



Thesis for the Degree of Master of Engineering

Comparison of molecular analysis and enzymatic characterization of phospholipase C-δ3A and C-δ3B from olive flounder

(Paralichthys olivaceus)

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by

Department of Biotechnology

The Graduate School

Pukyong National University

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(Paralichthys olivaceus)
 넙치로부터 인지질가수분해효소
 C-δ3A와 C-δ3B의 분자분석
 비교와 효소학적 특성 분석

Advisor: Prof. Hyung Ho Lee

by Jin Young Lee

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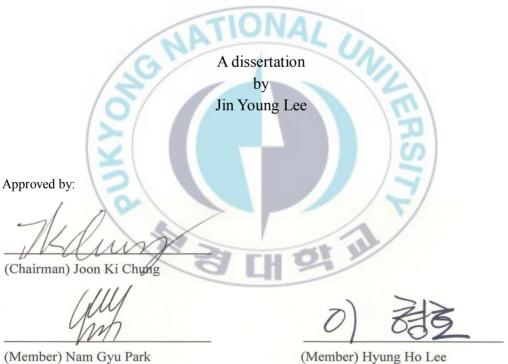
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(Member) Nam Gyu Park

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Comparison of molecular analysis and enzymatic characterization of phospholipase C-δ3A and C-δ3B from olive flounder (*Paralichthys olivaceus*)

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Abstract

The gene of phospholipase C-83 (PLC-83) encodes one of phospholipase C group which localizes to plasma membrane and catalyzes hydrolysis of phosphatidylinositol 4,5biphosphate (PIP_2) to produce second messengers, inositol 1,4,5-thriphosphate (IP_3) and diacylglycerol (DAG). Super-pathways related to this gene include signal transducer activity and calcium ion binding. In order to compare molecular characterization of Paralichthys olivaceus PLC-63, we identified duplicated PLC-63 genes, PLC-63A and PLC-63B, in olive flounder (P. olivaceus) from the previous studies. Similarity of amino acid sequence in two paralogous genes is 67.8% and regulatory domains discovered in mammalian PLC-δ are identically conserved in P. olivaceus PLC-83 isoforms. After performing RT-PCR and realtime PCR, we found out that the distribution of the tissue-specific expression and immune response by stimulation considerably varied in P. olivaceus PLC-83 isoforms. Although the recombinant proteins of PoPLC-83 isoforms were expressed with histidine-tag in *Escherichia coli*, PoPLC- δ 3B only showed the activity at pH 7.5 and high concentration of calcium ion. From the further PIP₂ hydrolyzing assay, we determined that the activity of PoPLC-δ3B was weakly inhibited by PLC inhibitor (U-73122) and strikingly regulated by spermine, spermidine and sphingosine. Moreover, PoPLC-83B has binding properties to phospholipid like mammalian PLC-\delta. Regarding PLC-\delta3 in aquatic organism, these results firstly provide the fundamental information of cellular signaling of PLC- δ 3B and the possibility that PoPLC- δ 3A has non-activity form *in vitro* or exists as non-processed pseudo gene.



1. Introduction

Phospholipase C (PLC) has significant role in cellular signal transduction involved in a variety of physiological functions, including hormone secretion, neurotransmitter transduction, grow factor signaling, membrane trafficking, ion channel activity, and cytoskeletal regulation. When signal molecules bind to paritular receptors on the cell membrane, PLC catalyzes the hydrolysis of membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP₂) into two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). These second messengers, IP₃ and DAG, mediate a variety of cellular responses to extracellular stimuli by inducing protein kinase C and increasing cytosolic Ca²⁺ concentrations. Several factors such as spermine, sphingosine and phospholipids affect enzymatic activation as well as calcium ion concentration (Rebecchi and Pentyala, 2000; Rhee and Bae, 1997). In addition, *neurologic diseases* and *huntington disease* are associated with PLC- δ as other physiological features has been reported (Runkel *et al.*, 2012).

PLC family in vertebrates is categorized as six classes (β , γ , δ , ε , ζ and η). Among them PLC- δ exists as three different isoforms (PLC- δ 1, - δ 3 and - δ 4) (Katan, 1998). The structure of all PLC δ -isozymes is primarily composed of four conserved domains such as catalytic domains (X and Y) and regulatory domains (C2, EF-hand and pleckstrin homology) (Rhee and Bae, 1997; Razzini *et al.*, 2000; Yamamoto *et al.*, 1999). Regarding intracellular location of PLC- δ group in the cell, PLC- δ 1 and PLC- δ 4, are primarily located in nucleus and cytosol



respectively has been reported (Liu *et al.*, 1996). In addition, PLC-δ3 has been found in plasma membrane (Pawelczyk and Matecki, 1998).

Despite important roles in cellular physiology including signal transduction of PLC family within all eukaryotic cells, molecular characterization of PLC- δ 3 has not been well known in aquatic animals unlike mammalian. Therefore, our previous studies defined that PLC- δ 1 and PLC- δ 3 have two paralogous genes respectively as a result of fish-specific genome duplication (FSGD) in teleostei, in other words, two paralogous genes of PLC- δ 3 were named as PoPLC- δ 3A and PoPLC- δ 3B by investigating cDNA sequence of *P. olivaceus* (Kim *et al.*, 2008). Furthermore, researches related into enzymatic properties of PoPLC- δ 1 isoforms and PoPLC- δ 4 have been recently published (Kim *et al.*, 2013; Bak *et al.*, 2013).

Here, we continuously tend to prove preliminary data about PLC- δ of a marine teleost through analysis of mRNA level expression and enzymatic characterization in PLC- δ 3A and PLC- δ 3B from olive flounder (*P. olivaceus*).

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2. Materials and methods

2.1. Sequence and phylogenetic analyses of PoPLC-δ3 isoforms

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Following the results of our previous studies, peptide sequences of PoPLC- δ 3A and - δ 3B were certified using BioEdit Sequence Alignment Editor and BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). After accumulating amino acid sequences of *PoPLC-\delta3A*, *C-\delta3B* and PLC isomerase of other species, multiple alignment and analysis was carried out with CLUSTAL W version 1.8 (Thompson *et al.*, 1994). The phylogenetic tree was constructed using MEGA version 4.0 with the neighbor-joining (NJ) method with 1000 bootstrap replicates (Kumar *et al.*, 2008). The GenBank and the Ensembl accession numbers of all species of PLC- δ used in the study are given in Table 1.

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Table 1

Sequences used in this study.

G		Accession n	Accession number	
Sequence for comparison	Species (common name)	Ensembl	Genbank	
ΡοΡLC-δ3Α	Paralichthys olivaceus (olive flounder)		ACA05827.1	
PoPLC-83B			ACA05828.1	
PfPLC-δ3A	Poecilia formosa (amazon molly)	ENSPFOP00000001225		
PfPLC-δ3B		ENSPFOP00000005641		
AmPLC-δ3A	Astyanax mexicanus (blind cave fish)	ENSAMXP00000018422		
AmPLC-δ3B		ENSAMXP0000006453		
TrPLC-δ3A	Takifugu rubripes (fugu)	ENSTRUP00000042343		
TrPLC-δ3B		ENSTRUP0000008073		
OIPLC-δ3A	Oryzias latipes (medaka)	ENSORLP0000003606		
OIPLC-δ3B	TIONA	ENSORLP00000023208		
XmPLC-δ3A	Xiphophorus maculatus (platyfish)	ENSXMAP0000008440		
XmPLC-δ3B	N	ENSXMAP00000014440		
GaPLC-83A	Gasterosteus aculeatus (stickleback)	ENSGACP00000012434		
GaPLC-83B		ENSGACP00000020164		
TnPLC-δ3A	Tetraodon nigroviridis (tetraodon)	ENSTNIP00000018000		
TnPLC-63B		ENSTNIP00000013383		
OnPLC-δ3A	Oreochromis niloticus (tilapia)	ENSONIP00000001092		
OnPLC-δ3B		ENSONIP00000010144		
DrPLC-δA	Danio rerio (zebrafish)	ENSDARP00000069390		
DrPLC-δB		ENSDARP00000074236		
LcPLC-63	Latimeria chalumnae (coelacanth)	ENSLACP0000003938		
XtPLC-δ3	Xenopus tropicalis (western clawed frog)		XP_002935564	
AcPLC-63	Anolis carolinensis (green anole)		XP_008111435.1	
GgPLC-63	Gallus gallus (chicken)	11/	XP_425839.4	
OoPLC-83	Orcinus orca (killer whale)		XP_004286005.1	
HsPLC-δ3	Homo sapiens (human)	ENSP00000313731		
HsPLC-δ1		ENSP00000335600		
HsPLC-64		ENSP00000251959		
HsPLC-β1		ENSP00000367908		
HsPLC-y1		ENSP00000362368		
HsPLC-E1		ENSP00000260766		
HsPLC-ζ1		ENSP00000266505		
HsPLC-ŋ1		ENSP00000345988		
ScPLC	Saccharomyces cerevisiae (yeast)		NP_015055.1	



2.2. mRNA isolation and cDNA synthesis

To confirm the expression pattern of the *PoPLC-* δ *3* genes in various tissues including brain, eye, gill, heart, gullet, liver, spleen, pyloric ceca, stomach, intestine, kidney, muscle from healthy olive flounder, total RNA was isolated using TRIzol® (Invitrogen, USA) in accordance with the manufacturer's instructions. Purified RNA was quantified by optical density at 260 nm with a UV spectrophotometer (Amersham Biosciences, NJ). Two micrograms of total RNA were reverse-transcribed with an oligo (dT)₁₈ and random hexamer primers and SuperscriptTM III reverse transcriptase (Invitrogen, USA), as per the manufacturer's instruction. Reverse transcription was carried out at 42 °C for 60 min.

2.3. Gene expression analysis by RT-PCR and quantitative real-time PCR

In order to analyze the tissue-specific expression of PoPLC- δ 3s mRNA, RT-PCR was conducted with the gene-specific primers. The *18s-rRNA* (EF126037) and *β-actin* (AU090737) genes of *P. olivaceus* were utilized as internal controls. The primers Po18s-rRNA-For and Po18s-rRNA-Rev were used to amplify the olive flounder *18s-rRNA* gene (Ahn *et al.*, 2013) and Poβ-actin-For and Poβ-actin-For for olive flounder *β-actin* gene (Ahn *et al.*, 2008). All PCRs cycles were performed as follows: 94 °C for 4 min, 30cyles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 20s, and a final 7 min elongation at 72 °C. The amplified PCR



products were separated on 1.2 % agarose/TAE gels containing ethidium bromide and visualized with a Gel Doc image analysis system (Bio-Rad, USA). The resultant products were purified via agarose gel extraction (QIAquick® Gel Extraction kit) and sequenced (COSMO Co. Ltd., DNA Sequencing Service, Seoul, Korea). Quantitative real-time PCR for the tissue-specific expression analysis of PoPLC- δ 3A and - δ 3B with the gene-specific primers (Table 2) was conducted via a LightCycler® 480 II using SYBR Green (Roche, Switzerland). Total RNA from various tissues was prepared as described above. The SYBR Green RT-PCR assay was carried out as described previously (Guan *et al.*, 2007). To calculate Δ C_t, mean C_t values of target genes in each sample was normalized to the housekeeping gene values (*18s-rRNA*), and relative expression values were determined by the 2^{- $\Delta\Delta$ Ct} method by comparing with detected expressions in the muscle (*PoPLC-\delta3B*) (Livak and Schmittgen, 2001).

2.4. mRNA expression profile after stimulation (LPS or Con A/PMA)

In order to examine the immune responses and expression regulation of PoPLC- δ 3A and PoPLC- δ 3B by quantitative real-time PCR, lipopolysaccharide (LPS) and concanavalin A/phorbol myristate acetate (Con A/PMA) were used to induce inflammatory responses. Gene expression of PoPLC- δ 3A and PoPLC- δ 3B in LPS (50 ug/mL) or Con A (50 ug/mL)/PMA (0.35 ug/mL)-stimulated spleen and kidney, which are known to be fish immune-tissues. LPS or Con A/PMA was injected intraperitoneally at a concentration of 0.02 mg/g body mass. The



expression of PoPLC- δ 3A and PoPLC- δ 3B and the immune responses *in vivo* were assessed in three fish (average mass: 100 g) that had been stimulated with LPS or Con A/PMA at 0, 1, 3, 6 and 24 h. Total RNA isolation, reverse transcription, PCR reaction, direct DNA sequencing and the quantitative PCR were performed as described above. *18s-rRNA* was utilized as an internal control for the housekeeping gene, and the relative fold change in gene expression as compared to the controls was determined by the $2^{-\Delta\Delta Ct}$ method as described previously (Giulietti *et al.*, 2001; Livak and Schmittgen, 2001).

Table 2

Oligonucleotide primers used for the RT-PCR and quantitative real-time PCR to analyze mRNA expression of olive flounder phospholipase C $-\delta$ 3A and $-\delta$ 3B.

Primer name	5'-3' sequence	Information
Poβ-actin-F	GACATGGAGAAGATCTGGCA	Primers for RT-PCR
Poβ-actin-R	ATCTCCTGCTCGAAGTCCAG	and real-time PCR
Po18s-rRNA-F	CACACGCTGATCCAGTCAGT	
Po18s-rRNA-R	CTTACTGGGAATTCCTCGT	
PoIL-1β-RT2-F2	GGTGCCAGCCAGAACATCATCCC	
PoIL-1β-RT2-R2	CAAAGTCTTTCCAGCAGACAGTGGTG	
PoPLC-δ3A-RT-F	GGCCCTTAGTGAAAACCCTCTG	
PoPLC-δ3A-RT-R	TCATTGGGCGGTTTTTCAGATGTAT	
PoPLC-δ3B-RT-F	CATCGGAGGTCTGGACCCTC	
PoPLC-δ3B-RT-R	GAGGGGTGTTTGCCTTCGG	



2.5. Expression and purification of recombinant proteins

The open reading frames (ORFs) of PoPLC-δ3A and PoPLC-δ3B which are amplified by PCR from P. olivaceus were inserted into pCold TF vector (Takara, Japan) for their expression in Escherichia coli BL21 (DE3). The PoPLC-83A and PoPLC-83B gene-specific primers allowing for the cloning of the amplified DNA in a predicted orientation into pCold expression vector are listed in Table 3. The amplified product was digested with *Eco*RI and *Xba*I, and inserted between the corresponding restriction sites of pCold-Self vector (Kim et al., 2004). The resulting expression vector was denoted as pCold-PoPLC-83A and PoPLC-83B, and the inserted DNA sequence was confirmed by DNA sequencing with the pCold-TF-For1 and pCold-TF-Rev (Table 3). To overexpress the polyhistidine (6xHis)-fusion and trigger factor (TF)-fused proteins (PoPLC-83A and PoPLCδ3B), transformed cells were grown in Luria Bertani medium containing100 µg/mL ampicillin at 37 °C. At a cell density of 0.6 (A₆₀₀), recombinant protein expression was induced by adding isopropyl-1-β-thiogalactoside (IPTG) at a final concentration of 1 mM. Cells were harvested after 5 h incubation at 15 °C and were then directly analyzed by SDS-PAGE. Collected cells were resuspended in ice-cold 1× homogenizing buffer (20 mM Tris/pH 7.9, 0.5 M NaCl) containing 200 μ g/mL lysozyme, and were incubated at 30 °C for 15 min, followed by centrifugation at 20,000 ×g at 4 °C for 20 min. The supernatant fraction was applied to the His-bind column (Novagen, USA). After washing the column twice with 1× wash buffer (20 mM Tris/pH 7.9, 60 mM imidazole, 0.5 M NaCl),



recombinantPoPLC- δ 3A and PoPLC- δ 3B were eluted in ten fractions with 1× elution buffer (20mMTris/pH 7.9, 1 M imidazole,0.5 M NaCl). The purified fractions were dialyzed and analyzed by SDS–PAGE and PLC activity assay. Protein concentration was measured using Bio-Rad protein assay reagent.

Table 3

Oligonucleotide primers used for the construction of expression vector of olive flounder phospholipase C $-\delta 3A$ and $-\delta 3B$.

Primer name	5'-3' sequence	Information
pCold-TF-F1	CCACTTTCAACGAGCTGATG	Sequencing primers
pCold-TF-R	GGCAGGGATCTTAGATTCT	
EcoRI/PoPLC-D3A-F	CG <u>GAATTC</u> ATGTTGGGCAGCGGCAAGTCAC	Primers for RT-PCR
XbaI/PoPLC-D3A-R	GC <u>TCTAGA</u> TCATGCCATGCTTTTGGCAATGG	and real-time PCR
EcoRI/PoPLC-δ3B-F	GAATTCATGTTGGGCCACCGAAAGAAAGCG	3
XbaI/PoPLC-D3B-R	GC <u>TCTAGA</u> TTAGGGTCCCTGGGCGGATGACTTG	0
1		



2.6. SDS-PAGE and Western blotting

Gel electrophoresis of protein was carried out by the manner of Laemmli (1970). The protein was denatured by boiling for 5 min in a buffer containing 60 mM Tris/pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 14.4 mM 2-mercaptoethanol and 0.1% (w/v) bromophenolblue, and finally separated by 10% SDS-PAGE (Bio-Rad, USA). Prestained molecular weight markers (Thermo, USA) were run as standards on each gel. The SDS-PAGE-separated proteins were electrotransferred to a nitrocellulose membrane (Schleicher & Schuell Co., USA)using a Hoefer transblotting system (Pharmacia Co., USA) according to the method as described by Towbin and Cordon (1984). The membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in TTBS (200 mM Tris/pH 7.0, 1.37 M NaCl, 1% (v/v) Tween-20) for 30 min at room temperature after transferring target protein to the membrane. The membrane was then incubated with mixed polyclonal anti-Histag antibody (Santa Cruz Biotechnology, USA) overnight at 4 °C, rinsed and washed as before, followed by incubation with alkaline phosphatase (AP)conjugated goat anti-rabbit IgG antibody (1:1000 dilution, Kirkegaard Perry Laboratories Co., USA) for 90 min at room temperature. The target proteins were visualized using an AP conjugation kit (Bio-Rad, USA) after the membrane was washed and rinsed.

2.7. Assay for recombinant PoPLC- δ 3A and PoPLC- δ 3B activity



PLC catalytic activity was analyzed by the method of Cifuentes et al. (1993). using $[{}^{3}H]$ phosphatidylinositol (PI) or $[{}^{3}H]$ PtdIns-4,5-P₂ (PIP₂) as the substrate. Briefly, the PIP₂-hydrolyzing activity was measured with mixed phospholipid micelles containing 40 μ M phosphatidylethanolamine (PE), 5 μ M PIP₂, and 1 µCi/mL PIP₂. The PI hydrolyzing activity was measured with mixed phospholipid micelles containing 100 µM PI and 0.26 µCi/mL PI. The lipids in the chloroform were mixed and dried under a stream of nitrogen gas. The dried phospholipid micelles were suspended in an assay buffer containing 50 mM Hepes/pH 7.0, 0.08% sodium deoxycholate (SDC), 1 mM ethylene glycol-bis (β-5-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 100 mM NaCl, 300 µM CaCl2, and an appropriate amount of recombinant protein in a total volume of 200 µL. The reaction mixture was incubated for 15 min at 30 °C and after incubation, the reaction was terminated by adding 1 mL of chloroform/methanol/HCl (100:100:0.6, v/v/v), followed by 0.3 mL of 5 mM EGTA in 1 N HCl. After vigorous vortexing for 30 s, the samples were centrifuged at 21,000 g for 5 min to separate the organic and aqueous phases. The separated aqueous phase was dissolved in 5 mL of liquid scintillation fluid, and counted in a liquid scintillation analyzer (Packard, USA).

The inhibitory effect of the recombinant proteins against PLC inhibitor, U-73122 (1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1Hpyrrole-2,5-dione), and its inactive analogue for negative control, 1-[6-[((17β)-3methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione (U-73343), were assayed in various concentrations (0, 10, 40, 80, 100, 200 and 400 μ M) of



U-73122 (Biomol) and U-73343 (Biomol) with the reaction mixture as described above.

The effect of sperimine (S3256, Sigma-Aldrich, USA), spermidine (S2626, Sigma-Aldrich, USA) and D-sphingosine (S7049, Sigma-Aldrich, USA) on PoPLC- δ 3B activity was assayed as described above. Reaction mixture containing purified PoPLC- δ 3B enzyme, 50mM Hepes/pH 7.0, 1 mM EGTA, 0.08% SDC, 100 mM NaCl, 300 μ MCaCl2, and various concentrations (0, 25, 50, 100, 200, 500 and 1000 μ M) of polyamine or sphingosine in a total volume of 200 μ L was incubated at 30 °C for 15 min.

2.8. Protein-lipid binding assay

The ability of the proteins to bind different phospholipids was examined using Protein–lipid overlay assay. Commercially available PIP-strip (P-6001; Echelon Biosciences, Salt Lake City, USA) were blocked with 3% non-fat skim milk in TTBS (200 mM Tris/pH 7.0, 1.37 M NaCl, 1% Tween-20) buffer for 1 h. After blocking, the membranes were incubated with purified proteins (0.3 μ g/mL), respectively, in blocking buffer for 14 h at 4 °C. After washing with TTBS buffer, the membranes were incubated with polyclonal anti-His-tag antibody (Santa Cruz Biotechnology, USA) for 2 h at room temperature, rinsed and washed as before, and then incubated with phosphatase-labeled goat anti-rabbit IgG antibody (1:1000 dilution, Kirkegard and Perry Laboratories Co., USA) at room temperature for 90 min. The membranes were washed and expressed proteins



were visualized by AP conjugated Kit (Bio-Rad). To quantify the spots of lipid, scanned images were digitally analyzed using NIH image analysis software (ImageJ version 1.48, National Institutes of Health, USA).

2.9. Statistical analysis

The expression data were tested with SPSS version 21.0 software. Significant differences between samples were analyzed via one-way ANOVA (analysis of variance) followed by Scheffe's post hoc. All Data were considered significantly different when P < 0.05 and presented as mean \pm standard error of the mean (SEM).

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3. Results and discussion

3.1. Identification of duplicated PoPLC-δ3 genes

Our previous studies have demonstrated that PLC- δ isozymes of *P. olivaceus* have two duplicated genes, PLC- δ 1 and PLC- δ 3 respectively and one PLC- δ 4 gene as a result of three rounds of fish-specific genome duplication (Kim *et al.*, 2008). *PoPLC-\delta3A* cDNA (3,816 bp) and *PoPLC-\delta3B* cDNA (2,868 bp) encode 787 amino acids (Fig. 1) and 792 amino acids (Fig. 2) respectively. The cDNA sequences of PoPLC- δ 3s were deposited in GenBank (*PoPLC-\delta3A*, Genbank accession number EU433322.1; *PoPLC-\delta3B*, Genbank accession number EU433323.1).

01 11



5' TGCT CGG GGT CTT TTA GGT GCT GCA CAA ATC CGC GTT TGC AGC AGG ACA CCT GCA CAC CTC AGA CTT CGT GCT CCC CCC AAC GAG GGC GAG TCC CTG AGC ATT AAA GTC 109 GCA ACC TEC AGE TTA TTT TCT CTT GGA AGT TTC AGE AAG TTC TTG ATA ATE TTE GEC AGE GGC AAG TCA CCE GCC ALC AGE CAC AGE GCA AGE GAE CGE GGA AGE GAA GAE GAE CGE CAE CGE AE CGE CAE G GAG AAG ACC ATG GAC CCC ATC AGG AAA CTC GGG CTC TTG GAC AAT GAG GAC ATA CAT TTG ATG ATG ATG GAC GGC TCC AAC ATG GTG AAA ATC CGC TCC CAA AGG TGG CAG 325 RKLGLLD D E K M D P I N Ι Н L M M K G S N M V K T 0 R 0 AG AGC CGA AAC CTG CGG CTG CTG GAG GAT GGA CTC ACC GTG TGG TGT GAA TCC ACC AAG AGC TCC CGC AAA GCT AAA GCC CAG CAG ACA TTC GCA GTG ACA GAG GTG 433 K S R N L R L L E D G L T V W C E S T K S S R K A K A Q Q T F A V T E V GAG TGC GTT CGT GAA GGC TGC CAA TCA GAG GCT CTG CGG GCG CTG TCG GGG TCA GTG CCG GAT AGC CGG TGC TTC ACG GTG GTC TTC AGG GGA GCC AGG AAG AGC CTG 541 E G C 0 \$ R R L V S D Α L. S G с. D D R V V R G ٨ D K L GAC CTG CTG TGC CCC GGT GAG GAC GAA GCC CAG CGC TGG GTG CGA GGG CTA CGC ACT TTG AAG GAG CGG GTG GCC AAC ATG ACT CAG AAG GAA AAA TTG GAC CAT 649 C P G E D E A Q R W V R G L R T L K TAC CTG AGG CGA GCG GAT CAG AAC CAA GAT GGC AAG ATG AGC TAC GAT GAA GTC E R V A N M T Q K E К T D 757 AAC ATT GAC TTG AGT GAG CAC TAT AAG CGG CTG TRGYLRRADONODGKMSYDEVKRLLOMTNTDLS н GCC CGC TCG CTA TTC AAG AGG TGT GAC CGA TCC GGC GAT AGC CGT CTG GAT CAT ATA GAG ATC GAG GAG TTC TGC AGG GAG CTG ATG CGG CCG GAG CTG GAG GAG GAG GAG ATG CGA GAG CTG ATG CGG CCG GAG CTG GAG CTG GAT GCC 865 R S L F K R C D R S G D S R L D H I E I E E F C R E м R n E D GTG TTC AGA CAC TAT TCA AGT AAT GGT TGT GTA CTC TCC ACT GCG GAG CTG CGC GAC TTC CTG GGA GAC CAG GGA GAC GAC GCC TCA TTG AAT CAT GCT CAG AGC CTC 973 0 Δ. E n 1 G D C D ATA CAC ACE TAT CAG ETT AAT GAC TGG GCC CAG AAG AAC CAG TTC ATG ACT CAA AAT GGC TTC ACT ATG TAC ATG CTG TAC ATG GAG AAT GAT GTG TTT AAC CCA GAC 1081 D 0 ¥. 0 M 0 N G D CAC GCC AGA GTC TAT CAA GAC ATG AGC CGA CCC CTG TCA CAC TAC TTC ATC TCC TCG TCG CAC AAC ACC TAC CTT ACC AAG GAC CAG GTC ACC AGC GCC AGC AGC ACA 1189 0 M н GA CCG TAC ATC AGG GET CTG CAT CAG GGC TGT CGC TGT CTG CAG CTG GAT GA GA GAA GAA GAT GAA GAT GAA CCC GTC ATT TAC CAC GGC CAC ACT CTC ACC TCC H W D R A 0 G R V E Ĩ. D C D G K G E Н MAN GTG CCC TTC ANG GAN GTC ATT GAN ACC ATC ANT CAG TAC GCC TTC ANG GCG TCC CCA TAC CCT CTA ATT CTA TCC TTG GAG AAC CAC TGT TCT GTG GAG CAG CAG K E NQYA K A SPYPL Н V T E Т T F Т L S L F N C S v F 0 GTG ATG GCT AAA CAC CTC CGC GCC ATC CTC GGC AGC AAA CTG CTC ACA AAG GCC CTT AGT GAA AAC CCT CTG AAA GAT CTG CCT TCT CCC GAA GAC CTG AAG GGC 1513 V M A K H L R A I L G S K L L T K A L S E N P L K D L P S P E D L K G ATT CTG GTA AAA GGG AAG AAG CAT ATC CCT CAC CTE GGC CAG CTG GGC AGC GGT GCC AGC TTC TCG TCC AGC GAC CTA GAC GAA CTA GCC ATC AGC AAT AAG GAC 1621 <u>R I L V K G K K</u> H I P H L G O L G S S A S F S S S S E D E L A I S N K D ACA CCC AAG AAG GAT CCC ACA AAG GTG TAT TCC AAA CTG AGC CCC GAG CTT TCC GAG CTG GTT GTG TAC TGC AGG AGT GTC TCC TTC TGT GGC TTT GAA AAT ACA TCT 1729 T P K K D P T K V Y <u>S K L S P E L S E L V V Y C R S V S F C G F E N T S</u> GAA AAA CCG CCC AAT GAA ATG TCC TCC TTC TCT GAA AGT GAA GCC CTC AGG CTC ATC AAA GAC TCG GGA AAG CCA TTT GTA AGA CAC AAC AGC AGG CAG CTG AGC CGG 1837 R H N K P P N E M S S F S E S E A L R L I K D S G K SRQL ATC TAC CCC TCT GGC CAG CGC CTC CAA TCA TCC AAC TAT GAT CCC CAG GAA ATG TGG AAC GGT GGA TGC CAG ATG GTG GCT CTG AAC TCC CAA ACA CCA GGG GAG CAG 1945 S G Q R L Q S S N Y D P Q E M W N G G C Q M V A L 0 T P G E O ATG GÁC CTG AÃC CĂG GGT CỘC TỪ CTT CÓ TÁC GỐT CÓC TGT GÁA TĂC ÁCC CTC AĂA CĆT AČC TỪ CTG TỐC AĞC CTC AĞC TỪ AĂC CỦA GÁG AĂC AČA GỔG M D L N Q G R F L P N G R C G Y T L K P S F L C S P T S N F N P E N T G GGA GGC CCT GGT CAC ATC CÓC AAC CTG ACA ATA CGA ATA ATA TCA GCG CAG CTG CCA AAA ATC AAC ACA GAA AAG GCG AGC TCC ATC CTG GAC CCA CAA GTG 2161 G G P G H I P T Q L T I R I I S A Q Q L P K I N T E K A S S I V D P Q V TGG GTG GAA ATT CAT GGG GTG GAT ATC GAT AAA GCA AGA GAC AAG AGG CAA GGC ATT GAC AAT GGT TTT AAT CCA CGC TGG GAC TGC ACA CTG AGC TTC CAG CTG 2269 <u>V E I H G V D I D K A R D K T Q R I D N N G F N P R W D C T L S F Q L</u> CAG GTC CCT GAT CTG GCC CTG GTG CGG TTT GTA GTG GAG GAC CAT GAC CAC ACT GCC AAA AAT GAC TTT GTG GGA CAA TTC ACT TTA CCT TTC ACA AGC CTT CGC ACA 2377 CAG GI CLI GAI CLI GUE GIG GIG GI CLI GUE TI GIE CAG GET CLI AGI CLI DE LI DI HI TI A KIN DI FIVIGI GIE CAG GET TI LI PIE TI SIL RITI GGI TATI CGA CAT GITI CAC TTA TTA AAG GCA GAT GGT ICT AGI CLI TAGI CLI TGCI CCC CCC ACC CLI CLI AGI CLI GIE CAC GEC AAA GEG AGT CCC AAA ACA GEG 2485 <u>G Y R H V H L L K A D G S S L S P A T L F V H V K V S R K G V P I K T V</u> TCC GAG CGT ATA GCC ATT GCC ATA AGC ATG GCA TGA ATG ATG TAC ATC TGA ATA TGA TGG AAA GAT GCA TTT TCT GAC GAA AAA GAG CTT TGG AAA CGG AGG TCT GAA 2593 S E R I A I A K S M A * AGA AAG GAG GAG AGG ACT AAT GCT GCT GCT GCT GCT GCC AGC CGG TCT GCC AGT CCA TAC CTC GAG TGG TCC AAT ATA AAT CGA GCC ACT TTC CAA CAC AGG 2701 2917 3025 3133 3241 3349 3457 3565 3673 3781

Fig. 1. Nucleotide and deduced amino acid sequence of *P. olivaceus* phospholipase C- δ 3A cDNA (*PoPLC-\delta3A*, GenBank accession number: EU433322.1). The *open box* in the amino acid sequence indicates the putative PH domain and the EF hand domain is indicated by a dark shaded box. The X-domain and Y-domain is underlined in the order named and a *dotted underline* indicates the C2 domain.



TC CTG GTT GAA ACC AAT CAG CAG TGG GAA GGT CCG GCC CGA CTC CTG TTC TCC TCC TCC GGT ATA AAA AGC ATG CGG AGC ACC GTG GAG CTC CAC GGA GAA GAG CGC 215 323 n GTG AAG CGG ATG TTG CAG GGC TCC AGT ATG GTG AAG GTT CGC TCA CCT CGC TGG CAG AAG CGA ACT CTA AAG CTG CTG GAA GAT GGA GTC ACT GTG TGG TGT CAG 431 M L Q G S S M V K V R S P R W Q K R R T L K L L E D G Q TCC CAN ANA ACC TCC TCC CCT GCC ANG GAG CAN CAN TCC TTC TCT GTC TTE GAG GTG GAG TGC GTT CCG GAG GGT TGC CAG TCA GAG ATG TTE CGG CAG TTG GGT GGG 530 Q Q 0 TCA GTA CCT GAG GGC CGG TGC TTG ACG GTG GTC TTC AAA GGG CCC AGA AAG AGC CTG GAT CTC CTC TGC CAG AGC CAG GAG GAG GCT CAA CAC TGG GCC CGG 647 GGC ATC К AGA GTG GAG AAC ATG AGC CAG AAG GAG AAA CTT 755 K L Q G R V E N M S Q K E GAA GAG GTC CAG ACT CTG CTG CAG ATG ATC AAT ATT GAT K L D HWIHAYLS RADONHDDKMS CTG AGC GAT CAA TAT GCC CGC AGC 863 V F F V O T L L O M T N T D L S D O Y A R S L F O K C D R S A D G R L D G GAG TTG TTA CGG AGA CCG GAG CTG GAC ACC GTG TTT ATC CGC TAC TCT GCA AAT GGC TGT GTA CTT TCC ACT GTG GAC CTG 971 HGELEVECR F R D GAC TTC CTG AAG GAC CAG GGG GAG GAT GCT TCT CTC ATC CAT GCC CAA AGC CTC ATA CTC ACA TAT GAA CTT AAT GAG TGG GCC CAG AAG AAC CAG TTC ATG ACC 1079 D n 0 C. 0 K CCC AAT GCC TTC ACC ATG TAC ATG CTG TCT AAG GAG AAC TGT GCC TTT AAC CCT GAA CAT GCG AGG GTT TAC CAA GAC ATG AAA CAC CCA CTG GCA CAC TAC TTC ATC Н Q M K Н н TCC TCC TCC CAC AAC ACA TAC CTC ACC AAG GAC CAG CTG ACT GGG GAC AGC AGC AGC AGC GAG CCA TAC ATA CGA GCT CTG AAT TAC GGT TGT CGC TGT GTG GAG CTG GAC 1295 DQ D K 1. G R A R E L TGG GAT GGA GAT ANA GGA GAG CCG GTC ATC TAC CAC GGA CAC ACA CTC ACC TCC ANA ATA CCG TTT GTG GAA GTC ATT GCG GTT ATC ANT GAG TAC GCT TTT ANA 1403 Κ Ē GCA TET CET TAC CET ETG ATC CTG TEC CTG GAG AAC CAC TEC TET GTG GAG CAG CAG ACA GTC ATG GCT CAA CAA CTG CGA TEC ATC CTG GGA GAC AAG CTC CTC ACC 1511 Q Q ANG CCC ATC GGA GGT CTG GAC CTT CAC AGC CTG CCC TCT CCG GAG GAT CTC ANA GGG ANA ATC CTT GTG ANA GGA ANG ANG GAG CAT GTG GAG GGC TCA TCG ANC AT K P I G G L D P H S L P S P E D L K G K I L V K G K K E H V E G S S S T 1619 K P I G G L D P H S L P S P E D L K G K I L V K G K K E H V E G S S S T TCA GAC CTC AGC TCC TCA GAT GAG GAG AGC AGC AGA GAC GAA GGC AAA CAC CCC TCC AAA AAA GGG GAC CCT AAG CCA AGT GTG TCT AAG CTG AGT CCC GAG CTG TCA 1729 S D L S S S D E E T S R T E G K H P S K K G D P K P S V <u>S K L S P E L S</u> GAG CTG GTT GTA TAC ACC CAC AGT GTT TCC TTT AAA AGC THT GAA CAC GCT GCC AGA AGC CCA GCG AGT GAA CTG TCC TCC TCC TCC TGC GAG GGT CTC AGA CAC 1835 ATT AND GAD TEA GED ATT TITE GTA GET CAC AND AND CAC CAG CTC AGE AGE AGE AGE AGE TAC CCC TEA GET CAG CGC CTC CAG TCC AAC TAC AAC CCT CAG GAG ATE 1943 I K D S G M Y F V R H N S H Q L S R I Y P S G Q R L Q S S N Y N P Q E M TGG AAT GGA GGC TGT CAG ATT GTC CCT CTG AAC TTC CAG ACC CCT GGT GAG CAG ATG GAC CTA AAC CAA GGG AGG TTC CTG CCA AAC GGT CAG TGT GGG TAC ATC CTG 2051 <u>W N G G C Q I V A L N F Q T P G E Q M D L N Q G R F L Q N G Q C G Y I L</u> AAA CCC CCC TTC ATG TGC CAG GCC GGC ACC ACC TTC AAC CCA GAG AAT GTT GGT GGA GGT CCT GGC CAG AGA CCT GTG CTG GTC ACT GTC CGG ATT ATT TCA GCT CAG K P P F N C Q P G T T F N P E N V G G G P G H R P V L F T V R I I S A Q CAG CTT CCC AAG CCA GAG TGG GAC AAG CCC AGT TG TG GG CCC CCT CAT GTG GTG GAG ATT CAT GGT OCT ACC ATC GAC AAC AAT AAG AAG AAG ACT CAT CAT 2267 <u>Q L P K P E V D K P S S I V D P H V V F I H G A T I D N N K K K T H H</u> GTT GAC AAT AAT GGC TTT AAC CCG CGG TGG GAC TGT ACA TTT AAC TTC ACC AT GCC CTT GAC TTG GCC CTT GAT GTC GAT GAC CAC GAC TAC ACC 2375 V D N N G F N P R W D C T F N F T I H A P D L A L V R F M V E D H D Y T TCC AGC AAT GAC TTC CTG GGG CAA TAC ACC CTG CCT TTC ACC AGT CTC CCC ACA GGC TAT CGC CAC GTC CGA CTG CTC AAG GTG GAC GGC TCC ACC CTT TCA CCC GCA 2483 <u>S S N D F L G Q Y T L P F T S L R T G Y R H V R L L K V D G S T L S P A</u> TCG CTC TTE GTC CAC GTC AGG CCC TGT GAG AGC AGT CCC CAC AAA TCG TCT GCA AAA TCA CCG GCC AGG TCA CCG ACC AAG TCA TCC GCC CAG GGA CCC TAA 2591 <u>S L F V H V T V</u> G P C E S S P H K S S A K S P A R S P T K S S A Q G P * TCC CCT GCC TCC GTC CAG GCA AGG GCA CTA AGG GAG GAG ATG AAA TCT TCC TGG TTC CAG GCG ATA AAA GAT TTC ATC AAA CTG GTT ATA AAG TAT GTA GCA TGT GAT 2699 2807

Fig. 2. Nucleotide and deduced amino acid sequence of *P. olivaceus* phospholipase C- δ 3B cDNA (*PoPLC-\delta3B*, GenBank accession number: EU433323.1). The *open box* in the amino acid sequence indicates the putative PH domain and the EF hand domain is indicated by a dark shaded box. The X-domain and Y-domain is underlined in the order named and a *dotted underline* indicates the C2 domain.



As shown Fig. 3, two paralogous of PoPLC- δ 3 genes have equally conserved regulatory domains containing PH, EF-hand, catalytic X and Y, and C2 domains PLC- δ 3 of *P. olivaceus* by comparing deduced amino acid sequences of PLC- δ of other typical species including coelacanth, western clawed frog, green anole, chicken, killer whale and human. As a consequence of comparison, amino acid sequence identity of *P. olivaceus* between PLC- δ 3A and PLC- δ 3B is 67.8%. The identical percentage of paralogous PLC- δ 3A genes ranged from 75.5% to 84.7%. and paralogous PLC- δ 3B genes showed lower identity ranged from 65% to 79.8% (Table 4).

The phylogenetic tree showed that phospholipase C clustered in four major groups such as two paralogous proteins (PLC- δ 3A and PLC- δ 3B), PLC- δ 3 of other species and human PLC isoforms (Fig. 4). The result also supports that PoPLC- δ 3A and PoPLC- δ 3B are grouped into duplicated form in teleostei, which is considered from a evolutional tendency. These results demonstrated the previous study (Kim *et al.*, 2008).



PoPLC- 8 3A	AIRI	LELLDNEDIHLAMKGSNWWAIRSQRWQASRNURULEDGLTWWCESTI	
PoPLC-δ 3B	3MLGHRKKAAPAAKNGAGTSSLKAIDPLR	LEVSEDEDVKRULQESSMVWVSPRWQWRWTLKULEDEVTVWCQSQ	KTSSRAKEQQSTSVLEVEOVREGCQSIMURQLGGSVREGROLTVVTKGPRKSLDDLCQSQDFAQH2
LcPLC- 8 3	MGSSHEKVQKKCCCPSTV1GLLTVFGPN1NLVFF	HCHAENED I RETURINGSKLING INSRRWOMEN I VALOD DEMSEMFETKI	RKSKKAPSMHTFSUNDTRVVWEGHQSEGLLKYGGTYNEKQOFTVVFKGQMKNLDLVAPTEEERKRC
XtPLC-δ3	MPGVFGTVCGGRKKKKKYKPESEVLEQEVQPVSSQGLKTFK	MCLSGDDDVNAMLCCSNLWATRPG-WHAARLYRDESDONTVWYESO	—AKKARSKQIISALHIBSVRECIUSECIQKUCRRPETCOFTIADUCRKNIDDAASSEKEALNU —FKKAYSOHVESILHIESVRECUSECIRKUCCCPECIQOFTIVBRCKRKNIDDAAISVNEAQQU
AcPLC-δ3 GgPLC-δ3	MICGKWKKSRSESHKRGRHRHHQQPPPPQIEPPVPAQITGGVAAAAAKLSGHRAFKI	LOD VOTEL CCUEDE	- FAMILSUNDSTLINESVARGE SEGUNATOGCHEDUCTI V SKONNALDJANI SVNESVC
GgPLC= δ 3 OoPLC= δ 3	MLCGRWRSRHRRREEPPVPAQVAAPVALPSPPAPQDGGNKRPGLRALK	LAUP NOTFLOSLEPS	
HsPLC- 6 3	MLCGRWR———————————————————————————————————	NGL I EDBUY RASEROSREGALASK LINNABAL LALADDELSY IF GRA IVELTENETVENUEVENE VELOSITATION I VELOSITATIONE	LCVPAVSVAHITAVHRGHISEGLIRVGRATEROFORTAR GORGENLAR GORGENLAR GARDOVILSU — IPRAPSOHTERVGHEAVREGIOSEGLIRRGGAFEPAROLITAR GORGENLAR TADEADRU — IPRAPSOHTERVGHEAVREGIOSEGLIRRGGAFAPAROLITAR GRXNI DIAA PTABEADRU
list LC 03	RECORDER ROARTEEST VARGENAGENAL SET IT SDOOTRA OLAALA	NOL TEDESTICKOR CONCERNING AND THE RESIDENT OF A	
			PH domain
PoPLC- 8 3A	WREERTIKERVANATOKERI DEWERGVERRADONODGKWSYDDVKRLEO/UNEDLSEE	YARST BURGESREDHT BUBBEREIMRRED DAVERHVSSNG	
PoPLC- 8 3B	ARCIERKLOGRVENMSQRERLEHWTHAYLSRADONHDDKMSVEEVOTULOMINTDLSDO	YARSLFOXCORSADGRUDHGELEVECRELLRRPELDTVFIRYSANG	VLSTVDURD TUKDOGED-ASU IHAOSU IL TYELNEW OKNOFVTPNOFTMYM SKENCAFNPETA
LcPLC- 8 3	IRGINILMEKAEAMSQLEKLDHWIHDYLHRADINKONKMSPKEIRDLLKMINIEMDD	YAYNLEKKCOKSNTNKLEDHEIVEFCTLUMRRPEVEBIEKHYSGED	LYLSDEBLIHFUKEQGEA-DTLENARRIUQKYELNEKANOHNFMLDGEMMYLLSREGDIFNQDHD
XtPLC-δ3	VCCLQKLMKRAEAMSQREKLVHWIWDYLRQADRDCDEKMTFQEVKDMLKMINIDLNDI	YAYNLFKACDRSNTNTLEDHETEEFCTKUMHRPEVEETFHHYSGED	RVLSAEELQEFLQAQGED-ATLERAKELLQKYELNETAKOHDLAMMECEMMYLLSDDCDIFNQKER
AcPLC- 8 3	VRGLKKLMARGEAMSQREKLEHWIHDMUHQADKNKONKMSFKEIKNMLRMINIDANDI	YAYRLEKE CORSNNOR DE YELEEFCTRUMERPELKELFHRYSGED	DVLSAEBLRETUHLOGBE-ATIKRATOIHQTYBLNBRARCHNLAMLDGRMAVILLSSAGDVLNQNHT
GgPLC-δ3	VRGLRTLRGRLRGMSQRERLDHWIHGMLQRADRDKDNRMCFQEVQIMLRMANIHMDN/	YTHQLFEECDHSGDGRLDDRELEDFCRRLLRRPELBELFGRYSGED	DVLSAEELRDFLODQGEE-GTLQQACAIIICAHELNEKARQQDLATLDGFTMVLLSPAGDILAQEIT
OoPLC-δ3	VRGLAKLRARLDAMSQRERLDHWIHFYLHRADSNODSKMSFKEIKNULRMVNVD/IND/	YAYGLFKECDRSNNEWLEGPETEEFLRRLLKRPELDEIFHRYSGED	RVLSTPELLETUEDQGEDDATLAHAQQLUHTYELNETAKCHELATLDGFMMYLLSPECAALAPATT
HsPLC- 8 3	VRGLTKURARLDAMSQRERLDHWIHSYLHRADSNODSKMSFREIKSLLRMVNVDMND	YAYLIIFKECDHSNNDRLEGAEITEEELRRULKRPELEEIFHQYSGED	RVLSAPELLERDEDQGEEGATLARAQQLUQTYELNETANQHELATLDGRMAVLLSPEGAALDNTHT
	I	F hand	
122			
PoPLC-δ 3A	RVVQDMSRPLSHYFISSSHNTYLTKDOVTSASSTEPYIRALHOGCRCVELDC0DGDK	EPVIYHGHTLTSKVPFKEVIETINQYAFKASPYPLILSLENHCSVE	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
PoPLC-δ3B	8 RVVQDMKHPLAHYFISSSHNTYLTKDQLTGDSSTEPYIRALNYGCRCVELDCwDGDK	EPVIYHGHTLTSKIPPVEVIAVINEVAFKASPYPLILSLENHCSVE	XXTVMAQQURSTLGDKULTKPTGGLDFHSLPSPEDLKGKTLVKGKKEHVEGSSSTSDLSSSDEE
LcPLC-δ3 XtPLC-δ3	CAYODAT RED SHYFTSSSENTYLTEN AFGCESST DATYRALAK GCRCVELDCANGVLC	EPHTYHGHALTSKILFROVI ATTINDCAROVSPYPVILSLENHCSVD	NUMERALIZATION CONTRACTOR CONTRAC
AcPLC- 0 3 AcPLC- 0 3	A YOUTOPICATE ISSSENT YOUNAL VEPSTERY I RECOVED OF EPST	EPVITYBENTLISKILFAUWTTTTRUYAFANSPTTVILSLENHOGLE	AVADANHI KETUGULUVI KPYPGADI AKUPSPDALAGKE U AGAN UNIKAS
GgPLC- 8 3	CUCONSOPLEMITISSINTTI TONOLOUSSTEATING ALOCCOCUTI DESIGN	EPHTHONILISKILPADVIISINDIAPARISSIPPTILSLENICAPE	VALUARIA MARTI COMULTARE LOOD TRACES SERVICES IL LAGRAT DE DORACLORCAT SAF DED
OoPLC-δ3	OPPOINTED ANY ISSENTED TO SOUCH STRATING AND CREDCING TO OPPOINT PLANY ISSENTED TO SOUCH STRATING AND CREDCING TO	EPHTOROTTUTSKTLENDVTOAVEDPARTISPVDVTI SLENDCOTE	VAAVAPHENTI ODUVIOU I DSONEEEL PSPEOLYOPVI VYOKP-II PAAPNE-DCPU SOPEED
HsPLC- 8 3	OPODINOPLAHVETSSSHITTU TOSOTOPPSSTEAVURAFAGCCRCVELDC/EPCC	EPVLYHCHTLTSKILERDWOAVRDHAFTLSPYPVILSLENHCOD	XXAAMARHUHTTILGDUUVTQLLDSQNPEELPSPEQIKORVUVKGKR-UPAARNE-DGRILSDREBD XXAAMARHUCTILGDUUVTQALDSPNPEELPSPEQIKORVUVKGKR-UPAARSE-DGRALSDREBE
1131 110 00			
		X domain	
PoPLC- 8 3A	ELAISNKDTPKK-DPTKVYSKLSPELSELVVYCRSVSFCGFENTSEKPPN-D	SSFSESBALRLIKDSCKPFVRHNSRQLSRIVPSCORLCSSNVDPOE	ATING COMMAINE AT THE BOMDING GRALPNER CENTRARS ALCS AT SMARLEN TEGEPCHIPTOLIT ATING COMMAINE AT THE BOMDING GRAL ON COMMISSION OF COMMISSION OF COMMISSION OF COMMISSION OF COMMISSION OF COM
PoPLC- 8 3B	B TSRTEGKHPSKKGDPKPSVSKLSPBLSELVVYTHSVSFKSFEHAARSPAT-D	SSFSESEALRHIKDSCMYFVRHVSHQLSRIYPSCORLQSSNYNPQE	WINCCCQTVALNEQTPGEOMDLINGGRELONGOCGYTLIKEPEMCOPGTTENPENVGGGPGHRPVLFT
LcPLC- 8 3	EEGYDNQKKTQWNSKLISKDFSDVIVYCKSVHFHGFEHAQNKQAVYD	SSFADRKARKLMRESC <mark>SSFVRINCRQLSRIIYP</mark> SGLRTHSSNYNPQEI	WNVGCQIVALNEQTPGYEMDLNQGKERONKNCGYILKEARMENEDAAEDPECIGQGGNHQMRTLI
XtPLC-δ3	EEGKPRMDTAK ELSDLVVVCQTVEYQGQPGNVCE	SSFSEDXVRRL1KDSGINLVRYNARQLSR11YPDGLRANSSNYCPQE	WNAGCQLVALNFQTPGYTMDLNRGRFQDNGLCGYWLKPEFLRKEDSYFNPDSPSKEPKLUF
AcPLC- 5 3	MQDEDDDRSISSLQEIKPLQAKDAIQISRDLSDVVVYCQAVPFQSLSRALHNQQSND	SSFSERKARKLIKESONOFVRYNTRHLSRUYPOGLKVNSSNYNPOE	MWAGCQLVALNFQTPGAEMDLNDGRFSVNGSCGYVLKFAFLENTQSSEDPEAPGRRAGQRPVAUT
GgPLC-δ3	EEEEDTEEEDRLQVAKDASHVAPDLSAMVVYCQATPFPGLAQALQHPRPYD	SSFSDRXARKLIKEACPARMRYTSROLSRWPLGLKMTSSNYNPORI	AWAGCQLVALNEQTPGYBMDLNTGRFLONGCCGWILKEQTLRSPHSGSPRPLVDR
0oPLC- δ 3	NED-DEEEEAGAPEQRRRAKQISPELSALALVCCASRLRTLRP4PAPPQPCQV	SSLSERKAKKLIREAGNSFIIRENACOLIRWYPLGLRMNSANYNPOE	WASSCOLVALNFOTPGYEMDLNAGRFLINGOCGYWLKFAOLROPDTTFDPECPGPPRTTFT
HsPLC-δ3	EEDDDEEEEEVEAAAQRRLAKQISP	SSLSER ANKING REACTION OF THE ACTIVITY OF THE SECOND	AND COLO ALLANDO COLLA CERTIFICACIÓN DE SUCCESSION SUPERIORDA CHIPTO DE MARCEN VALVENCIÓN COLLA CENTRE SUCCESSION DE SUCCESSION DE SUPERIORDA COLLA COLLA DE SUCCESSION D
		Y domain	
PoPLC- 8 3A		- ISOLOVERIAL VERVICE UNIVERVIEW AND STOREST PRESS	MILLY ADDOCT STATE DEFUSION DEVENUE DEVENUE DE LA LAKONA
PoPLC- 8 3B	VELUSACOL PEPEWDEPSSI VDPHVIPTHCATLONNKKKDHVD AVGENDED D	-ENERTHAPDLALVREWEDEDVTSSNDELCOVTLEFTSLATGRO	VELLKVDCSTI SPASLEVEVTVCPCPCSSPHKSSAKSPARSPTKSSAOCP
LcPLC- 5 3	VEW ITACOLPRIVING KINST VIDEL VRVIDVI (GVPK DTCKKODEVI LAVGENPS: KOSPI	RIVTENTOVPELAL TREVVEDYDTTSSNDEVCOPTI PETSLKK/CVP	ITHE SKOCAST SPATLEWITKISOS
XtPLC-δ3	VKWISACOLEKUAKERPASIVDEPVRVETHOVCKKK-EKERKVILANCENEVANEI-	- OLEVOVPELALVREWEDYDKTSSNDEVGOYTLPFLSLKOGYR	WHILSKOCASLSPATIEVOIRIEDL
AcPLC- 8 3	I KV ITAQQL PKLNKEKNSST VDP PVRI ETYGVPVDCCKKQTEVOLANGEN PRINET	LTEOVE IPELALVREVVEDYDTTSANDFVGQFTLPLLSLREGYR	HUHLLSKDGTSLSPATLFVHVRCKKM
GgPLC-δ3	IRVIDAQQLPKUNREKLSSIVDPEVRVETVGVTADCSKQCTAVRSNNGFNPRWEET-	LTFQLQVPELALVRFVVEDHDSTSCNDFVGQFTLPLGSMREGYR	HILLSKOCASLSPATLFVHVTCRCP
OoPLC- 8 3	IQVLTAQQLPKLNAEKPNSIVDPLVRTEIHGVPADCACKETNVVLNNGENPR#GQT-	-LOFOLRAPELVLVRFVVEDVDATSPNDFVGQFTLPLSSUKQGYR	HILLSKOCASLSPATLEVHICTORS
HsPLC- 8 3	IQVLTAQQLPKUNAEKPHSIVDPLVRIEIHGVPADCARQETDYVLNNGENPR#CQT	LQFQLRAPELALVRFVVED <mark>YDATSPNDFVGQFTLPLS</mark> SLKQ <mark>GYRI</mark>	IIHILSKOGASLSPATLFIQIRIQRS
		2 domain	

Fig. 3. Multiple alignment analysis of sequences of PLC- δ 3 proteins in various species. The identical conserved domains are expressed below the amino acid residues, respectively. The GenBank and the Ensembl accession numbers of other species of PLC- δ are given in Table 1.



Table 4

Comparison with sequential identity of amino acids among *P. olivaceus* PLC- δ 3 isoforms and PLC- δ 3 from a variety of species.

	PoPLC-δ3A	PoPLC-δ3B
ΡοΡΔC-δ3Α		0.678
PfPLC-δ3A	0.787	0.631
AmPLC-δ3A	0.765	0.664
TrPLC-δ3A	0.824	0.667
OIPLC-δ3A	0.755	0.605
XmPLC-δ3A	0.797	0.634
GaPLC-83A	0.832	0.65
TnPLC-δ3A	0.847	0.666
OnPLC-δ3A	0.837	0.65
DrPLC-83A	0.766	0.663
PoPLC-83B	0.678	
PfPLC-δ3B	0.668	0.798
AmPLC-δ3B	0.629	0.65
TrPLC-δ3B	0.649	0.767
OIPLC-δ3B	0.649	0.743
XmPLC-δ3B	0.665	0.794
GaPLC-83B	0.652	0.783
TnPLC-δ3B	0.668	0.765
OnPLC-δ3B	0.647	0.783
DrPLC-δ3B	0.635	0.677
LcPLC-δ3	0.508	0.497
XtPLC-δ3	0.501	0.483
AcPLC-83	0.502	0.486
GgPLC-δ3	0.472	0.455
OoPLC-δ3	0.495	0.494
HsPLC-δ3	0.492	0.478

The GenBank and the Ensembl accession numbers of a variety of PLC isoforms are given in Table 1.



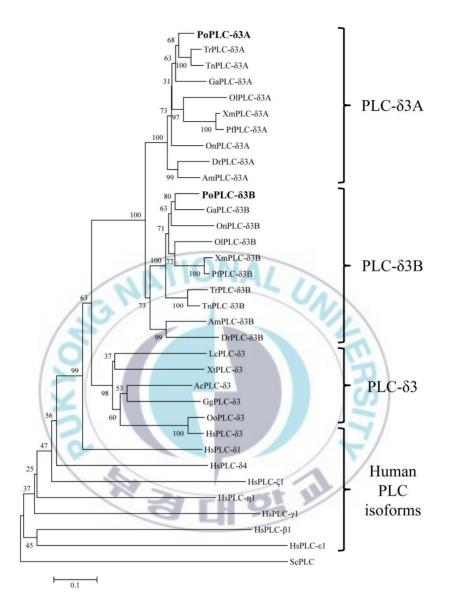


Fig. 4. The phylogenetic relationships of PoPLC- δ isoforms with human PLC family based on deduced amino sequences. A phylogenetic tree of the aligned sequences was constructed using neighbor-joining algorithm within MEGA (version 4.0). The GenBank and the Ensembl accession numbers of other species of PLC- δ are given in Table 1.

3.2. Tissue-specific distribution and immune interrelation of the PoPLC- δ 3A and PoPLC- δ 3B mRNAs established by RT-PCR and real-time PCR

We determined the distribution of *P. olivaceus* PoPLC- δ 3 isoforms mRNA in a variety of tissues including brain, eye, gill, heart, gullet, liver, spleen, pyloric ceca, stomach, intestine, kidney and muscle tissues by RT-PCR and quantitative real-time PCR with specific primer sets described in Table 2. The expression patterns of PoPLC- δ 3A and PoPLC- δ 3B mRNA were patently different as shown Fig. 5. The mRNA transcripts of PoPLC- δ 3A and PoPLC- δ 3B were widely present in all tissues and mostly distributed in myoneural tissues obtained from olive flounder. The mRNA transcripts of PoPLC- δ 3A were strongly expressed in liver followed by brain, eye, muscle, gill, gullet and stomach. While the mRNA transcripts of PoPLC- δ 3B were comparatively distributed in various tissues except in kidney and muscle, the level of mRNA expression was generally higher than that of PoPLC- δ 3A in eye followed by spleen, brain and heart (Fig. 5).



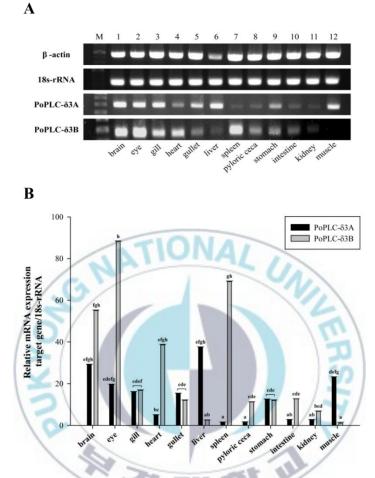


Fig. 5. Tissue-specific distribution of the *PoPLC-\delta3A* and *PoPLC-\delta3B* mRNA. (A) RT-PCR analysis of the *PoPLC-\delta3A* mRNA and *PoPLC-\delta3B* mRNA. (B) Quantitative real-time PCR of *PoPLC-\delta3A* and *PoPLC-\delta3B* mRNA in various tissues. Mean mRNA levels in olive flounder tissues were analyzed by real-time PCR, and $2^{-\Delta\Delta^{Ct}}$ levels were calculated relative to the tissue with the lowest expression (*PoPLC-\delta3B*; muscle) set to 1, and normalized against 18s-rRNA expression. The results are expressed as means \pm SEM (n = 3). Values with different letters indicate statistical difference (P < 0.05).



In order to identify the immune interrelation of *PoPLC-\delta 3A* and *PoPLC-\delta 3B* genes, three healthy olive flounders were stimulated by lipopolysaccharide (LPS) and concanavalin A/phorbol myristate acetate (Con A/PMA) in spleen and kidney as described above materials and methods. In all quantitative real-time PCR, relative gene expression levels were normalized using the olive flounder 18srRNA gene which was used as the internal control. In comparison with 18s-RNA gene which was uniformly expressed, $IL-1\beta$ gene was promptly expressed from 1 h to 6 h after stimulating with LPS and Con A/PMA, as shown in Fig. 6 and Fig. 7. Except for the expression of PoPLC-δ3B mRNA transcript from kidney stimulated by LPS at 6 h (Fig. 6B), the mRNA transcript of PoPLC-83A was more expressed than PoPLC-83B in spleen and kidney under any stimulus. From the results, we predict that PoPLC-83B may be related to early stage of immune response because mRNA transcripts were highly distributed in normal condition, that is non-stimulus (Fig. 5B), while PoPLC-δ3A might be more associated with HOIN late stage of immune response.



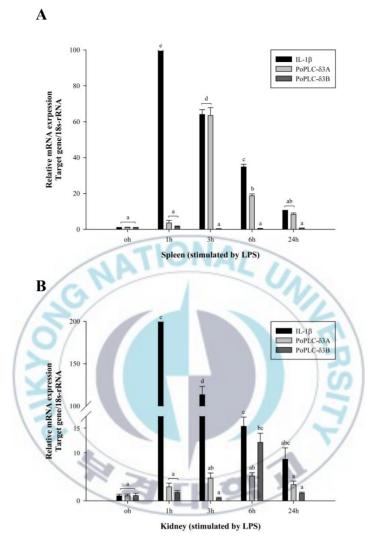


Fig. 6. Identification of immune response by stimulation of LPS. qPCR was performed using *PoPLC-\delta 3A*, *PoPLC-\delta 3B*, *IL-1\beta* and *18s-rRNA* of mRNA from spleen (A) and kidney (B) tissue at 0 to 24 h after LPS injection. The results are expressed as means \pm SEM (n = 3) using $2^{-\Delta\Delta^{Ct}}$ method calculated relative to the time zero (set to 1), and normalized against 18s-rRNA expression. Values with different letters indicate statistical difference (P < 0.05).

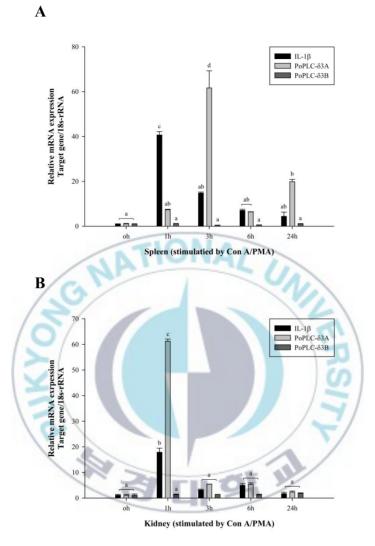


Fig. 7. Identification of immune response by stimulation of Con A/PMA. qPCR was performed using *PoPLC-\delta3A*, *PoPLC-\delta3B*, *IL-1\beta* and *18s-rRNA* of mRNA from spleen (A) and kidney (B) tissue at 0 to 24 h after Con A/PMA injection. The results are expressed as means \pm SEM (n = 3) using $2^{-\Delta\Delta^{Ct}}$ method calculated relative to the time zero (set to 1), and normalized against 18s-rRNA expression. Values with different letters indicate statistical difference (P < 0.05).

3.3. Expression, purification and activity assay of recombinant proteins

We have successfully expressed various recombinant *P. olivaceus* PLC- δ proteins such as PLC- δ 1A, PLC- δ 1B-Lf, PLC- δ 1B-Sf and PLC- δ 4 in our previous studies. Recombinant PoPLC- δ 3A and PoPLC- δ 3B were also expressed in *E. coli* BL21 (DE3) and purified by using Ni²⁺-NTA affinity column to study enzymatic characterization. Each expressed and purified proteins were analyzed by SDS-PAGE and western blotting to verify putative molecular mass based on the amino acid sequence of the recombinant proteins and binding capacity of commercial antibody by his-tag. Both of the recombinant proteins, PoPLC- δ 3A and PoPLC- δ 3B, appeared as a single band of 137 kDa (trigger factor-fused recombinant proteins). Although the recombinant PoPLC- δ 3A and PoPLC- δ 3B revealed enzymatic activity for substrates.



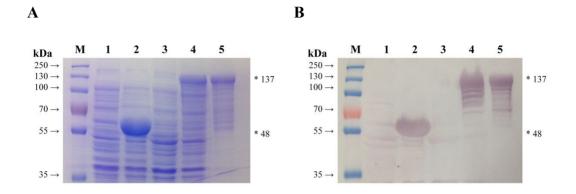


Fig. 8. Expression and purification of recombinant PoPLC- δ 3B from olive flounder (*P. olivaceus*). (A) Protein samples were separated by SDS-PAGE (10%) and were visualized by Coomassie R-250 blue staining. (B) Western blotting analysis using anti-His tag polyclonal antibody. Lane M, standard size marker; lane 1, pCold-self non-induced; lane 2, pCold-self induced with 1mM IPTG for 5 h at 15 °C; lane 3, pCold/PoPLC- δ 3B non induced; lane 4, pCold/PoPLC- δ 3B induced with 1mM IPTG for 5 h at 15 °C; lane 5, His-bind column purified fraction of pCold/PoPLC- δ 3B. The positions of standard size markers are shown on the left. The asterisk (*) indicates PoPLC- δ 3B and trigger factor of pCold TF vector.



The difference was shown where recombinant protein is induced by IPTG and vice versa, as is trigger factor of pCold-self (Fig. 8). The purified recombinant protein induced by IPTG was also confirmed by SDS-PAGE and western blotting after dialysis followed by enrichment. To ascertain the enzymatic activity of the recombinant PoPLC-83B protein, PI-PLC assay was carried out using the cholatemixed micelle assay containing $[^{3}H]$ PtdIns (PI) and $[^{3}H]$ PtdIns-4.5-P₂ (PIP₂). respectively. PIP₂-hydrolyzing activity was observed in fractions from E. coli expressing PoPLC-83B, but not in fractions from E. coli carrying the pCold-Self vector. On the other hand, PI-hydrolyzing activity rarely appeared in any fractions (Fig. 9). In comparison with activities of crude extract of PoPLC-83B and purified protein of PoPLC-83B, the activity of purified protein increased more than four times (Table 4). Unlike human PLC-83 from human fibroblasts (Ghosh et al., 1997; Pawelczyk and Matechi, 1999), optimal pH of the purified recombinant PoPLCô-3B for PIP₂ ranged from 7.5 to 8.0 (Fig. 10A). Human PLC-83 (Pawelczyk and Matecki, 1997) and rat PLC-81 (Asano et al., 1994) required 10^{-4} to 10^{-5} M Ca²⁺ as an optimal concentration, whereas 10^{-2} M Ca²⁺ was demanded for the recombinant PoPLC- δ 3B. This concentration is higher than the requirement of *P. olivaceus* PLC-δ1s (Kim *et al.*, 2013) and *P. olivaceus* PLCδ4 (Bak et al., 2013). As shown Fig. 10B, activity of PoPLC-δ3B was obviously differed on various Ca²⁺ concentration through a cholate-mixed micelle assay unlike most of mammalian PLC isozymes.



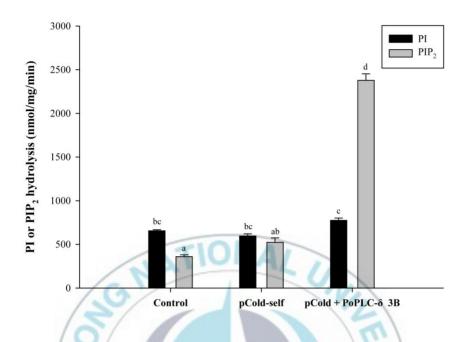


Fig. 9. Comparison with activity of the recombinant protein. Activities of PoPLC- δ 3B were assayed with the cholated-mixed micelles assay (PI and PIP₂). The results are expressed as means \pm SEM (n = 3). Values with different letters indicate statistical difference (P < 0.05).

Table 5

Purification of the recombinant PoPLC- δ 3B enzyme. The PLC activities of PoPLC- δ 3B protein was assayed using cholate-mixed micelle assay containing [³H]-PIP₂.

	Step		Total activity (nmol/mg/min)		Purification (fold)
PoPLC-ô3B	Crude extract	0.85	•25552	1815	1
	His-tag purification	0.65		8360	4.61



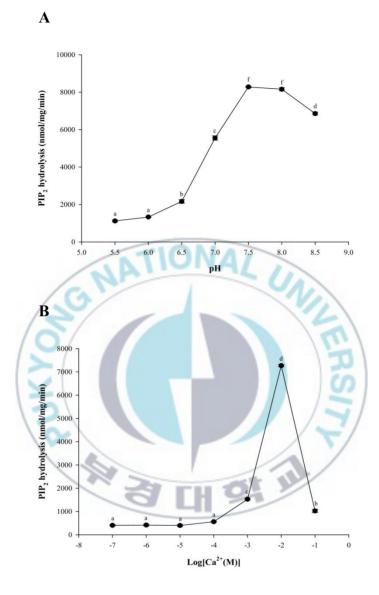


Fig. 10. Enzymatic characterization of the recombinant protein. The activity of recombinant PoPLC- δ 3B was assayed under various pH (A) and concentrations of calcium in buffer (B). The results are expressed as means \pm SEM (n = 3). Values with different letters indicate statistical difference (P < 0.05).



U-73122 known as a phospholipase C inhibitor has inhibitory effect against enzymatic activity of PoPLC- δ 3B assessed by hydrolyzing PIP₂. As a negative control of assay, U-73343 which has similar structural with U-73122 also used in the same condition (Wilsher *et al.*, 2007). The activity of PoPLC- δ 3B decreased in a concentration dependent manner by U-73122, in the contrary, U-73343 activated the recombinant PoPLC- δ 3B protein (Fig. 11A). The results also showed the differences that PoPLC- δ 3B demand higher concentration of U-73122 to inhibit inherent activity of the recombinant protein unlike PoPLC- δ 1s (Kim *et al.*, 2013) and PoPLC- δ 4 (Bak *et al.*, 2013),

To identify the regulatory properties of PoPLC- δ 3B, several cofactors were used for activity assay. Spermine and spermidine include in polyamine which is cationic molecule involved in cellular metabolism such as cell proliferation, protein synthesis and protein-DNA interaction in all eukaryotic cells (Tabor and Tabor, 1984). Sphingosine, long-chain sphingoid bases, involved in a variety of cell types and known as inhibitor of protein kinase C and activator of PLC and PLD (Chao *et al.*, 1994; Goñi and Alonso, 2006). The correlation between PoPLC- δ 3B and several cofactors was respectively confirmed from the assay of PIP2 hydrolysis with varied concentrations (0-1000 μ M). The recombinant PoPLC- δ 3B was activated by sphingosine as the concentration increases, on the contrary, the enzymatic activities were reduced in the presence of more than 200 μ M spermine and 100 μ M spermidine while increasing up to a certain concentration, respectively (Fig. 11B). Variation of activity by spermine and sphingosine tend to be relatively similar to mammalian PLC- δ (Pawelczyk and



Matecki, 1997) and *P. olivaceus* PLC-δ4 (Bak *et al.*, 2013), but the activity of recombinant PoPLC-δ3B protein effected by spermidine was differently revealed in the assay.





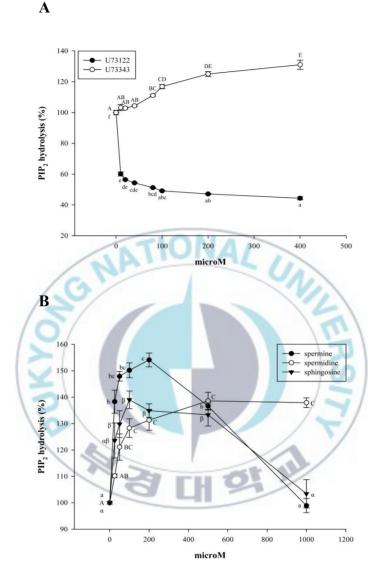


Fig. 11. The effect of inhibitor and activator on the activity of PoPLC- δ 3B. The activity of recombinant PoPLC- δ 3B was assayed with inhibitor (U73122) or structure analog of inhibitor (U73343) (A), and multifarious cofactor (spermine, spermidine or D-sphingosine) (B). The results are expressed as means ± SEM (n = 3). Values with different letters indicate statistical difference (P < 0.05).

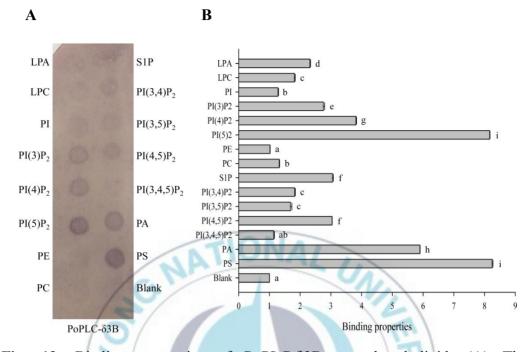


3.4. Binding properties of the purified proteins

The binding properties of recombinant PoPLC- δ 3B to phospholipid were observed by the lipid binding assay using PIP strip as described above materials and methods. PoPLC- δ 3B indicated strong binding affinity to PS followed by PI(5)P₂, PA and PI(4)P₂ but low binding affinity to PE, followed by PI(3,4,5)P₂, PI and PC (Fig. 12A and B). Although the recombinant PoPLC- δ 3B protein has weak affinity to PI(4,5)P₂ is the preferred substrate for human PLC- δ (Nagano et al., 1999; Coward *et al.*, 2007), PoPLC- δ 3B has similar binding properties with *P. olivaceus* PLC- δ 4 (Bak *et al.*, 2013) as well as *Mus musculus* PLC- δ 1 (Kim *et al.*, 2013).

As a result, our studies provide fundamental information based on analysis of sequences, tissue distribution and enzymatic characteristics by comparing PLC- δ 3A and PLC- δ 3B. Even though two paralogous PLC- δ 3 genes having high similarity for sequence homology were derived from the same ancestor, the expressed PoPLC- δ 3A protein has non-activity. Therefore, we surmise that PoPLC- δ 3A exists as non-processed pseudo gene or the condition for optimal activity of the recombinant protein has yet to be figured out so far. Although future studies are required to establish ambiguous hypotheses, these results may offer valuable information to study in PLC- δ isozymes of teleostei.





Binding properties of PoPLC-83B on phospholipid. (A) Fig. 12. The phospholipid binding assay was used to detect protein-lipid interactions (PIP strip: P-6001). PIP strips contain an array of acidic phospholipids, including lysophosphatidic acid (LPA); lysophosphocholine (LPC); phosphatidylinositol (PtdIns); PtdIns(3)P₂; PtdIns(4)P₂; PtdIns(5)P₂; phosphatidyl-ethanolamine (PE); phosphatidylcholine (PC); sphingosine-1-phosphate (S1P); $PtdIns(3,4)P_2;$ PtdIns(3,5)P₂; PtdIns(4,5)P₂; PtdIns(3,4,5)P₃; phosphatidic acid (PA); and phosphatidylserine (PS). PoPLC-83B was detected with a mixture of anti-His-tag antibody. (B) The spots were quantified using ImageJ described above materials and methods. The results are expressed as means \pm SEM (n = 3). Values with different letters indicate statistical difference (P < 0.05).

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넙치로부터 인지질가수분해효소 C-δ3A 와 C-δ3B 의 분자분석 비교와 효소학적 특성 분석

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인지질가수분해효소 C- δ 3 (PLC- δ 3)는 원형질막에 존재하며, PIP₂ 를 가수분해하여 2 차 전령체인 IP₃와 DAG 를 생성한다. 이 유전자가 연관된 super-pathway 는 신호 전달 활성과 칼슘 이온 binding 을 포함한다. 선행연구로부터 넙치에서 PLC-δ1, PLC-δ4 및 PLC-δ3 의 유전자를 동정하였고, 어류 특이적 게놈 중복을 통해 PLC-&1 와 PLC-&3 가 각각 두 가지 형태로 존재한다는 것을 밝혀내, PLC-δ3 의 경우 이를 PoPLC-δ3A 와 PoPLC-δ3B 라고 명명하였다. PoPLC-δ3A 의 cDNA 는 총 3,816bp 로 구성되어 있고, 787개의 아미노산으로 번역되는 ORF 는 2,361 bp 이다. PoPLC-δ3B 의 cDNA 는 2,868 bp 로 구성되어 있으며, 792 개의 아미노산으로 번역되는 ORF 는 2,376 bp 이다. 두 유전자의 번역된 아미노산 서열은 서로 67.8%의 유사성을 가지고 있으며, 포유류에서 발견되는 모든 조절적 도메인들이 잘 보존되어 있음을 밝혀냈다. RT-PCR 및 real-time PCR 을 통해, 두 유전자가 공통적으로 근신경계의 조직에 주로 발현하고 있으며, 면역과의 상관관계를 규명하기 위해 넙치의 외부로부터 LPS 및 Con A/PMA 를 처리한 결과, PoPLC-δ3A 후기 면역에 대해 연관성이 있으며, PoPLC-δ3B 가 정상상태의 넙치에서 비장에 많이 분포하므로 초기면역 대해서는 PoPLCδ3B 가 관여한다고 추측하였다. 두 유전자에 대해 효소학적 특성을 알아보기 위해 대장균에서 재조합 단백질을 만드는데 성공하였으나, PoPLC-δ3A 는 기질에 대해 활성을 나타내지 않았고, PoPLC-δ3B 만 활성을 나타내었다. PoPLC-δ3B 는 pH 7.5, 10⁻² M 의 칼슘 이온 버퍼에서 최적 활성을 나타내었고, PLC-δ 의 저해제에 대해 약한 감수성을 보였으며, 각종 활성인자들에 대해 다른 종의 PLC-δ 와 유사한 활성 변화를 보여주었다. PoPLC-δ3B 의 재조합 단백질은 기존에 PLC-δ 의 주요 기질로 알려진 PI(4,5)P2 외에 PS, PI(5)2 등에 높은 감수성을 나타내었다. PoPLC-δ3A 가 활성을 가지지 못한 것에 대해, PoPLC- δ 3A 가 non-processed pseudo gene 으로 존재하거나 PoPLC- δ 3B 보다 더 극적인 조건하에 활성을 가질 것이라는 가설을 세웠다. 이 연구는 최초로 해양생물 유래의 PLCδ3에 대해 특성분석을 하였으며, 세포 신호전달에 관련해 분자생물학적 기초자료를 제공한다.



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그리고 제 인생에 절대 빼놓을 수 없는 우리 모비딕스 가족들, 정우형, 봉수형, 영철이형,



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다들 행복하시고 항상 건강하세요.

다시 한 번 감사합니다.

