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A Succinyl-trialanine *p*-Nitroanilide Hydrolase in Hog Kidney Cytosol: Its Identification as Proline Endopeptidase[1)]

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A succinyl-trialanine *p*-nitroanilide [Suc–$(Ala)_3$–*p*NA] hydrolase which is able to hydrolyze an artificial elastase substrate, Suc–$(Ala)_3$–*p*NA, but unable to hydrolyze a naturally occurring substrate, elastin, was highly purified from hog kidney cytosol. The apparent molecular weight of the enzyme was estimated to be 65000 by gel filtration on Sephadex G-150 and 68000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the isoelectric point of the enzyme was 5.0. The enzyme is an endopeptidase which catalyzes the hydrolysis of peptides with the general structure Y-Ala (or Pro)-X (Y = peptide or N-protected amino acid; X = amino acid moiety, peptide or amide) at the carboxyl side of alanine and proline residues. The enzyme was markedly inhibited by diisopropyl fluorophosphate and *p*-chloromercuribenzoate. Ethylenediaminetetraacetate and 1,10-phenanthroline, however, were not inhibitors of the enzyme. The enzyme activity was retained on an affinity column having a proline endopeptidase [EC 3.4.21.26] inhibitor, Z–Gly–Pro, as ligand and could be eluted at 0.125 M NaCl (mean value). Suc–$(Ala)_3$–*p*NA-hydrolytic activity coincided with the peak of proline endopeptidase activity as determined with a sensitive fluorogenic substrate, succinylglycyl-L-proline 4-methylcoumaryl-7-amide (Suc–Gly–Pro–MCA). The optimum pH and k_{cat}/K_m values ($mM^{-1} \cdot s^{-1}$) were pH 7.5 and 5.4 for Suc–$(Ala)_3$–*p*NA and pH 6.8 and 24.8 for Suc–Gly–Pro–MCA, and the enzyme activity was competitively inhibited by Z–Ala–Ala and Z–Gly–Pro, as is the case with proline endopeptidase. These results suggest that Suc–$(Ala)_3$–*p*NA hydrolase in hog kidney cytosol may be identical with proline endopeptidase which was first found in human uterus as an oxytocin-degrading enzyme.

Keywords——succinyl-trialanine *p*-nitroanilide hydrolase; proline endopeptidase; hog kidney cytosol; substrate specificity; inhibition; affinity chromatography

An EDTA-resistant elastase-like enzyme which is able to hydrolyze an artificial elastase substrate, succinyl-trialanine *p*-nitroanilide [Suc–$(Ala)_3$–*p*NA], but unable to hydrolyze elastin, has been found to increase in the sera of patients suffering from severe hepatic disorders.[2)] A similar Suc–$(Ala)_3$–*p*NA hydrolase has been partly purified from human uterus.[3)] The real substrate and physiological roles, however, have not been clarified at all, because such Suc–$(Ala)_3$–*p*NA hydrolase in mammalian tissues has not been purified sufficiently to permit thorough characterization.

We have now highly purified Suc–$(Ala)_3$–*p*NA hydrolase from hog kidney cytosol, and have found that this enzyme may be identical with proline endopeptidase [EC 3.4.21.26].

Experimental

Materials——The following materials were commercially obtained. Bradykinin, angiotensin I, angiotensin II, Z–Gly–Pro, Z–Gly–Pro–Leu, Suc–$(Ala)_3$–*p*NA, Suc–$(Ala)_2$–*p*NA, Suc–Ala–*p*NA, Suc–Ala–Pro–Ala–*p*NA, succinylglycyl-L-proline 4-methylcoumaryl-7-amide (Suc–Gly–Pro–MCA), elastatinal, leupeptin, chymostatin and antipain from the Protein Research Foundation, Minoh, Japan; diisopropyl fluorophosphate (DFP), phenylmethanesulfonyl fluoride (PMSF), sodium *p*-chloromercuribenzoate (PCMB), Ala–*p*NA, Congo red-elastin, dithiothreithol (DTT) and soybean trypsin inhibitor from Sigma, St. Louis, Mo., U.S.A.; insulin B-chain from Serva, Heidelberg, West Germany; ACTH (1—24 analogue) from Vega, Tucson, Az., U.S.A.; Sephadex G-150, Con A-Sepharose,

DEAE-Sephadex A-150, AH-Sepharose 4B and Pharmalyte from Pharmacia, Uppsala, Sweden; hydroxyapatite from Seikagaku Kogyo, Tokyo, Japan. Other reagents used were of analytical grade. Z–Ala–Ala was synthesized in our laboratory and Z–Gly–Pro–AH–Sepharose was prepared by the method of Cuatrecasas.[4]

Enzyme Assays and Protein Concentrations——Suc–$(Ala)_3$–*p*NA–hydrolytic activity was measured by a modification of the method of Bieth *et al.*[5] The standard assay mixture was 0.2 M Tris-HCl (pH 7.5)/1 mM DTT/1 mM EDTA/2 mM substrate, to which enzyme solution was added to give a final volume of 250 μl. After incubation of the mixture at 37 °C for 15 min, 100 μl of 50% acetic acid was added to stop the reaction. The absorbance of the mixture was measured at 410 nm on a micro-flow spectrophotometer (Shimadzu UV-730). Suc–Gly–Pro–MCA–hydrolytic activity was measured according to the method of Kato *et al.*[6] One unit of the enzyme activity was defined as the amount of activity which released 1 nmol of *p*-nitroaniline or 1 nmol of 7-amino-4-methylcoumarin per min at 37 °C. Elastinolytic activity was detected by the method of Shotton using Congo red-elastin as a substrate.[7] The protein concentrations of column fractions were measured in terms of the absorbance at 280 nm and the protein concentrations used for the calculation of specific activities were measured by the Lowry method[8] using bovine serum albumin as a standard.

Molecular Weight Determination——The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-150 column (2 × 110 cm) according to the method of Andrews[9] and by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis as described by Weber and Osborn.[10] Catalase (Mr = 240000), aldolase (Mr = 158000), bovine serum albumin (Mr = 68000) and chymotrypsinogen (Mr = 25000) were used as standards.

Disc Gel Electrophoresis and Isoelectric Focusing——The purity of the enzyme preparation was examined by disc gel electrophoresis based on the method of Williams and Reisfeld[11] at pH 7.5. After gel electrophoresis, one of the gels was stained with Coomassie brilliant blue R-250. The other gels were used to evaluate the location of enzymatic activity by the standard assay method after being cut into 2 mm slices. To determine the isoelectric point, the enzyme was applied to an isoelectric focusing polyacrylamide gel (5%) according to the manufacturer's specifications using Pharmalyte as carrier ampholyte in the pH range of 3.0 to 10.0.

Identification of Cleavage Points in Peptides by the Enzyme——Cleavage points in naturally occurring peptides were identified by high performance liquid chromatography (HPLC) followed by amino acid analysis as described by Inokuchi and Nagamatsu.[12] In the case of synthetic peptide derivatives, cleavage points were identified by thin layer chromatography (TLC) on silica gel plates. The solvent was a mixture of *n*-butanol–acetic acid–water (4 : 1 : 2, v/v). The following reaction mixture components, in a final volume of 250 μl, were incubated for 2 h at 37 °C: 50 mM Tris-HCl (pH 7.5)/5 mM each substrate/1 mM EDTA/1 mM DTT/100 units of enzyme. After incubation, the reaction was stopped by heating in a boiling water bath for 5 min and 100 μl aliquots of the reaction mixtures were subjected to HPLC analysis, or 20 μl aliquots were subjected to TLC analysis.

pH Dependence of Enzyme Activity——The dependence of enzyme activity on pH was estimated using the following buffers: 100 mM sodium phosphate, pH 6.0—7.5, and 100 mM Tris-HCl, pH 7.5—9.0. The following assay components, in a final volume of 250 μl, were incubated for 15 min at 37 °C: 100 mM buffer/1 mM EDTA/1 mM DTT/2 mM substrate/10 units of enzyme.

Kinetic Studies——K_m and k_{cat} values of the enzyme for three substrates (given in Table IV) were determined from Lineweaver–Burk plots with substrate concentrations in the range of 0.1 to 3.0 mM. Initial velocities were determined from the amount of *p*-nitroaniline or 7-amino-4-methylcoumarin released after 2, 5 and 10 min of incubation. In the calculation of k_{cat}, the molecular weight of the enzyme was taken as 68000. When the inhibitory action of Z–Ala–Ala and Z–Gly–Pro was observed, four different concentrations of the inhibitors were assayed, and the assay mixtures were preincubated for 10 min before adding the substrate. Lineweaver–Burk plots were prepared to investigate the mechanism of inhibition. Inhibition constants (K_i) of Z–Ala–Ala and Z–Gly–Pro were calculated from Dixon plots[13] at the intersection of four lines obtained at different concentrations of inhibitors.

Purification of Suc–$(Ala)_3$–*p*NA Hydrolase from Hog Kidney——All steps were carried out at 4 °C and ultrafiltration was performed with a PM-10 membrane (Amicon). For purification studies, all buffers contained 1 mM EDTA and 1 mM DTT. Frozen kidneys having a total weight of 560 g were thawed by repeated washing with cold saline, sliced into pieces, freed from vascular and membranous tissue, and weighed. Tissue was homogenized in five volumes (w/v) of 10 mM Tris-HCl (pH 8.0)/0.25 M sucrose, twice for 30 s each, in a Polytron homogenizer. The supernatant centrifuged at 105000 × *g* for 90 min was subjected to fractionation with solid ammonium sulfate. The fraction precipitating between 35 and 85% saturation was suspended in 200 ml of 50 mM sodium phosphate buffer (pH 6.8) and dialyzed overnight against the same buffer. After removal of insoluble material by centrifugation, the dialysate was concentrated by ultrafiltration and applied to a DEAE-Sephadex A-50 column (5.0 × 35 cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.8). The column was washed with the equilibrating buffer and eluted by means of a concentration gradient of NaCl (0 to 0.4 M) in 800 ml of the same buffer at a flow rate of 30 ml/h. The active fractions were combined, and concentrated by ultrafiltration. One-fourth of this concentrate was applied to a hydroxyapatite column (2.5 × 25 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The column was washed with the equilibrating buffer, and eluted with a linear gradient formed from 400 ml of the same buffer and 400 ml of 0.2 M sodium phosphate buffer (pH 6.8) at a flow rate of 35 ml/h. This chromatographic step was repeated four times. The active fractions were combined and concentrated by ultrafiltration, then dialyzed overnight against

50 mM Tris-HCl buffer (pH 7.5) containing 1 M NaCl. The dialysate was applied to a Con A-Sepharose column (1.0 × 10 cm) equilibrated and washed with the above buffer at a flow rate of 35 ml/h. Suc–$(Ala)_3$–*p*NA–hydrolytic activity passed through the column, while the adsorbed proteins, which were eluted with the same buffer containing 0.5 M α-methyl-D-mannoside and 0.5 M α-methyl-D-glucoside, had no activity. The active fractions were combined, concentrated by ultrafiltration and dialyzed overnight against 50 mM sodium phosphate buffer (pH 6.8) containing 0.25 M NaCl. The dialysate was subjected to gel filtration on a Sephadex G-150 column (2.5 × 90 cm) equilibrated with the above buffer, and the column was eluted with the same buffer at a flow rate of 15 ml/h. The active fractions were pooled and concentrated by ultrafiltration. This enzyme preparation was stored in a freezer at −60 °C until use.

Results

Purification and Physicochemical Properties of Suc–$(Ala)_3$–*p*NA Hydrolase

Table I summarizes the purification of Suc–$(Ala)_3$–*p*NA hydrolase from hog kidney cytosol. Figure 1 shows the gel filtration pattern of the enzyme on Sephadex G-150 after purification by Con A-Sepharose chromatography. The enzyme was purified 1036-fold with 11% recovery. The final enzyme preparation gave a single protein band upon disc gel electrophoresis. Analytical isoelectric focusing in polyacrylamide gel gave a pI value of 5.0. The apparent molecular weight of the enzyme was estimated to be 65000 by gel filtration on Sephadex G-150, and 68000 by SDS-gel electrophoresis, suggesting that the enzyme exists as a monomer.

Effects of Reagents and Proteinase Inhibitors on Suc–$(Ala)_3$–*p*NA Hydrolase

As summarized in Table II, Suc–$(Ala)_3$–*p*NA hydrolase was not inhibited by EDTA, 1,10-phenanthroline, soybean trypsin inhibitor, leupeptin, chymostatin or antipain. Among serine–proteinase inhibitors, DFP strongly inhibited the enzyme at a concentration of 0.01 mM, while the enzyme was only partially inhibited by 0.2 mM PMSF. Furthermore, elastatinal (an elastase inhibitor) and PCMB (a sulfhydryl-modifying agent) were found to be potent inhibitors. On the other hand, the enzyme was significantly inactivated by dialysis against 0.2 M Tris-HCl buffer (pH 7.5) in the absence of DTT, and the activity was fully

TABLE I. Summary of the Purification of Suc–$(Ala)_3$–*p*NA Hydrolase from Hog Kidney

	Purification step	Total protein (mg)	Total units	Specific activity (units/mg)	Yield (%)	Purity (fold)
1.	105000 × *g* supernatant	20200	88000	4.4	100	1.0
2.	$(NH_4)_2SO_4$ fractionation	5448	45763	8.4	52	1.9
3.	DEAE-Sephadex A-50	791	36386	46	41	10
4.	Hydroxyapatite	50	14250	285	16	65
5.	Con A-Sepharose	12.5	12400	992	14	225
6.	Sephadex G-150	2.1	9576	4560	11	1036

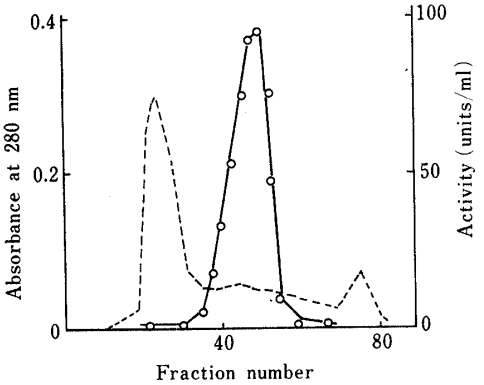

Fig. 1. Chromatography of Suc–$(Ala)_3$–*p*NA Hydrolase on a Sephadex G-150 Column

Fractions of 7.0 ml each were collected. The other procedures are described in Experimental. ----, absorbance at 280 nm; ○—○, Suc–$(Ala)_3$–*p*NA-hydrolytic activity.

Table II. Effects of Reagents and Proteinase Inhibitors on Suc–$(Ala)_3$–*p*NA Hydrolase[a]

Agent	Conc. (mM)	Relative activity (%)
None	—	100
EDTA	5	100
1,10-Phenanthroline	1	90
Soybean trypsin inhibitor	1 (mg/ml)	92
DFP	0.01	0
PMSF	0.2	65
PCMB	0.2	0
Elastatinal	0.08	10
Leupeptin	0.5	100
Chymostatin	0.05	100
Antipain	0.1	100

a) A mixture of purified enzyme and an agent in 0.2 M Tris-HCl (pH 7.5)/1 mM DTT was incubated at 37 °C for 15 min and then the remaining activity was determined as described in the text.

Table III. Cleavage Points in Peptides by Suc–$(Ala)_3$–*p*NA Hydrolase

Substrate and cleavage points[a]	
Ala–*p*NA[b]	(No hydrolysis)
Suc–Ala–*p*NA[b]	(No hydrolysis)
Suc–Ala–Ala↓–*p*NA[b]	
Suc–Ala–Ala–Ala↓–*p*NA[b]	
Suc–Ala–Pro↓–Ala↓–*p*NA[b]	
Arg–Pro–Pro↓–Gly–Phe–Ser–Pro↓–Phe–Arg (Bradykinin)[c]	
Asp–Arg–Val–Tyr–Ile–His–Pro↓–Phe–His–Leu (Angiotensin I)[c]	
Asp–Arg–Val–Tyr–Ile–His–Pro↓–Phe (Angiotensin II)[c]	
Z–Gly–Pro↓–Leu[b]	
Suc–Gly–Pro↓–MCA[b]	
Insulin B-chain[c]	(No hydrolysis)
ACTH (1—24 analogue)[c]	(No hydrolysis)
Congo red-elastin[d]	(No hydrolysis)

a) Cleavage points are indicated by arrows.
b) Identified by TLC as described in the test.
c) Identified by HPLC followed by amino acid analysis as described in the test.
d) Detected by the method of Shotton as described in the test.

recovered by addition of 1 mM DTT (data not shown).

Substrate Specificity

As shown in Table III, the enzyme was inactive toward Congo red-elastin, Ala–*p*NA and Suc–Ala–*p*NA. However, Suc–$(Ala)_2$–*p*NA and Suc–$(Ala)_3$–*p*NA were hydrolyzed with release of *p*-nitroaniline. In addition, the enzyme catalyzed hydrolysis of prolyl linkages of bradykinin, angiotensin I, angiotensin II and synthetic substrates for proline endopeptidase such as Z–Gly–Pro–Leu and Suc–Gly–Pro–MCA. On the other hand, the enzyme was unable to digest insulin B-chain or ACTH (1—24 analogue) in spite of the presence of the prolyl

linkage.

Affinity Chromatography on Z–Gly–Pro–AH–Sepharose Column

The observed substrate specificity of the enzyme suggests that Suc–$(Ala)_3$–*p*NA hydrolase in hog kidney cytosol may be identical with proline endopeptidase. To confirm this, affinity chromatography was examined using a proline endopeptidase inhibitor, Z–Gly–Pro,[14] as the ligand. Figure 2 shows the elution pattern obtained with a gradient from 0 to 0.25 M NaCl. Suc–$(Ala)_3$–*p*NA–hydrolytic activity coincided with peak of Suc–Gly–Pro–MCA–hydrolytic activity. Furthermore, the enzyme recovered from the affinity column gave a single protein band upon disc gel electrophoresis, and both hydrolytic activities were detected only in the region of the single band, as shown in Fig. 3.

pH Optimum

The activity of the enzyme was examined in phosphate and Tris-HCl buffers covering a pH range of 6.0 to 9.0. For Suc–$(Ala)_3$–*p*NA the pH optimum was at 7.5 and for Suc–Gly–Pro–MCA at 6.8. The optimum pH for Suc–$(Ala)_3$–*p*NA was similar to that of the human uterus hydrolase,[3] but the optimum pH for Suc–Gly–Pro–MCA was somewhat higher than that of proline endopeptidase in rat brain.[7]

Kinetic Studies

As shown in Table IV, K_m and k_{cat}/K_m values for Suc–$(Ala)_2$–*p*NA were caclulated to be 2.0 (mM) and 0.15 ($mM^{-1} \cdot s^{-1}$), respectively. When the peptide chain length was elongated to Suc–$(Ala)_3$–*p*NA, the K_m value decreased to half and the k_{cat}/K_m value increased thirty-six-fold. On the other hand, the enzyme showed four-fold lower K_m value and about five-fold higher k_{cat}/K_m value for Suc–Gly–Pro–MCA than for Suc–$(Ala)_3$–*p*NA, respectively. The addition of Z–Ala–Ala or Z–Gly–Pro to both assay systems caused an increase in the apparent K_m, while the maximum velocity remained unchanged (data not shown). These data indicate that Z–Ala–Ala and Z–Gly–Pro act as competitive inhibitors of the enzyme reaction.

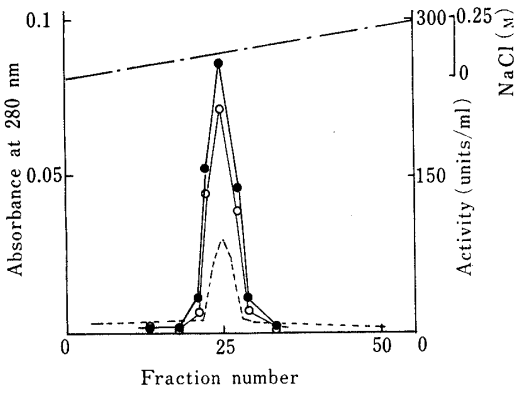

Fig. 2. Affinity Chromatography of Purified Suc–$(Ala)_3$–*p*NA Hydrolase on a Z–Gly–Pro–AH–Sepharose Column

The purified enzyme preparation (1.0 mg protein) was applied to a Z–Gly–Pro–AH–Sepharose column (1.0 × 10 cm) equilibrated previously with 50 mM sodium phosphate (pH 6.8)/1 mM EDTA/1 mM DTT. The column was first washed with the same buffer and then eluted with a linear gradient of NaCl (0—0.25 M) in 180 ml of the buffer. Fractions of 3.5 ml each were collected at a flow rate of 25 ml/h.

----, absorbance at 280 nm; — · —, NaCl concentration; ○—○, Suc–$(Ala)_3$–*p*NA–hydrolytic activity; ●—●, Suc–Gly–Pro–MCA–hydrolytic activity.

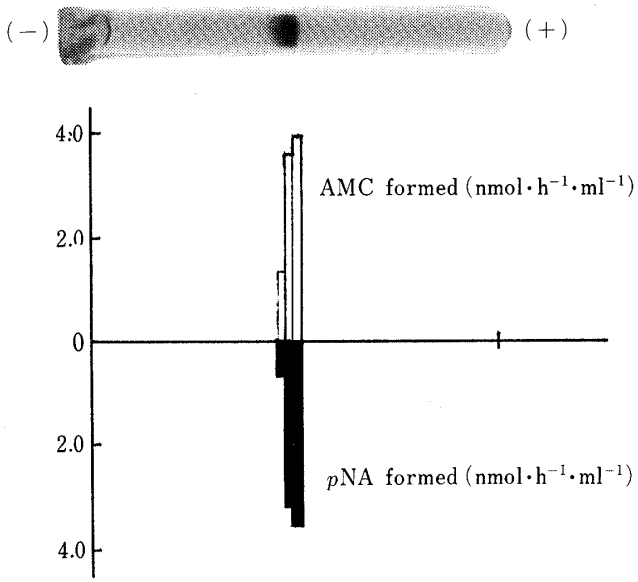

Fig. 3. Disc Gel Electrophoresis of Suc–$(Ala)_3$–*p*NA Hydrolase Recovered from Affinity Column

Suc–$(Ala)_3$–*p*NA–hydrolytic and Suc–Gly–Pro–MCA-hydrolytic activities were determined with standard assay mixtures after extraction from 2 mm gel slices. *p*NA, *p*-nitroaniline; AMC, 7-amino-4-methylcoumarin.

TABLE IV. Kinetic Parameters of Suc–$(Ala)_3$–*p*NA Hydrolase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Suc–$(Ala)_2$–*p*NA[a]	2.0	0.3	0.15
Suc–$(Ala)_3$–*p*NA[a]	1.0	5.4	5.4
Suc–Gly–Pro–MCA[b]	0.25	6.2	24.8

The assay mixture was composed of 100 mM Tris-HCl (pH 7.5)[a] or 100 mM sodium phosphate (pH 6.8)[b], 1 mM EDTA, 1 mM DTT, 0.1—0.3 mM substrate and the enzyme (10 units).

TABLE V. Inhibition of Suc–$(Ala)_3$–*p*NA Hydrolase by Z–Ala–Ala and Z–Gly–Pro

Peptide	(mM)	Substrate	K_i (mM)
Z–Ala–Ala	(0.25—2.0)	Suc–$(Ala)_3$–*p*NA[a]	2.0
		Suc–Gly–Pro–MCA[b]	3.5
Z–Gly–Pro	(0.25—2.0)	Suc–$(Ala)_3$–*p*NA[a]	0.15
		Suc–Gly–Pro–MCA[b]	0.3

The assay mixture was composed of 100 mM Tris-HCl (pH 7.5)[a] or 100 mM sodium phosphate (pH 6.8)[b], 1 mM EDTA, 1 mM DTT, 0.25—1.0 mM substrate and the enzyme (10 units).

The K_i values of both inhibitors calculated by the method of Dixon[13] are shown in Table V. Z–Gly–Pro was a more potent inhibitor than Z–Ala–Ala.

Discussion

In the present work, an elastase-like enzyme Suc–$(Ala)_3$–*p*NA hydrolase, was purified for the first time from hog kidney cytosol. The most important finding was that the enzyme may be identical with proline endopeptidase, which was first found in human uterus,[15] and was subsequently purified from lamb kidney,[16] rat brain,[6,17] rabbit brain[18] and bovine pituitary.[19]

The enzyme obtained from hog kidney cytosol was EDTA-resistant and was markedly inhibited by DFP, indicating that the enzyme can be classified as a serine–proteinase. In addition, the inhibitory effect of PCMB and the activation by DTT established that the enzyme contains an essential sulfhydryl group. Ishida *et al.*[20] reported that the hydrolysis of Suc–$(Ala)_3$–*p*NA in human kidney was performed by joint action of an endopeptidase and an aminopeptidase, but we have confirmed that Suc–$(Ala)_3$–*p*NA hydrolase in hog kidney cytosol directly cleaves the substrate with release of *p*-nitroaniline. With molecular weight and pH optimum of the enzyme, our observations agree with those of Ito *et al.*[3] on Suc–$(Ala)_3$–*p*NA hydrolase in human uterus, except that the enzyme in hog kidney cytosol was inhibited by elastatinal but the hydrolase in human uterus was not.

On the other hand, the study on the substrate specificity of the enzyme in hog kidney cytosol indicates that the enzyme is able to hydrolyze not only alanyl linkages but also prolyl linkages. Yoshimoto *et al.*[14] have noted that proline endopeptidase isolated from lamb kidney catalyzes the hydrolysis of prolyl and alanyl linkages. Furthermore, cleavage points in peptides such as bradykinin and angiotensin II by the enzyme obtained here correspond to the results with proline endopeptidases.[16,18,19] Therefore, it is suggested that Suc–$(Ala)_3$–*p*NA hydrolase purified from hog kidney cytosol may be identical with proline endopeptidase. To test this possibility, affinity chromatography was attempted using a proline endopeptidase inhibitor, Z–Gly–Pro, as the ligand. Suc–$(Ala)_3$–*p*NA–hydrolytic activity was retained on the

affinity column and the peak of activity toward Suc–$(Ala)_3$–*p*NA was identical with that toward Suc–Gly–Pro–MCA. Furthermore, both activities migrated to the same position on polyacrylamide gel electrophoresis, indicating that Suc–$(Ala)_3$–*p*NA hydrolase and proline endopeptidase were the same or very similar proteins. The molecular weight of the enzyme (68000) approximates to that of Suc–$(Ala_3$–*p*NA hydrolase in human uterus (71000),[3] and that of proline endopeptidase in rat brain (70000)[6,17] and in rabbit brain (66000).[18] As indicated in Table II, the enzyme was markedly inhibited by DFP and PCMB but was hardly affected by metal chelators. These results are similar to those obtained with proline endopeptidases,[6,18,19,21] except for the enzyme isolated from lamb kidney,[16] which is strongly inhibited by DFP but partially inhibited by PCMB only at high concentration.[22]

The enzyme in hog kidney cytosol has higher K_m values and lower k_{cat}/K_m values for Suc–$(Ala)_2$–*p*NA and Suc–$(Ala)_3$–*p*NA as compared with those for Suc–Gly–Pro–MCA. These kinetic parameters indicate that the primary specificity of the enzyme may be hydrolysis of the prolyl linkage rather than the alanyl linkage. Z–Ala–Ala and Z–Gly–Pro act as competitive inhibitors of the enzyme reaction and the latter compound is more potent than the former. These kinetic parameters for inhibition of the enzyme agree well with those for proline endopeptidase in lamb kidney, reported by Yoshimoto *et al.*[14] Their studies of the lamb kidney enzyme demonstrated that the K_i values of Z–Ala–Ala and Z–Gly–Pro are 1.5 mM and 0.22 mM using Z–Gly–Pro–ON*p* (ON*p* : *p*-nitrophenol) as a substrate, or 4.3 mM and 0.53 mM using Z–Gly–Pro–Leu–Gly as a substrate, respectively.

In conclusion, the present study indicates that the EDTA-resistant Suc–$(Ala)_3$–*p*NA hydrolase in hog kidney cytosol may be identical with proline endopeptidase, and that its real function may be the hydrolysis of peptides with the general structure Y–Pro–X (Y = peptide or N-protected amino acid; X = amino acid moiety, peptide or amide) at the carboxyl side of proline residue. Furthermore, it is suggested that Suc–$(Ala)_3$–*p*NA hydrolase in human uterus[3] may also be identical with proline endopeptidase, although the substrate specificity has not been studied in detail.

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