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An Inhibitor of Proline Endopeptidase: Purification from Rat Brain and Characterization

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A proline endopeptidase inhibitor was purified from rat brain cytosol by ion exchange chromatography on SP-Sephadex C-25 and repeated gel filtrations on Sephadex G-50. The purified inhibitor appeared to be homogeneous on polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate and displayed no multiple forms. The inhibitor showed $M_r = 7000$ by gel filtration and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and contained 61.6% polar amino acid residues, 3.3% aromatic amino acid residues and no tryptophan. The inhibitor was highly specific for proline endopeptidase from rat brain and hog kidney, and inhibited the enzyme competitively. It did not act on proline-specific exopeptidase such as dipeptidyl aminopeptidase IV, or on usual endopeptidases such as trypsin, elastase and plasmin, or thiol proteases such as papain and ficin.

Keywords——proline endopeptidase; inhibitor; rat brain cytosol; purification

Since proline endopeptidase (EC 3.4.21.26) catalyzes the hydrolysis of several biologically active peptides, *i.e.* oxytocin, vasopressin, thyroliberin, substance P, neurotensin, angiotensin I and angiotensin II,[1-4] the enzyme has been deduced to play an important role in the regulation of the biological activity of these peptides. However, there is no conclusive evidence that the enzyme has such a role.

In order to investigate the reaction mechanism and the biological role of proline endopeptidase, the finding and utilization of naturally occurring inhibitors would be very useful. Thus, we attempted to purify the inhibitor from rat brain. Previously, Yoshimoto *et al.*[5] had attempted to screen the inhibitor in mammalian tissues, and found that the inhibitor is widely distributed in several mammalian tissues. They partially purified the inhibitor from hog pancreas.

The present paper deals with the purification and characterization of the inhibitor from rat brain cytosol.

Experimental

Materials——The following materials were obtained commercially: succinylglycyl-L-proline 4-methylcoumaryl-7-amide (Suc-Gly–Pro–MCA), Gly–Pro–MCA, benzoyl-L-arginine MCA (Bz-Arg–MCA), Leu–MCA and succinyltrialanine *p*-nitroanilide [Suc-$(Ala)_3$–*p*NA] (Protein Research Foundation, Minoh, Japan); D-Val–Leu–Lys–*p*NA and human plasminogen (AB Kabi, Stockholm, Sweden); 2-mercaptoethanol, hog pancreatic elastase Type III, bovine pancreatic tyrpsin Type XII and papain Type IV (Sigma, St. Louis, Mo., U.S.A.); ficin and leucine aminopeptidase (Boehringer Mannheim Yamanouchi, Tokyo, Japan); SP-Sephadex C-25 and Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). Plasmin was prepared from human plasminogen according to the method of Wiman and Wallen.[6] Dipeptidyl aminopeptidase IV was prepared from hog kidney as described by Kenny *et al.*[7] Rat brain proline endopeptidase was purified according to the method of Kato *et al.*[2] and hog kidney proline endopeptidase was purified as previously described by us.[4] Female Wistar rats were obtained from the Animal Center

of Fukuoka University.

Enzyme Assay——Hydrolysis of peptide- or amino acid-MCA was assayed as follows: 50 μl of 0.2 mM substrate solution and 50 μl of enzyme solution were added to 150 μl of 0.1 M phosphate buffer (pH 6.8 for proline endopeptidase, pH 7.8 for dipeptidyl aminopeptidase IV, trypsin and leucine aminopeptidase, and pH 6.0 for papain and ficin) at 37 °C. The fluorescence of 7-amino-4-methylcoumarin released was monitored by using a Hitachi 650-10S fluorophotometer thermostatically controlled at 37 °C with excitation at 370 nm and emission at 440 nm. Suc-Gly–Pro–MCA was used as the substrate for the assay of proline endopeptidase. Gly–Pro–MCA and Leu–MCA were used as the substrates for dipeptidyl aminopeptidase IV and leucine aminopeptidase, respectively. Bz-Arg–MCA was used as the substrate for assays of trypsin, papain and ficin. Suc-$(Ala)_3$–*p*NA-hydrolytic activity of elastase was measured by the method of Bieth *et al.*[8] The activity of plasmin-catalyzed hydrolysis of D-Val–Leu–Lys–*p*NA was determined as described by Teger-Nilson *et al.*[9]

Inhibitor Activity Assay——Proline endopeptidase (0.3 unit) was incubated with 50 μl of inhibitor solution and 100 μl of phosphate buffer at 37 °C for 5 min, and the remaining activity of the enzyme was assayed by the standard activity assay method. One unit was defined as the amount of inhibitor which reduces the activity of 1.0 unit of enzyme by 50% under the above conditions.

Estimation of Molecular Weight——The apparent molecular weight of the inhibitor was estimated by gel filtration on a Sephadex G-50 column (1.9 × 90 cm) according to the method of Andrews[10] and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described by Weber and Osborn.[11] Aprotinin (M_r = 6500), cytochrome c (M_r = 12500) and soybean trypsin inhibitor (M_r = 21500) were used as reference proteins.

Determination of Protein Concentration——Protein concentration was determined by the method of Lowry *et al.*[12] with bovine serum albumin as a standard.

Gel Electrophoresis——The purity of the inhibitor preparation was examined by disc gel electrophoresis based on the method of Williams and Reisfeld[13] at pH 7.6. After gel electrophoresis, one of the gels was stained overnight with Coomassie brilliant blue R-250 solution containing 25% isopropyl alcohol and 10% acetic acid.[14] The other gels were used to evaluate the location of inhibitory activity by the standard assay method after being cut into 2 mm slices. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Weber and Osborn.[11]

Chemical Analysis of Amino Acids and Carbohydrate——The inhibitor was hydrolyzed in 4 M methanesulfonic acid containing 0.2% tryptamine at 110 °C for 24 h and 72 h in order to estimate tryptophan as well as other amino acids.[15] Amino acids were analyzed in a Hitachi 638-30 high-performance liquid chromatograph (HPLC). The N-terminal amino acid residue was determined as the dansylated derivative by two-dimensional thin layer chromatography (TLC) on polyamide sheets.[16] Determination of carbohydrate was performed by the method of Dubois *et al.*[17]

Results

Purification of Inhibitor

All steps were carried out at 4 °C. The proline endopeptidase inhibitor was purified from the soluble fraction of rat whole brain at 5 to 7 weeks of age. A sample of 50 g of rat brain was homogenized in 9 volumes of 50 mM Tris–HCl buffer, pH 7.5, containing 0.25 M sucrose. The

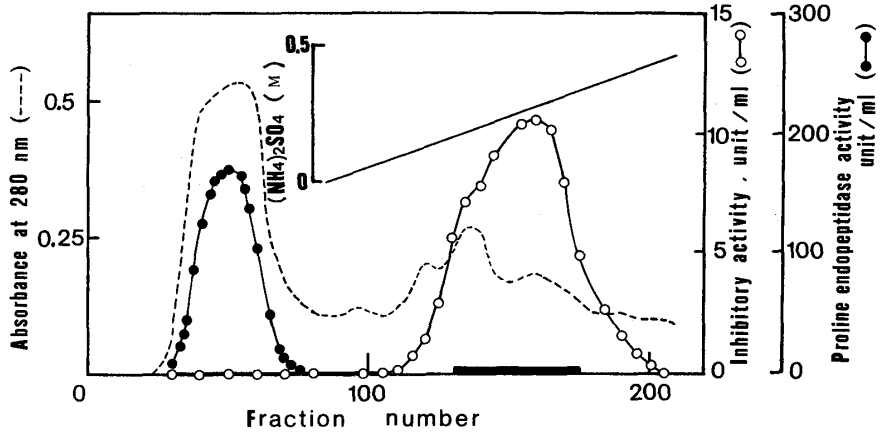

Fig. 1. SP-Sephadex C-25 Chromatography of Rat Brain Cytosol

Fractions of 4.9 ml each were collected. The other procedures are described in Results.

homogenate was centrifuged at 105000 × *g* for 90 min, then the supernatant (435 ml) was concentrated to one-third volume by ultrafiltration with a YM-5 membrane (Amicon). The pH of the concentrate was adjusted to 5.25 with 5% acetic acid and the precipitate was centrifuged off. The supernatant was applied to an SP-Sephadex C-25 column (5 × 15 cm) equilibrated with 50 mM ammonium acetate buffer, pH 5.25, containing 0.01% Tween 80. The column was washed with the equilibrating buffer and eluted by means of a concentration gradient of $(NH_4)_2SO_4$ (0 to 0.5 M) in 400 ml of the same buffer at a flow rate of 30 ml/h. Proline endopeptidase activity in the soluble fraction of rat brain passed through the column. The inhibitory activity was adsorbed, and was eluted by increasing the $(NH_4)_2SO_4$ concentration as shown in Fig. 1. The active fractions were combined, and concentrated by ultrafiltration with a YM-5 membrane. The concentrate was subjected to gel filtration on a Sephadex G-50 column (2.5 × 90 cm) equilibrated with 50 mM phosphate buffer, pH 6.8, containing 0.25 M NaCl, and the inhibitor was eluted with the same buffer at a flow rate of 15 ml/h. As shown in Fig. 2-A, the inhibitory activity appeared in 3 peaks (S-1, S-2 and S-3). The most active fractions (S-2) were concentrated by ultrafiltration. The concentrate was rechromatographed on the same Sephadex G-50 column (Fig. 2-B). The active fractions were combined and concentrated by ultrafiltration. The concentrate was stored at −80 °C. The

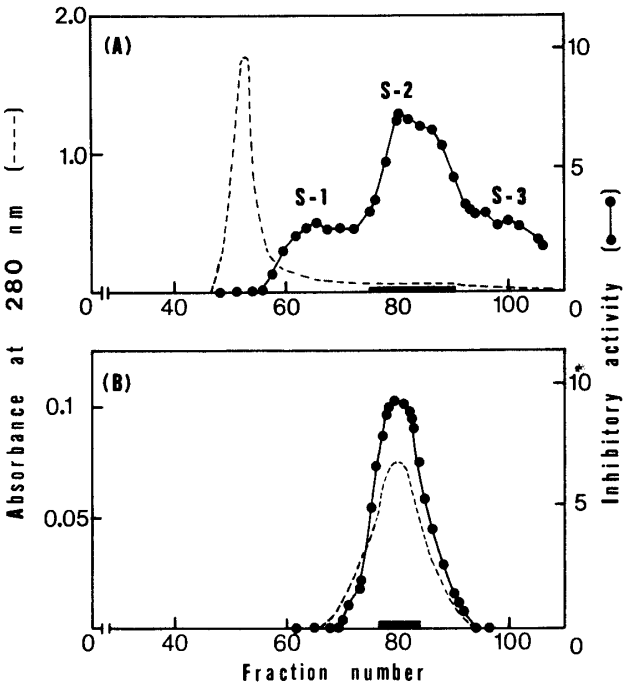

Fig. 2. Chromatography of Proline Endopeptidase Inhibitor on a Sephadex G-50 Column

Fractions of 3.9 ml each were collected. The other procedures are described in Results.

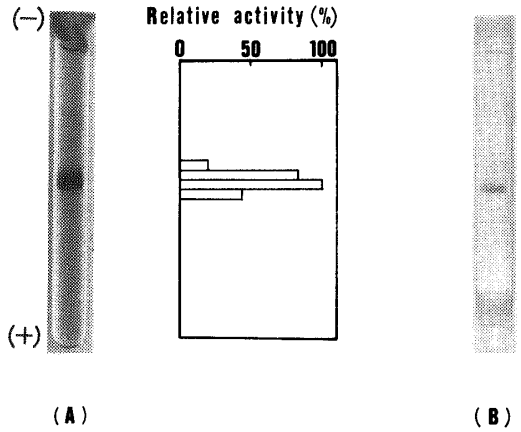

Fig. 3. Disc Gel Electrophoresis of Proline Endopeptidase Inhibitor

A, disc gel electrophoresis of proline endopeptidase inhibitor in the absence of sodium dodecyl sulfate. The inhibitory activity was determined after extraction of the inhibitor from 2 mm gel slices. B, disc gel electrophoresis of proline endopeptidase inhibitor in the presence of sodium dodecyl sulfate.

TABLE I. Purification of the Proline Endopeptidase Inhibitor from Rat Brain

Steps	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification factor	Yield (%)
Cytosol fraction	1240	660	0.52	1	100
SP-Sephadex C-25	91	396	4.4	8.5	60
Sephadex G-50 1st	5.5	215	39.0	75	33
Sephadex G-50 2nd	3.2	185	57.8	111	28

purification procedures are summarized in Table I. By the use of these procedures, the inhibitor was purified about 111-fold from the soluble fraction of rat brain with a recovery of 28%. The apparent molecular weight of the inhibitor was estimated to be 7000 by gel filtration on Sephadex G-50 and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The purified inhibitor gave a single protein band upon disc gel electrophoresis, and the inhibitory activity was detected only in the region of the band, as shown in Fig. 3-A. The inhibitor also gave a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fig. 3-B).

Stability

The inhibitor was quite stable to heat. When the inhibitor was heated at 80 °C for 30 min at pH 6.8, more than 95% of the inhibitory activity remained. The inhibitor was also stable to usual proteinases; it retained almost full activity even after incubation at 37 °C for 30 min with trypsin, chymotrypsin, elastase and pepsin.

Amino Acid Composition

The amino acid composition of the inhibitor is shown in Table II. The inhibitor did not contain tryptophan. The minimum molecular weight, calculated assuming 60 amino acid residues per molecule, was 6837. This value is in good agreement with the molecular weight values determined by gel filtration and by disc gel electrophoresis. The inhibitor contained 61.6% polar and 3.3% aromatic amino acid residues. Homogeneity of the inhibitor preparation was checked by analysis of the N-terminal amino acid residue. The only residue found was phenylalanine. Less than 5.2% carbohydrate was detected by the phenol–sulfuric acid method.[17]

Inhibitory Effect of Inhibitor on Proline Endopeptidase and Various Proteinases

Inhibition of proline endopeptidase by various amounts of the inhibitor is shown in Fig.

TABLE II. Amino Acid Composition of Proline Endopeptidase Inhibitor from Rat Brain

Amino acid	Time of hydrolysis 24	72	Average	Nearest integer
Asp	6.95	6.71	6.83	7
Thr	4.00	4.12	4.06	4
Ser	2.06	2.30	2.18	2
Glu	13.00	13.15	13.08	13
Gly	4.92	4.93	4.93	5
Ala	3.04	2.98	3.01	3
Val	2.07	2.42	2.25	2
Met	0.87	0.85	0.86	1
Ile	3.80	4.04	3.92	4
Tyr	0.88	0.83	0.86	1
Phe	0.95	1.01	0.98	1
Lys	6.93	6.89	6.91	7
His	0.92	0.94	0.94	1
Arg	3.04	3.22	3.13	3
Pro	1.11	1.06	1.09	1
Trp	0	0	0	0
1/2 Cys	0	0	0	0
Total				60

Values were calculated assuming the molecular weight of the inhibitor to be 7000. Half-cystine was determined as *S*-sulfocysteine by treating the hydrolysate with dithiothreitol followed by an excess of tetrathionate.

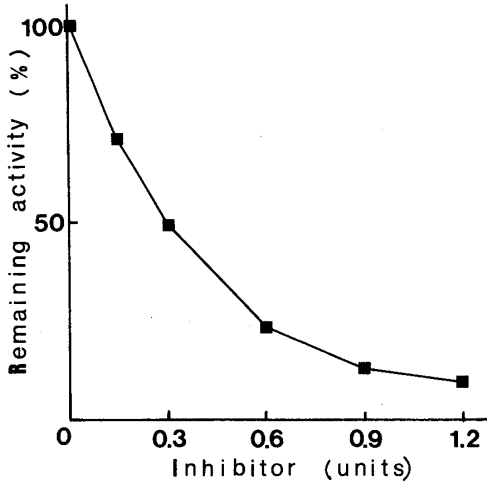

Fig. 4. Inhibition of Activity of Proline Endopeptidase by the Inhibitor

Test conditions were as described in Experimental.

TABLE III. Inhibitory Spectrum of Proline Endopeptidase Inhibitor from Rat Brain

Enzyme	Enzyme activity (unit or μg)	Substrate	% inhibition
Proline endopeptidase (rat brain)	0.3 unit	Suc-Gly-Pro-MCA	90
Proline endopeptidase (hog kidney)	0.3 unit	Suc-Gly-Pro-MCA	86
Dipeptidyl aminopeptidase IV	10.0 μg	Gly-Pro-MCA	0
Trypsin	5.0 μg	Bz-Arg-MCA	0
Elastase	5.0 μg	Suc-$(Ala)_3$-*p*NA	0
Plasmin	10.0 μg	D-Val-Leu-Lys-*p*NA	0
Leucine aminopeptidase	50.0 μg	Leu-MCA	0
Ficin	2.0 μg	Bz-Arg-MCA	0
Papain	1.5 μg	Bz-Arg-MCA	10

The enzyme was preincubated at 37 °C for 5 min with the inhibitor (1.5 units), then the reaction was initiated by the addition of substrate (incubation time; 10 min). The final incubation volume was 250 μl.

4; maximum inhibition was almost 90%. The inhibitory spectrum of the inhibitor is summarized in Table III. Proline endopeptidases from rat brain and hog kidney were inhibited by the inhibitor. However, dipeptidyl aminopeptidase IV, trypsin, elastase, plasmin, leucine aminopeptidase, papain and ficin were not inhibited by the inhibitor. As shown in Fig. 5, proline endopeptidase was inhibited competitively. The K_i value of the inhibitor calculated by the method of Dixon[18] was 2.6 μM.

Changes of Proline Endopeptidase-Inhibitory Activity during Maturation of Rat Brain

As shown in Fig. 6, the inhibitory activity per brain towards proline endopeptidase rapidly increased until 2 weeks of age, and then decreased during maturation. The inhibitory activity in a 2-week-old rat brain was about three times that in a 4-week-old brain.

Discussion

Yoshimoto *et al.*[5] reported the presence of proline endopeptidase inhibitor in hog and rat tissues. They partially purified an inhibitor with $M_r = 6500$ from hog pancreas, though it was still heterogeneous. In the present work, the proline endopeptidase inhibitor was purified from rat brain cytosol by chromatographies on SP-Sephadex C-25 and Sephadex G-50. In the 1st gel filtration step, the inhibitory activity appeared in 3 peaks as shown in Fig. 2-A. The mean molecular weights of S-1, S-2 and S-3 in two experiments were below 5000, 7000 and

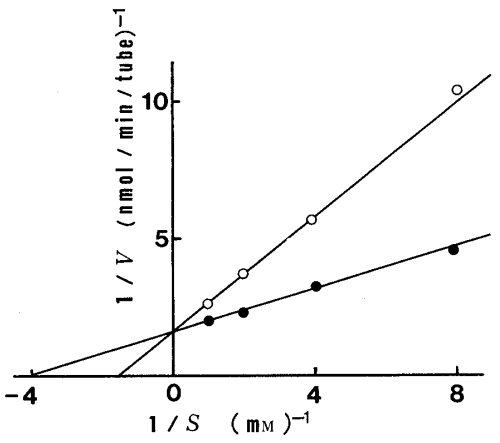

Fig. 5. Kinetic Analysis of the Reaction of Proline Endopeptidase with the Inhibitor

Lineweaver–Burk plots of Suc–Gly–Pro–MCA concentration against proline endopeptidase activity in the presence (—○—) and absence (—●—) of the inhibitor. Test conditions were as described in Experimental.

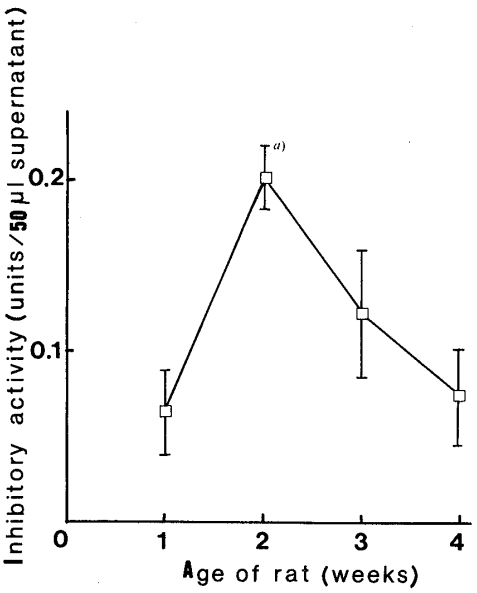

Fig. 6. Developmental Changes of Proline Endopeptidase-Inhibitory Activity in Rat Brain

Rat brain cytosol was heated for 20 min at 80 °C to inactivate the enzyme activity of rat brain. After removal of the coagulated materials by centrifugation, 50 μl of the supernatant was assayed for inhibitor activity. The other test conditions were as described in Experimental.

Each value represents the mean ± S.D. ($n = 5$).
a) $p < 0.001$ *versus* the 4-week-old group.

12500, respectively. For further purification, S-2 fractions were used because these fractions contained the largest amount of the inhibitor. S-1 and S-3 fractions have not been studied in the present work. The purified inhibitor from S-2 was homogeneous on polyacrylamide gel electrophoresis in the absence and in the presence of sodium dodecyl sulfate, and from the results of analysis of the N-terminal amino acid.

As has been reported for the inhibitor from hog pancreas,[5] the proline endopeptidase inhibitor from rat brain was very stable to heat. It was also stable to usual proteinases. The molecular weight of the inhibitor, 7000, was close to that of hog pancreatic inhibitor. The inhibitor was highly specific for proline endopeptidase from rat brain and hog kidney. It had no effect on dipeptidyl aminopeptidase IV from hog kidney, although this enzyme shows specificity for proline residues.

Kato *et al.*[2] have found that the proline endopeptidase activity in 2-week-old rat brain, where neurones develop rapidly, was the highest among the activities at all the ages studied and was about twice that in adult brain. In the present work, developmental changes of proline endopeptidase-inhibitory activity in rat brain were found to be very similar to those of the enzyme activity. The inhibitory activity in rat brain rapidly increased up to 2 weeks of age, and then decreased during maturation; the inhibitory activity in a 2-week-old rat brain was about three times that in a 4-week-old brain. Therefore, the physiological roles of rat brain proline endopeptidase and its inhibitor may be related to the development of neurones in the brain. However, further studies are required to clarify the physiological role of the inhibitor.

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