the C-terminal region. Hatching enzyme cleaved 100% at Asn^2-Leu^3 and 47% at Lys^{12}-Ile^{13}, without any cleavage of Ala^5-Leu^6 and Ala^8-Leu^9 bonds. Furthermore, unexpectedly, it showed unspecific cleavage at the palindromic center Ala^7-Ala^8 (100%) and to a lesser extent at Ala^{10}-Lys^{11} (47%).

Serum thymic factor and δ-sleep inducing peptide that contain no hydrophobic residues except at the N-termini were not cleaved at all by HEz, while thermolysin moderately cleaved them at the amino sides of -Ser, -Gln, -Gly, and -Ala. Somewhat peculiarly, LHRH containing three hydrophobic residues, -Trp, -Tyr, and -Leu, in the sequence was not cleaved by HEz, while it was a good substrate for thermolysin. The failure of HEz to cleave at Gly^6-Leu^7 is interpreted as the same case as [Sar^9]substance P, because it contains Pro^9 at the P_3' position. The failure to cleave the other two bonds is difficult to interpret, but might be due to the presence of a Trp residue.

The above results demonstrated that HEz cleaved preferentially the peptide bonds followed by bulky hydrophobic amino acid residues, -Leu, -Ile, and -Phe as well as -Tyr, with a few exceptions. The Xaa-Val bonds in the peptides tested were not cleaved by HEz, at least by 12-h incubation, while thermolysin cleaved well the Arg^2-Val^3 bond in angiotensin I and moderately two of the three -Val bonds in insulin B chain. The primary specificity of HEz, thus, was revealed to

Table II: Species Specificity of *H. pulcherrimus* Hatching Enzyme As Examined by Its Activity To Dissolve the Fertilization Envelopes of Ethanol-Fixed Embryos from Six Species of Sea Urchin

species origin of ethanol-fixed embryos	% of denuded embryos after an incubation time (h) of						
	0.5	0.75	1.0	1.5	3.0	6.0	24.0
H. pulcherrimus	0	100					
A. crassispina (F)	0	0	0	0	0	0	0
G. crenularis (O)	0	20	60	100			
C. japonicus (SC)	0	0	0	0	<5	20	40
A. manni (SC)	0	0	0	0	0	<5	40
P. japonica (SC)	0	0	0	0	0	0	20

[a] The five sea urchin species other than the homologous *H. pulcherrimus* are listed in the order of decreasing taxonomical relatedness. The symbols in parentheses indicate the difference from *H. pulcherrimus* by family (F), order (O), or subclass (SC).

be very close to that of thermolysin if not identical. However, the number of bonds cleaved by HEz was much smaller than by thermolysin, indicating the higher dependence on the amino acid sequence around the scissile bond (secondary specificity). A very clear example is the suppressed cleavage of the P_1–P_1' bond by the presence of imino acid (proline or sarcosine) at the P_2' or P_3' position for HEz and at the P_2' position for thermolysin. Hatching enzyme and α-protease, therefore, have higher secondary specificity than thermolysin.

In supplementary experiment, the pentapeptide Cbz-Gly-Pro-Leu-Gly-Pro, a substrate for bacterial collagenase (cleavage at Leu-Gly) and for a neutral metalloproteinase contaminating the commercial collagenase sample, as well as for thermolysin (Pro-Leu cleavages; Nomura, 1980), was tested for the cleavage by HEz and α-protease. The peptide was not cleaved at all by HEz (20 °C, 24 h), nor by α-protease (37 °C, 8 h). This confirms the P_3' restriction for both enzymes, in sharp contrast with thermolysin.

Species Specificity. The susceptibility of the ethanol-fixed embryos from six species of sea urchins to the *H. pulcherrimus* HEz is summarized in Table II. The homologous embryos were denuded completely by 45 min, and those of *G. crenularis*, belonging to a different taxonomical order, were 100% denuded by 90 min. In contrast, the embryos of *A. crassispina* that belong to a neighborning taxonomical family of the same suborder were not denuded even ater 24 h. The other three species, *C. japonicus*, *A. manni*, and *P. japonica* (sand dollars), which are taxonomically far from *H. pulcherrimus*, belonging to a diffrent subclass, were moderately (20–40%) denuded after 24-h incubation. Therefore, sea urchin HEz can dissolve the FE of various heterologous species, some of which may be very distantly related.

Discussion

The investigation of the HEz of sea urchin has a long history spanning more than half a century from the discovery that it was a protease (Ishida, 1936; Sugawara, 1943) to the recently accomplished purification, characterization, and cDNA cloning (Lepage & Gache, 1989, 1990; Roe & Lennarz, 1990). During this period of slow progress major interest has focused on the regulation of gene expression and enzyme biosynthesis (Barrett & Angelo, 1969; Showman & Whiteley, 1980), as well as purification, crystallization (Yasumasu, 1961), and characterization. Investigations from the late 1960s through the late 1980s mainly by two groups, Barrett's and Hoshi's, laid the cornerstone for today's knowledge of the basic aspects of sea urchin HEz.

Purification of HEz. The difficulty in isolating the HEz has mainly resided in the unusually strong binding of the enzyme to its substrate and products, the FE and its fragments. Most trials to dissociate the enzyme–products complex with chaotropic agents induced autolysis into inactive forms and reduced the yield of native enzymes. Furthermore, those HEzs claimed to be purified to homogeneity were devoid of hatching activity in seawater. We isolated and purified the HEz of *H. pulcherrimus* for the first time to almost an electrophoretically homogeneous level and retained the hatching activity at high salt concentration (0.5 M NaCl) as in seawater. Hydrophobic chromatography on a column of reactive red 120–agarose, first used by Lepage and Gache (1989) for a HEz under the name of Procion–agarose, simplified the purification procedure. We started the purification of HEz from the supernatant fluid of naturally hatched blastulae without prior dissolution of FE with crude HEz (Lepage & Gache, 1989), intending to avoid the autolysis prior to chromatography. After the further purification on a Superose 12 HR column, however, the final yield was not as high as those achieved by Lepage and Gache (1989), presumably due to autolysis caused by storage at 4 °C for several days between the chromatography steps. The specific activity of our purified enzyme (1612 units/mg) cannot be directly compared with that of Lepage and Gache (1989), since they did not follow the purification by hatching activity on ethanol-fixed embryos but rather by caseinolytic activity. They showed only in summary that their purified enzyme (100 ng) completely denuded the 200 embryos at 20 °C in 20 min. Our enzyme (83 ng) denuded the same number of embryos at 20 °C in 75–90 min. Although the hatching activity assays were not performed under the same conditions, these time values might allow one to conclude that the two purified enzyme samples have comparable, if not identical, specific activities.

Structural Features of HEz. The molecular weight of our active HEz was estimated as 37 000. According to Lepage and Gache (1990) HEz is synthesized as a 587 amino acid long preproenzyme with M_r 65 000, processed by truncation of a 18 residue long signal peptide into a proenzyme (569 residues, M_r 63 000) that is further processed in a Ca^{2+}-dependent manner to an active enzyme (420 residues, M_r 48 000). An autolysis product of M_r 30 000 is devoid of the N-terminal 180 residues including the HEXXH motif corresponding to the zinc binding residues at the active site of a number of metalloproteinases (Jongeneel et al., 1989), thus confirming that the autolysis product is inactive. Our present result and that of Roe and Lennarz (1990) demonstrated, however, that the molecular species of M_r 37 000 and 33 000, respectively, are fully active, although they are doubtlessly the products of proteolytic cleavage by autolysis or by other proteinase(s). This discrepancy may be compromised by assuming that these small active enzymes are derived from the 50–60-kDa secreted enzymes by appropriately releasing a C-terminal sequence, although it is left to be directly proved by determination of the amino acid sequence of the small active enzyme. The missing sequence is possibly the hemopexin domain, similar to PUMP-1 with M_r 28 000 [Quantin et al., 1989; for a short recent review, see Matrisian (1990)]. There are a couple of other examples of small molecular weight metalloproteinases. An *Astacus* metalloprotease originally described to have M_r 11 000 has been reported to have M_r 22 614 (Titani et al., 1987). A hemorrhagic metalloproteinase, HT-2, from snake venom with 202 residues was sequenced and found to contain a HEXXH motif at residues 142–146 (Takeya et al., 1990). Thermolysin has an M_r of 34 400 (Titani et al., 1972).

Inhibitors of HEz. The HEz of *H. pulcherrimus*, both crude and purified, was inhibited by the three chelating agents

as well as α_2-macroglobulin, but not in the least by most other inhibitors for serine and thiol proteinases. These inhibitor spectra established the metalloproteinase nature of HEz. Exceptionally, chymostatin and pepstatin inhibited the crude enzyme significantly and the purified one slightly. The chymostatin inhibition of crude HEz appears much stronger than the result of Hoshi et al. (1979), partly due to its 3-fold higher concentration. This finding, combined with the results on crude HEz by Hoshi et al. (1979) and Post et al. (1988), suggests the presence in the hatching supernatant of another metalloproteinase with sensitivity to the above two inhibitors higher than HEz. Indeed, in the preliminary stage of our experiment, we found a protease fraction with caseinolytic activity but without hatching activity during Sephadex G-100 chromatography of the concentrated hatching supernatant fluid (Nomura et al., 1988). This enzyme was completely inhibited by 10 mM EDTA and 1 mM chymostatin when β-neoendorphin was used as a substrate, while chymotrypsin and subtilisin were fully inhibited by chymostatin but not by EDTA at all. However, since the purified enzyme was also significantly inhibited by chymostatin when substance P was used as substrate, it should be kept in mind that the extent of inhibition of an inhibitor depends on the nature of substrate used. This inhibition appears to be due to substrate competition rather than mechanism-based inhibition, because HEz is proved to be a metalloproteinase but not a serine enzyme, and hence the aldehyde group of the inhibitor is not likely to react with the catalytic site residues of HEz. The structure of chymostatin and pepstatin, comprising mostly hydrophobic residues but without an arginine residue, supports this. It is of interest that reactive red 120, a triazine dye, which was used as a ligand for affinity chromatography of HEz, was an inhibitor of it more potent than 1,10-phenanthroline (Roe & Lennarz, 1990). This dye, if properly derivatized, may be a highly specific inhibitor of HEz or of stromelysin.

Primary Specificity. The purified HEz exhibited highly specific cleavage patterns on the 15 peptides tested in this experiment. This indicates the high degree of purification of this enzyme sample free from contamination of any other protease. On the basis of those results the primary specificity of HEz was determined, for the first time, as almost the same as that of thermolysin, both cleaving the peptide bonds on the amino side of hydrophobic amino acid residues with bulky side chains, -Leu, -Ile, and -Phe, as well as -Tyr (Matsubara, 1970), although none of the Xaa-Val bonds cleaved by thermolysin were significantly cleaved by HEz in the 15 peptides tested.

Exceptional cleavages by thermolysin at -Thr, -Ser, -Glu, -Gln, -Ala, and -Gly were observed in the peptides used in this experiment, consistently with the previous report [Matsubara (1970) and references therein]. These exceptional or unspecific cleavages are apt to occur in the substrates where no or few specific scissile bonds are present. When small peptides are used to determine the primary and secondary specificity of a protease, care should be taken to choose peptides with sufficient length and variety of amino acid residues. Otherwise, it may lead to an erroneous conclusion by taking the unspecific cleavages as specific.

Thus, the claim by Barrett and Edwards (1976) that the *Strongylocentrotus purpuratus* HEz is specific to Glu- and Asp- bonds is hardly acceptable, because it is based on indirect evidences, e.g., inhibition by Cbz-Glu, cleavage of Cbz-Glu-Tyr, Cbz-Glu-Phe, and Cbz-Glu-Leu, etc. They should have used larger peptides such as oxidized insulin B chain to determine the cleavage site precisely as was done in our experiments. There is, however, left a possibility that the HEz of this particular species *S. purpuratus* really has such specificity, since no one has thus far determined the cleavage sites in larger peptides by isolating the product fragments.

According to the result of cDNA cloning for *P. lividus* prepro-HEz with 587 amino acid residues, its signal peptide is released by cleavage of the Thr^{18}-Val^{19} bond, the autocatalytic activation site is Pro^{166}-Phe^{167}, and the autolytic inactivation occurs by cleavage of the Ser^{324}-Leu^{325} bond (Lepage & Gache, 1990). The latter two cleavages by HEz itself at -Phe and -Leu are consistent with the primary specificity of HEz established in the present work, as well as the secondary specificity and P_3' restriction discussed below.

Secondary Specificity. The secondary specificity of HEz was much narrower than that of thermolysin, as was shown in the limited cleavage pattern on the various peptides. The most significant finding is that HEz and α-protease can hardly, if at all, cleave the Gln^6-Phe^7 bond in [Sar^9]substance P, while it easily cleaves the same bond in substance P, whose ninth residue is Gly. This tendency is very similar to that of human fibroblast stromelysin that has much narrower substrate specificity against small peptides, cleaving only the Gln^6-Phe^7 bond in substance P, among various peptides such as angiotensin I, bradykinin, synthetic collagenase substrate, etc. (Teahan et al., 1989). This highly specific enzyme, stromelysin (MMP-3), is capable of degrading extracellular matrix proteins such as fibronectin, laminin, type IV collagen, proteoglycan, and elastin and belongs to a collagenase gene family with high sequence homology to collagenase (Chin et al., 1985; Whitham et al., 1986; Saus et al., 1988). Although HEz is not as specific as stromelysin, cleaving the above peptides as well as casein and the natural substrates FE proteins, its low capability in cleaving the Gln^6-Phe^7 bond in the substance P analogue rationally leads to the conclusion that HEz belongs to a stromelysin subfamily or more widely to an MMP family rather than to a family of bacterial metalloproteinases represented by thermolysin (EC 3.4.24.4). In this regard α-protease is also concluded as a member of stromelysin subfamily, although it exhibited exceptional cleavages at Xaa-Ser and Xaa-Gln bonds in an encephalitogenic fragment of guinea pig myelin basic protein (Nomura et al., 1977). MMP-7, extracted from rat uterus and supposedly identical with PUMP-1, exhibited cleavage patterns very close to that of HEz. It cleaved insulin B chain at two points, Ala^{14}-Leu^{15} and Tyr^{16}-Leu^{17}, and moreover cleaved the synthetic collagenase substrates DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH_2 and DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg, but not PZ-Pro-Leu-Gly-Pro-D-Arg (Woessner & Taplin, 1988; the mistyped sequences of DNP-peptides were corrected). These results are in favor of our conclusion, although the authors emphasized the difference of the uterus enzyme from stromelysin.

P_2'/P_3' Restriction. The discrimination of HEz from thermolysin is based on the difference in the cleavage patterns revealed on small peptides: the suppressive effect of the imino acid (Pro and Sar) at either the P_2' or P_3' position for HEz, as compared with only P_2' for thermolysin. The resistance of [Sar^9]substance P to stromelysin is also ascribed to the suppression by the P_3' imino acid, thus allowing the classification of HEz, stromelysin, and possibly α-protease into one group. Human fibroblast collagenase may also be a member of it, since its cleavage sites demonstrated in collagens, α_2-macroglobulins, and some other related proteins are consistent with the P_2'/P_3' restriction except for a single case in the autolytic cleavage of itself: Pro-Ile cleavage in the sequence -Pro-

Val-Gln-Pro-Ile-Gly-Pro-Gln- (Sottrup-Jensen & Birkedal-Hansen, 1989). There are, also in HEz cleavages, two cases contrary to the above hypothesis. It attacked bradykinin and peptide T on the peptide bonds with the amino group of the residue penultimate to the C-terminal, where no P_3' residue can be assumed. In bradykinin the bond Gly^4-Phe^5 cannot be cleaved because of the presence of Pro^7 three residues downstream, and hence the only potentially scissile bond left is Pro^7-Phe^8, even lacking the P_3' residue. The same interpretation is possible for peptide T where Asn^6-Tyr^7 was moderately cleaved and, in addition, Thr^3-Thr^4 was cleaved slightly. As noted above, exceptional or unspecific cleavages are apt to occur in the substrates in which no or few specific scissile bonds are present.

The catalytic restriction by the presence of imino acids or by the absence of any amino acid appears to be based on the inability of forming a catalytically essential hydrogen bond between the NH of P_3' residue (Gly vs Sar/Pro) of the substrate and the catalytic group of the enzyme, as suggested by Teahan et al. (1989).

We would like to emphasize the P_2'/P_3' restriction as a distinctive characteristic for the stromelysin subfamily, or more widely for the MMP family (collagenase gene family), first revealed in this experiment in comparison with the simple P_2' restriction of thermolysin. It might be concluded that in general eukaryotic metalloproteinases have P_2'/P_3' restriction, while prokaryotic metalloproteinases have the simple P_2' restriction.

Now that the sea urchin HEz is classified as a metalloproteinase with the specificity between thermolysin and stromelysin, we propose the name "envelysin" for the HEz of sea urchin, or of wider origins, if established. It might also be called "MMP-11". We propose furthermore a correction of the description on HEz (EC 3.4.24.12) in *Enzyme Nomenclature* (International Union of Biochemistry, Nomenclature Committee, 1984) that it has a specificity for Glu- and Asp-residues, on the basis of the paper of Barrett and Edwards (1976). The description on the specificity of α-protease (*Crotalus atrox* metalloproteinase, EC 3.4.24.1) should also be corrected.

Mechanism of FE Dissolution. Since our primary interest is the mechanism of hatching, i.e., dissolution of the FE by HEz, the next target of the specificity study is its natural substrate. Un-cross-linked FE, prepared from the embryos fertilized in the presence of 3-amino-1,2,4-triazole, an inhibitor of ovoperoxidase, exhibits various protein bands on SDS–PAGE, ranging from 15- to ca. 300-kDa molecular mass (Nomura et al., 1990a). Among those FE component proteins, both cross-linked and un-cross-linked, the proteins of 96–91, 74, and 50 kDa from *S. purpuratus* gradually decreased (Uher & Carroll, 1987) and finally disappeared. Apparently, the opposite result was obtained on the same species by Roe and Lennarz (1990), i.e., that only the high molecular mass proteins (>100 kDa) were degraded rapidly, producing a prominent 40-kDa intermediate that may be further degraded into smaller fragments. Moreover, a subset of proteins of low molecular mass (<100 kDa) were not significantly degraded. These results indicate that HEz has a relatively high degree of protein substrate specificity. This, along with the species specificity, is an intriguing aspect of this enzyme and should be examined in more detail. Actually, it is currently in progress in our laboratories using isolated individual FE proteins and other mammalian extracellular matrix proteins.

Since HEz is demonstrated as a member of the MMP family, it is highly probable that it is inhibited by tissue inhibitors of metalloproteinases, TIMP and TIMP-2 (Cawston et al., 1981; Stetler-Stevenson et al., 1989), the verification of which is planned in our next experiment.

Species Specificity. Another question of high interest is the species specificity as demonstrated for *H. pulcherrimus* HEz against the ethanol-fixed embryos from six species of sea urchin. Among the FEs from the five heterologous species, that of *A. crassispina*, which belongs to a neighboring family and hence the most closely related one, was resistant at least for 24 h, while that of *G. crenularis*, the second most related one, was much more susceptible to HEz action. The species *H. pulcherrimus* has a different breeding season (early spring) than that of *A. crassispina* (summer), and its habitat (middle and southern parts of Honshu Island) is different from that of *G. crenularis* (Aomori Bay, northern end of Honshu Island). Thus the species specificity among the three species is not necessarily essential for their successful hatching. In general, a protease cannot exert species specificity on a certain substrate protein from various species if it has the same structure represented by amino acid sequence, three-dimensional structure, and the mode of association. Therefore, the difference in susceptibility revealed in this experiment depends on and reflects the variety in the component proteins, their mode of association into mature FE, and the extent of cross-links. Indeed, the FEs from the three species revealed different molecular weights of major compnent proteins and various contents of Tyr-derived cross-links (Nomura et al., 1990a). However, it cannot be an answer, but rather another question emerges whether the FE of each species has a key protein that is a specific target of its homologous HEz and plays an essential role in maintaining the integrity of the FE structure. Anyway, the knowledge of the component proteins of FE and their mode of association as well as the structure of vitelline coat as a scoffold for the FE is the prerequisite for the detailed elucidation of the mechanism of FE dissolution by HEz.

Physiological Roles of Metalloproteinases. There are some other metalloproteinases involved in the early development of sea urchin: a Zn^{2+}-dependent metalloendopeptidase involved in acrosome reaction (Farach et al., 1987) and that involved in gamete fusion (Roe et al., 1988), both of which thus far have been left for isolation and characterization.

From the teleost *Oryzias latipes* (medaka) two forms of HEz, designated as high and low choriolytic enzymes (HCE and LCE), were isolated and characterized as Zn^{2+} proteases (Yasumasu et al., 1989a,b). LCE can digest the inner layer of the chorion only after it is swollen by the action of HCE.

Metalloproteinases of wide origin appear to play a major role in degradation of structural proteins. Among them, MMPs and their specific inhibitors, with a wide variety of physiological and pathological implications in mammals, have attracted much attention for the last decade. The fact that sea urchin HEz belongs to the MMP family (collagenase gene family), and especially to the stromelysin subfamily, is of great interest from the standpoint of nonmammalian biology and biochemistry as well as general protein chemistry and enzymology.

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Registry No. LHRH, 33515-09-2; Hez, 50812-13-0; MMP, 79955-99-0; angiotensin I, 484-42-4; bradykinin, 58-82-2; dynorphin A [1–13], 72957-38-1; β-neoendorphin, 77739-21-0; neurotensin, 39379-15-2; peptide T, 106362-32-7; physalaemin, 2507-24-6; collagenase substrate, 63014-08-4; substance P, 33507-63-0; [Sar^9]-substance P, 77128-75-7; mastoparan, 72093-21-1; serum thymic

factor, 63958-90-7; δ-sleep inducing peptide, 69431-45-4; insulin, 9004-10-8.

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