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Note

High-performance liquid chromatographic separation of peptides possessing a proline residue in the amino-terminal penultimate position, and their products generated by enzymatic hydrolysis

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Dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5) is a brush border membrane glycoprotein enzyme [1,2] that specifically hydrolyses Xaa-Pro-Yaa- peptides (Xaa and Yaa are non-specified amino acids) into Xaa-Pro + Yaa-, except when the Yaa position is occupied by Pro or Hyp [3]. Conversely, amino peptidase-P (AP-P, EC 3.4.11.9) specifically hydrolyses the peptide bond between Xaa and Pro residues of such peptides to produce Xaa + Pro-Yaa- [4,5]. For determination of these enzyme activities, synthetic chromophore-bearing peptides, such as Gly-Pro-pNA [6] and Gly-Pro-MCA [7], are generally available for DPP-IV; *p*-nitro-L-phenylalanyl-Pro-Pro-HN-CH_2-CH_2-NH-*o*-aminobenzoyl is used for AP-P [5].

To determine the steps involved in the degradation of peptides in specialized tissues or sub-cellular fractions, however, utilization of native peptides as substrate should be an advantage for considering the hydrolytic process physiologically. In this paper we report the conditions for the separation by high-performance liquid chromatography (HPLC) of peptides possessing a proline residue in the amino-terminal penultimate position and the application of the method in an enzyme assay using the microvillous fraction of rabbit kidney.

EXPERIMENTAL

Materials

Gly-Pro-L-Arg diacetate and Gly-Pro-Arg-L-Pro acetate were purchased from Bachem (Bubendorf, Switzerland). L-Pro-L-Hyp, Gly-L-Pro-L-Ala and Gly-L-

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Pro-L-Hyp acetate were obtained from Sigma (St. Louis, MO, U.S.A.). Gly-L-Pro was obtained from the Peptide Institute (Minoh, Osaka, Japan). HPLC-grade acetonitrile and sodium 1-octanesulphonate for ion-pair chromatography were purchased from Junsei Chemical and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. The other reagents used in this work were reagent grade.

Apparatus

A Shimadzu LC-4A liquid chromatograph equipped with a Rheodyne sample injector (20-μl sample loop) and a Shimadzu SDP-24S UV monitor was used. The chromatograms were recorded with a Shimadzu C-R3A Chromatopac graphic data system monitoring the absorbance (0.04 or 0.08, a.u.f.s.) of peptides at 210 nm. The column (15 cm$\times$4.6 mm I.D.) was packed with Zorbax ODS.

Chromatography

The mobile phases [8,9] were 2.0, 10.0, and 20.0 m*M* potassium dihydrogenphosphate adjusted to pH 2.1, 2.5, or 3.0 with orthophosphoric acid. When necessary, 1-octanesulfonate was added at a concentration of 1.0 m*M* to an aliquot of this solution. Acetonitrile was also used to obtain good separation of the peptides.

The HPLC system was operated at a flow-rate of 1.0 ml/min and a temperature of 50°C throughout the determination [9].

The standard solutions of the peptides (0.1 m*M*) were directly injected after dilution with water of 3.0 m*M* stock solutions. The mean values for retention time and peak areas were determined by analysing 10 μl of each solution three times.

Application to determination of DPP-IV and AP-P activities

In order to determine the enzyme activity in kidney microvillous fraction, the substrate and the peptides produced were separated by the above-mentioned methods. The microvillous fraction was prepared according to the method of Booth and Kenny [10].

The assay medium (500 μl) contained 0.03 mmol of Tris–HCl buffer (pH 8.0), 0.30 μmol of peptide substrate, an appropriate amount (μg) of microvillous fraction and water. When AP-P activity was determined, 1.0 μmol of manganese chloride was added. Three different assay mixtures were incubated with different protein concentrations at 37°C for 30 min, and the reactions were stopped by the addition of 400 μl of 10% perchloric acid to each tube. After centrifugation of the assay tubes at 3000 *g* for 10 min, the concentration of the remaining and/or formed peptides in the clear medium solution was determined by HPLC. Incubation time dependency (0–30 min) of the enzyme activities was also observed in the same assay mixture.

Michaelis constant (K_M) values to Gly-Pro-Ala for DPP-IV and to Gly-Pro-Hyp for AP-P in rabbit kidney microvillous fraction were calculated by a Y.H. P-97 computer programmed for Michaelis–Menten enzyme kinetics [11].

Protein concentrations were determined by the method of Hartree [12] with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Separation of standard proline-containing peptides by HPLC

To examine the substrate specificity of DPP-IV and AP-P, the HPLC separation of the standard peptides Gly-Pro-Arg, Gly-Pro-Hyp and Gly-Pro-Arg-Pro and their hydrolytic products Gly-Pro and Pro-Hyp was studied (Table I).

The retention time of Gly-Pro-Arg-Pro, which inhibits fibrinogen aggregation [13], was clearly different from that of Gly-Pro with 2.0 m*M* potassium dihydrogenphosphate at pH 2.1, while Gly-Pro-Arg and Gly-Pro were separated with the same mobile phase but at a slightly higher pH.

The complete separation of Gly-Pro-Ala and Gly-Pro was achieved using ion-pair chromatography with 10.0 m*M* potassium dihydrogenphosphate (pH 2.5) containing 1.0 m*M* 1-octanesulphonate, although the peaks became rather broad at the longer retention time. To obtain more rapid elution of the peptides with fairly good separation, 20.0 m*M* potassium dihydrogenphosphate (pH 3.0) was found to be suitable.

Gly-Pro-Hyp could be useful as a substrate for AP-P. The separation of Gly-Pro-Hyp and Pro-Hyp with a mobile phase of 10.0 m*M* potassium dihydrogenphosphate containing 1.0 m*M* 1-octanesulphonate (pH 2.1) was possible, but the peaks of Gly-Pro and Pro-Hyp could not be separated. A mobile phase consisting of 10.0 m*M* potassium dihydrogenphosphate containing 1.0 m*M* 1-octanesulphonate (pH 2.1) and acetonitrile (15:85, v/v) was the best for the separation of Gly-Pro, Pro-Hyp and Gly-Pro-Hyp, though the retention times of their peaks became retarded.

Application of the method to the determination of enzyme activity

For study of the hydrolysis of a peptide in a specific tissue or sub-cellular fraction, it is necessary to make a quantitative determination of liberated and residual portions of the substrate.

It is accepted that cytosol and microsomal aminopeptidases cannot hydrolyse the peptide bonds formed between the carbonyl group of an N-terminal amino acid residue and the secondary amine group of a proline residue, such as is found in a Gly-Pro-Y-peptide. In order either to initiate or to complete the degradation of such peptides to their constituent amino acids, a specific aminopeptidase must attack the peptide bonds of the N-terminal or C-terminal side of proline residues in the peptides.

The HPLC method of separation of the peptides was applied to the identification and/or determination of hydrolytic enzymes in the microvillous fraction of rabbit kidney. Using Gly-Pro-Ala as the substrate, the hydrolytic activity of DPP-IV was determined to depend on the microvillous protein concentration (0–250 μg). For an enzyme blank, the reaction was carried out without substrate, and after 30 min the reaction was stopped with perchloric acid. No peaks were identified at the retention times of the peptides formed.

Typical chromatograms obtained with the rabbit kidney microvillous fractions and those of the blanks are shown in Fig. 1. The retention times for peaks 1 and 2 were 6.4 and 4.9 min, respectively. The peak for Gly-Pro was identified on the

TABLE I

RETENTION TIMES OBSERVED WITH PROLINE-CONTAINING PEPTIDES WITH DIFFERENT MOBILE PHASES

Mobile phase	pH	Retention time (min)					
		Gly-Pro	Pro-Hyp	Gly-Pro-Ala	Gly-Pro-Arg	Gly-Pro-Hyp	Gly-Pro-Arg-Pro
2.0 m*M* KH_2PO_4	2.1	2.183	2.174	2.333	2.183	2.780	7.365
	2.5	2.468		2.698	3.115		
10.0 m*M* KH_2PO_4	2.1	2.177	2.129	2.307	2.150	2.704	
	2.5	2.224	2.208	2.390	2.382		
10.0 m*M* KH_2PO_4–1.0 m*M* 1-octanesulphonate	2.1	4.042	3.788			5.372	
	2.5	4.992		6.433			
10.0 m*M* KH_2PO_4–1.0 m*M* 1-octanesulphonate–acetonitrile (15:85)	2.1	5.867	6.573			8.598	
20.0 m*M* KH_2PO_4	3.0	2.153		2.372			

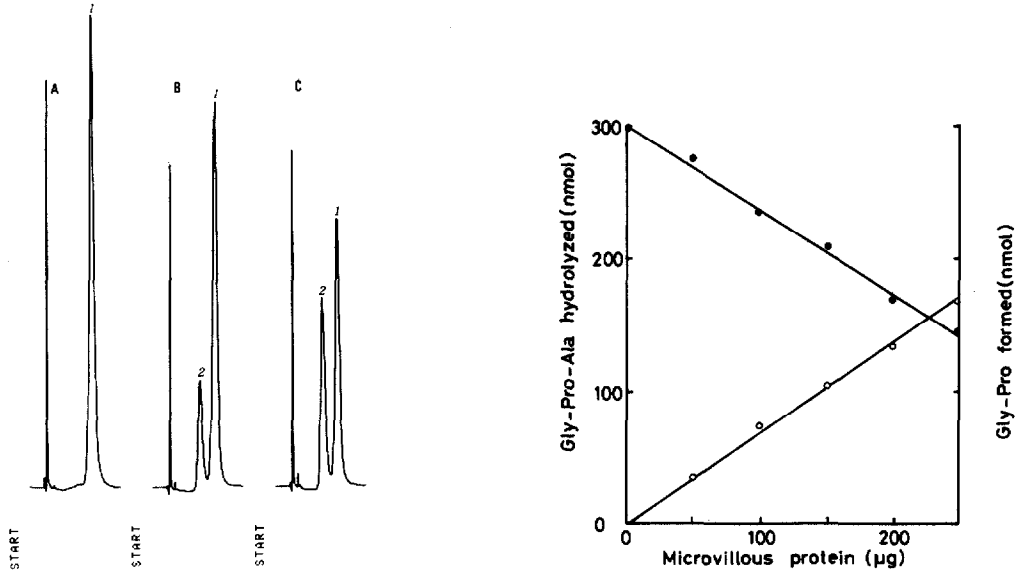

Fig. 1. Typical chromatographic patterns of the hydrolysis of Gly-Pro-Ala with different concentrations (A, 0 μg; B, 100 μg; C, 200 μg) of rabbit kidney microvillous fractions. Peak 1 (A), Gly-Pro-Ala (3.3 nmol, retention time 6.4 min) decreased, and peak 2 (B,C), Gly-Pro (retention time 5.0 min) increased at each protein concentration. Mobile phase, 10.0 m*M* potassium dihydrogenphosphate containing 1.0 m*M*, 1-octanesulphonate (pH 2.5).

Fig. 2. Dependence of microvillous protein concentration of Gly-Pro formation from Gly-Pro-Ala catalysed by DPP-IV. Each value of the sample concentration represents the mean of three different determinations. Hydrolysis of Gly-Pro-Ala (●) and formation of Gly-Pro (○) were stoichiometrically related under the analytical conditions described in Experimental.

basis of its retention time and by co-chromatography with the standard. The peak for Pro-Ala was not identified.

DPP-IV activity was linear up to 250 μg of microvillous protein concentration (Fig. 2). The amount of substrate degradation corresponded stoichiometrically to Gly-Pro formation. Gly-Pro-Ala in the concentration range 0.1–2.0 m*M* gave a maximum and constant activity of DPP-IV in kidney microvillous preparation, with K_M values (mean ± S.E., $n=3$ each) of 0.27 ± 0.01 m*M* (data not shown).

On the other hand, AP-P activity in the microvillous fraction of rabbit kidney was also determined using Gly-Pro-Hyp as substrate, since it was not attacked by DPP-IV [3]. Typical chromatograms of AP-P activity are shown in Fig. 3. The retention times for peaks 1 and 2 were 5.4 and 3.8 min, respectively. The peak of Pro-Hyp was identified on the basis of its retention time and by co-chromatography with the standard. As shown in Fig. 4, the extent of formation of Pro-Hyp in the assay mixture increased linearly with microvillous protein concentration, and the amount of substrate degradation corresponded to the Pro-Hyp formation. The K_M value (mean ± S.E., $n=3$ each) of AP-P was determined to be 1.08 ± 0.18 m*M* towards Gly-Pro-Hyp in a concentration range 0.1–5.0 m*M* (data not shown).

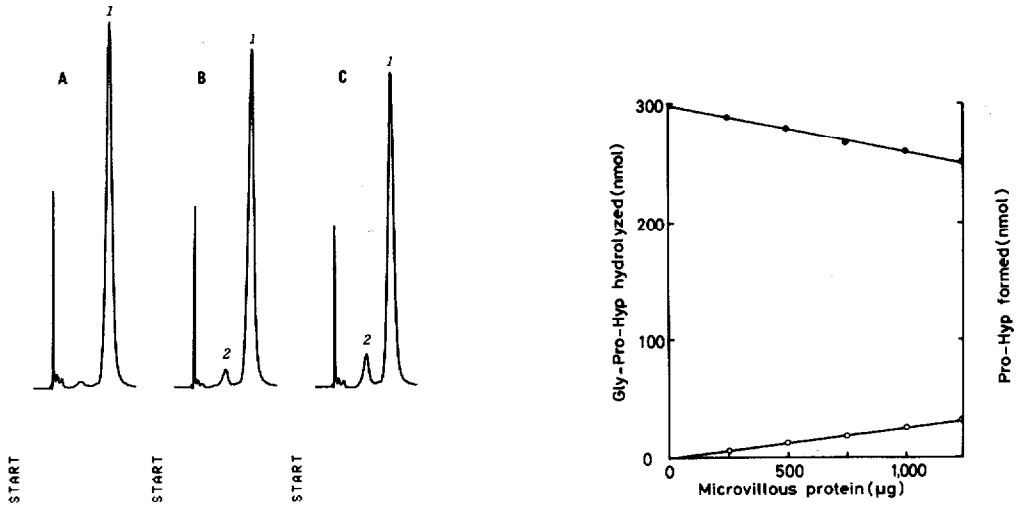

Fig. 3. Typical chromatographic patterns of the hydrolysis of Gly-Pro-Hyp with different concentrations (A, 0 μg; B, 500 μg; C, 1000 μg) of rabbit kidney microvillous fractions. Peak 1 (A), Gly-Pro-Hyp (3.3 nmol, retention time 5.4 min) decreased, and peak 2 (B,C), Pro-Hyp (retention time 3.8 min) increased, at each protein concentration. Mobile phase, 10.0 m*M* potassium dihydrogenphosphate containing 1.0 m*M* 1-octanesulphonate (pH 2.1).

Fig. 4. Dependence of microvillous protein concentration of Pro-Hyp formation from Gly-Pro-Hyp catalysed by AP-P. Each value of the sample concentration represents the mean of three different determinations. Hydrolysis of Gly-Pro-Hyp (●) and formation of Pro-Hyp (○) were stoichiometrically related under the analytical conditions described in Experimental.

For calibration curves of the peptides, linear relationships were observed between the peak area of each standard peptide and the concentration over the range from 0.5 nmol per 5 μl to 2.0 nmol per 20 μl at 0.04 a.u.f.s. The detection limit for Gly-Pro or Pro-Hyp formed enzymatically was 4–5 nmol per assay mixture or 50 pmol per 10 μl of injection volume at 0.01 a.u.f.s.

Using Gly-Pro-Ala as the substrate, we found the microvillous fraction to hydrolyse the peptide and produce only one new peak with the retention time of Gly-Pro, and this peak also gradually decreased with time (data not shown). From these results it appears that DPP-IV in the microvillous fraction first attacks the peptide bond between Pro and Ala to form Gly-Pro and that prolidase then splits the bond between Gly and Pro.

AP-P activity was also determined in the microvillous fraction when Gly-Pro-Hyp was used as the substrate, but the activity was less than 10% of the DPP-IV activity. Therefore, the Pro-Ala peptide was not detected under the experimental conditions described above.

Recently, Holtzman et al. [14] detected AP-P activity in human serum, using a new fluorescent substrate with only 10 μl of serum. Our present assay procedure could not be applicable to the determination of enzyme activity in serum directly. The concentration of the enzyme fraction in serum will be required for the determination of serum activity with this procedure.

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