POST-PROLINE ENDOPEPTIDASE. PARTIAL PURIFICATION AND CHARACTERIZATION OF THE ENZYME FROM PIG KIDNEYS

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Post-proline endopeptidase was isolated from pig kidneys and partially purified. The procedure consisted of fractionation with ammonium sulphate, ion exchange chromatography on DEAE--Sephadex A-50, gel filtration on Sephadex G-200 and rechromatography on DEAE-Sephadex A-50. The preparation had 55 times higher specific activity than the crude extract and did not contain any contaminating enzymic activities. The enzyme cleaved a number of proline-containing peptides and was strictly specific in catalyzing the hydrolysis of the peptide bond on the carboxyl side of the proline residue. The optimum pH for the hydrolysis of the synthetic peptides benzyloxycarbonylglycyl-prolyl-leucyl-glycinamide and benzyloxycarbonyl-glycyl-proline β-naphthylamide was 7·8—8·0 and, in the case of benzyloxycarbonylglycyl-proline *p*-nitroanilide, 7·2 to 7·5. For the hydrolysis of the tetrapeptide benzyloxycarbonylglycyl-prolyl-leucyl-glycinamide, the K_m value of 75 μ mol l^{-1} was obtained.

A new group of enzymes called "proline-specific endopeptidases" [EC 3.4.21.26] has recently[1] been included among serine proteinases. These peptidases are able to hydrolyse specifically the peptide bond on the carboxyl side of the proline residue inside the peptide chain: Y—Pro$\overset{\downarrow}{-}$X, where Y is a peptide or a protected amino acid* and X can be an amino acid, peptide, amide, aromatic amine or alcohol. The first representative of this group, the so-called "post-proline cleaving enzyme", was discovered in the human uterus[3,4] in 1971. A similar enzyme was subsequently found in lamb kidneys[5,6] and purified by affinity chromatography. The enzyme was characterized as a serine proteinase by using specific inhibitors[7] and its substrate specificity was studied in detail[8,9]. After a highly specific method for detecting the peptidase activity had been developed, based on the use of the synthetic substrate benzyloxycarbonylglycyl-proline 4-methylcoumarinyl amide, studies were made of the distribution of the enzyme in rat organs and human body fluids[10].

Enzymes with similar specificity have lately been isolated from a number of animal tissues. Orlowski and coworkers[11] isolated a so-called prolyl endopeptidase from rabbit brain and Oliviera and coworkers[12] described kininase B as the enzyme responsible for the degradation of bradykinin in this tissue. Although the two preparations differ in some respects, they can be considered to contain the same enzyme. Moreover, the so-called "thyroliberin-deamidating enzyme" isolated from rat brain[13] was found to have post-proline cleaving specificity[14–16].

* The nomenclature and symbols are in accordance with the published recommendation[2]. All optically active amino acids are of L-configuration. Additional abbreviations used: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate.

Another similar enzyme was recently isolated from chicken liver[17] and a "proline-specific endopeptidase" was purified from *Flavobacterium meningosepticum*[18,19].

Opinions on the physiological role of post-proline endopeptidase differ. Apart from its possible participation in the general metabolism of proteins and peptides, it is thought to have a specific function in the regulation of the action of peptide hormones[1]. The highest levels of post-proline endopeptidase activity were found in organs of synthesis and target tissues of proline-containing peptide hormones. For example, post-proline endopeptidase can participate in the degradation of oxytocin and vasopressin in kidneys.

The present paper offers the first results of studies of the properties of post-proline endopeptidase from pig kidneys and its ability to split neurohypophysial hormones.* It draws upon previous studies concerned with the properties of post-proline endopeptidase from lamb kidneys[20] and with the inactivation mechanisms of neurohypophysial hormones and their analogues in rat tissues[21,22].

EXPERIMENTAL

Material

DEAE-Sephadex A-50, Sephadex G-25 and Sephadex G-200 were purchased from Pharmacia (Uppsala, Sweden), UM-10 dialysation membranes from Amicon (Lexington, U.S.A.) and Silufol sheets from Kavalier (Votice, Czechoslovakia).

Oxytocin, [8-lysine]vasopressin, [8-tert-leucine]oxytocin[23], glycinamide, leucyl-glycinamide, prolyl-leucyl-glycinamide, S-benzylcysteinyl-prolyl-leucyl-glycinamide (*I*) and benzyloxycarbonyl-S-benzylcysteinyl-prolyl-leucyl-glycinamide (*II*) were synthetized at the Department of Organic Synthesis of this Institute. The synthesis of benzyloxycarbonylglycyl-prolyl-leucyl-glycinamide (*III*) was published in a previous paper[20]. Leucine *p*-nitroanilide was kindly supplied by Dr E. Kasafírek, Research Institute for Pharmacy and Biochemistry, Prague. Benzyloxycarbonyl-glycyl-proline *p*-nitroanilide (*IV*) was prepared according to Yoshimoto and coworkers[8].

Benzyloxycarbonylglycyl-proline β-naphthylamide (*V*) was synthetized as follows. To a solution of benzyloxycarbonylproline (5 g) in tetrahydrofuran (30 ml), cooled to 0°C, dicyclohexylcarbodiimide (2 g) was added. The mixture was stirred for 3 h at 0°C. Precipitated dicyclohexylurea was then removed by filtration and β-naphthylamine (1·43 g) was added to the solution of symmetric anhydride. After stirring for 3 h at 0°C and 12 h at room temperature, the solution was evaporated and the residue was crystallized from an ether–light petroleum mixture and from methanol. The yield was 2·1 g (57%) of product, m.p. 133—135°C. The sample for analysis was recrystallized from methanol and had a m.p. of 134—136°C. $[\alpha]_D$ −81° (*c* 0·1, dimethylformamide). For $C_{23}H_{21}N_2O_3$ (373·4) calculated: 73·97% C, 5·67% H, 7·50% N; found: 73·88% C, 5·84% H, 7·57% N.

Benzyloxycarbonyl-proline β-naphthylamide (1·5 g) obtained in this way was dissolved in 4M-HBr in acetic acid (10 ml). After 10 min at room temperature, hydrobromide was precipitated by ether, the crystalline portion was filtered off, washed with ether and dried. $E^{His}_{2,4}$ 0·83, $E^{His}_{5,7}$ 0·93. To a solution of hydrobromide in dimethylformamide (20 ml), N-ethylpiperidine was added until the pH value of 7·5 was obtained (moist pH paper). Benzyloxycarbonylglycine *p*-nitro-

* The results have been presented in part at the symposium: "Modern Approach in Applied Enzymology", Reinhardsbrunn, German Democratic Republic, 1980.

phenyl ester (1·3 g) was then added, the mixture was stirred for 6 h at room temperature, and another portion of active ester (0·4 g) was added. After 12 h, the mixture was evaporated and the residue was triturated with light petroleum and ether. Crystallization from ethanol yielded 1·06 g (61%) of product, m.p. 126—130°C. The sample for analysis was recrystallized in the same way; m.p. 140—142°C; $[\alpha]_D$ —122·8° (*c* 0·1, dimethylformamide). Amino acid composition: Pro 1·00, Gly 1·00. For $C_{25}H_{25}N_3O_4$ (431·5) calculated: 69·58% C, 5·84% H, 9·74% N; found: 69·48% C, 5·83% H, 9·84% N. Published data state[8] a m.p. of 139—141°C and $[\alpha]_D$ —124° (*c* 2, dimethylformamide).

Methods

Purification procedure. The enzyme was purified by modification of a method described earlier[20]. The following buffers were used during purification: buffer A: 50 m mol l^{-1} Na-phosphate, pH 6·8, containing 2 m mol l^{-1} EDTA and 1 m mol l^{-1} DTT; buffer B: 20 m mol l^{-1} Na-phosphate, pH 7·8, containing 1 m mol l^{-1} EDTA and 1 m mol l^{-1} DTT. All the purification steps were performed at 4°C, centrifugations were carried out for 30 min at 10 000 *g* and 4°C.

Extraction and fractionation with ammonium sulphate. Fresh or frozen pig kidney (1 kg) were freed of fat and connective tissue and homogenized in a blendor for 3 times 30 s with 3 l of buffer A. The homogenate was stirred for 1 h and centrifuged. The supernatant was subjected to fractionation with solid ammonium sulphate. The fraction precipitating between 30—60% saturation was suspended in a minimum volume of buffer B and dialyzed for 4 times 16 h against 10 l of the same buffer.

Ion exchange chromatography on DEAE-*Sephadex*. The dialyzed preparation (approximately 5 g of protein) was applied on a column (3·5 × 50 cm) of DEAE-Sephadex A-50, equilibrated with buffer B. The column was washed with 1000 ml of buffer B and the proteins that remained bound to the column were eluted by means of a concentration gradient of NaCl (0—0·4 mol l^{-1}) in buffer B (2 × 1000 ml) at a flow rate of 40—50 ml/h. The active fractions were combined, dialyzed overnight against 10 l of buffer B and concentrated by ultrafiltration on a UM-10 membrane; the resultant volume was approximately 30 ml. The final protein concentration was 20 mg/ml. An alternative procedure was based on the precipitation of the proteins with ammonium sulphate and desalting on a column of Sephadex G-25.

Gel filtration on Sephadex G-200. 9 ml of the partially purified enzyme preparation (20 mg protein/ml) were transferred to a column of Sephadex G-200 (3·5 × 50 cm) equilibrated with buffer B. The column was eluted with the same buffer at a flow rate of 10 ml/h and the active fractions were combined.

Rechromatography on DEAE-*Sephadex*. The combined active fractions were transferred to a column (1·6 × 80 cm) of DEAE-Sephadex A-50 equilibrated with buffer B. The column was washed with 250 ml of buffer B and then eluted with a 0—0·4 mol l^{-1} NaCl concentration gradient in buffer B (total volume 500 ml). The active fractions were combined, dialyzed overnight against 10 l of buffer B and concentrated by one of the above-mentioned procedures.

Determination of protein concentration was performed according to the method of Lowry and coworkers[24] or by the biuret reaction[25]; bovine serum albumin was used as a standard. In the course of chromatography on columns, the protein concentration in the eluted fractions was estimated by measuring the absorbance at 280 nm.

Enzyme assays. Three methods were used for determining the activity of post-proline endopeptidase, differing in accordance with the substrate used.

Peptide *III*. The incubation mixture (1 ml) contained 20 m mol l^{-1} Na-phosphate, pH 7·7, 1 m mol l^{-1} EDTA, 1 m mol l^{-1} DTT, 1 m mol l^{-1} peptide *III*, 2·5% dimethylformamide and 0·5—5·0 mU enzyme activity. The incubation was carried out for 30 min at 37°C and was terminated by adding 1 ml of ninhydrin reagent according to the method published earlier[20]. After adding 1 ml of parboiled distilled water, the samples were placed for 15 min in a boiling water bath and then cooled in running water. The amount of leucyl-glycinamide released was read from the calibration plot of absorbance at 570 nm.

Substrates *IV* and *V*. The incubation mixture (1 ml) contained 40 m mol l^{-1} Na-phosphate, pH 7·5, 1 m mol l^{-1} EDTA, 1 m mol l^{-1} DTT, 0·25 m mol l^{-1} substrate and 0—1 mU of enzyme activity. 20% of dimethyl sulphoxide was added in order to dissolve the substrates. The incubation was carried out at 37°C for 30 min and was terminated by adding 2·0 ml of 1 mol l^{-1} Na-acetate buffer, pH 4·2. The amount of *p*-nitroaniline released was calculated from measurements of absorbance values of the incubation mixture at 406 nm ($\varepsilon_{406} = 9600$). The amount of β-naphthylamine released was determined spectrofluorometrically (Spectromom 203, $\lambda_{exc} =$ = 345 nm), using a standard β-naphthylamine solution for reference.

The unit of activity was expressed as that amount of enzyme which catalyses the hydrolysis of 1 μmol of substrate in 1 min at 37°C and the appropriate pH.

The aminopeptidase activity of the enzyme preparation was estimated under the same conditions, using 0·5 m .mol l^{-1} leucine *p*-nitroanilide as substrate. The concentration of liberated *p*-nitroaniline was calculated from absorbance values at 406 nm ($\varepsilon_{406} = 9\,600$).

Thin layer chromatography. The reaction products resulting from the incubation of post-proline endopeptidase with tetrapeptide *III* and the C-terminal fragments of the oxytocin molecule were analysed by means of thin layer chromatography on Silufol sheets in a solvent system of 1-butanol–pyridine–acetic acid–water (30 : 20 : 6 : 12). Aliquots were taken from the incubation mixture at several intervals (0, 15, 30, 60 and 120 min); leucyl-glycinamide, leucyl-glycine, glycinamide and glycine were used as standards. The chromatograms were visualized by spray application of a ninhydrin solution.

Hydrolysis of neurohypophysial hormones and their analogue. [8-Lysine]vasopressin, oxytocin and [8-tert-leucine]oxytocin were incubated with post-proline endopeptidase for 0—60 min at 37°C. The incubation mixture (100 μl) contained 0·01—0·5 m mol l^{-1} substrate, 20 m mol l^{-1} Na-phosphate (pH 7·7), 1 m mol l^{-1} EDTA, 1 m mol l^{-1} DTT and 0—0·5 mU of enzyme. The reaction was terminated by heating for 3 min in a boiling water bath. The amount of intact hormone was determined by the uterotonic assay *in vitro* according to Holton's method[26], as modified by Munsick[27] (oxytocin and [8-tert-leucine]oxytocin) and the pressor assay on pithed rats[28] ([8-lysine]vasopressin). Hormones incubated under the same conditions in the absence of the enzyme were used as standards.

pH *Dependence of enzyme activity and kinetic measurements*. The dependence of enzyme activity on pH was estimated using the following buffers: 50 m mol l^{-1} Na-citrate-phosphate, pH 3·0—7·5 and 50 m mol l^{-1} Na-borate, pH 8·0—10·0, both containing 1 m mol l^{-1} EDTA and 1 m mol l^{-1} DTT. The kinetics were measured at the optimum pH value with respect to the hydrolysis of the individual substrates. The incubation mixtures contained 0—1 m mol l^{-1} substrate and 0—5 mU of enzyme activity in 1 ml of buffer. In the case of substrate *V*, the initial velocity of the enzyme reaction was estimated from spectrofluorometric recordings (Spektromom 203, $\lambda_{exc} = 345$ nm) of the time dependence of the concentration of β-naphthylamine in the incubation mixture at 30°C. In experiments with the other substrate, the methods described under Enzyme assays were used. The duration of incubation and enzyme concentration were chosen to ensure that the measurement was performed in the linear part of the time dependence of product concentration.

RESULTS

The purification procedure is summarized in Table I. Fig. 1 shows the elution profile of ion exchange chromatography of the crude fraction of the kidney extract on a column of DEAE-Sephadex A-50. The active peptides were eluted in a sharp peak and the specific activity of the preparation increased 14 times after this step, as determined with substrate *III*. However, the preparation was still not homogeneous and was strongly contaminated by aminopeptidases. Fig. 2 shows the elution diagram of the following gel chromatography on Sephadex G-200. The proteins were distributed in three peaks, the last of which was identical with the maximum post-proline endopeptidase activity. The highest aminopeptidase activity was found in the first peak. The specific activity of post-proline endopeptidase after this purification step was 34 times higher than in the kidney extract and the preparation was only slightly contaminated by aminopeptidases. Aminopeptidase activity was removed completely by rechromatography on DEAE-Sephadex A-50 (Fig. 3). The final preparation had 55 times higher specific activity than the crude extract (substrate *III* was used in measurements of activity) and was not contaminated by any other peptidase activity. Post-proline endopeptidase was stored at −18°C.

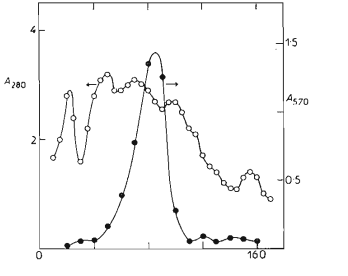

FIG. 1

Chromatography of the enzyme on a DEAE-Sephadex A-50 column (step 3 of the purification procedure). Ordinate: protein concentration — absorbance at 280 nm (○—○), enzymic activity — absorbance at 570 nm (●—●). Abscissa: fraction number. See text for details

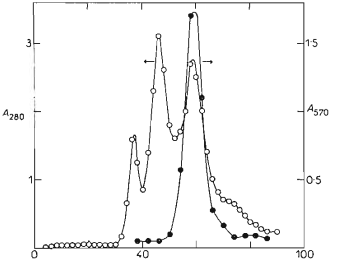

FIG. 2

Chromatography of the enzyme on a Sephadex G-200 column (step 4 of the purification procedure). Ordinate: protein concentration — absorbance at 280 nm (○—○), enzymic activity — absorbance at 570 nm (●—●). Abscissa: fraction number. See text for details

TABLE I

Summary of the purification of post-proline endopeptidase

Purification step	Total protein mg	Total activity U	Yield %	Specific activity mU/mg	Degree of purification
1. Tissue extract	51 200	143	100	2·8	1
2. Ammonium sulphate fractionation	14 300	67	47	4·7	1·7
3. DEAE-Sephadex A-50 chromatography	1 600	62	43	39	14
4. Sephadex G-200 chromatography	535	51	36	96	34
5. DEAE-Sephadex A-50 rechromatography	220	34	24	154	55

Note added in proof: Our most recent findings suggest that the real values of the degree of purification are about 3 times higher than those given in Table I.

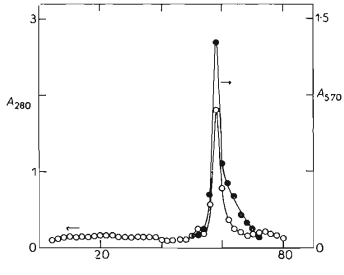

FIG. 3

Rechromatography of the enzyme on a DEAE-Sephadex A-50 column (step 5 of the purification procedure). Ordinate: protein concentration — absorbance at 280 nm (○—○), enzymic activity — absorbance at 570 nm (●—●). Abscissa: fraction number. See text for details

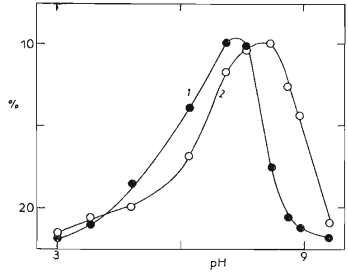

FIG. 4

pH Dependence of the activity of post-proline endopeptidase. Enzymic activity was determined as stated in the text. Ordinate: enzymic activity in percentages of maximum activity; substrate *IV* (●—●), substrate *V* (○—○). Abscissa: pH values

Properties of the enzyme. The activity of post-proline endopeptidase was considerably lowered by oxidation and by the presence of heavy metals. For this reason, we used buffers containing EDTA and DTT. The enzyme activity decreased by approximately 20% each time the preparation was thawed. In the course of storage at 4°C, the enzyme activity decreased to half the initial level in about a week, and at 20–25°C in 12 h.

Specificity of hydrolysis. The dipeptide leucyl-glycinamide was the only product detected after the incubation of post-proline endopeptidase with tetrapeptide *III*. The other reaction product benzyloxycarbonylglycyl-proline could not be visualized by means of the ninhydrin reagent. The incubation mixture did not contain any other reaction products, *i.e.* neither proline, leucine, nor glycinamide. This finding provides evidence that the preparation was not contaminated by peptidyl carboxyamidase, dipeptidase or aminopeptidase activities.

Hydrolysis of neurohypophysial hormones and related peptides. The peptides that were tested as possible substrates of post-proline endopeptidase are summarized in Table II. The tripeptide prolyl-leucyl-glycinamide was not split; tetrapeptide *I*,

TABLE II

Cleavage of proline-containing peptides by the post-proline endopeptidase from pig kidneys

A Not hydrolyzed:	Pro-Leu-Gly-NH_2
B Hydrolyzed sparingly:	↓
I	Cys(Bzl)-Pro-Leu-Gly-NH_2
C Hydrolyzed:	↓
II	Z-Cys(Bzl)-Pro-Leu-Gly-NH_2
	↓
III	Z-Gly-Pro-Leu-Gly-NH_2
	↓
IV	Z-Gly-Pro-pNA
	↓
V	Z-Gly-Pro-Nap
	↓
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
	↓
[8-Lysine]vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2
	↓
[8-Tert-leucine]oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-tert-Leu-Gly-NH_2

Abbreviations: Z, benzyloxycarbonyl; Bzl, benzyl; pNA, *p*-nitroanilide; Nap, β-naphthylamide.

containing an unprotected α-amino group, was degraded only slightly. Peptides with protected N-terminal amino groups, as well as the neurohypophysial hormones and their analogue, were found to be very good substrates. The rate of hydrolysis of oxytocin was highest, that of [8-lysine]vasopressin was approximately 3 times lower and [8-tert-leucine]oxytocin 10 times lower than that of oxytocin.

pH *Dependence and kinetic measurements.* Fig. 4 shows the pH dependence of the hydrolysis of substrates *IV* and *V* catalysed by post-proline endopeptidase. The optimum pH was in the neutral region, in the case of substrate *V* between 7·8 and 8·0 and in the case of substrate *IV* between 7·2 and 7·5. The optimum pH value for the hydrolysis of substrate *III* and oxytocin was 7·8–8·0. The K_m value for substrate *III*, determined by means of the Lineweaver–Burk plot, was 75 μ mol l^{-1}.

DISCUSSION

Following our work with post-proline endopeptidase from lamb kidneys[20], which had been previously isolated and characterized in detail by Walter[5], Koida and Walter[6] and others[7–9], we now aimed at purifying post-proline endopeptidase from pig kidneys. It was necessary to develop a new isolation procedure, since the endopeptidase from pig kidneys had somewhat different properties than the enzyme from lamb kidneys. Problems arose when choosing suitable specific substrates. The substrates considered to be sufficiently specific, *i.e.* substrates *IV* and *V*, had very low solubility in aqueous media; furthermore, the rate of their hydrolysis, catalysed by post-proline endopeptidase, was low. We could not use these substrates in the initial phases of the purification procedure because the specific activity of post-proline endopeptidase was not high enough. The solubility of tetrapeptide *III* was higher and the rate of its hydrolysis sufficient. However, it can also be hydrolysed by other peptidases that are present in kidney extracts (*e.g.* peptidyl carboxyamidase). The dipeptide leucyl-glycinamide, released in the course of hydrolysis, can also be split by aminopeptidases or dipeptidases, possibly by amidases. The action of the above-mentioned enzymes could contribute to an increase in the concentration of free amino groups in the incubation mixture and thus interfere with the accurate determination of the reaction products of hydrolysis catalysed specifically by post-proline endopeptidase. The presence of peptidases in the crude fractions resulted in higher values determined by the ninhydrin method and thus in a seemingly higher specific activity of post-proline endopeptidase. The calculations of the total balance of the purification procedure were therefore unfavourably influenced because they were based on the assumption of high initial levels of specific post-proline endopeptidase activity in the crude fractions.

The preparation of kidney post-proline endopeptidase catalysed with strict specificity the hydrolysis of the peptide bond on the carboxyl side of the proline

residue inside the peptide chain. Moreover, it had other properties similar to those of the other proline-specific endopeptidases described so far. For example, an SH--group was found to be necessary for the activity of the enzyme, the optimum pH of the reaction was in the neutral region, the enzyme had a higher affinity for longer peptides, *etc.*

Interesting results were obtained by studying the rate of hydrolysis of oxytocin, [8-lysine]vasopressin and [8-tert-leucine]oxytocin. [8-Lysine]vasopressin was hydrolysed at a 3 times lower rate and the analogue containing tert-leucine instead of leucine in position 8 of the oxytocin molecule at a 10 times lower rate than that of oxytocin. It can be seen that the rate at which substrates are hydrolysed is strongly influenced by the properties of the amino acid residue following proline in the peptide chain. Further studies of the hydrolysis of analogues of neurohypophysial hormones with modifications in position 8 could reveal substances that would be resistant to the action of post-proline endopeptidase and have suitable biological activities.

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