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Stereo-specific inhibition of sea urchin envelysin (hatching enzyme) by a synthetic autoinhibitor peptide with a cysteine-switch consensus sequence

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Inhibition of envelysin, a metalloproteinase which dissolves the fertilization envelope of sea urchin embryo, was studied using a synthetic autoinhibitor peptide, Ac-Pro-Arg-Cys-Gly-Val-Pro-Asp-Val-NH_2, with a 'cysteine-switch' consensus sequence. Although its effect is reversible, the hatching of sea urchin embryos was effectively delayed by 0.5 mM of the peptide. When α_1-proteinase inhibitor was used as the substrate, envelysin was inhibited by the autoinhibitor and an Ala^6 analogue, but not by a D-Cys^3 analogue. However, envelysin was weakly inhibited by both D- and L-cysteines to the same extent. Snake venom α-protease exhibited cleavage and inhibition behavior similar to envelysin with a little weaker stereo-specificity. The results suggest that the coordination of the autoinhibitor Cys residue with the envelysin active site Zn is established only after the amino acid residues on both sides of the Cys residue get into an appropriate interaction with the catalytic site residues, and that the precise orientation of the cysteine SH group is essential. By contrast, thermolysin was weakly inhibited by the three peptide non-stereo-specifically. Furthermore, thermolysin cleaved the autoinhibitor at the Cys^3 Gly^4 bond when incubated without substrate.

Envelysin; Hatching enzyme; Sea urchin, Autoinhibitor; Cysteine switch, Stereo-specificity, D-Cysteine

1. INTRODUCTION

After about 12 h of fertilization, the sea urchin embryo secretes envelysin (EC 3.4.24.12, historically called 'hatching enzyme'), which dissolves the protective fertilization envelope and allows it to escape for free swimming with cilia. Although this enzyme has a long research history covering over half a century [1], its nature as a member of matrix metalloproteinase (MMP) family had not been elucidated until cDNA cloning of the enzyme isolated from the Mediterranean sea urchin *Paracentrotus lividus* was accomplished by Lepage and Gache in 1990 [2]. We purified a 37 kDa enzyme from *Hemicentrotus pulcherrimus,* designated it as envelysin, and determined its specificity at the cleavage site level using various peptide substrates [3]. The amino acid sequence and cDNA structure of *H. pulcherrimus* envelysin are still left to be determined; the enzyme is expected to have a domain structure very similar to that of *P. lividus*. In the propeptide domain, it has a region encompassing the sequence, Pro-Arg-Cys-Gly-Val-Pro-Asp-Val, highly conserved among MMPs [4,5]. The Cys residue coordinates the Zn atom in the catalytic center of MMP to achieve the latency of each proenzyme, and when the propeptide is removed, the Cys–Zn coordination is terminated and the active enzyme emerges. This is denoted the 'cysteine switch' mechanism [6,7]. The above sequence in the pro-domain acts as an 'autoinhibitor', and some synthetic peptides having the above sequence with or without some substitutions were shown to inhibit type IV collagenase and transin (rat stromelysin), typical MMP family members [8,9]. As a member of MMP family, sea urchin envelysin is also expected to be inhibited by the autoinhibitor. We report here the effects the synthetic autoinhibitor peptide and its D-Cys^3 and Ala^6 analogues, as well as D- and L-cysteines on the limited cleavage of human α_1-proteinase inhibitor by envelysin, snake venom α-protease and thermolysin by a semi-quantitative method using SDS-PAGE.

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2. MATERIALS AND METHODS

2.1. *Preparation of envelysin and autoinhibitor peptides*

Envelysin was isolated from the supernatant of the incubation medium of the hatched blastulae of *H. pulcherrimus* by the method previously reported [3]. In short, the enzyme in the supernatant was adsorbed to the column of Reactive Red 120-Agarose (Sigma, Type 3000), and the bound enzyme was eluted with a gradient of ethylene glycol (0–60%) in buffer A (10 mM HEPES (pH 8.2) containing 10 mM $CaCl_2$, 1 M NaCl, and 0.1% CHAPS). The fractions with the fertilization envelope-dissolving activity, assayed on ethanol-fixed 4-cell stage embryos, were concentrated with an Amicon PM-10 membrane and then chromatographed on a Superose 12HR column, and active fractions were collected. The enzyme solution in buffer A was kept frozen. It exhibited a major 37 kDa band with faint 32 kDa and 15 kDa bands on SDS-PAGE. About 100 μg of purified enzyme was obtained from 20 female urchins.

The autoinhibitor peptide, Ac-Pro-Arg-Cys-Gly-Val-Pro-Asp-Val-NH_2, and its D-Cys^3 and Ala^6 analogues were synthesized on a peptide

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synthesizer Applied Biosystems model 431A and purified by reverse-phase HPLC on a Senshupak column (6×250 mm) with an acetonitrile gradient in 0.1% trifluoroacetic acid. Snake venom α-protease was a product of P-L Biochemicals Inc. (Milwaukee, WI, USA), and thermolysin was purchased from Peptide Institute Inc. (Osaka, Japan). HEPES, CHAPS and human serum α_1-proteinase inhibitor (α_1-PI) were from Sigma Chemical Co. (St. Louis, MO, USA). D- and L-cysteines and other reagents of analytical grade were from Wako Pure Chemicals Inc. (Osaka, Japan).

2.2. *Effect of autoinhibitor on sea urchin hatching*

The eggs and sperm of *H. pulcherrimus* were prepared by the previously described method [3] and the eggs were fertilized, washed and incubated in Millipore-filtered seawater at 20°C with gentle stirring. After about 11 h, aliquots of the embryo suspension (about 200 embryos in 100 μl) were withdrawn and mixed with 400 μl autoinhibitor solutions in microplate wells, and further incubated at 20°C in a moistured chamber. At adequate time intervals the number of hatched (completely denuded) embryos were counted and the percentage was calculated.

2.3. *Assay of proteolytic activity*

Among the proteins tested for susceptibility to envelysin, α_1-PI was the best in terms of the rate and the limitedness of cleavage, and was therefore used throughout this experiment. The reaction mixture consisted of 0.2 μg envelysin, 60 μg α_1-PI, 0.5 mM inhibitor peptide or 1 mM D- or L-cysteine, 50 mM Tris-HCl (pH 7.5) and 5 mM $CaCl_2$ in a total 160 μl volume. The reaction was started by the addition of enzyme solution (5 μl) and continued up to 24 h at 30°C. At adequate time intervals, 15 μl aliquots were withdrawn and blown into plastic micro-tubes prefrozen on dry ice to stop the reaction. They were analyzed by SDS-PAGE on 12% gels and stained with Coomassie brilliant blue R-250.

The effects of the peptides and cysteines on α-protease (20 μg) and thermolysin (0.5 μg) were tested in a similar way to envelysin. In the case of thermolysin, the aliquots withdrawn were mixed with 2 μl of 0.1 M EDTA and quickly frozen on dry ice-acetone

2.4. *Cleavage of autoinhibitor by thermolysin*

Thermolysin turned out to cleave the autoinhibitor when incubated without α_1-PI. A 150 μl reaction mixture of 132 μg autoinhibitor and 10 μg thermolysin, after 3 h incubation at 37°C, was subjected to reverse-phase HPLC. The peaks were isolated, lyophilized and hydrolyzed with 6 N HCl for 20 h for analysis on a Hitachi 835–50 automatic amino acid analyzer

3. RESULTS

3.1. *Autoinhibitor peptides*

Synthetic autoinhibitor peptides were purified by HPLC and small portions were analyzed for amino acid composition. The results for three peptides were consistent with the respective theoretical values except for Cys which was not derivatized to a stable form such as cysteic acid. For example, the original autoinhibitor peptide had the following composition (ratio to Gly): Asp 1.04, Gly *1.00*, Cys 0.31, Val 1.91, Arg 0.99, and Pro 1.86. Stock solutions of 2 mM were made and used throughout the experiments.

3.2. *Inhibition of sea urchin hatching by autoinhibitor*

As depicted in Fig. 1, the peptide inhibited the hatching of sea urchin embryos in a concentration-dependent manner. Especially above 0.5 mM, hatching was effectively delayed by the peptide, although hatching finally reached completion after a long incubation. The hatched embryos developed normally at least up to the gastrula stage, indicating that the peptide has no cytotoxicity.

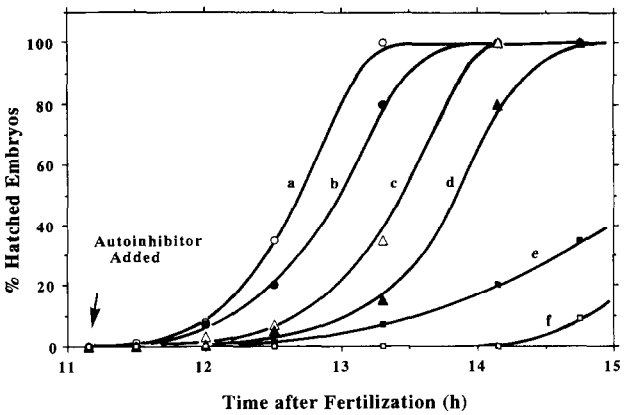

Fig. 1. Inhibition of hatching of the sea urchin, *H. pulcherrimus*, by the autoinhibitor peptide. At the time indicated by the arrow, the autoinhibitor peptide, Ac-Pro-Arg-Cys-Gly-Val-Pro-Asp-Val-NH_2, at various concentrations was added. The final concentrations in mM are: a, 0 mM; b, 0.01mM; c, 0.05mM; d, 0.10mM; e, 0 50mM; f, 1.0mM.

3.3. *Effects of autoinhibitor and its analogues on metalloproteinases*

The effects of the peptides and cysteines on α_1-PI hydrolyses by envelysin, α-protease and thermolysin were analyzed by SDS-PAGE as depicted in Figs. 2–4. Human α_1-PI was heavily contaminated with serum albumin, as shown by a 66 kDa band, but it was not cleaved by envelysin nor by α-protease. Only α_1-PI of 52 kDa was cleaved in a highly limited manner producing a 48 kDa band by the two enzymes. Cleavage of α_1-PI by envelysin was almost over after 1 h despite its low amount. Its action was effectively inhibited by 0.5 mM autoinhibitor and its Ala^6 analogue, delaying its complete cleavage to after 8 h or later (Fig. 2b,d), while no inhibition was observed on D-Cys^3-peptide (Fig. 2c). The inhibition was not irreversible and the limited cleavage reached completion after a long incubation. At this point the inhibitor appeared to be mostly dimerized through a cystine crosslink by spontaneous oxidation, as revealed by the shift of elution position on reverse-phase HPLC without any change in amino acid composition (data not shown). Both D- and L-cysteines were equally inhibitory to envelysin, although weaker than the peptides (Fig. 2e,f).

Snake venom α-protease was equally inhibited by the autoinhibitor and the Ala^6 analogue, and only slightly inhibited by the D-Cys^3 analogue as compared with the control. It was almost equally inhibited by both D- and L-cysteines (Fig. 3). The specific activity of this enzyme was revealed to be much lower than envelysin. It may

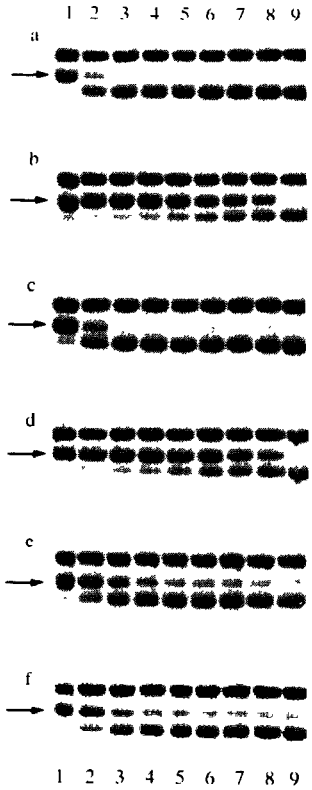

Fig. 2. Effects of inhibitors on the cleavage of human α_1-proteinase inhibitor by envelysin as detected by SDS-PAGE. The arrows indicate the bands of α_1-PI (52 kDa). The bands above them are contaminating serum albumin (66 kDa) due to incomplete purification. The inhibitors were: a, none; b, 0.5 mM autoinhibitor; c, 0.5 mM D-Cys^3 analogue; d, 0.5 mM Ala^6 analogue; e, 1.0 mM L-cysteine; f, 1 0 mM D-cysteine. The reaction times were: lane 1, 0 min, lane 2, 10 min; lane 3, 30 min; lane 4, 1 h; lane 5, 2 h; lane 6, 3 h, lane 7, 4 h; lane 8, 6 h; and lane 9,24 h.

be due to partial denaturation induced by prolonged storage in a freezer, or to its own substrate specificity.

In contrast to the above two proteases, thermolysin cleaved both α_1-PI and serum albumin, the former rapidly and the latter much more slowly. The cleavage of serum albumin was revealed by the appearance of new bands of about 56 kDa after 1 h incubation. Thermolysin was inhibited almost equally by both the autoinhibitor and the D-Cys^3 analogue (Fig. 4b,c). Inhibition by the Ala^6 analogue appears rather weaker than the other peptides. L-Cysteine exhibited apparently weaker inhibition than D-cysteine (Fig. 4e,f).

3.4. *Cleavage of autoinhibitor by thermolysin*

The reaction mixture of autoinhibitor and thermolysin after 3 h incubation was subjected to HPLC, and the collected peaks were analyzed for amino acid composition. More than 80% of the pepetide was cleaved into fragments. With the known sequence of the autoinhibitor, the peaks other than the parent peptide were identified as Ac-Pro-Arg-Cys and Gly-Val-Pro-Asp-Val-NH_2, indicating that the cleavage occurred at the Cys^3–Gly^4 bond, which is not typical for the known specificity of thermolysin.

4. DISCUSSION

Since the Ishida's finding of sea urchin hatching enzyme as a protease in 1936 [1], many reports have been published on the purification, specificity and classification among the four basic classes of proteases. However, most of them led to erroneous conclusion, mainly because of incomplete purification of the enzyme, which presumably reflects its high tendency to autolytic degradation. Purification and isolation of the enzyme, in the true sense of word, was attained by the use of procion (red) agarose [10], and it allowed for cDNA cloning to find that it is a member of mammalian MMP family [2].

In this letter we first demonstrated that the synthetic autoinhibitor peptide corresponding to the highly conserved region in the pro-domain of MMPs indeed inhibited the hatching of the sea urchin, *H. pulcherrimus*. Although the IC_{50} value for the inhibition of the hatching process cannot be precisely defined, it appears to be in the range of 0.1–0.5 mM, comparable to the K_i value reported on transin (rat stromelysin) [9]. This is much higher than the K_i value of 8 mM for a 14-residue peptide in the inihibition of type IV collagenase [8]. So far

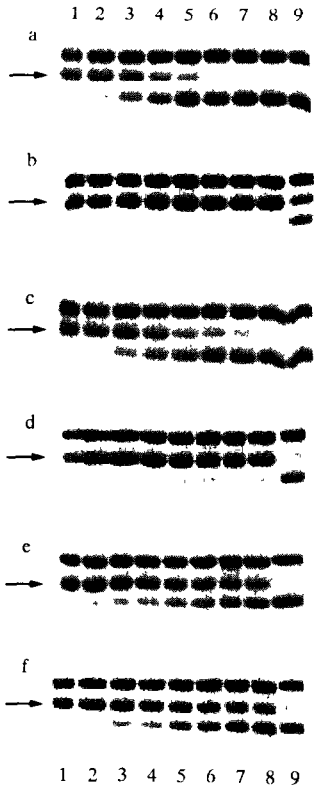

Fig. 3. Effects of inhibitors on the cleavage of human α_1-proteinase inhibitor by snake venom α-protease as detected by SDS-PAGE. The inhibitors (a–f) and the reaction times (lanes 1–9) are the same as for envelysin (Fig. 2)

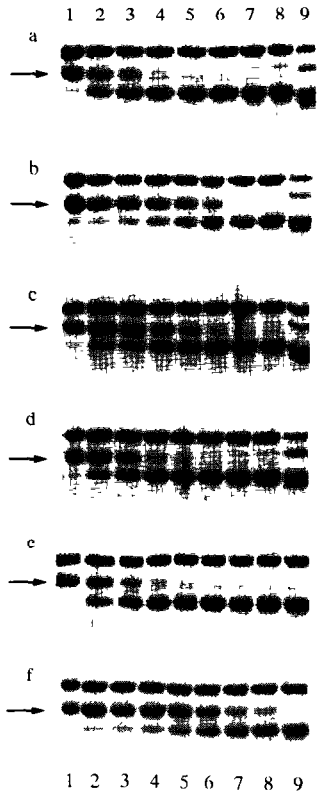

Fig. 4. Effects of inhibitors on the cleavage of human α_1-proteinase inhibitor by thermolysin as detected by SDS-PAGE. The inhibitors (a–f) are the same as Figs. 2 and 3. The reaction times are: lane 1, 0 min; lane 2, 5 min; lane 3, 10 min; lane 4, 20 min; lane 5, 30 min; lane 6, 1 h; lane 7, 2 h; lane 8, 3 h; and lane 9, 24 h.

there has only been a single report describing the inhibition of any physiological phenomenon by the autoinhibitor peptide: inhibition of tumor cell invasion that is ascribed to 72 kDa type IV collagenase [11].

The limited cleavage of α_1-PI by purified envelysin was also inhibited by the autoinhibitor and the Ala^6 analogue, but not by the D-Cys^3 analogue, clearly indicating that the inhibition is stereo-specific with respect to the essential Cys. This means that the interaction of the Cys residue in the peptide with the Zn atom in the enzyme catalytic site is stereo-specific. Since both D- and L-cysteines equally inhibited envelysin, stereo-specific inhibition by the autoinhibitor indicates that appropriate interactions of the amino acid residues on both sides of the Cys residue in the autoinhibitor peptide with the active site residues are a prerequisite for the effective coordination of the Cys residue and Zn atom leading to the inhibition by the peptide. In the case of the D-Cys^3 analogue, after binding to the active site, the SH group of the D-Cys residue is presumably oriented to the direction opposite to the Zn atom, making the Cys–Zn coordination impossible. Alternatively, the binding affinity of the D-Cys^3 analogue to the active site may be too low to ensure the effective Cys–Zn coordination. The finding that the inhibition by reduced glutathione is weaker than free cysteine [8] confirms that appropriate amino acid residues on both sides of the cysteine play an essential role in the interaction with the active site amino acid residues, which then ensures the Cys–Zn coordination. Furthermore, the strength of the interaction of the autoinhibitor peptide with the active site seems to be dependent on the number of residues on both sides of the Cys residue [8]. The present results strongly suggest, if not confirm, that envelysin has the cysteine switch sequence in its prodomain for the latency of the proenzyme similar to the *P. lividus* enzyme and other MMPs. Other members of mammalian MMP family that share the common cysteine switch sequence, e.g. interstitial collagenase, type IV collagenases (72 kDa and 92 kDa), stromelysins 1–3, transin, matrilysin, are also expected to be stereo-specifically inhibited by the autoinhibitor peptides.

Snake venom α-protease was also inhibited by the autoinhibitor and the Ala^6 analogue in a similar manner to envelysin. The D-Cys^3 analogue was very faintly inhibitory as compared with the control. This small deviation from the strict stereo-specific inhibition of envelysin may reflect the fact that the snake venom metalloproteases are not members of the MMP family but make up a distinct gene family of their own [12]. On this standpoint, it is of great interest to know whether or not other metalloproteinases from snake venom are inhibited by the autoinhibitor peptides and to what extent the inhibition is stereo-specific. Among them are hemorrhagic toxin e (trolysin e) [13] and hemorrhagic proteases Ht-c and Ht-d [14] from Western diamondback rattlesnake, *Crotalus atrox*, or non-hemorrhagic H2-proteinase from Habu snake, *Trimeserus flavoviridis* [15]. Moreover, it is also of great interest to know if our synthetic peptide inhibits the gamete lytic enzyme of the unicellular biflagellated alga, *Chlamydomonas reinhardtii*, which has high homology with MMP and with the sequence PRCNVPRA in its prodomain [16]. Since the Asp^7 in other MMPs is replaced by an oppositely charged Arg^7 in this enzyme, the inhibition may not be so strong, if present at all.

Another metalloproteinase arouses the same interest: the hatching enzyme (choriolytic enzymes, HCE and LCE) which synergistically dissolve the fertilization envelope (chorion) of medaka, *Oryzias latipes* [17,18]. They are not members of the MMP family, rather they belong to the astacin family [19]. They do not have a cysteine switch sequence in the pro-domains, but instead they have two Cys residues at positions 5 and 10, and 5 and 12, respectively, in the mature enzymes HLE and LCE.

Thermolysin was not inhibited by the Ala^6 analogue as much as the autoinhibitor. The reason is presumably that the analogue was cleaved at Gly^4–Val^5 faster than the autoinhibitor, due to the absence of Pro at position 6.

As previously reported, envelysin cleaves various bio-

active peptides such as oxidized insulin B chain and substance P [3]. However, they were not appropriate for this kinetic study because the hydrolysis reactions could not be followed continuously. Detailed quantitative kinetic analyses of the stereo-specific inhibition, to determine the inhibitor constant, K_i, and some other parameters, will be achieved by the use of intramolecularly quenched fluorogenic peptide substrates, such as a coumarine-labelled hepta-peptide substrates by continuous fluorometric assay [20], which we are going to perform in the next experiment.

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