Purification, characterization and molecular cloning of group I phospholipase A2 from the gills of the red sea bream, Pagrus major

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Purification, Characterization, and Molecular Cloning of Group I Phospholipases A_2 from the Gills of the Red Sea Bream, *Pagrus major*

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ABSTRACT: Phospholipase A_2 (PLA_2) activity was investigated in various tissues of male and female red sea bream. In both male and female fishes, the specific activity of PLA_2 in the gills was 70 times higher than that in other tissues, such as the adipose tissue, intestine, and hepatopancreas. Therefore, we tried to purify PLA_2 from the gill filaments of red sea bream to near homogeneity by sequential chromatography on Q-Sepharose Fast Flow, Butyl-Cellulofine, and DEAE-Sepharose Fast Flow columns, and by reversed-phase high-performance liquid chromatography. Two minor and one major PLA_2, tentatively named G-1, G-2 and G-3 PLA_2, were purified, and all showed a single band with an apparent molecular mass of approximately 15 kDa by sodium dodecylsulfate-polyacrylamide gel electrophoresis. The exact molecular mass values of G-1, G-2, and G-3 PLA_2 were 14,040, 14,040 and 14,005 Da, respectively. G-1, G-2, and G-3 PLA_2 had a Cys 11 and were all identical in N-terminal amino acid sequences from Ala-1 to Glu-56. A full-length cDNA encoding G-3 PLA_2 was cloned by reverse transcriptase-polymerase chain reaction and rapid amplification of cDNA ends methods, and G-3 PLA_2 was found to be classified to group IB PLA_2 from the deduced amino acid sequence. G-1, G-2, and G-3 PLA_2 had a pH optimum in an alkaline region at around pH 9–10 and required Ca^{2+} essentially for enzyme activity, using a mixed-micellar phosphatidylcholine substrate with sodium cholate. These results demonstrate that three group I PLA_2, G-1, G-2, and G-3 PLA_2, are expressed in the gill filaments of red sea bream.

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Phospholipase A_2 (phosphatide 2-acyl hydrolase, EC 3.1.1.4) (PLA_2) hydrolyzes the fatty-acyl ester bond at the *sn*-2 position of glycerophospholipids. PLA_2 has now become recognized as being a large superfamily of distinct enzymes that play a central role in diverse cellular processes including phospholipid digestion and metabolism, host defense, and signal transduction (1). New groups of Ca^{2+}-dependent low molecular mass enzymes, so-called secretory PLA_2, have recently been purified and are now classified into six groups, group I, II, III, V, IX, and X, based on their primary structures (1–3). Mammalian pancreatic type PLA_2, group IB PLA_2, was originally found in large amounts in the pancreas and was considered to function in the digestion of dietary phospholipids. However, this enzyme was later found to express in nonpancreatic tissues including the spleen, lung, kidney, and ovary (4–12). It has now been proposed to function as a kind of cytokine/growth factor in several cell lines by interacting with specific binding sites on the cell surface (13,14). Two major types of receptors, N-type and M-type receptors, have been recently identified for secretory PLA_2, including snake venom group IA PLA_2 and mammalian group IB PLA_2, and the involvement of mammalian group IB PLA_2 *via* M-type receptor in various physiological and pathophysiological responses such as cell proliferation, cell contraction, lipid mediator release, acute lung injury, and endotoxic shock has been proposed (13,15,16). These aspects indicate that the physiological importance of group I PLA_2 in the nondigestive tissues and cells is higher than considered previously.

On the other hand, there is little information about the enzymology of fish PLA_2. Attempts have been made to purify PLA_2 from liver, hepatopancreas, and muscles of fish such as rainbow trout *(Salmo gairdneri)* (17,18) and cod *(Gadus morhua)* (19,20). However, only one PLA_2 has been purified from cod muscle (19). Zambonino Infante and Cahu (21) recently analyzed the mRNA level of PLA_2 in *Dicentrarchus labrax* larvae cultured with diets containing different lipid levels but did not obtain the purified enzyme. To understand the enzymology of digestive lipolysis in fish, we purified three secretory PLA_2 from the digestive organs including the pyloric ceca and hepatopancreas of red sea bream, and two PLA_2, DE-1 and DE-2 PLA_2, purified from the hepatopancreas were found to be classified as group I PLA_2 based on the amino acid sequence (22–24). We also found that an antiserum against *Naja naja* venom PLA_2 reacted weakly with

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Abbreviations: *p*-APMSF, *p*-amidinophenylmethylsulfonyl fluoride; *p*-BPB, *p*-bromophenacyl bromide; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CTAB, cetyl trimethylammonium bromide; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PLA_2, phospholipase(s) A_2; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; RACE, rapid amplification of cDNA ends; RP–HPLC, reversed-phase–HPLC; RT, reverse transcriptase; SDS, sodium lauryl sulfate; TFA, trifluoroacetic acid.

the partially purified PLA_2 from the hepatopancreas of red sea bream and that it labeled zymogen granules of the pancreatic acinar cells and secretory materials of certain epithelial cells of epithelial crypts in the pyloric ceca of red sea bream (25). In the present report, we investigated the distribution of PLA_2 activity in the various tissues of red sea beam and found extremely high PLA_2 activity in the nondigestive tissue, gills. We further purified two minor and one major PLA_2, tentatively named G-1, G-2, and G-3 PLA_2 from the gill filaments of red sea bream. The N-terminal amino acid sequences of G-1, G-2, and G-3 PLA_2 show that they belong to group I PLA_2. Furthermore, we isolated a full-length cDNA clone of G-3 PLA_2 by polymerase chain reaction (PCR) methods and showed that G-3 PLA_2 is classified to group IB PLA_2 from the deduced amino acid sequence.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Sodium cholate was purchased from Nacalai Tesque (Kyoto, Japan). 9-Anthryldiazomethane was purchased from Funakoshi Co., Ltd. (Tokyo, Japan) and *n*-heptadecanoic acid from Tokyo Kasei Organic Chemicals (Tokyo, Japan). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and *p*-bromophenacyl bromide (*p*-BPB) were obtained from Dojindo Laboratories (Kumamoto, Japan). DEAE-Sepharose Fast Flow and Q-Sepharose Fast Flow were purchased from Pharmacia (Uppsala, Sweden). Butyl-Cellulofine was purchased from Seikagaku Kogyo (Tokyo, Japan), Asahipak ODP-50 was obtained from Showa Denko (Tokyo, Japan), YMC-Pack Protein-RP from YMC Co., Ltd. (Tokyo, Japan), and TSKgel Octadecyl-NPR from Tosoh (Tokyo, Japan). High-performance liquid chromatography (HPLC) grade CH_3CN was purchased from Nacalai Tesque, and 2-mercaptoethanol, 4-vinylpyridine, cetyl trimethylammonium bromide (CTAB), *p*-amidinophenylmethylsulfonyl fluoride (*p*-APMSF), and a silver staining kit were from Wako Pure Chemicals (Osaka, Japan). The DC Protein Assay Kit was obtained from Bio-Rad Laboratories (Richmond, CA).

Standard assay for PLA_2. PLA_2 activity was determined as described previously (23). The standard incubation systems (100 μL) for the assay of PLA_2 contained 2 mM POPC, 6 mM sodium cholate, 100 mM NaCl, 50 mM glycine-NaOH (pH 9.5), and 5 mM $CaCl_2$. One unit (U) was defined as the liberation of 1 μmol of free fatty acid/min as measured by reversed-phase (RP)–HPLC. In assays without detergent, the substrate was sonicated for 2 min in a water bath sonicator (Transsonic T460; Elma GmbH & Co. KG, Singen, Germany).

Preparation of dialyzate. Gill filaments, heart, spleen, adipose tissue, testis or ovary, hepatopancreas, stomach, pyloric ceca, and intestine were collected from five freshly killed farm-raised male and female red sea bream, *Pagrus (Chrysophrys) major* (0.8–1.0 kg), and were immediately frozen using dry ice. Frozen tissues were lyophilized, and the delipidated powder was prepared as described previously (23). Each 0.5 g of the resulting delipidated powder was solubilized in 5 mL of 5 mM Tris-HCl buffer (pH 7.4) for 1 h to yield a crude extract. The pH of the crude extract was adjusted to 4 by adding 6 N HCl, and the acidified extract was then heated at 80°C for 3 min. The cooled extract was centrifuged at $10,000 \times g$ for 15 min at 4°C. The supernatant was dialyzed overnight against 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at $10,000 \times g$ for 15 min at 4°C. The resulting dialyzate was used for the determination of PLA_2 activity in the various red sea bream tissues.

For the purification of PLA_2, gill filaments were collected from 58 freshly killed farm-raised red sea bream (0.8–1.0 kg) and were immediately frozen using dry ice. The delipidated powder (125.5 g) of the gill filaments was solubilized in 10 vol of 5 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM *p*-APMSF and a dialyzate was prepared as described above.

Purification of gill PLA_2. All steps were carried out at 4°C except for the HPLC runs, which were performed at room temperature. The dialyzate was loaded onto a Q-Sepharose Fast Flow column (5×5.5 cm) which was equilibrated with 5 mM Tris-HCl buffer (pH 7.4). The column was washed extensively with the same buffer, and PLA_2 activity was eluted with 5 mM Tris-HCl buffer (pH 7.4) containing 2 M NaCl at a flow rate of 5 mL/min. The active fractions were directly applied to a Butyl-Cellulofine column (2.6×19 cm) which was equilibrated with 5 mM Tris-HCl buffer (pH 7.4) containing 2 M NaCl. The column was washed extensively with the same buffer, followed by elution with 5 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and with 5 mM Tris-HCl buffer (pH 7.4), respectively, at a flow rate of 0.6 mL/min. PLA_2 activity was eluted with 5 mM Tris-HCl buffer (pH 7.4). The active fractions were pooled, lyophilized, and dissolved in 20 mM Tris-HCl buffer (pH 8.0) containing 0.5% CHAPS. This solution was applied to a DEAE-Sepharose Fast Flow column (1.6×15 cm) which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 0.5% CHAPS. The PLA_2 activity was bound to the column and was eluted with a linear gradient of NaCl. The active fractions were pooled. Trifluoroacetic acid (TFA) was added to the active fractions at a final concentration of 0.1%, and the resulting solution was applied to a YMC-Pack Protein RP column (4.6×150 mm) which was equilibrated with 0.1% TFA/10% CH_3CN. The PLA_2 activity was bound to the HPLC column, and the column was eluted with a CH_3CN linear gradient. The active fractions were pooled. Disodium hydrogenphosphate was added to the active fractions to a final concentration of 10 mM. The resulting solution was applied to an Asahipak ODP-50 column (6×250 mm) that was pre-equilibrated with 10 mM Na_2HPO_4. PLA_2 activity was bound to the HPLC column. The column was then washed with 10 mM Na_2HPO_4 and eluted with a linear gradient from 10 mM Na_2HPO_4 to 0.5 mM Na_2HPO_4/60% CH_3CN. Peaks 1, 2, and 3 showing

PLA_2 acivity were pooled and stored as final enzyme preparations, tentatively named G-1, G-2 and G-3 PLA_2, respectively. During the purification step, the protein concentration was measured with a DC Protein Assay Kit with bovine serum albumin as a standard. The protein concentration of G-3 PLA_2 was measured as described above. For G-1 and G-2 PLA_2, an HPLC protein determination method was devised. G-1 and G-2 PLA_2 were injected on a TSK gel Octadecyl-NPR (4.6 × 35 mm) column that had been equilibrated with 10 mM Na_2HPO_4. The column was eluted with a linear gradient from 10 mM Na_2HPO_4 to 0.5 mM Na_2HPO_4/60% CH_3CN, and the eluate was analyzed at 220 nm. G-3 PLA_2 was used for HPLC analysis as a protein standard.

Molecular weight determination of the purified enzymes. The molecular weights of purified enzymes were determined by sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometer (Dynamo, Finnigan-MAT, San Jose, CA). SDS-PAGE was carried out as described by Laemmli (26) using a 16% polyacrylamide gel in the presence of 2-mercaptoethanol, and the protein bands were stained with Coomasie Brilliant Blue-R250.

For MALDI-TOF mass spectrometry, the purified enzymes were desalted by RP–HPLC with a TSKgel Octadecyl-NPR column having a linear gradient of CH_3CN from 10% CH_3CN/0.1% TFA to 70% CH_3CN/0.1% TFA, at a flow rate of 0.8 mL/min. One microliter (approximately 0.3 pmol) of the desalted enzyme solution was mixed with an equal volume of matrix solution [10 mg/mL 2,5-dihydroxybenzoic acid/10 mg/mL 5-methoxysalicylic acid (9:1, vol/vol)] on a target disk and allowed to dry. Subsequently, spectra were obtained using a MALDI-TOF mass spectrometer, with horse apomyoglobin (16,951 Da) as a calibration standard.

N-terminal amino acid sequence determination. The purified PLA_2 were reduced with 2-mercaptoethanol and were S-pyridylethylated with 4-vinylpyridine as described previously (24). The amino acid sequence of the S-pyridylethylated PLA_2 was analyzed with a Hewlett-Packard G1005A protein sequencing system (Palo Alto, CA).

Extraction of mRNA. Gill filaments were removed immediately from freshly killed red sea bream (approximately 200 g) and were stored in liquid nitrogen. Total RNA was extracted from the gill filaments using Isogen (Nippon Gene, Tokyo, Japan), and mRNA was isolated using Oligotex-dT30 <Super> (Roche Diagnostics K.K., Tokyo, Japan), according to the manufacturer's protocol.

cDNA amplification of gill G-3 PLA_2 by PCR. Primers P20 and P2a are 5′-ATCTGGCAGTTCGGCGACATGATCGAG-3′ and 5′-GC(A/G)GCCTT(C/T)CTGTCGCACTC(A/G)CA-3′, that were derived from possible cDNA sequences corresponding to parts of amino acid sequence of red sea bream gill G-3 PLA_2, IWQFGDMIE (Residues 2–10, Fig. 5), and that of conserved amino acid sequence of mammalian pancreatic PLA_2 (27) and red sea bream hepatopancreas DE-2 PLA_2 (DDBJ/EMBL, Accession No. AB009286), CECDRKAA (Residues 96–103), respectively. One microgram of red sea bream gill mRNA was used to prepare first-stranded cDNA with a RNA PCR kit (Takara, Tokyo, Japan), employing molony murine leukemia virus reverse transcriptase. An internal cDNA fragment encoding PLA_2 was generated by PCR from first-stranded cDNA of red sea bream gill using degenerated primers P20 and P2a and *Pyrococcus kodakaraensis* KOD1 (KOD dash) DNA polymerase (Toyobo, Tokyo, Japan). After an initial denaturation for 160 s at 94°C, 30 cycles of amplification were carried out, with 30 s at 94°C, 10 s at 58°C, 30 s at 74°C, followed by incubation at 74°C for 10 min. Then the PCR product was isolated from an agarose gel using Ultrafree R-MC centrifugal filter unit (0.45 μm) (Millipore, Bedford, MA) and was sequenced. From the determined nucleotide sequence of the internal cDNA, new oligomers, primers P26 (5′-CATGACGACTGCTATGGA-GCACA-3′, identical to nt 211–233) and AP22 (5′-GGTC-GCTGAGCAGGTCACCTTGCG-3′, complementary to nt 307–330) were designed for 3′- and 5′-end amplifications, respectively. A first- and second-stranded cDNA were synthesized using red sea bream gill mRNA and Marathon cDNA Amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Briefly, 1 μg of red sea bream gill mRNA was reverse-transcribed using 10 μM of cDNA synthesis primer and 20 units of avian myeloblastosis virus reverse transcriptase at 42°C for 1 h. Second-stranded cDNA synthesis was carried out using *Escherichia coli* DNA polymerase I (24 units), DNA ligase (4.8 units), and RNase H (1 unit) at 16°C for 1.5 h, followed by T4 DNA polymerase (10 units) at 16°C for 45 min. The resulting second-stranded cDNA was precipitated and ligated to Marathon cDNA Adaptor (2 μM) using 1 unit of T4 DNA ligase for 40 min at room temperature. The 5′-end amplification of PLA_2 cDNA was carried by reverse transcriptase-polymerase chain reaction (RT-PCR) with adaptor-ligated double-stranded cDNA, Adaptor primer 1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′), AP22 antisense primer and KOD Dash, and 3′-end amplification of PLA_2 cDNA was with adaptor-ligated double stranded cDNA, Adaptor primer, P26 sense primer, and KOD Dash. PCR conditions of 5′- and 3′-end amplification were as follows. After an initial denaturation for 160 s at 94°C, 30 cycles of amplification were carried out, with 30 s at 94°C, 10 s at 66°C, 30 s at 74°C, followed by incubating at 74°C for 10 min. The resulting PCR products were subcloned into pGEM-T vector (Promega, Madison, WI) and were transformed into JM109 cells, and positive clones were selected on LB/ampicillin/IPTG/X-Gal plates according to the manufacturer's protocol. Plasmid DNA was purified from positive clones with QIAprep Spin Miniprep Kit (Qiagen, Tokyo, Japan) and was sequenced.

Sequencing of PCR products. Cycle sequencing reaction of PCR products was performed using Dye terminator cycle sequencing kit (PerkinElmer, Norwalk, CT), according to the manufacturer's protocol, and the sequences of PCR products were determined with an Applied Biosystems 373A DNA sequencer (Foster, CA).

TABLE 1
Distribution of Phospholipase A_2 (PLA_2) Activity in the Various Tissues of Red Sea Bream[a]

Tissues	Specific activity (mU/mg protein)		Specific activity (mU/g tissue)	
	Male	Female	Male	Female
Gills	1000	1040	14,659	13,769
Heart	1	7	6.7	49.3
Spleen	0.4	0.4	4.3	7.1
Muscle	0.1	0.8	1.5	3.1
Adipose tissue	14.5	10	19.1	8.1
Testis	1.5	—	13.4	—
Ovary	—	0.4	—	3.9
Hepatopancreas	3	1.5	15.2	16
Stomach	0.4	0.6	2.3	11.2
Pyloric ceca	0.7	0.5	13.7	12.9
Intestine	4.2	3	69.3	34.3

[a]The reaction mixture consisted of 50 mM glycine-NaOH (pH 9.5), 2 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 6 mM sodium cholate, 5 mM $CaCl_2$, and 0.1 M NaCl in a final volume of 0.1 mL. Data were obtained in duplicate.

RESULTS

Distribution of PLA_2 activity in the various tissues of red sea bream. The dialyzate was prepared from the various tissues of red sea bream, and PLA_2 activity in the dialyzate was measured using the mixed-micellar POPC substrate with sodium cholate. As shown in Table 1, the specific activity (mU/mg protein) of PLA_2 in the gills was extremely high, followed by adipose tissue, intestine, and hepatopancreas of male fish; PLA_2 activity of gills was over 70 times higher than that of adipose tissue and 200 times higher than that of other tissues, such as intestine, hepatopancreas and so on. Also in female fish, PLA_2 activity in the gills was exceedingly high, followed in order by adipose tissue, heart, and intestine. In comparison with PLA_2 activity per gram of tissue, specific activity of PLA_2 in the gills was 200 times higher than that in other tissues of male and female fishes.

Purification of PLA_2 from the gills of red sea bream. The purification procedure is summarized in Table 2. In starting from the crude extract, G-1, G-2, and G-3 PLA_2 were purified more than 10,000-fold, and the yields of G-1, G-2, and G-3 PLA_2 were 0.4, 0.3, and 5.5%, using mixed-micellar POPC substrate, respectively. Figure 1 documents the results obtained from a first RP–HPLC. The active fractions (fraction No. 62–70) were pooled and applied to a second RP–HPLC (Fig. 2). Three peaks of PLA_2 activity appeared in fraction No. 126–129, 133–136, and 141–148, respectively. Peaks 1, 2, and 3 (shown by the bars in Fig. 2) were pooled and were further applied to a TSKgel Octadecyl-NPR column. Peaks 1, 2, and 3 were eluted separately, and PLA_2 activity was also found in these peaks (data not shown). When peaks 1, 2, and 3 were mixed and applied to the same column, each peak eluted separately (Fig. 3). Therefore peaks 1, 2, and 3 were pooled as G-1, G-2, and G-3 PLA_2 to yield final enzyme preparations. G-1, G-2, and G-3 PLA_2 yielded a single protein band by SDS-PAGE under reducing conditions with an estimated molecular mass of approximately 15 kDa, identical to that of porcine pancreatic PLA_2 (Fig. 4). From the analysis of MALDI-TOF mass spectrometry, the exact molecular mass values of G-1, G-2, and G-3 PLA_2 were calculated to be 14,040, 14,040, and 14,005 Da, respectively. The specific activities of G-1, G-2, and G-3 PLA_2 amounted to 1294, 1143, and 1293 μmol/min/mg protein, respectively (Table 2).

N-terminal amino acid sequence of G-1, G-2, and G-3 PLA_2. The purified PLA_2 were reduced, S-pyridylethylated, and subjected to automated Edman analysis. Then, the N-terminal amino acid sequences of S-pyridylethylated G-1, G-2, and G-3 PLA_2 were determined as in the following: AIWQFGDMIECVQPGVDPINYNNYGCYCGLGGKGTPVDDLDRCCKVHDDCYGAQME.

G-1, G-2, and G-3 PLA_2 contained Cys 11 and were all identical in amino acid sequences from Ala1 to Glu56.

cDNA cloning of gill G-3 PLA_2. A full-length cDNA clone of gill G-3 PLA_2 was isolated by RT-PCR and rapid amplification of cDNA ends (RACE) methods. The nucleotide se-

TABLE 2
Purification of G-1, G-2, and G-3 PLA_2 from the Gills of Red Sea Bream[a]

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	46,176	5,082	0.11	1	100
Dialyzate	25,111	3,570	0.14	1	70.2
Q-Sepharose Fast Flow	3,132	2,442	0.78	7	48.1
Butyl-Cellulofine	26.55	1,662	62.6	569	32.7
DEAE-Sepharose Fast Flow	8.48	1,794	211.6	1,923	35.3
YMC-Pack PROTEIN-RP	1.06	679	640.6	5,823	13.4
Asahipak ODP-50					
G-1 PLA_2	0.017	22	1,294	—	0.4
G-2 PLA_2	0.014	16	1,143	—	0.3
G-3 PLA_2	0.215	278	1,293	11,754	5.5

[a]The reaction mixture consisted of 50 mM glycine-NaOH (pH 9.5), 2 mM POPC, 6 mM sodium cholate, 5 mM $CaCl_2$, and 0.1 M NaCl, in a final volume of 0.1 mL. Data were obtained in duplicate. Protein concentration was determined with a DC Protein assay kit. For further information on purification see the Materials and Methods section. See Table 1 for abbreviations.

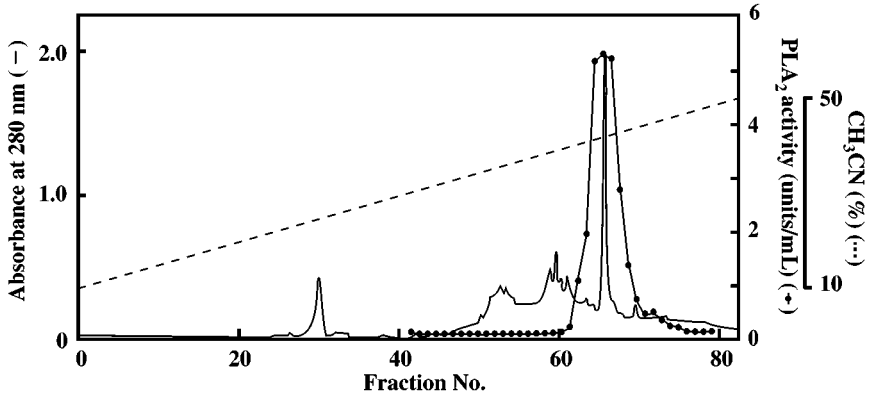

FIG. 1. Elution profile of phospholipase A_2 (PLA_2) on first reversed-phase–high-performance liquid chromatography (RP–HPLC). The pooled active fraction from the DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) column was applied to a YMC-Pack Protein RP (YMC Co. Ltd., Tokyo, Japan) column (4.6 × 250 mm) pre-equilibrated with 10% CH_3CN/0.1% trifluoroacetic acid (TFA). The elution of protein was followed by monitoring the absorbance at 280 nm (–). The dashed line indicates the linear gradient of CH_3CN. The flow rate was 1.0 mL/min. The fraction volume was 0.5 mL. The PLA_2 activity (●) of a 2 μL aliquot of each fraction was measured as described in the Materials and Methods section.

quence of gill G-3 PLA_2 cDNA (1084 bp) included a 444 bp open reading frame that encoded for a signal peptide of 24 amino acids, followed by a mature protein of 124 amino acids, as shown in Figure 5. The calculated molecular mass and isoelectric point of the mature protein were 14,007 Da and 5.17, respectively. The 3′-noncoding region contained two putative polyadenylation signals located upstream of the poly A tail. An alignment of the mature amino acid sequences of bovine pancreatic PLA_2 (28), porcine pancreatic PLA_2 (29), and *N. naja kaouthia* venom PLA_2 (30) with the sequence of gill G-3 PLA_2 is presented in Figure 6. The sequence of gill G-3 PLA_2 has common characteristics with mammalian pancreatic type, group IB PLA_2, including the presence of Cys11 and the alignment of other Cys residues; residues of N-terminal helix Gln4, Phe5, and Ile9, and the presence of the absolutely conserved active-site His48, Tyr52, Tyr73 and Asp99; a "pancreatic loop" of residues 63–67 that are conserved in group IB PLA_2; the conserved sequence of the calcium-binding segment Tyr25-Gly33.

Properties of G-1, G-2, and G-3 PLA_2. Effects of pH and concentrations of Ca^{2+} and sodium cholate on the purified PLA_2 activity are shown in Figure 7. G-1, G-2, and G-3 PLA_2 hydrolyzed POPC efficiently in an alkaline pH region, and optimal activities for all PLA_2 were found to be at around pH

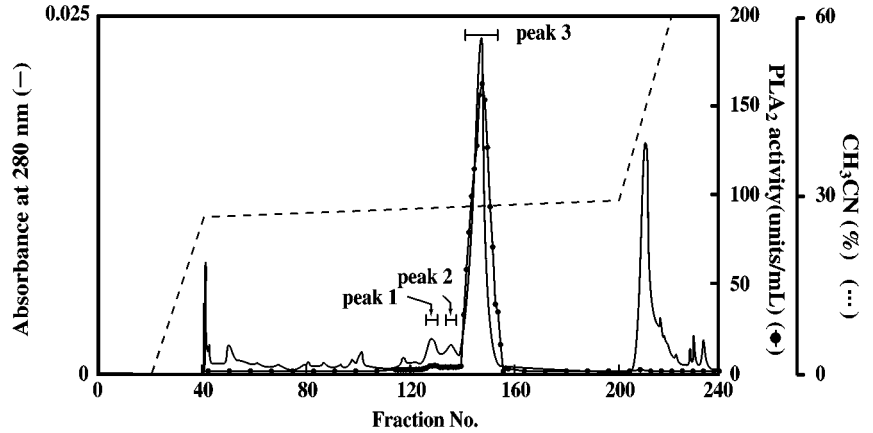

FIG. 2. Elution profile of PLA_2 on second RP–HPLC. The pooled fraction from the YMC-Pack Protein RP column was applied to an Asahipak ODP-50 (Showa Denko, Tokyo, Japan) column (6.0 × 250 mm) pre-equilibrated with 10 mM Na_2HPO_4. The elution of protein was followed by monitoring the absorbance at 280 nm (–). The dashed line indicates the linear gradient of CH_3CN. The flow rate was 1.0 mL/min. The fraction volume was 0.25 mL. Each fraction was diluted 50 times with 30% CH_3CN/0.1% TFA, and the PLA_2 activity (●) of a 2 μL aliquot of each diluent was measured as described in the Materials and Methods section. For abbreviations and other manufacturer see Figure 1.

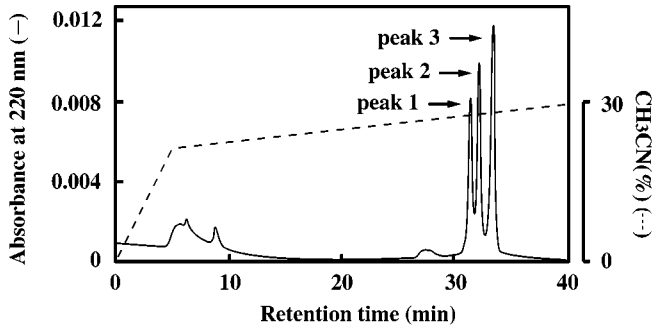

FIG. 3. Elution profile of G-1, G-2, and G-3 PLA_2 on RP–HPLC. The pooled fractions of peak 1, peak 2, and peak 3 eluted from the Asahipak ODP-50 column were mixed and were applied to a TSKgel Octadecyl-NPR (Tosoh, Tokyo, Japan) column (4.6 × 35 mm) pre-equilibrated with 10 mM Na_2HPO_4. The elution of protein was followed by monitoring the absorbance at 220 nm (–). The dashed line indicates the linear gradient of CH_3CN. The flow rate was 0.8 mL/min. For other abbreviations and manufacturer see Figures 1 and 2.

9–10 (Fig. 7A). The activities of G-1, G-2, and G-3 PLA_2 were barely detected in the presence of 1 mM EGTA (Fig. 7B). The maximal activities were observed in the presence of 2–40 mM Ca^{2+}. Activities of G-1, G-2, and G-3 PLA_2 toward POPC were not detectable in the absence of bile salts. However, they were stimulated dramatically by the addition of sodium cholate, and their optimal activities were found at cholate concentrations of about 6–8 mM (cholate/POPC molar ratio of 3–4) (Fig. 7C). The effects of various agents on the activity of purified PLA_2 were investigated using a mixed-micellar POPC substrate (Table 3). The activities of

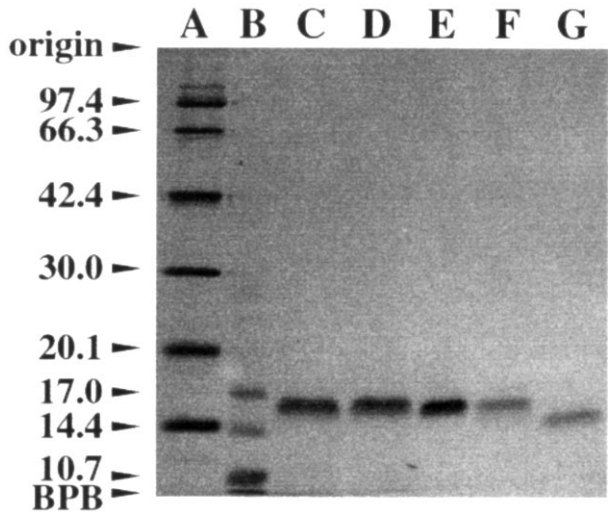

FIG. 4. Sodium lauryl sulfate-polyacrylamide gel electrophoresis of red sea bream gill G-1 (lane C), G-2 (lane D), and G-3 (lane E) PLA_2, porcine pancreatic PLA_2 (lane F), and *Naja naja kaouthia* venom PLA_2 (lane G). Molecular masses of marker proteins (lane A) from top to bottom: 97.4, 66.3, 42.4, 30.0, 20.1, and 14.4 kDa, and those of marker peptides (lane B) from top to bottom: 17.0, 14.4, and 10.7 kDa. BPB, bromophenacyl bromide; for other abbreviation see Figure 1.

G-1, G-2, and G-3 PLA_2 toward the POPC substrate were almost completely inhibited by the addition of 1 mM EGTA or EDTA, and Mg^{2+}, Zn^{2+}, Fe^{3+}, and Cu^{2+} could not replace Ca^{2+}. CTAB and SDS greatly inhibited the activities of G-1, G-2, and G-3 PLA_2. *p*-APMSF, a serine protease inhibitor, did not inhibit the activities of G-1, G-2, or G-3 PLA_2 (data not shown). *p*-BPB, an alkylating reagent of His, inhibited all three enzymes but was less sensitive than porcine pancreatic and *N. naja kaouthia* venom PLA_2. Mixed micelles of sodium cholate and phospholipids containing various head groups (POPC, POPE and POPG) or aqueous phospholipid dispersions (without sodium cholate) were prepared and were used as substrates for examining the substrate specificity of the purified enzymes (Table 4). Porcine pancreatic PLA_2 was the most active for the micellar POPG substrate, and *N. naja kaouthia* venom PLA_2 was for micellar POPC. On the other hand, red sea bream gill G-1, G-2, and G-3 PLA_2 hydrolyzed efficiently both POPC and POPG micelles at similar rates. G-1, G-2, and G-3 PLA_2 were active for POPE and POPG liposomes, but not for POPC liposome. All PLA_2 tested were inactive for both micellar and dispersed POPS (data not shown).

DISCUSSION

We investigated the distribution of PLA_2 activity in the various tissues of red sea bream and found extremely high activity in the gills (Table 1). We had found previously that PLA_2 activity in the crude enzyme extract of pyloric ceca and hepatopancreas of red sea bream increased significantly due to dialysis (23,24). This suggests the presence of PLA_2 inhibitory materials in the crude enzyme extract. Therefore, we used a dialyzate as a sample for measuring PLA_2 activity in the present experiment. In mammals, pancreatic PLA_2 are stored as a form of proenzyme, which are converted into the active form by limited tryptic proteolysis in the intestinal lumen (31,32). Also, in red sea bream, PLA_2 activity in the hepatopancreas increased dramatically during autolysis (22). Therefore, it exists as a proenzyme in the hepatopancreas of red sea bream, similarly to porcine pancreatic PLA_2 (33). As the crude enzyme extract of hepatopancreas was not autolyzed in the present experiment, it is inappropriate to compare the PLA_2 activities of the hepatopancreas and gills. However, the specific activity of PLA_2 in a gill dialyzate (Table 1) was over 150-fold higher than that of the hepatopancreas (7 mU/mg protein) after autolysis (24). As we could not find any PLA_2 activity in the plasma of red sea bream, PLA_2 activity in the gills originated from the gills. It remains unclear whether PLA_2 exists as a proenzyme or active enzyme in other tissues in addition to hepatopancreas. However, it is thought that somewhat higher PLA_2 activity expresses in the gills of red sea bream. Therefore, we tried to purify PLA_2 from red sea bream gill filaments.

Two minor and one major PLA_2, tentatively named G-1, G-2, and G-3 PLA_2, were finally purified with second RP–HPLC (Figs. 2 and 3) and were all identical in their N-ter-

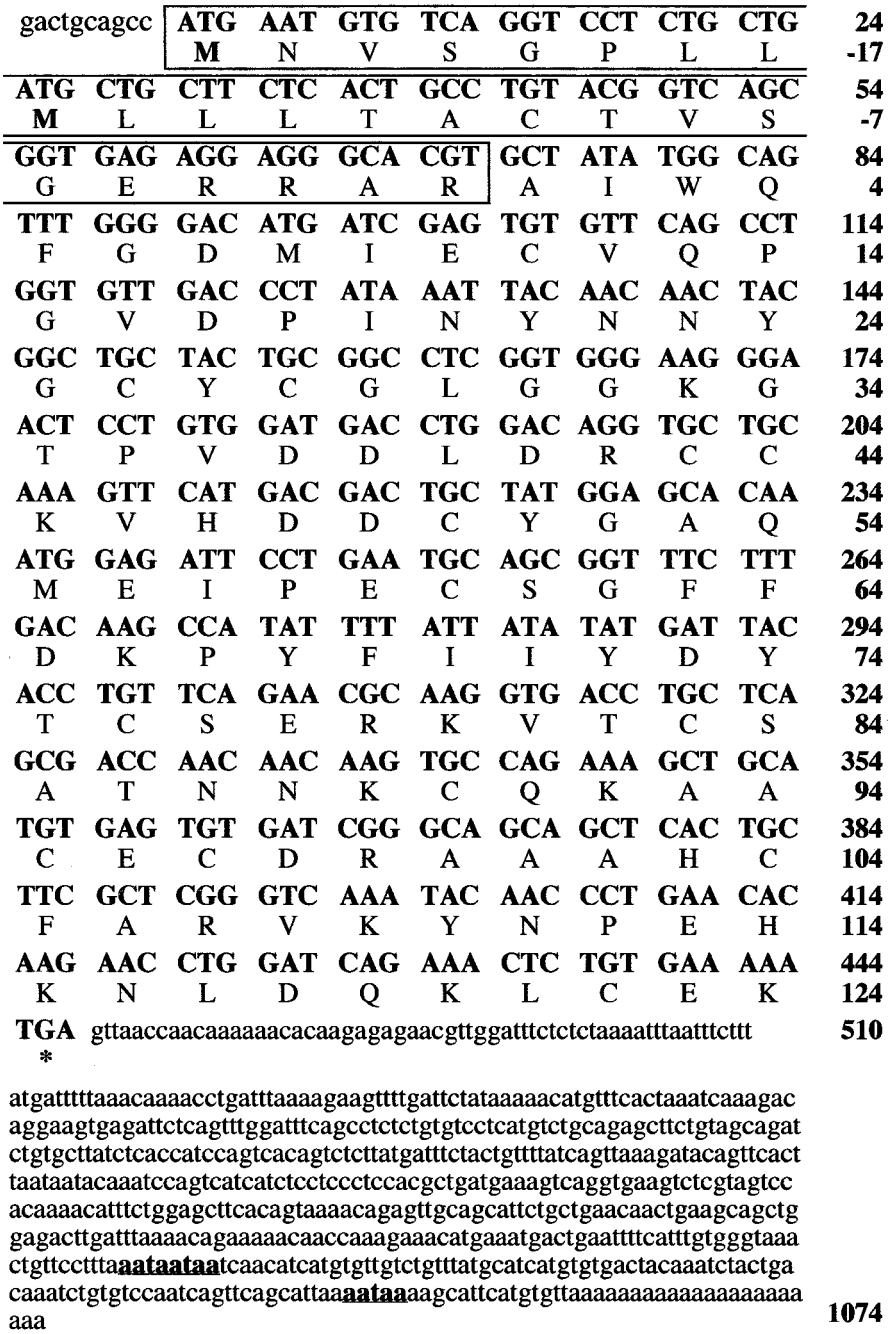

FIG. 5. Nucleotide and deduced amino acid sequences of red sea beam gill G-3 PLA_2. The predicted preprosegment is boxed and possible initiator methionines are shown in **bold**. The putative polyadenylation signal is underlined and in **bold**. Asterisk shows termination codon. For abbreviation see Figure 1.

minal amino acid sequences from Ala1 to Glu56. A full-length cDNA clone for G-3 PLA_2 was isolated by RT-PCR and RACE methods (Fig. 5), and the molecular mass (14,005 Da) of G-3 PLA_2 obtained by time of flight mass spectrometric analysis almost coincided with that by predicted amino acid sequence of mature enzyme (14,007 Da, Fig. 6). G-3 PLA_2 contains 14 cysteines including Cys11 and Cys77 and a pancreatic loop of residues 63–67, which are commonly conserved in group IB PLA_2. In addition, G-3 PLA_2 had a pH optimum in an alkaline region at around pH 9–10 and required the presence of millimolar concentrations of Ca^{2+} for enzyme activity, using a mixed-micellar POPC substrate with sodium cholate. These results indicate that red sea bream gill G-3 PLA_2 belongs to group IB PLA_2. The sequence similarity and hydropathy profiles suggest that the N-terminal 24 residues are prepropeptide sequence (Fig. 5). SignalP computer analy-

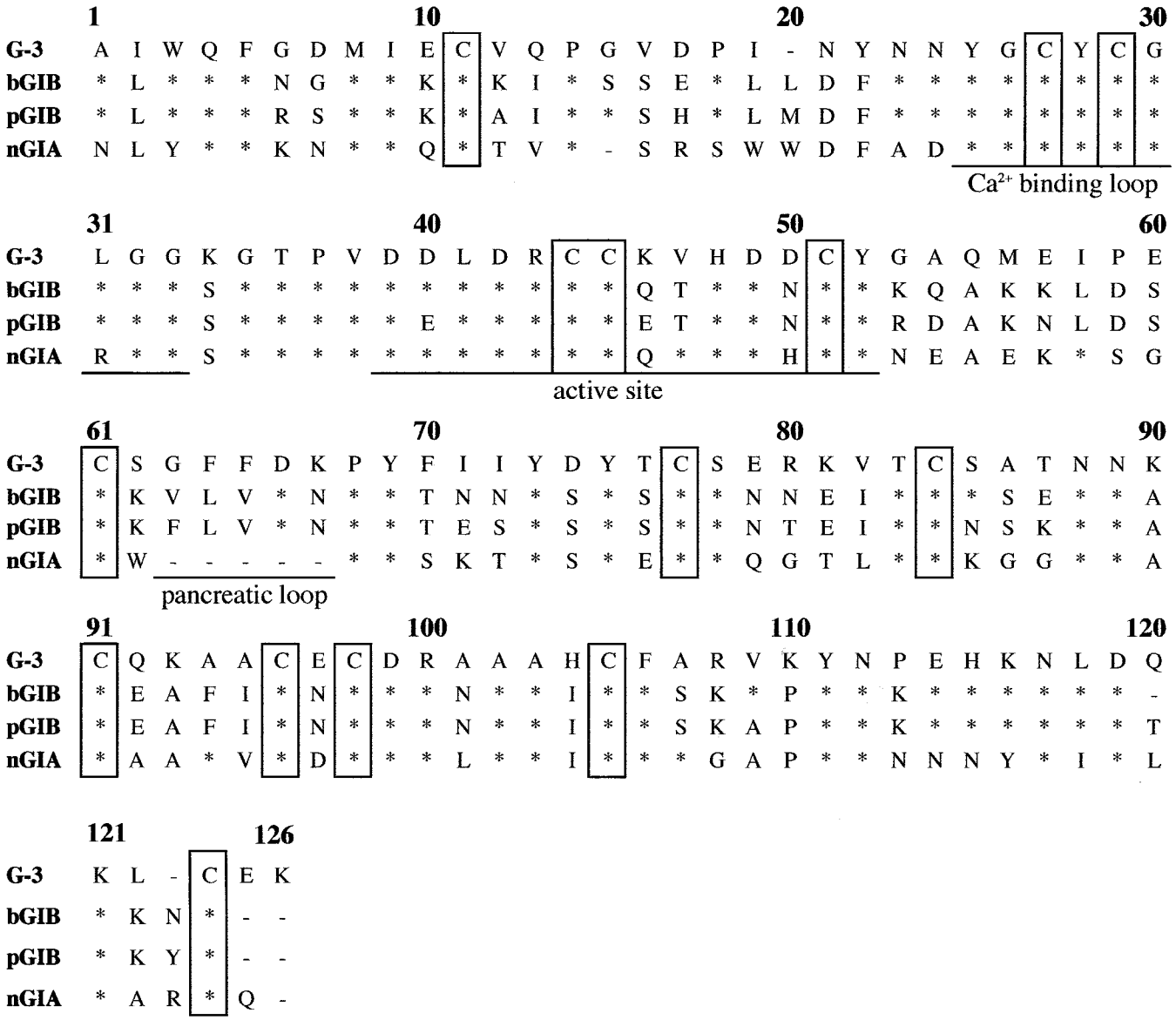

FIG. 6. Alignment of the amino acid sequences of red sea bream gill G-3 PLA_2 with other group I PLA_2. Only mature protein sequences of PLA_2 are shown. G-3, red sea bream gill G-3 PLA_2; bGIB, bovine pancreatic group IB PLA_2 (19); pGIB, porcine pancreatic group IB PLA_2 (29); nGIA, *N. naja kaouthia* venom group IA PLA_2 (30). Asterisks indicate the amino acid residues identical to those of red sea bream gill G-3 PLA_2. Cysteines that are conserved in all PLA_2 are boxed.

sis (34) for the potential cleavage positions in its signal sequence suggests that the signal peptide cleavage site is present between position Gly-6 and Glu-5 preceding the mature protein. Furthermore, arginine doublet, which is the cleavage site of subtilisin-like convertase in the Golgi apparatus (35), is also included at positions –3 and –4 preceding the mature protein. Further studies are needed to understand the maturation process of gill G-3 PLA_2.

Red sea bream gill G-3 PLA_2 hydrolyzed efficiently both mixed-micellar POPG and POPC at similar rates (Table 4). It is well known that snake venom and porcine pancreatic PLA_2 show high affinity toward zwitterionic substrate POPC and negatively charged substrate POPG, respectively (31). A similar result was obtained for *N. naja kaouthia* venom and porcine pancreatic PLA_2 in the present experiment (Table 4). It has been shown that ionic residues 53 through 58 (Fig. 6) located at the end of a long α-helix loop of group IA, IB, IIA, and V PLA_2 are involved in binding to the head group of the phospholipid substrate (36–41). The cationic and anionic charges of residues 53 and 56 in mammalian pancreatic and *N. naja kaouthia* venom PLA_2 (Fig. 6) appear to be responsible for the anionic and zwitterionic phospholipid preference of these enzymes, but substitution of cationic residues 53 and/or 56 to nonionic residues of mammalian pancreatic PLA_2 improves higher preference for zwitterionic substrate but keeps the higher activity toward anionic substrate. In

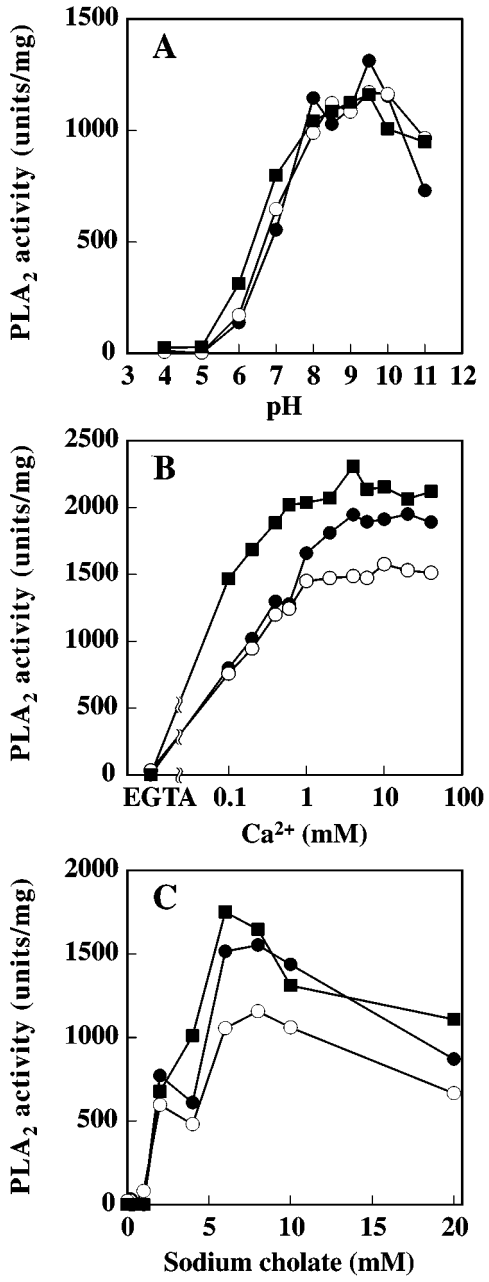

FIG. 7. Effect of pH (A), concentrations of Ca^{2+} (B) and sodium cholate (C) on the activities of red sea bream gill G-1 (●), G-2 (○), and G-3 (■) PLA_2. (A) Reaction mixtures containing purified G-1, G-2, or G-3 PLA_2, 2 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 6 mM sodium cholate, 100 mM NaCl, and 5 mM $CaCl_2$ were incubated for 15 min at 37°C in a total volume of 100 μL. The buffers used were 50 mM acetate buffer from pH 4.0 to 5.0, 50 mM Tris-maleate buffer from pH 5.0 to 8.0, 50 mM Tris-HCl from pH 8.0 to 9.0, and 50 mM glycine-NaOH from pH 9.0 to 11.0. (B) Reaction mixtures containing purified G-1, G-2, or G-3 PLA_2, 2 mM POPC, 6 mM sodium cholate, 100 mM NaCl, 50 mM glycine-NaOH (pH 9.5), and 1 mM EGTA or 0–40 mM $CaCl_2$ were incubated, and PLA_2 activity was measured as described in the Materials and Methods section. (C) In the presence of various concentrations of sodium cholate, the activity of purified G-1, G-2, or G-3 PLA_2 was examined in a reaction mixture containing 2 mM POPC, 100 mM NaCl, 50 mM Tris-HCl (pH 8.5), and 10 mM $CaCl_2$, as described in the Materials and Methods section. Results are shown as means of two separate experiments. For abbreviation see Figure 1.

bovine and porcine pancreatic PLA_2, the methionine mutant (53M or 56M) shows a large increase in activity toward zwitterionic substrate and a slight decrease toward anionic substrate (36,39,42). Interestingly, red sea bream gill G-3 PLA_2 contains nonionic residues at residues Gly53 and Met56. As N-terminal helix Gln4, Phe5 and Ile9, Ca^{2+}-binding loop (Y25-G33) and active-site His48, Tyr52, Tyr73, and Asp99 are all conserved in red sea bream gill G-3 PLA_2 (Fig. 7), residues Gly53 and Met56 of gill G-3 PLA_2 may be interacted with choline moiety of POPC, in addition to keep the higher affinity toward POPG micelles. However, it cannot be denied that other residues of gill G-3 PLA_2 are involved in the preference for both POPC and POPG micelles, in addition to Gly53 and Met56. It is necessary to define the substrate specificity of gill G-3 PLA_2 for POPC and POPG micelles, using gill G-3 PLA_2 mutants such as G53R, M56K and G53RM56K.

In the absence of bile salts, aqueous dispersions of POPG and POPE (liposome) were appreciably hydrolyzed, whereas those of POPC were hardly hydrolyzed at all by G-3 PLA_2, similar to porcine pancreatic PLA_2 (Table 4) and rat splenic group I PLA_2 (5). The low affinity of gill PLA_2 toward POPC liposomes remains to be established.

Multiple group I PLA_2 enzymes are found in snake venom and also in the mammalian pancreas. Of 14 group I PLA_2 in the venom of the Australian king brown snake, *Pseudechis australis*, six group I PLA_2, PG-1Ga and -1Gb, Pa-3a and -3B, and Pa-15a and -15b showed microheterogeneity at the 103rd position with Thr/Ala, Thr/Pro, and Ala/Pro, respectively (43). Two group I PLA_2 isoforms exist in the porcine pancreas (44). In addition, commercially available porcine pancreatic PLA_2 consists of at least four enzymes within a range of 13,036 to 14,001 Da, and they are assumed to be genetic variants of PLA_2 (45). The N-terminal amino acid sequences of two minor PLA_2, G-1 and G-2 PLA_2, were all identical to that of G-3 PLA_2 from Ala1 to Glu56, but molecular masses of G-1 and G-2 PLA_2 (14,040 Da) were slightly higher than that of G-3 PLA_2 (14,005 Da). In addition, the enzyme properties of G-1 and G-2 PLA_2 were almost similar to that of G-3 PLA_2 (Fig. 7, Tables 3,4). Concerning the above aspects, G-1 and G-2 PLA_2 may be genetic variants of G-3 PLA_2 in which replacement or modification of amino acids occurred in the amino acid sequences from the 57th position to the carboxyl-terminus.

We recently purified two group I PLA_2 isoforms, DE-1 and DE-2 PLA_2, from the hepatopancreas of red sea bream (24). In the present report, we found one major and two minor group I PLA_2 variants in the gills of red sea bream. As we could not find a large difference in the molecular masses and amino acid sequences among three group I PLA_2 in the gills, we conclude that one group I PLA_2 isoform exists in the gills. These aspects indicate the existence of at least three structurally distinct group I PLA_2 isoforms in the hepatopancreas (DE-1 and DE-2 PLA_2) and gills (G-3 PLA_2) of red sea bream. In addition, Ca^{2+}-dependent low molecular mass PLA_2 was also found in the pyloric ceca of red sea bream (23). It is well known that

TABLE 3
Effect of Various Compounds on the Activities of Red Sea Bream Gill G-1, G-2, and G-3 PLA_2, Pig Pancreas PLA_2, and *Naja naja kaouthia* Venom PLA_2[a]

Compounds	Final concentration	Red sea bream gill G-1	Red sea bream gill G-2	Red sea bream gill G-3	Porcine pancreas	*N. naja kaouthia* venom
		Remaining activity (%)				
Complete		100	100	100	100	100
EGTA	1.0 mM	ND	ND	ND	ND	ND
EDTA	1.0 mM	ND	ND	ND	ND	ND
$CaCl_2$		17.8	23.3	31.9	ND	33.3
$MgCl_2$	5.0 mM	ND	ND	ND	ND	ND
$ZnCl_2$	5.0 mM	26.8	34.2	47.5	ND	16.4
$FeCl_3$	5.0 mM	ND	ND	ND	ND	ND
$CuCl_2$	5.0 mM	ND	ND	ND	ND	ND
$CuSO_4$	5.0 mM	ND	ND	ND	ND	ND
CTAB	0.01%	ND	ND	ND	ND	ND
	0.10%	2.7	ND	6.5	ND	ND
	0.20%	ND	ND	ND	ND	ND
SDS	0.01%	3.5	3.3	8.0	5.7	17.0
	0.10%	ND	ND	ND	ND	ND
	0.20%	ND	ND	ND	ND	ND
Triton X-100	0.01%	ND	ND	ND	ND	63.6
	0.10%	16.5	17.2	35.0	ND	118.9
	0.20%	26.4	23.4	46.6	ND	140.3
p-BPB	1.0 mM	63.0	74.8	76.1	24.1	15.0

[a]The activity of PLA_2 was measured in reaction mixtures containing 50 mM glycine-NaOH (pH 9.5), 2 mM POPC, 5 mM $CaCl_2$, 0.1 M NaCl, and 6 mM sodium cholate. To determine the effect of ions, $CaCl_2$ was depleted or replaced by EGTA, EDTA, $MgCl_2$, $ZnCl_2$, $FeCl_3$, $CuCl_2$, or $CuSO_4$, and the effect of detergents, sodium cholate was replaced by cetyl trimethylammonium bromide (CTAB), sodium lauryl sulfate (SDS) or Triton X-100. To determine the effect of *p*-bromophenacyl bromide (*p*-BPB), it was added to PLA_2 and incubated for 10 min at 37°C before starting the reaction. ND, not detectable; for other abbreviations see Table 1.

multiple group I PLA_2 variants locate in the same tissue or organ such as porcine pancreas and snake venom. However, it remains unclear whether group I PLA_2 isoforms distribute in different tissues and cells of mammals and snakes. This may be the first report indicating that structurally different group I PLA_2 isoforms distribute in different tissues such as the hepatopancreas and gills of red sea bream.

Finally, this study demonstrates that three group I PLA_2 variants exist in the gills, an organ not part of the digestive system like the hepatopancreas and pyloric ceca. Fish gills

TABLE 4
Comparison of Substrate Specificities of Red Sea Bream Gill G-1, G-2, and G-3 PLA_2, Porcine Pancreatic PLA_2, and *Naja naja kaouthia* Venom PLA_2 for Various Phospholipid Head Groups[a]

Origin	POPC Micelle	POPC No bile salts	POPE Micelle	POPE No bile salts	POPG Micelle	POPG No bile salts
	(units/mg protein)					
Red sea bream gill						
G-1 PLA_2	1,369	ND	170	137	1,155	128
G-2 PLA_2	1,166	ND	153	142	872	118
G-3 PLA_2	1,592	ND	357	161	1,211	119
Pig pancreas PLA_2	101	ND	278	243	1,861	250
N. naja kaouthia venom PLA_2	563	66	212	93	135	100

[a]The reaction mixture consisted of 50 mM glycine-NaOH (pH 9.5), 2 mM phospholipid, 5 mM $CaCl_2$, and 0.1 M NaCl with or without 6 mM sodium cholate in a final volume of 0.1 mL. Data were obtained in duplicate. POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; for other abbreviations see Tables 1 and 3.

constitute a multifunctional and complex organ. The branchial epithelium consists of various cell types such as pavement cells, mucous cells, neuroepithelial cells, and ionocytes that are involved in respiratory, osmoregulatory, and excretory functions (46). We obtained monoclonal antibodies raised against purified gill PLA_2. We are now investigating the localization of PLA_2 proteins and expression of PLA_2 mRNA in the gills and other tissues of red sea bream using immunohistochemical staining and *in situ* hybridization.

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