wongsa, unpublished). The observation that collagen content and prolyl hydroxylase activity did not increase further, but in fact were decreasing, after 13 weeks of infection suggests no further stimulation of collagen biosynthesis after long-term infection. On the other hand, the possibility that more degradation than synthesis might occur in chronic infection has not yet been excluded. Prolyl hydroxylase activity decreased to almost normal level at 16–22 weeks postinfection; the high collagen content may cause a decline in the enzyme activity, since it has been reported that the propeptide released during conversion of procollagen to collagen may act as negative regulator of collagen synthesis[13]. Alternatively, the effect of cell death and damage caused by chronic infection of liver fluke may also have an effect. The present investigation, although indicates an enhancement of collagen deposition, but, further studies on genetic type of collagen would provide more information on changes in liver collagen during liver fluke disease.

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Characterization of a soluble form of dipeptidyl peptidase IV from pig liver

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Summary. Soluble dipeptidyl peptidase IV (EC 3.4.14.5) was purified from the 100,000 × g supernatant fraction of pig liver homogenate. The purified enzyme had the same properties as, and immunological identity with, the membrane-bound enzyme which was described previously. However, the purified enzyme had a pattern of molecular heterogeneity different from the membrane-bound enzyme; this was shown by isoelectric focusing. Carbohydrate analysis revealed that the soluble enzyme contained glucose, which is not found in the membrane-bound one, and less fucose, mannose, and sialic acid than the latter. From these results, we conclude that the soluble form of dipeptidyl peptidase IV in pig liver is closley related to the membrane-bound enzyme, but is not simply a proteolytically solubilized product of it.

Dipeptidyl peptidase IV (EC 3.4.14.5; DPP IV), discovered and partially purified from rat liver by Hopsu-Havu and Sarimo[3], liberates N-terminal glycyl-proline from either glycyl-proline-2-naphthylamide or peptides. It has been shown to be mainly associated with the brush border membrane in mammalian intestine[4] and kidney[5] using a method of subcellular fractionation, and to be immunohistochemically[6] localized in the plasma membrane in rat liver, kidney, and submaxillary glands. However Gly-Pro-p-nitroanilide hydrolyzing activity is found in human serum[7] and in the soluble fractions of several mammalian organs; for example, the hydrolyzing activity of the soluble fraction is 5% in rat liver[3], 22% in human submaxillary glands[8], 44–57% in bovine oral tissues[9], and 10–30% in several pig organs[2].

In our previous work[2], we showed that the Gly-Pro-p-nitroanilide hydrolyzing activity in pig liver was distributed in both soluble (28.6%) and microsomal (35.0%) fractions, and purified the microsomal enzyme. In this paper, we describe the purification of the enzyme from the soluble fraction of pig liver in order to compare its properties with the membrane-bound form of DPP IV. Also the possible origin of the soluble form is discussed.

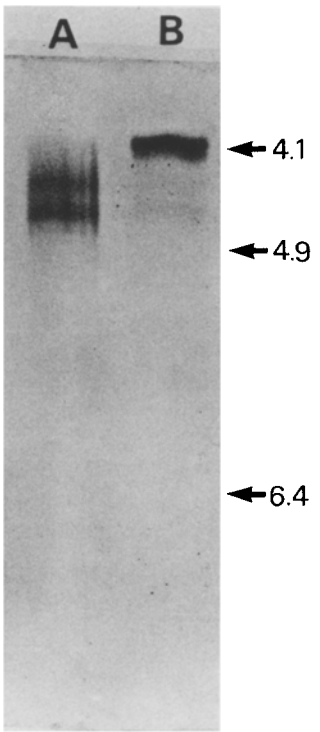
Materials and methods. Fresh pig liver obtained from a slaughterhouse was stored at −80 °C until used. Gly-Pro-p-nitroanilide was obtained from Ajinomoto Co, Inc., Tokyo, Japan.

Enzyme activity was assayed by the photometric method of Nagatsu et al.[10], using Gly-Pro-p-nitroanilide tosylate as substrate. Protein was measured by the method of Lowry et al.[11] using bovine serum albumin as standard. Amino acid and carbohydrate analyses were carried out by the same methods as described in the previous paper[12]. Antiserum to pig kidney DPP IV and Sepharose 4B conjugated anti-DPP IV were produced by the same methods as described in previous papers[2,12,13]. Isoelectric focusing in polyacrylamide gel was carried out by the thin layer-slab gel technique[14] which was described previously[13]. Disc gel electrophoresis was carried out as described by Davis[15]. Double immunodiffusion analysis was performed by the method of Ouchterlony[16].

The pig liver was homogenized with an Ultra Turrax homogenizer in 9 vol. of 0.25 M sucrose. The homogenate was centrifuged at 100,000 × g for 1 h to separate soluble and particulate fractions. The soluble fraction 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 anti-pig kidney DPP IV antibodies, as described by Svensson et al.[4], followed by chromatography on a Sephadex G-200 column

equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The active fractions were pooled and concentrated in a collodion bag to 5 mg protein/ml solution.

Results and discussion. The dipeptidyl peptidase IV purified mainly by immunoaffinity chromatography had a specific activity of 38.5 U/mg with a purification factor of about 4000 and a 16.6% overall yield. The purified enzyme showed 2 bands stained with Coomassie brilliant blue G-250 on polyacrylamide gel electrophoresis, and both protein bands showed enzyme activity using the staining method described by Oya et al.[18]. The specific activity was the same as that of the membrane-bound dipeptidyl peptidase IV which was reported previously[2].



Isoelectric focusing of soluble and membrane-bound dipeptidyl peptidase IV in thin-layer polyacrylamide gel (pH 3.5–9.5 gradient). 20 µg of each enzyme were applied on the gel. *A* Soluble form, *B* membrane-bound form.

Amino acid and carbohydrate compositions of the soluble and membrane-bound dipeptidyl peptidase IV from pig liver

Amino acid	Soluble form	Membrane-bound form[2]
	Residues/1000 residues	
Lysine	57	50
Histidine	29	24
Arginine	46	45
Aspartic acid	113	121
Threonine	59	78
Serine	81	90
Glutamic acid	121	114
Proline	52	42
Glycine	62	66
Alanine	61	56
Valine	65	63
Isoleucine	57	53
Leucine	78	76
Tyrosine	73	76
Phenylalanine	46	46
Carbohydrate	Weight percent (%)	
Fucose	0.9	2.5
Mannose	4.3	10.3
Galactose	1.5	0.9
Glucose	5.4	ND
Glucosamine	4.8	3.3
Sialic acid	2.6	5.8

ND, not detected.

The purified enzyme showed a single, symmetrical peak by gel filtration on Sephadex G-200 and the mol.wt was estimated to be 360,000, the same as that of the membrane-bound enzyme. However, the patterns produced by isoelectric focusing clearly differentiated between the 2 forms of the enzyme (fig.). The purified enzyme from the soluble fraction showed a diffuse protein band from pH 4.4 to pH 4.9, while the membrane-bound enzyme had a single band at pH 4.2.

Many glycoproteins, such as alkaline phosphatase[17] and acetyl-CoA acetyl-transferase[18], have been indicated to be microheterogeneous by isoelectric focusing. We showed previously that dipeptidyl peptidase IV enzymes purified from microsomal fractions of pig kidney and liver were heterogeneous glycoproteins[2]. The soluble form of dipeptidyl peptidase IV in pig liver was a glycoprotein and had a different pI value from those of the membrane-bound forms in pig liver and kidney and was shown to be microheterogeneous itself by isoelectric focusing.

Some enzymic properties of the purified enzyme from the soluble fraction were studied. The pH optimum and the K_m value for Gly-Pro-p-nitroanilide were at 7.8–8.0 and 6.7×10^{-4} M, respectively, and enzyme activity was completely inhibited by a 10-min preincubation in the presence of 0.1 mM diisopropylfluorophosphate. These properties are the same as those found for the membrane-bound enzyme. Therefore we concluded that the conformation of the active sites of the 2 enzymes was identical. Immunochemical reactivity of the 2 forms of the enzyme was examined by double immunodiffusion analysis. A single precipitin line was formed between antiserum against dipeptidyl peptidase IV purified from pig kidney and each form of the enzyme purified from pig liver, and the 2 precipitin lines were completely confluent without any spur formation (results not shown). This suggests the immunological identity of the 2 forms of the pig liver enzyme.

The table shows the amino acid and carbohydrate compositions of the soluble enzyme compared with those of the membrane-bound one. Amino acid compositions and the total carbohydrate contents of the 2 forms of the enzyme were quite similar. However, the carbohydrate compositions of the 2 were clearly different from each other, especially in that glucose residues were not detected in the membrane-bound enzyme. The carbohydrate chains of the membrane-bound enzyme are rich in mannose; it is not surprising that it does not contain glucose residues, considering the fact that oligosaccharides of an asparagine-linked class of glycoproteins undergo a number of processing reactions resulting in removal of all glucosyl residues[19–21] in the Golgi apparatus. It is therefore unlikely that the soluble enzyme is simply a solubilized form of the membrane-bound enzyme; it may be reasonable to propose that the soluble form of the enzyme is not a released form, but a precursor of the membrane-bound enzyme.

It has been shown that in the case of aminooligopeptidase[22], or sucrase-isomaltase[23], which are intestinal microvillous membrane proteins like dipeptidyl peptidase IV, there is a cytosol precursor of each membrane-bound enzyme. But the present results are not sufficient for us to conclude that the soluble enzyme is a precursor of the membrane-bound enzyme. Therefore we will study further the biosynthetic

relationship between the 2 forms of the pig liver dipeptidyl peptidase IV using tracer techniques.
These data demonstrate that the soluble and membrane-bound forms of dipeptidyl peptidase IV from pig liver are not distinct isozymes. They have different pI values but the same mol.wts as estimated by gel filtration, the same enzymic and immunoreactivity properties, and similar amino acid compositions.

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Stimulation-dependent uptake of an extracellular marker to subcellular fractions of isolated neurohypophysial tissue[1]

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Summary. Slices of ox neurohypophyses and groups of isolated rat neurointermediate lobes were incubated in a medium containing horseradish peroxidase (HRP) and stimulated by high K^+ concentrations in the medium. After washing, the tissue was homogenized and subjected to subcellular fractionation in a Percoll/sucrose gradient. HRP was exclusively taken up by particles banding at a low density of the gradient. The HRP containing particles located to this region included vacuoles of a size comparable to secretory vesicles.

Some essential facts are known about the mechanism by which the neurohypophysial hormones vasopressin and oxytocin are released. It has been reported that on stimulation of isolated rat neurohypophyses, vesicles containing oxytocin and the binding protein (neurophysin) release their contents to the medium in a roughly stoichiometric way[4], whereas axoplasma enzyme markers are not released[5]. These facts support the hypothesis that release occurs by exocytosis. Since the area of the cell membrane in the terminal dilatations is not increased after strong stimulation[6,7] retrieval of membrane (vesicle membrane and/or cell membrane of a size similar to the total area of membrane secretory vesicles incorporated) must take place after stimulation. On the basis of morphological studies the prevailing hypothesis has been that secretory vesicles are retrieved as microvesicles[8,9], although early histological investigations have provided evidence that membrane is retrieved as vacuoles similar in size to the secretory vesicles[10,11]. In recent years, an increase in the vacuole population on stimulation without increase in the microvesicle population has been shown in several morphometric studies[6,12-14].

Stimulation-dependent uptake of isotope-labeled extracellular markers has been observed when whole rat neurohypophyses were incubated in media containing such markers[15]. No studies have been reported on larger animals and/or on attempts to isolate the vesicles retrieved after exocytosis. Such isolation would be necessary to answer the question whether the vacuole membrane is similar to that of the secretory veiscle membrane or that of the cell membrane and might be of use in studies of the fate of the vacuoles.
In the present work we report on stimulation-dependent uptake of HRP into subcellular fractions from rat and bovine neurohypophyses. As analyzed in density gradients the material containing the extracellular marker can be isolated from both species.
Materials and methods. Isolated rat neurointermediate lobes. For each of 3 experiments, 60 isolated neurointermediate lobes from female Wistar rats (300 g b.wt) killed by decapitation, were divided into 2 groups of 30. Both groups were washed twice in 'control' medium without HRP (table 1). The 2 groups were then incubated in 'control' medium and 'stimulation' medium, respectively, for 60