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Purification and characterization of enkephalinase B from rat brain membrane

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Enkephalinase B from rat brain membrane which hydrolyzes enkephalin at the Gly-Gly bond was purified about 9400-fold to apparent electrophoretic homogeneity. The enzyme, which has a molecular weight of 82 000, consists of a single polypeptide chain. The enzyme has a pH optimum of 6.0–6.5 and is stable in the neutral pH region. The K_m values of Met-enkephalin and Leu-enkephalin for this enzyme were $5.3 \cdot 10^{-5}$ M and $5.0 \cdot 10^{-5}$ M, respectively. The enzyme was inactivated by metal chelators, EDTA and *o*-phenanthroline and restored by the addition of divalent metal ions, Zn^{2+}, Mn^{2+} or Fe^{2+}, but was not inhibited by bestatin, amastatin, phosphoramidon or captopril. The enzyme hydrolyzed Met-enkephalin and Leu-enkephalin effectively. Although the enzyme belongs to the dipeptidyl aminopeptidase class, enkephalin-related peptides such as Leu-enkephalin-Arg, dynorphin (1–13) or α-endorphin and other biologically active peptides examined were hardly, or not at all, hydrolyzed. It was assumed that enkephalinase B functions mainly in enkephalin degradation in vivo.

Introduction

Enkephalins (Met-enkephalin and Leu-enkephalin) are degraded after administration in vivo and their analgesic activity does not last [1]. These two peptides are hydrolyzed by brain enzymes designated as enkephalinases [2,3] and aminopeptidases [4–7] at three distinct sites [8]. One of these enzymes, enkephalinase B, which hydrolyzes at the Gly-Gly bond, exists in rat brain membrane and was first prepared by Gorenstein and Snyder [9]. Also, dipeptidyl aminopeptidase III (DAP-III) from the soluble fraction of rat brain cleaved the Gly-Gly bond of enkephalin as well as enkephalinase B [10]. This enzyme has been classified as a serine enzyme. But neither enzyme has been purified completely.

Recently, we found two potent specific inhibitors of enkephalinase B, named propioxatins A and B, from microbiological products [11]. Propioxatins A and B have a hydroxamic acid moiety [12] which is characteristic of metallo-proteinase inhibitors. Therefore, it might be thought that enkephalinase B belongs to a metallo-proteinase class. In order to clarify the characteristics and action of membrane-bound enkephalinase B, we purified the enzyme homogeneously from rat brain and discuss here the function of enkephalinase B on the degradation system of enkephalin.

Abbreviations: DAP-III, dipeptidyl aminopeptidase III; Arg-Arg-βNA, Arg-Arg-β-naphthylamide.

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Materials and Methods

Materials. Met-enkephalin, Leu-enkephalin, [D-Ala^2,D-Leu^5]-enkephalin, [D-Ala^2,Met^5]-enkepha-

lin, α-neo-endorphin, dynorphin (1–13), α-endorphin, amastatin, bestatin, phosphoramidon, diprotin A and other biologically active peptides were purchased from Peptide Institute, Inc. (Osaka Japan). N^{α}-Acetyl-Leu-enkephalin was prepared by acetylation of Leu-enkephalin with acetic anhydride in 10% methanol/4.5 M sodium acetate and was purified on a Lobar column of Lichroprep RP-8, size A, (Merck) with a linear concentration gradient of acetonitrile to 50% in 0.1% trifluoroacetic acid. Arg-Arg-β-naphthylamide (Arg-Arg-βNA), Asp-Phe-NH_2, Tyr-Gly, Tyr-Gly-Gly, Phe-Leu, Met-enkephalinamide, Leu-enkephalinamide and other enkephalin related peptides were obtained from Sigma. Develosil ODS-5 (4.6 × 250 mm) was obtained from Nomura Chemicals (Seto Aiichi, Japan). Mono P HR 5/20, Superose 12 HR 10/30 and polybuffer 74 were obtained from Pharmacia Fine Chemicals. DEAE-Toyopearl 650 s, Toyopearl HW-55 (superfine) and TSK gel ODS-120T (4.6 × 250 mm) were obtained from Toyo Soda MFG Co., Ltd. (Tokyo). All other chemicals and reagents were of the purest grade available commercially.

Enzyme assay. The assay of enkephalinase B was carried out using Met-enkephalin, as reported previously [11]. Briefly, 1 mM of Met-enkephalin was incubated with enzyme for 10 min in 100 μl of 0.1 M sodium phosphate buffer (pH 6.5) and stopped by adding 10 μl of 2 M HCl. A resultant reaction product, Tyr-Gly, was separated by HPLC, monitored with an emission maximum at 304 nm of Tyr on excitation at 275 nm using a fluorescence spectromonitor and calculated with a known concentration of Tyr-Gly.

The activity for Gly-Gly-Phe-Leu or Trp-Met-Asp-Phe-NH_2 was calculated by measuring the amount of Phe-Leu or Asp-Phe-NH_2 released using HPLC. Aliquots of the reaction product incubated at 37 °C for 10 min were applied to a Develosil ODS-5 column (4.6 × 250 mm) and elution was carried out with 27% CH_3CN in the case of Gly-Gly-Phe-Leu or 14% CH_3CN in the case of Trp-Met-Asp-Phe-NH_2 in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The above dipeptide was monitored by absorbance at 210 nm with an ultraviolet detector and calculated with a known concentration of a standard sample.

Purification of enkephalinase B. Extraction and separation of enkephalinase B was carried out according to the method of Gorenstein and Snyder [9] with slight modifications as reported previously [11]. 100 g of brains from young male Wister rats was used as starting material. Other enkephalin-degrading enzymes were separated from enkephalinase B first by column chromatography using DEAE-Sephacel and then partially purified by column chromatography using DEAE-Toyopearl as reported previously [11]. Further purification was carried out as follows: the enzyme fractions from a DEAE-Toyopearl column were collected and dialyzed thoroughly against 25 mM imidazole-HCl (pH 7.4). The dialyzed solution was applied to a Mono P column (0.5 × 20 cm) previously equilibrated with the same buffer and the enzyme was eluted with Polybuffer 74-HCl (pH 4.0) diluted with distilled water 9-fold. Enkephalinase B was eluted in a fraction of pH 4.2 and the p*I* was therefore estimated to be about 4.2. The fraction of the enzyme from the Mono P column was directly applied to a Toyopearl HW-55 column (1.6 × 85 cm) previously equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and eluted with the same buffer. As shown in Fig. 1a, the fractions containing enkephalinase B (fractions 71–75) were pooled and used in subsequent studies.

Determination of protein concentration. Protein concentration in the presence or absence of Triton X-100 was determined according to Sugawara [13]. Bovine serum albumin was used as a standard protein.

Polyacrylamide gel electrophoresis. Electrophoretic analysis of native enzyme was carried out in 7.5% acrylamide gels according to the method of Davis [14] and Ornstein [15]. Matched pairs of gels were run. One of the gels was stained for protein, the other was sliced into 2–3-mm segments for determination of enzyme activity. Electrophoresis of protein in the presence of SDS (sodium dodecyl sulfate) was carried out in 10% acrylamide gels according to the method of Weber and Osborn [16]. Phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α-lactalbumin (14400) were used as a standard mixture.

Estimation of molecular weight by gel filtration.

The native molecular weight of the enzyme was determined by gel filtration on a Superose 12 column (1.0 × 30 cm). The elution was carried out with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl at a flow rate of 0.3 ml/min. The elution was monitored by absorbance at 280 nm with a UV-1 (Pharmacia Fine Chemicals). Glutamate dehydrogenase (280 000), lactate dehydrogenase (132 000), enolase (67 000), adenylate kinase (21 000) and cytochrome *c* (13 000) were used as a standard mixture.

Identification of cleavage sites in enkephalin, enkephalin-related and biologically active peptides. The enzyme and a peptide (1 mM) in 100 μl of 0.1 M sodium phosphate buffer (pH 6.5) were incubated for 60 min at 37 °C. Aliquots of the reaction mixture were applied to a TSK gel ODS-120T column (4.6 × 250 mm) previously equilibrated with 5% acetonitrile in 0.1% trifluoroacetic acid. Elution was carried out at 30 °C with a linear concentration gradient of acetonitrile to 60% in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The elution was monitored by absorbance at 225 nm with an ultraviolet detector. The peaks corresponding to peptides were collected and their amino acid compositions were determined to identify the peptide fragment. Amino acid analysis was carried out with a Hitachi 835 amino acid analyzer. Samples for analysis were prepared by hydrolysis with 6 M HCl at 110 °C for 20 h in evacuated sealed tubes.

Assay and preparation of DAP-III from rat brain. DAP-III activity was measured by monitoring the release of β-naphthylamine from the substrate Arg-Arg-βNA according to the method of Swanson et al. [17]. The increase of fluorescence by β-naphthylamine was recorded continuously with a Hitachi MPF-4 fluorescence spectrometer using excitation and emission wavelengths of 335 nm and 410 nm, respectively. The assay mixture (1 ml) containing 100 μM Arg-Arg-βNA, 0.1 M Tris-HCl buffer (pH 8.5) and enzyme preparation was maintained at 37 °C in a water jacket cuvette holder. The amount of β-naphthylamine was calculated with a standard sample. DAP-III activity was also measured using Met-enkephalin as a substrate by the same method as that used for enkephalinase B.

DAP-III was prepared from the soluble fraction of rat brain according to the method of Lee and Snyder [10] with modifications. Brains from young male Wistar rats (5 g) were homogenized in 50 ml of 50 mM sodium phosphate buffer (pH 7.0) with a Polytron. The homogenate was centrifuged at 50 000 × *g* for 20 min. The supernatant was obtained and fractionated by ammonium sulfate precipitation (30–70% saturation). The pellet obtained by centrifugation was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Toyopearl 650s column (1.0 × 11 cm) previously equilibrated with the same buffer. The enzyme was eluted with the same buffer containing a linear gradient of sodium chloride to 0.4 M (200 ml + 200 ml). Fractions containing DAP-III activity were pooled and used for some of the experiments. This enzyme preparation could not release Tyr from substrate Met-enkephalin.

Results

Purification of enkephalinase B

As reported previously [11], the elution pattern of enkephalin-degrading enzymes from the DEAE-Sephacel column was similar to that found by Gorenstein and Snyder, and enkephalinase B activity was well separated from other enkephalin-degrading enzymes. Enkephalinase B from the DEAE-Sephacel column was further purified by a DEAE-Toyopearl, Mono P and Toyopearl HW-55 column. A typical purification procedure of enkephalinase B is summarized in Table I. Enkephalinase B was purified 9400-fold with a recovery of 18%. As shown in Fig. 1 (b), disc gel electrophoresis of purified enzyme showed apparent homogeneity on staining with Coomassie blue and the protein band corresponded to enkephalinase B activity on the gel (arrow indication in Fig. 1b). We considered the enzyme from this method of preparation to be homogeneous.

Molecular weight

A molecular weight of 82 000 was found in experiments with gel filtration on a calibrated Superose 12 column. Similar results were obtained by SDS-polyacrylamide gel electrophoresis (Fig. 1c), which suggests that the enzyme consists of a

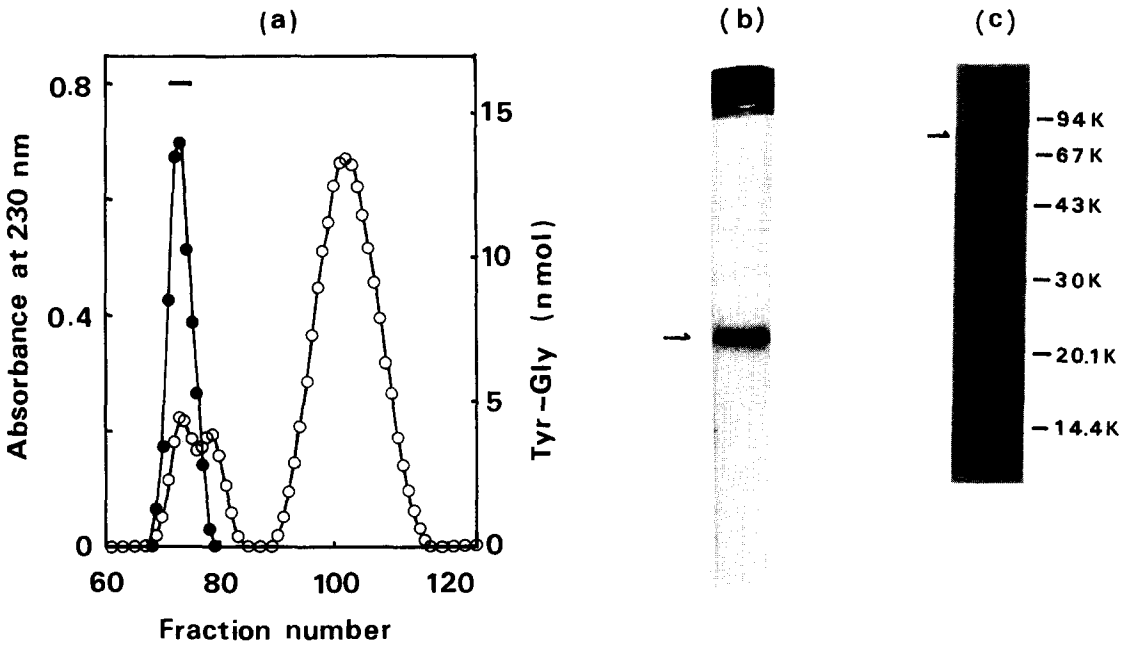

Fig. 1. (a) Gel filtration of enkephalinase B from a Mono P column on Toyopearl HW-55. Fractions of 1.0 ml were collected and 10 μl-aliquots of each fraction were assayed for enkephalinase B activity (●). Fractions 71–75 were pooled. ○, absorbance at 230 nm. (b) Disc electrophoresis of purified enkephalinase B (5 μg) on 7.5% acrylamide gel. (c) SDS-polyacrylamide gel electrophoresis of purified enzyme and molecular weight markers.

single polypeptide chain with a molecular weight of 82000.

pH optimum

The effect of pH on enzyme activity was examined in the pH 4–10 region using 0.1 M sodium acetate buffer (pH 4–6), sodium phosphate buffer (pH 6–8) and sodium borate buffer (pH 8–10). The maximum activity of enkephalinase B was found at pH 6.0–6.5 (Fig. 2).

Stabilities with respect to pH and temperature

The enzyme was incubated at various pH levels at 37 °C for 15 min and then activity was measured at the optimum pH (pH 6.5). The enzyme was stable in the pH 6.5–8.0 region. The enzyme was incubated at various temperatures at pH 6.5 for 15 min and then the activity was measured at 37 °C. The enzyme was stable in the range 4–40 °C.

TABLE I

A TYPICAL PURIFICATION OF ENKEPHALINASE B FROM RAT BRAIN MEMBRANE.

Purification step	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Purification factor	Yield (%)
Triton X-100 extract	2610	2.000	0.00077	1	100
DEAE-Sephacel	135	2.483	0.0184	24	124
DEAE-Toyopearl	10.3	2.009	0.195	253	100
Mono P HR 5/20	n.d.	0.418	n.d.	n.d.	21
Toyopearl HW-55	0.05	0.363	7.26	9429	18

n.d., protein concentration was not determined because of disturbance of Polybuffer 74.

TABLE II

EFFECTS OF VARIOUS PROTEINASE INHIBITORS ON ENKEPHALINASE B ACTIVITY

The enzyme and inhibitor in 90 μl of sodium phosphate buffer (pH 6.5) were preincubated at 37 °C for 5 min and the reaction was started by adding 10 μl of 10 mM Met-enkephalin in the same buffer. Subsequent procedures are described in Materials and Methods. Inhibitors are indicated at a final concentration in 100 μl reaction mixture.

Compound	Concentration (mM)	Inhibition (%)
DFP	0.1	17
PMSF	1.0	0
EDTA	1.0	100
o-Phenanthroline	1.0	100
PHMB	1.0	100
Iodoacetic acid	1.0	0
Dithiothreitol	1.0	0
Puromycin	1.0	0
Amastatin	0.1	0
Bestatin	1.0	0
Phosphoramidon	1.0	0
Captopril	1.0	0
Diprotin A	1.0	0

TABLE III

RESTORATION OF ENKEPHALINASE B WITH DIVALENT IONS.

The enzyme was treated with 1 mM EDTA at 37 °C for 5 min and dialyzed against 5 mM Tris-HCl buffer (pH 7.0), instead of sodium phosphate buffer, to prevent formation of precipitation by metal ions. The enzyme and metal ions in 90 μl of Tris-HCl buffer (pH 7.0) were incubated at 37 °C for 5 min and the reaction was started by adding 10 μl of 10 mM Met-enkephalin in the same buffer. Subsequent procedures are described in Materials and Methods. Divalent ions are indicated at a final concentration in 100 μl reaction mixture. Percent activity was calculated from 100% of enzyme activity not treated with EDTA and metal ions.

Metal ions	Activity (%)		
	Concn.: 1 mM	100 μM	10 μM
$CaCl_2$	26	21	0
$ZnSO_4$	0	68	21
$MgCl_2$	16	0	0
$MnCl_2$	47	89	0
$FeSO_4$	0	52	0
$CoCl_2$	1295	1453	32
$HgCl_2$	16	0	0
$CuSO_4$	0	0	11

Effects of various inhibitors

Although Gorenstein and Snyder have already reported the effects of certain reagents on the activity [18], in order to obtain further information the effects of various inhibitors on the enzyme activity were examined (Table II). The enzyme was completely inhibited by metal chelators such as EDTA or *o*-phenanthroline and *p*-hydroxymercuribenzoate, but diisopropylfluorophosphate and phenylmethylsulphonyl fluoride, inhibitors of serine proteinases, had no effect on the activity. All inhibitors of other enkephalin-degrading enzymes, puromycin, amastatin and bestatin (aminopeptidase inhibitor), phosphoramidon (enkephalinase A inhibitor) and captopril (angiotensin-converting enzyme inhibitor) had no effect on the activity. Recently, we found two potent inhibitors of this enzyme, named propioxatins A and B, from microbiological products [11]. Both inhibitors have a hydroxamic acid [12], which is characteristic for metallo-proteinase inhibitors. A structurally similar compound to propioxatin B, diprotin A (Ile-Pro-Ile, dipeptidyl aminopeptidase IV inhibitor) had no effect on the activity. From the above results (inhibition by metal chelators and propioxatins), it was concluded that enkephalinase B could be classified as a metallo-proteinase.

Restoration of the enzyme activity by metal ions

In order to confirm that enkephalinase B was a metallo-proteinase, restoration of the enzyme activity by metal ions was examined (Table III). A solution of the enzyme containing 1 mM EDTA was thoroughly dialyzed against 5 mM Tris-HCl buffer (pH 7.0) to remove the chelating agent and the activity disappeared. But addition of 100 μM of Zn^{2+}, Mn^{2+} or Fe^{2+} to a dialyzed solution containing the inactive enzyme caused restoration of 50–90% of the original enzyme activity. Furthermore, the fact that the enzyme was activated by addition of 100 μM Co^{2+} more than 10-fold compared with the original activity is worth noting. Orlowski et al. [19] reported that a soluble metallo-endopeptidase from rat brain was also activated by addition of Co^{2+}. It is not clear why these metallo-enzymes were activated by addition of Co^{2+}.

TABLE IV

KINETIC PARAMETERS FOR HYDROLYSIS OF TETRA- AND PENTAPEPTIDES BY ENKEPHALINASE B.

K_m values were estimated by Lineweaver-Burk plots.

Substrate ($P_2P_1\downarrow P_1'P_2'P_3'$)	K_m [a] (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($min^{-1} \cdot \mu M^{-1}$)
Tyr-Gly-Gly-Phe	15 ± 2 (3)	240	16
Gly-Gly-Phe-Leu	380 ± 20 (3)	3000	7.9
Tyr-Gly-Gly-Phe-Leu	50 ± 3 (3)	790	16
Tyr-Gly-Gly-Phe-Met	53 ± 6 (4)	760	14
Tyr-Gly-Gly-Phe-Leu-NH_2	150 ± 32 (3)	180	1.2
Tyr-Gly-Gly-Phe-Met-NH_2	130 ± 12 (3)	180	1.4
Trp-Met-Asp-Phe-NH_2	65 ± 6 (3)	1000	15

[a] Data are mean values ± S.D. of three or four determinations given in parentheses.

Hydrolysis of enkephalin, enkephalin-related peptides and biologically active peptides

In order to clarify the substrate specificity of enkephalinase B, cleavage sites of substrates were examined using enkephalins, enkephalin-related peptides and some biologically active peptides.

Enkephalin pentapeptides and their amidated forms were hydrolyzed only at the Gly-Gly bond, and Tyr-Gly and other tripeptide fragments were detected by HPLC. K_m values for the Met-enkephalin and Leu-enkephalin were $5.3 \cdot 10^{-5}$ M and $5.0 \cdot 10^{-5}$ M, respectively. No clear differences were found in the K_m values for Met- and Leu-enkephalin. Tetrapeptides, Tyr-Gly-Gly-Phe lacking the C-terminal Met of Met-enkephalin and Gly-Gly-Phe-Leu lacking the N-terminal Tyr of Leu-enkephalin were hydrolyzed at the Gly-Gly bond and the Gly-Phe bond, respectively. (Tyr-Gly and Gly-Phe fragments were detected for Tyr-Gly-Gly-Phe, and Gly-Gly and Phe-Leu fragments were detected for Gly-Gly-Phe-Leu.) The kinetic parameters for the above peptides are compared in Table IV. The rate of reaction (K_{cat}) and the specificity constant (K_{cat}/K_m) for Met- or Leu-enkephalinamide were about 4- and 10-fold lower, respectively, than that of Met- or Leu-enkephalin. This result suggested that the C-terminal-blocked enkephalins were difficult to hydrolyze by enkephalinase B.

We further examined hydrolysis of other enkephalin-related peptides by the enzyme. Tripeptide, Tyr-Gly-Gly which lacks the Phe-Met of Met-enkephalin, was not hydrolyzed. This result supported the supposition that enkephalins are hydrolyzed only at the Gly-Gly bond and that Gly-Phe-Met (-NH_2) and Gly-Phe-Leu (-NH_2) are not hydrolyzed. N^{α}-acetyl-Leu-enkephalin, which was blocked at the N-terminal Tyr of Leu-enkephalin, [D-Ala^2,Met^5]-enkephalin and [D-Ala^2,D-Leu^5]-enkephalin were not hydrolyzed. Although the activity of the enzyme to Met-enkephalinamide or Leu-enkephalinamide still remained, the enkephalin-related peptides to which amino acid or peptides were added to the C-terminal side (Leu-enkephalin-Arg, Met-enkephalin-Arg-Phe, α-neo-endorphin, dynorphin (1–13) and α-endorphin) were not susceptible to the action of the enzyme.

Some other biologically active peptides were examined. Tetrapeptide, Trp-Met-Asp-Phe-NH_2, was hydrolyzed at the Met-Asp bond as shown in Table IV and Trp-Met and Asp-Phe-NH_2 fragments were detected by HPLC. Longer peptides such as angiotensin I, substance P, bradykinin and delta sleep-inducing peptide were not hydrolyzed by the enzyme even after incubation for periods of longer than 1 h. Angiotensin II and III were not hydrolyzed after 10 min incubation, but when the reaction was carried out for longer than 1 h, these two peptides were slightly hydrolyzed. To identify the peptide fragments, the reaction products were separated by HPLC and the amino acid composition of the fragments obtained were determined by an amino acid analyzer. Val-Tyr and Pro-Phe were detected from a hydrolyzate of angiotensin II (Asp-Arg-$\downarrow$Val-Tyr-$\downarrow$Ile-His-$\downarrow$Pro-Phe) and Arg-Val, Tyr-Ile, His-Pro-Phe and Tyr-Ile-His-Pro-Phe were detected from a hydrolyzate of angiotensin III (Arg-Val-$\downarrow$Tyr-Ile-$\downarrow$His-Pro-Phe). It was confirmed that the enzyme was classified as dipeptidyl aminopeptidase.

From the above results, it was concluded that a free NH_2 terminal was required for the hydrolysis of peptide by the enzyme and although this enzyme belongs to the dipeptidyl aminopeptidase class, peptides longer or shorter than tetra- or pentapeptides were not so much hydrolyzed. Short peptides such as enkephalin are good substrates for effective hydrolysis. Longer peptides than enkephalin are not hydrolyzed due to a steric hind-

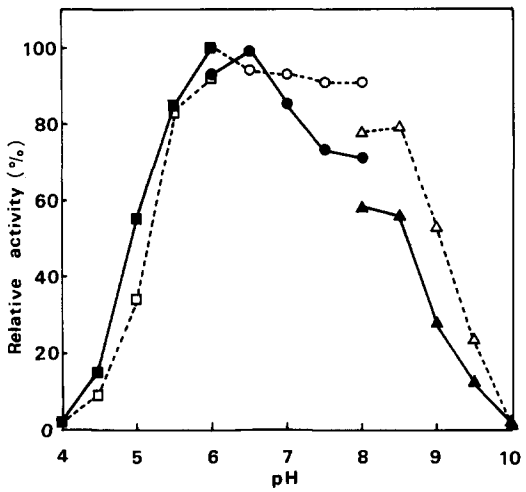

Fig. 2. pH activity profiles of enkephalinase B (closed symbols) and DAP-III (open symbols). The enzyme activity was measured using Met-enkephalin as a substrate in 0.1 M sodium acetate buffer (■, □), sodium phosphate buffer (●, ○) or sodium borate buffer (▲, △).

rance of the active site and not due to interference of interaction between the free carboxyl group and the enzyme as enkephalinamides were hydrolyzed. We concluded that enkephalinase B effectively catches and hydrolyzes enkephalin as a substrate.

Differences between soluble DAP-III and membrane-bound enkephalinase B

Additional experiments were carried out in order to clarify that enkephalinase B is distinct from DAP-III. The pH activity profile of DAP-III using Met-enkephalin as a substrate was compared with that of enkephalinase B as shown in Fig. 2. DAP-III activity did not change much in the pH 6.0–8.0 region (sodium phosphate buffer) and was higher than that of enkephalinase B in the basic region. The pH showing maximum activity also differed.

DAP-III hydrolyzed well angiotensin II as reported by Lee and Snyder [10], but enkephalinase B hardly hydrolyzed it, as mentioned above. K_i values of propioxatins A and B for DAP-III were determined by a Dixon plot [20] for comparison with those of enkephalinase B. As shown in Fig. 3, K_i values of propioxatins A and B for DAP-III were $1.3 \cdot 10^{-8}$ M and $5.6 \cdot 10^{-9}$ M, respectively. Propioxatin A inhibited both enzymes to the same degree (the K_i value for enkephalinase B was $1.3 \cdot 10^{-8}$ M) but propioxatin B inhibited DAP-III more strongly (the K_i value for enkephalinase B was $1.1 \cdot 10^{-7}$ M). From these results it was suggested that membrane-bound enkephalinase B is somewhat different from soluble DAP-III.

Discussion

Since the degradation system of enkephalin has generated considerable interest on the study of the action of substances such as opioid peptides, some

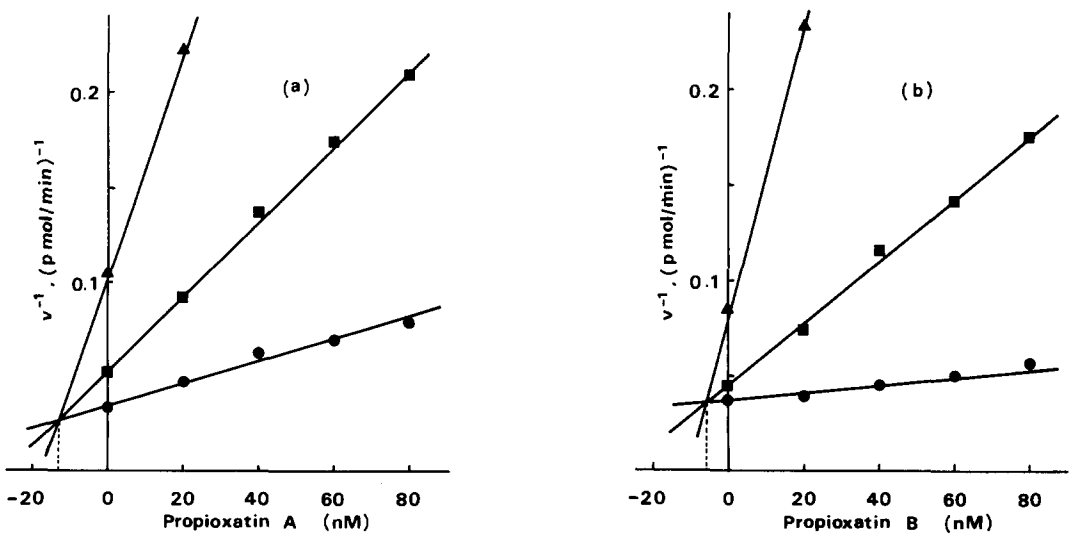

Fig. 3. Dixon plots for the inhibition by propioxatins A (a) and B (b) of hydrolysis of Arg-Arg-βNA by DAP-III. Substrate concentration (μM); 100, ●; 25, ■; 10, ▲.

enkephalin-degrading enzymes (aminopeptidases and enkephalinase A) from the mammalian brain [21–25] or kidney [26–29] have been purified and their characteristics have been investigated. However, enkephalinase B has not become the object of much study because it has been considered to be a minor enzyme in the enkephalin degradation system. Now we report the first complete purification of the enzyme from rat brain membrane and describe its properties.

Enkephalinase B was determined to be a metallo-enzyme like enkephalinase A, as metal chelators and peptide hydroxamic acids inhibited the activity. Several peptide hydroxamic acids were examined as inhibitors of thermolysin by Nishino and Powers [30,31]; hydroxamic acid being involved in coordinating the zinc ion at the active site of thermolysin in a bidentate fashion. Kelatorphan [32], which inhibited activities of both enkephalinase A and enkephalinase B, also contains a hydroxamic acid moiety. Just as in the case of a thermolysin, the hydroxamic acid function group of propioxatins and the carbonyl oxygen of the enkephalin Gly-Gly bond are involved in coordinating the metal ion at the active site of enkephalinase B. It remains to be seen whether the metal ion at the active site is zinc or not. X-ray crystallography of a propioxatin A derivative showed that the backbone of the molecule is folded [33]. If this folded molecule is essential for tight binding between the inhibitor and the enzyme and fit for the active site of the enzyme, the size of the active site pocket will be rather small and, as a result, longer peptides will not be hydrolyzed. The study on structure-activity relationships of propioxatin will clarify the properties of subsites, S_1', S_2' and S_3', against amino acid residues, P_1', P_2' and P_3', shown in Table IV.

In the case of calf brain enkephalinase B, it was reported that membrane-bound and soluble enzyme were identical [34]. But rat brain membrane-bound enkephalinase B is somewhat different from soluble DAP-III. It is not known whether membrane-bound and soluble enzyme consist of the same polypeptide chain or not. (For example, membrane-bound enzymes have an additional hydrophobic peptide which can bind to cell membrane.) The molecular weights of these two enzymes were very similar. But, even if the two enzymes were similar (or identical), since a considerable amount of activity was present in the membrane-bound form, the physiological role of enkephalinase B may be distinct from that of DAP-III.

Several dipeptidyl aminopeptidases III were inhibited by EDTA or *o*-phenanthroline [17,35–37]. If these enzymes are inhibited by propioxatins, it will be clear that a metal ion in the enzyme is essential for catalysis. As DAP-III is inhibited by *o*-phenanthroline [10] and propioxatins A and B, this enzyme may be a metallo-proteinase. The *p*-hydroxymercuribenzoate-reactable SH group of cysteine [17,37] or the diisopropylfluorophosphate-reactable OH group of serine [35–37] in the enzyme are somewhat involved in the activity.

At present, enzymes known to be involved in degradation of enkephalin include aminopeptidase [4–7], dipeptidyl aminopeptidase [9] and enkephalinase A [2,3]. It is suggested that the enzyme designated as enkephalinase A contributes to the main metabolic pathway of enkephalin on the basis of its regional distribution and relatively high affinity for enkephalin [2]. But the high specificity with which enkephalinase B explicitly requires enkephalin as a substrate cannot be overlooked. It can be assumed that enkephalinase B plays an important role in the degradation system of enkephalin.

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