Increased liver prolyl hydroxylase activity in hamsters infected with the human liver fluke *Opisthorchis viverrini*[1]

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Summary. A parallel increase in liver collagen content and prolyl hydroxylase activity was observed in hamsters infected with the human liver fluke *Opisthorchis viverrini.* They were elevated at 2 weeks after infection, gradually increased to approximately 2-fold at 7–11 weeks of infection, and then declined as with duration of infection time increasing from 11 to 22 weeks.

Infection by *Opisthorchis viverrini,* a liver fluke, causes a disease which is characterized by necrosis of liver parenchyma in the portal area and multilobular cirrhosis as well as periductal fibrosis[2]. Hepatic fibrosis is associated with elevated collagen deposition in the liver as observed in hepatosplenic schistosomiasis[3,4] and experimentally induced liver injuries[5,6]. The increased collagen synthesis has been shown in schistosome-infected livers by an increased incorporation of radioactive proline into liver collagen[3,4]. In addition, the activity of liver prolyl hydroxylase, required for post-translational modification of collagen, has been widely used as a measure of collagen biosynthetic rate in hepatic injuries induced by different methods[5,8]. The factor(s) responsible for the increased enzyme activity are, however, still unclear. The present work reports preliminary studies on the biochemistry of liver fluke disease. Prolyl hydroxylase activity and collagen content were measured in livers of hamsters at various times after infection with *O. viverrini.*

Materials and methods. Two-month-old Syrian golden hamsters were infected by intragastric inoculation with 50 viable metacercariae of *O. viverrini* which were removed from muscles of infected cyprinoid fishes[2]. Infected hamsters and controls of the same age were maintained *ad libitum* on normal basal diet and sacrificed at different times after infection. Worms were removed from infected livers before the subsequent analysis. The liver was homogenized in 5 vols of Krebs-Ringer phosphate buffer, pH 7.4. One-half of the homogenate was used for extraction of collagen and protein determination[9]. The remaining homogenate was centrifuged at 15,000 × g to obtain liver supernatant which was subsequently used for determination of prolyl hydroxylase activity. Collagen was extracted from liver homogenate with 5% trichloroacetic acid at 90 °C for 1 h according to the method of Rojkind and De León[10]. The extraction mixture was centrifuged at 500 × g for 15 min and the resulting supernatant containing collagen was dialyzed extensively against several changes of 0.5 N acetic acid. The collagen content was then measured by assaying for the amount of hydroxyproline after acid hydrolysis in 6 N HCl at 100 °C for 18 h[11]. Prolyl hydroxylase activity was measured in the 15,000 × g liver supernatant by using [^{3}H] protocollagen prepared from chick embryos as a substrate[12]. The assay was performed for 30 min at 37 °C in 1 ml reaction mixture containing 50 mM Tris-HCl buffer pH 7.8, 0.08 mM $FeSO_4$, 2 mM ascorbic acid, 0.5 mM α-ketoglutarate, 0.2 mg catalase, 2 mg bovine serum albumin, 15,000–20,000 cpm of [^{3}H] protocollagen substrate and appropriate amount of 15,000 × g liver supernatant. The reaction after termination by addition of 1 ml of 12 N HCl was hydrolyzed at 110 °C for 18 h. The hydrolysate was then analyzed for the radioactivity [^{3}H] hydroxyproline[11] formed after the enzymatic reaction. Enzyme activity was expressed as cpm of [^{3}H] hydroxyproline formed/min/mg of 15,000 × g supernatant protein.

Results and discussion. Hamsters infected with *Opisthorchis viverrini* showed an increase in liver collagen content, whereas there were no significant changes ($p > 0.05$) in the liver wet weights and protein content, when compared to those in the uninfected controls (data are not shown). The time course of collagen deposition in the infected liver is shown in the table. At 3 weeks after inoculation with metacercariae, the infected liver collagen content was gradually raised, while liver collagen in the control group remained unchanged. At this point in the infection, the parasite is maturing, a process which is completed at 4 weeks postinfection. Liver collagen content increased approximately 2 times between the 5th and 13th week postinfection; however, a slight decrease was observed at longer term of infection (table). The increase in collagen coincides with the formation of histopathological lesions in the liver as observed by Bhamarapravati et al.[2]. These lesions begin about 15 days postinfection and increase with infection time.

To investigate whether elevated collagen content is due to an increase in its synthetic rate, the activity of liver prolyl hydroxylase, required for collagen biosynthesis was assayed at various infection times. As shown in the table, the increase in prolyl hydroxylase activity occurred as early as 2 weeks after infection and increased approximately 2-fold by 7–11 weeks after infection. The parallel increase in collagen content and prolyl hydroxylase activity, as demonstrated by a linear relationship, with a correlation coefficient of 0.904 (table) suggests an enhancement of liver collagen synthetic rate in liver fluke infection. Hence, activation of liver collagen biosynthesis may be responsible for deposition of collagen in the liver tissue, although the effect of previously synthesized collagen has not been considered. The result is not unexpected, as stimulation of in vitro collagen synthesis has also been observed in murine schistosomiasis. The extent of such stimulation in opisthorchiasis as measured by prolyl hydroxylase activity is quite comparable to that measured by the in vitro incorporation of proline into liver collagen (N. Hutadilok and P. Ruen-

Liver collagen and propyl hydroxylase activity in hamsters infected with 50 metacercariae of *O. viverrini*

Infection time (weeks)	Collagen content[e,g]		Prolyl hydroxylase activity[f,g]	
	Control	Infected	Control	Infected
1	1.52 ± 0.19	1.54 ± 0.45[a]	135 ± 4	126 ± 7[a]
3	1.55 ± 0.24	2.08 ± 0.29[b]	155 ± 9	231 ± 12[d]
5	1.95 ± 0.10	4.39 ± 0.27[c]	225 ± 13	332 ± 9[b]
7	2.5 ± 0.55	8.0 ± 0.20[c]	205 ± 4	395 ± 25[c]
9	1.77 ± 0.18	6.5 ± 1.05[c]	204 ± 21	372 ± 9[c]
11	3.09 ± 0.17	5.77 ± 0.15[c]	190 ± 16	353 ± 15[c]
13	1.88 ± 0.12	5.52 ± 0.35[c]	201 ± 19	315 ± 36[d]
16	2.7 ± 0.40	4.8 ± 0.39[c]	200 ± 12	260 ± 27[d]
22	2.62 ± 0.43	5.07 ± 0.65[c]	235 ± 17	281 ± 22[b]

Values shown are means ± SEM (n - No. of hamsters = 4). [a]$p > 0.05$; [b]$p < 0.05$; [c]$p < 0.01$; [d]$p < 0.025$. [e]Expressed as mg/g liver protein. [f]Expressed as cpm [^{3}H] hydroxyproline/min/mg 15,000 × g liver supernatant protein. [g]There is a linear relationship between collagen content and prolyl hydroxylase activity with a correlation coefficient, $r = 0.904$.

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