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Prolyl Endopeptidase from Bovine Testis: Purification, Characterization and Comparison with the Enzymes from Other Tissues

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Prolyl endopeptidase (EC 3.4.21.26) was purified from bovine testis by chromatographies on carboxymethyl (CM)-cellulose, diethylaminoethyl (DEAE)-Sephadex, hydroxyapatite, *p*-chloromercuribenzoate (PCMB)-Sepharose and Sephadex G-150. The enzyme appeared homogeneous as judged by disc gel electrophoresis. The molecular weight of the enzyme was estimated to be 76000 by gel filtration. The optimum pH of the activity was 7.2. The enzyme was inactivated with diisopropylphosphorofluoridate, Z–Gly–Pro–CH_2Cl, Z–Pro-prolinal and *p*-chloromercuribenzoate. The enzyme hydrolyzed biologically active peptides at the carboxyl side of prolyl residues. All of the characteristics of the testis prolyl endopeptidase are quite similar to those of the enzymes isolated from bovine brain, liver, kidney and muscle. These enzymes were immunologically indistinguishable: the antiserum raised against bovine brain enzyme gave a fused precipitation line against the enzymes from bovine testis, brain, liver and kidney, and the testis and brain enzymes gave the same immunoelectrophoretic profile.

Keywords——prolyl endopeptidase; post-proline cleaving enzyme; bovine testis; immunoelectrophoresis

Introduction

Prolyl endopeptidase (post-proline cleaving enzyme: EC 3.4.21.26) was discovered by Walter *et al.*[1] in human uterus. The enzyme is widely distributed in animal tissues and body fluids,[2] and has been purified from lamb kidney[3-5] and brain,[6] bovine brain[7] rabbit brain,[8] bovine pituitary,[9] rat brain[10] and other sources.[11-20] Most of the studies have been focused on hydrolysis of biologically active peptides by the enzyme in the brain, though the role of the enzyme *in vivo* is still obscure. We showed that intravenous injection of inhibitors, such as N^{α}-benzyloxyl carbonyl–L-prolyl–L-prolinal (Z–Pro–prolinal), specific for prolyl endopeptidase resulted in a remarkable reduction in the enzyme activity of rat brain and prevention of amnesia induced by scopolamine.[21]

Recently, prolyl endopeptidase was purified from sperm of the ascidian, *Halocynthia roretzi*, by Yokosawa *et al.*[23] and they proposed that the enzyme has a role in fertilization. High enzyme activities were also observed in the testis of rat and in human semen by using several synthetic substrates.[2]

To elucidate the biological significance of the testis enzyme and for comparative studies of the enzymes from different tissues of the same animal, prolyl endopeptidase was purified from bovine testis and other tissues, and the enzymatic properties were compared.

Materials and Methods

Materials——Bovine testis was supplied by a local slaughter house. Ethylenediaminetetraacetate (EDTA), *p*-chloromercuribenzoate (PCMB) and *o*-phenanthroline were from Nakarai Kagaku Co., Kyoto. Fast Garnet GBC

salt, bovine pancreatic trypsin and alanyl-β-naphthylamide (Ala-2-NNap) were from Sigma Chemical Co., U.S.A. Horse heart muscle catalase, bovine pancreatic chymotrypsinogen and rabbit heart muscle aldolase were obtained from Boehringer-Mannheim, Germany. Z–Gly–Pro–CH_2Cl, Gly–Pro–2-NNap, Z–Gly–Pro–2-NNap, Z–Phe–2-NNap, Leu–Tyr, Gly–Leu–Tyr and bovine brain prolyl endopeptidase were the same batches used in previous studies.[4-7] PCMB-Sepharose was prepared by the method described by Yokosawa *et al.*,[23] except that triethylenetetramine (T) was used as the spacer instead of ethylenediamine, and hydroxyapatite was prepared by the method of Bernardi.[24] Inhibitors specific for proly endopeptidase, prolinal derivatives, were synthesized as described previously.[25]

Assays of Prolyl Endopeptidase Activity——The arylamidase activity was determined by the method of Yoshimoto *et al.*[7] with Z–Gly–Pro–2-NNap as the substrate; 100 μl of enzyme solution was added to 0.8 ml of 20 mM Tris–HCl buffer, pH 7.0, containing 10 mM each of EDTA and 2-mercaptoethanol, and the mixture was preincubated at 37 °C for 3 min. The reaction was initiated by adding 100 μl of 5 mM Z–Gly–Pro–2-NNap in 40% dioxane. After 15 min of incubation at 37 °C, the reaction was stopped by adding 1 ml of 25% trichloroacetic acid and, after 5 min, the solution was centrifuged at 3000 rpm for 10 min. Then 0.5 ml of 0.1% $NaNO_2$ was added to 0.5 ml of the supernatant, and, after incubation at 37 °C for 3 min, 0.5 ml of 0.5% ammonium sulfamate and 1 ml of 0.05% *N*-(1-naphthyl) ethylenediamine dihydrochloride in ethanol were further added. The mixture was left for 25 min at 37 °C and then the absorbance was measured at 570 nm. One unit of the enzyme activity was defined as the amount of the enzyme that released 1 μmol of β-naphthylamine per min under the standard conditions. Dipeptidyl aminopeptidase IV was assayed by the method of Yoshimoto *et al.*[2] using Fast Garnet GBC salt.

Purification of Prolyl Endopeptidase from Bovine Testis——All procedures were carried out at 4 °C. Bovine testis, 1.0 kg, was homogenized with 2 l of 20 mM Tris–HCl buffer containing 10 mM EDTA and 2-mercaptoethanol, pH 7.0, for 2 min using an electric mixer. The homogenate was centrifuged at 8000 rpm for 20 min, and the supernatant was fractionated by ammonium sulfate precipitation at 45 to 70% saturation. The precipitate was dissolved in a small volume of the above buffer, desalted by gel filtration on Sehadex G-25 and then applied to a column of carboxymethyl (CM)-cellulose (3 × 23 cm). The flow-through solution was directly applied on a diethylaminoethyl (DEAE)-Sephadex A-50 column (4 × 35 cm) equilibrated with the same buffer. The adsorbed enzyme was eluted with an increasing linear gradient of NaCl concentration from 0 to 1 M. The active fractions were combined and, after concentration by ultrafiltration using an Amicon ultrafilter (PM-10) and desalting by gel filtration, the enzyme was rechromatographed on a column (3 × 30 cm) of DEAE-Sephadex A-50 as above. The active fractions were combined, concentrated and then applied to a Sephadex G-25 column (2 × 20 cm) equilibrated with 5 mM phosphate buffer, pH 7.0. The enzyme solution was applied to a hydroxyapatite column (2 × 7 cm) and the adsorbed enzyme was eluted with a linear gradient of phosphate from 5 to 500 mM at pH 7.0. The active fractions were combined, concentrated and desalted on a Sephadex G-25 column as above, except that 20 mM Tris–HCl buffer, pH 7.0, containing 10 mM EDTA was used. The enzyme solution was applied onto a PCMB-T-Sepharose column (1 × 6 cm), and after the column had been washed with the same buffer, the adsorbed enzyme was eluted with 20 mM Tris–HCl buffer, pH 7.0, containing 10 mM 2-mercaptoethanol. The active fractions were combined, concentrated and applied to a Sephadex G-150 column (2 × 100 cm) equilibrated with 20 mM Tris–HCl buffer, pH 7.0, containing 10 mM each of EDTA and 2-mercaptoethanol and 0.1 M KCl, pH 7.0. After gel filtration on Sephadex G-150, the enzyme was precipitated by the addition of ammonium sulfate, and dissolved in 5 ml of 10 mM phosphate buffer. The enzyme fractions were combined and kept at −20 °C until use. The prolyl endopeptidases from bovine liver, muscle and kidney were similarly purified.

Disc Gel Electrophoresis——Polyacrylamide gels (7.5% acylamide) were prepared as described by Davis.[26] Electrophoresis was carried out at 1 mA per tube until the sample had entered the gel and then at 3 mA per tube and 4 °C. Enzyme activities on the gels were detected by the standard assay method after extraction from sliced gels.

Estimation of Molecular Weight——Molecular weight was estimated by gel filtration on a column of Sephadex G-150 following the method of Andrews.[27] Dipeptidyl aminopeptidase IV (M.W. 230000) from lamb kidney, proline-specific endopeptidase (M.W. 76000) from *F. meningosepticum*, *Bacillus subtilis* α-amylase (M.W. 54000) and bovine pancreatic trypsin (M.W. 24000) were used as molecular weight markers.

Isoelectric Focusing——This was carried out with a 250 ml column according to the method of Vesterberg and Svensson.[28] Carrier ampholyte in the pH range of 3.5 to 10.0 with a sucrose density gradient was used. Focusing conditions were 300 V at 4 °C for 48 h.

Substrate Specificity and Kinetic Analysis——Hydrolysis of biologically active peptide was assayed as follows. A 100 μl aliquot of 1 mM peptide was mixed with 10 μl of enzyme (0.02 unit) at 37 °C for 3 h. The digest was separated by HPLC with an ODS column (0.46 × 25 cm) at a flow rate of 0.7 ml/min and 18 °C. The products were eluted with a gradient system of 0.1% trifluoroacetic acid (TFA) in H_2O–0.08% TFA in CH_3CN/2-propanol (3 : 1) and monitored by following the absorbance at 214 nm. Identification was based on the amino acid composition. In the case of thyrotropin releasing hormone (TRH), the products were separated by high voltage electrophoresis and identified by comparison with a parallel run of an authentic marker of acid TRH, Pyr–His–Pro. Electrophoresis was carried out at pH 6.2, 1000 V for 2 h. After drying of the filter paper, products were visualized by the use of the Pauly reaction.[29]

Hydrolysis of peptide-β-naphthylamide was assayed by the standard method except that the substrate

concentration was varied from 0.05 to 0.8 mM. Liberation of *p*-nitroaniline was measured by following the increase in absorbance at 450 nm. Hydrolysis of Z-Gly-Pro-Leu-Gly was assayed by the ninhydrin method. K_m and K_i values were calculated by a microcomputer program.[30]

Immunochemical Analysis——An antiserum was prepared by using the prolyl endopeptidase purified from bovine brain. This enzyme (0.5 mg) was emulsified with 0.5 ml of Freund's complete adjuvant. The emulsion was injected into the hind foot pads of rabbit weighing about 2.5 kg. Two boosters of 0.25 mg of protein were given subcutaneously at intervals of 2 weeks. The rabbit was fed commercial diet and bled 6 weeks after the first injection. Double immunodiffusion analysis[31] was performed overnight in 1.2% agarose gel in 70 mM phosphate buffer, pH 7.5, containing 0.9% NaCl and precipitated protein was stained with Amido Black 10B after soaking the agar plate in water for 2 d. The plate was destained with 2% acetic acid. Immunoelectrophoresis was carried out for brain, testis and liver enzymes by the method described by Laurell[32] using an antiserum against bovine brain enzyme in 1% agar plate containing 20 mM Tris HCl, pH 7.0. After electrophoresis at 4 °C and 3 mA/cm for 2 h, two channels were made between testis and brain, and brain and liver enzymes in the plate, and antiserum against bovine brain enzyme was added to these channels. The plate was incubated for 20 h at 30 °C, and stained as done for immunodiffusion analysis.

Results

Purification of Prolyl Endopeptidase from Bovine Testis——The enzyme was purified from the supernatant of bovine testis homogenate by ammonium sulfate fractionation and sequential chromatography. After the ammonium sulfate fractionation, the enzyme was applied to a DEAE-Sephadex column. The concentration of NaCl needed for enzyme elution was 0.5 M, which was similar to that in the case of the bovine brain enzyme. The chromatogram on DEAE-Sephadex is shown in Fig. 1. After rechromatography on DEAE-

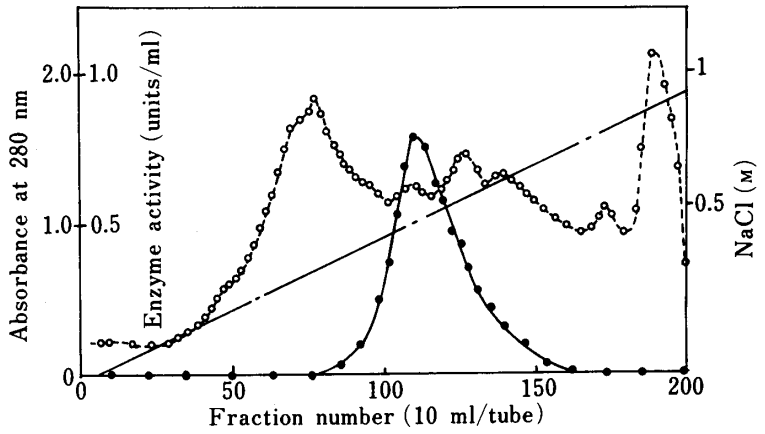

Fig. 1. Chromatogram of a Crude Preparation of Prolyl Endopeptidase from Bovine Testis on DEAE-Sephadex

The column (3.5 × 24 cm) was equilibrated with 20 mM Tris-HCl buffer, pH 7.0. Elution was performed using a linear gradient of NaCl (0.0 to 1 M) in 20 mM Tris-HCl buffer (— - —). Absorbance was measured at 280 nm (○---○). Enzyme activity was determined under the standard conditions (●—●).

TABLE I. Purification of Prolyl Endopeptidase from Bovine Testis

Step	Protein	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
Supernatant of homogenate	10714	754	100	0.007	1
Ammonium sulfate fractionation	7240	362	48	0.05	7
1st DEAE-Sephadex	475	257	34	0.54	77
2nd DEAE-Sephadex	119	193	26	1.62	231
Hydroxyapatite	34	140	19	4.12	589
PCMB-T-Sepharose	5.2	70	9	13.5	1929
Sephadex G-150	1.9	37	5	19.5	2786

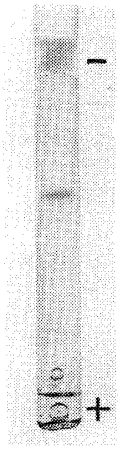

Fig. 2. Disc Gel Electrophoresis of Prolyl Endopeptidase from Bovine Testis

About 5 μg of enzyme was applied to a 7.5% gel, pH 9.4. Electrophoresis was carried out with a current of 2.5 mA per tube for 1.2 h. Staining and destaining methods were described in Materials and Methods.

TABLE II. Enzymatic Properties of Prolyl Endopeptidases Purified from Bovine Tissues

	Testis	Brain	Liver	Muscle	Kidney
Optimum pH	7.0	6.9	7.1	7.0	6.9
pH stability	5.0—9.0	5.5—9.0	5.5—9.5	5.5—9.0	5.5—9.0
Optimum temperature	40 °C	40 °C	40 °C	40 °C	40 °C
Thermal stability	48 °C	47.5 °C	46 °C	48 °C	47 °C
Molecular weight	76000	76000	76000	76000	76000
Isoelectric point	5.1	4.8	5.1	4.6	4.8

TABLE III. Comparison of Effects of Chemicals and Metal Ions on Prolyl Endopeptidases from Bovine Tissues

Chemicals	Conc. (mM)	Remaining activity				
		Testis	Brain	Liver	Kidney	Muscle
None	—	100	100	100	100	100
DFP	0.5	0	0	0	16	10
PMSF	1	101	97	73	101	86
PCMB	0.5	0	5	3	10	2
Iodoacetate Na	1	84	71	73	88	82
N-Ethylmaleimide	1	60	59	38	52	43
EDTA	1	104	110	100	110	100
o-Phenanthroline	1	100	105	103	105	100
Z–Pro–prolinal	0.001	8	25	5	11	12
Boc–Pro–prolinal	0.001	12	0	6	0	5
Z–Pyr–prolinal	0.01	3	9	10	0	5
Zn^{2+}	1	98	85	91	95	83
Hg^{2+}	1	2	3	2	0	2
Co^{2+}	1	105	78	100	102	95
Ni^{2+}	1	57	24	48	55	51

Boc, *tert*-butyloxycarbonyl; Pyr, L-pyroglutamyl; metal salts used were the chlorides.

Sephadex, the specific activity increased 160 times. Affinity chromatography with PCMB-Sepharose was also effective for purification, and after gel filtration on Sephadex G-150, the specific activity was increased about 2800-fold with an activity recovery of 5% (Table I). As shown in Fig. 2, the purified enzyme appeared homogeneous upon disc gel electrophoresis.

Table IV. Kinetic Studies of Prolyl Endopeptidases

	K_m (mM)				
	Testis	Brain	Liver	Kidney	Muscle
Z-Gly-Pro↓2-NNap	0.19	0.23	0.20	0.19	0.15
Z-Gly-Pro↓*p*-NA	0.23	0.29	0.24	0.18	0.24
Z-Ala-Gly-Pro↓2-NNap	0.30	0.36	0.40	0.41	0.45
Z-Gly-Pro↓Leu-Gly	0.12	0.15	0.10	0.20	0.14
	K_i (mM)				
Z-Ala-Pro	0.037	0.065	0.057	0.023	0.033

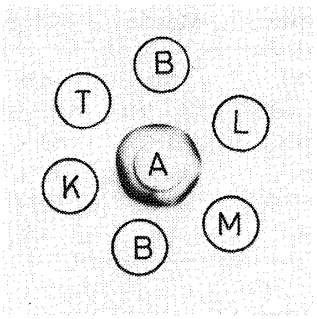

Fig. 3. Double Immunodiffusion Analysis of Bovine Prolyl Endopeptidases

The center well (A) contained 30 μl of antiserum against bovine brain enzyme. The outer wells contained about 100 units of enzymes from different tissues. T, testis; B, brain; L, liver; M, muscle; K, kidney.

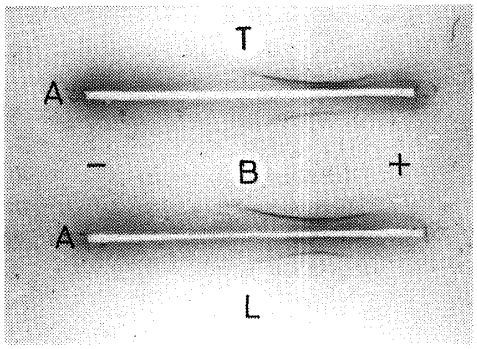

Fig. 4. Immunoelectrophoresis of Prolyl Endopeptidases from Bovine Testis, Brain and Liver

Immunoelectrophoreses were carried out in 1% agarose containing 20 mM Tris-HCl buffer, pH 7.0. Wells T, B and L contained about 120 units of enzymes from bovine testis, brain and liver, respectively. After electrophoresis, antiserum was added to the channels (A) and the plate was incubated for 20 h at 30 °C.

The enzyme activity was detected at the same position as that shown by protein staining. The enzymes from bovine liver, muscle and kidney were purified by similar procedure, but the final preparations were still contaminated with minor impurities.

Enzymatic and Physicochemical Properties——Some properties of bovine testis prolyl endopeptidase are summarized in Table II in comparison with those of the enzymes from brain, liver, muscle and kidney. Maximal activity of the enzyme was observed at pH 7.2 with Z-Gly-Pro-2-NNap. The enzyme was stable between pH 5.0 and 9.0 for 30 min at 30 °C. The optimum temperature for the activity of the enzyme was 45 °C. The properties of these five enzymes are almost the same except for optimum pH and isoelectric point.

Effects of Chemical Reagents, Metal Ions and Inhibitors on Enzyme Activity——The enzymes were markedly inhibited by diisopropylphosphorofluoridate (DFP), Z-Gly-Pro-CH_2Cl, Z-Pro-prolinal, PCMB and Hg^{2+}, whereas they were not affected by monoiodoacetate, phenylmethanesulfonyl fluoride (PMSF) and metal chelators such as EDTA and *o*-phenanthroline. These results coincide with those obtained for the enzyme from lamb brain. The enzyme was resistant to a trypsin inhibitor from pancreas (Table III).

Substrate Specificity——The K_m and V_{max} values of testis enzyme toward Z-Gly-Pro-2-NNap were 0.19 mM and 0.24 μmol/min/unit, respectively. K_m values of bovine enzymes from

several tissues for some substrates and K_i values of Z–Ala–Pro are summarized in Table IV. These enzymes were also active toward Ala–X bonds (X: L-amino acids), although the rates of hydrolyses were very low as compared with that for Pro–X bond hydrolysis (data not shown). The enzymes hydrolyzed biologically active peptides; TRH, neurotensin, vasopressin, oxytocin and bradykinin were easily split at the carboxyl side of prolyl residues (data not shown).

Immunological Comparison——By Ouchterlony's double immunodiffusion, a completely fused precipitin line was observed between antiserum against the enzyme from bovine brain and enzymes from testis, brain, kidney, liver and muscle (Fig. 3). Immunoelectrophoreses were also done between the enzymes from brain, testis and liver. The precipitin lines showed the same migration profiles (Fig. 4).

Discussion

We[2] have previously reported that high activities of prolyl endopeptidase are observed in the brain and testis of rats. Similarly, high activity was also observed in bovine testis.

Various workers[3-19,33,34] have reported the properties of prolyl endopeptidases purified from several mammalian tissues. There are some discrepancies in the reported physicochemical properties of these enzymes, although the enzymatic characteristics of most prolyl endopeptidases from several tissues of different animals are basically indistinguishable. It remains unclear, however, whether such discrepancies are dependent upon the kind of tissue or species of animal examined. Thus, a systematic comparison of prolyl endopeptidases from different tissues of the same animal is desirable. Two papers that compare prolyl endopeptidases from the same animals have appeared; the enzymes were purified from rat brain and kidney by Andrews *et al.*[33] and from bovine pituitary and brain by Hersh.[34] They found no significant difference in the properties of the prolyl endopeptidases among different tissues in the same animals.

We characterized the prolyl endopeptidase purified from bovine testis and compared it with the enzymes from bovine brain, liver, muscle and kidney. The optimum pHs of these enzymes for hydrolysis of Z–Gly–Pro–2-NNap are somewhat different, but the differences seem to be within the experimental error. No significant difference was observed in pH stability, optimum temperature or thermal stability among these enzymes.

The molecular weights of these bovine enzymes were measured by the gel filtration method using a prolyl endopeptidase from *Flavobacterium* as one of the markers, the molecular weight of which has been estimated to be 76000 by ultracentrifugal analysis.[35] The molecular weights of prolyl endopeptidases from bovine testis and other tissues were the same as those of the brain enzymes of lamb[6] and bovine.[7]

There were some minor differences in the isoelectric point of bovine enzymes (Table II). These may reflect a difference in the degree of amidation of glutamyl and aspartyl residues.

We have previously indicated that prolyl endopeptidases are serine enzymes,[4-7,20] regardless of their origin, and this conclusion was confirmed by many workers.[8,36,37] Nevertheless, the enzymes from mammals[6,7] and plants[38] are very sensitive to PCMB as well as DFP, while the microbial enzymes are insensitive to PCMB.[20,35] As shown in Table III, the prolyl endopeptidases from bovine tissues were exceptionally inactivated by PCMB, DFP, Z–Pro–prolinal and other prolinal derivatives.

Immunochemical analyses by Ouchterlony's method and electrophoresis demonstrated that the prolyl endopeptidases of bovine brain, testis, liver, muscle and kidney were immunologically indistinguishable (Figs. 3 and 4). The same result was previously reported for prolyl endopeptidases from rat brain and kidney by Andrews *et al.*[33]

In conclusion, there was no significant difference in enzymatic and immunochemical properties among the prolyl endopeptidases from bovine testis, brain, kidney, and muscle.

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