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Purification and Characterization of Proline Endopeptidase from Rat Liver

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Proline endopeptidase (PEPase) was purified 71800-fold from the soluble fraction of rat liver with a yield of 65.2%. Ammonium sulfate fractionation and chromatographies on diethyl aminoethyl (DEAE)-Sepharose CL-6B, DE-52, blue Sepharose CL-6B, carbobenzoxyglycyl-L-prolyl-AH-Sepharose 4B and Mono-Q were used for the purification of the enzyme. Among the purification procedures, blue Sepharose CL-6B chromatography was the most effective step to eliminate various contaminants. The final enzyme preparation had a high specific activity of 12200 unit/mg protein and showed maximal activity at pH 5.9 toward succinylglycyl-L-proline 4-methylcoumaryl-7-amide (Suc–Gly–Pro–MCA). The K_m and V_{max} values for Suc–Gly–Pro–MCA were 0.43 mM and 52.0 μmol/min/mg protein, respectively. The apparent molecular weight of rat liver PEPase was estimated to be 68000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and 59000 by gel filtration on TSK-Gel G-4000 SW. The enzyme was extremely sensitive to diisopropyl fluorophosphate, but no effect was observed with aprotinin, soybean trypsin inhibitor or ethylenediaminetetraacetate. In addition, the enzyme activity was strongly inhibited by *p*-chloromercuribenzoate and Hg^{2+}. Elastatinal and 1,10-phenanthroline also significantly inactivated the enzyme. These results indicate that rat liver PEPase was similar to PEPase purified previously from rat brain, porcine liver and other sources in some enzymatic properties and in molecular nature. The distribution of the enzyme was similar to that of the cytosol marker enzyme, lactate dehydrogenase.

Keywords——proline endopeptidase; rat liver cytosol; purification; blue Sepharose CL-6B chromatography; subcellular localization

Since proline endopeptidase (PEPase, EC 3.4.21.26) catalyzes the hydrolysis of several biologically active peptides, *i.e.*, oxytocin, vasopressin, substance P, neurotensin and angiotensin II, the enzyme has been studied in relation to the metabolism of neuropeptide hormones.[1-12] Recently, Andrews *et al.*[13] suggested that PEPase may function not only in the metabolism of hormones but also in the overall process of intracellular protein degradation. In fact, PEPase activity is widely distributed in mammalian organ and body fluids.[14] However, the properties of PEPase in tissues, except for those of brain[1-4] and kidney,[5-8] remain to be fully established.

In this work, we chose rat liver as an enzyme source for studies on the properties of PEPase in peripheral tissues. In addition, we attempted to improve the purification method of PEPase. The present paper deals with the purification and some properties of liver PEPase.

Materials and Methods

Materials——The following materials were commercially obtained: succinylglycyl-L-proline 4-methylcoumaryl-7-amide (Suc–Gly–Pro–MCA), Gly–Pro–MCA, succinyl-L-alanine *p*-nitroanilide (Suc–Ala–*p*NA), Suc–Ala–Ala–*p*NA, Suc–Ala–Ala–Ala–*p*NA, carbobenzoxy (Z)–Gly–Pro–Leu–Gly, Z–Gly–Pro, angiotensin I, bradykinin, substance P, adrenocorticotropic hormone (ACTH) (1—24), α-neo-endorphin and elastatinol from the Protein

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Research Foundation, Minoh, Osaka, Japan; 1,10-phenanthroline from E. Merck A. G. Darmstadt, West Germany; aprotinin and soybean trypsin inhibitor from Boehringer Mannheim Yamanouchi, Tokyo, Japan; Congo-red elastin, *p*-chloromercuribenzoate (PCMB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), ethylenediaminetetraacetate (EDTA) and diisopropyl fluorophosphate (DFP) from Sigma, St. Louis, Mo., U.S.A.; diethyl aminoethyl (DEAE)-Sepharose CL-6B, blue Sepharose CL-6B, AH-Sepharose 4B and Mono Q from Pharmacia Fine Chemicals, Uppsala, Sweden; DE-52 from Whatman, England; TSK-Gel G-4000 SW from Toyo Soda Manufacturing Co., Tokyo, Japan. All other chemicals were of the purest grade available from Wako Pure Chemicals, Osaka, Japan. Z–Gly–Pro–AH-Sepharose 4B was prepared by the method of Cuatrecasas.[15] Male Wistar rats were obtained from the Animal Center of Fukuoka University.

Enzyme Assay——PEPase activity was determined using Suc–Gly–Pro–MCA as a substrate. Fifty μl of 0.5 mM substrate solution and 50 μl of enzyme solution were added to 100 μl of 50 mM sodium phosphate buffer, pH 5.9, containing 2 mM DTT and 1 mM EDTA. After an incubation for 10 min at 37 °C, the reaction was terminated by the addition of 2.0 ml of 1 M sodium acetate buffer, pH 4.0, and 7-amino-4-methylcoumarin (AMC) released was fluorometrically measured as described previously.[16] One unit was defined as the amount of enzyme that released 1 nmol of AMC from the substrate per min.

Protein Concentration——Protein concentration was determined by the method of Lowry *et al.*,[17] using bovine serum albumin as the standard.

Electrophoresis——Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn.[18] Polyacrylamide gel electrophoresis was carried out as described by Williams and Reisfeld.[19] All gels were stained with Coomassie brilliant blue R-250.

Molecular Weight Determination——The molecular weight of the undenatured enzyme was determined by gel filtration on a TSK-Gel G-4000 SW (7.5 × 600 mm) with a high-performance liquid chromatography (HPLC) apparatus from Pharmacia Fine Chem. Co. The column, pre-equilibrated with 100 mM sodium phosphate buffer, pH 6.8, containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 300 mM NaCl, was loaded with 10 μg of protein in 50 μl of the equilibration buffer and eluted with the buffer at a flow rate of 0.5 ml/min. Catalase (M_r = 240000), aldolase (M_r = 158000), bovine serum albumin (M_r = 68000) and chymotrypsinogen (M_r = 25000) were used as standards. SDS-polyacrylamide gel electrophoresis of the denatured enzyme was performed as described above. SDS-PAGE Marker II (Seikagaku Kogyo Co., Tokyo, Japan) was used as a calibrating agent.

Identification of Cleavage Points in Peptides by the Enzyme——Cleavage points in naturally occurring peptides (angiotensin I, bradykinin, substance P, ACTH (1—24) and α-neo-endorphin) and Z–Gly–Pro–Leu–Gly were identified by HPLC followed by amino acid analysis as described by Inokuchi and Nagamatsu.[20] Reaction mixtures contained peptide (0.8—1.0 mM), sodium phosphate buffer (20 mM, pH 7.0), DTT (1 mM), EDTA (1 mM) and enzyme (1.0 unit) in a final volume of 250 μl. The mixture was incubated at 30 °C for 8 h. In the case of Suc–$(Ala)_3$–*p*NA, Suc–$(Ala)_2$–*p*NA, Suc–Ala–*p*NA, Gly–Pro–MCA and Suc–Gly–Pro–MCA, cleavage points were identified by thin layer chromatography (TLC, Silica gel $60F_{254}$, Merck) as described previously.[4] Elastinolytic activity of the enzyme was determined by the method of Shotton,[21] with Congo-red elastin as a substrate.

Optimal pH——The pH–activity profile for PEPase was measured with Suc–Gly–Pro–MCA according to the method described above except that 0.1 M sodium phosphate buffer containing 1 mM EDTA and 2 mM DTT for the pH range 4.5—7.5 and 0.1 M Tris–HCl buffer containing 1 mM EDTA and 2 mM DTT for the pH range 7.5—8.0 were used.

Kinetic Studies——K_m and V_{max} values of the enzyme for Suc–Gly–Pro–MCA were determined from a Lineweaver–Burk plot with substrate concentrations in the range of 0.12 to 1.0 mM.

Results

Subcellular Localization

The subcellular localization of PEPase in rat liver was examined by sucrose density gradient centrifugation, and 96% of the enzyme activity toward Suc–Gly–Pro–MCA was recovered in the soluble fraction. The distribution of the enzyme was similar to that of the cytosol marker enzyme, lactate dehydrogenase, and distinct from those of cytochrome c oxidase (mitochondria), reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome c reductase (endoplasmic reticulum), acid phosphatase (lysosome) and 5′-nucleotidase (plasma membrane).

Purification

All steps were carried out at 0 to 4 °C and ultrafiltration or equilibration of enzyme solution was performed with a YM-10 membrane or YM-30 membrane (Amicon).

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Step 1. Crude Extract——PEPase was purified from the soluble fraction of rat liver at 5 to 6 weeks of age. The liver (300 g) was homogenized with four volumes (w/v) of 50 mM phosphate buffer, pH 7.2, containing 0.25 M sucrose for 90 s in a Polytron homogenizer. The homogenate was centrifuged at $105000 \times g$ for 90 min, and the supernatant (crude extract) was collected.

Step 2. Ammonium Sulfate Fractionation——The crude extract (1360 ml) was subjected to fractionation with solid ammonium sulfate. The fraction precipitating between 40% and 70% saturation was collected by centrifugation at $10000 \times g$ for 20 min, dissolved in a small volume of 50 mM sodium phosphate buffer, pH 6.8, containing 1 mM 2-mercaptoethanol and 1 mM EDTA (buffer A), and then dialyzed overnight against the same buffer. The insoluble materials in the dialysate were removed by centrifugation at $10000 \times g$ for 20 min.

Step 3. DEAE-Sepharose CL-6B Chromatography——The dialysate (265 ml) was applied to a DEAE-Sepharose CL-6B column (5.0×12 cm) equilibrated with buffer A. The column was washed with buffer A (100 ml) and eluted with a linear gradient of 0 to 0.8 M NaCl in buffer A (1000 ml) at a flow rate of 80 ml/h. PEPase activity was eluted with NaCl concentrations ranging from 0.08 to 0.35 M, as a broad peak. The active fractions were combined and concentrated by ultrafiltration on a YM-30 membrane. The concentrate was equilibrated with 20 mM sodium phosphate buffer, pH 7.2, containing 1 mM 2-mercaptoethanol and 1 mM EDTA (buffer B).

Step 4. DE-52 Chromatography——The equilibrated active fraction (50 ml) was applied to a DE-52 column (2.8×26 cm) equilibrated with buffer B. The column was eluted with a linear gradient of NaCl (0 to 0.4 M) in buffer B (800 ml) at a flow rate of 50 ml/h. The enzyme activity was eluted at around 0.1 M NaCl, as a sharp peak. The active fractions (44 ml) were concentrated and equilibrated through a YM-30 membrane against buffer B.

During DEAE-Sepharose CL-6B and DE-52 chromatographies, the specific activity of the enzyme increased 11- and 10-fold as compared with the preceding step, respectively.

Step 5. Blue Sepharose CL-6B Chromatography——The concentrated enzyme solution (2.0 ml) from step 4 was applied to a column of blue Sepharose CL-6B (1.0×42 cm) equilibrated with buffer B. The column was developed with the same buffer. As shown in Fig. 1, PEPase activity passed through the column, while the absorbed proteins, which were eluted

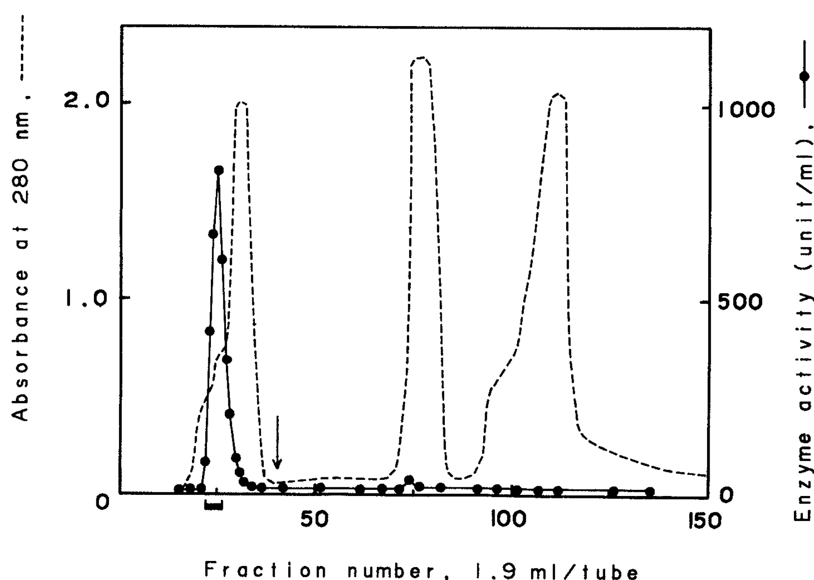

Fig. 1. Blue Sepharose CL-6B Chromatography of Partially Purified PEPase from Rat Liver Cytosol

The arrow indicates the replacement of the initial buffer with 1.5 M KCl. The flow rate was 30 ml/h. The other procedures are described in Results. Fractions 23 to 26 were pooled.

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with 1.5 M KCl, had no activity. The pooled active fractions (9.8 ml) were equilibrated against 50 mM sodium phosphate buffer, pH 5.9, containing 5 mM 2-mercaptoethanol and 1 mM EDTA (buffer C) by the use of a YM-10 membrane.

During this step, the specific activity of the enzyme increased 147-fold as compared with that of the enzyme preparation from step 4. On the other hand, the enzyme activity was apparently inhibited by the fractions eluted with 1.5 M KCl (data not shown). Thus, the blue Sepharose CL-6B chromatography was very useful to separate liver PEPase from an endogeneous inhibitor.

Step 6. Z–Gly–Pro–AH-Sepharose 4B Chromatography——The equilibrated enzyme solution was applied to a Z–Gly–Pro–AH-Sepharose 4B column (1.25 × 18 cm) equilibrated with buffer C. The column was eluted with a linear gradient of NaCl (0 to 0.25 M) in buffer C (400 ml) at a flow rate of 50 ml/h. PEPase activity was eluted at around 0.09 M NaCl, as a single peak. The active fractions (39.8 ml) were collected and concentrated to 2 ml by ultrafiltration on a YM-10 membrane. The concentrate was equilibrated against 50 mM sodium phosphate buffer, pH 7.2, containing 1 mM 2-mercaptoethanol and 1 mM EDTA (buffer D).

Step 7. Mono-Q Chromatography——The active fraction (500 μl × 4) was loaded on Mono-Q column (0.5 × 5 cm) equilibrated with buffer D. As shown in Fig. 2, the enzyme was

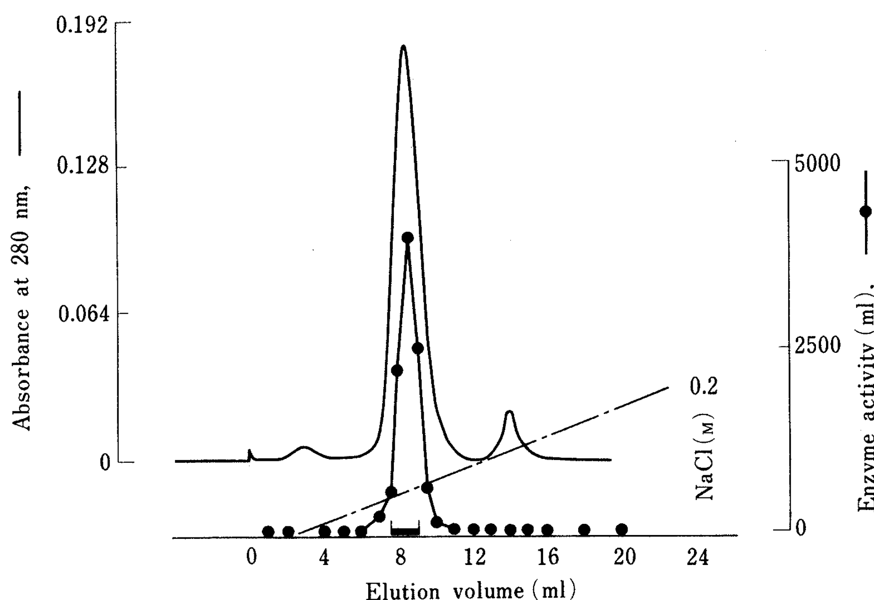

Fig. 2. Mono-Q Column Chromatography of Rat Liver PEPase

The column (0.5 × 5 cm) was equilibrated with 50 mM phosphate buffer (pH 7.2, containing 1 mM 2-mercaptoethanol and 1 mM EDTA), using a high performance liquid chromatograph (Pharmacia). Elution was performed with a linear gradient of NaCl (0 to 0.2 M). The flow rate was 60 ml/h. The fractions under the bar were collected.

TABLE I. Purification of PEPase from Rat Liver

Purification	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Purity (fold)	Yield (%)
1. 105000 × g supernatant	5389	31700	0.17	1	100
2. $(NH_4)_2SO_4$ fractionation	1062	11800	0.09	0.53	19.7
3. DEAE-Sepharose CL-6B	1596	1650	0.97	5.7	29.6
4. DE-52	3960	395	10.0	59	73.5
5. Blue Sepharose CL-6B	5890	4.0	1469	8640	109
6. Z–Gly–Pro–AH-Sepharose	5207	0.85	6097	35700	96.6
7. Mono-Q	3510	0.29	12200	71800	65.2

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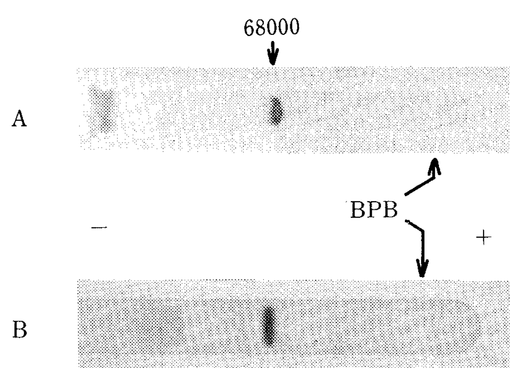

Fig. 3. Polyacrylamide Gel Electrophoresis of Purified PEPase

A: Disc-gel electrophoresis of PEPase in the presence of SDS. B: Disc-gel electrophoresis of PEPase in the absence of SDS.

About 10 μg of the enzyme was applied to each gel.

TABLE II. Cleavage Points in Various Peptides by PEPase from Rat Liver

Substrates and cleavage points[a]	Products found
Angiotensin I:	
Asp–Arg–Val–Tyr–Ile–His–Pro↓Phe–His–Leu[b]	Asp–Arg–Val–Tyr–Ile–His–Pro, Phe–His–Leu
Bradykinin:	
Arg–Pro–Pro↓Gly–Phe–Ser–Pro↓Phe–Arg[b]	Arg–Pro–Pro, Gly–Phe–Ser–Pro, Phe–Arg
Substance P:	
Arg–Pro–Lys–Pro↓Gln–Gln–Phe–Phe–Gly–Leu–Met[b]	Arg–Pro–Lys–Pro, Gln–Gln–Phe–Phe–Gly–Leu–Met
α-Neo-endorphin:	
Tyr–Gly–Gly–Phe–Leu–Arg–Lys–Tyr–Pro↓Lys[b]	Tyr–Gly–Gly–Phe–Leu–Arg–Lys–Tyr–Pro, Lys
ACTH (1—24)[b]	Not detectable
Z–Gly–Pro↓Leu–Gly[b]	Z–Gly–Pro, Leu–Gly
Suc–Ala–Ala–Ala↓*p*NA[c]	Suc–Ala–Ala–Ala, *p*-nitroaniline
Suc–Ala–Ala↓*p*NA[c]	Suc–Ala–Ala, *p*-nitroaniline
Suc–Ala–*p*NA[c]	Not detectable
Gly–Pro–MCA[c]	Not detectable
Bovine serum albumin[d]	Not detectable
Congo-red elastin[e]	Not detectable

a) Cleavage points are indicated by arrows. *b*) Identified by HPLC as described in Materials and Methods. *c*) Identified by TLC as described in Materials and Methods. *d*) Detected by SDS-polyacrylamide gel electrophoresis. *e*) Detected by the method of Shotton as described in Materials and Methods.

eluted at NaCl concentrations ranging from 0.06 to 0.08 M, as a sharp symmetrical peak.

The increases in the degree of purification on steps 6 and 7 were small. However, these steps were effective to eliminate contaminants from the enzyme. PEPase was finally purified 71800-fold from the soluble fraction of rat liver with 65.2% recovery. These results are summarized in Table I.

Homogeneity and Molecular Weight

The final enzyme preparation gave a single protein band on polyacrylamide gel electrophoresis in the presence or absence of SDS, as shown in Fig. 3. The apparent molecular weight of the purified liver enzyme was estimated to be 59000 by gel filtration on TSK-Gel G-4000 SW, and 68000 by SDS-polyacrylamide gel electrophoresis.

pH Optimum

The activity of liver PEPase was examined in phosphate and Tris–HCl buffers covering a pH range of 4.5 to 8.0. The purified enzyme showed a pH optumum of 5.9 for the hydrolysis of Suc–Gly–Pro–MCA. The optimum pH for Suc–Gly–Pro–MCA was similar to that of PEPase in rat brain.[6]

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TABLE III. Effects of Various Reagents and Proteinase Inhibitors on the Activity of PEPase from Rat Liver

Effector		Remaining activity (%)
DFP	0.1 mM	0[a]
PCMB	0.1 mM	0[b]
DTNB	1 mM	0[b]
EDTA	4 mM	100
1,10-Phenanthroline	0.5 mM	52[a]
Soybean trypsin inhibitor	1 mg/ml	91
Aprotinin	0.4 mg/ml	94
Elastatinol	50 μg/ml	72
$CaCl_2$	5 mM	106[c]
$MgCl_2$	5 mM	104[c]
$HgCl_2$	1 mM	0[b]

a) Against control containing 1.0 mM DTT, 0.5 mM EDTA and 0.5% methanol. *b*) Against control containing 25 μM DTT and 25 μM EDTA. *c*) Against control containing 1.0 mM DTT and 0.1 mM EDTA. The enzyme activity was determined with Suc–Gly–Pro–MCA as described in Materials and Methods. The enzyme (0.08 unit) and each effector were incubated without preincubation.

Substrate Specificity

As shown in Table II, the enzyme catalyzed hydrolysis of prolyl linkages of angiotensin I, bradykinin, substance P, α-neo-endorphin and synthetic substrates for PEPase such as Z–Gly–Pro–Leu–Gly and Suc–Gly–Pro–MCA. In addition, Suc–Ala–Ala–Ala–*p*NA and Suc–Ala–Ala–*p*NA were hydrolyzed by the enzyme, but Suc–Ala–*p*NA, Congo-red elastin and bovine serum albumin were not. On the other hand, the enzyme was unable to digest ACTH (1—24) in spite of the presence of the prolyl linkage.

Effects of Heavy Metals and Various Inhibitors

The effects of various reagents on liver PEPase were investigated by the use of Suc–Gly–Pro–MCA as a substrate. The results are shown in Table III.

The enzyme was strongly inhibited by a serine protease inhibitor, DFP, and sulfhydryl-blocking reagents, PCMB and DTNB. However, soybean trypsin inhibitor and aprotinin hardly affected the enzyme activity. On the other hand, elastatinal and 1,10-phenathroline had significant inhibitory effects. Among the divalent metal ions examined, Hg^{2+} was a potent inhibitor, whereas no inhibitory effect on the enzyme activity could be observed with Mg^{2+} and Ca^{2+}. Furthermore, the enzyme was not inhibited by EDTA.

Kinetic Studies

The kinetic parameters of the purified enzyme toward a PEPase-specific substrate, Suc–Gly–Pro–MCA, were determined by means of a Lineweaver–Burk plot. The K_m and V_{max} values for liver PEPase were calculated to be 0.43 mM and 52.0 μmol/min/mg protein, respectively.

Discussion

We attempted the purification of PEPase from rat liver cytosol for the first time, using Suc–Gly–Pro–MCA as PEPase-specific substrate. Up to the present, PEPase has generally been purified from various enzyme sources by ammonium sulfate fractionation and chromatographies on DEAE-Sephadex, Sephadex, PCMB-Sepharose, Z–Gly–Pro–Leu–Gly–AH-Sepharose, Con A-Sepharose, phenyl-Sepharose and hydroxyapatite columns. However, the combinations of these procedures take a long time to perform, and sometimes failed to give

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good results. During the purification of liver PEPase, we noted that: 1) PEPase activity considerably decreases with increase in its purity, 2) the use of phenyl-Sepharose column chromatography causes a reduction of the enzyme activity, 3) hydroxyapatite column chromatography gives a low recovery of the enzyme, 4) the reduction in working time is still an important problem for effective purification. In addition, it is essential to eliminate an endogeneous inhibitor, which was partially purified from rat liver by us (Yamakawa *et al.*, unpublished data). Finally, we used the purification procedures summarized in Table I. PEPase inhibitor was absorbed on a blue Sepharose CL-6B column, while PEPase activity passed through the column (Fig. 1). Therefore, this chromatography was most effective in eliminating the inhibitor and decreasing the working time. The inhibitor was completely removed from the enzyme fraction by Z–Gly–Pro–AH-Sepharose 4B and Mono-Q chromatographies. Finally, the purified enzyme had a specific activity of 12200 unit/mg of protein, which corresponded to 71800-fold purification with a recovery of 65.2%.

The enzyme purified from rat liver cleaved angiotensin I, bradykinin, substance P, α-neoendorphin and Z–Gly–Pro–Leu–Gly on the carboxyl side of proline (Table II). The enzyme also released *p*-nitroaniline from Suc–Ala–Ala–Ala–*p*NA and Suc–Ala–Ala–*p*NA as previously indicated by us using PEPase from hog kidney cytosol.[4] The K_m value of the enzyme toward Suc–Gly–Pro–MCA was 0.43 mM. This value approximates to that obtained with PEPase purified from rat brain.[6] As reported for PEPase from rat[6,22] and bovine[8] brain, lamb[2] and hog[4] kidney and porcine liver,[23] the rat liver enzyme was susceptible not only to DFP but also to sulfhydryl-blocking reagents, PCMB and DTNB. In addition, the enzyme was EDTA-resistant and was not inhibited by soybean and pancreatic trypsin inhibitors (Table III). Thus, we consider that the enzyme is a serine protease and that one or more cysteinyl groups probably exist near the active site seryl residue of the enzyme. The molecular weight and pH optimum of the liver enzyme were also similar to those of rat brain PEPase.[6] These results indicate that the enzyme purified from rat liver may be identical with PEPase from rat brain. Furthermore, we examined the subcellular localization of the enzyme activity using sucrose density gradient centrifugation and found that most of the enzyme activity was recovered in the soluble fraction of liver.

Recently, we reported that two molecular species of PEPase (PEP I, 130000; PEP II, 62000) were concurrently isolated from normal human plasma.[16] The physicochemical and enzymatic properties of the liver enzyme were very similar to those of PEP II. Therefore, the possibility cannot be ruled out that at least one of the plasma PEPases is derived from the liver in the case of hepatobiliary disorders.

PEPase has been deduced to play an important role in the regulation of the biological active peptides such as angiotensin I, angiotensin II, bradykinin, substance P and thyroliberin. Previously, we purified a native PEPase inhibitor from rat brain[24] and found that inhibition of brain PEPase produced by this inhibitor was restored by the addition of polyamine.[25] A similar restoration of the inhibition by polyamine was observed using liver enzyme and its native inhibitor (Soeda *et al.*, unpublished data). Therefore, we assume that the physiological function of PEPase could be greatly affected by the native inhibitor and polyamine. However, the physiological role of PEPase remains to be fully elucidated.

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