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Note

High-performance liquid chromatographic determination of aminopeptidase P activity in Fischer F344 rat serum and kidney

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It is well accepted that cytosolic and microsomal aminopeptidases cannot hydrolyse the peptide bond formed between the carbonyl group of an N-terminal amino acid residue and the secondary amine of a proline residue such as found in Xaa–Pro–Yaa peptide (Xaa and Yaa indicate amino acids). In order either to initiate or to complete the degradation of such peptides to their constituent amino acids, specific amino peptidases must attack the peptide bonds of the N-terminal and C-terminal side of proline residues in these peptides.

Aminopeptidase P (AP-P; EC 3.4.11.9) specifically hydrolyses the peptide bond linking the N-terminal amino acid residue to proline in oligopeptides [1,2], whereas dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) specifically hydrolyses Xaa–Pro–Yaa peptides into Xaa–Pro + Yaa peptides, except when the Yaa position is occupied by Pro or Hyp [3,4]. One of the physiological roles of DPP IV in the brush border of kidney was recently demonstrated using β-casomorphin as being involved in the uptake of small peptides into vesicles by the peptide transport system of the kidney [5].

Fischer F344 rats have been widely used for testing possible carcinogens ever since the NCI (U.S.A.) established guidelines [6] recommending the use of this strain for such tests. In this rat several organs were reported to be deficient in DPP IV not only in terms of its activity but also in terms of the protein itself [7]. One problem with such a deficiency might be the impaired metabolism of Xaa–Pro–Yaa peptides, as these peptides might not be degraded, owing to the

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low DPP IV activity, and hence not be reabsorbed by the kidney brush border membrane.

Because AP-P can also attack the peptides, the activity of this enzyme was determined in the F344 rat serum and kidney by a recently established high-performance liquid chromatographic (HPLC) method [8]. In this study, the AP-P activity in F344 rat serum and microsomal fraction of the kidney was demonstrated to be similar to that formed in control CRJ:CD(SD) rats and we suggest that it plays an important role in hydrolysing Xaa–Pro–Yaa peptides instead of DPP IV activity.

EXPERIMENTAL

Chemicals

L-Pro–L-Hyp, L-Pro–L-Ala, Gly–L-Pro–L-Hyp acetate and Gly–L-Pro–L-Ala were obtained from Sigma (St. Louis, MO, U.S.A.). Gly–Pro–pNA was kindly provided by Ajinomoto (Tokyo, Japan).

Preparation of rat materials for enzyme source

Male Fischer F344/DU Crj rats (5–6 weeks old) and male CRJ:CD(SD) rats (5–6 weeks old) as the control were purchased from Charles River Japan.

The animals were anaesthetized with diethyl ether and whole blood was drawn by cardiac puncture. The sera were separated by centrifugation of the blood samples at 3000 *g* for 10 min. After blood had been collected, the kidneys were carefully removed. The sera and kidneys were kept frozen at −80°C until used.

DPP IV and AP-P have both been purified from the microsomal fraction of kidney [9,10]; therefore, the microsomal fraction of rat kidney was used for the determination of the activities. The method of Hogeboom [11] was employed for its preparation.

Chromatographic determination of AP-P activity

AP-P activity was determined chromatographically using Gly–Pro–Hyp and Gly–Pro–Ala as substrates with an application of the new method [8]. Enzymatically formed Pro–Hyp or Pro–Ala from the substrates in the assay mixture was chromatographically separated by HPLC with a mobile phase composed of 10.0 m*M* potassium dihydrogenphosphate containing 1.0 m*M* 1-octanesulphonate, of pH 2.1 for Gly–Pro–Hyp and pH 2.5 for Gly–Pro–Ala, and determined by the absorbance of the peptides at 210 nm. The column (15 cm × 4.6 mm I.D.) was packed with Zorbax ODS, particle size 5 μm, supplied by DuPont (Wilmington, DE, U.S.A.).

To determine the AP-P activity in serum, 100–200 μl of serum were required owing to its low activity. Therefore, quantitatively concentrated (two-fold) serum, obtained using collodion bags (Ultra-Thimbles UH 100/75; Schleicher

& Schüll, Dassel, F.R.G.) in a cold room, was employed. This simple procedure enabled us to observe the AP-P activity in serum. For the determination of AP-P activity in kidney, about 500 μg of microsomal protein were used for both rat strains.

Other enzyme assay and protein determination

DPP IV activity was determined by the method of Nagatsu et al. [12] using Gly–Pro–pNA as substrate, except that 10 m*M* Tris–HCl buffer (pH 8.0) and a scaled-down volume of the assay mixture of 0.2 ml were used [13]. For the determination of the enzyme activity in serum with Gly–Pro–pNA as substrate, 10 μl of CRJ : CD rat serum and 20 μl of F344 rat serum were used, and about 1.5 and 35 μg of microsomal protein were required for CRJ : CD and F344 rats, respectively.

Protein concentrations were determined by the method of Hartree [14] with bovine serum albumin as standard. In addition, the DPP IV protein content in the sera was determined using an enzyme immunoassay (EIA) method [15] with anti-DPP IV (rat kidney) immunoglobulin G (IgG) conjugated with β-D-galactosidase (*Escherichia coli*).

RESULTS AND DISCUSSION

AP-P activity in rat serum and kidney

For the determination of AP-P activity in the rat serum, at least 100–200 μl of the serum were required in the assay medium (500 μl). If the volume of the assay medium exceeds 500 μl after addition of the serum, it is recommended that the serum is concentrated two-fold accurately with a collodion bag.

Typical chromatograms obtained with the rat serum and the blank are shown in Fig. 1. The retention times for enzymatically formed Pro–Hyp (3.8 min) from Gly–Pro–Hyp (5.4 min) corresponded to those cited in a previous report [8]. The areas of the peaks of the product (Pro–Hyp) formed from Gly–Pro–Hyp corresponded to two different volume of serum, viz., 916 and 1952 in CRJ : CD rat and 823 and 1724 in F344 rat, respectively.

AP-P activities in the serum and kidney in both rat strains are summarized in Table I. No difference in enzyme activity against Gly–Pro–Hyp as substrate in either serum or microsomal fraction was observed between CRJ : CD and F344 rats. AP-P activity against Gly–Pro–Ala was also determined in the microsomal fraction of F344 rat kidney (Table I) in order to demonstrate the production of Pro–Ala from Gly–Pro–Ala and its activity compared with Gly–Pro–Hyp as substrate.

Gly–Pro–pNA hydrolytic activity in rat serum and kidney

In order to consider the physiological significance of AP-P and DPP IV in F344 rat, the enzyme activities of serum and kidney microsomal fractions to-

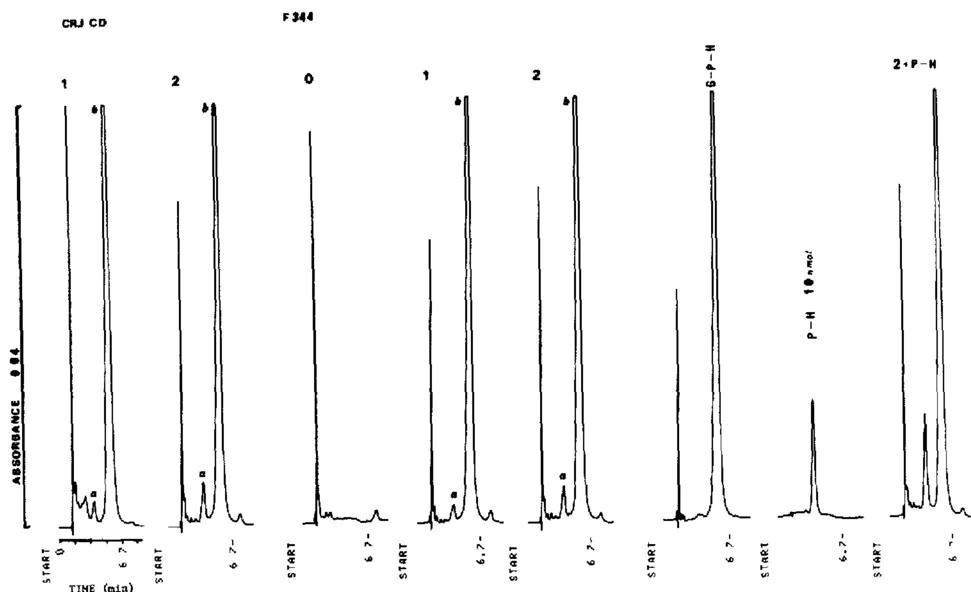

Fig. 1. Typical chromatographic patterns of the hydrolysis of Gly–Pro–Hyp and determination of produced Pro–Hyp (nmol) with different volumes of rat serum (μl). CRJ·CD: 1, 0.159 nmol per 80 μl; 2, 0.339 nmol per 160 μl. F344: 0, 140 μl without substrate; 1, 0.143 nmol per 70 μl; 2, 0.299 nmol per 140 μl. G-P-H, standard Gly–Pro–Hyp (3.3 nmol); P-H, standard Pro–Hyp (1.0 nmol); 2+P-H, F344 2 mixed with standard Pro–Hyp. Mobile phase, 10.0 m*M* potassium dihydrogenphosphate containing 1.0 m*M* 1-octanesulphonate (pH 2.1).

TABLE I

AMINOPEPTIDASE P ACTIVITY IN RAT SERUM AND KIDNEY

Values are means ± S.D. Four animals were used in each instance.

Rat strain	Serum ($nmol\ min^{-1}\ ml^{-1}$)	Kidney microsomal fraction ($nmol\ min^{-1}\ mg^{-1}$ of protein)	
	Gly–Pro–Hyp	Gly–Pro–Hyp	Gly–Pro–Ala
CRJ:CD	5.9 ± 0.8	11.9 ± 1.7	N.D.[a]
F344	5.3 ± 1.1	11.6 ± 0.9	7.9 ± 1.1

[a]Not determined.

wards Gly–Pro–pNA as substrate were determined in CRJ:CD and F344 rats (Table II).

The activity observed from the absorbance at 385 nm indicates the formation of *p*-nitroaniline from Gly–Pro–pNA. In the kidney microsomal fraction of CRJ:CD rats, the activity ($nmol\ min^{-1}\ mg^{-1}$ of protein, mean ± S.D.) was high (660 ± 60) compared with that of F344 rats (4.9 ± 0.2). Comparing the serum activity ($nmol\ min^{-1}\ ml^{-1}$), CRJ:CD rats showed higher activity

TABLE II

Gly-Pro-pNA HYDROLYTIC ACTIVITY IN RAT SERUM AND KIDNEY

Values are means ± S.D. Values in parentheses are the number of animals used.

Rat strain	Serum ($nmol\ min^{-1}\ ml^{-1}$)	Relative activity (%)	Kidney microsomal fraction ($nmol\ min^{-1}\ mg^{-1}$ of protein)	Relative activity (%)
CRJ·CD	30.32 ± 4.06 (9)	100	660 ± 60 (4)	100
F344	4.51 ± 0.54 (9)	14.9	4.9 ± 0.2 (4)	0.74

(30.32 ± 4.06) than F344 rats (4.51 ± 0.54), but the difference was much smaller than that found between their kidney microsomal fraction values.

In CRJ:CD rats the values depended on the DPP IV activities, whereas in F344 rats the values could indicate AP-P activities, as DPP IV activity was not found [7]. This result seems to indicate the presence of relatively high AP-P activity in the F344 rat serum, which is contrary to the extremely low activity of the microsomal fraction in the kidney.

The AP-P activities in both serum and microsomal fractions of the F344 rat are similar to or slightly higher than the hydrolytic activities determined with Gly–Pro–pNA as substrate (Tables I and II). This hydrolytic activity for Gly–Pro–pNA should be attributed to the AP-P activity in F344 rats, which is considered to be activated by Mn^{2+} in the assay mixture [2].

As reported previously [7], the DPP IV protein could not be demonstrated in F344 rats by gel electrophoresis and immunodiffusion analysis, and we have now obtained enzyme immunoassay data (not shown) that support those findings.

From these results we consider that in F344 rat serum and kidney, Xaa–Pro–Yaa peptides are hydrolysed first by AP-P to yield Xaa + Pro–Yaa, then prolyl dipeptidase (EC 3.4.13.8) or non-specific dipeptidase [16] hydrolyses Pro–Yaa into Pro and Yaa. Although the AP-P activity is lower than the DPP IV activity in CRJ:CD rats, it plays an important role in F344 rats in hydrolysing Xaa–Pro–Yaa peptides which could be derived from collagen catabolism. There was a highly significant correlation between ageing and severity of the renal disease in F344 rat [17]. Deficiency of DPP IV in F344 rat may cause the defect of the reabsorption of dipeptides in the proximal tubules of the microvillous membrane of the kidney [18].

As proline carboxypeptidase (EC 3.4.16.2) cannot attack Gly–Pro–Hyp [19] under the conditions used, this hydrolytic sequence for Xaa–Pro–Yaa peptide degradation proposed above is most probable in the F344 rat. It would be interesting to devise experiments to study the relationship between deficiency of DPP IV and various physiological aspects of the F344 rat.

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