Thesis for the Degree of Master of Science

Cloning and Characterization of Phospholipase D from Olive Flounder *(Paralichthys olivaceus)*

by

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Department of Fish Pathology

The Graduate School

Pukyong National University

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Cloning and Characterization of Phospholipase D from Olive Flounder *(Paralichthys olivaceus)*

넙치 인지질가수분해효소 PLD 의 클로닝 및 특성분석

Advisor : Joon Ki Chung

by

Soo Jin Jeon

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Cloning and Characterization of Phospholipase D from Olive Flounder *(Paralichthys olivaceus)*

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Abstract

The gene encoding phospholipase D (PLD) in Olive flounder (*Paralichthys olivaceus*), designated PoPLD, was cloned and sequenced. PoPLD cDNA was obtained using a combination of cloning cDNA obtained from olive flounder brain, and the 3'- and 5'-rapid amplification of cDNA ends (RACE) method. The full-length PoPLD gene is 4656-bp long and contains an open reading frame of 3162 bp from the first ATG start codon through the TAA stop codon flanked by the 194 bp 5'- and 1300 bp 3'-noncoding regions. This gene was predicted to encode 1053 amino acids with a molecular mass of 120.5kDa. The deduced amino acid sequence shows 65.30% and 49.38% identity to *Homo sapiens* PLD1a (HsPLD1a) and *Homo sapiens* PLD2 (HsPLD2), respectively. The phylogenic analysis and amino acid sequence comparison suggest that PoPLD is closely related to the PLD1b isozyme. The tissue expression analysis of PoPLD showed that the mRNA of PoPLD was predominantly expressed in the eye, liver, stomach and duodenum. In addition, the recombinant protein of PoPLD (GFP-PoPLD), which demonstrated a phosphatidylcholine (PC)-hydrolyzing activity, was partially localized as a distinct ring-shaped form surrounding the rim of the nucleus in EPC cells. Together, our results suggest that PoPLD is similar to mammalian PLD1b isoform and is generally widespread within olive flounder tissue.

Keyword(s): Phospholipase D (PLD); Olive flounder (*Paralichthys olivaceus*)

1. Introduction

Phospholipase D (PLD) is an enzyme that is widely distributed in bacteria, fungi, plants and animals [1,2]. It has been suggested that PLD results in modification of various lipid constituents of the membrane, either by degradation or by phosphorylation, and generation of one or more products ('messengers') that are able to recruit or modulate specific target proteins [3]. PLD has been implicated in several physiological processes and diseases including signal transduction, membrane trafficking, mitosis regulation, proliferation, secretion, respiratory burst, inflammation, diabetes and neuronal and cardiac stimulation [4,5]. PLD catalyzes the hydrolysis of phospholipids, usually phosphatidylcholine (PC), to produce phosphatidic acid (PA) and choline in response to a variety of agents including hormones, neurotransmitters, growth factors, and phorbol esters [4]. PA has been shown to act directly as a signaling molecule and can be converted into diacylglycerol (DAG) by PA phosphohydrolase, which activates members of the Ca^{2+}- and phospholipid-dependent protein kinase C family [6]. In the presence of primary alcohols, PLD also catalyzes a transphosphatidylation reaction in which the phosphatidyl moiety is preferentially transferred to the alcohol to generate phosphatidylalcohol. This reaction is a unique characteristic of PLD and thus the accepted index of PLD activity [7].

There are lots of evidence for the existence of multiple isoforms of PLD in

mammalian tissue, and PLD is known to be activated by several factors including unsaturated fatty acid [8], phosphatidylinositol 4,5-bisphosphate (PIP_2) [9], monomeric GTP-binding proteins (G proteins) such as ADP-rybosylation factor 1 (ARF1) [10, 11] and RhoA [12,13], protein kinase C [14], and calmodulin [15]. ARF has been implicated in mediating agonist induced activation of PLD in vivo [3] and reported to synergistically stimulate PLD activity in concert with small G proteins of the Rho family including RhoA, Cdc42, and Rac1 [16]. Ral A, another member of the small G protein family, was reported to play a role in v-Src, a non-receptor protein-tyrosine kinase, induced PLD activation [17]. In addition to the small G proteins, PKC-α was shown to stimulate PLD activity in a phosphorylation-independent manner, through its regulatory domain [3], and PLD activation by PKC-α occurs synergistically with ARF and RhoA [18,19]. Another important component in the PLD activation complex is PIP_2, a phospholipid that acts as a cofactor in PLD activation by the above molecules [9,16,20]. On the other hand, PLD activity has been reported to inhibit by several proteins. The polyphosphoinositide 5-phosphatase synaptojanin inhibits the activity of phosphoinositide dependent PLD enzymes in vitro by hydrolysis of the activator PIP_2 [21]. Also, fodrin (a non-erythroid form of spectrin) and the synapse specific clathrin assembly protein AP3 were reported to inhibit ARF activated PLD in permeabilized HL-60 cells [22] and PLD1 [23], respectively. Based on the sensitivity to various activators, PLD isoforms can be classified into two large

groups, a small G-protein-dependent form and oleate-dependent form [24-26].

Many PLD genes have been cloned from bacteria, plants, yeasts and mammalian species including humans. These genes have been well characterized by two mammalian PLD genes [25,27] that have been termed PLD1 and PLD2. Two distinct mammalian PLD genes have been isolated from human, rat, and mice [24,27-31]. They share an approximately 50% amino acid sequence identity and consist of phox homology (PX), pleckstrin homology (PH), four conserved sequences (I-IV), and carboxyl-termini (CT) domains in tandem at their N-termini. However, PLD1 encodes a region of 100-150 amino acids, designated loop sequences, at the center of the protein that is not present in PLD2 or in PLDs from lower organisms [27.31]. The loop region is attractive to distinguish PLD1 from PLD2 and PLDs from other species. Furthermore, PLD1 is expressed as two splice variants, namely, PLD1a and PLD1b. The major difference between PLD1a and PLD1b is deletion of 38 amino acids residue in the middle of the PLD1b sequence. However, the splicing does not alter the catalytic activity or the regulation of these isoenzymes [32]. PLD1 and PLD2 also differ in molecular weight, intracellular location, as well as their sensitivity to activators. PLD1, a 1074-aminoacid protein, exhibits a low basal activity but is markedly stimulated in a synergistic manner by protein kinase C, ARF1, or RhoA in the presence of PIP_2, a cofactor that is required for PLD1 activation [10,33]. PLD2, a 933-amino acid protein, is also dependent on PIP_2 but differs from PLD1 in that it exhibits constitutively high activity and it

appears to be weekly activated by ARF1 but not activated by RhoA [28,30,34]. In addition, PLD1 and PLD2 are found in discrete subcellular locations. PLD1 has been reported to be located in endoplasmic reticulum, secretary granules, and lysosomes and PLD2 in plasma membrane [31,35].

Although PLD has been well characterized in mammalians, little is known about the PLD in fish except for the EST gene of fish PLD. Therefore this paper reports the cloning, primary structure and characterization of PLD cDNA from olive flounder.

2. Material and methods

2.1. cDNA synthesis from olive flounder brain and amplification by PCR

To amplify the clone (694-bp) containing the CRII-CRIV region of PoPLD, we obtained an EST sequence (accession no. AU050587) for olive flounder PLD from GenBank. This sequence, which was chosen because it exhibits significant sequence homology with the CRII region of the mammalian PLD1 protein family at the nucleotide and amino acid levels, was used to design the forward primer (Fig. 1). Alignment of the amino acid sequences of all known PLD1s revealed several highly conserved regions within the CRIV regions. The conserved amino acids (SMLGKRDS) in this region were selected for synthesis of the reverse primers. The following primers were used to conduct nested PCR; 1st forward primer PoPLD-1F, 5′-CATCGACAGATACACCACTCC-3′, 2nd nested forward primer PoPLD-2F, 5′-CCAGACACTTCATTCAACACTG-3′ and reverse primer PoPLD-R, 5′-CTGTCNCGCTTYCCSAGCAWGC-3′. PCR was conducted using a GeneAmp PCR system 2400 (Perkin Elmer). Poly(A)$^+$ RNA was isolated from flounder brain using the PolyATtrack® System 1000 (Promega) according to the manufacturers instructions and cDNA was synthesized from this isolated mRNA using the GeneRacer™ kit (Invitrogen) and then used as the template for amplification. The PCR reaction mixture was comprised of 2μl of the cDNA, 10pmol of each primer (PoPLD-1F and PoPLD-R for 1st PCR, and PoPLD-2F and PoPLD-R for 2nd PCR),

2.5 mM dNTP mix, 2.5 units of Taq polymerase (Promega), and the manufacturer′s buffer in a final volume of 50μl. PCR was conducted under the following conditions: initial denaturation for 3 min at 94 °C, followed by 35 cycles of a 30-s denaturation step at 94 °C, a 30-s annealing step at 45 °C, and a 40-s extension step at 72 °C, with a final extension step of 10 min at 72 °C. Amplified DNA products were separated on a 1% agarose gel. A 694-bp cDNA fragment was eluted from the gel, purified and ligated into pCR®2.1-TOPO vector (Invitrogen). DNA sequencing was then conducted according to the BigDye™ Terminator Cycle Sequencing method on an automated ABI 377 Sequencer (Perkin Elmer).

2.2. 5′ and 3′ rapid amplification of cDNA ends (RACE)

RACEs for the 3′- and 5′- cDNA end were conducted using the GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. The first strand cDNA was synthesized from mRNA, which was isolated by using the method described above. The second-strand synthesis for 3′ and 5′ RACEs were primed with PoPLD-3RACEF1 or PoPLD-5RACER1 primer, respectively, which were designed from the cloned partial PLD gene (694-bp) (Fig. 1). Primary PCR was performed using PLD-specific primer1 and GeneRacer primer on 3′ and 5′ RACE, according to the manufacturer's instructions. The following primer sets were used for the primary reaction: for the 3'RACE, PoPLD-3RACEF1 5′-

CAGGCATAAAGTACCACGAGGA-3′; and for the 5′ RACE, PoPLD-3RACEF1 5′-CTTGCTGTGGATGTAGATGAGCTC-3′. Nested PCR was then conducted on 1ul of the primary-reaction product for the secondary 3′ RACE- and 5′ RACE-using the following primer sets: for 3′ RACE-PCR, PoPLD-3RACEF2 (5′-CAACGCCTACATCCAGGTCAT-3′) and GeneRacer 3′ Nested primer (5′-CGCTACGT-AACGGCATGACAGTG-3′); for 5′ RACE-PCR, PoPLD-5RACER2 (5′-GTGACCAGGCGT-CCCTCCAGCTC-3′) and GeneRacer 5′ Nested primer (5′-GGACACTGACATGGACTGAA-GGAGTA-3′). After amplification using PLD-specific primer2 and GeneRacer nested primer on each RACE, the DNA products were cloned into pCR4-TOPO vector (Invitrogen) and then sequenced as above.

2.3. Sequence and phylogenetic analysis

Nucleotide and predicted protein sequences were analyzed using DNAsis for Windows version 2.5 (Hitachi software engineering), BioEdit Sequence Alignment Editor version 5.0.9. [36] and BLAST programs in non-redundant databases of the National Center for Biotechnology Information (NCBI BLAST, http://www.ncbi.nlm.nih.gov/BLAST/). For phylogenetic analysis, the protein sequences of PoPLD and other PLD enzymes obtained from GenBank were aligned using CLUSTAL W version 1.8 [37] as included in BioEdit [36] with default

parameters and a phylogenetic tree was constructed using MEGA version 3.1 [38] using maximum parsimony and neighbor-joining methods

2.4. Cell culture

Epithelioma papillosum of carp (EPC) cells were grown in minimum essential medium (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 350 μg/ml sodium bicarbonate, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 27℃.

2.5. Construction and transfection of GFP-PoPLD expression vector

To obtain a contiguous PoPLD open reading frame (ORF), full-length cDNA was constructed as described in Fig. 1. Two sequences encompassing the 5′ region from the 5′ RACE clone and 3′ region from 3′ RACE were combined using the megaprimer-PCR method [39] and then cloned into pCR2.1-TOPO (Invitrogen). The pEGFP-C1 (Clontech Laboratories, Palo Alto, CA) vector was used to construct the GFP-fused flounder PLD expression vector. PCR was conducted using the cloned full-length cDNA as a template to remove the 5′- and 3′- untranslated regions from full-length PoPLD cDNA and introduce a contiguous PoPLD ORF into the pEGFP-C1 vector. The amplified product (about 3.1-kb) was digested with EcoRI and XhoI and inserted between the corresponding restriction sites of pEGFP-C1. The resulting expression vector was denoted pGFP-PoPLD. Transfection into EPC cells was done at 80% confluence in a 75cm flask using electroporation as

previously described [40]. EPC cells were electrically pulsed at 350V in the presence of 5ug of plasmid to induce transfection. As a control, pEGFP-C1 vector alone was subjected to the same conditions. Cells were incubated in the 75 cm flask for 24 hours at 27 ℃, and then examined using a fluorescent microscope (Olympus) with FITC dichromic filter set.

2.6. PLD activity assay from the transfected EPC cells

After transfection, EPC cells were harvested and washed twice with ice cold phosphate buffer saline. The cells were then resuspended in lysis buffer (50 mM Hepes, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor) and sonicated for 30s five times. The cell lysate was then centrifuged at 500 g for 10 min to remove unbroken cells. The supernatant was then removed and centrifuged at 40,000 rpm for 60 min at 4℃ to separate the cytosolic and membrane fractions. The pellet was resuspended with lysis buffer containing triton X-100 and centrifuged again at 40,000 rpm for 60 min at 4℃. Protein concentrations in the cytosolic and membrane fractions were measured by the Bradford method and used for an in vitro PLD assay. PLD activity was measured using an assay as described previously [10] with a minor modification. Briefly, 25ul of mixed lipid vesicles containing PE, PIP_2, and PC in a molar ratio of 16:1.4:1 with [*choline-methyl*-3^H]$(pam)_2$PC to yield 200,000

cpm per assay were added 10ul of PLD source to a total volume of 125ul with a final concentration of 50 mM Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl, 2.5 mM $MgCl_2$, and 2 mM $CaCl_2$. Assays were incubated at 37°C for 60 min before addition of 1 ml of stop solution ($CHCl_3$, CH_3OH, and concentrated HCl 50:50:0.3, v/v) and 0.35 ml of 1 M HCl in 5 mM EGTA. After separation of the organic and aqueous phases by centrifugation, the released [3^H]choline in 0.5 ml of the aqueous phase was quantified by liquid scintillation spectrometry.

2.7. RT-PCR amplification

To confirm the expression pattern of the PLD gene obtained from various tissues of olive flounder or the transfected EPC cells. Total RNA was isolated using TRIzol® (invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using oligo-$(dT)_{15}$ and Superscript II reverse transcriptase (Life Technologies). The resulting cDNA was subjected to PCR analysis using primers for the flounder PLD or β-actin gene. PCR was also conducted on Olive flounder β-actin (GenBank accession no. AU090737) as a housekeeping gene to confirm the steady-state level of expression. The condition for PCR amplification consisted of a 3 min pre-denaturation at 95 °C, followed by 35 cycles of 1 min denaturation at 92 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C with a final 5 min extension at 72 °C. The primer sequences

employed were as follows: PLD-1 (5′-CATCGACAGATACACC-ACTCC-3′) and PoPLD-ORF-R (5′-CGTCCAGATCTCAGTTGGGACC) for the flounder PLD; fAct-a (5′-TCCTCCCTGGAGAAGAGCTA-3′) and fAct-b (5′-GATCCAGACAGAGT-ATTTACGC-3′) for the flounder β-actin. Semi-quantitative PCR results were generated using densitometry to determine the ratio of the target gene (PLD) to the housekeeping enzyme (β-actin).

2.8. Electrophoresis and immunoblotting

Samples were resuspended in the sample buffer (60 mM Tris, 10% glycerol, 45 mM mercaptoethanol, 80 mM sodium dodecyl sulfate, pH 6.8) and boiled for 5 min. Proteins were then separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using 8% polyacrylamide gels and transferred to the nitrocellulose membrane then incubated with antibody. Antigen-antibody complexes were detected using an AP conjugate substrate kit (Bio-Rad).

3. Results

3.1. Isolation and characterization of cDNA encoding PoPLD

We obtained an EST sequence for olive flounder PLD from GenBank to clone the PLD gene from olive flounder brain. The sequence (GenBank accession no. AU050587) contained a partial sequence of Conserved Region II (CRII) in comparison with the mammalian PLD1 family. Primer sets were designed based on comparison of this sequence with mammalian PLD genes. These primers generated a 694-bp fragment of olive flounder cDNA that exhibited significant homology with CRII-CRIV from the mammalian PLD1 family based on both nucleotide sequence and amino acid levels. The clone was used as the starting point for isolation of the full-length PLD1 gene from olive flounder cDNA by using 3′ and 5′ Gene Race (Fig. 1). A 2800-bp clone and a 2237-bp clone were amplified using 5′ Gene Race and 3′ Gene Race, respectively. To obtain the full-length PoPLD gene from these sequences, two sequences encompassing the 5′ region of the 5′ RACE clone and the 3′ region of the 3′ RACE were combined using megaprimer-PCR [40] (Fig. 1). The complete 4656-bp sequence was then cloned into PCR2.1-TOPO vector, and sequenced on an automated ABI 377 Sequencer (Perkin Elmer). The nucleotide and deduced amino acid sequences are depicted in Fig. 2. The full-length cDNA of olive flounder PLD (PoPLD) yielded a 4656-bp sequence that included an initiation codon ATG, an in-frame stop codon, and a poly (A) tail, as well as most structural

features found in eukaryotic mRNA. The cDNA also comprised a 194-bp 5′-untranslated region (5′-UTR) and a 3162-bp coding region, followed by a 1297-bp 3′-untranslated region (3′-UTR) containing one potential poly adenylation signal, AATAAA (nt 4624-4630) (Fig. 2). The nucleotide of PoPLD was predicted to encode 1053 amino acids with a calculated molecular mass of 120.5 kDa starting from the first methionine according to universal codon usage (Fig. 2). A translational termination codon (nt 3354-3356, TAA) was observed in-frame following codon 1053. (Fig. 2). The PoPLD showed 68.67% amino acid identity homology to house mouse (*Mus musculus*) PLD1 and also PH, PX and PLD motifs, conserved motifs of mammalian PLD (Fig. 1). The full-sequence of PoPLD was deposited in GenBank under the accession number AY396567.

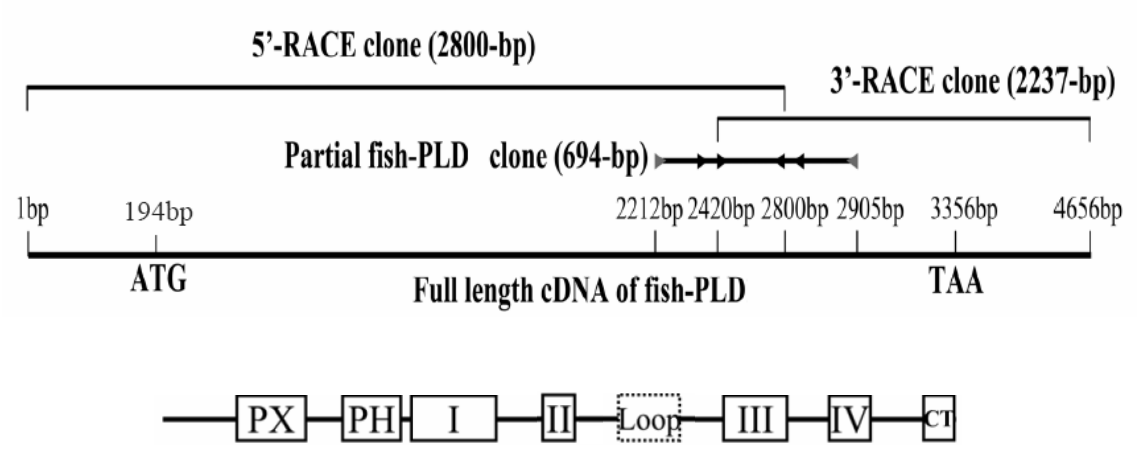


Fig. 1. Strategy for cloning of *Paralichthys olivaceus* PLD, and conserved and unique features for PoPLD. The PoPLD amino acid sequences encode regions that are conserved among mammalian PLD and some or all PLDs from nonmammalian species (boxed regions). PX, phox; PH, pleckstrin homology; CR, conserved region; Loop, loop region; CT, carboxyl terminus.

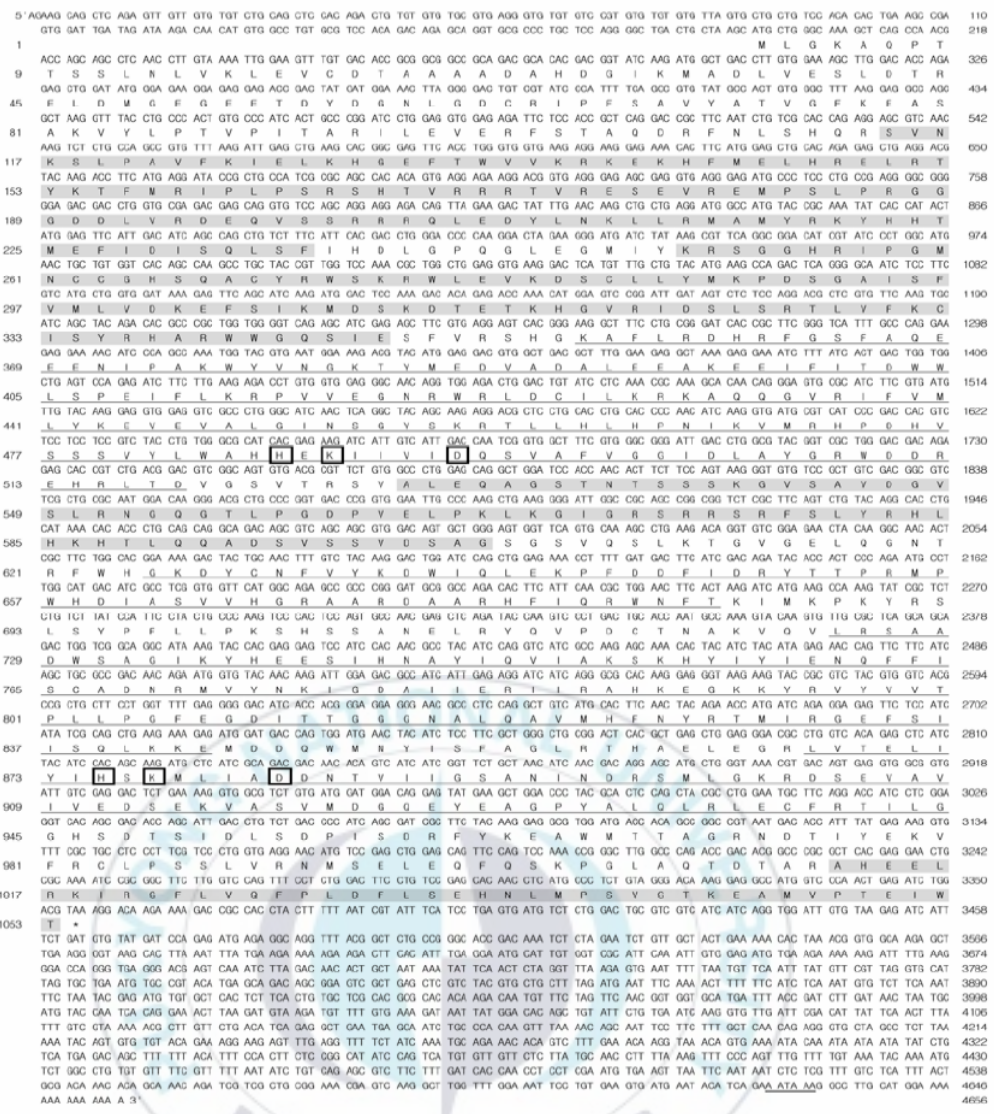


Fig. 2. Nucleotide and deduced amino acid sequence of PoPLD. Underlined amino acids indicate the locations of four conserved regions found in all known PLD homologues. The regions marked with shaded boxes express the locations of PX, PH, and CT domains. Boxes indicate the duplicated motif (HKD motif) that is presumed to form the active site required for catalysis. This sequence was registered on GeneBank (accession number AY396567).

3.2. Comparison of amino acid sequences

The deduced amino acid sequence of PoPLD demonstrated that PoPLD, like mammalian PLD families, contains a PX domain (aa 114-235), a PH domain (aa 250-346), a conserved region I (CR I; aa 354-518), a CR II (aa 625-683), a CR III (aa 724-843), a CR IV (aa 867-944) and Carboxyl terminus (CT) domain (aa 1012-1053), which were all previously defined as conserved elements in the extended PLD and phospholipid synthesis family [24,31,41]. However, the loop region of PoPLD (aa 527-602) showed relatively low similarity with that of mammalian PLD1. Mammalian PLD2 lacks this region and part of the human PLD1 loop region undergoes alternative splicing, removing 38 amino acids to generate the PLD1b isoforrm [27,42]. This regulated splicing is conserved in mammalian PLD1s (mouse, rat, and human) and confers no obvious changes in regulation to human PLD1 [42], although it has been suggested that there are subtle changes in Rho responsiveness for rat PLD1 [10]. The loop region of PoPLD is more closely related to PLD1b isotypes than to PLD1a isotypes when compared with mouse and rat PLD1 sequences (Fig. 3). Among the PoPLD amino acids, HKD motifs, which are duplicated in CRI and CRIV, are relatively invariant in all known PLDs and have been demonstrated to be necessary for catalytic activity [24,43]. All of these HKD motifs are similarly conserved in PoPLD as well. Based on the extensive conservation of PoPLD and other PLDs, it is likely that these are cognate genes that encode functionally identical phospholipases with indistinguishable biochemical

properties and cellular roles. A comparison of the amino acid sequence of PoPLD with other PLD enzymes from a variety of other species revealed that PoPLD showed 16-68% overall identities with all known PLD families (Table 2). Among them, PoPLD shares a relatively high similarity with other PLD1 isoforms (61-68%), and a relatively low identity with ScPLD (Table 2). The amino acid sequence identities in the conserved domain also revealed that PoPLD is relatively similar to PLD1 isoforms (Table 3).

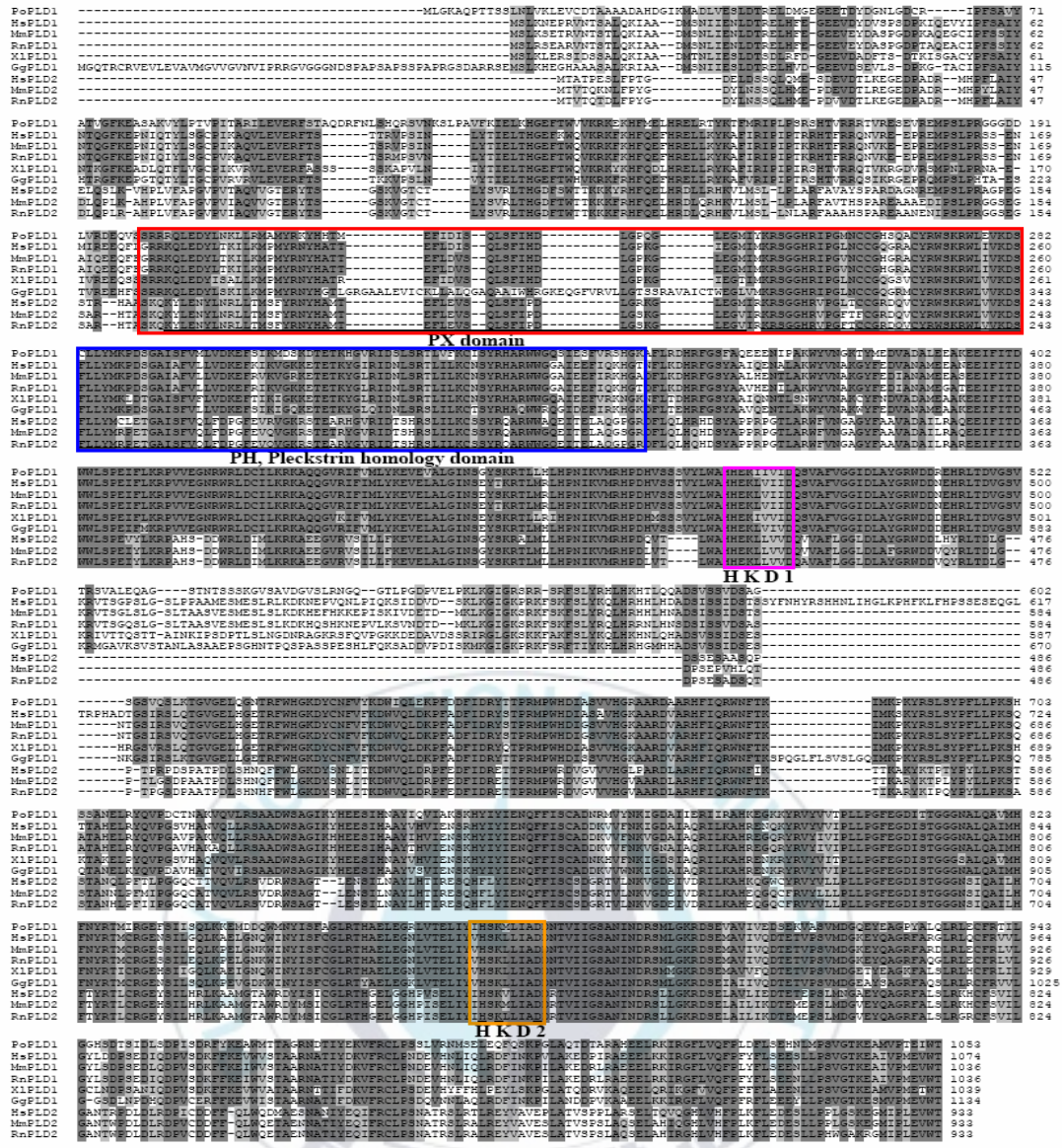


Fig. 3. Comparison of the deduced amino acid sequence of PoPLD with PLDs of other species. Identical amino acids are indicated in the black box and similar amino acids are lightly shaded. Dashes were introduced to obtain maximal alignment.

3.3. Phylogenetic analysis

To determine the evolutionary relationship of PoPLD with other PLD isozymes, phylogenetic analysis was performed using the amino acid sequences of 15 PLD isozymes obtained from GenBank using maximum parsimony and neighbor-joining methods. Both methods produced similar results, therefore only the neighbor-joining tree is presented (Fig. 4). These data indicate that PoPLD is more closely related to PLD1 isotypes than to PLD2 isotypes.

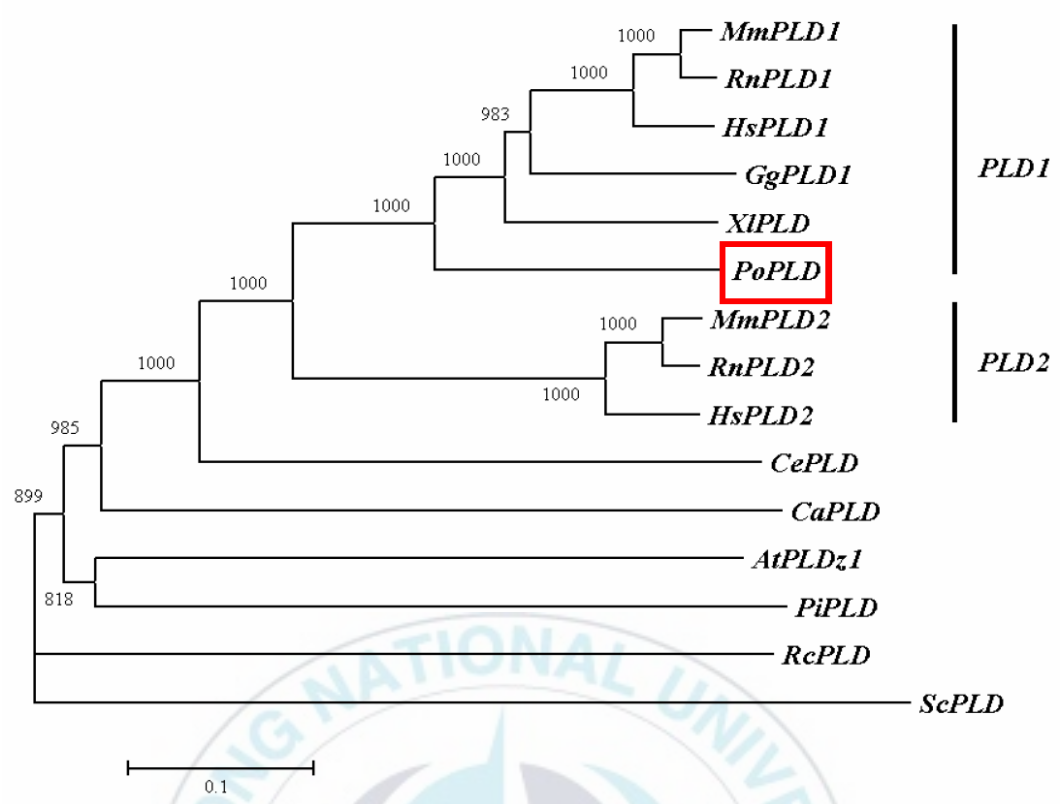


Fig. 4. Phylogenetic relationships of PoPLD with other PLD families. In this neighbor-joining phylogram, all individuals are represented and the branches are based on the number of inferred substitutions as indicated by the bar. Bootstrap values from 1000 replicates are indicated at the nodes.

3.4. Tissue distribution of flounder PLD

It has been reported that while mammalian PLD1 is highly expressed in kidneys and lungs, it is also detectable levels in other tissues [30]. In rats, the predominant isoform of PLD expressed is PLD1b which is found in high levels in the kidneys, small intestine, colon and liver, whereas the PLD1a is only detectable in the lung, heart and spleen [29]. To determine if the expression pattern of mammalian PLD1 differed from that of flounder, we examined the expression pattern of PoPLD in various flounder tissues using RT-PCR with PoPLD specific primers. As shown in Fig. 5, PoPLD was observed in most of the flounder tissues examined. Expression of PoPLD appeared to be highest in the eye, liver, stomach and duodenum, moderately expressed in the spleen, heart and intestine, and expressed in the lowest levels in the brain, head kidney and body kidney. This expression pattern was similar to that of mammalian PLD1 except in the kidney. In kidney, expressional difference between flounder and mammalian can be inferred that the expression pattern of PoPLD may be an evolutional result for adapting in the unique aquatic environment.

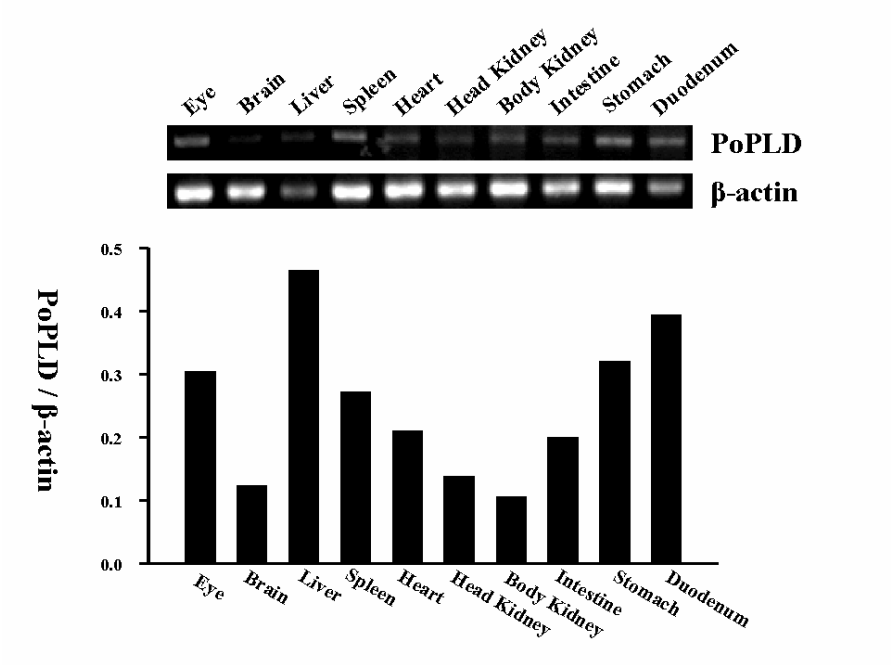


Fig. 5. Tissue distribution of the flounder PLD mRNA. Total RNA was isolated from various tissues of *Paralichthys olivaceus*, and 0.5μg were subjected to RT-PCR analysis using the amplimers for the flounder PLD or β-actin. mRNA levels are normalized to the mRNA levels of β-actin and plotted as a ratio in each bar.

3.5. Expression of GFP-PoPLD in EPC cell line

To determine the subcellular localization of PoPLD within cells, GFP-PoPLD was constructed and then expressed in EPC cells. It was observed that while both GFP and GFP-PoPLD were expressed in EPC cells, expression of GFP was much higher than that of GFP-PoPLD. As previously reported [44], GFP was equally distributed throughout the cell (Fig. 6A), while GFP-PoPLD was localized in cells, forming a distinct ring surrounding the rim of the nucleus (Fig. 6B). Additionally little GFP-PoPLD was present in cytosol throughout the cell. These results indicate that GFP-PoPLD may be localized in the perinuclear membrane, such as endoplasmic reticulum, Golgi and secretory granules, and lysosomal/endosomal compartment, similar to previous reports [45]. GFP-PoPLD mRNA was confirmed using RT-PCR with specific PLD primers as described in Material and Methods (Fig. 7). It shows the results of electrophoresis of the RT-PCR products. In the lane containing the EPC/PoPLD cDNA, the only signal present corresponded to the GFP-PoPLD gene. This suggests that the mRNA for PoPLD is present and that GFP-PoPLD is expressed in EPC cells.

A

B

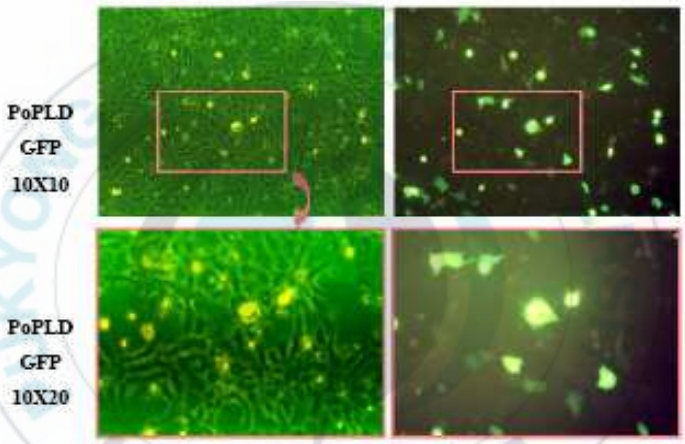


Fig. 6. Expression of GFP-PoPLD in EPC cell line. (A) As a control, pEGFP-C1 vector alone was transfected into EPC cells. (B) GFP-PoPLD plasmid DNA was transfected into EPC cells. Top lower, images of the GFP-PoPLD are shown under the fluorescence microscope, 10×10 magnification; Bottom lower, boxes were enlarged to twice, 10×20 magnificaton.

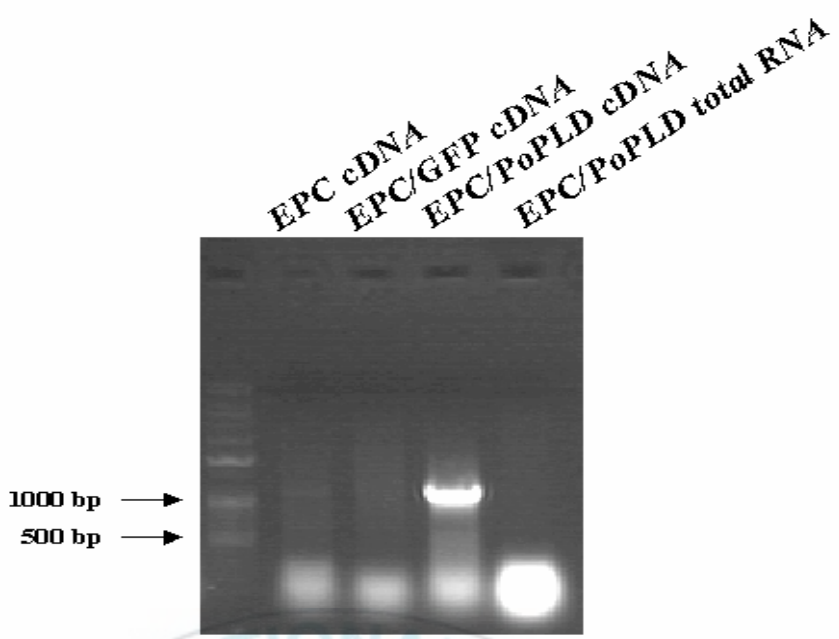


Fig. 7. RT-PCR analysis of GFP-PoPLD in EPC cell line. An ethidium bromide-stained 1.5% agarose gel is shown after electrophoretic separation of RT/PCR products. In lane 1 EPC cDNA was used as template whereas in lane 2 EPC transfected with pEGFP-C1 was used as template DNA. EPC transfected with plasmid DNA containing GFP-PoPLD was used in lane 3 for PCR. Lane 4 is the control.

3.6. PLD activity of the expressed PoPLD in EPC cells

To examine the PC-hydrolyzing phospholipase activity of GFP-PoPLD, [^{3}H]choline-releasing activity from [choline-methyl-^{3}H](pam)$_2$PC was determined in the membrane and cytosol fraction of cell homogenate. We confirmed that PoPLD was capable of hydrolyzing phosphatidylcholine in both the cytosol and membrane fractions. The basal activity in membrane fraction was relatively higher than that of the cytosol fraction. We have also examined the effect guanosine 5′-O-(thio)triphosphate(GTPγS) on the PLD activity in the membrane fraction. The activity was insensitive to GTPγS, indicating its independence of GTP-binding protein (data not shown). This characterization of PoPLD is similar to that of protozoan PLD [46]. It has been reported that Tetrahymena PLD activity was not affected by PMA and GTPγS, which is distinguishable from mammalian PLD1.

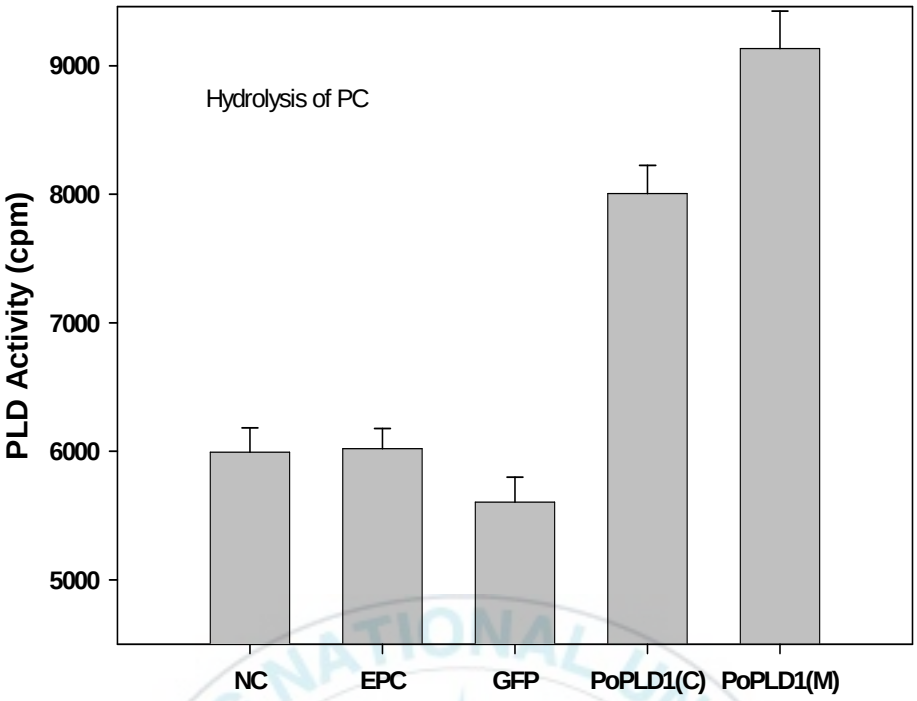


Fig. 8. Hydrolysis of PC for phospholipase D activity. Phospholipase D activity was measured in cell homogenates in vitro. Assays were performed as described in Section 2.6. Bar graphs represent the mean of three determinations ±S.D.

4. Discussion

It is well known that PLD plays an important role in the regulation of cell function and cell fate by extracellular signal molecules, as well as in diseases including inflammation, diabetes and neuronal and cardiac stimulation [4,5]. In this study, the full-length sequence of 3162 bp PoPLD cDNA was cloned from the *Paralichthys olivaceus*. The open reading frame encodes a protein of 1053 amino acids. GenBank homology search and CLUSTAL W multiple sequence alignment confirmed that the cloned cDNA sequence encodes a PLD. This sequence contains the conserved regions of the PX, PH and CRI-IV. Furthermore, the sequence alignment of PoPLD revealed that the predicted amino acid sequence was homologous to other PLD1 family in the NCBI GenBank and showed similarity of 68%, 67% and 66% to PLD from house mouse *Mus musculus*, African clawed frogs *Xenopus laevis* and manchurian norway rat *Rattus norvegicus*, respectively (table 2). These data indicate that PoPLD is clearly a member of the PLD1 subfamily. On the other hand, it was well known that mammalian PLD1 subdivided into two isotypes, PLD1a and PLD1b [3], which these were 99.9% identical based on their cDNA sequences, except for 38 amino acid residues that were not found in PLD1b that are present in the middle of PLD1a, indicating that they are produced by alternative splicing [22]. The 38 amino acid residues absent from mammalian PLD1b were also not found in PoPLD (Fig. 3), suggesting that PoPLD belongs to the PLD1b subfamily.

PoPLD and hPLD1b resemble each other considerably and share a sensitivity to PIP_2. However, their responses to GTPγS are clearly distinguishable. hPLD1b is stimulated by GTPγS, but PoPLD is not. This result indicates that PoPLD is differently regulated from mammalian PLDs.

PoPLD was ubiquitously distributed in various flounder tissues including brain, liver, heart, head kidney, body kidney, intestine and duodenum. The mRNA of PoPLD was predominantly expressed in eye, liver, stomach and duodenum, but it was weekly expressed in brain, head kidney and body kidney. According to previous studies, PLD1 is highly expressed in kidney, lung, brain, spleen, uterus, small intestine, pancreas and heart [3,30]. Furthermore, PLD1b is the major expressed form in rat and is found at high level in kidney, small intestine, colon and liver, whereas PLD1a is mainly expressed in the lung, heart and spleen [3,29]. Therefore, it can be suggested that the tissue distribution of PoPLD is similar to that of rat PLD1b, except that PoPLD transcript is expressed at significantly low levels in kidney.

A number of studies have examined localization by tagging PLDs with GFP or HA to demonstrate their localization. The results revealed that PLD1 was found in the Golgi, ER, endosomes or lysosomes, while PLD2 was predominantly found in the plasma membrane [31,35]. Also, to examine the location of PLD1 variants, subcellular localization of PLD1a expressed in several types of mammalian cells was compared with that of PLD1b. These results show that both PLD1a and PLD1b

are localized to late endosomes and lysosomes in NRK, Vero and HeLa [4]. Taken together, this indicates that PLD isotypes have specific expression sites which are associated with their activity, and that no differences in localization exist between the PLD1 splicing variants. To confirm expression of the recombinant protein, the cDNAs encoding PoPLD were cloned into the pEGFP-C1 (Clontech) vectors and their expression was detected in EPC cells using a fluorescent microscope (Olympus) with a FITC dichromic filter set. Unlike the control cells, we observed that GFP-PoPLD existed in the submembranous vesicular structures and circular endocytic structures (Fig. 6B). The vesicles were again separated from the cells and the location of the characteristic PoPLD was found to be similar to that of PLD1b [35,47]. Our findings were consistent with a report on localization of GFP-PLD1b and HA-PLD1b in RBL-mast cells [26]. This suggests that the vesicles have a possible function in regulated exocytosis. The location of the fusion protein was not clearly determined in EPC cells because we did not use highly specific PLD1 antibodies in combination with immunoelectron and fluorescence microscopy. However, these results sufficiently indicate that PoPLD exists in the intracellular membrane and is not found in the plasma membrane.

This report represents the first report on PLD from an aquatic organism. Further studies to understand the diverse role of PLD would provide a better overall understanding of the finfish.

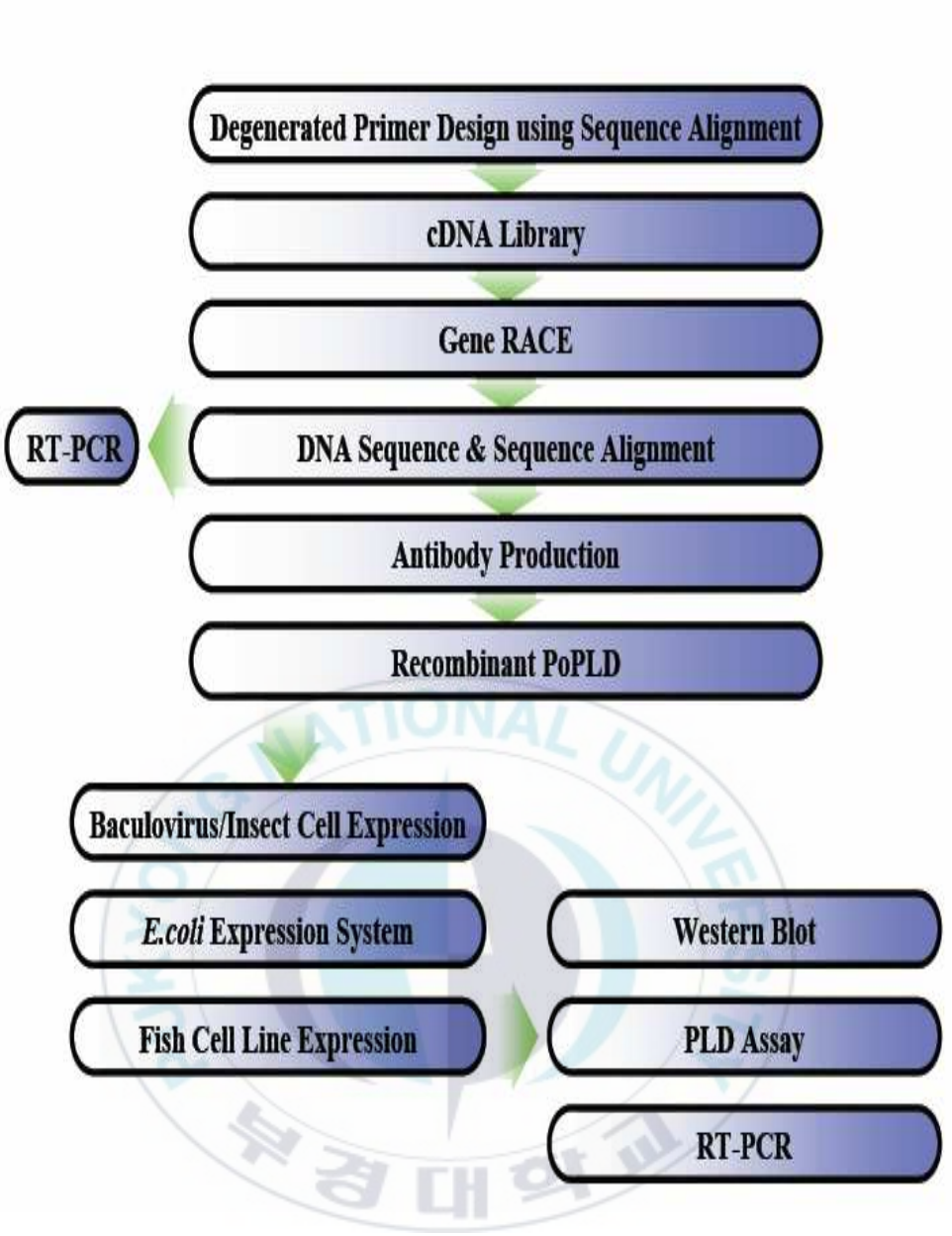
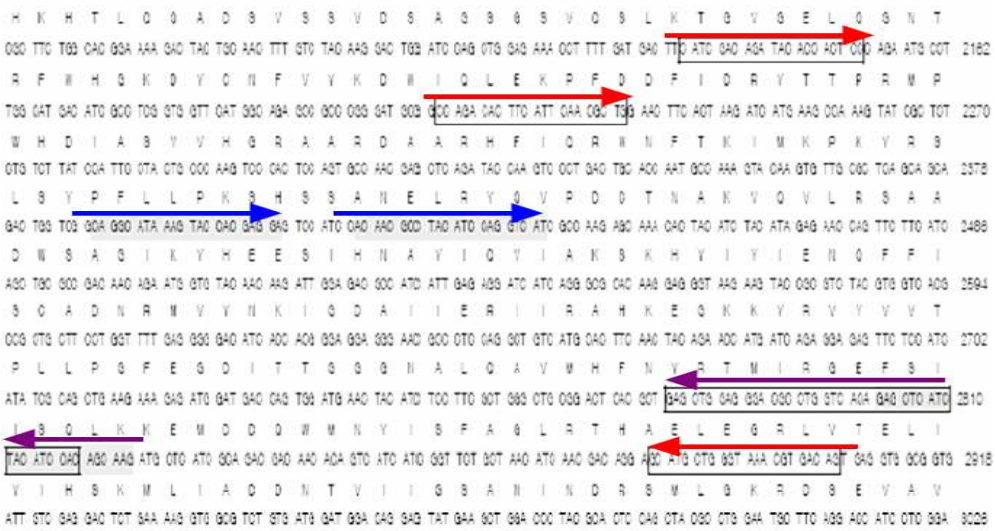


Fig. 9. Diagram showing the experiments in this study.



Name name	Sequences (5’ to 3’)	Position (nt)	Purpose
PoPLD-1F	5’-CATCGACAGATACACCACTCC-3’	2132-2152	Partial PLD clone (694-bp)
PoPLD-2F	5’-CCAGACACTTCATTCAACACTG-3’	2212-2233	
PoPLD-R	5’-CTGTCNCGCTTYCCSAGCAWGC-3’	2884-2905	
PoPLD-3RACEF1	5’-CAGGCATAAAGTACCACGAGGA-3’	2389-2410	3’-RACE
PoPLD-3RACEF2	5’-CAACGCCTACATCCAGGTCAT-3’	2420-2440	
PoPLD-5RACER1	5’- CTTGCTGTGGATGTAGATGAGCTC-3’	2802-2825	5’-RACE
PoPLD-5RACER2	5’- GTGACCAGGCGTCCCTCCAGCTC-3’	2778-2800	

Table 1. Primer sets for cloning of a full-length PoPLD sequence.

Sequence for comparison	Identity (%)	Similarity (%)	Genbank
HsPLD1a	65.30	77.84	AAB49031
MmPLD1	68.67	81.52	NP_032901
RnPLD1a	64.74	77.85	BAA24076
MmPLD1b	68.01	80.68	AAB81245
RnPLD1b	66.95	80.62	BAA24077
GgPLD1	61.76	74.39	XP_422793
XlPLD1	67.79	79.72	AAH77188
HsPLD2	49.38	63.98	O14939
MmPLD2	48.81	64.29	P97813
RnPLD2	48.86	63.95	P70498
CePLD	33.29	47.36	AAA98011
SPO14	22.62	35.97	AAA74938
RcPLD	21.83	37.12	Q41142
AtPLDz1	29.94	47.66	AAL06337
PiPLD	20.95	31.56	AAX28839
ScPLD	16.05	27.35	BAA75216

Table 2. The amino acid sequence identity and similarity between PoPLD from olive flounder (*Paralichthys olivaceus*) and other PLD isoforms.

Sequence for comparison	Domains (%)															
	PX		PH		CRI		Loop		CRII		CRIII		CRIV		CT	
	I	S	I	S	I	S	I	S	I	S	I	S	I	S	I	S
HsPLD1a	59.8	74.6	70.1	80.4	84.2	90.9	24.1	39.7	86.4	94.9	79.2	88.3	79.5	89.7	76.2	88.1
RnPLD1a	59.0	73.8	71.0	81.3	84.2	90.3	29.4	45.4	86.4	94.9	71.7	85.0	78.8	86.3	81.0	88.1
RnPLD1b							41.0	63.9								
GgPLD1	49.3	67.9	67.3	79.4	85.5	91.5	33.7	55.4	88.1	94.9	80.0	87.5	76.9	88.5	73.8	83.3
XlPLD1	61.5	77.9	69.2	78.5	84.9	87.9	34.2	58.5	88.1	94.9	80.8	89.2	78.2	88.5	71.4	85.7
HsPLD2	40.2	60.7	55.1	66.4	62.5	72.6	n.i.	n.i.	72.9	83.1	61.5	71.3	70.5	79.5	50.0	73.8

Table 3. The amino acid sequence identity (I) and similarity (S) in the conserved domains between PoPLD from olive flounder (*Paralichthys olivaceus*) and other PLD isoforms.

KOREAN ABSTRACT

넙치 인지질가수분해효소 PLD의 클로닝 및 특성분석

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요약

Phospholipase D는 포유류 조직과 여러가지 cell lines을 포함하여 다양한 원핵생물과 진핵생물에 존재한다. 이러한 효소는 증식, 분비, 호흡폭발을 일으키는 수용체의 신호전달 과정에서 중요한 역할을 할 뿐만 아니라, 염증, 당뇨, 신경 및 심장자극과 같은 질병에서도 효소로써 작용하는 것으로 알려져 있다. 우리는 넙치 PLD 유전자(PoPLD)를 클로닝 하였고, 서열을 확인하였다. PoPLD에 관한 연구는 넙치 뇌의 cDNA를 조사하는 것으로부터 시작되어졌고, 5' 과 3' RACE로 완성 되어졌다. PoPLD의 전체길이는 4656 bp로써, 5' - 194 bp와 3'-1300 bp의 UTR을 가지며, 시작 코돈 ATG부터 종결 코돈 TGA까지 3162 bp의 ORF를 가진다. 또한 PoPLD는 1053개의 아미노산을 가지며, 120.5 kDa의 크기로 확인되었다. 아미노산 서열 분석에서 hPLD1과 hPLD2는 PoPLD와 각각 65.30%, 49.38%의 상동성을 보였다. Phylogenetic tree와 서열분석을 통해, 다른 PLD와 비교한 결과, PoPLD는 PLD1 isozyme과 모든 구조적 특성을 공유하고 있었다. PoPLD의 조직 발현량 분석은 RT-PCR로 수행되었다. 이것은 뇌, 간, 심장, 두신, 체신, 장, 식도를 포함하는 모든 조직에 분포하였으며, 눈과 간, 위, 식도에서 높은 발현량을 나타냈다. PLD는 pEGFP-C1 벡터에 삽입되어 EPC에서 발현되어졌고, GFP-PoPLD를 형광현미경으로 관찰하였다. 이러한 결과들은 PoPLD가 PLD1의 isozyme이며, 다양한 조직에서 발현된다는 것을 보여주고 있다.

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