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Two Molecular Species of Proline Endopeptidase in Human Plasma: Isolation and Characterization

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Two molecular species of proline endopeptidase (PEP I and II) were concurrently isolated from normal human plasma. They were clearly separated on ammonium sulfate fractionation of plasma followed by chromatographies on DEAE-Sephadex and Con A-Sepharose and thereafter were partly purified by separate but almost identical procedures which included chromatographies on Sephacryl S-300, hydroxyapatite and carbobenzoxy-Gly-Pro-AH-Sepharose. The apparent molecular weights of PEP I and PEP II were 130 and 62 kilodaltons, respectively, as judged by gel filtration on TSK-Gel G 4000 SW. PEP I had a high affinity for Con A-Sepharose, but PEP II did not. Both enzymes were apparently highly specific for peptides such as angiotensin I, bradykinin, substance P and succinyltrialanine *p*-nitroanilide, cleaving them after proline or alanine residues. However, both enzymes were inert toward elastin and albumin. The K_m and optimal pH values toward succinylglycyl-L-proline 4-methylcoumaryl-7-amide were 2.3 mM and 5.5—6.5 for PEP I and 0.71 mM and 6.0—7.0 for PEP II. Both enzymes were extremely sensitive to diisopropyl fluorophosphate, but no effect was observed with phenylmethanesulfonyl fluoride, aprotinin, soybean trypsin inhibitor, iodoacetamide or ethylenediaminetetraacetate. Elastatinal significantly inhibited PEP II while PEP I activity was only slightly affected. In addition, PEP II was strongly inactivated by *p*-chloromercuribenzoate and divalent metal ions such as Hg^{2+} and Zn^{2+}, whereas PEP I was only slightly inactivated by these reagents. Thus, the enzymes seem to be quite distinct. PEP II appears to be very similar to PEPs purified previously from brain, pituitary and kidney tissues in terms of enzymatic properties, physicochemical properties and substrate specificity. On the other hand, PEP I is different from other PEPs in molecular nature and some enzymatic properties, but is indistinguishable in substrate specificity and enzyme classification.

Keywords——proline endopeptidase; human plasma; affinity chromatography; substrate specificity; inhibition

Proline is widely distributed in biologically active peptides such as various hormones and the catabolism of proline-containing peptides is thought to be a unique process in intracellular protein degradation. Proline endopeptidase (EC 3.4.21.26, PEP) has received a great deal of attention in recent years. It has been purified mainly from kidney,[1-4] brain[5-8] and pituitary[9] tissues and studied in relation to the metabolism of neuropeptide hormones.[1-12] However, reports on the physiological and pathological roles of PEP are limited in scope. Recently, Andrews *et al.*[13] suggested that PEP may function not only in the metabolism of hormones but also in the overall process of intracellular protein degradation. In fact, PEP is ubiquitously distributed in mammalian organs and body fluids,[14] but the properties of PEP present in tissues and body fluids, except for those of brain and kidney, remain to be fully established.

In this work, we chose normal human plasma as an enzyme source for studies on the properties of PEP in body fluid, and we isolated two molecular species of PEP in parallel by almost identical methods. The purification methods are described and some properties of the two enzymes are compared.

Materials and Methods

Materials——*p*-Chloromercuribenzoate (PCMB), iodoacetamide, phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), ethylene diaminetetraacetate (EDTA), diisopropyl fluorophosphate (DFP) and Congo-red elastin were purchased from Sigma Chem. Co., St. Louis, Mo., U.S.A. 1,10-Phenanthroline was from E. Merck AG, Darmstadt, Germany. Aprotinin and soybean typsin inhibitor were from Boehringer Mannheim Yamanouchi Co., Tokyo, Japan. Succinylglycyl-L-proline 4-methylcoumaryl-7-amide (Suc–Gly–Pro–MCA), Gly–Pro–MCA, Suc–Ala–Ala–*p*-nitroanilide (*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)), Tyr–Gly–Gly–Phe–Met and elastatinal were from the Protein Research Foundation, Minoh, Japan. Outdated human plasma was from the Red Cross Blood Center, Fukuoka, Japan. Sephacryl S-300, DEAE-Sephadex A-50, blue-Sepharose CL-6B, Con A-Sepharose and AH–Sepharose 4B were products of Pharmacia Co., Uppsala, Sweden. Hydroxyapatite was obtained from Seikagaku Kogyo, Tokyo, Japan. All other chemicals were of the purest grade available from Wako Pure Chemicals, Osaka, Japan. Z–Gly–Pro–AH–Sepharose was prepared by the method of Cuatrecasas.[15]

Buffers——The following buffers were used in the chromatographic procedures for the purification of the two proteases: 50 mM sodium phosphate, pH 7.5, containing 5 mM 2-mercaptoethanol and 1 mM EDTA (buffer A); 50 mM Tris-HCl, pH 7.5, containing 0.5 mM 2-mercaptoethanol and 1 M NaCl (buffer B); 20 mM sodium phosphate, pH 7.0, containing 5 mM 2-mercaptoethanol (buffer C); 50 mM sodium phosphate, pH 7.0, containing 0.5 mM 2-mercaptoethanol and 50 mM NaCl (buffer D).

Purification Procedures——Purification of PEP I and PEP II required six different procedures in the following sequence: ammonium sulfate fractionation, and chromatographies on DEAE-Sephadex A-50, Con A-Sepharose, Sephacryl S-300, hydroxyapatite and Z–Gly–Pro–AH–Sepharose. All procedure were performed at 4 °C. Flow rate, column dimensions, buffer systems and the amounts of the protein loaded on the chromatographic columns used in this study are given under Results and in the legends to the figures.

Enzyme Assay——PEP activity was determined with Suc–Gly–Pro–MCA as the substrate. A typical reaction mixture contained 1.5 mM substrate, 50 mM sodium phosphate buffer, pH 6.5, containing 1 mM DTT and 1 mM EDTA in a total volume of 200 μl. After incubation for 30 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 the fluorescence intensity was measured at 440 nm with excitation at 370 nm. One unit (U) was defined as the enzyme activity releasing 1 nmol of 7-amino-4-methylcoumarin per min.

Identification of Cleavage Products of Peptides and Proteins by the Protease——For the identification of the cleavage points in peptides such as angiotensin I, bradykinin, substance P, ACTH (1—24), Tyr–Gly–Gly–Phe–Met and Z–Gly–Pro–Leu–Gly, reaction mixtures contained a substrate (0.8—1.0 mM), sodium phosphate buffer (20 mM, pH 7.0), 2-mercaptoethanol (1 mM), EDTA (1 mM) and enzyme (90—120 mU) in a final volume of 250 μl. All incubations were performed at 30 °C for 8 h. Products of the reaction were separated on a Waters high performance liquid chromatography (HPLC) system, followed by amino acid analysis as described by Inokuchi and Nagamatsu.[16] Analysis of enzymatic products formed from Suc–Ala–Ala–Ala–*p*NA or Suc–Ala–Ala–*p*NA was performed on silica gel plates as described previously.[4] Elastinolytic activity of the protease was determined by the method of Shotton,[17] with Congo-red elastin as a substrate. Cleavage of bovine serum albumin by the protease was determined by analyzing the hydrolysate on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Taylor and Dixon.[12]

Inhibitors——The following solutions were used for the inactivation of PEP: DFP in 2-propanol; PMSF in 10% dioxane; 1,10-phenanthroline in 100% ethylene glycol. All other reagents were used in water.

Optimal pH——The pH-activity profile for PEP 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 1 mM DTT for the pH range 5—7 and 0.1 M Tris-HCl buffer containing 1 mM EDTA and 1 mM DTT for the pH range 7—9 were used.

Kinetic Studies——The K_m value of the protease toward the synthetic substrate, Suc–Gly–Pro–MCA, was determined in 50 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA and 1 mM DTT at 37 °C. Substrate levels were varied between 0.1 and 3.0 mM. For evaluation of the hydrolytic rates, the fluorescence of 7-amino-4-methylcoumarin released was recorded.

Molecular Weight Determination——The molecular weight of the protease was determined by gel filtration on a TSK-Gel G 4000 SW column (7.5 × 600 mm) with an 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 0.25 mg of protein in 50 μl and eluted with the equilibration buffer at a flow rate of 0.5 ml/min. Catalase (Mr = 240000), aldolase (Mr = 158000), bovine serum albumin (Mr = 68000) and chymotrypsinogen (Mr = 25000) were used as standards.

Protein Determination——Protein concentration was determined by the method of Lowry *et al.*,[18] using bovine serum albumin as the standard.

Electrophoresis——Polyacrylamide disc gel electrophoresis with SDS was performed by the method of Weber and Osborn.[19] The molecular weight of the protease was estimated by using a molecular weight calibration kit from Boehringer Mannheim Co. All gels were stained with Coomassie brilliant blue.

Results

Enzyme Purification

PEP I and PEP II were isolated by almost identical methods. Solid ammonium sulfate was gradually added to about 500 ml of outdated human plasma while the mixture was stirred gently. The solution was brought to 20% saturation and allowed to stand for 1 h at 0 °C. The cloudy suspension was separated by centrifugation. The supernatant was brought to 55% saturation by further addition of solid ammonium sulfate, left to stand at 0 °C for 1 h, and centrifuged. The pellets were dissolved and dialyzed overnight against buffer A. The dialyzed protein was applied to a column of DEAE-Sephadex A-50 (5.0 × 15 cm) pre-equilibrated with

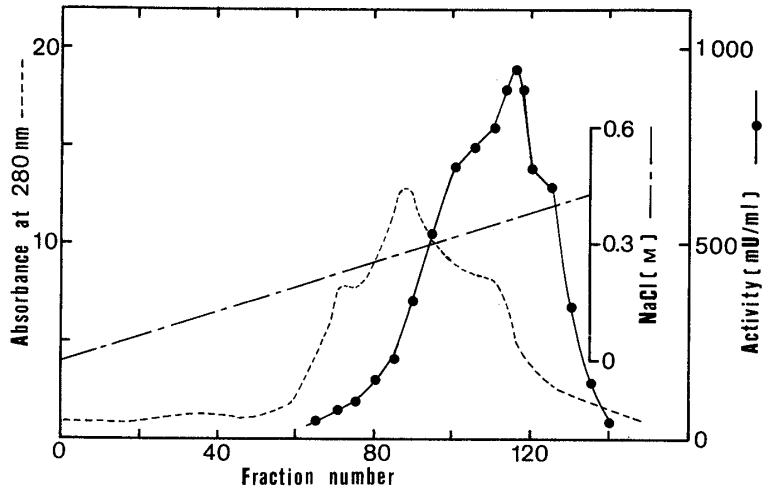

Fig. 1. DEAE-Sephadex A-50 Chromatography of Human Plasma Extract

A column of DEAE-Sephadex A-50 (5 × 15 cm) was loaded with 20—55% $(NH_4)_2SO_4$-fractionated human plasma extract containing 19 g of protein in 200 ml and eluted with one liter of buffer A having a linear gradient of 0 to 600 mM NaCl at a rate of 60 ml/h. Fraction volume, 5 ml. Fractions 93 to 130 were pooled.
------, absorbance at 280 nm; —●—, proline endopeptidase activity.

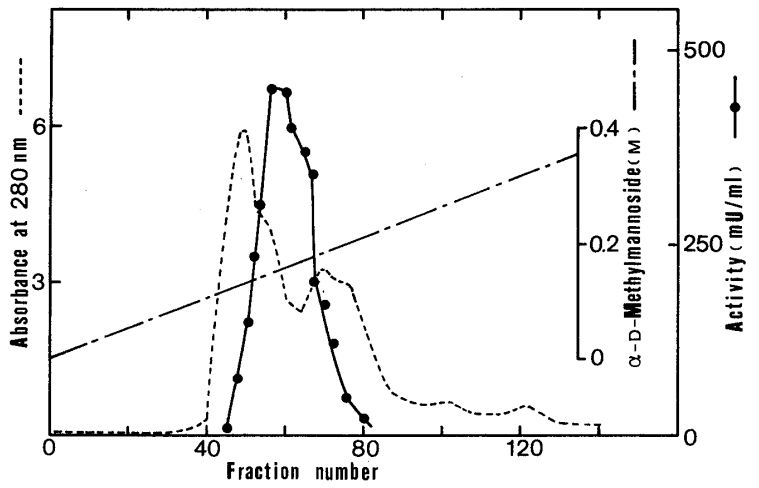

Fig. 2. Con A-Sepharose Chromatography of Partially Purified PEP from Human Plasma

A column of Con A-Sepharose (2.5 × 20 cm) was loaded with the PEP recovered from the DEAE-Sephadex column (Fig. 1, 30 ml, 3.9 g of protein) and eluted with 500 ml of buffer B having a linear gradient of 0 to 400 mM of α-D-methylmannoside at a rate of 40 ml/h. Fraction volume, 3 ml. Fractions 50 to 70 were pooled.
------, absorbance at 280 nm; —●—, PEP activity.

buffer A. The column was washed extensively with the same buffer until the absorbance at 280 nm decreased to a negligible level. No PEP activity was observed in the flow-through fraction. The adsorbed protein was eluted with a linear gradient of NaCl, 0 to 600 mM, in a total volume of one liter. As shown in Fig. 1, PEP activity was eluted at NaCl concentrations ranging from 200 to 450 mM, as a broad peak. The active fractions were collected and concentrated by ultrafiltration on a PM-10 membrane (Amicon). The concentrate (30 ml, 3.9 g of protein) was dialyzed against buffer B and applied to a column of Con A-Sepharose (2.5 × 20 cm) pre-equilibrated with buffer B. During washing of the column with the same buffer, about 60% of the total activity applied to the column was eluted in the flow-through fraction. The unadsorbed PEP (PEP II) was concentrated by ultrafiltration after removing albumin from the solution by using a blue-Sepharose CL-6B column (2.5 × 10 cm) according to the method of Travis *et al.*[20] On the other hand, the adsorbed PEP (PEP I) was eluted with a linear gradient of α-D-methylmannoside, 0 to 400 mM, in a total volume of 500 ml. The PEP I was eluted at α-D-methylmannoside concentrations ranging from 150 to 200 mM, as a single peak (Fig. 2). The two PEPs were completely separated by this Con A-Sepharose chromatography.

The following purification steps were performed separately for each PEP. The concentrate (7.0 ml, 0.5 g of protein for PEP I and 7.0 ml, 1.2 g of protein for PEP II) was applied to a column of Sephacryl S-300 (2.5 × 90 cm), pre-equilibrated with buffer A containing 300 mM NaCl. PEP activity was eluted with the same buffer as a sharp symmetrical peak (Fig. 3, A and B). The active fraction were collected and dialyzed against buffer C. The dialysate (43 ml, 219 mg of protein for PEP I and 20 ml, 138 mg of protein for PEP II) was applied to a column of hydroxyapatite (2.5 × 15 cm), pre-equilibrated with buffer C. The column was washed with the same buffer and then the PEP activity was eluted with a linear gradient of sodium phosphate, 20 to 200 mM, in a total volume of 600 ml (Fig. 3, C and D). The active fractions were collected and dialyzed overnight against buffer D. The dialysate (12 ml, 46.2 mg of protein for PEP I and 8 ml, 28 mg of protein for PEP II) was applied to a column of Z–Gly–Pro–AH–Sepharose (1.5 × 13 cm), pre-equilibrated with buffer D. The column was washed

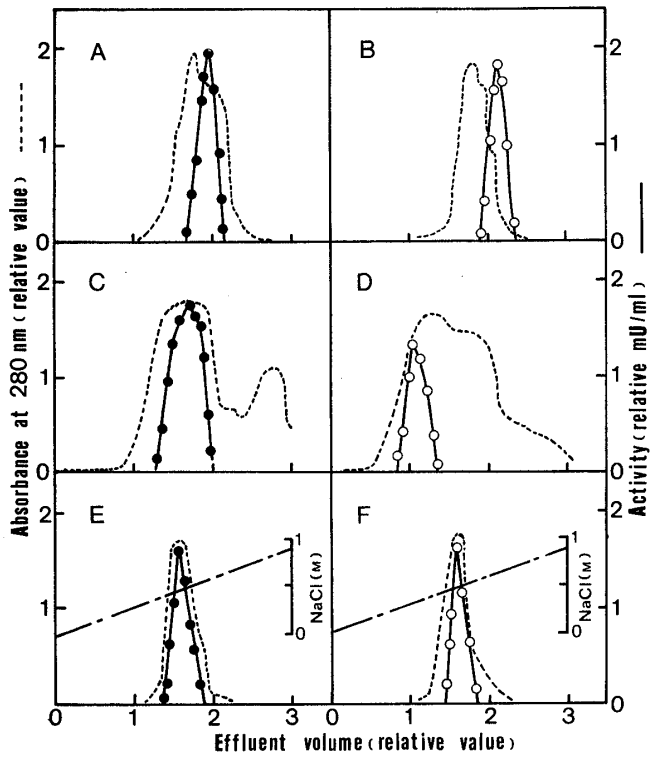

Fig. 3. Comparison of Chromatographic Patterns of PEP I and PEP II from Human Plasma

Abscissae and ordinates are scaled in relative values. To obtain true values, the relative values should be multiplied by the following factors. For absorbance: A, 2.0; B, 2.0; C, 0.3; D, 0.2; E, 0.2; F, 0.1. For enzyme activity: A, 400; B, 400; C, 200; D, 200; E, 400; F, 400. For effluent volume: in A and B, 1 represents 150 ml, 2 300 ml and 3 450 ml; in C and D, a factor of 1.2 should be used; in E and F, a factor of 0.33 should be used. A, C and E are for PEP I and B, D and F are for PEP II. The elution profiles are: A and B, from Sephacryl S-300 (2.5 × 90 cm); C and D, from hydroxyapatite (2.5 × 15 cm); E and F, from Z–Gly–Pro–AH–Sepharose 4B (1.5 × 13 cm). The flow rates (per h) were: 20 ml in A and B; 30 ml in C and D; 40 ml in E and F. The details of the chromatographic procedures are described in Results. ------, absorbance at 280 nm; —●—, PEP I activity; —○—, PEP II activity.

TABLE I. Purification of PEP I and PEP II from Human Plasma

Step and protease		Total activity (mU)	Protein (mg)	Specific activity (mU/mg)	Purity (fold)	Yield (%)
1. Plasma (PEP I and PEP II)		148960	41820	3.8		
2. $(NH_4)_2SO_4$ fractionation (PEP I and PEP II)		131675	19364	6.8		
3. DEAE-Sephadex (PEP I and PEP II)		112632	3920	24.7		
4. Con A-Sepharose	PEP I	30240	540	55.5	1.0	100[a]
	PEP II	27945[b]	1242	22.5	1.0	100[a]
5. Sephacryl S-300	PEP I	30660	219	140	2.5	101
	PEP II	13193	138	95.6	4.2	47
6. Hydroxyapatite	PEP I	25493	46.2	552	9.9	84
	PEP II	7784	28	278	12.4	28
7. Z–Gly–Pro–AH–Sepharose						
	PEP I	12898	3.1	4161	75	43
	PEP II	5403	2.3	2350	104	19

a) Activities after Con A-Sepharose chromatography were taken as 100% for each enzyme.
b) Value represents the enzyme activity after removal of albumin and other proteins on blue-Sepharose CL-6B.

TABLE II. Comparison of Some Properties of PEP I and PEP II from Human Plasma

Properties	PEP I	PEP II
Molecular weight (TSK-Gel G 4000 SW)	130000 daltons	62000 daltons
Affinity for Con A-Sepharose	Yes	No
Temperature stability (for 10 min at 58 °C)		
in the presence of 1 mM DTT	Full activity left	Less than 20% left
in the absence of DTT	More than 90% left	No activity left
K_m value	2.3 mM	0.71 mM
Optimal pH	5.5—6.5	6.0—7.0

The enzyme activity was determined with Suc–Gly–Pro–MCA as described in Materials and Methods.

with the same buffer and eluted with a linear gradient of NaCl, 0.05 to 1 M, in a total volume of 180 ml. PEP activity was eluted as a single peak (Fig. 3, E and F). These results are summarized in Table I.

Characterization of the Purified Enzymes

The molecular nature and physical properties of PEP I and PEP II are compared in Table II. The molecular weights of both species were determined by comparing their elution positions on TSK-gel G 4000 SW with those of molecular weight standard proteins. PEP I was eluted at the position of apparent Mr = 130000 and PEP II at the position of Mr = 62000, each as a sharp symmetrical peak (graphic data not shown). As shown in Fig. 4, the purified PEP I and PEP II appeared to be homogeneous upon SDS-gel electrophoresis, and the molecular weights were estimated to be 140000 and 65000 daltons, respectively. PEP I had a strong affinity for Con A-Sepharose in the presence of 1 M NaCl, but PEP II had no affinity. These results indicate that PEP I is a glycoprotein and is distinct from PEP II.

When both enzymes were incubated in the presence of 1 mM DTT at 58 °C for 10 min,

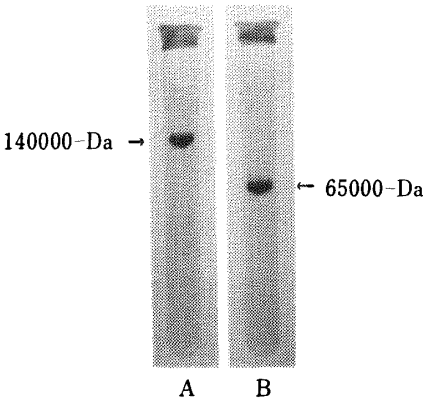

Fig. 4. SDS-Gel Electrophoresis of PEP I and PEP II

A, SDS-gel electrophoresis of PEP I; B, SDS-gel electrophoresis of PEP II.
About 20 μg of enzyme was applied to a 7.5% gel, pH 7.0 and subjected to electrophoresis at 8 mA per tube for 2 h.

TABLE III. Cleavage Points in Various Peptides by the Two Proline Endopeptidases from Human Plasma

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
Z–Gly–Pro↓Leu–Gly[b]	Z–Gly–Pro, Leu–Gly
ACTH (1—24)[b]	Not detectable
Met-enkephalin: Tyr–Gly–Gly–Phe–Met[b]	Not detectable
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 as described in Materials and Methods.
e) Detected by the method of Shotton as described in Materials and Methods.

PEP I activity remained unchanged, while more than 80% of PEP II activity was lost. On the other hand, more than 90% of PEP I activity remained in the absence of DTT, while PEP II activity was completely lost. PEP I had an optimal pH of 5.5 to 6.5 while that of PEP II was 6.0 to 7.0, when determined with Suc–Gly–Pro–MCA as the substrate. By means of Lineweaver–Burk plots, the kinetic parameters of both enzymes toward a PEP-specific substrate, Suc–Gly–Pro–MCA, were determined. The K_m and V_{max} values for PEP I and PEP II were calculated to be 2.3×10^{-3} M, 11.2 nmol/min/mg protein and 7.1×10^{-4} M, 3.6 nmol/min/mg protein, respectively.

As shown in Table III, both enzymes were apparently highly specific for peptides such as angiotensin I, bradykinin, substance P and Z–Gly–Pro–Leu–Gly, cleaving them after the proline residues. However, the enzymes could not hydrolyze ACTH (1—24). In agreement with the substrate specificity of PEP from hog kidney cytosol,[4] both enzymes were capable of

TABLE IV. Effects of Various Inhibitors on the Activities of PEP I and PEP II

Addition		% inhibition of	
		PEP I	PEP II
DFP	0.005 mM	100[a]	100[a]
Elastatinal	50 μg/ml	15[b]	75[b]
PMSF	0.05 mM	0[b]	0[b]
Soybean trypsin inhibitor	250 μg/ml	0[b]	0[b]
Aprotinin	200 μg/ml	0[b]	0[b]
Human plasma	20-fold dilution	0[b]	0[b]
EDTA	5 mM	0[b]	0[b]
1,10-Phenanthroline	0.5 mM	47[a]	40[a]
PCMB	0.01 mM	15[c]	75[c]
Iodoacetamide	0.5 mM	0[c]	0[c]
$HgCl_2$	0.05 mM	20[c]	75[c]
$ZnCl_2$	0.1 mM	15[d]	55[d]
$MgCl_2$	0.1 mM	0[d]	0[d]
$CaCl_2$	0.1 mM	0[d]	0[d]

a) Against control containing 1.0 mM DTT, 1.0 mM EDTA and 0.5% methanol.
b) Against control containing 1.0 mM DTT and 1.0 mM EDTA.
c) Against control containing 10.0 μM DTT, no EDTA.
d) Against control containing 1.0 mM DTT, no EDTA.
The enzyme activity was determined with Suc–Gly–Pro–MCA as described in Materials and Methods. The enzyme (50 mU) was preincubated at 37 °C for 10 min with the inhibitors to be tested before the reaction was initiated by the addition of substrate.

releasing *p*-nitroanilide from Suc–Ala–Ala–Ala–*p*NA or Suc–Ala–Ala–*p*NA, but were incapable of hydrolyzing Suc–Ala–*p*NA, Tyr–Gly–Gly–Phe–Met and Gly–Pro–MCA. Proteins such as bovine serum albumin and Congo-red elastin were not attacked by these enzymes.

Effects of various reagents on the activities of PEP I and PEP II are shown in Table IV. Both enzymes were extremely sensitive to DFP. Addition of only 5 μM DFP resulted in complete loss of the enzymatic activities, indicating the presence of serine residues as essential functional groups in these enzymes. However, PMSF and trypsin inhibitor from soybean or pancreas did not inhibit either enzyme. Human plasma, which probably contains several serine protease inhibitors such as α_1-antitrypsin, α_2-antitrypsin and α_2-macroglobulin, had no inhibitory effect on the enzyme activities. Elastatinal significantly inhibited PEP II, whereas the activity of PEP I was only slightly affected. As reported for PEP isolated from brain,[6,8,21] pituitary[9] and kidney[2,4] tissues, we observed a significant inhibition of PEP II activity by PCMB. However, the reagent was less effective toward PEP I activity. Another sulfhydryl-blocking reagent, iodoacetamide, had no inhibitory effect on either enzyme activity. Among the divalent metal ions tested, Hg^{2+} and Zn^{2+} were more effective toward PEP II, whereas no inhibitory effect on either enzyme activity could be observed with Mg^{2+} or Ca^{2+}. Neither enzyme was inhibited by EDTA. 1,10-Phenanthroline had a significant inhibitory effect on both enzyme activities. Whether or not this inhibition is related to the chelating effect of 1,10-phenanthroline remains to be determined.

Discussion

We have concurrently isolated two molecular species of PEP (PEP I and PEP II) from human blood plasma, using Suc–Gly–Pro–MCA as a PEP-specific substrate. Both enzymes cleaved angiotensin I, bradykinin, substance P and Z–Gly–Pro–Leu–Gly on the carboxyl side

of proline (Table III) according to the defined substrate specificity of PEP.[1,2,7,8,22,23] They also released *p*-nitroaniline from synthetic elastase substrates Suc–Ala–Ala–Ala–*p*NA and Suc–Ala–Ala–*p*NA as previously indicated by us using PEP purified from hog kidney cytosol.[4] The K_m values of PEP I and PEP II toward Suc–Gly–Pro–MCA were 2.3×10^{-3} M and 7.1×10^{-4} M, respectively. These K_m value are 10-fold or about 3-fold higher, respectively than that ($K_m = 2.5 \times 10^{-4}$ M) obtained with PEP purified from hog kidney.[4] The peptidase activities of both enzymes were completely lost after DFP treatment. Proteins such as elastin and albumin were not substrates for the two enzymes. In addition, the enzymes could not hydrolyze Met-enkepharin or Gly–Pro–MCA. Thus, it is reasonable to conclude that the two enzymes are serine-type prolyl endopeptidases. However, the enzymes were distinguishable in apparent molecular weight on gel filtration, affinity for Con A-Sepharose, stability to heating (58 °C, 10 min), optimal pH (Table II) and susceptibility to several inhibitors (Table IV). As reported for PEP from rat[6,21] and bovine[8] brain, bovine anterior pituitary[9] and lamb[2] and hog[4] kidney, PEP II was susceptible not only to DFP but also to a sulfhydryl-blocking reagent, PCMB. Thus, one or more cysteinyl groups probably exists near the active site seryl residue of PEP, and the introduction of a bulky group such as PCMB at the cysteinyl residue may cause steric hindrance in binding of the substrate to the enzyme.[8,9] However, the susceptibility of PEP I to PCMB was less than that of PEP II. The apparent molecular weights of PEP preparations from the soluble fraction of various tissues are around 70000 daltons (76000 for PEP from bovine pituitary,[9] 66000 for PEP from rat brain,[24] 74000 for PEP from lamb kidney[3] and 65000 for PEP from hog kidney[4]), similar to that of PEP II. As regards the molecular weight of PEP I, such a large molecular value as 130000 daltons has not been reported except in a paper by Hersh[25] giving the molecular weight of PEP in bovine brain extract as 110000 daltons. Further, PEP I was clearly separated from PEP II on a Con A-Sepharose column in the presence of 1 M NaCl (Fig. 2 and Table II). Recently, we have purified PEP from hog kidney cytosol by similar purification procedures including Con A-Sepharose chromatography. The hog kidney PEP had no affinity for Con A-Sepharose.[4] These results indicate that PEP II more closely resembles PEPs previously purified from various tissues[1–9] in its enzymatic properties and physicochemical properties.

As regards the difference in molecular nature of PEP I and PEP II, the following questions must be considered. (1) Are the enzymes which we have isolated from human plasma really distinct enzymes? (2) Is there a possibility that PEP may be a complexed form of PEP II with a protein from plasma? As regards the first question, it remains to be determined whether or not the two enzymes are immunochemically distinguishable. However, we isolated PEP I and PEP II from the same source by separate but almost identical procedures, and PEP activity was eluted as a single symmetrical peak in each chromatographic step as shown in Fig. 3. Further, upon reduced or unreduced SDS-polyacrylamide gel electrophoresis, PEP I and PEP II were clearly distinguishable and gave a main protein band corresponding to 140000 or 65000 daltons, respectively. In addition, both enzymes fully active forms and their activities were not affected by the addition of plasma, which contains many protease inhibitors (Table IV). Therefore, it seems unlikely that PEP I is a precursor form or a complexed form of PEP II.

In this study, we also found that the two PEPs from human plasma are able to hydrolyze Suc–Ala–Ala–Ala–*p*NA. Recently, an EDTA-resistant elastase-like enzyme with Mr = 200000 which is able to hydrolyze a synthetic elastase substrate, Suc–Ala–Ala–Ala–*p*NA, but unable to hydrolyze elastin has been reported to show increased activity in the sera of patients suffering severe hepatic disorders,[26] but its enzymatic properties are not yet known in detail. Further work is necessary on the properties and subcellular localization of PEP in liver, because most plasma proteins are mainly synthesized in the liver and released into the circulation.

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