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Short communication

High-performance liquid chromatographic determination of prolylcarboxypeptidase activity in monkey kidney

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Abstract

A simple HPLC procedure for the determination of prolylcarboxypeptidase activity in monkey kidney was established with Cbz-Pro-Ala used as substrate. Decrease of the substrate and increase of the product were stoichiometrically related to each other. Heat treatment at 60°C freed the enzyme preparation of contaminating activities. Data on substrate specificity and influence of inhibitors suggested this method was sensitive for the determination of prolylcarboxypeptidase without the use of a radioactive substrate.

1. Introduction

Prolylcarboxypeptidase (lysosomal Pro-X carboxypeptidase, EC 3.4.16.2, PCP) was first detected in the lysosomal fraction of pig kidney by Yang et al. as an enzyme that could inactivate angiotensin II [1,2]. The enzyme catalyzes the hydrolysis of C-terminal amino acid residues from peptides with the general structure R_1-Pro-R_2-OH, where R_1 is either a blocking group, another protected amino acid, or a peptide, and R_2 is an aromatic or aliphatic amino acid with a free carboxyl group [3]. The enzyme was named angiotensinase C because it cleaves and inactivates the C-terminal Pro-Phe bond in angiotensin II and angiotensin III [1–4]. It is of importance in the renin-angiotensin system and is in the etiology of high blood pressure. The enzyme was detected in the lysosomal fraction of homogenized pig kidney [5], human leucocytes [2,6] and in urine [2]. In addition, the same or similar enzyme is also present in rat liver and bovine spleen [7,8].

PCP is distinguishable from cathepsin A (serine-type carboxypeptidase, EC 3.4.16.1) and carboxypeptidase P (membrane Pro-X carboxypeptidase, EC 3.4.17.16) by its stability to heating [1,7,8] and optimum pH [4].

Radioassay utilizing a radiolabeled substrate [9] or measurement with an amino acid analyzer [4,5] is generally used for assaying PCP [3]. The measurement of the enzyme activity in large numbers of samples has been hampered by the lack of an easy assay procedure. In this study we used a high-performance liquid chromatographic (HPLC) method utilizing *N*-benzyloxycarbonyl-L-prolyl-L-alanine (Cbz-Pro-Ala) for the easy determination of PCP activity. The present communication describes this simple HPLC procedure for the determination of prolylcarboxypep-

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tidase as a part of our series [10–13] of studies on proline-specific exopeptidase activities.

2. Experimental

2.1. Chemicals

Cbz-Pro-Ala, Cbz-Pro, Cbz-Pro-Phe, *p*-chloromercuriphenylsulfonic acid (Sigma, St. Louis, MO, USA), Cbz-Glu-Tyr (Peptide Institute, Minoh, Osaka, Japan), and phenylmethylsulfonyl fluoride (Wako Pure Chem., Osaka, Japan) were purchased from the sources listed. All the other products were of the highest purity available.

2.2. Enzyme preparation

Monkey (Japanese monkey, *Macaca fuscata*) organs were kindly supplied by Dr. Y. Yoshikawa, Department of Orthodontics, Matsumoto Dental College, and kept frozen at −80°C until used. PCP was prepared from monkey kidney and other organs by the method of Odya et al. [4]. Tissue was homogenized in 2 volumes of 20 m*M* sodium phosphate buffer, pH 6.8, that contained 0.1 *M* NaCl and 1 m*M* EDTA with an Ultra-turrax homogenizer, and the homogenate was then centrifuged for 15 min at 12 000 *g*. The supernatant was decanted, and its pH adjusted to 4.5 by the addition of 40% citric acid solution. After 10 min at ambient temperature, precipitated proteins were removed by centrifugation as before. The pH of the retentate was adjusted at room temperature to 7.0 with 2 *M* Tris base and then heated at 60°C for 30 min. Precipitated proteins were removed by centrifugation as before, and the resulting preparation was used as the enzyme source. This fraction was frozen at −20°C, and enzyme activities were tested within two months after collection.

Protein concentrations were determined by the method of Hartree [14] with bovine serum albumin (Calbiochem, La Jolla, CA, USA) as standard.

2.3. Chromatographic determination of PCP activity

Incubation was carried out in all instances in a standard reaction mixture (400 μl) containing 125 mmol of sodium acetate buffer (pH 5.0), 0.40 μmol of Cbz-Pro-Ala, 0–100 μl of tissue homogenate, and water. The temperature was 37°C, and the period of incubation was 1 h. When a low level PCP activity was detected in a tissue homogenate, neomycin sulfate was added to a final concentration of 0.2% in the assay mixture, and incubation time was extended to 18 h. The reaction was terminated by addition of 200 μl of 10% perchloric acid followed by centrifugation for 5 min at 10 000 *g* to remove the insoluble material. A 20-μl amount of 5 *M* NaOH was added to the 180-μl supernatant for neutralization before analysis. The concentration of substrate in the clear medium solution was determined by injection of a 20-μl sample into an HPLC apparatus equipped with an autosampler (Jasco 850-AS with 801-SC). Cbz-Pro-Ala and Cbz-Pro, enzymatically formed from the Cbz-Pro-Ala in the assay mixture, were chromatographically separated by HPLC with 10.0 m*M* potassium phosphate buffer (pH 6.6) containing 3% acetonitrile as the mobile phase, at a flow-rate of 1.0 ml/min at a temperature of 55°C, and the eluate was monitored for the substrate or the product at 210 nm. The column (150 × 4.6 mm I.D.) was packed with Zorbax ODS, particle size 5 μm, supplied by DuPont (Wilmington, DE, USA). A guard column (50 × 4.0 mm I.D.) containing Permaphase ETH of particle size of 30 μm, also supplied by DuPont, was connected to the separation column.

3. Results and discussion

The retention times of Cbz-Pro-Ala and Cbz-Pro were determined to be 6.4 and 4.9 min, respectively, with 10 m*M* potassium phosphate buffer (pH 6.6) as the mobile phase (Figs. 1A, 1B).

The activity of PCP was detected in monkey kidney. Incubation of Cbz-Pro-Ala with increas-

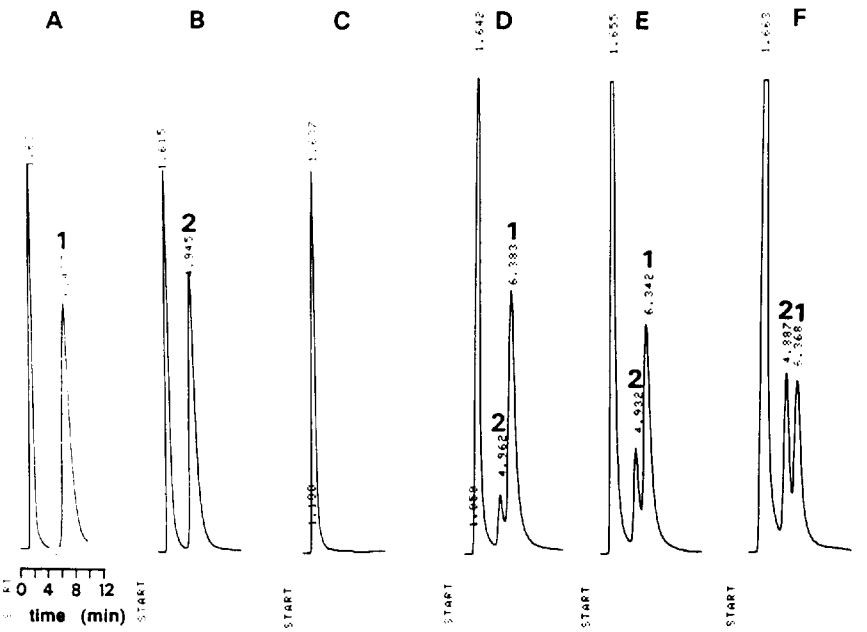

Fig. 1. Typical chromatographic patterns of the authentic peptides and Cbz-Pro-Ala with different concentrations of monkey kidney homogenate. (A) Cbz-Pro-Ala, 0.4 μmol; retention time 6.4 min (peak 1); (B) Cbz-Pro, 0.4 μmol; retention time 4.9 min (peak 2); (C) without substrate; (D) Cbz-Pro-Ala with 150 mg of monkey kidney homogenate; (E) with 300 mg of monkey kidney homogenate; (F) with 600 mg of monkey kidney homogenate.

ing amounts of kidney homogenate (Figs. 1D–1F) resulted in a gradual decrease in the Cbz-Pro-Ala peak (peak 1) with a concomitant increase in the 4.9-min peak corresponding to Cbz-Pro (peak 2). The total value for substrate, reaction product, and the amount of each detected were plotted against the amount of homogenate protein used (Fig. 2). As can be clearly seen, the Cbz-Pro-Ala and Cbz-Pro concentrations were stoichiometrically related to each other. The activity of PCP in the crude homogenate of monkey kidney was 0.378 ± 0.004 (mean of three times experiments ± standard error) μmol per mg protein per hour.

Cbz-Pro-Ala-hydrolyzing activity was fairly stable in heat, with a loss of only 28% of the activity and 38% of the protein concentration after treatment at 60°C for 30 min (Fig. 3). The C-terminal attack on Cbz-Pro-Ala cannot be attributed to cathepsin A, which is able to cleave the Pro-Phe bond of angiotensin II at an acidic pH [15]. We determined the cathepsin A activity by the HPLC technique, which was the same as for PCP except that a pH 5.5 buffer containing 2% acetonitrile was used as the mobile phase at 50°C. The retention times of Cbz-Glu-Tyr, which

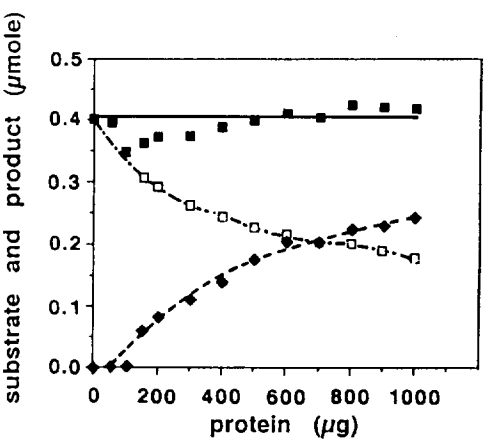

Fig. 2. Dependence of Cbz-Pro formation on monkey kidney homogenate concentration. Cbz-Pro (◆) formed from Cbz-Pro-Ala (□) by PCP activity. Individual values and the total amount (■) of these two peptides were plotted against the amount of homogenate protein used.

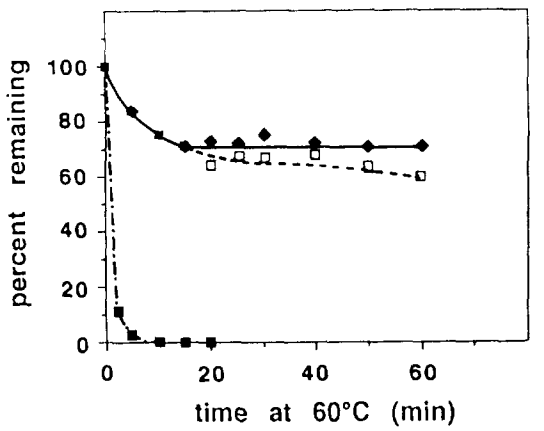

Fig. 3. Effect of heat treatment on Cbz-Pro-Ala- (□) and Cbz-Glu-Tyr- (■) hydrolyzing activities in homogenate of kidney. Aliquots were removed at various times and centrifuged to remove denatured protein. Activities and soluble protein concentration (◆) contained in the supernatant were assayed as described in the text.

is known as a pepsin substrate that can also be used as a substrate for cathepsin A [16], and the product, Cbz-Glu, were determined to be 5.9 and 2.4 min, respectively. As shown in Fig. 3, cathepsin A activity disappeared quickly by heat treatment at the temperature used in preparation of the enzyme source. Within 15 min, this activity in the crude homogenate of kidney promptly decreased to less than 0.1% of that before treatment (12.25 μmol per mg protein per hour).

The substrate specificity of PCP was studied at pH 5.0 by comparing the rate of hydrolysis of Cbz-Pro-Ala, Cbz-Pro-Phe, and Cbz-Glu-Tyr (Table 1). The Cbz-Pro-Phe-hydrolyzing activity was determined by the same HPLC technique as used for Cbz-Pro-Ala except that buffer containing 8% acetonitrile was used as the mobile phase, resulting in a retention time of Cbz-Pro-Phe of 9.7 min. Cbz-Pro-Ala represents the C-terminal end of the angiotensin II antagonist saralasin and is cleaved by purified human PCP at a rate 4.2-fold faster than Cbz-Pro-Phe, representing the C-terminal end of angiotensins II and III [4]. Our crude PCP preparation of monkey kidney similarly cleaved Cbz-Pro-Ala at a rate 2.5-fold faster than that for Cbz-Pro-Phe cleavage. Table 1 also shows that when the rate of hydrolysis of Cbz-Pro-Ala at optimum pH was

Table 1
Substrate specificity of monkey kidney homogenate and percent inhibition of PCP activity by several inhibitors

Substrate	Rate of hydrolysis (%)[a]	
	Monkey kidney homogenate	Purified human PCP[b]
Cbz-Pro-Ala	100	100
Cbz-Pro-Phe	40.2	23.8
Cbz-Glu-Tyr	0	0
Cbz-Pro-Ala (pH 7.8)	0	–
Inhibitor (1 mM)		
Phenylmethylsulfonyl fluoride	31.4	0
p-Chloromercuri-phenylsulfonic acid	99	85
EDTA	98	100

[a] Each value is the mean of duplicate experiments.
[b] Data from Odya et al. [4]

taken as 100%, the rate dropped to 0% at pH 7.8, the pH employed when Cbz-Pro-Ala is used as a substrate of carboxypeptidase P. Since prolyl oligopeptidase (EC 3.4.21.26) has no activity toward Cbz-Pro-Ala and Cbz-Pro-Phe either at neutral and acidic pH [17], we think our method can detect true PCP activity.

The data also show the similarity in chemical properties between crude monkey PCP and purified human PCP; that is, both enzymes were inhibited by 1 m*M* phenylmethylsulfonylfluoride, but not by an SH enzyme inhibitor, i.e., *p*-chloromercuriphenylsulfonic acid, and EDTA had no effect on either PCP.

We examined the distribution of PCP activity in eight organs of the monkey. A high level of PCP activity was found in the kidney and a low level in the liver (0.040 μmol per mg protein per hour) and in the spleen (0.027 μmol per mg protein per hour), but no activity could be detected in the submaxillary gland, parotid gland, lung, brain, or heart. Kumamoto et al. reported that the human kidney and lung were rich in PCP [18]. It is not clear why we could not detect the activity in monkey lung.

Also we could not detect PCP in rat kidney

when we used the present method. Rat [15] and pig [5] kidney homogenates are reported to have a low PCP activity, being almost one-twentieth that of humans, and a long incubation time at 37°C is required for the enzyme assay. Actually, the physiological concentration of angiotensin II in human plasma is 10^{-10} to 10^{-9} M, which is one to two orders of magnitude greater than that of rat plasma [19]. As PCP has a role of inactivating angiotensin II or III, its low level of activity in rat kidney, and probably in pig kidney also, is not surprising.

Because the biochemical properties of components in the monkey renin-angiotensin system are very similar to those of the human system, monkeys have been utilized in many studies. Therefore, we think that primates are also suitable for study of PCP and that the present method can replace the radioassay method.

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