Occurrence of Two High Molecular Phospholipases with Different Isoelectric Points in Porcine Pancreas and Their Reversible Interconversion

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Two high molecular weight proteins with phospholipase activity (Phospholipase-I and Phospholipase-II) were found to be present in the homogenate of porcine pancreas without delipidation and heat treatments. Phospholipase-I had an isoelectric point (pI) of about 4.5, while Phospholipase-II about 6.0. Both the Phospholipase-I and -II were purified by isoelectric focusing and gel filtration on Sephadex G-200. The molecular weights of both enzymes are about 5×10^5 . Two enzymes differed greatly from each other in the heat stability and the effect of pH on the activities. Phospholipase-I (pI 4.5) was converted to the fraction with pI 6.0 by extraction of lipids, while Phospholipase-II (pI 6.0) to the fraction with pI 4.5 by addition of the extracted lipids in a yield of about 50%. These converted enzymes were very similar to the corresponding native enzymes in the properties above described, respectively. These results suggest that the Phospholipase-I may exist as a Phospholipase-II-lipid complex. Furthermore, the relationship among the Phospholipase-I, Phospholipase-II, and the phospholipase A_2 reported by de Haas *et al.* are discussed.

Pancreatic phospholipase A (EC 3. 1. 1. 4) has been the subject of considerable research in the past. The enzymes of pancreatic tissues of porcine,^{1~6)} ox,^{7,8)} man,^{9,10)} horse,¹¹⁾ and rat¹²⁾ have been purified and characterized. Especially, porcine pancreatic phospholipase A_2 was completely purified,^{1~3)} and the enzymatic properties,^{1~3)} the primary structure,^{4,5)} and the active site⁶⁾ of the purified enzyme were examined. Furthermore, it is known that phospholipases exist in pancreatic tissues as zymogens, which are converted to active enzymes via limited proteolysis by trypsin.^{2,8,9,12)} Wezel and de Haas also reported that two phospholipase A_2 isoenzymes with isoelectric points of 6.3 and 5.9 exist in porcine pancreas in the ratio of about 95: 5, respectively.¹³⁾ However, almost all the purified phospholipases described above were extracted and isolated from the delipidated and heat-treated homogenate of porcine pancreas.

On the other hand, it is described that a high molecular weight lipase purified from human pancreas is converted to another low molecular weight lipase either by the extraction

of endogeneous lipids with acetone or by digestion with phospholipase A, and the latter is converted to the former by adding pure bile, phosphatidylcholine, or phosphatidylserine but neither by lysophosphatidylcholine nor by sodium taurocholate.¹⁴⁾ From these observations it is speculated that the properties of the phospholipases of pancreas may be changed by the extraction of the lipids as well as heat treatment. However, with respect to porcine pancreas phospholipase, no such report has so far been published.

In the present study, the authors describe that two high molecular weight phospholipases with different isoelectric points, each of which differs significantly from the phospholipase A_2 reported by de Haas *et al.*,^{1,2)} exist in the homogenate of porcine pancreas without delipidation and heat treatments. Furthermore, it is reported that these two enzymes are purified by means of isoelectric focusing and gel filtration on Sephadex G-200, and are reversibly converted by the extraction or the addition of the endogeneous lipids.

MATERIALS AND METHODS

Materials. Porcine pancreas was obtained from the local slaughterhouse, usually within 30 min after the death of the animal. The tissue, after being freed of most of the fat and connective tissue, was stored frozen at -20°C .

Reagents. Carrier ampholyte was obtained from LKB-Productor AB, Sweden. Sephadex G-200 and G-100 was purchased from Pharmacia Fine Chemicals AB, Sweden. Sodium deoxycholate, ethyldiaminetetraacetic acid (EDTA), sodium dodecylsulfate, cetyltrimethylammonium chloride, *p*-chloromercuribenzoate, *N*-ethylmaleimide, cysteine, and mercaptoethanol were obtained from Wako Pure Chemical Industries Ltd., and diisopropylfluorophosphate from Kishida Chemicals Ltd. Pure reference proteins were purchased from Boehringer Mannheim, W. -Germany. All other reagents used were of analytical grade.

Substrates. Phosphatidylcholine was purified from egg lipids.¹⁶⁾ Triglyceride was prepared from olive oil.¹⁰⁾

Enzyme assays. Phospholipase and lipase activities were assayed by determining free fatty acids released from a substrate, phosphatidylcholine or triglyceride, respectively, according to the method previously reported.^{17,18)} The substrate solutions used were now previously dispersed with sodium deoxycholate by ultrasonic treatments. The assay mixtures for phospholipase activity contained 0.5 mM phosphatidylcholine, 2 mM sodium deoxycholate, and 100 mM CaCl_2 for phospholipase-I or 0.1 mM CaCl_2 for phospholipase-II in 0.1 M Tris-HCl buffer (pH 8.0). The assay mixture for lipase contained 10 mM triglyceride, 2 mM sodium deoxycholate, and 10 mM CaCl_2 in 0.1 M Tris-HCl buffer (pH 7.0). The mixtures (total volume, 1 ml) were incubated with shaking at 35°C for 20 min. The enzyme activities were corrected by subtracting the rates of the enzymatic digestion of the endogeneous lipids contained in the enzyme preparations, which were obtained by the incubation without the substrates. The assay mixture for a low molecular weight phospholipase contained 0.5 mM phosphatidylcholine, 6 mM sodium deoxycholate, and 10 mM CaCl_2 in 0.1 M Tris-HCl buffer (pH 8.0). The enzyme activities were expressed as enzyme unit, where one unit of enzymes was defined as the amount of the enzyme which releases 1 μmol of free fatty acid from the substrate per min. The specific activities were expressed as the enzyme unit per mg of enzyme protein. Protein was determined by the Lowry method modified by Hartree.¹⁰⁾

Isoelectric focusing. Isoelectric focusing was carried out in the 110 ml-apparatus of LKB, using a carrier ampholyte (the pH range of 3~6) at a final con-

centration of 0.5% and a stepwise sucrose gradient (0~50%).²⁰⁾ Electrophoresis was performed at 900 V for 48 hr at 4°C .

Gel filtration. A Sephadex G-200 column (3 $\times$ 20 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0) was used, and applied proteins were eluted with the same buffer.

Heat treatment. The enzyme preparation was heat-treated at pH 4.0 and 100°C for 5 min according to the method described by de Haas *et al.*¹⁾ The remaining activity was then assayed by the standard methods.

Extraction and addition of lipids. Five ml of the purified Phospholipase-I solution was added to 9 vol of acetone at -50°C . The mixture was centrifuged at $15,000 \times g$ for 30 min. The precipitate was further treated with acetone and with diethyl ether at -50°C by the same method. The resulting precipitate was immediately dried under vacuum at 0°C to remove the solvent. The delipidated enzyme was dissolved in the same volume of 5 mM Tris-HCl buffer (pH 8.0) (crude delipidated Phospholipase-I), and was subjected to isoelectric focusing. The extracted lipids were combined, dried by an evaporator, and dispersed in 5 ml of the same buffer by ultrasonic treatment. The extracted lipid solution obtained was incubated with the purified delipidated Phospholipase-I obtained by isoelectric focusing or the purified Phospholipase-II at 4°C for 24 hr (reconstituted Phospholipase-I or lipid-added Phospholipase-II, respectively).

Positional specificity. Positional specificity of a low molecular weight phospholipase was determined by the method as previously described.¹⁰⁾

RESULTS

Occurrence and purification of high molecular weight phospholipases

Unless otherwise mentioned, all experiments were performed at about 4°C .

Fresh porcine pancreas without delipidation were homogenized in 3 vol (v/v) of 10 mM Tris-HCl buffer (pH 8.0) in a Waring blender. The homogenate was filtered through two layers of nylon cloth. The filtrate was centrifuged at $15,000 \times g$ for 60 min. The enzyme was further extracted from the pellet by the same method. The combined supernatant was concentrated by freeze-drying to give a crude extract.

FIG. 1. Isoelectric Focusing of the Crude Extract. The crude extract obtained from the non-delipidated and non-heat-treated homogenate of porcine pancreas was subjected to isoelectric focusing. The isoelectric focusing was carried out for 48 hr at 900 V and 4°C in a LKB 8101 column using ampholyte in the pH range of 3~6 with the cathode at the bottom. After electrophoresis, the content of the column was fractionated into 3 ml portions. Each fraction was assayed for pH, optical density at 280 nm, and the enzymic activities. ●—●, pH; ●—●, optical density at 280 nm; ○—○, phospholipase activity; ▲—▲, lipase activity.

The crude extract was subjected to electric focusing. As Fig. 1 shows, phospholipase activity was separated into two peaks with different isoelectric points of 4.5 (Phospholipase-I) and 6.0 (Phospholipase-II). The combined fractions of Nos. 19~25 (Phospholipase-I) and Nos. 4~8 (Phospholipase-II) gave the yields of the enzyme activities of 47 and 44%, respectively, of the total activity applied to the column. Almost all lipase activity, however, existed in the Phospholipase-I fraction.

Each of Phospholipase-I and Phospholipase-II obtained by isoelectric focusing was loaded on a Sephadex G-200 column (3×20 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0) containing 1 mM mercaptoethanol and 1 mM CaCl₂ and was eluted with the same buffer. As is indicated in Fig. 2, both of the phospholipases were eluted after the void volume on the column, indicating that they had a similar high molecular weight. Lipase

FIG. 2. Gel Filtrations of Phospholipase-I and -II on a Sephadex G-200 Column.

The Phospholipase-I and -II fractions obtained by isoelectric focusing were separately loaded on a Sephadex G-200 column (3×20 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0) containing 1 mM mercaptoethanol and 1 mM CaCl₂. Fractions of 5 ml were collected and subjected to enzyme assay. A, Phospholipase-I fraction; B, Phospholipase-II fraction. ○—○, Phospholipase activity; ▲—▲, lipase activity.

TABLE I. PURIFICATIONS OF PHOSPHOLIPASE-I AND -II FROM NON-DELIPIDATED AND NON-HEAT-TREATED HOMOGENATE OF PORCINE PANCREAS

Stages	Specific activity (mU/mg)	Purification (-fold)
Crude extract	2.85	1
Isoelectric focusing		
Phospholipase-I	74.1	26
Phospholipase-II	433.2	152
Gel filtration on Sephadex G-200		
Phospholipase-I	103	36
Phospholipase-II	599	210

activity, however, could not be separated from Phospholipase-I activity (Fig. 2A). The active fractions (fraction Nos. 9~15 for both phospholipase-I and -II) were separately combined, concentrated by freeze-drying, and dialyzed against 5 mM Tris-HCl buffer (pH 8.0) for 12 hr. Phospholipase-I and Phospholipase-II were purified 36- and 210-folds, re-

spectively, as summarized in Table I. The ratio of phospholipase activity to lipase activity in the partially purified Phospholipase-I fraction was 1:10. Phospholipase-II fraction contained no lipase activity.

Properties of Phospholipase-I and Phospholipase-II

Molecular weight. The molecular weight of each enzyme was determined by gel filtration on a Sephadex G-200 column according to the method of Andrews.²¹ Both molecular weights were about 5×10^5 .

FIG. 3. Effect of pH on the Activities of Purified Porcine Pancreas Phospholipase-I and -II.

The enzyme activities were measured by the standard methods in 100 mM citrate buffer (pH 4.0~6.5), 100 mM Tris-HCl buffer (pH 7.0~9.0), and 100 mM Sodium carbonate buffer (pH 9.5~11). ●---●, Phospholipase-I; ○---○, Phospholipase-II.

Effect of pH on the activities. The pH dependent profiles of activities of both enzymes are shown in Fig. 3. Optimal pH for Phospholipase-II was in pH range of 8.0~8.5, while that for Phospholipase-I spread over a broader pH range of 6.5~9.0.

Heat stability. By heating at pH 4.0 and 100°C for 5 min, Phospholipase-II lost 90% of its original activity, whereas Phospholipase-I retained more 95% of the activity. Even by heating for 10 min, 45% of the activity of Phospholipase-I remained. These results are demonstrated in Fig. 4. The extracted lipids (0.2~1.2 mg) from Phospholipase-I and bovine serum albumin (0.1 mg) protected considerably Phospholipase-II (1.0 mg) from the inactivation by the heat treatment. However, they had no effect on the restoration of the enzyme inactivated.

FIG. 4. Heat Stability of Porcine Pancreas Phospholipase-I and -II.

Each enzyme solution was brought to pH 4.0 with 1 N HCl and heated at 100°C for 5 min. The solutions after being rapidly cooled to 0°C, were freed from insoluble material by centrifugation and then subjected to enzyme assays. ●---●, Phospholipase-I; ○---○, Phospholipase-II.

FIG. 5. Effects of Ca^{2+} and EDTA on Porcine Pancreas Phospholipase-I and Phospholipase-II.

The enzymes were preincubated with EDTA for 5 min at 0°C and then the remaining activities were assayed by the standard methods. Furthermore, the enzymes preincubated with 0.1 mM EDTA were dialyzed against 5 mM Tris-HCl buffer (pH 8.0) for 12 hr, and then were assayed at various concentrations of Ca^{2+} by the standard methods. ●---●, Phospholipase-I; ○---○, Phospholipase-II.

Effects of Ca^{2+} and EDTA on phospholipase activities. As Fig. 5 shows, Phospholipase-I and -II exhibited progressive decreases of the activities with increasing the amount of EDTA added to the assay systems. Activities of both phospholipases were fully restored and enhanced by Ca^{2+} . Phospholipase-II activity was remarkably stimulated by 0.1 mM

TABLE II. EFFECTS OF VARIOUS REAGENTS ON PORCINE PANCREAS PHOSPHOLIPASE-I AND-II

Reagents	Conditions		Phospholipase-I	Phospholipase-II
	Conc.	pH		
			Relative activity (%)	
None	—		100	100
Sodium dodecylsulfate	0.01 %	pH 8.0	43	11
	0.1 %	pH 8.0	9	0
Cetyltrimethylammonium chloride	0.1 mM	pH 8.0	24	0
<i>p</i> -Chloromercuribenzoate	0.1 mM	pH 5.0	50	87
N-Ethylmaleimide	10 mM	pH 7.0	65	65
Cysteine	100 mM	pH 8.0	69	20
Mercaptoethanol	100 mM	pH 8.0	52	13
Diisopropylfluorophosphate	10 mM	pH 8.0	103	91

The enzymes were preincubated with each reagent for 20 min at 0°C. After preincubation, the remaining activities were measured by the standard methods.

CaCl₂ but inhibited by the cation at a higher concentration, while Phospholipase-I activity was markedly stimulated by the cation at a higher concentration (100 mM).

Effects of detergents and reagents on both the activities. As Table II shows, both enzymes were strongly inhibited by detergents, sodium dodecylsulfate (anionic detergent) and cetyltrimethylammonium chloride (cationic detergent). Phospholipase-I was considerably inactivated by *p*-chloromercuribenzoate, N-ethylmaleimide, cysteine, and mercaptoethanol, but was not inhibited by diisopropylfluorophosphate. Phospholipase-II was remarkably inactivated by cysteine and mercaptoethanol, but was not inhibited by diisopropylfluorophosphate and *p*-chloromercuribenzoate. Phospholipase II was generally more sensitive than Phospholipase-I to the reagents.

Reversible interconversion between Phospholipase-I and Phospholipase-II

The delipidated Phospholipase-I and the reconstituted Phospholipase-I or the lipid-added Phospholipase-II were prepared as described in MATERIALS AND METHODS. These treated enzymes and the native enzyme were separately subjected to isoelectric focusing. The results are summarized in Fig. 6. Almost all Phospholipase-I with an isoelectric point of 4.5 was shifted to the fractions corresponding to Phospholipase-II with an isoelectric point of 6.0 by the delipidation. Furthermore, the re-

sulting delipidated Phospholipase-I and the native Phospholipase-II were converted to the fractions with an isoelectric point of 4.5 by

FIG. 6. Interconversions between Phospholipase-I and Phospholipase-II by Extraction and Addition of the Endogeneous Lipids.

Phospholipase-I preparation was delipidated as described in MATERIALS AND METHODS, and the resulting delipidated enzyme was applied to isoelectric focusing. Furthermore, the reconstituted Phospholipase-I and the lipid-added Phospholipase-II preparations prepared as described in MATERIALS AND METHODS were also separately subjected to electrophoresis. Electric focusing was performed under the same conditions as described in Fig. 1. ○—○, Purified Phospholipase-I; △---△, delipidated Phospholipase-I; ●---●, reconstituted Phospholipase-I; ▲---▲, lipid-added Phospholipase-II; ●•●, pH.

addition of the extracted lipids in yields of about 50%. Such shift of the isoelectric point was also observed when the crude extract of porcine pancreas was delipidated with the same method, as shown in Fig. 7. The delipidated

and the reconstituted enzymes did not contain lipase activity. Lipase activity in Phospholipase-I preparation was shifted to the fractions with an isoelectric point of 5.4 (Matsuda and Hirayama, unpublished data).

FIG. 7. Isoelectric Focusing of the Crude Extract of Delipidated Porcine Pancreas.

The crude extract obtained from the delipidated homogenate of porcine pancreas was subjected to isoelectric focusing. The isoelectric focusing was carried out under the same conditions as described in Fig. 1. ○—○, Phospholipase activity, ●—●, optical density at 280 nm; ●—●, pH.

Comparison of properties of the native and the converted phospholipases

The properties of the native and the converted phospholipases are summarized in Table III. The delipidated Phospholipase-I and the purified reconstituted Phospholipase-I obtained in the experiment were very similar to native Phospholipase-II and native Phospholipase-I, respectively, in the properties such as isoelectric point, the behavior on gel filtration on Sephadex G-200, and heat stability. The lipid-added Phospholipase-II was also very similar to native Phospholipase-I.

The composition of the extracted lipids

The extracted lipids of Phospholipase-I were analyzed by the method previously described.²³⁾ As demonstrated in Fig. 8 and Table IV, the extracted lipids consisted of free fatty acids, unknown lipids I and II, phospholipids, and other lipids. The unknown lipids and the effective components for the inter-conversion are not yet clear. They will be re-

TABLE III. COMPARISON OF PROPERTIES OF THE NATIVE AND THE CONVERTED PHOSPHOLIPASES

Enzymes	Yield ^a (%)	Isoelectric point	Heat stability ^b Relative remaining activity (%)	Molecular ^c weight
Native Phospholipase-I	100	4.5	95	5×10^5
Delipidated Phospholipase-I	75	6.0	16	5×10^5
Reconstituted Phospholipase-I	38	4.5	88	5×10^5
Native Phospholipase-II	100	6.0	11	5×10^5
Lipid-added Phospholipase-II	48	4.5	93	5×10^5

^a The yields of the enzyme activities were expressed as percentages of the activity of the each native Phospholipase-I or -II.

^b The enzymes were heated for 5 min at 100°C and pH 4. Relative remaining activities were expressed as a percentage of the each native enzyme activity.

^c The molecular weight was estimated by gel filtration on a Sephadex G-200 column according to the method of Andrews.²¹⁾

FIG. 8. Thin-layer Chromatogram of the Extracted Lipids.

The extracted lipids were developed on a silica gel plate with a solvent mixture, *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v). The lipids separated were detected with 50% H_2SO_4 containing 1% dichromate.²³⁾ Standard lipids: SE, sterol ester; TG, triglyceride; DG, diglyceride; MG, monoglyceride; FFA, free fatty acid. Numbers in figure show band number.

TABLE IV. COMPOSITION OF THE EXTRACTED LIPIDS

Band No.	Composition (%)	Lipids identified
1	23	Unknown lipid I
2	14	Unknown lipid II
3	41	Free fatty acids
4	10	diglyceride
5	7	Polar lipids (Phospholipids)
Others	5	

The composition of the extracted lipids was determined by the densitometry of the thin-layer chromatogram visualized by charring with 50% H_2SO_4 containing 1% dichromate.²²⁾

ported in near future.

Preparation of a low molecular weight phospholipase from Phospholipase-I

The purified Phospholipase-I was delipidated with acetone and diethyl ether as described in MATERIALS AND METHODS. The precipitate was collected by centrifugation ($15,000 \times g$, 30 min), and was dissolved in the small volume of 5 mM Tris-HCl buffer (pH 8.0). The resulting enzyme solution was adjusted to pH 4.0 with 1 N HCl, and was heated at $100^\circ C$ for 5 min. After centrifugation ($10,000 \times g$,

FIG. 9. Gel Filtration of Phospholipase Obtained from Phospholipase-I by Delipidation and Heat treatments on a Sephadex G-100 Column.

The phospholipase obtained from Phospholipase-I by delipidation and heat-treatment was loaded on a Sephadex G-100 column (3×20 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$, and eluted with the same buffer. Fractions of 5 ml were collected and subjected to enzyme assay. ○—○, Phospholipase activity; ●—●, optical density at 280 nm.

FIG. 10. Isoelectric Focusing of Low molecular Weight Phospholipase.

The low molecular weight phospholipase preparation obtained from gel filtration on Sephadex G-100 was subjected to isoelectric focusing using ampholyte in the pH range of 3~10. The electrophoresis was performed under the same conditions as described in Fig. 1. ○—○, Phospholipase activity; ●—●, optical density at 280 nm; ●—●, pH.

TABLE V. COMPARISON OF THE FATTY ACID COMPOSITIONS OF HYDROLYSIS PRODUCTS OBTAINED BY A LOW MOLECULAR WEIGHT PHOSPHOLIPASE AND SNAKE VENOM PHOSPHOLIPASE A_2 AS A REFERENCE ENZYME

Enzymes	Hydrolysis products	Fatty acids (mol%)					
		16:0	18:0	18:1	18:2	Saturated	Unsaturated
Snake venom phospholipase A_2	LPC (C-1)	75	25	trace	trace	100	trace
	FFA (C-2)	4	1	73	22	5	95
low molecular weight phospholipase	LPC (C-1)	77	23	trace	trace	100	trace
	FFA (C-2)	5	2	71	22	7	93

Egg phosphatidylcholine was hydrolyzed with a low molecular weight phospholipase and snake venom phospholipase A_2 . Hydrolysis products were separated by thin-layer chromatography, and the resulting FFA and LPC-bound FFA were analyzed by gas chromatography as previously described.¹⁸⁾ C-1 and C-2 show the positions of the lipid glycerol. LPC; lysophosphatidylcholine, FFA; free fatty acid.

20 min), the supernatant was dialyzed against 5 mM Tris-HCl buffer (pH 8.0) for 12 hr. The dialyate was loaded on a Sephadex G-100 column (3×20 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and was eluted with the same buffer. As Fig. 9 shows, phospholipase activity was eluted as a single peak, indicating that it had a low molecular weight. The active fractions were collected and concentrated by freeze-drying, and was subjected to electric focusing. As Fig. 10 indicates, the phospholipase had an isoelectric point of about 6.0. The active fractions were collected and dialyzed against 5 mM Tris-HCl buffer (pH 8.0) for 12 hr to give a purified low molecular weight phospholipase. The phospholipase was purified about 15-fold from Phospholipase-I (536-fold from crude extract). The enzyme was estimated to be a molecular weight of about 14,000 by gel filtration on a Sephadex G-100 column,²⁰⁾ as shown in Fig. 11, and had also a high heat stability. Furthermore, the positional specificity of the enzyme was examined according to the method described previously.¹⁸⁾ As Table V demonstrates, the fatty acid compositions of hydrolysis products such as free fatty acid and lysophosphatidylcholine by the phospholipase digestion were very similar to those of hydrolysis products by snake venom phospholipase A_2 used as a reference enzyme. Therefore, the low molecular weight phospholipase seems to be an enzyme of A_2 type. These properties

FIG. 11. Determination of the Molecular Weight of Phospholipase obtained from Phospholipase-I by Delipidation and Heat Treatments.

The purified phospholipase was loaded on a Sephadex G-100 column (0.9×22 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0) containing 1 mM NaCl, and eluted with the same buffer.²⁰⁾ The fractions of 0.5 ml were collected. The reference proteins were filtrated under the same condition. A, Cytochrome C (M. W.=12,500); B, chymotrypsinogen (M. W.=25,000); C, ovalbumin (M. W.=43,000); D, serum albumin (M. W.=68,000).

were in agreement with those of porine pancreas phospholipase A_2 described by de Haas *et al.*,^{1,2)} and van Wezel and de Haas,¹³⁾ as shown in Table VI.

Relationship among Phospholipase-I, Phospholipase-II, and low molecular weight phospholipase A_2

Relationship among these three phospholi-

FIG. 12. Relationship Among High Molecular Weight Phospholipase-I and Phospholipase-II, and Low molecular Weight Phospholipase A₂.

➔, Route in this experiment; →, Route reported by de Haas *et al.*^{1,2)}

TABLE VI. COMPARISON OF PROPERTIES OF LOW MOLECULAR WEIGHT PHOSPHOLIPASE WITH PHOSPHOLIPASE A₂

	Low molecular weight phospholipase	Phospholipase A ₂ ^a
Optimum pH	8.0	8.0
Molecular weight	14,000	14,000 ^b
Isoelectric point	about 6.0	6.3
Heat stability	stable	stable
Positional specificity	A ₂ type	A ₂

^a From de Haas *et al.*^{1,2)} and van Wezel and de Haas.¹³⁾

^b By gel filtration on Sephadex G-75¹³⁾.

phases may be illustrated in Fig. 12 from the results described above. Fresh porcine pancreas contained two high molecular weight phospholipases, and these phospholipases seem to be reversibly converted to each other by delipidation or by incubating it with the extracted lipids. Low molecular weight phospholipase A₂ was obtained from high molecular weight phospholipase-I by delipidation and heat treatments.

DISCUSSION

Two high molecular weight Phospholipase-I and Phospholipase-II with different isoelectric points of 4.5 and 6.0, respectively, were found in the homogenate without delipidation and heat treatments of porcine pancreas (Fig. 1).

Phospholipase-I was converted to the fractions corresponding to Phospholipase-II by delipidation, while Phospholipase-II was converted to the fractions corresponding to Phospholipase-I by incubating it with the extracted lipids. Furthermore, a low molecular weight phospholipase was obtained from the Phospholipase-I by delipidation and heat treatments. The enzyme obtained was very similar to phospholipase A₂ purified from porcine pancreas with delipidation and heat treatments.^{1,2)} These results suggested that Phospholipase-I exist as a lipoprotein which is composed of Phospholipase-II and unknown lipids. The proteins of both phospholipases may be the same. Furthermore, the two high molecular weight phospholipases seem to be in more native and physiological forms than low molecular weight phospholipase in the pancreas.

It is known that all the lipases obtained from human pancreas,^{14,23)} human adipose tissue,²⁴⁾ and rat liver²⁵⁾ exist as a protein-lipid complex. Erlanson *et al.*,²³⁾ reported that human pancreatic lipase forms macromolecular aggregates in intestinal content. Furthermore, Kimura *et al.*,¹⁴⁾ described that a high molecular weight Lipase-F exists as a low molecular weight Lipase-S-phosphatidylcholine complex, and they further suggested that Lipase-S is synthesized in the human pancreas and secreted to the pancreatic duct, and then

is modified in the duodenum in the presence of bile to form Lipase-F. Since the porcine pancreas phospholipases changed the isoelectric point by the extraction or addition of endogeneous lipids, they differed from the human pancreatic lipase¹⁴⁾ and adipose tissue lipase,²⁴⁾ which showed a change in molecular weights. Although the physiological roles of Phospholipase-I and -II are not clear, the greater stabilities of Phospholipase-I against heat-denaturation (Fig. 4) seem to have a physiological importance. Furthermore, much broader optical pH of Phospholipase-I (Fig. 3) may give a better property to the enzyme for working in intestinal content. The physiological importance of the modification of the enzyme by lipids also should be emphasized.²⁵⁾

Almost all Phospholipase-I was converted to the fractions corresponding to Phospholipase-II by delipidation, but only about 50% of Phospholipase-II was converted to the fractions corresponding to Phospholipase-I by incubating it with the extracted lipids (Fig. 6 and Table III). The yield of the conversion might be increased more than 50% under more suitable conditions. However, since Phospholipase-I and -II were usually found at the ratio of about 1:1 in the crude extract, it seems likely that an equilibrium is established for the conversion under the conditions tested.

Human pancreatic Lipase-S was converted to Lipase-F by incubating with pure bile, phosphatidylcholine, or phosphatidylserine, but neither with lysophosphatidylcholine nor with sodium taurocholate.¹⁴⁾ Effective components required for the conversion of porcine pancreas phospholipases are not yet clear. However, there may be a possibility that the effective components are anionic substances, e.g., anionic phospholipids, because the isoelectric point of Phospholipase-II shifted to more acidic pH by addition of the extracted lipids.

De Haas *et al.*, also reported that the highly purified pancreatic lipase has a strong lipase activity and a slight phospholipase A₁ activity.²⁶⁾ As Figs. 1 and 2 show, Phospholipase-

I fractions contained a high activity of lipase (the ratio of the activity of phospholipase to that of lipase is about 1:10). However, the lipase in the Phospholipase-I fractions (isoelectric point of 4.5) was converted to other fractions with an isoelectric point of 5.4 by extracting lipids with acetone and diethyl ether (Matsuda and Hirayama, unpublished data). Native Phospholipase-II and reconstituted Phospholipase-I preparations contained no lipase activity. Therefore, it seems that the phospholipase A₁ activity of the purified lipase is independent of the conversion in this experiment.

The modification of the enzyme by the endogeneous lipids is of physiological importance,^{14,25)} and the further properties and structures of such phospholipases are very interesting. The substrate and positional specificities, the mode of action, and the subunit structure of the phospholipases will be reported in near future.

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