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Purification of lysosomal prolylcarboxypeptidase angiotensinase C

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SUMMARY

Prolylcarboxypeptidase (angiotensinase C) was purified 247-fold from homogenized hog kidney cortex by the sequential application of ammonium sulfate precipitation, DEAE-Sephadex and hydroxylapatite column chromatographies. The enzyme was relatively heat stable and free of cathepsin A contamination. Disc gel electrophoresis revealed only a single band of protein indicating homogeneity. The molecular weight of the enzyme was estimated to be 210 000. The K_m of prolylcarboxypeptidase with Bz-Pro–Phe substrate was $1.3 \cdot 10^{-3}$ M. After pretreatment with 2-mercaptoethanol, urea and sodium dodecyl sulfate prolylcarboxypeptidase dissociated to subunits.

Prolylcarboxypeptidase hydrolyzes peptide bonds of C-terminal amino acids[1-3] with proline. Initially we used angiotensin II as substrate, since prolylcarboxypeptidase inactivated this peptide by cleaving the Pro^7–Phe^8 bond. Consequently we followed the terminology adopted by others[4] calling the partially purified enzyme angiotensinase C (refs 1,2) in our earlier publications. Because this enzyme attacks proline bonds in peptides other than angiotensin II, we renamed it prolylcarboxypeptidase. We detected prolylcarboxypeptidase in a lysosomal fraction of swine kidney[2], and leucocytes[2,5,6]. This paper deals with the purification of the kidney enzyme as reported briefly at a scientific meeting[7].

Hog kidney cortices were homogenized[2] in a Waring blender (1:1.7, w/v) in 0.02 M phosphate buffer (pH 6.8), containing 10^{-3} M EDTA (Table I). The supernatant was collected in the refrigerated centrifuge at 10 000 × *g* in 30 min. The active enzyme was precipitated

Abbreviations: DNS-, 1-dimethylaminonaphthalene-5-sulfonyl; Bz-, benzoyl; PCMS, *p*-chloromercuriphenylsulfonic acid.

from the supernatant between 30 % and 70 % saturation with solid $(NH_4)_2SO_4$. The precipitate was dialyzed overnight against 0.05 M phosphate buffer (pH 6.8) and concentrated with an Amicon PM-30 membrane filter under nitrogen pressure. The enzyme dissolved in the phosphate buffer was adsorbed on a DEAE-Sephadex A-50 column (4.5 cm × 35 cm). It was then eluted at the rate of 30 ml per h with a linear gradient of NaCl increasing in molarity from 0.05 to 0.5 M in the same phosphate buffer. Fractions of 10 ml each were collected. Prolylcarboxypeptidase was eluted from the column between 0.1 and 0.2 M concentrations of NaCl. The enzyme was concentrated again with the membrane filter and dialyzed against the buffer overnight. Prolylcarboxypeptidase was further purified on a hydroxylapatite column (2 cm × 43 cm). Using stepwise increasing concentrations of the phosphate buffer from 0.05 to 0.5 M, prolylcarboxypeptidase was eluted in a sharp peak of activity at 0.35 M concentration of the buffer. The active fraction was concentrated again with the membrane filter. The homogeneity of prolylcarboxypeptidase was investigated in disc gel electrophoresis in Tris–glycine buffer for 2 h at 2 mA per tube[8]. The molecular weight was also determined in electrophoresis on polyacrylamide gel, but in the presence of sodium dodecyl sulfate[9]. Prolylcarboxypeptidase was incubated for 1 h at 37 °C with sodium dodecyl sulfate, 2-mercaptoethanol, and urea in phosphate buffer or for 16 h at 60 °C. Electrophoresis was carried out for 4 h at 6 mA per tube[9].

The activity of prolylcarboxypeptidase was determined by measuring the hydrolysis of Bz-Pro–Phe (ref. 2). The reaction mixture contained 0.1 ml of $1 \cdot 10^{-2}$ M of peptide in 40 % dimethylsulfoxide, 0.15 ml of 0.2 M potassium phosphate buffer (pH 5.8) and 0.1 ml of enzyme. The reaction rate progressed in a linear fashion at 37 °C for 3 h when samples were withdrawn. The liberated phenylalanine was assayed in an automatic amino acid analyzer (BioCal 2000) equipped with a sample injector or by a manual ninhydrin determination technique[10]. In addition, qualitatively the cleavage of a peptide bond was shown in thin-layer chromatography on silica gel G with the fluorescent substrate DNS-Pro–Phe (refs 2, 11). The substrate was prepared as described previously[2]. Cathepsin A activity was measured with a similar technique, but with Bz-Glu–Tyr as substrate[12]. The concentrations of the proteins were determined according to the method of Lowry *et al.*[13].

A unit of the enzyme is the amount of protein needed to release 1 nmole of phenylalanine in 1 h. When inhibitors were used they were preincubated with the enzyme for 20 min.

The purification of prolylcarboxypeptidase of hog kidney is shown in Table I. After DEAE-Sephadex column chromatography two activity peaks were observed. The first major peak was eluted with 0.15 M NaCl, which contained prolylcarboxypeptidase and was purified further. Most of the activity in the second protein peak which was eluted with 0.2 M NaCl was attributed to heat sensitive cathepsin A present in the preparation. The purification of this fraction was not pursued.

After hydroxylapatite column chromatography 30 μg of purified enzyme was applied to polyacrylamide gel in disc electrophoresis. Only a single major band was detected after the gel was stained with Coomassie blue indicating that the prolylcarboxypeptidase preparation was essentially homogenous.

TABLE I

PURIFICATION OF PROLYLCARBOXYPEPTIDASE OF SWINE KIDNEY CORTEX

Substrate, Bz-Pro–Phe.

	Vol. (ml)	*Total units*	*Protein (mg/ml)*	*Units★/mg protein*	*Yield (%)*	*Purification*
Homogenate	720	33 750	38	1.25	100	1
$(NH_4)_2SO_4$ 70% satn	150	20 143	72	1.9	60	1.5
DEAE-Sephadex column chromatography	480	17 450	1.5	24.2	52	19
Hydroxylapatite column chromatography	21	710	0.11	309	2.1	247

★ Unit = 1 nmole/h per mg.

The molecular weight of renal prolylcarboxypeptidase was determined in sodium dodecyl sulfate electrophoresis against protein standards of known molecular weight (bovine serum albumin tetramer, trimer, dimer and monomer, β-galactosidase, ovalbumin, pepsin, α and β chains of hemoglobin[9]). Prolylcarboxypeptidase has a molecular weight of 210 000. The band due to the protein of 210 000 molecular weight was detected when applied to the gel without preincubation with sodium dodecyl sulfate. After preincubation with sodium dodecyl sulfate, mercaptoethanol and urea to varying lengths of time and at various temperatures, prolylcarboxypeptidase dissociated to several lower molecular weight subunits. The estimated molecular weight of the unit that migrated farthest in the gel was about 22 000–25 000. If this estimate is correct then the other subunits were multiples of the subunit of the smallest molecular weight. Accordingly, prolylcarboxypeptidase may contain as many as eight subunits.

The K_m of the purified enzyme Bz-Pro–Phe was $1.3 \cdot 10^{-3}$ M as calculated by plotting $1/v$ against $1/S$. Purified prolylcarboxypeptidase was stable when heated at 60 °C for 10 min. It was inhibited 100 % by 10^{-3} M DFP, but not at all by the same concentration of *p*-chloromercuriphenylsulfonic acid (PCMS). Bz-D-Phe also inhibited the enzyme 60 % at a $1 \cdot 10^{-3}$ M concentration. Prolylcarboxypeptidase cleaved DNS-Pro–Phe as shown in thin-layer chromatography[2]. The purified enzyme did not hydrolyze Bz-Glu–Tyr, the typical substrate of cathepsin A (ref. 12).

Prolylcarboxypeptidase was discovered by Erdös and Yang[14] during their studies on the metabolism of bradykinin by the kidney cortex. They observed that a renal enzyme cleaved the –Pro^7–Phe^8-OH bond in the des-Arg^9 derivative of the nonapeptide. Because the C-terminal end of angiotensin II was also phenylalanine this peptide was the substrate in subsequent studies. Prolylcarboxypeptidase was soon found, however, to hydrolyze many other peptide substrates[2] with a general structure of R_1-Pro–R_2-OH. Here, R_1 can be either a blocking group, a DNS- group, another protected amino acid, or a peptide. R_2 is an aromatic or aliphatic amino acid with a free carboxyl group. Substrates in which

proline is replaced by another amino acid were not cleaved. The enzyme is most active at pH below neutrality. It is inhibited by DFP, but not by EDTA or *p*-chloromercuriphenylsulfonic acid[2].

In this report we described the purification of the enzyme from hog kidney cortex, but PCP occurs elsewhere such as in human urine[2] or in a granular fraction of human leucocytes[5,6,11]. (The appearance of prolylcarboxypeptidase in biological fluids will be the subject of a later publication.) Prolylcarboxypeptidase is absent from dog kidney medulla (Sorrells and Erdös, unpublished), but it is present in rat kidney lysosomes[15]. An enzyme quite similar to prolylcarboxypeptidase was detected in a spleen extract[16]. The soluble fraction of homogenized rabbit liver contained an enzyme that was active above neutral pH and cleaved the C-terminal phenylalanine in angiotensin II (ref. 17). The identity of the liver enzyme with prolylcarboxypeptidase has not yet been explored.

We obtained a prolylcarboxypeptidase preparation that appeared to be homogenous in disc gel electrophoresis because it showed a single protein band. These experiments also indicated that prolylcarboxypeptidase has a molecular weight of 210 000. When the protein was preincubated with sodium dodecyl sulfate, urea and 2-mercaptoethanol, it dissociated to subunits, the smallest of them having a molecular weight of approximately 25 000. This was taken as an indication that prolylcarboxypeptidase may consist of 8 units.

Recently, cathepsin A at a high concentration has also been shown to cleave prolylphenylalanine bond in angiotensin II (ref. 18). Our preparation of prolylcarboxypeptidase was contaminated by cathepsin A (ref. 1) in the initial stages of purification, but prolylcarboxypeptidase was separated from the catheptic enzyme during column chromatography. Although the two enzymes have some properties in common, they can be readily distinguished. Cathepsin A is easily destroyed by heating, while prolylcarboxypeptidase resists 60–65 °C temperature[1,16,19]. Because of this prolylcarboxypeptidase was purified first from a heated homogenate of kidney cortex[1].

Cathepsin A can act either as an endopeptide (peptidyl peptide hydrolase) or as a carboxypeptidase (peptidyl-L-amino acid hydrolase[12,18,20]), and does not require proline in the penultimate position of the substrate. A commonly used substrate of cathepsin A is Bz-Glu–Tyr (ref. 12), a peptide not cleaved by prolylcarboxypeptidase[2]. Cathepsin A is inhibited by PCMS, while the enzyme we purified is not[2,21]. Recently the suggestion was made that a spleen enzyme that is probably identical with prolylcarboxypeptidase should be called "catheptic carboxypeptidase C" (ref. 16). Prolylcarboxypeptidase, however, lacks some of the general properties of catheptic enzymes[1,2]. An enzyme extracted from citrus peel had already been named carboxypeptidase C (refs 22, 23). This enzyme cleaves substrates which are not hydrolyzed prolylcarboxypeptidase. Because of these considerations, we prefer to retain the term prolylcarboxypeptidase for the enzyme described in this publication.

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REFERENCES

1 Yang, H.Y.T., Erdös, E.G. and Chiang, T.S. (1968) *Nature* 218, 1224–1226
2 Yang, H.Y.T., Erdös, E.G., Chiang, T.S., Jenssen, T.A. and Rodgers, J.G. (1970) *Biochem. Pharmacol.* 19, 1201–1211
3 Yang, H.Y.T. and Erdös, E.G. (1971) in *Immunopathology of Inflammation* (Forscher, B.K. and Houck, J.C., eds), pp. 146–148, Excerpta Medica, Amsterdam
4 Erdös, E.G. (1971) in *Handbook of Experimental Pharmacol.* (Brodie, B.B. and Gillette, J.R., eds), Vol. XXVIII, Part B, pp. 620–653, Springer-Verlag, Heidelberg
5 Sorrells, K. and Erdös, E.G. (1972) in *The Fundamental Mechanisms of Shock* (Hinshaw, L.B., ed.), pp. 393–397, Plenum Press, New York
6 Sorrells, K. and Erdös, E.G. (1971) *Fed. Proc.* 30,600
7 Sorrells, K., Kakimoto, T. and Erdös, E.G. (1972) *Abstr. 5th Int. Congr. Pharmacol., San Francisco, Calif.* 219
8 Davis, B.J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427
9 Shapiro, A.L., Vinuela, E. and Meizel, Jr, J.V. (1967) *Biochem. Biophys. Res. Commun.* 28, 815–819
10 Yemm, E.W. and Cocking, E.C. (1955) *Analyst* 80, 209–213
11 Sorrells, K., Erdös, E.G. and Massion, W.H. (1972) *Proc. Soc. Exp. Biol. Med.* 140, 310–313
12 Iodice, A.A., Leong, V. and Weinstock, I.M. (1966) *Arch. Biochem. Biophys.* 117, 477–486
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275
14 Erdös, E.G. and Yang, H.Y.T. (1966) in *Hypotensive Peptides* (Erdös, E.G., Back, N. and Sicuteri, F., eds), pp. 235–250, Springer-Verlag, New York
15 Matsunaga, M., Kira, J., Saito, N., Ogino, K. and Takayasu, M. (1968) *Jap. Circ. J.* 32, 137–143
16 McDonald, J.K., Zeitman, B.B. and Ellis, S. (1972) *Biochem. Biophys. Res. Commun.* 46,62–70
17 Johnson, D.C. and Ryan, J.W. (1968) *Biochim. Biophys. Acta* 160, 196–203
18 Logunov, A.I. and Orekhovich, V.N. (1972) *Biochem. Biophys. Res. Commun.* 46, 1161–1168
19 Matsunaga, M. (1971) *Jap. Circ. J.* 35, 333–338
20 Iodice, A.A. (1967) *Arch. Biochem. Biophys.* 212, 241–242
21 Misaka, E. and Tappel, A.L. (1971) *Comp. Biochem. Physiol.* 38B, 651–662
22 Zuber, H. (1964) *Nature* 201, 613
23 Sprössler, B., Heilmann, H.-D., Grampp, E. and Uhlig, H. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* 352, 1524–1530