

Identification and Characterization of a Novel fish Phospholipase D1B in olive flounder (*Paralichthys oliveceus*)

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넙치(*Paralichthys oliveceus*)로부터 새로운 어류 인지질가수분해효소 PLD1B의 동정 및 특성분석

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Abstract

Phospholipase D (PLD) is an important enzyme that plays a pivotal role in the phospholipid metabolism and signaling, including membrane trafficking, cytoskeletal reorganization, receptor-mediated endocytosis, exocytosis, and cell migration. In this study, we identified an additional PLD1 gene in the olive flounder (*Paralichthys oliveceus*), which emerged through fish-specific genome duplication (FSGD), and designated it as PoPLD1B. Notably, the expression pattern of PoPLD1B differs significantly from that of both PoPLD1A and mammalian PLD1 isoforms. PoPLD1B exhibits high expression in digestive organs, including the gullet, pyloric ceca, stomach, and intestine. Furthermore, recombinant PoPLD1B protein, overexpressed using the pCold TF vector, demonstrated phosphatidylcholine (PC)-hydrolyzing activity in the presence of ADP-ribosylation factor 1 (ARF1). Collectively, these findings indicate that ARF1-dependent activation of fish PLD1B plays a crucial role in digestive organs and gill function, particularly in osmotic and ionic regulation, acid-base balance, nitrogenous waste excretion, and calcium ion homeostasis.

Key words : Phospholipase D1 (PLD1), Olive flounder (*Paralichthys oliveceus*), ADP-ribosylation factor (ARF), Phosphatidylcholine (PC)

I. Introduction

Phospholipase D(PLD) is a widely distributed

enzyme in many organisms, including bacteria, viruses, yeast, plants, and animals (Exton, 1997; Liu et al., 2010; McDermott et al., 2004). It plays

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a key role in several cellular processes including signal transduction, membrane trafficking and mitosis regulation (Liscovitch et al., 2000). PLD is located in the plasma membrane and catalyzes the hydrolysis of phosphatidylcholine (PC) to form phosphatidic acid (PA) and choline (Kodaki & Yamashita, 1997). In mammals, two PLD isozymes, PLD1 and PLD2, which are approximately 50% identical, have been identified. These isozymes consist of Phox homology (PX) domain, pleckstrin homology (PH) domain, four conserved sequences (I-IV), and CT (carboxyl-termini) domains in tandem at their N-termini. However, PLD1 contains a loop region between CR I and II domain when mammalian PLD2 is not present (Cockcroft, 2001; Frohman et al., 1999; Panda et al., 2018; Sung et al., 1999). In mammalian tissues, PLD is known to be activated by several factors including unsaturated fatty acid, phosphatidylinositol 4,5-bisphosphate (PIP2), monomeric GTP-binding proteins (G proteins) such as ADP-ribosylation factor 1 (ARF1) (Brown et al., 1993; Massenburg et al., 1994), Ras homolog gene family member A (RhoA) (Kuribara et al., 1995; Yokozeiki et al., 1996), protein kinase C (Kiss, 1996), and calmodulin (Takahashi et al., 1996). ARF has been implicated in mediating agonist induced activation of PLD, and synergistically stimulated PLD activity in concert with small G proteins, and PLD activation by PKC- α occurs synergistically with ARF and RhoA (Ohguchi et al., 1996; Singer et al., 1996). 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 (Bowling et al., 2021; Brandenburg et al., 2014; Kasai et al., 1998).

Teleost genomes contain more copies of genes than tetrapods (the lobed-finned fishes, amphibians,

reptiles, birds and mammals) as the result of ancient fish-specific genome duplication(FSGD). This has been attributed to FSGD which probably occurred just in an ancestor of teleost fish after the split from the "non-teleosts". For instance, seven unlinked Hox gene clusters were identified in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), whereas in mammalian genomes only four of these gene clusters have been identified. The recently completed characterization of pufferfish (*Tetraodon nigroviridis*) genome has also confirmed whole-genome duplication occurred in the teleost fish lineage, subsequent to its divergence from mammals (Amores et al., 1997; Braasch et al., 2007; Jaillon et al., 2004; Taylor et al., 2001).

In this study, we isolated a unique clone that is similar to olive flounder PLD1 (PoPLD1) reported previously. Also, we found that the homologs identified here are shared by the zebrafish, fugu and tetradon fish genomes in addition to stickleback and medaka genomes. This finding suggests that the olive flounder genome (and potentially all teleost) might possess more PLD1 gene than in the entire mammalian. We report here that a novel duplicated phospholipase D1 underwent a unique gene evolution in early vertebrate. Furthermore, we conducted on activity assay for enzymatic characterization of recombinant PoPLD1B in presence of ARF 1 (ADP-ribosylation factor 1), and expression pattern in tissues of olive flounder PLD1B, as well as comparison of IL-1 β , IL-6 and Mx expression patterns after stimulation with Lipopolysaccharide (LPS), concanavalin A (ConA)/ Phorbol 12-myristate 13-acetate (PMA), and polyinosinic:polycytidylic acid (poly I:C) which innate immune inducers leading to cytokine production and inflammation.

II. Research method

1. Molecular Cloning and Sequence Analysis of Olive Flounder PLD1B

The specimen used in this study was purchased from Namcheon fishery market in Busan. Total RNA was extracted from olive flounder tissue samples using TRIzol[®] reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA was used as a template for first-strand cDNA synthesis, which was performed using the SuperScript[™] First-Strand Synthesis System (Invitrogen). Reverse transcription was

carried out at 42°C for 60 minutes. The resulting cDNA was subsequently used to clone the initial cDNA fragments of the olive flounder PLD1B gene.

Initial cDNA fragments were obtained by nested polymerase chain reaction (PCR) amplification. Primers were designed to span highly conserved regions of known PLD family sequences in teleost fishes and other vertebrates, referencing sequences cloned, predicted, or identified as full-length ORFs in GenBank (NCBI, <http://www.ncbi.nlm.nih.gov>) and the Ensembl genome database (<http://www.ensembl.org>) (<Table 1>).

<Table 1> PLD sequences in teleost fishes and other vertebrates used in this study

Sequence for comparison	Species (common name)	Ensembl protein ID
HsPLD1	<i>Homo sapiens</i> (Human)	ENSP00000342793
HsPLD2		ENSP00000263088
MmPLD1	<i>Mus musculus</i> (Rat)	ENSMUSP00000113810
MmPLD2		ENSMUSP00000018429
GgPLD1	<i>Gallus gallus</i> (Chicken)	ENSGALP00000015041
XtPLD1	<i>Xenopus tropicalis</i> (Western clawed frog)	ENSXETP00000020063
XtPLD2		ENSXETP000000045447
DrPLD1A	<i>Danio rerio</i> (Zebrafish)	ENSDARP00000073147
DrPLD1B		ENSDARP00000074178
DrPLD2		ENSDARP00000039697
TrPLD1A	<i>Takifugu rubripes</i> (Japanese pufferfish)	SINFRUP00000175182
TrPLD1B		SINFRUP00000176609
TrPLD2		SINFRUP00000177427
TnPLD1A	<i>Tetraodon nigroviridis</i> (Spotted green pufferfish)	GSTENP000012719001
TnPLD1B		GSTENP000007932001
TnPLD2		GSTENP000022141001
GaPLD1A	<i>Gasterosteus aculeatus</i> (Three-spined stickleback)	ENSGACP00000014504
GaPLD1B		ENSGACP00000016732
GaPLD2		ENSGACP00000026957
OiPLD1A	<i>Oryzias latipes</i> (Medaka)	ENSORLP00000014666
OiPLD1B		ENSORLP00000003484
OiPLD2		ENSORLP00000010066
CiPLD	<i>Ciona intestinalis</i> (Sea squirt)	ENSCINP00000005818
CsPLD	<i>Ciona savignyi</i> (Pacific sea squirt)	ENSCSAVP00000012603
SPO14	<i>Saccharomyces cerevisiae</i> (Baker's yeast)	YKR031C
		NCBI accession number
PoPLD1A	<i>Paralichthys olivaceus</i> (Olive flounder)	AAR29590.1

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The PLD-DegF1 and PLD-DegR primers were used for the first round of nested PCR targeting the PoPLD1B gene. The PCR reaction mixture consisted of 1 μ l of cDNA, 10 pmol of each primer, 2.5 mM dNTP mix, 2.5 units of Taq polymerase (Promega), and the manufacturer's buffer, in a final volume of 50 μ l. PCR was conducted under the following conditions: initial denaturation at 94°C for 3 minutes; 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 1

minute; followed by a final extension at 72°C for 10 minutes.

The amplified PCR product was used as the template for a second round of PCR with the PLD-DegF2 and PLD-DegR primer sets. Amplified DNA products were separated by 1% agarose gel electrophoresis, purified, ligated into the pGEM[®]-T Easy vector (Promega), and cloned. DNA sequencing was performed using the BigDye[™] Terminator Cycle Sequencing Kit on an ABI 377 automated sequencer (PerkinElmer).

<Table 2> Oligonucleotide primers used in PCR amplification of PoPLD1B gene. qRT-PCR, quantitative reverse transcription-polymerase chain reaction; F, Forward; R, reverse; S, C or G; M, A or C; Y, C or T; R, A or G

Primer name	5'-3' sequence	Description
PLD-DegF1	GAYTQQTQGYTVAYGTYCHGA	Degenerated primers used in the 1st and 2nd round PCR of nested PCR to obtain initial fragments of PoPLD1B
PLD-DegF2	TGGGSCAYCAYGARAA	
PLD-DegR	RTCRTTDA TRTRTRGCNGANCEDAT	
5'PoPLD1B-R1	GATGAAGTGCTGGCTGTTCTGGATGCCG	Specific primers for 5'RACE of PoPLD1B
5'PoPLD1B-R2	CTCGCTCTGCTGGGTGGGTGAGACTT	
3'PoPLD1B-F1	AAGCTCCACACCACAGCAGAGAGACAG	Specific primers for 3'RACE of PoPLD1B
3'PoPLD1B-F2	CGACACTATTACCGAGCGAAATCATCAGAGC	
Po β -actin F	GAC ATG GAG AAG ATC TGG CA	For qRT-PCR
Po β -actin R	ATG TCC TGC TCG AAG TCC AG	GenBank accession no: AU090737
Po1b rRNA F	ATGGCCGTTT TTAGTTGGTG	For qRT-PCR
Po1b rRNA R	CACACGC TGA TCCAGTCAGT	GenBank accession no: AB070835
IL-1 β F	AACAGCCAAGCAAAGAGATG	For qRT-PCR
IL-1 β R	AATGTCCTGCCTCCTTCA	GenBank accession no: AB070835
IL-8 F	TGGGCATCAGCTCGGCTAC	For qRT-PCR
IL-8 R	CGAAGGCCACAGATTGGTC	GenBank accession no: DQ287937
PoMx F	AACAGCCAAGCAAAGAGATG	For qRT-PCR
PoMx R	AATGTCCTGCCTCCTTCA	GenBank accession no: AB110446
PoPLD1B RT F	TGGGCATCAGCTCGGCTAC	For qRT-PCR and real time PCR
PoPLD1B RT R	CGAAGGCCACAGATTGGTC	
KpnI PoPLD1B-F	CGGGTACCATGCTGCACGCATTCCCAAC	Primers for the construction of expression vector
PoPLD1B-XhoI R	GCCTCGAGTCAGGTCCAGAC CTCCATGGGC	

To identify the full-length coding region of the PoPLD1B gene, rapid amplification of cDNA ends (RACE) was performed using the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc.), along with gene-specific primers designed from initial fragment sequences (<Table 2>). PCR products from 5' and 3' RACE were cloned into the pGEM®-T Easy vector and sequenced.

Nucleotide and predicted protein sequences were analyzed using DNAsis for Windows (version 2.5,

Hitachi Software Engineering), BioEdit Sequence Alignment Editor, and BLAST searches against non-redundant NCBI databases. For phylogenetic analysis, sequences were aligned using CLUSTAL W version 1.8 and phylogenetic trees were constructed using the neighbor-joining method with 1,500 bootstrap replicates, employing the Kimura two-parameter model in MEGA 6 software (<https://www.megasoftware.net>) (<Table 3>).

<Table 3> The amino acid sequence identity and similarity between PoPLD1B from olive flounder (*Paralichthys olivaceus*) and other PLD isoforms

Sequence comparison	Identity	Similarity	NCBI Accession number
PoPLD1A	74.13%	83.57%	AAR29590.1
PoPLD1B	ID	ID	ACF94295.1
			Ensembl Protein ID
HsPLD1	62.61%	75.14%	ENSP00000342793
HsPLD2	48.05%	61.16%	ENSP00000263088
MmPLD1	64.84%	77.69%	ENSMUSP00000113810
MmPLD2	47.29%	61.06%	ENSMUSP00000018429
GgPLD1	64.49%	78.19%	ENSGALP00000015041
XtPLD1	63.61%	76.20%	ENSXETP00000020063
XtPLD2	46.93%	59.70%	ENSXETP00000045447
DrPLD1A	73.00%	82.73%	ENSДАРP00000073147
DrPLD1B	77.64%	86.56%	ENSДАРP00000074178
DrPLD2	47.34%	59.22%	ENSДАРP00000039697
TrPLD1A	73.09%	83.57%	SINFRUP00000175182
TrPLD1B	71.94%	77.18%	SINFRUP00000176609
TrPLD2	48.00%	61.24%	SINFRUP00000177427
TnPLD1A	70.44%	80.70%	GSTENP000012719001
TnPLD1B	29.27%	31.77%	GSTENP000007932001
TnPLD2	47.05%	59.13%	GSTENP000022141001
GaPLD1A	67.34%	74.26%	ENSGACP00000014504
GaPLD1B	73.10%	80.79%	ENSGACP00000016732
GaPLD2	46.31%	60.49%	ENSGACP00000026957
OiPLD1A	69.22%	77.06%	ENSORLP00000014666
OiPLD1B	77.37%	86.29%	ENSORLP00000003484
OiPLD2	46.71%	59.68%	ENSORLP00000010066
CiPLD	38.52%	52.19%	ENSCINP00000005818
CsPLD	35.00%	49.49%	ENSCSAP00000012603
SPO14	16.31%	26.57%	YKR031C

2. Gene Expression Analysis of PoPLD1B Under Healthy Conditions

To confirm the tissue-specific expression pattern of PoPLD1B, total RNA was isolated from various tissues of healthy olive flounder using TRIzol® (Invitrogen). First-strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) at 42°C for 60 minutes.

RT-PCR analysis was conducted using PoPLD1B-specific primers (Table 2). PCR targeting β -actin and 18S rRNA served as internal controls. PCR conditions included: pre-denaturation at 94°C for 5 minutes; 25–35 cycles of denaturation at 98°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds; and a final extension at 72°C for 7 minutes.

Amplified products were separated by 1% agarose gel electrophoresis containing ethidium bromide and visualized using the Gel Doc image analysis system (Bio-Rad, USA). Products were purified using the QIAquick® Gel Extraction Kit and sequenced (COSMO Co. Ltd., Seoul, Korea).

3. Gene Expression Analysis of PoPLD1B in Response to Immune Stimulation

To assess the immune responsiveness of PoPLD1B, healthy juvenile olive flounder (100 ± 15 g) were intraperitoneally injected with 500 μ l of the following solutions: LPS (500 μ g/ml), ConA (50 μ g/ml) plus PMA (0.35 μ g/ml), Poly(I:C) (50 μ g/ml) and PBS (control).

Fish were anesthetized with MS-222 (Sigma) prior to injection. Tissues (brain, gill, heart, spleen, stomach, intestine, kidney, and muscle) were collected at 0, 1, 3, 6, 24, and 48 hours post-injection. Total RNA was extracted using TRIzol®, and first-strand cDNA was synthesized as

described previously.

Gene expression levels of PoPLD1B, IL-1 β , IL-6, and Mx were quantified by RT-PCR and real-time PCR using SYBR Green chemistry on a LightCycler® 480 II (Roche). Expression was normalized to β -actin or 18S rRNA. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± S.D. Statistical significance was determined using unpaired Student's t-tests, with p-values < 0.05 considered significant.

4. Expression and Purification of Recombinant PoPLD1B Protein

The open reading frame (ORF) of PoPLD1B was amplified by PCR using primers PoPLD1B-F (5'-ATGCTGCAGCATTCCCAACCAA-3') and PoPLD1B-R (5'-TCAGGTCCAGACCTCCATGGGC-3'). The resulting 3117 bp fragment was cloned into the pCold™ TF expression vector (Takara) and transformed into E. coli BL21(DE3) cells.

Transformed cells were grown in LB broth containing ampicillin (50 μ g/ml) at 37°C, induced with 0.5 mM IPTG, and harvested. Cells were lysed via sonication, and the cytosolic and membrane fractions were separated by ultracentrifugation. Recombinant proteins were analyzed by SDS-PAGE, Western blotting, and activity assays. Protein concentrations were determined using a Bio-Rad protein assay kit.

5. PLD Activity Assay of Recombinant PoPLD1B Protein

PLD activity was assayed following a modified method of Brown et al. (1993). Briefly, 25 μ l of mixed lipid vesicles containing phosphatidylethanolamine (PE), phosphatidylinositol 4,5-bisphosphate (PIP2),

and phosphatidylcholine (PC) (molar ratio 16:1.4:1) with [choline-methyl- ^3H]-labeled PC (~200,000 cpm per assay) were mixed with 10 μl of the PLD source. The final assay buffer contained 50 mM Hepes (pH 7.5), 3 mM EGTA, 80 mM KCl, 2.5 mM MgCl_2 , and 2 mM CaCl_2 .

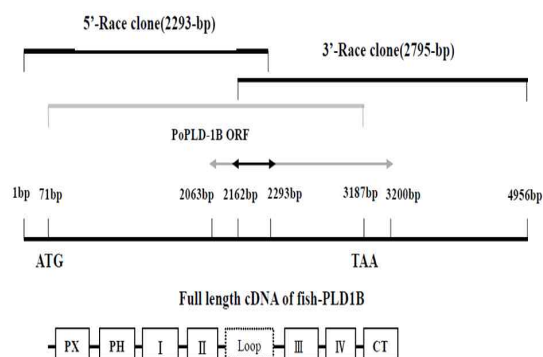
Reactions were incubated at 37°C for 60 minutes and terminated with a stop solution (chloroform:methanol:HCl, 50:50:0.3 v/v) and 1 M HCl containing 5 mM EGTA. After phase separation by centrifugation, the aqueous phase containing [^3H]choline was quantified by liquid scintillation counting. Phosphatidylcholine-hydrolyzing activity was assayed in the presence of 150 μM GTP γ S.

III. Research results

1. Isolation and Characterization of cDNA Encoding PoPLD1B Gene

To isolate and characterize the duplicated PoPLD1 gene, we identified putative PLD1B sequences in the Ensembl Genome Browser. These sequences exhibited homology to PLD1 genes from zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), fugu (*Takifugu rubripes*), stickleback (*Gasterosteus aculeatus*), frog (*Xenopus tropicalis*), chicken (*Gallus gallus*), mouse (*Mus musculus*), and human (*Homo sapiens*) (<Table 1>). PCR primer sets were designed to amplify the initial cDNA fragments corresponding to each PLD1 gene. To obtain the full-length coding sequence of PoPLD1B, rapid amplification of cDNA ends (RACE) was performed using gene-specific primers (<Table 2>, [Fig. 1]) designed based on the initial cDNA fragments.

The full-length cDNA sequence of PoPLD1B was successfully obtained, revealing a 4958-bp



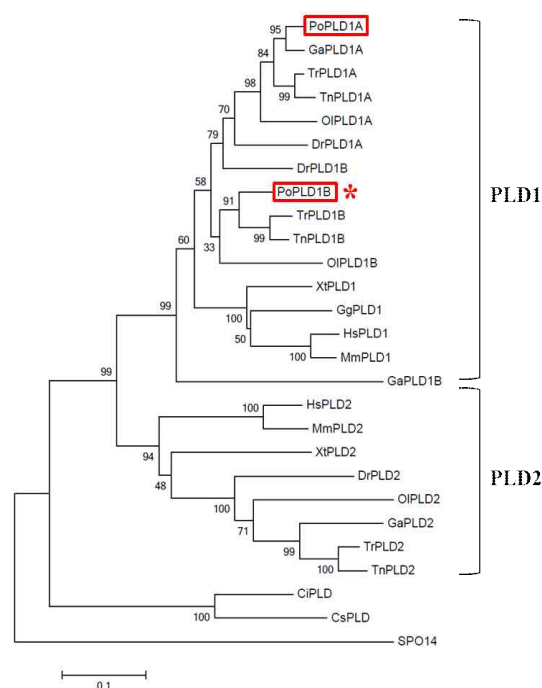
[Fig. 1] Strategy for cloning of *P. olivaceus* PLD1B, and conserved and unique features for PoPLD1B. The PoPLD1B amino acid sequences encode regions that are conserved among mammalian PLD and some or all PLDs from non-mammalian species (boxed regions). PX, phox; PH, pleckstrin homology; CR, conserved region; Loop, loop region; CT, carboxyl terminus.

sequence comprising an open reading frame of 3117 bp encoding 1039 amino acids, with an estimated molecular mass of 167 kDa. Structural features, including a 5'-untranslated region (70 bp), a 3'-untranslated region (1771 bp), and conserved eukaryotic mRNA elements, were present. PoPLD1B exhibited 77.64% amino acid sequence identity to *D. rerio* PLD1B (<Table 3>). Additionally, conserved structural motifs, including four phospholipase D (PLD) conserved regions (CR I-IV), PX, and PH domains, were identified ([Fig. 1, Table 3]), confirming PoPLD1B as a member of the PLD family. The PoPLD1B sequence has been deposited in GenBank under accession number EU872186.

2. Phylogenetic and Evolutionary Relationship of PoPLD1B

Previous studies reported the first isolation of PoPLD1 cDNA from *Paralichthys olivaceus* (Jeon et

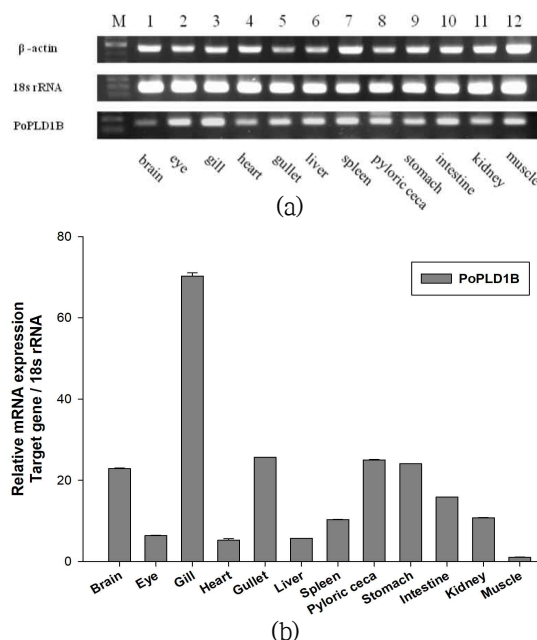
al., 2008). During subsequent investigations, we identified a duplicated PLD1 gene, designated as PoPLD1B, likely arising from fish-specific genome duplication (FSGD) (Meyer et al., 2005; Pasquier et al., 2016; Volff, 2005). Our phylogenetic analysis, using the neighbor-joining method ([Fig. 2]), demonstrated that PoPLD1B shares an evolutionary origin with tetrapod PLDs, with strong bootstrap support (99%).



[Fig. 2] Phylogenetic relationships of PoPLD1B 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 1500 replicates are indicated at the nodes.

Phylogenetic clustering indicated that PoPLD1A and PoPLD1B form distinct subclades within the vertebrate PLD1 lineage, suggesting that their

duplication occurred within the teleost lineage. PLDs from vertebrates are clearly distinct from those of tunicates (*Ciona intestinalis* and *Ciona savignyi*), indicating that the PLD1 subfamily in bony vertebrates diverged from early vertebrate ancestors.



[Fig. 3] Phylogenetic relationships of PoPLD1B 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 1500 replicates are indicated at the nodes.

3. Expression Analysis of PoPLD1B in Different Tissues and Immune Challenges

The tissue-specific expression of PoPLD1B was assessed using real-time PCR and RT-PCR ([Fig. 3]). PoPLD1B was broadly expressed across various tissues, with predominant expression in the brain, gill, gullet, pyloric ceca, and stomach. Notably,

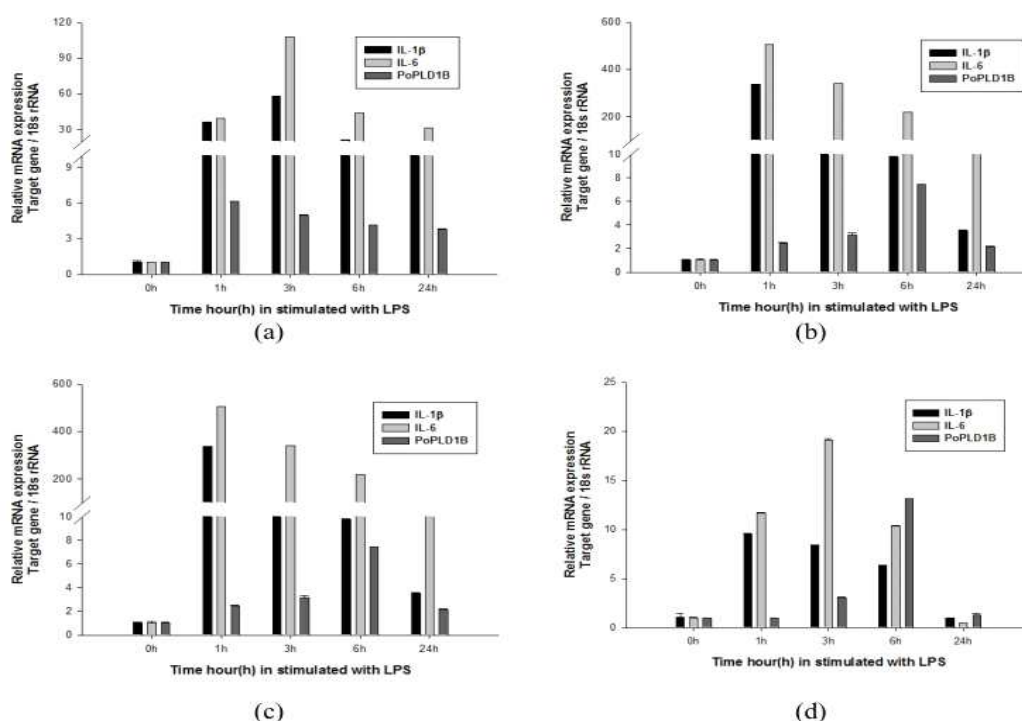
expression in gill tissue was significantly elevated. Conversely, the lowest expression was observed in muscle, consistent with findings in mammalian PLD1B orthologs (Jaafar et al., 2013; Kim et al., 2010).

The high expression of PoPLD1B in digestive organs suggests a functional adaptation to the aquatic environment.

PLD has been implicated in phagocytosis and innate immunity, but the molecular mechanisms regulating fish PLD isoforms remain unclear. We analyzed the expression of PoPLD1B alongside pro-inflammatory cytokines (IL-1 β , IL-6) and the antiviral Mx gene following stimulation with lipopolysaccharide (LPS), Concanavalin A

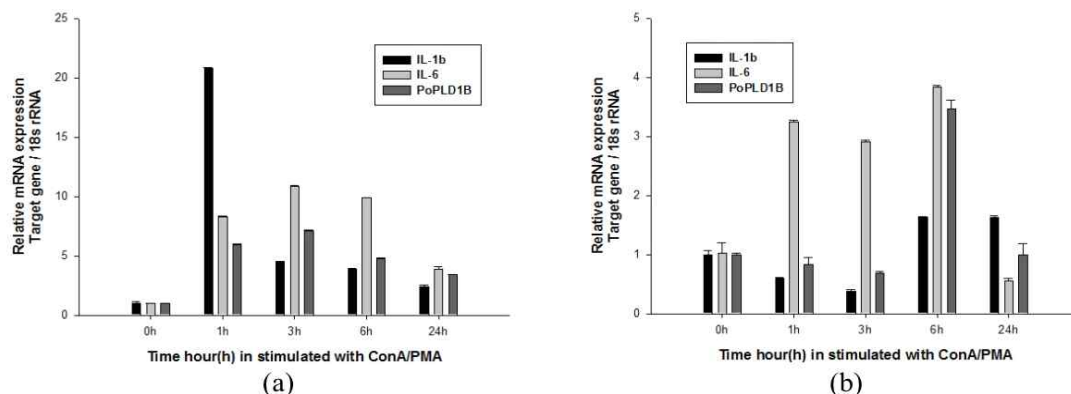
(ConA)/phorbol 12-myristate 13-acetate (PMA), and polyinosinic-polycytidylic acid (poly I:C). Significant upregulation of PoPLD1B was observed in gill, spleen, stomach and muscle tissues at 1–24 hours post-injection (HPI) with LPS ([Fig. 4]).

ConA/PMA stimulation induced a notable increase in PoPLD1B expression in gill tissue at 3 HPI and in the heart at 6 HPI ([Fig. 5]). Following poly I:C stimulation, PoPLD1B, IL-1 β , and Mx expression increased significantly lipopolysaccharide (LPS), across multiple tissues, particularly in gill and intestine between 1–6 HPI ([Fig. 6]). These findings suggest that piscine PLD1B may play a critical role in immune responses and inflammation.

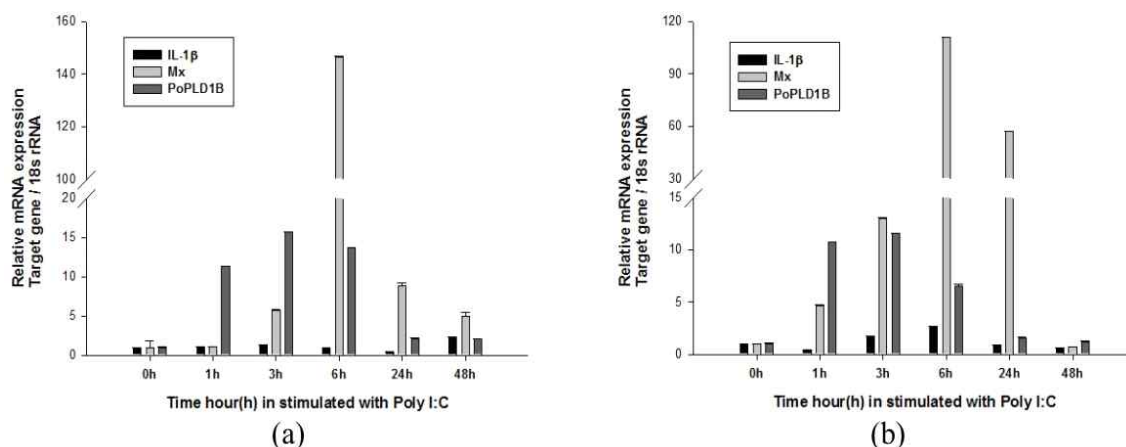


[Fig. 4] Real-time PCR analysis of PoPLD1B, IL-1 β , IL-6 and 18S rRNA following LPS treatment from gill (a), spleen (b), stomach (c), and muscle (d) tissues at 0, 1, 3, 6 and 24h. The expression level of 18S rRNA was used as an internal control for real-time PCR. Each experiment was conducted in triplicate.

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[Fig. 5] Real-time PCR analysis of PoPLD1B, IL-1 β , IL-6 and 18s rRNA following ConA/PMA treatment from gill (a) and heart (b) tissues at 0, 1, 3, 6 and 24h. The expression level of 18S rRNA was used as an internal control for real-time PCR. Each experiment was conducted in triplicate.



[Fig. 6] Real-time PCR analysis of PoPLD1B, IL-1 β , Mx and 18s rRNA following Poly I:C treatment from gill (a) and intestine (b) tissues at 0, 1, 3, 6 and 24h. The expression level of 18S rRNA was used as an internal control for real-time PCR. Each experiment was conducted in triplicate.

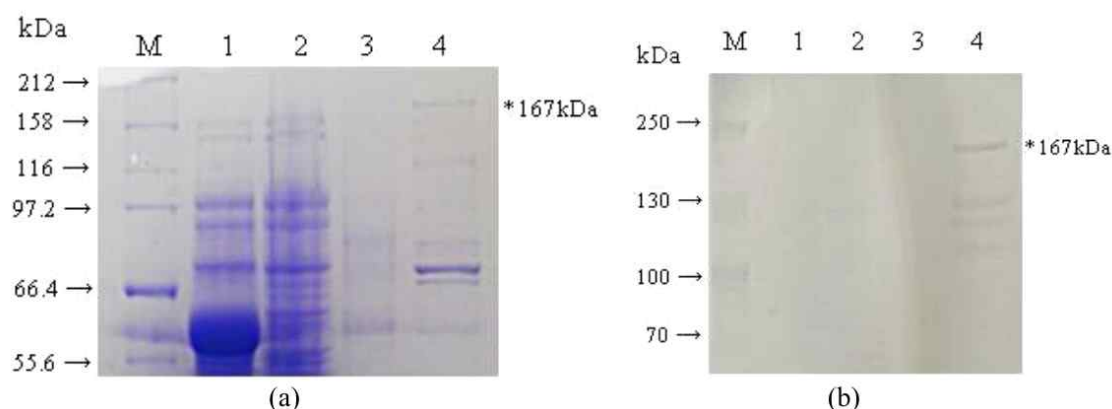
4. Enzymatic Characterization of Recombinant PoPLD1B Protein

Despite extensive characterization of mammalian PLD isozymes, fish PLD isozymes remain poorly understood. To address this, recombinant PoPLD1B was expressed in *E. coli* and purified for enzymatic analysis. SDS-PAGE and Western blot analysis confirmed the expression of recombinant PoPLD1B, which exhibited a molecular mass of 167 kDa,

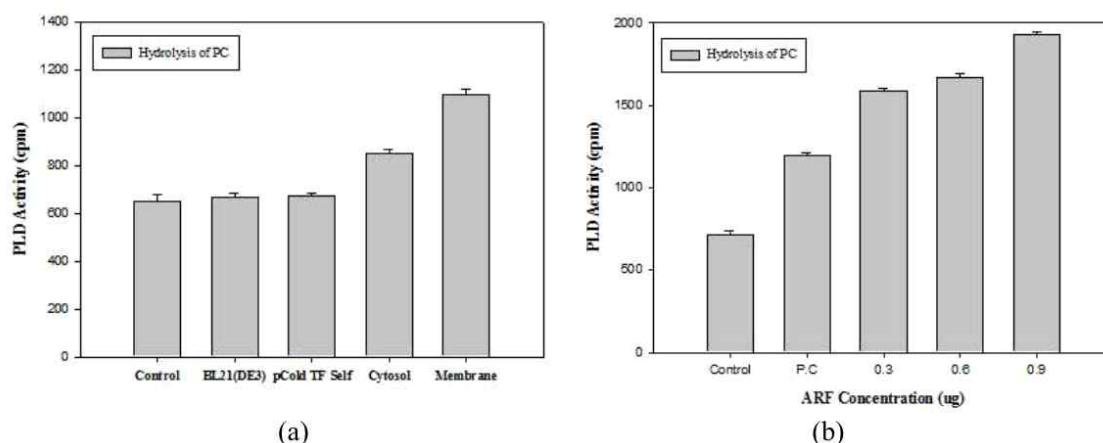
consistent with the predicted size([Fig. 7]). The enzymatic activity of purified PoPLD1B was assessed using phosphatidylcholine (PC)-hydrolyzing assays. Recombinant PoPLD1B demonstrated PC-hydrolyzing phospholipase activity in both membrane and cytosolic fractions, with basal activity higher in membrane fractions([Fig. 6]). Additionally, PoPLD1B activity was enhanced in the presence of ADP-ribosylation factor 1 (ARF1)

([Fig. 8]), consistent with mammalian PLD1 regulatory mechanisms (Kim et al., 1998). The mechanism for the ARF1-mediated activation of PLD1 has been elucidated, involving an interaction

between the PLD1 catalytic domain and ARF1 (Kim et al., 1998). This finding indicates that PoPLD1B might be similarly regulated through this structural interaction.



[Fig. 7] Expression of recombinant PoPLD1B protein. (a) Coomassie blue staining after SDS-PAGE. The asterisk (*) indicates the purified PoPLD1B protein (167 kDa). Lane M, standard size marker; lane 1, whole cell lysate from over-expressed pCold TF-Self expressing *E. coli* BL21(DE3); lane 2, whole cell lysate from IPTG-uninduced PoPLD1B-expressing *E. coli* cells; lane 3, whole cell lysate from 0.5mM IPTG-induced PoPLD1B-expressing *E. coli* cells; lane 4, purified PoPLD1B protein. (b) Western blot analysis of the SDS-PAGE-separated proteins using anti-His-tag polyclonal antibody.



[Fig. 8] Hydrolysis of PC for phospholipase D activity. Phospholipase D activity was measured in cell homogenates in vitro. Bar graphs represent the mean of three determinations $\pm$ S.D. (a) Hydrolysis of PC for phospholipase D activity according to condition. (b) Hydrolysis of PC for phospholipase D activity according to ARF concentration.

IV. Conclusion

In this study, we identified and characterized PoPLD1B, a duplicate of the PLD1 gene found in various teleost species. PoPLD1B exhibited high sequence identity (62.61%) with human PLD1 and retained conserved structural domains, including a Pleckstrin Homology (PH) domain, four conserved regions (CR I-IV), and a C-terminal domain. Tissue-specific expression analysis revealed significant expression in digestive organs, particularly the gill, highlighting its potential adaptation to the aquatic environment. Furthermore, functional studies demonstrated that PoPLD1B expression is modulated in response to immune challenges, implicating it in host defense mechanisms. Finally, enzymatic characterization of recombinant PoPLD1B confirmed its phosphatidylcholine-hydrolyzing activity and regulatory interaction with ARF1.

These findings contribute to the broader understanding of piscine PLD1 isoforms in osmoregulation, calcium homeostasis, and cellular signaling pathways, including membrane trafficking, signal transduction, coagulation, mitosis, and apoptosis.

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