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SEPARATION OF ENKEPHALIN-DEGRADING ENZYMES FROM LONGITUDINAL MUSCLE LAYER OF BOVINE SMALL INTESTINE

ENZYME INHIBITION BY ARPHAMENINE A

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Abstract—The enkephalin-degrading enzymes, such as aminopeptidase, dipeptidyl aminopeptidase, dipeptidyl carboxypeptidase and carboxypeptidase, were purified partially by DEAE-cellulose column chromatography, using longitudinal muscle from bovine small intestine. These enzymes were inhibited by EDTA and *o*-phenanthroline. Several protease inhibitors of microbial origin, and synthetic compounds, were tested for their abilities to inhibit these enkephalin-degrading enzymes. Among them, arphamenine A, a new potent inhibitor for aminopeptidase B, was shown to be a useful compound in inhibiting all of the enkephalin-degrading enzymes in small intestine.

Enkephalins of opioid peptides have been detected as immunoreactive substances not only in brain but also in digestive organs, serum, and adrenal gland [1, 2]. In the digestive organs, they seem to control intestinal motility [3–9], but the mechanism of action has not been studied successfully. In this kind of study, the fact that enkephalins are degraded rapidly by specific enzyme systems is an obstacle [10, 11]. The purification of enkephalin-degrading enzymes has been performed mainly with brain tissue. Recently, these enzymes were also separated from rat kidney and pancreas and from porcine kidney. On the other hand, studies of enzymes in digestive organs have been rarely reported [8, 12, 13], and the aminopeptidases have been primarily examined.

In this paper, we report the separation of enkephalin-degrading enzymes, such as aminopeptidase, dipeptidyl aminopeptidase, dipeptidyl carboxypeptidase and carboxypeptidase, from longitudinal muscle layer of bovine small intestine, together with the activity of some inhibitors towards these enzymes, especially the activity of arphamenine A (Fig. 1) which is a new specific inhibitor for aminopeptidase B [14].

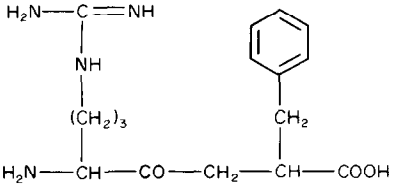

Fig. 1. Structure of arphamenine A.

MATERIALS AND METHODS

Chemicals. DE-52 cellulose from Whatman, [^{3}H] Leu-enkephalin (21.0 Ci/mmole) from the New England Nuclear Corp. (Boston, MA) and Leu-enkephalin from the Protein Research Foundation (Osaka) were used for these experiments. Protease inhibitors of microbial origin were supplied by Dr. H. Umezawa, Institute of Microbial Chemistry (Tokyo). The other chemicals used were of the best quality available from commercial sources.

Preparation of enkephalin-degrading enzymes. Bovine small intestines (jejunum and ileum) were obtained from a slaughterhouse. They were cooled in an icebox for less than 1 hr before they reached our laboratory. All operations were performed at 0–4°. The longitudinal muscle layer was separated from the small intestine. About 300 g of muscle was homogenized with 1.2 liters of buffer A (25 mM Tris–HCl, pH 7.0) and then centrifuged at 30,000 *g* for 30 min. The supernatant was obtained as the soluble fraction of the muscle. The pellet was solubilized with the same buffer, containing 1% Triton X-100 and was centrifuged at 100,000 *g* for 1 hr; the supernatant thus obtained was treated as the membrane-bound fraction. Each fraction was applied to a DE-52 cellulose column (4.2 × 22.0 cm) equilibrated with buffer A for the soluble fraction, and with buffer A containing 0.1% Triton X-100 for the membrane-bound fraction; step-wise gradient elution of 50, 75, 100 and 150 mM NaCl in buffer A was carried out.

Assay methods of enzyme activity. A 50 μl sample

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was placed in a solution containing 2 μCi of [^{3}H]Leu-enkephalin, 10 μmoles of unlabeled Leu-enkephalin and 25 mM Tris–HCl buffer, pH 7.0, in the presence or absence of 30 μg bestatin, in a total volume of 100 μl. After incubation for 1 hr at 37°, 20 μl of 30% acetic acid was added to stop the reaction, and then 100 μl of the reaction mixture was placed on a column containing 80 mg of Porapak Q. The fragments from Leu-enkephalin, such as [^{3}H]tyrosine, [^{3}H]tyrosyl-glycine and [^{3}H]tyrosyl-glycyl-glycine, were eluted with 1 ml of 25 mM Tris–HCl buffer, pH 7.0, from the column. After the addition of ATOMLIGHT scintillation fluid, the amount of radioactivity was measured in a liquid scintillation counter.

The fractions that showed enkephalin-degrading activity were next subjected to autoradiography for analysis of fragments. Twenty microliters of the reaction mixture was spotted on a silica gel thin-layer plate and developed by chloroform–methanol–acetic acid–water (45:30:6:9, by vol.). The air-dried plate was sprayed with 2,5-diphenyloxazole in ethyl ether and exposed to Kodak XS-1 film at −80° for 2 days. Authentic samples of Leu-enkephalin, tyrosine, tyrosyl-glycine, tyrosyl-glycyl-glycine and tyrosyl-glycyl-glycyl-phenylalanine were used as reference compounds that were detected by ninhydrin.

Enzyme activities were assayed according to (1) the method of Suda *et al.* [15] using tyrosine-β-naphthylamide as the substrate for aminopeptidase, (2) the method of Hazato *et al.* [16] using Arg-Arg-β-naphthylamide for dipeptidyl aminopeptidase, and (3) the method of Hayakari *et al.* [17] using hippuryl-His-Leu for dipeptidyl carboxypeptidase and hippuryl-Phe for carboxypeptidase.

RESULTS

When the reaction mixture of [^{3}H]Leu-enkephalin and the soluble fraction was developed on a silica gel thin-layer plate, three spots appeared which were identified as [^{3}H]tyrosine, [^{3}]tyrosyl-glycine and [^{3}H]-tyrosyl-glycyl-glycyl-phenylalanine (Fig. 2-1). When the enzyme reaction was performed in the presence of bestatin, the radioactive spot of tyrosine did not appear and the other two spots were stronger (Fig. 2-2). The reaction mixture with [^{3}H]Leu-enkephalin and the membrane-bound fraction produced two radioactive spots (Fig. 2-3), one of which was [^{3}H]-tyrosine, and the other [^{3}H]tyrosyl-glycyl-glycyl-phenylalanine. With the addition of bestatin, the tyrosine spot did not appear and three new radioactive spots developed which were identified as [^{3}H]-Leu-enkephalin, [^{3}H]tyrosyl-glycine and [^{3}H]tyrosyl-glycyl-glycine (Fig. 2-4). These results suggested that the enkephalin-degrading enzymes in the longitudinal muscle layer of bovine small intestine consisted of an aminopeptidase, a dipeptidyl aminopeptidase, a dipeptidyl carboxypeptidase and carboxypeptidase, although the activity of dipeptidyl carboxypeptidase was found only in the membrane-bound fraction.

The soluble fraction was applied to a DE-52 cellulose column. The enkephalin-degrading enzymes were separated by step-wise elution with 50, 75, 100 and 150 mM NaCl in the 25 mM Tris–HCl buffer, pH 7.0 (Fig. 3). Autoradiography, with or without addition of 30 μg bestatin, indicated that two different enkephalin-degrading enzymes were contained in the fraction that eluted with 50 mM NaCl.

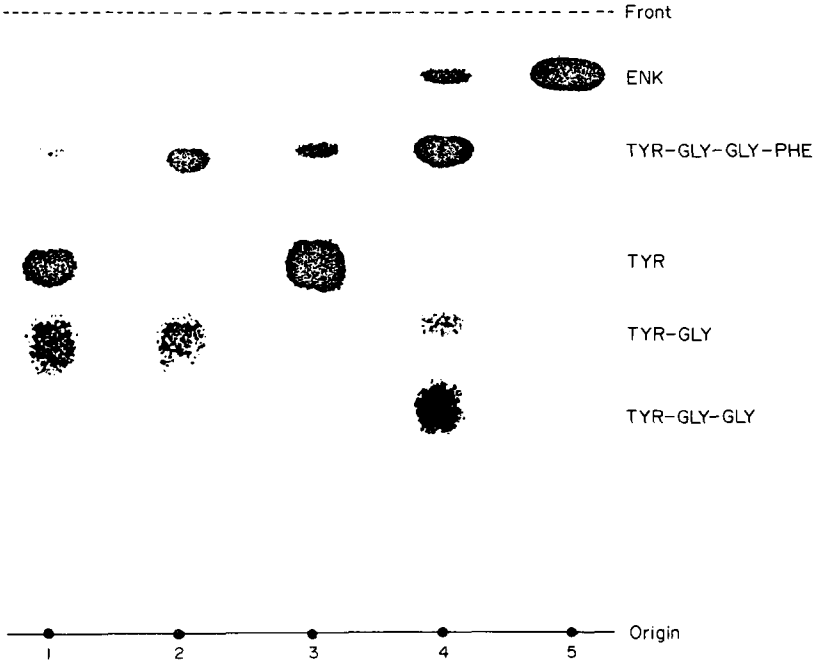

Fig. 2. Autoradiogram of fragments from degraded enkephalin using silica gel thin-layer chromatography. Chromatography profile of a sample incubated with (1) [^{3}H]Leu-enkephalin and soluble fraction, (2) [^{3}H]Leu-enkephalin, soluble fraction and bestatin, (3) [^{3}H]Leu-enkephalin and membrane-bound fraction, (4) [^{3}H]Leu-enkephalin, membrane-bound fraction and bestatin and (5) [^{3}H]Leu-enkephalin. The method is described in the text.

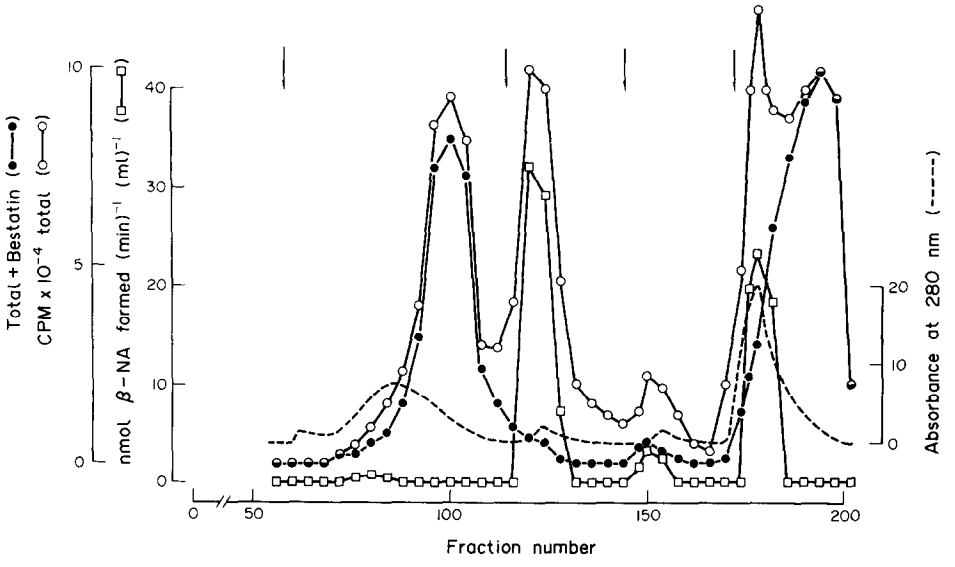

Fig. 3. DEAE cellulose chromatography of enkephalin-degrading enzymes in the soluble fraction from longitudinal muscle layer of bovine small intestine. Elution was performed by a step-wise method of NaCl in 25 mM Tris–HCl buffer (pH 7.0). NaCl at concentrations of (a) 50, (b) 75, (c) 100 and (d) 150 mM was added in the buffer at the position of each arrow. Each fraction was 15 ml. A dashed line (- - - -) indicates absorbance at 280 nm. Key: total cpm of enkephalin-degrading activities in the absence (—○—) and the presence (●) of 30 μg bestatin in the assay system described in Materials and Methods, (—□—) aminopeptidase activity with tyrosine-β-naphthylamide.

One of them was an aminopeptidase that was not inhibited by bestatin, and the other was a carboxypeptidase that cleaved both enkephalin and hippuryl-Phe. In the fraction eluted with 75 mM NaCl, aminopeptidase activity, which was inhibited by bestatin, was observed. An aminopeptidase that had almost the same characteristics as the enzyme in the fraction that eluted with 75 mM NaCl, and a dipeptidyl aminopeptidase that hydrolyzed both enkephalin and Arg-Arg-β-naphthylamide, were contained in the fraction that eluted with 150 mM NaCl in the buffer. In the fraction with 100 mM NaCl in the buffer, enkephalin-degrading enzyme activity was observed, but the activity was so weak that the characteristics of the enzyme(s) in this fraction could not be studied.

When the membrane-bound fraction was applied on a DE-52 cellulose column, which had been equilibrated with 25 mM Tris–HCl buffer, pH 7.0, containing 0.1% Triton X-100, dipeptidyl carboxy-

Table 1. Effects of various protease inhibitors on enkephalin-degrading enzymes* in longitudinal muscle layer of bovine small intestine

Compound	Concn (mM)	Inhibition (%)				
		AP-I	AP-II	DPP	CP	DCP
EDTA	5.0	49.3	41.6	95.8	+†	91.7
o-Phenanthroline	0.5	72.2	77.9	90.2	+	93.8
Bacitracin	0.1	57.0	28.4	20.1	–	—
Puromycin	0.2	—	70.4	—	–	—
Bestatin	0.2	—	88.5	—	–	—
Amastatin	0.2	56.3	85.5	—	–	10.0
Arphamenine A	0.2	44.6	32.0	65.0	+	45.1
Phosphoramidon	0.2	35.2	—	67.8	–	32.5
SQ 14225 (captopril)	0.2	—	15.4	—	–	52.0
Phe-Ala	0.2	56.7	21.5	68.2	–	—

* AP-I, aminopeptidase that was eluted from a DE-52 cellulose column with 50 mM NaCl in the buffer; AP-II, aminopeptidase that was eluted from the column with 75 mM NaCl; DPP, dipeptidyl aminopeptidase; CP, carboxypeptidase; and DCP, dipeptidyl carboxypeptidase in membrane-bound fraction.

† The activities of tested inhibitors were judged as positive, when the spot of tyrosyl-glycyl-glycyl-phenylalanine was not detected on the autoradiogram after the addition of each inhibitor.

Table 2. Inhibitory activity of arphamenine A on the enzymes in longitudinal muscle layer of bovine small intestine

Enzymes	Substrate	IC_{50} (10^{-4} M)
Aminopeptidase (AP-I)	Enkephalin	2.8
(AP-II)	Enkephalin	7.6
Dipeptidyl aminopeptidase	Enkephalin	1.0
Dipeptidyl carboxypeptidase	Enkephalin	2.9
	Hippuryl-His-Leu	−*
Carboxypeptidase	Hippuryl-Phe	1.9

* No inhibition.

peptidase activity eluted with the same buffer without Triton X-100.

The partially purified enkephalin-degrading enzymes, obtained as above, were used for further experiments.

The characteristics of these enzymes were not studied thoroughly, but the activities of some inhibitors towards these enzymes were tested. The results are shown in Table 1. The activity of carboxypeptidase toward enkephalin as a substrate was not measurable quantitatively by our assay method. Metal chelators, such as EDTA and *o*-phenanthroline, acted as inhibitors for all the enzymes. However, the activities toward these enzymes of the inhibitors of microbial origin were different from each other. The activity of an aminopeptidase (AP-1), eluted with 50 mM NaCl from the DE-52 cellulose column, was not inhibited by 30 μg bestatin. Arphamenine A, a new inhibitor for aminopeptidase B, was inhibitory to all the enzymes; the inhibitory activity toward these enzymes is shown in Table 2. When Leu-enkephalin was used as a substrate, arphamenine A inhibited the dipeptidyl aminopeptidase most strongly, with an IC_{50} value of 100 μM, and also weakly inhibited the dipeptidyl carboxypeptidase. However, when hippuryl-His-Leu was used as a substrate, arphamenine A did not show any inhibitory activity for the dipeptidyl carboxypeptidase.

DISCUSSION

Biological activities of enkephalins toward digestive organs have been demonstrated by their binding with morphine receptors [4, 9, 10]. The activities were almost equal to that of morphine and were abolished by pretreatment of the organs with a morphine antagonist such as naloxone [4, 9].

It is known that enkephalins are rapidly hydrolyzed by specific enzyme systems, and that the duration of enkephalin activity is very short. Therefore, if the activities of these specific enzymes were inhibited, experiments on the mechanisms of enkephalins would be performed more easily.

Heretofore, the enkephalin-degrading enzymes have been separated mainly from the brains of animals, and the enzymes have been classified as aminopeptidase [18], dipeptidyl aminopeptidase [17] and dipeptidyl carboxypeptidase [19]. These enzymes were found also in other tissues, but those in digestive organs were merely reported. Other investigators have studied the enkephalin-degrading enzymes in digestive organs, but have paid attention only to aminopeptidase [8, 12, 13].

In our experiments, several kinds of enkephalin-degrading enzymes, including a carboxypeptidase, which has not been reported for brain tissue, were separated from the longitudinal muscle layer of bovine small intestine. Whether these enzymes play an important role in the degradation of enkephalins *in situ* or are related to the metabolism of some other bioactive substance awaits further investigation.

The enzymes that we obtained were not purified thoroughly, but the activities of some inhibitors on these enzymes were tested. The results show that all the enzyme activities tested were inhibited by metal chelators, such as EDTA or *o*-phenanthroline. However, these metal chelators are not suited for use in bioassay systems to test the biological activities of enkephalin.

The activities of the inhibitors of microbial origin were different from each other. Bestatin, which is a powerful inhibitor of aminopeptidases, did not affect the aminopeptidases that we obtained, although it has shown inhibitory activity to another aminopeptidase in digestive organs and all of the aminopeptidases in brain [18]. Since aminopeptidases are assumed to play an important role in enkephalin degradation *in vivo*, it is interesting that an aminopeptidase which is not inhibited by bestatin was found in the digestive organs. However, this result may be in contradiction to the observation that the [^{3}H]tyrosine spot on the autoradiogram of the reaction mixture of [^{3}H]Leu-enkephalin and soluble fraction did not appear at all after the addition of bestatin to the reaction system. Therefore, it may be considered that the content of bestatin-resistant aminopeptidase was very slight compared to that of the bestatin-sensitive one.

Arphamenine A, purified from the culture medium of *Chromobacterium violaceum* by Umezawa and Aoyagi [20], is a specific, potent inhibitor for aminopeptidase B. It showed inhibitory activity toward all the enkephalin-degrading enzymes from the longitudinal muscle layer of bovine small intestine; a 2 mM concentration completely inhibited the enkephalin-degrading enzyme activities in both the soluble and the membrane-bound fractions. Since all enkephalin-degrading enzymes obtained from the muscle layer were metal enzymes, it may be speculated that arphamenine A had metal-chelating activity. However, aminopeptidase B inhibition by

arphamenine A was not reversed by bivalent metal ions (Ogawa *et al.*, personal communication). Therefore, it is possible that the inhibitory activity of arphamenine A on the enzymes was not due to its metal-chelating activity.

Recently, Bouboutou *et al.* [21] reported that a new dipeptide compound, designated Ketalorphan, shows inhibitory potency against all enkephalin-degrading enzymes, with IC_{50} values in the 4 to 15 nM range. However, they used enzymes that were purified from rat brain and rabbit kidney. Therefore, it is uncertain whether Ketalorphan has inhibitory activity toward the enzymes of digestive organs.

From the results mentioned above, it is expected that the addition of arphamenine A to bioassay systems that examine the effect of enkephalin on digestive organs may produce good results by preventing its degradation.

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