Partial Purification and Characterization of Phospholipase A₂ from the Hepatopancreas of Red Sea Bream

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Although apparent phospholipase A₂ activity is very low in freshly prepared hepatopancreas homogenates of red sea bream, its activity increased considerably during autolysis. Hence, phospholipase A₂ was purified about 14,500-fold from the dialyzate of delipidated powder of frozen hepatopancreas homogenate by the sequential use of column chromatographies on DEAE-sepharose CL-6B, Toyopearl HW-55F, reversed phase HPLC, and TSK-GEL G3000SW. The final enzyme preparation was nearly homogeneous in SDS-polyacrylamide gel electrophoresis, and its molecular weight was estimated to be approximately 20,000. Purified enzyme had a pH optimum of 8.0 for phosphatidylethanolamine and essentially required Ca²⁺ for activity. These properties of red sea bream hepatopancreas phospholipase A₂ were similar to those of the secretory enzymes of mammalian pancreatic or snake venom phospholipase A₂.

Phospholipase A₂ [EC 3.1.1.4] specifically hydrolyze the fatty acyl ester bond at sn-2 position of glycerophospholipid. In mammals, dietary phospholipids are absorbed into the intestinal epithelial cells as free fatty acids and lysophospholipids after hydrolysis by pancreatic phospholipase A₂ (PLA₂).^{1,2} It is recognized that pancreatic PLA₂ is secreted into the intestinal lumen in the form of an inactive precursor (zymogen), and then activated by tryptic hydrolysis of an Arg-Ala bond in its N-terminal chain.²⁻⁴ Pancreatic PLA₂'s have been isolated from several animals,⁴⁻¹² and their complete amino acid sequences have already been determined.^{4,7,9-11} Moreover, the reaction mechanism between the enzyme and micellar substrates was thoroughly investigated on the basis of the three-dimensional structure of the porcine pancreatic PLA₂.¹³

On the other hand, the study of lipolytic enzymes in fish has been hampered by the lack of a discrete pancreas in most teleosts including red sea bream.¹⁴ Although the muscle tissue of rainbow trout was used as the source of PLA₂ and the enzyme has been partially purified,^{15,16} digestive PLA₂ has not been investigated yet.¹⁷

In the present paper, we tried to purify the PLA₂ from the hepatopancreas of red sea bream and to investigate its properties in order to clarify the mechanism of phospholipid digestion and absorption in fish. This may be the first report concerning the purification of PLA₂ from fish hepatopancreas.

Materials and Methods

Materials

1-Palmitoyl-2-[1-¹⁴C]arachidonylphosphatidylcholine (52.9 mCi/mmol, [1-¹⁴C]-2-acyl PC) was purchased from New England Nuclear through the Nippon Isotope Association. Egg yolk phosphatidylcholine (PC) and 1,2-dioleoylglycerophosphoethanolamine (PE) were purchased from Sigma and Avanti Polar Lipids, Inc., respectively. DEAE-sepharose CL-6B was purchased from Pharmacia Fine Chemicals. Toyopearl HW-55F was obtained from Toso Co., Ltd. All other chemicals were of the highest purity available from commercial sources.

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Fish

Red sea bream *Pagrus major* weighing 300–500 g were maintained in a tank (1 ton) with commercial pellets (Nippon Haigo Shiryō Co., Ltd.). Fish starved for two days were decapitated and transported on ice to the laboratory as soon as possible. Hepatopancreas was rinsed in ice-cold 0.85% (w/v) NaCl solution to remove adhering blood and used as fresh sample.

Hepatopancreas samples of red sea bream, 0.3–2 kg of body weight, were obtained from a fish dealer or fish processing plant. They were transported on ice, rinsed as described above, and stored -20°C for 3 to 6 months.

Assay of PLA_2

Method 1: The assay of PLA_2 was performed according to the method of Teramoto *et al.*¹⁸⁾ [$1\text{-}^{14}\text{C}$]-2-acyl-PC (22,000 dpm) and 100 nmol of egg yolk PC in chloroform-methanol (2:1, v/v) were mixed in a screw-capped tube, and the solvent was evaporated under a stream of nitrogen. To this tube CaCl_2 , sodium deoxycholate, and glycine-NaOH buffer (pH 9.5) were added at final concentrations of 5 mM, 0.3%, and 50 mM, respectively, to make a final volume of 0.5 ml. The tube was sonicated for about 5 min with an Arnst Hansen model Transonic T460 sonicator, and the substrate micellar solution was prepared. The enzyme solution (50 μl) was added to initiate the reaction. Incubation was carried at 20°C for 30 min, and the reaction was terminated by adding 50 μl of 100 mM EDTA. To the reaction mixture, 2 ml of isopropanol-*n*-heptane-1 N sulfuric acid (40:10:1, by vol.) was added, and the tube was vortically mixed. Water (0.75 ml) and *n*-heptane (1.2 ml) were then added, and the tube was centrifuged at $500\times g$ for 10 min. One ml of upper heptane layer was mixed well with 100 mg silicic acid and 2 ml of heptane to adsorb the unreacted PC. After centrifugation at $500\times g$ for 10 min, 1 ml of heptane was transferred to a scintillation vial, and the radioactivity was measured in 10 ml of scintillation cocktail, which consists 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene and 4 g of 2,5-diphenyloxazole per l of toluene, with a liquid scintillation spectrometer (Aloka model LSC-903). One unit of enzyme activity was defined as nmol of fatty acids released per min. Specific activity was expressed as units per mg of protein.

Method 2: PLA_2 activity was measured essentially according to the method of Teramoto *et al.*¹⁸⁾ and Anderson and McCarty.¹⁹⁾ The as-

say mixture contained 2 mM 1,2-dioleoyl PE, 5 mM CaCl_2 , 0.3% sodium deoxycholate, 0.1 M NaCl, 50 mM glycine-NaOH (pH 9.5), and the enzyme solution, in a final volume of 1 ml. For the incubation with ethylenediaminetetraacetic acid, disodium salt (EDTA) or metals, and cetyl trimethylammonium bromide, sodium dodecyl sulfate (SDS), or Triton X-100, those compounds were added instead of CaCl_2 and sodium deoxycholate, respectively. The enzyme reaction was carried at 30°C for 30 min. The termination of the reaction and the extraction of the released fatty acids were performed as described above, and 2 ml of heptane after the adsorption with silicic acid was mixed gently with 2 ml of 0.01% (w/v) rhodamine 6G (Merck)-benzene.¹⁸⁾ The absorbance of 515 or 530 nm was measured at 15 min after the addition of the dye solution, and the released fatty acids were measured by using a calibration curve obtained with an authentic oleic acid.

Method 1 was used for the autolysis experiment and preliminary purification steps, and method 2 was used in duplicate for routine assay at the purification steps and for the specific activity and the enzyme properties. For the assay of specific activity and the enzyme properties, enzyme activity was corrected by subtracting the value of 0 min incubation after addition of enzyme solution. One unit of enzyme activity was defined as nmol of fatty acids released per min, and specific activity was expressed as units per mg of protein.

High-Performance Liquid Chromatography

The HPLC system consisted of Toso Model 803D, a Model Gradient mixer GE-4, and a Rheodyne 7125 sample injector with a 1 ml sample loop. A column of Synchropak RP-P (250 $\times$ 4.1 mm) and a precolumn (50 $\times$ 4.1 mm) were obtained from Synchro, Inc., and a column of ULTRON 300-C₄ (150 $\times$ 4.6 mm) from Shinwa Kako Co., Ltd. The eluents used were as follows: A, 0.1% trifluoroacetic acid (TFA) in water; B, 0.1% TFA in the presence of 49.5% acetonitrile. The post-column eluate was monitored at 280 nm with a Toso spectrophotometer, UV-8 Model II. A column of TSK-GEL G3000SW (600 $\times$ 7.5 mm) was purchased from Toso Co., Ltd.

Other Analytical Procedure

Protein concentration was measured by the method of Lowry *et al.*²⁰⁾ or Bio-Rad protein assay with bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis (PAGE)

was performed by the method of Laemmli^[21] using 15% (w/v) polyacrylamide gel. Amino acid analysis was performed by an Amino acid analyzer (Model 835, HITACHI, Ltd.). Acid hydrolysis was carried out in 4 N methanesulfonic acid *in vacuo* at 110°C for 24 h and 72 h, respectively.

Results

Preliminary Experiments

The PLA₂ activity was measured by Method 1 with [1-¹⁴C]-2-acyl-PC in the preliminary experiment. Fresh hepatopanceras tissue of red sea bream was homogenized in four volumes of Buffer A; 30 mM Tris-HCl (pH 8.0) containing 0.1% (w/v) triton X-100, 100 units/ml of penicillin G potassium, 0.01% (w/v) kanamycin HCl, and 0.006% (w/v) streptomycin, and the homogenate was incubated at 20°C for 72 h. The homogenate was centrifuged at 1,500 × g for 10 min at the selected intervals, and the PLA₂ activity of supernatant was measured. As shown in Fig. 1, although PLA₂ activity was low until 24 h, thereafter its activity rapidly increased up to 72 h.

Fig. 1. Increase in PLA₂ activity of a fresh hepatopanceras homogenate of red sea bream. The hepatopanceras homogenate was incubated at 20°C. The homogenate at the selected interval was centrifuged, and the PLA₂ activity of the supernatant was measured by using [1-¹⁴C]-2-acyl PC as described in Method 1.

Fig. 2. Increase of PLA₂ activity of a frozen hepatopanceras homogenate of red sea bream. The homogenate of hepatopanceras stored for 3 to 6 months was incubated at 20°C, and the PLA₂ activity of the supernatant was measured at the selected interval by using [1-¹⁴C]-2-acyl-PC as described in Method 1.

The effect of pH on the enzyme activity and the stability on heat treatment or acid-organic solvent treatment were investigated by using the supernatant after 72 h autolysis as crude enzyme solution. The optimum activity was found in the range of pH 9.0 to 9.5, and this enzyme was stable for heating at 70°C for 5 min or in a solution of 0.1% TFA containing 49.5% acetonitrile (data not shown).

Purification of PLA₂

Step 1. Autolysis, heat treatment and delipidation: Frozen hepatopanceras of red sea bream was used for the purification of PLA₂. Before purification procedure, the time course change of PLA₂ activity of frozen tissue during autolysis at 20°C was investigated with [1-¹⁴C]-2-acyl-PC as described in Method 1, and it was recognized that the enzyme activity slowly increased and showed the maximum value around 5 to 6 days (Fig. 2). Therefore, the pooled frozen tissues (1 kg) were homogenized with four volumes of Buffer A and incubated at 20°C for 5 days according to the same procedure as described above. Then, after 5 days autolysis the pH of tissue homogenate was adjusted to pH 4.0 by adding 6 N HCl, and the homogenate was heated at 70°C for 3 min. The cooled homogenate was centrifuged at 5,900 × g for 10 min, and then the supernatant was lyophilized. The lyophilized powder was delipidated with three volumes of chloroform-butanol mixture (−10°C) according to the method of Verger *et al.*^[22] The delipidated powder dissolved in a small volume of

5 mM Tris-HCl (pH 7.4) was dialyzed overnight against 5 mM Tris-HCl (pH 7.4), and the dialyate was used as the source for the purification of PLA₂.

Step 2. DEAE-sepharose column chromatography: A half of the resultant dialyate from step 1 was applied to a CM-sepharose CL-6B column (1.6 × 35 cm) connected with DEAE-sepharose CL-6B column (1.6 × 35 cm) which had been equilibrated with 5 mM Tris-HCl (pH 7.4) at 4°C. In the

preliminary experiment, we checked that PLA₂ was adsorbed to DEAE-sepharose CL-6B, and not to CM-sepharose CL-6B at pH 7.4. The columns were exclusively washed with the same buffer, and then the PLA₂ was eluted from DEAE-sepharose CL-6B column with linear gradient of increasing concentration of NaCl from 0 to 1 M at a flow rate of 1 ml/min. The protein was continuously monitored by the absorbance at 280 nm (Fig. 3). Then the same purification pro-

Fig. 3. Elution profile of PLA₂ (—○—) from a DEAE-sepharose CL-6B column. A half of pooled dialyate (316 ml) was applied to a CM-sepharose CL-6B column connected with DEAE-sepharose column. Washing of the column and elution of the enzyme are described in Results. The elution of protein was followed by monitoring the absorbance at 280 nm (—). The dashed line indicates the linear concentration gradient of NaCl (0–1 M). The fraction volume was 7 ml. The PLA₂ activity of a 100 μ l aliquot of each fraction was measured by using Method 2 with 1, 2-dioleoyl-PE.

Fig. 4. Elution profile of protein (—●—) and PLA₂ activity (—○—) from a Toyopearl HW-55F column. The pooled fraction from the DEAE-sepharose column was applied to a Toyopearl HW-55F column. The fraction volume was 5 ml. The PLA₂ activity of a 100 μ l aliquot of each fraction was measured by Method 2 with 1, 2-dioleoyl-PE as described in Method 2.

cedure of the residual dialyate was repeated once more, and the fractions (tube number 31–49) containing PLA₂ activity were combined.

Step 3. Toyopearl HW-55F column chromatography: The fractions with PLA₂ activity which had been recovered from step 2 were lyophilized and dissolved in a small volume of 5 mM Tris-HCl (pH 7.4). Then, the sample was loaded on a Toyopearl HW-55F column (2.2 × 90 cm) pre-

equilibrated with 5 mM Tris-HCl (pH 7.4) containing 0.3 M NaCl. The column was eluted with the same buffer at a flow rate of 0.5 ml/min (Fig. 4). The fractions (elution volumes 294–318 ml) showing high PLA₂ activity were pooled for further purification.

Step 4. Reversed phase HPLC: TFA was added to the pooled sample obtained from step 3 to make a final concentration of 0.1 % (w/v), and

Fig. 5. Elution profile of PLA₂ (—○—) from a reversed phase HPLC. The pooled PLA₂ fraction recovered from the Toyopearl HW-55F column was applied to a Synchropak RP-P column. The elution of protein was followed by monitoring the absorbance at 280 nm (—). The dashed line indicates the linear gradient of the elution solvent. The flow rate was 1 ml/min, and the fraction volume was 1 ml. The PLA₂ activity of a 50 μ l aliquot of each fraction was measured by Method 2 using 1, 2-diolyol-PE.

Fig. 6. Elution profile of PLA₂ (—○—) from a TSK-GEL G3000SW column. The pooled PLA₂ fraction recovered from a reversed phase HPLC was lyophilized after neutralized with 1 M Tris. The lyophilized powder was dissolved in a small volume of 50 mM sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl and loaded onto the TSK-GEL G3000SW column. The elution of protein was followed by measuring the absorbance of 280 nm (—). The PLA₂ activity of a 50 μ l aliquot of each fraction was measured by Method 2 with 1, 2-dioleoyl-PE.

Table 1. Purification of PLA₂ from the hepatopancreas of red sea bream

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)	Purification (fold)	Yield (%)
Dialysis	4605	1018	0.2	1	100
DEAE-sepharose CL-6B	80.7	324	4.0	18	31.9
Toyopearl HW-55F	34.1	334	9.0	44	32.8
Reversed phase HPLC	0.19	280	1474	7370	27.5
TSK-GEL G3000SW	0.18	523	1906	14530	51.4

Fig. 7. SDS-polyacrylamide (15%) gel electrophoresis of the purified PLA₂. Lane 1 contains protein standards; aprotinin (molecular weight 6,500), trypsin inhibitor (20,100), and ovalbumin (45,000). Lane 2 contains cytochrome C (12,500) and trypsin inhibitor. Lane 3 contains the purified PLA₂ from the hepatopancreas of red sea bream.

the sample was applied to a Synchropak RP-P column which was equilibrated with 0.1% TFA. The column was eluted with a linear gradient of increasing concentration (eluant B), from 0 to 20% in 5 min and subsequently from 20 to 80% in 70 min. A sharp peak of PLA₂ activity was obtained in the fractions in tube numbers 46–49 as shown in Fig. 5.

Step 5. High performance gel-filtration chromatography (HPGC): The resultant sample from step 4 was applied to a TSK-GEL G3000SW column which was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl. The column was eluted with the same buffer at a

Fig. 8. Effect of pH on the purified PLA₂ activity. The PLA₂ activity was measured by Method 2 using 1, 2-dioleoyl-PE. The buffers used were 50 mM Tris-HCl (pH 7.0–9.0) and 50 mM glycine-NaOH (pH 9.0–11.0).

flow rate of 0.5 ml/min, and the eluent was monitored by the absorbance at 280 nm (Fig. 6). The protein was eluted as one major peak with PLA₂ activity. The fractions (tube numbers 44–47) showing high PLA₂ activity was collected and stored at –40°C until further use.

Table 1 summarizes the recovery of protein and enzyme activity at various purification steps of PLA₂ activity. Protein concentrations of the purification steps 1, 2, and 3 (dialyate, DEAE-sepharose and Toyopearl HW-55F column chromatographies) were measured by the method of Lowry *et al.*,²⁰ and those of steps 4 and 5 (reversed phase HPLC and HPGC with TSK-GEL G3000SW) were analyzed by Bio-Rad protein assay. The activity of the final PLA₂ preparation increased about 14,500-fold as high as that of the dialyate obtained from step 1, and the purified

PLA₂ was judged to be nearly homogeneous by SDS-PAGE (Fig. 7). The molecular weight of the purified PLA₂ was estimated to be approximately 20,000 by SDS-PAGE and HPGC with TSK-GEL G3000SW.

Some Properties of PLA₂

Properties of the purified PLA₂ were studied. The pH dependence of the activity of the purified PLA₂ is shown in Fig. 8. The substrate PE was hydrolyzed well in an alkaline pH region, and the optimum activity was found at pH 8.0.

The effect of Ca²⁺ on the PLA₂ activity is shown in Fig. 9. In the absence of Ca²⁺, the enzyme activity was hardly detected. Although the PLA₂ activity reached maximum at 5 mM Ca²⁺, its activity gradually increased from 10 to 50 mM Ca²⁺. The effects of various agents on the activity gradually increased from 10 to 50 mM Ca²⁺. The effects of various agents on the activity of the purified enzyme were investigated, and the results are shown in Table 2. The enzyme activity was inhibited completely by the addition of 1 mM EDTA. It was very low by the addition of Mg²⁺, Zn²⁺, Fe³⁺, or Cu²⁺ to replace Ca²⁺. Phenylmethylsulfonyl fluoride considerably inhibited the enzyme activity. Both SDS and cetyl trimethylammonium bromide completely inhibited the PLA₂ activity at high concentrations (0.1–0.2%). The activity did not change in the presence of Triton X-100 at the concentration of 0.01–0.2%; however, its activity decreased at the low level of sodium deoxycholate (0.2 and 0.3%).

The PLA₂ preparation obtained after HPGC with TSK-GEL G3000SW was analyzed by reversed phase HPLC with ULTRON 300 C₄, and it was recognized that the PLA₂ preparation consisted of one major and three minor peaks. The main peak, which showed high PLA₂ activity, was collected by reversed phase HPLC and used for amino acid analysis determination. The result is shown in Table 3. The amino acid composition of PLA₂ suggests that the enzyme is composed of 193 or 194 residues with a calculated molecular weight of 21,667 or 21,738 in agreement with the apparent molecular weight of 20,000 calculated by SDS-PAGE. Moreover, PLA₂ contains 10 cysteines and rich hydrophobic amino acids.

Discussion

In mammals, pancreatic PLA₂ is produced in the form of inactive pro-phospholipase A₂ (zymogen)

Fig. 9. Effect of calcium ion on the purified PLA₂ activity. The assay conditions were described in Materials and Methods, except that the reaction was carried out in 50 mM Tris-HCl buffer (pH 8.0) containing various concentrations of CaCl₂. The enzyme activity was measured by Method 2 with 1, 2-dioleoyl PE.

Table 2. Effects of various compounds on the purified PLA₂.

Compounds	Final concentration	Remaining activity (%)
Control	(—)	100
EDTA* ¹	1 mM	0
MgCl ₂	5 mM	10.3
ZnCl ₂	5 mM	18.8
FeCl ₃	5 mM	17.1
CuCl ₂	5 mM	12.8
CuSO ₄	5 mM	24.8
PMSF* ²	0.5 mM	51.7
	1 mM	25.2
	2 mM	25.8
CTAB* ³	0.01%	114
	0.1%	0
	0.2%	0
SDS	0.01%	98.1
	0.1%	45.5
	0.2%	0
Triton X-100	0.01%	107
	0.1%	107
	0.2%	107
Sodium deoxycholate	0.2%	24.8
	0.25%	37.2
	0.3%	61.0

*¹ EDTA, ethylenediaminetetraacetic acid, disodium salt.

*² PMSF, phenylmethylsulfonyl fluoride.

*³ CTAB, cetyl trimethylammonium bromide.

which is stored in the secretory granules. The activation of pro-phospholipase A₂ (pro-PLA₂) occurred by limited tryptic proteolysis only after secreted in the intestinal caeca.³⁷ We recognized that PLA₂ in the hepatopancreas of red sea bream

Table 3. Amino acid composition of PLA₂ from the hepatopancreas of red sea bream

Amino acid	Red sea bream hepatopancreas		Porcine pancreas* ¹
	mol (%)	nearest integer	
Asx	16.32	32	22
Glx	9.69	19	8
Arg	3.72	7	4
Lys	4.12	8	11
His	2.92	6	3
Ser	5.82	11	8
Thr	2.69	5	7
Gly	6.52	13	7
Ala	7.99	15 or 16	4
Val	4.00	8	4
Ile	4.15	8	6
Leu	4.80	9	6
Cys	5.28	10	14
Met	2.72	5	1
Pro	8.26	16	5
Phe	5.25	10	4
Tyr	5.18	10	9
Try	0.52	1	1
Total		193 or 194	124
Calculated molecular weight		21,667 or 21,738	13,981

*¹ Cited from the data of Evenberg *et al.* (10)

also existed as pro-PLA₂ (Fig. 1) like mammalian pancreatic PLA₂.^{2,3)} In the preliminary experiment, we tried to activate the proenzyme by incubating the hepatopancreas homogenate with porcine pancreatic trypsin at various intervals, but we did not succeed. This was probably because it was essential to perform autolysis before extraction of the enzyme. Moreover this enzyme was stable by heat treatment at 70°C for 5 min or in the solution of 0.1% TFA containing 49.5% acetonitrile (data not shown) like mammalian organic PLA₂.^{18,23,24)}

Initially, we attempted to purify the PLA₂ by measuring the enzyme activity with [1-¹⁴C]-2-acyl-PC and by using a series of purification procedures from the fresh hepatopancreas of red sea bream; autolysis (3 days at 20°C), heat treatment (70°C for 3 min), DEAE-sepharose column chromatography (5 mM Tris-HCl, pH 7.4, 0–1 M NaCl gradient), the first reversed phase HPLC (0.1% TFA, 0–49.5% acetonitrile), and the second reversed phase HPLC. At the purification step of the first reversed phase HPLC, the enzyme was purified about 2,650-fold in a yield of 9.6%. However, at the second reversed phase HPLC a recovery of enzyme was lowered only 0.3% and the protein concentration could not be measured by the method of Lowry *et al.*²⁰⁾ Therefore we

tried to purify the enzyme from 1 kg of frozen hepatopancreas by using the similar purification steps beside the final step. When the frozen tissue was used as the enzyme source, the increase of the PLA₂ activity by autolysis was not so rapid and high, when compared with that in fresh tissue (Figs 1 and 2). It was assumed from these results that autolysis proceeded during storage from 3 to 6 months at –20°C.

PLA₂ preparation was finally purified approximately 14,500-fold from the dialyzate of delipidated powder of frozen hepatopancreas homogenates by using the different chromatographies. The molecular weight of the purified PLA₂ preparation was estimated to be about 20,000 by SDS-PAGE. This enzyme was assumed to be a monomer, because its molecular weight did not change both with and without 2-mercaptoethanol by SDS-PAGE. Moreover, the amino acid analysis gave a minimum molecular weight of 21,667 or 21,738 and high cysteine contents (Table 3). The molecule was estimated to contain 193 or 194 amino acids residues. The activity of the enzyme is essentially dependent on Ca²⁺ ions (Fig. 9), and it had a pH optimum at alkaline region (pH 8.0) as shown in Fig. 8.

From the above results, four points can be summarized about the properties of PLA₂ of red sea

bream hepatopancreas: a) PLA₂ exists as inactive precursor (zymogen) in the hepatopancreas, b) PLA₂ is stable against acid and heat treatment and in the solution of 0.1% TFA-49.5% acetonitrile, c) purified PLA₂ contains 10 cysteines and rich hydrophobic in its molecules, d) purified PLA₂ activity is dependent for Ca²⁺ ion concentration, and it has an optimum pH in the alkaline region. Although the molecular weight (20,000) of the purified PLA₂ was larger than those from mammalian pancreas (MW 13,000–14,000),^{4–12)} the above properties of the purified PLA₂ such as pH optimum, Ca²⁺ requirement, heat stability, high cysteine contents and the existence as inactive precursor are similar to mammalian pancreatic PLA₂.^{2,5)} Therefore, we suggest that this enzyme is secreted in the intestinal lumen as pro-PLA₂ from the hepatopancreas, and then activated by limited tryptic hydrolysis in the same manner of mammalian pancreatic PLA₂.^{2–4,25)}

Ono *et al.*¹²⁾ recognized that rat pancreatic PLA₂ purified as a single band on SDS-PAGE was separated into active PLA₂ and pro-PLA₂ by reversed phase HPLC. Also in the present report the final PLA₂ preparation after HPGC with TSK-GEL G300SW was separated into major one and minor three components by reversed phase HPLC. Although the major component showed a high PLA₂ activity, we have not yet measured the PLA₂ activity of the three minor components. Further investigation about the properties of three minor components is in progress.

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