Prolyl Endopeptidase Inhibitors Derived from Actinomycetes

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Four prolyl endopeptidase inhibitors isolated from actinomycetes, named propeptin, SNA-8073-B, staurosporine, and enduracidin were classified into 3 groups on the basis of their inhibition potency against prolyl endopeptidase from a bacterium (Flavobacterium) and a mammal (human placenta). Staurosporine inhibited the enzyme from Flavobacterium more strongly than that from human placenta. Enduracidin inhibited the enzyme from human placenta more strongly than that from Flavobacterium. Propeptin and SNA-8073-B, both new compounds, inhibited the enzymes from both origins to the same extent.

Key words: prolyl endopeptidase; inhibitor; actinomycetes; natural product

Prolyl endopeptidase (PEP; post-proline cleaving enzyme, the latest name is prolyl oligopeptidase; EC 3.4.21.26) was first isolated from human uterus as an oxytocin-inactivating enzyme, and cleaves peptide bonds at the carboxyl side of proline residues.1–3 It is distributed in a wide range of species, especially in human brain4 and prolyl endopeptidase-like immunoreactivity was detected in the mouse hippocampus.5 PEP, a new type of serine proteinase, has been proposed to play a role in degradation of proline-containing biologically active peptides such as oxytocin, vasopressin, substance P, Bradykinin, LH-RH, neurotransin, and angiotensins.6–7 Thus it would be important in the biological functions of these peptides in various organs, especially in the brain. Vasopressin has been suggested to be involved with learning and memory processes.8,9 Also the PEP activity of Alzheimer’s patients was significantly higher than the normal10 and a putative amyloid A4-generating enzyme in Alzheimer’s disease is identified as a PEP.11 Moreover it was reported that the neurodegenerative effects of β amyloid could be prevented by intracerebral or systematic administration of substance P.12 Thus specific inhibitors for PEP are expected to have anti-amnesic effects and some inhibitors have been synthesized as anti-amnesic drugs.13,14

The enzymes from animals and a plant were susceptible to PCMB (p-chloromercuribenzoic acid), while the enzyme from Flavobacterium was insensitive to PCMB.13 In addition, the isoelectric point of microbial PEP was different from those of the enzyme from animals and a plant.15,16 Recently, bacterial (Flavobacterium) and mammalian (porcine brain) enzyme genes were cloned and their amino acid sequences had 38.2% identity.6,17 Thus, it would be very interesting to clarify and compare the inhibition susceptibility against both enzymes. In fact, Yoshimoto et al. and Saito et al. compared the effects of synthetic inhibitors, prolinal-containing peptide and pyrrolidine derivatives on enzymes from bovine brain and Flavobacterium.14,18 Z-Pyrrolinal and Boc-Pro-prolinal strongly inhibited the mammalian PEP and alkyacyl-peptidyl-pyrrolidine derivatives strongly inhibited only the bacterial enzyme, showing a variety of enzyme/inhibitor specificities. However, there is no report concerning the actions of natural inhibitors against the bacterial and mammalian enzymes, excluding the case of eurystatins.19

In the course of screening for a new type of PEP inhibitor from natural origins, we have discovered novel compounds named propeptin20 and SNA-8073-B,21 and known compounds named staurosporine22 and enduracidin23 from actinomycetes. In this time, our study indicates that those inhibitors were classified into 3 groups on the basis of inhibition potency against PEP originated from Flavobacterium and human placenta.

Bacterial prolyl endopeptidase (Flavobacterium, Seikagaku Kogyo Co. Ltd.) and human placental cystosol fraction24,25 were used as the enzyme for the characterization of inhibitors. Z-Gly-Pro-7AMC (Genosys Biotechnologies, Inc.) was used as a substrate. Z-Pro-Prolinol and Z-Pro-Prolinol were synthesized by the method of Wilk and Orlowski.26 Enduracin and bacitracin were purchased from Wako Pure Chemicals and Sigma Co., Ltd., and other compounds were isolated from actinomycetes as described previously.20,21,27 PEP T was prepared by the trypsin digestion of propeptin. All compounds were dissolved in methanol. The enzyme activity was assayed by the slightly modified method of Yoshimoto et al.,15 in which 0.1 ml Tris-HCl, pH 7.0 (0.96 ml), PEP (0.01 ml of 0.085 unit/ml) (Flavobacterium) or 0.01 ml (human placental cystosol fraction, 273 µg protein) and test samples in methanol (0.01 ml) were mixed and incubated at 37°C for 5 min, then the reaction was started by adding 0.02 ml of 2-mm Z-Gly-Pro-7AMC (in 40% 1,4-dioxane). After incubation at 37°C for 10 min, 0.5 ml of stop solution (10 g Triton X-100/95 ml 1 M acetate buffer, pH 4.0) was added and the fluorescence intensity of the solution was measured at 460 nm with excitation at 380 nm. IC50 values were estimated by this method with various concentrations of test compounds. The percentage of inhibition can be calculated from the fluorescence intensity with (A) and without (B) inhibitor, by the following equation: Percentage of inhibition = [(B – A)/B] × 100.

Bacitracin, enduracidin, and propeptin are peptide antibiotic, but SNA-8073-B is an isosteretaceneone (angucyclinone) and staurosporine is an indrocarbazole antibiotic (Fig. 1). The concentration of these compounds causing 50% inhibition against PEP from Flavobacterium and from human placenta are shown in the Table. They are classified into 3 types on the basis of their inhibition potency against PEP from a bacterium (Flavobacterium) and a mammal (human placenta). Staurosporine inhibited the enzyme from Flavobacterium more strongly than that from human placenta. Enduracidin inhibited the enzyme from human placenta more potently than that from Flavobacterium. Propeptin and SNA-8073-B, novel compounds, inhibited the enzyme from both
(1) Staurosporine

(2) Enduracidin

(3) Propeptin

(4) Propeptin T

(5) SNA-8073-A

(6) SNA-8073-B

(7) Bacitracin

Fig. 1. Chemical Structures of Prolyl Endopeptidase Inhibitors.

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<th>Table</th>
<th>Prolyl Endopeptidase Inhibition Activity of Various Compounds</th>
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<tr>
<td></td>
<td>PEP inhibition (IC&lt;sub&gt;50&lt;/sub&gt;, μM)</td>
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<tr>
<td></td>
<td>Flavobacterium (F)</td>
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<tr>
<td>Staurosporine</td>
<td>0.75</td>
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<tr>
<td>Enduracidin</td>
<td>10</td>
</tr>
<tr>
<td>Propeptin</td>
<td>0.35</td>
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<tr>
<td>Propeptin T</td>
<td>0.43</td>
</tr>
<tr>
<td>SNA-8073-A</td>
<td>&gt;60</td>
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<tr>
<td>SNA-8073-B</td>
<td>8.9</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>21</td>
</tr>
<tr>
<td>Z-Pro-Prolinal</td>
<td>175</td>
</tr>
<tr>
<td>Z-Pro-Prolinal</td>
<td>0.00064</td>
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origins to the same extent as well as synthetic inhibitors such as Z-Pro-Prolinal and Z-Pro-Prolinal. Bacitracin obtained from Bacillus sp. also inhibited the enzyme from both origins to the same extent (Table).

We have already shown that staurosporine and SNA-8073-B were non-competitive inhibitors and propeptin was a competitive inhibitor of the PEP of Flavobacterium when Z-Gly-Pro-pNA was used as a substrate. In this experiment, we examined the inhibition mechanism of enduracidin against PEP of human placenta when Z-Gly-Pro-7AMC was used as a substrate. Enduracidin inhibited PEP non-competitively and the K<sub>i</sub> was 0.25 μM, as shown in Fig. 2. As propeptin T, which is a linear type of propeptin (Fig. 1), inhibited the enzyme from both origins to the same extent as propeptin, the cyclic structure of propeptin was not important. SNA-8073-A (named fujiamycin B, rubiginone A<sub>2</sub>), a stereoisomer of SNA-8073-B at C-4 (Fig. 1) did not inhibit the enzyme from either origin at all (Table), indicating that the stereochemistry at the C-4 position was crucial for the PEP inhibition of this molecule. In the structural comparison, peptide antibiotics had rather strong inhibition with or without their proline residue in the enzyme against mammal PEP. While non-peptide antibiotics were stronger inhibitors of bacterial PEP under our conditions.

During the submission of this paper, it was reported that a novel lipohepapeptide containing t-proline, lipohexin specifically inhibited PEP at about ten-fold better inhibition of the enzyme from human placenta than that from Flavobacterium. Enduracidin not containing t-proline inhibited the PEP of human placenta at about fifty-fold better than that of Flavobacterium in this report. As both compound is classified to lipopeptide, fatty acyl moiety might be important for specific inhibition against the PEP of human placenta. We have no precise explanation of
Fig. 2. Dixon Plot of the Inhibition of Human Placental Prolin Endopeptidase by Enduracidin.

Enduracidin was incubated for 5 min at 37°C with the enzyme and the reaction was started by addition of Z-Gly-Pro-7-AMC as described. 1/ V was defined as 1/A 460 nm with excitation at 380 nm.

- - - , Z-Gly-Pro-7-AMC 0.01 mM; ▲ ▲ ▲ , 0.02 mM; ● ● ● , 0.04 mM.

This exact mechanism at this point, but if the natural product has structural diversity as seen above, it would give various good leading compounds for investigating the PEP inhibition mechanism and PEP action in various organs in detail. Moreover these results might be useful for development of a new type of PEP inhibitor from natural products for future work.

References