POST-PROLINE ENDOPEPTIDASE. FURTHER CHARACTERIZATION OF THE ENZYME FROM PIG KIDNEYS

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A post-proline endopeptidase (EC 3.4.21.26) was isolated from pig kidneys using a modified method described earlier. The enzyme was further purified by ion exchange chromatography on DEAE-Sephacel. The final product contained about 95% of post-proline endopeptidase. The enzyme molecule consisted of one peptide chain with a relative molecular mass of 65 600 to 70 000, containing a large proportion of acidic and alifatic amino acids (glutamic acid, aspartic acid and leucine) and the N-terminus was formed by aspartic acid or asparagine. In order to prevent losses of enzyme activity, thiol compounds had to be added.

"Proline-specific endopeptidases" (EC 3.4.21.26) are serine proteases with a high degree of specificity for catalysing the hydrolysis of the peptide bond between a proline residue, localised inside a peptide chain, and the following amino acid. In the course of the first decade since the discovery of the endopeptidase in the human uterus[1], the enzyme has been found in other tissues of many vertebrate species and even in some strains of bacteria (*cf.* review[2]).

Nowadays, attention is mainly paid to the possible involvement of post-proline endopeptidase in the catabolism of proline-containing peptide hormones. For instance, it has been reported that bradykinin (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9)* is inactivated in the rabbit brain[4]; a proline-specific enzyme splits the bond between Pro^7 and Phe^8 and also, though at a lower rate, the bond between Pro^3 and Gly^4. Thyroliberin (<Glu-His-Pro-NH_2) is inactivated in the bovine brain[5] by specific deamidation of the C-terminal proline amide. A comparison was made of enzymes that were isolated from different tissues of the same animal species[6]. The possible role of the enzyme in lung physiology and pathology was also investigated[7]. Most post-proline endopeptidases catalysed the hydrolysis of peptide bonds between proline and the following amino acid residue when localised inside a peptide chain, regardless of the type of amino acids in the vicinity of the hydrolyzed peptide bond. Only one exception has been described so far, *i.e.* a post-proline endopeptidase from bovine brain[8] that catalysed the hydrolysis of peptide bonds after a proline residue only if a basic amino acid (such as histidine, lysine or arginine) preceded proline in the peptide chain.

In our previous communications[9,10] we presented a method for purifying post-proline endopeptidase from pig kidneys and described some properties of the isolated

* The nomenclature and symbols are in accordance with the published recommendation[3]. All optically active amino acids are of L-configuration. Additional abbreviations used: dansyl, 5-dimethylaminophthalene-1-sulfonyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylendiaminetetraacetate; DTT, dithiothreitol, β-ME, 2-mercaptoethanol.

enzyme. In the present paper we report on some further physico-chemical properties of the enzyme.

EXPERIMENTAL

Material

Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden); the same firm kindly supplied us with a sample of DEAE-Sephacel. DEAE-Cellulose DE 32 was from Whatman (Mainstone, England). Fast Garnet GBC salt (diazotized *o*-aminoazotoluene) was purchased from Fluka AG (Buchs, Switzerland) as Echtgranatsalz GBC; dansyl chloride was obtained from the same firm. Triton X-100 was purchased from Serva (Heidelberg, Germany), and polyamide layer sheets from BDH (Poole, England). Benzyloxycarbonylglycyl-proline 2-naphthylamide was synthetized as published elsewhere[9], benzyloxycarbonylglycyl-proline 4-nitranilide was prepared according to Yoshimoto and coworkers[11].

Methods

Purification procedure. The purification of the enzyme was performed by methods described earlier[9,10], with some modifications. The following buffer (A) was used in all the purification steps: Na-phosphate, pH 6·5, 20 mmol l^{-1}, containing EDTA, 2 mmol l^{-1}, and β-ME, 2 mmol . . l^{-1}. All the purification steps were performed at 4°C, unless stated otherwise. Centrifugations were carried out at 10 000*g* and 4°C for 30 min.

Combined acetone—ammonium sulphate precipitation. The homogenate of pig kidneys in bufferA (30% w/v) was centrifuged and to the clear supernatant, cooled acetone (−15°C) in an amount resulting in 40% of acetone in the mixture was gradually added while constantly stirring and cooling. The precipitate was removed by centrifugation and discarded and solid ammonium sulphate was added to the supernatant, reaching 80% of saturation of the aqueous phase. After 1 h of intensive stirring at room temperature, the precipitate formed was collected by centrifugation and extracted with a solution of EDTA and β-ME, both 2 mmol l^{-1} in water. The clear extract was then dialysed against buffer A.

Ion exchange chromatography on DEAE-*Cellulose.* The dialysed preparation (about 200 ml, containing approximately 5 g of proteins) was applied on a column (3·2 × 20 cm) of DEAE-Cellulose, equilibrated with buffer A. The column was washed with 400 ml of buffer A and the proteins that remained adsorbed were eluted with 100 ml of buffer A, containing 0·1 mol l^{-1} NaCl, at a flow rate of 150 ml h^{-1}. The combined active fractions were dialysed against a solution of EDTA and β-ME, both 2 mmol l^{-1} in water and freeze-dried.

Ion exchange chromatography on DEAE-*Sephacel.* The freeze-dried enzyme (50—500 mg dissolved in 10—30 ml of water) was applied on a column of DEAE-Sephacel (2 × 15 cm) equilibrated with buffer A. The column was washed with 100 ml of buffer A and the proteins that remained adsorbed were eluted by means of a linear concentration gradient of sodium chloride (0—0·25 mol l^{-1}) in buffer A (2 × 250 ml) at a flow rate of 30 ml h^{-1}. The combined active fractions were dialysed twice for 16 h against 1 l of a solution of EDTA (1 mmol l^{-1}) and β-ME (2 mmol l^{-1}) in water and freeze-dried. The dry enzyme was stored at 4°C without loss of activity for three months.

Rechromatography on DEAE-Sephacel was done under the same conditions as had been used during the first ion exchange chromatography on DEAE-Sephacel.

Enzyme assays. Two synthetic substrates were used for measuring the activity of post-proline endopeptidase: benzyloxycarbonylglycyl-proline 2-naphthylamide and benzyloxycarbonylglycyl--proline 4-nitranilide.

1) Benzyloxycarbonylglycyl-proline 2-napthylamide: The incubation mixture (1 ml) contained Na-phosphate buffer (20 mmol l^{-1}, pH 7·0), benzyloxycarbonylglycyl-proline 2-naphthylamide (250 μmol l^{-1}), 15% dimethyl sulphoxide and 0—20 pkat of enzyme activity. The incubation was carried out for 0—40 min at 30°C and was terminated by adding 1 ml of a 0·1% solution of Fast Garnet GBC salt in Na-acetate buffer, pH 4·0, 1 mol l^{-1}, containing 10% of Triton X-100. After standing at room temperature for 30 min, the volume of the incubation mixture was adjusted by adding 1 ml of water and the absorbance of the samples was measured at 525 nm. The amount of 2-naphthylamine released was read from the calibration plot.

2) Benzyloxycarbonylglycyl-proline 4-nitranilide. The incubation mixture (4 ml) contained Na-phosphate buffer (20 mmol l^{-1}, pH 7·5), benzyloxycarbonylglycyl-proline 4-nitranilide (250 μmol l^{-1}), 15% dimethyl sulphoxide and 0—100 pkat of enzyme activity. The incubation was carried out at 30°C and the absorbance of the mixture was measured at 406 nm at intervals of 0—80 min. The amount of 4-nitraniline released was calculated from the absorbance values, assuming that $\varepsilon_{406} = 9\,600$.

In both cases the reaction rate was calculated from the curve constructed from the time course of product concentration. Enzyme activity, measured in the assay system specified above, was expressed in pkat, in accordance with the recommendation[12].

Electrophoresis in polyacrylamide gel. The purity of the enzyme preparation was checked by means of polyacrylamide gel electrophoresis in discontinuous buffer systems: at pH 9·5 according to the method of Davis[13], at pH 8·0 according to Williams and Reisfeld[14] and at pH 3·8 according to Reisfeld and coworkers[15]. When the electrophoresis was run at pH 8·0 in the presence of EDTA (1 mmol l^{-1}) and DTT (1 mmol l^{-1}) in the electrophoretic buffers, the enzyme activity could be detected directly in the gels as follows: The gels were incubated for 15 min in a reaction mixture containing Na-phosphate buffer (pH 7·0, 20 mmol l^{-1}), benzyloxycarbonylglycyl-proline 2-naphthylamide (0·5 mmol l^{-1}), 30% dimethyl sulphoxide, and then stained in a solution containing 0·1% Fast Garnet GBC, 10% Triton X-100 and Na-acetate buffer (pH 4·0, 1 mol l^{-1}) for 1 h at room temperature.

Determination of protein concentration was performed according to the method of Lowry and coworkers[16], or according to Bradford[17], using bovine serum albumin as a standard. In the course of column chromatography, the protein concentration in the eluted fractions was estimated by measuring the absorbance at 280 nm, assuming that $\varepsilon_{280}^{1\%} = 10$.

The relative molecular mass of the enzyme was estimated by means of gel electrophoresis in the presence of sodium dodecyl sulphate according to the method of Weber and Osborn[18] and Neville[19]. For the same purpose, pore-gradient gel electrophoresis, according to the methods of O'Farrell[20] and Margolis and Kenrick[21], was also used.

Ultracentrifugation analysis. Sedimentation velocity analysis was performed on a Spinco Model E ultracentrifuge equipped with a schlieren optical system at 59 780 revolutions per minute. The enzyme concentration was 0·5 and 1·0%.

Sedimentation equilibrium studies were performed using the same device at 23 150 revolutions per minute and a 0·03% enzyme solution. The relative molecular mass was calculated from sedimentation equilibrium data according to the method of Yphantis[22], assuming a partial specific volume $\bar{v} = 0{\cdot}73$.

Amino acid analysis. Samples were hydrolysed in the sealed tubes under N_2 at 110°C with 6 mol l^{-1} HCl for 20 and 70 h and analysed in a Spinco-Beckman Model 120 amino acid analyser. Cysteine was estimated as cysteic acid after oxidation of the sample by performic acid[23]. Corrections were made for the destruction of serine and threonine.

The N-terminal amino acid was determined by the dansylation method in a modification suitable for proteins as described by Hartley[24]. Dansyl-amino acids were identified by thin-layer chromatography on polyamide sheets[25].

Effect of thiol compounds on the activity of post-proline endopeptidase. Benzyloxycarbonyl-glycyl-proline 4-nitranilide was used as a substrate and the enzyme activity was assayed as described under "Enzyme assays", except that the enzyme was desalted on a column of Sephadex G-25 before the assay and phosphate buffer was not used. Instead, the following buffers (20 mmol . l^{-1}) were used in the preparation of the reaction mixtures: Tris-maleate, pH 6·5, Tris-maleate, pH 7·5, Tris-HCl, pH 8·5. Cysteine or DTT were present in the incubation mixtures in a concentration of 1, 10, 100, and 1 000 μmol l^{-1}.

RESULTS

Purification procedure. The method employed for the isolation of post-proline endopeptidase from pig kidneys was described in the Methods. During the first three steps, *i.e.* the extractions of proteins from the tissue, followed by precipitation with acetone and ammonium sulphate, a significant portion of the inactive proteins was removed. The resulting enzyme preparation had 20 times higher specific activity than that of the tissue extract. Further purification was performed by applying two ion exchange chromatography procedures. Chromatography on a column of DEAE-Cellulose performed by stepwise elution was followed by chromatography on DEAE-Sephacel, using a linear gradient of ionic strength. The specific activity of the resulting enzyme preparation was about 280 times higher than that of the crude extract. This preparation was used for the estimation of the properties of the enzyme. However, the active enzyme was not stable enough, especially in solution, and was gradually digested, probably owing to autolysis[26]. The elution profile of the repeated ion exchange chromatography of the enzyme preparation on DEAE-Sephacel is shown in the Fig. 1.

Properties of the enzyme. The progress in the purification of the enzyme was checked by means of polyacrylamide gel electrophoresis. Fig. 2 documents the distribution of the protein bands in gels after electrophoresis (for details, see Methods). Post-proline endopeptidase activity was present in the widest protein band. On the basis of this finding, we assumed that the final enzyme preparation contained more than 95% of post-proline endopeptidase. The relative molecular mass of post-proline endopeptidase was 67 000 when estimated by electrophoresis in the presence of sodium dodecyl sulphate and 70 000 when established by pore-gradient gel electrophoresis. These results led us to conclude that the enzyme is not composed of subunits.

Sedimentation analysis. The relative molecular mass calculated from results of sedimentation analysis was 65 600 which accords well with the above-mentioned results. The sedimentation constant $s^0_{20,w}$ equalled 4·25 in a 1% solution and 4·35 in a 0·5% solution of enzyme.

The results of amino acid analysis are presented in Table I. The dansylation method proved that the N-terminal amino acid is either aspartic acid or asparagine.

The effect of organic solvents on the activity of post-proline endopeptidase. An organic solvent had to be added to the incubation mixture, in order to achieve the required concentrations of the synthetic substrates, both of which were only poorly soluble in water. A comparison was therefore made of the influence of the solvents used, *e.g.* 10% dimethyl sulphoxide and 10% dimethylformamide, on the activity of the enzyme. Under the same conditions, the enzyme activity was about three times higher in the presence of 10% dimethyl sulphoxide than in the presence

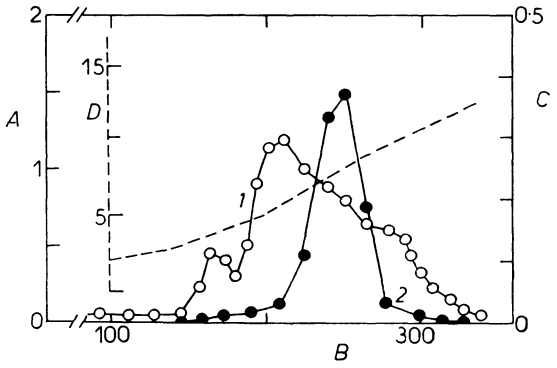

FIG. 1

Chromatography of the enzyme on DEAE-Sephacel. *A* Protein content, absorbance at 280 nm (○); *B* elution volume (ml); *C* enzyme activity determined by method 2., absorbance at 406 nm (●); *D* conductivity of the fractions (mS). For details, see text

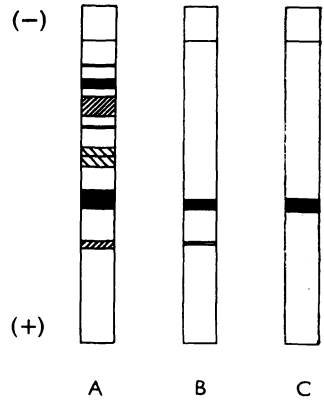

FIG. 2

Discontinuous electrophoresis in polyacrylamide gel. A Active protein fraction obtained by combined precipitation with acetone and ammonium sulphate; B enzyme preparation obtained by chromatography on DEAE-Cellulose; C enzyme preparation obtained by chromatography on DEAE-Sephacel. Electrophoresis was carried out at 4 mA per gel for 2 h, using a discontinuous buffer system according to Davis[1,2] (pH 9·5)

of 10% dimethylformamide. Dimethyl sulfoxide was therefore used in all the other incubation experiments.

The effect of thiol compounds on the activity of post-proline endopeptidase. The enzyme activity was assayed in the presence of different concentrations (0, 1, 10, 100, 1 000 μmol l^{-1}) of cysteine or dithiothreitol. The presence of cysteine at the given concentrations in the pH range of 6·5–8·5 did not affect enzyme activity. At pH 6·5, dithiothreitol at the given concentrations was also without influence. By contrast, at pH value of 7·5 and 8·5, dithiothreitol at concentrations of 100 and 1 000 μmol l^{-1} remarkably increased the rate of benzyloxycarbonylglycyl-proline 4-nitroanilide hydrolysis.

DISCUSSION

The method for the isolation of post-proline endopeptidase from pig kidneys presented in this communication differs from the previously published method[9]. The

TABLE I

Amino acid composition of post-proline endopeptidase from pig kidneys. Mean values from six measurements; corrections were made for 9·46% water content. The figures in brackets give the number of amino acid residues expressed in the nearest whole integer

Amino acid	Amino acid residues (mol per 66 000 g protein)
Aspartic acid (asparagine)	44·4 (44)
Threonine	22·9 (23)
Serine	23·2 (23)
Glutamic acid (glutamine)	67·2 (67)
Proline	30·2 (30)
Glycine	19·1 (19)
Alanine	41·2 (41)
Cysteine	24·9 (25)
Valine	28·0 (28)
Methionine	1·8 (2)
Isoleucine	18·7 (19)
Leucine	50·6 (51)
Tyrosine	18·1 (18)
Phenylalanine	23·4 (23)
Histidine	17·5 (17)
Lysine	42·8 (43)
Arginine	23·0 (23)
Tryptophan	n.d.[a]

[a] Not determined.

finding that the enzyme is easily dissolved in aqueous solutions of acetone made it possible to remove the major part of inactive proteins by precipitation with acetone and ammonium sulphate in the first stages of the purification procedure[10]. This yielded 90% of the active enzyme and increased its specific activity 20 times. Ion exchange chromatography using modified cellulose produced better results than the formerly used purification on DEAE-Sephadex. Ion exchange chromatography usually yielded more than 100% of enzyme activity. Similar results have already been described and explained to be due to the removal of a hypothetical inhibitor that is present in the tissue extract[27]. Results of analytic electrophoresis indicate that our final product contains about 95% of post-proline endopeptidase.

Assuming this to be so, then the total yield of the enzyme would be remarkably higher than the yields obtained by other methods used so far for the isolation of post-proline endopeptidase from other animal sources. The total degree of purification is lower than that given by other authors. In one of our previous papers[9], we considered the possibility that the values of the degree of purification in the individual steps of the procedure could be influenced by the fact that the enzyme activity was assayed using an unspecific substrate. In our present work we used benzyloxycarbonylglycyl-proline 2-naphthylamide, a sufficiently specific substrate for hydrolysis by post-proline endopeptidase. As expected, the values of the degree of purification measured in this way were about three times higher than the results of the previous experiments using benzyloxycarbonylglycyl-prolyl-leucyl-glycinamide as substrate[9]. We must also consider the possibility that a part of the thiol groups of the enzyme, necessary for its activity, might be oxidised or otherwise modified in the course of the isolation of the enzyme. Thus, a portion of the enzyme in the final enzyme preparation might be in inactive form which we are unable to detect by the methods used.

The relative molecular mass of post-proline endopeptidase from various sources[2,4,5,6,8] was found to be in the range of 66 000–77 000. The values that we obtained for post-proline endopeptidase from pig kidneys (67 000, 70 000, and 65 600) agree well with the data published on post-proline endopeptidase from other sources. The molecule of post-proline endopeptidase contains a relatively large number of leucine and dicarboxylic acids, as documented by the results of amino acid analysis. To our knowledge, no results have been published on the amino acid composition of post-proline endopeptidase obtained from other sources. However, the pI values of post-proline endopeptidases from other sources[2,4,6,8] are in the range of 4·5–4·9. These findings seem to indicate that the enzyme contains a large portion of acidic amino acids.

The hydrolytic activity of the enzyme was assayed in the presence of thiol compounds. The results indicate that the molecule of post-proline endopeptidase contains a thiol group which is necessary for the hydrolytic activity of the enzyme. Although the effect of the presence of cysteine and dithiothreitol on the enzyme

activity differed (dithiothreitol increased the activity, whereas cysteine did not), both compounds had the same ability to protect the thiol group of the enzyme against oxidation. The role of the thiol group of the post-proline endopeptidase molecule in the catalytic action of the enzyme and its interaction with the disulphide bridge of some substrates (*e.g.* neurohypophysial hormones) is being investigated.

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REFERENCES

1. Walter R., Shlank H., Glass J. D., Schwartz I. L., Kerenyi T. D.: Science *173*, 827 (1971).
2. Walter R., Simmons W. H., Yoshimoto T.: Moll. Cell. Biochem. *30*, 111 (1980).
3. *Biochemical Nomenclature and Related Documents.* International Union of Biochemistry, London 1978.
4. Orlowski M., Wilk E., Pearce S., Wilk S.: J. Neurochem. *33*, 461 (1979).
5. Knisatschek H., Bauer K.: J. Biol. Chem. *254*, 10 936 (1979).
6. Yoshimoto T., Simmons W. H., Kita T., Tsuru D.: J. Biochem. *90*, 325 (1981).
7. Orlowski M., Orlowski J., Lesser M., Kilburn K. H.: J. Lab. Clin. Med. *97*, 467 (1981).
8. Tate S. S.: Eur. J. Biochem. *118*, 17 (1981).
9. Hauzer K., Servítová L., Barth T., Jošt K.: This Journal *47*, 1139 (1982).
10. Hauzer K., Polgár L.: Acta Biochim. Biophys. Acad. Sci. Hung., in press.
11. Yoshimoto T., Fischl M., Orlowski R. C., Walter R.: J. Biol. Chem. *253*, 3708 (1978).
12. Recommendation of NC-IUB: *Units of Enzyme Activity.* Eur. J. Biochem. *97*, 319 (1979).
13. Davis B. J.: Ann. N. Y. Acad. Sci. *121*, 404 (1964).
14. Williams D. E., Reisfeld R. A.: Ann. N. Y. Acad. Sci. *121*, 373 (1964).
15. Reisfeld R. A., Lewis U. J., Williams D. E.: Nature (London) *195*, 281 (1962).
16. Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1961).
17. Bradford M. M.: Anal. Biochem. *72*, 248 (1976).
18. Weber K., Osborn M.: J. Biol. Chem. *244*, 4406 (1969).
19. Neville D. M.: J. Biol. Chem. *246*, 6328 (1971).
20. O'Farrell P. H.: J. Biol. Chem. *250*, 4007 (1975).
21. Margolis J., Kenrick K. G.: Anal. Biochem. *25*, 347 (1968).
22. Yphantis D. A.: Biochemistry *3*, 297 (1964).
23. Moore S.: J. Biol. Chem. *238*, 235 (1963).
24. Hartley B. S.: Biochem. J. *119*, 805 (1970).
25. Weiner A. M., Platt T., Weber K.: J. Biol. Chem. *247*, 3242 (1972).
26. Hauzer K.: Unpublished result.
27. Koida M., Walter R.: J. Biol. Chem. *251*, 7593 (1976).

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