Biochimica et Biophysica Acta, 657 (1981) 179—189

BBA 69172

COMPARISON OF DIPEPTIDYL PEPTIDASE IV PREPARED FROM PIG LIVER AND KIDNEY

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(Received June 24th, 1980)

Key words: Dipeptidyl peptidase IV; Glycoprotein; (Pig liver, Kidney)

Summary

Dipeptidyl peptidase IV (dipeptidylpeptide hydrolase, EC 3.4.14.-) has been purified from the microsomal fraction of pig liver, using an immunoaffinity chromatography, and its properties compared with those of the enzyme purified from pig kidney. The amino acid compositions of both enzymes were similar. The same kinds of carbohydrates were found in both enzymes, but there were differences in the molar concentrations of individual sugars. The liver enzyme had greater concentrations of mannose, fucose and sialic acid than the kidney enzyme, while the concentrations of galactose and glucosamine were greater in the kidney enzyme. The carbohydrates accounted for approx. 18.3 and 22.7% of the weight of the kidney and liver enzymes, respectively. The pH optima, molecular weights, substrate specificities and K_m values of the two enzymes and the effects of diisopropylfluorophosphate on their activities were nearly identical. The liver enzyme was heat- and pH-sensitive, but not attacked by proteinases.

Introduction

In our previous studies [1,2], it was shown that dipeptidyl peptidase IV (dipeptidylpeptide hydrolase, EC 3.4.14.-) was a glycoprotein and that the kidney enzyme had an immunological identity with the enzymes in different organs (liver, submaxillary gland and serum) but had a different p*I* value. In order to elucidate the distinction between the enzymes of kidney and liver, we have purified dipeptidyl peptidase IV from pig liver using immunoaffinity chromatography and compared its enzymatic properties with those of the enzyme in pig kidney.

Materials and Methods

Fresh pig liver obtained from a slaughterhouse was stored at −80°C until used. Gly-Pro-*p*-nitroanilide was obtained from Ajinomoto Co. Inc. Tokyo, Japan. The dipeptide-*p*-nitroanilides used were a gift from Professor T. Nagatsu (Dept. of Life Chemistry, Graduate School at Nagatsuta, Tokyo Institute of Technology), these were Gly-Leu-, Gly-Hyp-, Lys-Pro-, Glu-Pro- and Ala-Ala-*p*-nitroanilide. Pro-β-naphthylamide, Leu-β-naphthylamide, benzyloxycarbonyl (Z)-Gly-Pro-Leu were purchased from Protein Research Fundation, Japan. Trypsin (Sigma, Type III), chymotrypsin A (Boehringer Mannheim), pronase P (Kaken Chem. Co.), papain (Sigma), neuraminidase (Nakarai Chem.), CNBr-activated Sepharose 4B (Pharmacia Fine Chem.) and Ampholine PAG plate (LKB) were also purchased. The laboratory reagents were obtained from Nakarai Chem. Co. Ltd., Japan.

Enzyme and protein assay. Enzyme activity was assayed by the photometric method of Nagatsu et al. [3] using Gly-Pro-*p*-nitroanilide tosylate as substrate. 1 unit enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol *p*-nitroanilide/min at 37°C. Dipeptidyl peptidase activity was assayed by the same method using the other dipeptide-*p*-nitroanilides as substrates. Aminopeptidase activity was assayed by the colorimetric method of Nagatsu et al. [4] using Pro-β-naphthylamide or Leu-β-naphthylamide as substrate. Endopeptidase activity was assayed by the ninhydrin method [5]. A 0.4-ml aliquot of enzyme solution was mixed with 0.1 ml 5 mM substrate (Z-Gly-Pro-Leu) and 0.5 ml 0.1 M Tris-maleate buffer (pH 7.0) and the mixture was incubated at 37°C for 30 min.

Protein was measured by the method of Lowry et al. [6], using bovine serum albumin as standard.

Neuraminidase treatment. 0.2 units neuraminidase were added to 1000 μg of enzymes in 0.1 M acetate buffer (pH 5.0). The mixtures were incubated for 24 h at 37°C, then subjected to determination of some enzymic and physicochemical properties of the desialized enzymes.

Immunodiffusion and various electrophoreses. Double-immunodiffusion analysis and immunoelectrophoresis were performed by the method of Ouchterlony [7] and Scheidegger [8], respectively.

Isoelectric focusing in polyacrylamide gel was carried out by the thin-layer slab gel technique [9] which was described in the previous paper [2].

Disc electrophoresis and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were carried out as described by Davis [10] and by Weber and Osborn [11], respectively.

Preparation of antiserum. Antiserum against dipeptidyl peptidase IV from pig kidney was produced by immunization of rabbits using the same method as in the previous paper [2]. The antibodies were purified by affinity chromatography on Sepharose 4B conjugated to the antigen (1.0 × 2.0 cm) equilibrated with 0.02 M borate buffer/0.15 M NaCl (pH 8.0). The column was washed with 200 ml of the same buffer and the antibody was eluted with 0.17 M glycine-HCl buffer (pH 2.0) and neutralized immediately with 0.5 M $NaHCO_3$ buffer (pH 9.0). The antibodies were stored at −80°C.

Preparation of affinity chromatograms. The purified enzyme from pig

kidney (3 mg) and the antibodies (30 mg) were coupled to 1 ml and 10 ml CN-Sepharose 4B, respectively, according to the method of Axen et al. [12]. Essentially all the proteins were bound to the Sepharose, as judged from absorbance measurements at 280 nm before and after coupling.

Digestion with various proteinases. A solution containing 10 μg proteinase was added to 100 μg dipeptidyl peptidase IV in 0.2 M phosphate buffer, pH 7.0. The reaction mixture was incubated at 37°C and aliquots were withdrawn at given intervals and assayed for enzyme activities.

Amino acid and carbohydrate analyses. Amino acid and carbohydrate analyses were carried out by same methods as described in the previous paper [1].

Purification of dipeptidyl peptidase IV from pig liver. The livers were homogenized with an Ultra Turrax homogenizer in 9 vol. 0.25 M sucrose. The homogenate was centrifuged at 700 × *g* for 10 min, then at 5000 × *g* for 10 min and finally at 100 000 × *g* for 60 min, to separate nuclear, mitochondrial, microsomal and soluble fractions, respectively. The microsomal fraction was suspended in 0.1 M Tris-maleate buffer, pH 6.0 and then stirred at 37°C for 7 h. The suspension was centrifuged at 11 000 × *g* for 15 min and the supernatant was fractionated by $(NH_4)_2SO_4$ precipitation (40—80% saturation) using solid $(NH_4)_2SO_4$. The active fraction was purified by chromatography on Sepharose 4B conjugated to the antibodies, essentially as described by Svensson et al. [13], followed by chromatography on a Sephadex G-200 column equilibrated with 50 mM Tris-HCl buffer, pH 7.4/0.15 M NaCl.

The enzyme from pig kidney was prepared as described in the previous paper [1].

Results

Purification of dipeptidyl peptidase IV from pig liver

The intracellular distribution of the enzyme in pig liver and kidney is shown in Table I. The enzyme activity in pig kidney was concentrated in the microsomal fraction, whereas it was distributed to every fraction in pig liver.

It was proved that the solubilization of the enzyme from pig liver was ineffective, because the recovery of the activity was low due to autodigestion at pH 3.8 (Fig. 1). Therefore, solubilization from pig liver was achieved by auto-

TABLE I

SUBCELLULAR DISTRIBUTION OF DIPEPTIDYL PEPTIDASE IV IN PIG LIVER, KIDNEY AND SUBMAXILLARY GLAND

Subcellular fraction	Distribution of the activity (%)		
	Liver	Kidney	Submaxillary grand
Homogenate	1.6 *, 100	19.5 *, 100	7.0 *, 100
Nuclear	16.1	10.5	7.3
Mitochondrial	19.3	19.8	5.6
Microsomal	35.0	60.2	60.5
Soluble	28.6	9.3	26.6

* Total activity shown units/g wet weight.

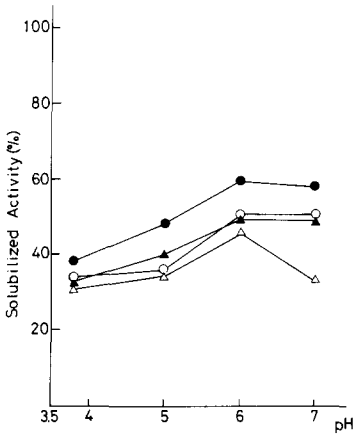

Fig. 1. Effect of time and pH on the solubilization of the enzyme from pig liver microsomes by autodigestion. The microsomal preparation was adjusted to each pH, then incubated at 37°C for varying times. The suspension was centrifuged at 100 000 × *g* for 60 min and the supernatant was assayed for enzyme activity. The solubilized activity was calculated from the original activity in the microsomal preparation. Incubation periods were expressed as: ▲———▲, 2.5 h; ●———●, 5.0 h; ○———○, 10 h and △———△, 21 h.

digestion at pH 6.0 for 7 h, at 37°C, and proved to be very effective in solubilizing about 50% of the enzyme activity. Then, solid $(NH_4)_2SO_4$ was added to the supernatant of autodigestion without adjusting the pH of the solution and over 80% of the enzyme activity was recovered in the 40—80% $(NH_4)_2SO_4$ fraction. The same elution profile as that of the intestinal enzyme [13] was observed on the immunoaffinity chromatography and specific activity of the active fraction increased 150-fold. The combined active fractions were concentrated to 5 ml in a collodion-bag (Sartorius membrane filter) and further purified by Sephadex G-200 chromatography, to eliminate the trace amount of inactive protein. Only a symmetrical protein peak with a slight shoulder on both sides was seen in the elution profile and the enzyme activity corresponded exactly to the peak. Therefore, the symmetrical peak region was pooled as the purified enzyme fraction. A typical example of the purification is summarized in Table II.

TABLE II

PURIFICATION OF DIPEPTIDYL PEPTIDASE IV FROM PIG LIVER

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification coefficient
Homogenate	328 000	3196	0.0097	100	1
Microsomal fraction	70 000	1120	0.0160	35.04	1.6
Extraction by autolysis for 7 h at 37°C, at pH 6.0	33 800	575	0.0170	17.99	1.8
$(NH_4)_2SO_4$ fraction (40—80%)	2136	470	0.2200	14.70	22.7
Immunoaffinity pooled preparation	8.86	294	33.18	9.19	3420
Sephadex G-200 pooled preparation	6.58	250	37.99	7.82	3916

The starting material was 2 kg pig liver.

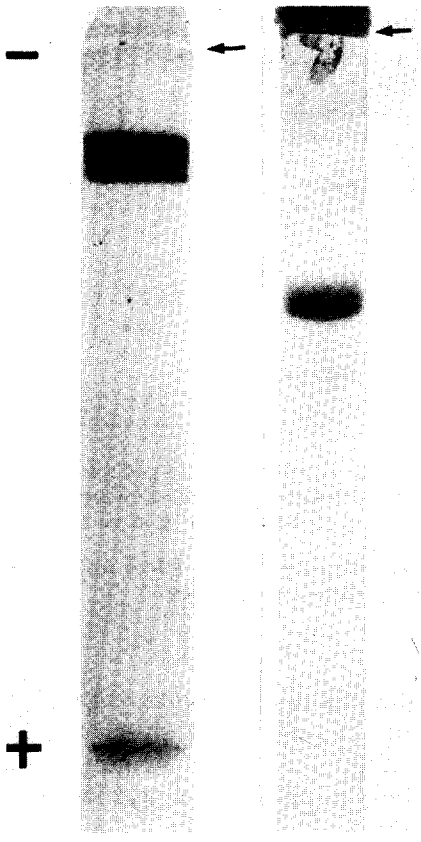

Fig. 2. Polyacrylamide gel electrophoresis in the absence (A) and presence (B) of SDS, of the purified enzyme from pig liver; 100 μg (A) and 20 μg (B) of protein were applied to each gel. Protein was stained with 0.05% Coomassie brilliant blue G-250. Arrows indicate origin.

The purified enzyme showed a single broad band on polyacrylamide gel electrophoresis (7.5% gel, pH 9.5) and we found that the enzyme activity coincided with the protein band, using the staining method described by Oya et al. [14]. The enzyme also showed a single band in the presence of SDS (5% gel) (Fig. 2).

Comparison of the properties of both enzymes

Some enzymatic and physicochemical properties of the enzymes from liver and kidney are summarized in Table III. Both enzymes exhibited the same pH optima, K_m values, molecular weights and the effect of diisopropylfluorophosphate on their activities. On the substrate specificity, they also showed similarities, namely, an N-terminal X-Pro dipeptide was liberated from X-Pro-*p*-nitroanilides (X=Gly (100%), Lys (34%), Glu (27%), each value represents relative activity), while the substrates with X-Y-*p*-nitroanilides (X-Y=Gly-Hyp (7%), Gly-Leu (0%), Ala-Ala (0%)), with no N-terminal free amino group (Z-Gly-Pro-Leu) and with Pro- or Leu-β-naphthylamide were not hydrolyzed.

The effect of H^+ concentration on the enzyme stability was studied for 45

TABLE III

SOME ENZYMATIC AND PHYSICOCHEMICAL PROPERTIES OF THE PURIFIED AND DESIALIZED ENZYMES

	Liver		Kidney	
	Native	Desialized	Native	Desialized
Optimum pH *	7.8—8.0	—	7.8—8.0	—
pH stability **	5.8—10	—	4.6—11	—
Molecular weight (SDS electrophoresis)	130 000	—	130 000	—
K_m ($1 \cdot 10^{-4}$ M)	6.7	6.7	6.7	6.7
p*I*	4.2	5.3	5.2	5.3
DFP ($1 \cdot 10^{-5}$ M) ***	68.7%	—	75.9%	—
($1 \cdot 10^{-4}$ M)	0	—	5.8	—

* Buffer used: 0.1 M Tris-maleate pH 6.5—8.6.

** Buffer used: pH 3.5—5.5 in 0.2 M acetate, pH 7.5—8.5 in 0.2 M Tris-HCl, pH 9—11 in 0.2 M borate/NaOH, in each case incubated at 37°C for 45 min. Assays for residual activities were carried out under standard conditions. Residual activity was over 75% after incubation at 37°C at the pH indicated.

*** The enzyme was preincubated in the presence of diisopropylfluorophosphate (DFP) for 10 min before the substrate was added. Results are given as percentage of the control values.

min, at 37°C, in buffers of different pH values. The kidney enzyme retained at least 75% of its activity between pH 4.6 and 11, while the liver enzyme retained 75% of its activity between pH 5.8 and 10 (Table III). Although the purified kidney enzyme retained 60% of its activity at pH 3.5, the purified liver enzyme lost all of its activity at pH 4.0, for 45 min.

For studies on the heat stability of the enzymes, samples were incubated at a certain temperature in 0.02 M Tris-HCl buffer, pH 7.4, and aliquots were transferred to an ice bath after varying periods of time and assayed for activity. Results are shown in Fig. 3. The kidney enzyme was found to be fairly stable at 66°C but the liver enzyme was rapidly inactivated.

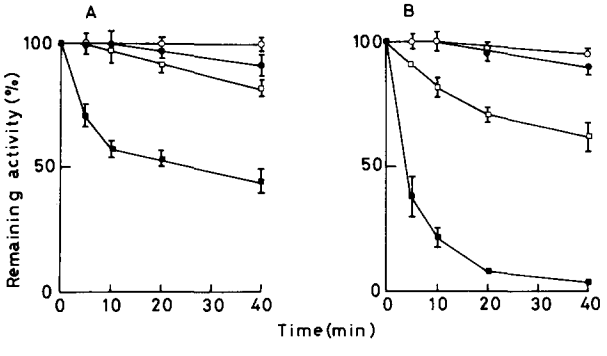

Fig. 3. Heat stability of the kidney (A) and the liver (B) enzymes. Each activity was expressed as a percentage of the activity of a control sample. Temperatures were expressed as follows. ○———○, 55°C; ●———●, 60°C; □———□, 65°C and ■———■, 66°C. Vertical bars indicate standard error.

Digestion with various proteinases

The effect of treatment with various proteinases on these enzyme activities is shown in Fig. 4. Treatment with trypsin, chymotrypsin A, pronase P and papain clearly decreased the activity of the kidney enzyme. But the liver enzyme was strongly activated by chymotrypsin A and papain, and treatment with trypsin and pronase P showed little if any effect on its activity.

Amino acid and carbohydrate analyses

The amino acid and carbohydrate analyses of the enzymes are summarized in Table IV. Amino acid and carbohydrate composition are expressed as residues per 1000 total residues and weight percentage, respectively. Molecular weight of the subunit was not able to be determined because the amount of purified liver enzyme was insufficient for the quantitative determination of the N-terminal amino acid. The amino acid composition of the liver enzyme is similar to that of kidney enzyme while the carbohydrate composition is remarkably different. In particular, the sialic acid content of the liver enzyme is 8-fold higher than that of the kidney enzyme.

Some properties of the desialized enzymes

In order to examine the effect of sialic acid residues on the enzyme activities and the immunochemical reactions, the purified enzymes were treated with neuraminidase. The enzyme activity did not change significantly before or after

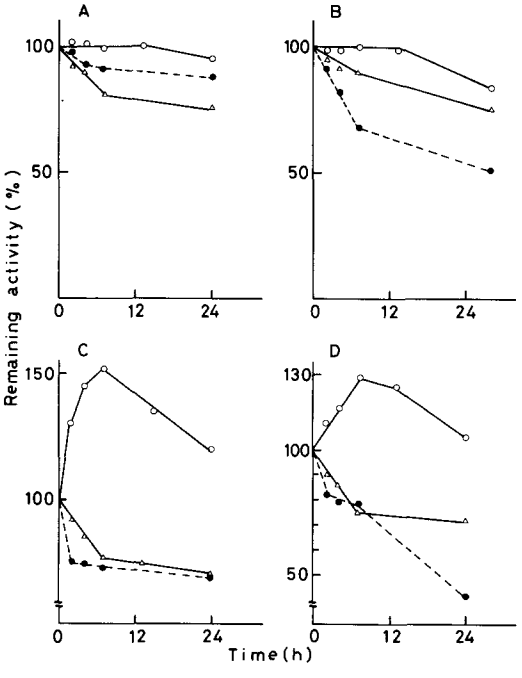

Fig. 4. Effect of treatment with trypsin (A), pronase P (B), chymotrypsin A (C) and papain (D) on the purified enzymes from kidney (△———△), liver (○———○) and on the desialized liver enzyme (●———●).

TABLE IV

AMINO ACID AND CARBOHYDRATE COMPOSITIONS OF THE PURIFIED ENZYMES

Amino acid was analyzed after 24, 48, 72 and 96 h of hydrolysis in 6 N HCl at 105 ± 0.5°C except methionine, cystine, cysteine and tryptophan.

	Residues/1000 residues	
	Liver	Kidney
Amino acid		
Lysine	50	47
Histidine	25	26
Arginine	35	41
Aspartic acid	112	107
Threonine	62	63
Serine	85	79
Glutamic acid	101	96
Proline	38	48
Glycine	59	58
Alanine	55	55
Valine	65	53
Isoleucine	50	55
Leucine	61	75
Tyrosine	78	59
Phenylalanine	48	45
Methionine [a]	12	33
1/2 Cystine [a]	20	35
Cysteine [b]	1	1
Tryptophan [c]	44	25
Amide [d]	142	98
	Weight percent (%) [e]	
Carbohydrate		
Fucose [f]	2.45 ± 0.05	0.89 ± 0.07
Mannose [f]	10.30 ± 0.18	3.41 ± 0.21
Galactose [f]	0.92 ± 0.04	5.09 ± 0.11
Glucosamine [g]	3.30 ± 0.07	8.18 ± 0.09
Sialic acid [h]	5.75 ± 0.63	0.72 ± 0.04

[a] Determined as methionine sulfone and cysteic acid after performic acid oxidation.
[b] Estimated by the colorimetric method based on the reaction with 5,5′-dithio-bis(2-nitrobenzoic acid).
[c] Determined spectrophotometrically.
[d] Determined after 8 h hydrolysis in 1 N HCl at 105 ± 0.5°C.
[e] Values are average ± S.E. of three analyses.
[f] Determined as alditol acetates.
[g] Determined after 4 h hydrolysis in 4 N HCl at 100 ± 0.5°C.
[h] Determined after enzymic hydrolysis with neuraminidase at 37°C for 24 h.

incubation with neuraminidase and the K_m values of the desialized enzymes were identical with those of the native enzymes (Table III). Therefore, sialic acid moiety of the liver enzyme as well as the kidney enzyme was not related to the activity of the enzyme. By double-immunodiffusion analysis a single precipitin line was formed between antiserum and the desialized enzymes and the precipitin lines of the native and desialized enzymes were completely confluent without any spur formation (Fig. 5A). By immunoelectrophoresis the two precipitin arcs of the desialized enzymes were located at almost the same position (Fig. 5B). It was evidenced that the desialized enzymes of liver

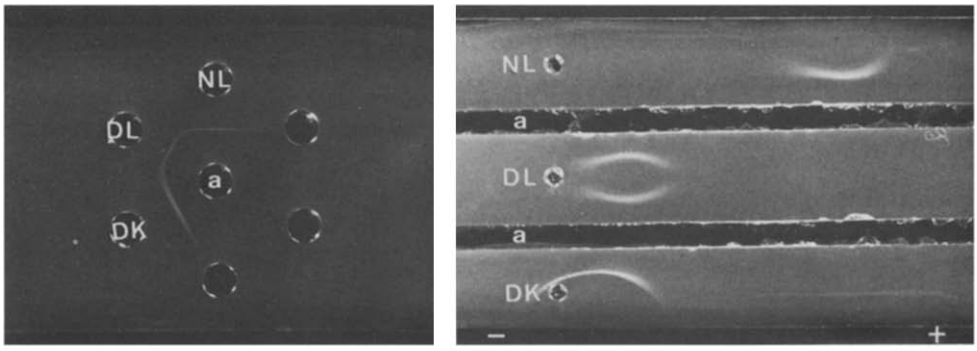

Fig. 5. Double-immunodiffusion analysis and immunoelectrophoresis. A; Cross-reaction between liver enzyme (NL) and desialized liver enzyme (DL) and desialized kidney enzyme (DK) against antiserum to the kidney enzyme (a). B; Electrophoresis was run for 1 h at 6 mA/gel (2.5 × 7.5 cm) in 20 mM sodium barbital buffer pH 8.6. And then the antiserum to the kidney enzyme was added to the channel (a). The plate was allowed to develop for 24 h at 4°C.

and kidney had similar p*I* values of 5.3 determined by isoelectric focusing (Table III).

The effect of treatment with the four proteinases on the activity of the desialized liver enzyme was studied. Like the native kidney enzyme, the desialized liver enzyme became susceptibly to attack by these proteinases (Fig. 4).

Discussion

Dipeptidyl peptidase IV has been purified from several mammalian sources such as pig kidney [1,15,16], pig intestine [13], rat liver [17], lamb kidney [18] and human submaxillary gland [14] and the enzymic properties have been described. In our previous study the partially purified enzymes from pig kidney, liver, submaxillary gland and serum exhibited an immunochemical identity but had different p*I* values [2].

In the present study we purified dipeptidyl peptidase IV from pig liver and clarified the differences in chemical properties between the enyzmes from pig liver and kidney. In pig liver, the proportion of the enzyme activity in the microsomal fraction was relatively low in contrast to other organs such as kidney and submaxillary gland (Table I). An attempt to extract the enzyme from the microsomal fraction of liver by autodigestion at pH 3.8, used as an ordinary method for other sources such as rat liver [17] and pig kidney [16], was unsuccessful in pig liver because the liver enzyme was very unstable under the H^+ concentration. Therefore, we chose the effective H^+ concentration (pH 6.0) for extracting the enzyme from the microsomal fraction according to the solubilization experiment (Fig. 1).

Svensson et al. [13] purified dipeptidyl peptidase IV from pig intestine about 500-fold using immunoaffinity chromatography. Using the same technique we were able to purify dipeptidyl peptidase IV from pig liver about 4000-fold (Table II). In the case of liver or serum, the enzyme activity is low

and likely to be decreased by the action of many proteolytic enzymes, so the use of immunoaffinity technique on purification is very effective.

The chemical and enzymic properties of the enzymes from liver and kidney were nearly identical. A similar result was reported on rat intestinal brush-border membrane peptidases [19]. The most remarkable chemical difference between the purified enzymes from liver and kidney was found in the higher quantity of mannose and sialic acid in the liver enzyme than in that of the kidney enzyme. The liver enzyme had a sialic acid content 8-times higher than the kidney enzyme and it is a heat- and pH-sensitive protein. Alternatively, in case of the multiple forms of alkaline phosphatase, the temperature and the pH stability increased with an increase in negative charge [20].

When both enzymes were treated with neuraminidase, the sialic acid was removed from the enzymes and the electrophoretic mobility toward the anode was markedly reduced in the liver enzyme, as compared to the kidney enzyme. As reported for several other glycoprotein enzymes [21,22], the desialized enzymes were found to retain full enzymatic activity.

The protective effect of the sialic acid moiety against proteolytic attack was proved by treatment with trypsin, pronase P, chymotrypsin A and papain on the two enzymes and the desialized liver enzymes. The kidney enzyme and the desialized liver enzyme were more susceptible than the native liver enzyme. A similar finding was reported in the case of deglycosilated yeast invertase that was more susceptible to intracellular proteolytic digestion [23].

It was suggested that dipeptidyl peptidase IV in serum may be derived from liver [24]. The liver enzyme could easily be released from the microsomal fraction in weak acid condition and became soluble. Therefore, it is conceivable that dipeptidyl peptidase IV from liver is ultimately released into the blood, where it exists in a stable form as a consequence of protection from proteases provided by its sialic acid moiety.

This paper suggests that dipeptidyl peptidase IV in pig liver and kidney exhibits a multiple form having a different postsynthetic modification of carbohydrate moieties.

Acknowledgements

We wish to thank Dr. S. Sakakibara (Institute for Protein Research, Osaka University, Osaka) for the synthesis of dipeptide-*p*-nitroanilides. The capable technical assistance of Miss. K. Yanagisawa is gratefully acknowledged.

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