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*)

by

Jin Young Lee

Department of Biotechnology

The Graduate School

Pukyong National University

February 2015

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

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

Advisor: Prof. Hyung Ho Lee

by

Jin Young Lee

A thesis submitted in partial fulfillment of the requirements
For the degree of
Master of Engineering
in Department of Biotechnology, The Graduate School,
Pukyong National University

February 2015

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

A dissertation
by
Jin Young Lee

Approved by:

(Chairman) Joon Ki Chung

(Member) Nam Gyu Park

(Member) Hyung Ho Lee

February 27, 2015

CONTENTS

LIST OF TABLES

LIST OF FIGURES

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

Jin Young Lee

Department of Biotechnology, The Graduate school,

Pukyong National University

Abstract

The gene of phospholipase C-δ3 (PLC-δ3) encodes one of phospholipase C group which localizes to plasma membrane and catalyzes hydrolysis of phosphatidylinositol 4,5-biphosphate (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-δ3, we identified duplicated PLC-δ3 genes, PLC-δ3A and PLC-δ3B, 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-δ3 isoforms. After performing RT-PCR and real-time PCR, we found out that the distribution of the tissue-specific expression and immune response by stimulation considerably varied in *P. olivaceus* PLC-δ3 isoforms. Although the recombinant proteins of PoPLC-δ3 isoforms were expressed with histidine-tag in *Escherichia coli*, PoPLC-δ3B only showed the activity at pH 7.5 and high concentration of calcium ion. From the further PIP_2 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-δ3B has binding properties to phospholipid like mammalian PLC-δ. Regarding PLC-δ3 in aquatic organism, these results firstly provide the fundamental information of cellular signaling of PLC-δ3B and the possibility that PoPLC-δ3A 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 (PIP_2) into two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers, IP_3 and DAG, mediate a variety of cellular responses to extracellular stimuli by inducing protein kinase C and increasing cytosolic Ca^{2+} 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-δ1, -δ3 and -δ4) (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-δ1 and PLC-δ4, 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-δ3 has not been well known in aquatic animals unlike mammalian. Therefore, our previous studies defined that PLC-δ1 and PLC-δ3 have two paralogous genes respectively as a result of fish-specific genome duplication (FSGD) in teleostei, in other words, two paralogous genes of PLC-δ3 were named as PoPLC-δ3A and PoPLC-δ3B by investigating cDNA sequence of *P. olivaceus* (Kim *et al*., 2008). Furthermore, researches related into enzymatic properties of PoPLC-δ1 isoforms and PoPLC-δ4 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-δ3A and PLC-δ3B from olive flounder (*P. olivaceus*).

2. Materials and methods

2.1. Sequence and phylogenetic analyses of PoPLC-δ3 isoforms

Following the results of our previous studies, peptide sequences of PoPLC-δ3A and -δ3B 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-δ3A*, *C-δ3B* 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.

Table 1

Sequences used in this study.

Sequence for comparison	Species (common name)	Accession number	
		Ensembl	Genbank
PoPLC-δ3A	*Paralichthys olivaceus* (olive flounder)		ACA05827.1
PoPLC-δ3B			ACA05828.1
PfPLC-δ3A	*Poecilia formosa* (amazon molly)	ENSPFOP00000001225	
PfPLC-δ3B		ENSPFOP00000005641	
AmPLC-δ3A	*Astyanax mexicanus* (blind cave fish)	ENSAMXP00000018422	
AmPLC-δ3B		ENSAMXP00000006453	
TrPLC-δ3A	*Takifugu rubripes* (fugu)	ENSTRUP00000042343	
TrPLC-δ3B		ENSTRUP00000008073	
OlPLC-δ3A	*Oryzias latipes* (medaka)	ENSORLP00000003606	
OlPLC-δ3B		ENSORLP00000023208	
XmPLC-δ3A	*Xiphophorus maculatus* (platyfish)	ENSXMAP00000008440	
XmPLC-δ3B		ENSXMAP00000014440	
GaPLC-δ3A	*Gasterosteus aculeatus* (stickleback)	ENSGACP00000012434	
GaPLC-δ3B		ENSGACP00000020164	
TnPLC-δ3A	*Tetraodon nigroviridis* (tetraodon)	ENSTNIP00000018000	
TnPLC-δ3B		ENSTNIP00000013383	
OnPLC-δ3A	*Oreochromis niloticus* (tilapia)	ENSONIP00000001092	
OnPLC-δ3B		ENSONIP00000010144	
DrPLC-δA	*Danio rerio* (zebrafish)	ENSDARP00000069390	
DrPLC-δB		ENSDARP00000074236	
LcPLC-δ3	*Latimeria chalumnae* (coelacanth)	ENSLACP00000003938	
XtPLC-δ3	*Xenopus tropicalis* (western clawed frog)		XP_002935564
AcPLC-δ3	*Anolis carolinensis* (green anole)		XP_008111435.1
GgPLC-δ3	*Gallus gallus* (chicken)		XP_425839.4
OoPLC-δ3	*Orcinus orca* (killer whale)		XP_004286005.1
HsPLC-δ3	*Homo sapiens* (human)	ENSP00000313731	
HsPLC-δ1		ENSP00000335600	
HsPLC-δ4		ENSP00000251959	
HsPLC-β1		ENSP00000367908	
HsPLC-γ1		ENSP00000362368	
HsPLC-ε1		ENSP00000260766	
HsPLC-ζ1		ENSP00000266505	
HsPLC-η1		ENSP00000345988	
ScPLC	*Saccharomyces cerevisiae* (yeast)		NP_015055.1

2.2. mRNA isolation and cDNA synthesis

To confirm the expression pattern of the *PoPLC-δ3* 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 Superscript™ III reverse transcriptase (Invitrogen, USA), as per the manufacturer's instruction. Reverse transcription was carried out at 42 ℃ 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-δ3s 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 ℃ for 4 min, 30cyles of 94 ℃ for 30 s, 56 ℃ for 30 s and 72 ℃ for 20s, and a final 7 min elongation at 72 ℃. 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-δ3A and -δ3B 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 ΔC_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-δ3B*) (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-δ3A and PoPLC-δ3B 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-δ3A and PoPLC-δ3B 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-δ3A and PoPLC-δ3B 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 -δ3A and -δ3B.

Primer name	5'-3' sequence	Information
Poβ-actin-F	GACATGGAGAAGATCTGGCA	Primers for RT-PCR and real-time PCR
Poβ-actin-R	ATCTCCTGCTCGAAGTCCAG	
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-δ3A and PoPLC-δ3B 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-δ3A and PoPLC-δ3B, 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-δ3A and PoPLC-δ3B), transformed cells were grown in Luria Bertani medium containing100 μg/mL ampicillin at 37 °C. At a cell density of 0.6 (A_{600}), 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 μg/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-δ3A and PoPLC-δ3B 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 -δ3A and -δ3B.

Primer name	5'-3' sequence	Information
pCold-TF-F1	CCACTTTCAACGAGCTGATG	Sequencing primers
pCold-TF-R	GGCAGGGATCTTAGATTCT	
EcoRI/PoPLC-D3A-F	CGGAATTCATGTTGGGCAGCGGCAAGTCAC	Primers for RT-PCR
XbaI/PoPLC-D3A-R	GCTCTAGATCATGCCATGCTTTTGGCAATGG	and real-time PCR
EcoRI/PoPLC-δ3B-F	GAATTCATGTTGGGCCACCGAAAGAAAGCG	
XbaI/PoPLC-D3B-R	GCTCTAGATTAGGGTCCCTGGGCGGATGACTTG	

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) for30 min at room temperature after transferring target protein to the membrane. The membrane was then incubated with mixed polyclonal anti-His-tag 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-δ3A and PoPLC-δ3B 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-P_2 (PIP_2) as the substrate. Briefly, the PIP_2-hydrolyzing activity was measured with mixed phospholipid micelles containing 40 μM phosphatidylethanolamine (PE), 5 μM PIP_2, and 1 μCi/mL PIP_2. 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 in5 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]-1H-pyrrole-2,5-dione), and its inactive analogue for negative control, 1-[6-[((17β)-3-methoxyestra-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 μM) 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-δ3B activity was assayed as described above. Reaction mixture containing purified PoPLC-δ3B enzyme, 50mM Hepes/pH 7.0, 1 mM EGTA, 0.08% SDC, 100 mM NaCl, 300 μMCaCl2, and various concentrations (0, 25, 50, 100, 200, 500 and 1000 μM) of polyamine or sphingosine in a total volume of 200 μL 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 μg/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 ± standard error of the mean (SEM).

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-δ1 and PLC-δ3 respectively and one PLC-δ4 gene as a result of three rounds of fish-specific genome duplication (Kim *et al*., 2008). *PoPLC-δ3A* cDNA (3,816 bp) and *PoPLC-δ3B* cDNA (2,868 bp) encode 787 amino acids (Fig. 1) and 792 amino acids (Fig. 2) respectively. The cDNA sequences of PoPLC-δ3s were deposited in GenBank (*PoPLC-δ3A*, Genbank accession number EU433322.1; *PoPLC-δ3B*, Genbank accession number EU433323.1).

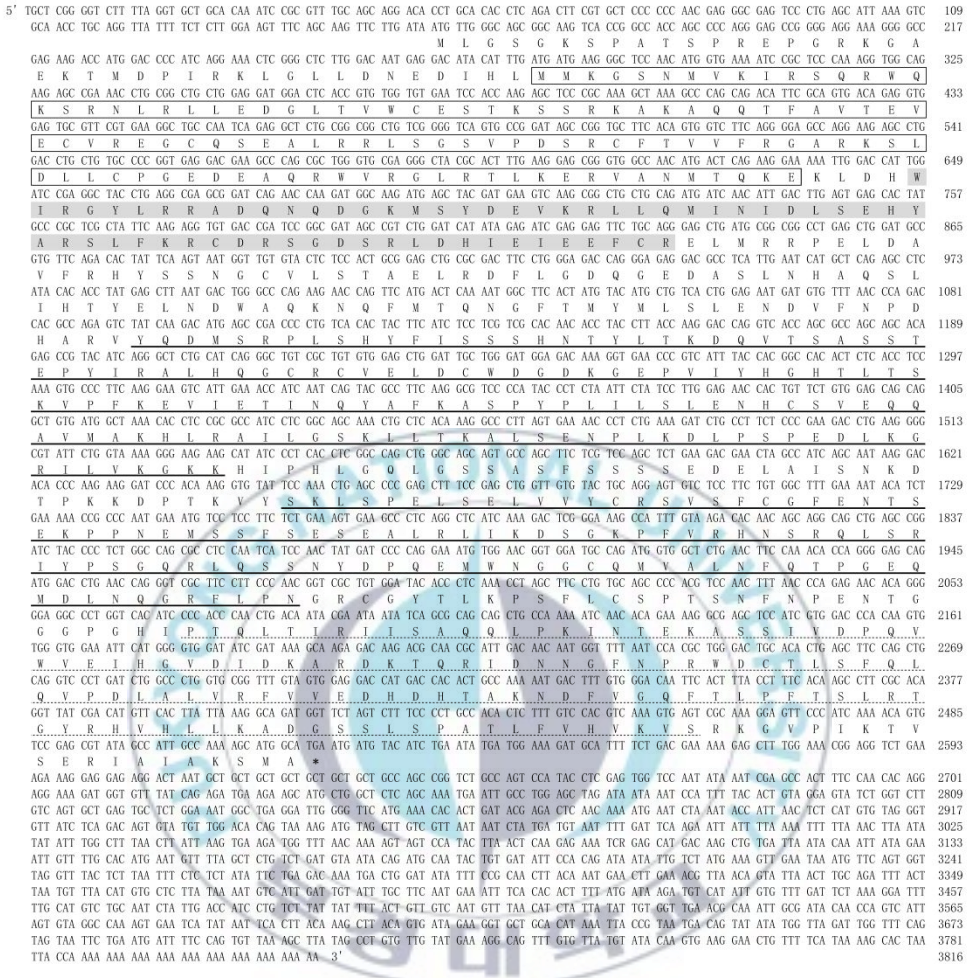

Fig. 1. Nucleotide and deduced amino acid sequence of *P. olivaceus* phospholipase C-δ3A cDNA (*PoPLC-δ3A*, GenBank accession number: EU433322.1). The *open box* in the amino acid sequence indicates the putative PH domain and the EF hand domain is indicated by a dark shaded box. The X-domain and Y-domain is underlined in the order named and a *dotted underline* indicates the C2 domain.

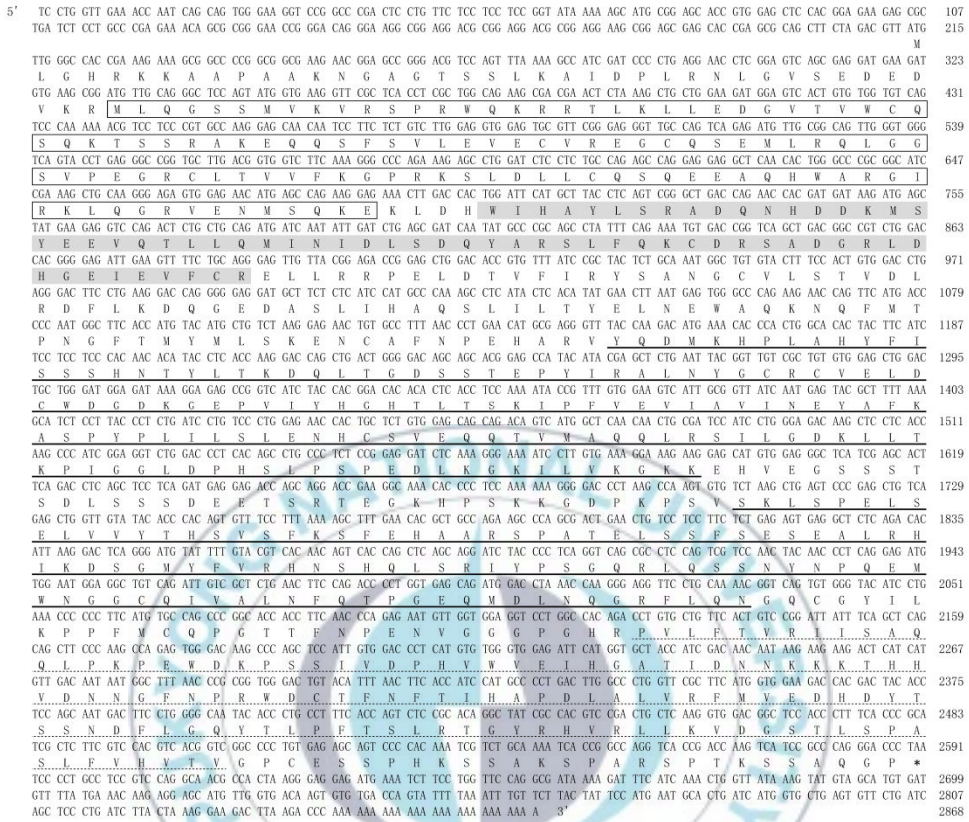

Fig. 2. Nucleotide and deduced amino acid sequence of *P. olivaceus* phospholipase C-δ3B cDNA (*PoPLC-δ3B*, GenBank accession number: EU433323.1). The *open box* in the amino acid sequence indicates the putative PH domain and the EF hand domain is indicated by a dark shaded box. The X-domain and Y-domain is underlined in the order named and a *dotted underline* indicates the C2 domain.

As shown Fig. 3, two paralogous of PoPLC-δ3 genes have equally conserved regulatory domains containing PH, EF-hand, catalytic X and Y, and C2 domains PLC-δ3 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-δ3A and PLC-δ3B is 67.8%. The identical percentage of paralogous PLC-δ3A genes ranged from 75.5% to 84.7%. and paralogous PLC-δ3B 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-δ3A and PLC-δ3B), PLC-δ3 of other species and human PLC isoforms (Fig. 4). The result also supports that PoPLC-δ3A and PoPLC-δ3B are grouped into duplicated form in teleostei, which is considered from a evolutional tendency. These results demonstrated the previous study (Kim *et al*., 2008).

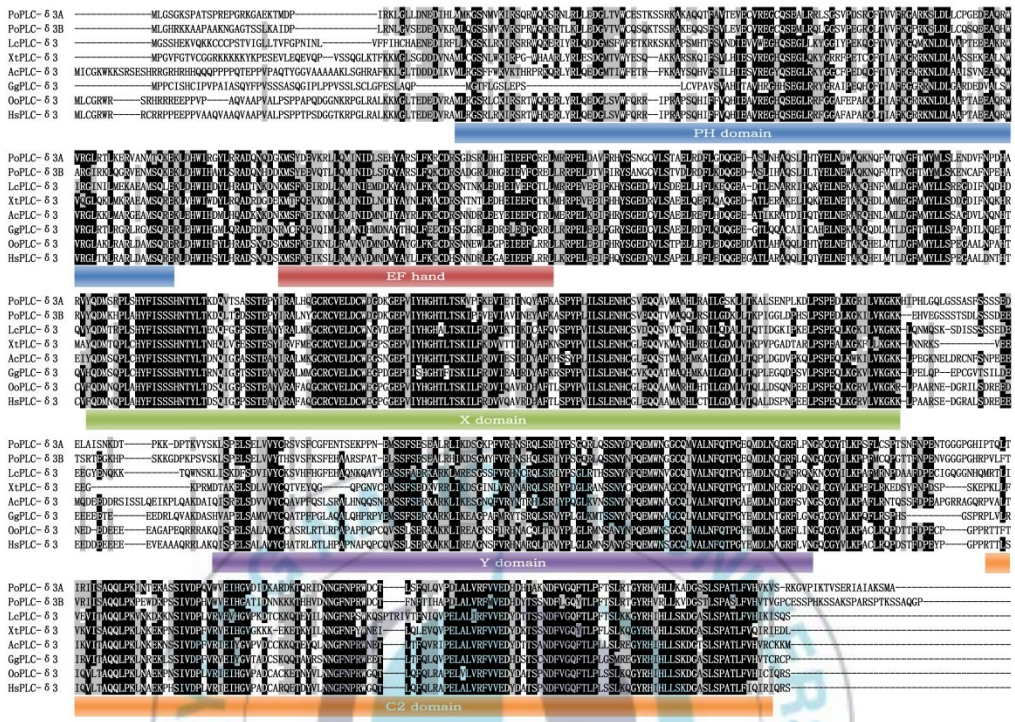

Fig. 3. Multiple alignment analysis of sequences of PLC-δ3 proteins in various species. The identical conserved domains are expressed below the amino acid residues, respectively. The GenBank and the Ensembl accession numbers of other species of PLC-δ are given in Table 1.

Table 4

Comparison with sequential identity of amino acids among *P. olivaceus* PLC-δ3 isoforms and PLC-δ3 from a variety of species.

	PoPLC-δ3A	PoPLC-δ3B
PoPLC-δ3A		0.678
PfPLC-δ3A	0.787	0.631
AmPLC-δ3A	0.765	0.664
TrPLC-δ3A	0.824	0.667
OlPLC-δ3A	0.755	0.605
XmPLC-δ3A	0.797	0.634
GaPLC-δ3A	0.832	0.65
TnPLC-δ3A	0.847	0.666
OnPLC-δ3A	0.837	0.65
DrPLC-δ3A	0.766	0.663
PoPLC-δ3B	0.678	
PfPLC-δ3B	0.668	0.798
AmPLC-δ3B	0.629	0.65
TrPLC-δ3B	0.649	0.767
OlPLC-δ3B	0.649	0.743
XmPLC-δ3B	0.665	0.794
GaPLC-δ3B	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-δ3	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.

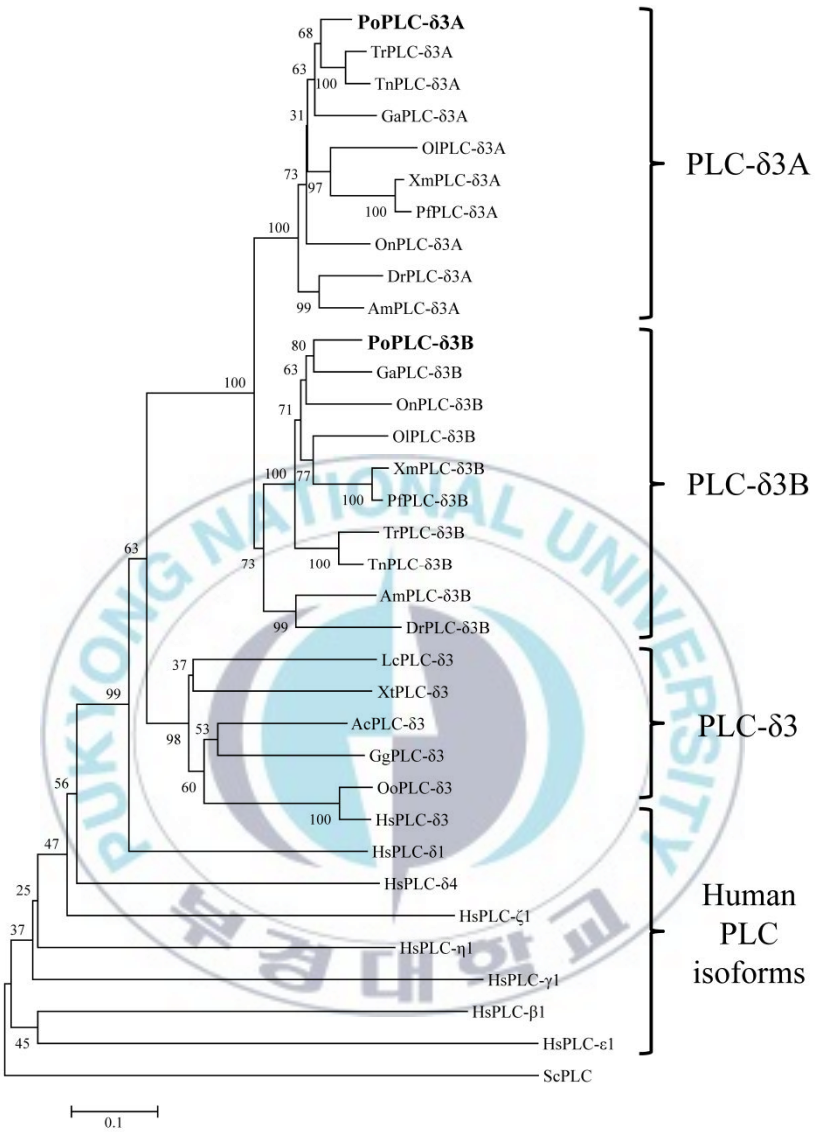

Fig. 4. The phylogenetic relationships of PoPLC-δ isoforms with human PLC family based on deduced amino sequences. A phylogenetic tree of the aligned sequences was constructed using neighbor-joining algorithm within MEGA (version 4.0). The GenBank and the Ensembl accession numbers of other species of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

3.2. Tissue-specific distribution and immune interrelation of the PoPLC-δ3A and PoPLC-δ3B mRNAs established by RT-PCR and real-time PCR

We determined the distribution of *P. olivaceus* PoPLC-δ3 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-δ3A and PoPLC-δ3B mRNA were patently different as shown Fig. 5. The mRNA transcripts of PoPLC-δ3A and PoPLC-δ3B were widely present in all tissues and mostly distributed in myoneural tissues obtained from olive flounder. The mRNA transcripts of PoPLC-δ3A were strongly expressed in liver followed by brain, eye, muscle, gill, gullet and stomach. While the mRNA transcripts of PoPLC-δ3B were comparatively distributed in various tissues except in kidney and muscle, the level of mRNA expression was generally higher than that of PoPLC-δ3A in eye followed by spleen, brain and heart (Fig. 5).

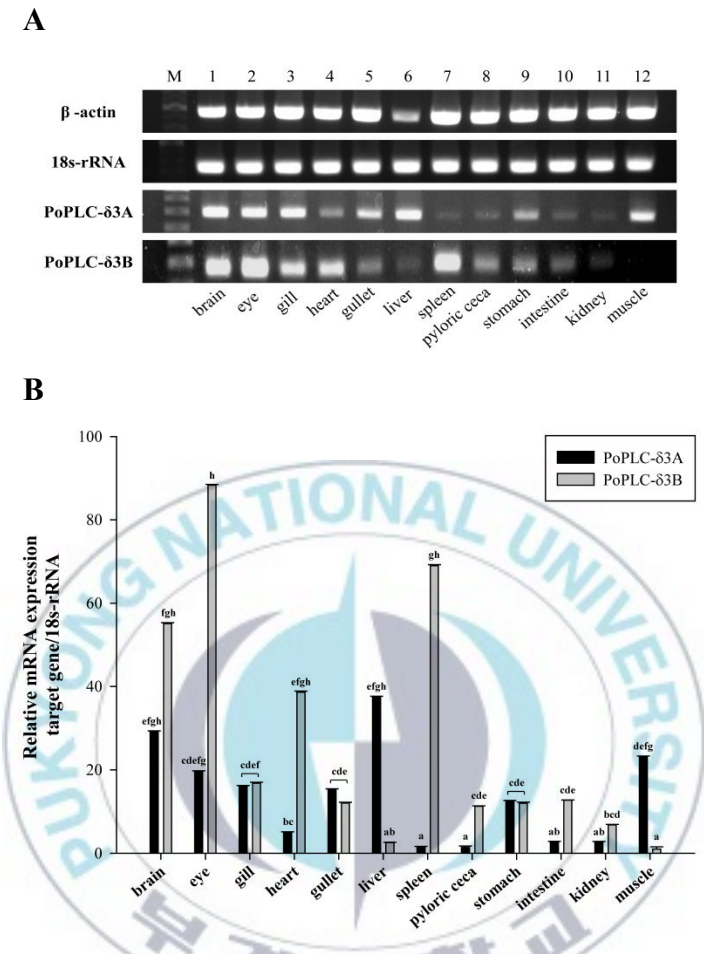

Fig. 5. Tissue-specific distribution of the *PoPLC-δ3A* and *PoPLC-δ3B* mRNA. (A) RT-PCR analysis of the *PoPLC-δ3A* mRNA and *PoPLC-δ3B* mRNA. (B) Quantitative real-time PCR of *PoPLC-δ3A* and *PoPLC-δ3B* mRNA in various tissues. Mean mRNA levels in olive flounder tissues were analyzed by real-time PCR, and $2^{-\Delta\Delta Ct}$ levels were calculated relative to the tissue with the lowest expression (*PoPLC-δ3B*; muscle) set to 1, and normalized against 18s-rRNA expression. The results are expressed as means ± SEM (n = 3). Values with different letters indicate statistical difference ($P < 0.05$).

In order to identify the immune interrelation of *PoPLC-δ3A* and *PoPLC-δ3B* 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 *18s-rRNA* gene which was used as the internal control. In comparison with *18s-RNA* gene which was uniformly expressed, *IL-1β* 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-δ3A was more expressed than PoPLC-δ3B in spleen and kidney under any stimulus. From the results, we predict that PoPLC-δ3B 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 late stage of immune response.

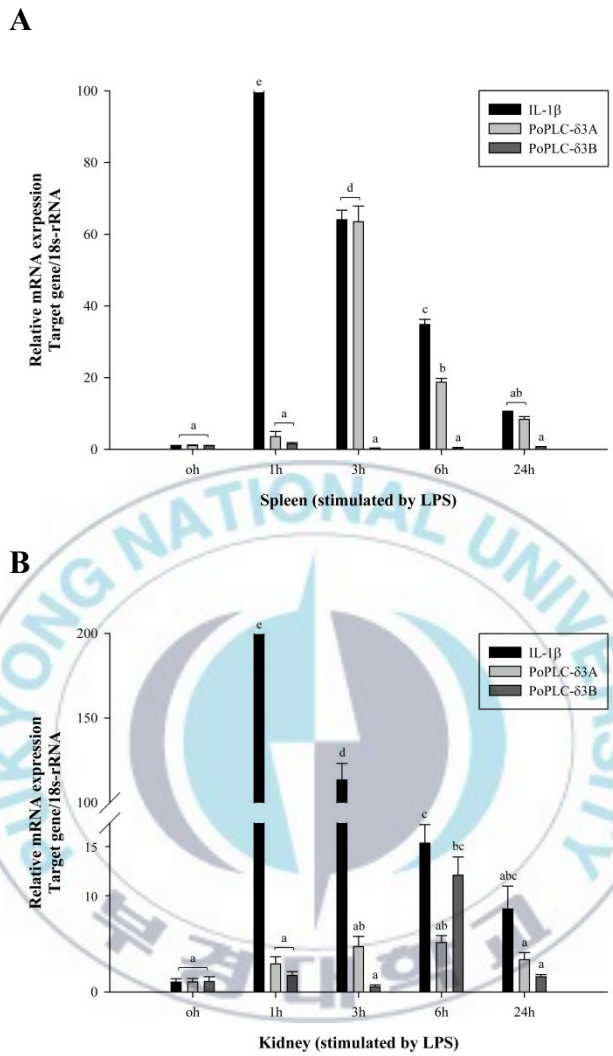

Fig. 6. Identification of immune response by stimulation of LPS. qPCR was performed using *PoPLC-δ3A*, *PoPLC-δ3B*, *IL-1β* and *18s-rRNA* of mRNA from spleen (A) and kidney (B) tissue at 0 to 24 h after LPS injection. The results are expressed as means ± SEM (n = 3) using $2^{-\Delta\Delta Ct}$ method calculated relative to the time zero (set to 1), and normalized against 18s-rRNA expression. Values with different letters indicate statistical difference ($P < 0.05$).

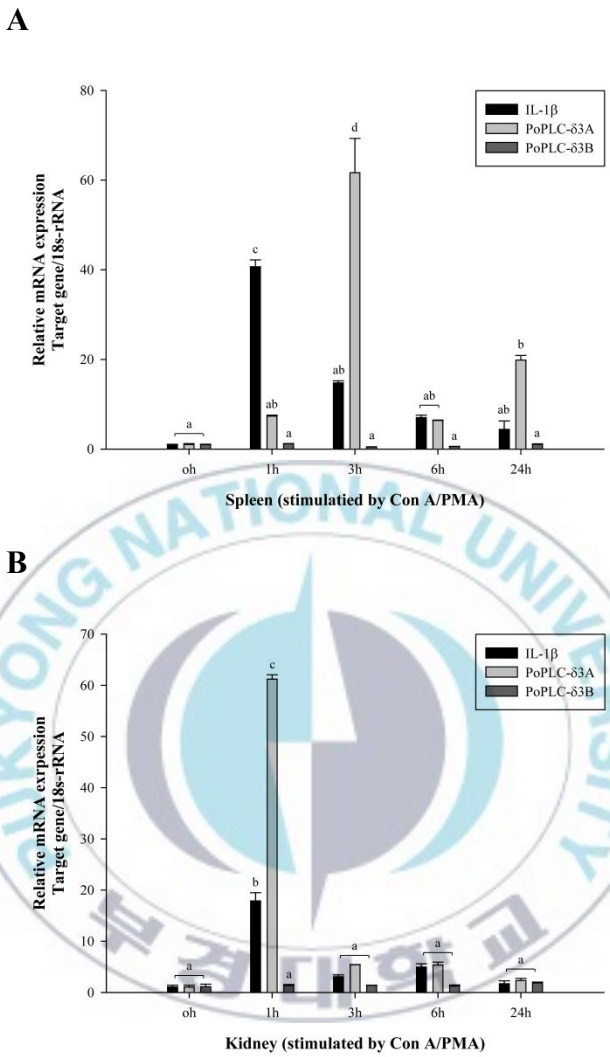

Fig. 7. Identification of immune response by stimulation of Con A/PMA. qPCR was performed using *PoPLC-δ3A*, *PoPLC-δ3B*, *IL-1β* and *18s-rRNA* of mRNA from spleen (A) and kidney (B) tissue at 0 to 24 h after Con A/PMA injection. The results are expressed as means ± SEM (n = 3) using $2^{-\Delta\Delta Ct}$ 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-δ1A, PLC-δ1B-Lf, PLC-δ1B-Sf and PLC-δ4 in our previous studies. Recombinant PoPLC-δ3A and PoPLC-δ3B were also expressed in *E. coli* BL21 (DE3) and purified by using Ni^{2+}-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-δ3A and PoPLC-δ3B, appeared as a single band of 137 kDa (trigger factor-fused recombinant proteins). Although the recombinant PoPLC-δ3A and PoPLC-δ3B proteins were expressed in *E. coli* BL21 (DE3), only PoPLC-δ3B revealed enzymatic activity for substrates.

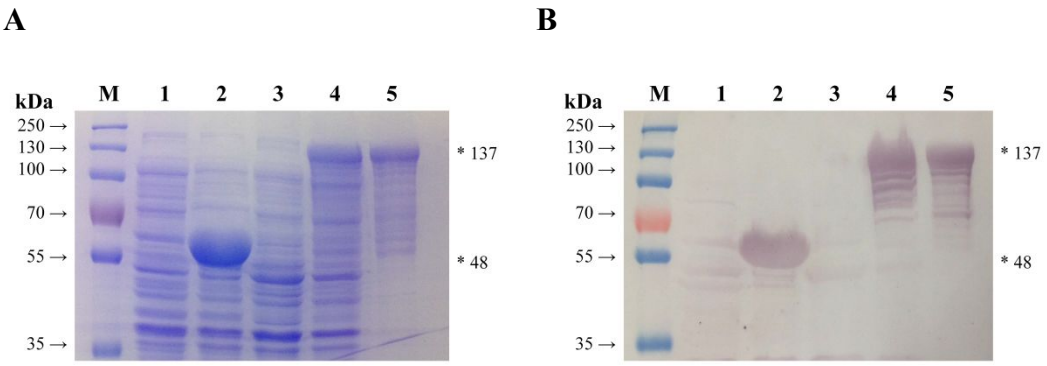

Fig. 8. Expression and purification of recombinant PoPLC-δ3B from olive flounder (*P. olivaceus*). (A) Protein samples were separated by SDS-PAGE (10%) and were visualized by Coomassie R-250 blue staining. (B) Western blotting analysis using anti-His tag polyclonal antibody. Lane M, standard size marker; lane 1, pCold-self non-induced; lane 2, pCold-self induced with 1mM IPTG for 5 h at 15 ^{o}C; lane 3, pCold/PoPLC-δ3B non induced; lane 4, pCold/PoPLC-δ3B induced with 1mM IPTG for 5 h at 15 ^{o}C; lane 5, His-bind column purified fraction of pCold/PoPLC-δ3B. The positions of standard size markers are shown on the left. The asterisk (*) indicates PoPLC-δ3B 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-δ3B protein, PI-PLC assay was carried out using the cholate-mixed micelle assay containing [^{3}H] PtdIns (PI) and [^{3}H] PtdIns-4,5-P_2 (PIP_2), respectively. PIP_2-hydrolyzing activity was observed in fractions from *E. coli* expressing PoPLC-δ3B, 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-δ3B and purified protein of PoPLC-δ3B, the activity of purified protein increased more than four times (Table 4). Unlike human PLC-δ3 from human fibroblasts (Ghosh *et al*., 1997; Pawelczyk and Matechi, 1999), optimal pH of the purified recombinant PoPLCδ-3B for PIP_2 ranged from 7.5 to 8.0 (Fig. 10A). Human PLC-δ3 (Pawelczyk and Matecki, 1997) and rat PLC-δ1 (Asano *et al*., 1994) required 10^{-4} to 10^{-5} M Ca^{2+} as an optimal concentration, whereas 10^{-2} M Ca^{2+} was demanded for the recombinant PoPLC-δ3B. This concentration is higher than the requirement of *P. olivaceus* PLC-δ1s (Kim *et al*., 2013) and *P. olivaceus* PLC-δ4 (Bak *et al*., 2013). As shown Fig. 10B, activity of PoPLC-δ3B was obviously differed on various Ca^{2+} concentration through a cholate-mixed micelle assay unlike most of mammalian PLC isozymes.

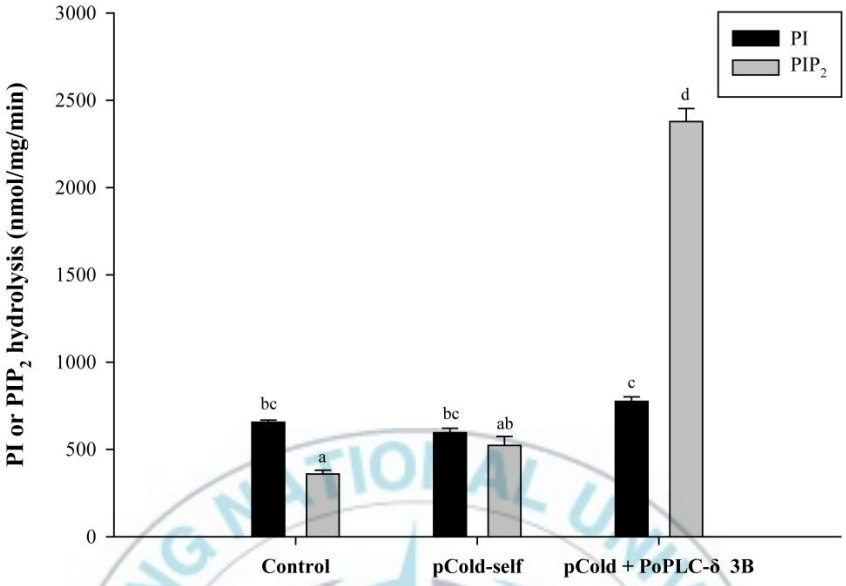

Fig. 9. Comparison with activity of the recombinant protein. Activities of PoPLC-δ3B were assayed with the cholated-mixed micelles assay (PI and PIP_2). The results are expressed as means ± SEM (n = 3). Values with different letters indicate statistical difference ($P < 0.05$).

Table 5

Purification of the recombinant PoPLC-δ3B enzyme. The PLC activities of PoPLC-δ3B protein was assayed using cholate-mixed micelle assay containing [^{3}H]-PIP_2.

	Step	Total protein (mg)	Total activity (nmol/mg/min)	PIP_2 activity (nmol/mg/min)	Purification (fold)
PoPLC-δ3B	Crude extract	0.85	25552	1815	1
	His-tag purification	0.65		8360	4.61

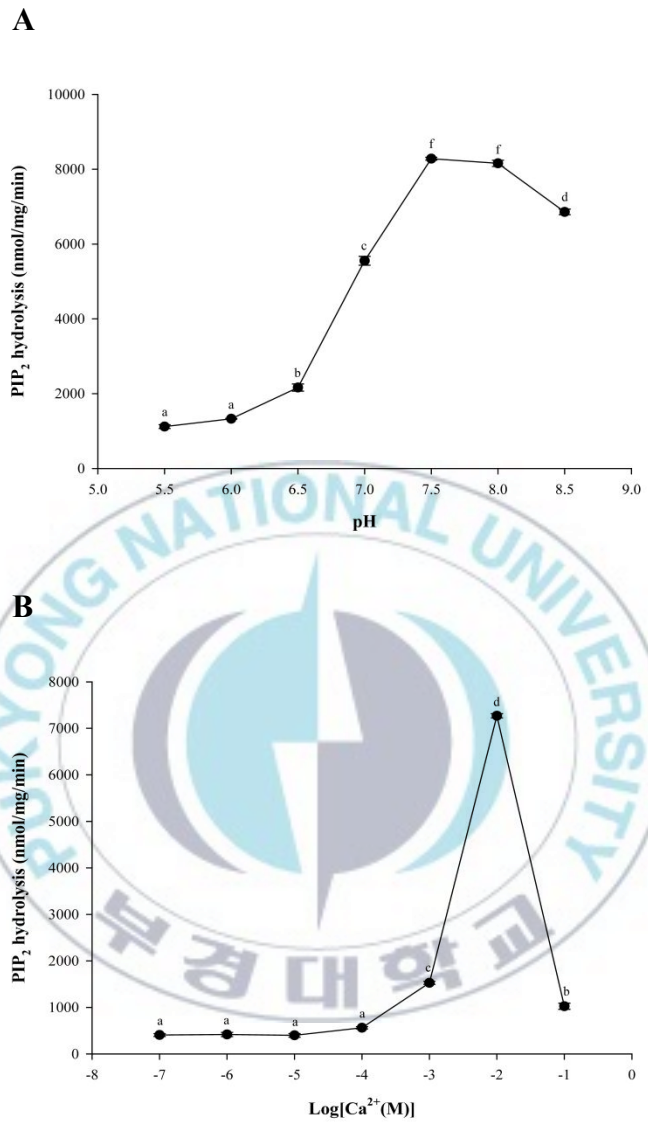

Fig. 10. Enzymatic characterization of the recombinant protein. The activity of recombinant PoPLC-δ3B was assayed under various pH (A) and concentrations of calcium in buffer (B). The results are expressed as means ± SEM (n = 3). Values with different letters indicate statistical difference ($P < 0.05$).

U-73122 known as a phospholipase C inhibitor has inhibitory effect against enzymatic activity of PoPLC-δ3B assessed by hydrolyzing PIP_2. 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-δ3B decreased in a concentration dependent manner by U-73122, in the contrary, U-73343 activated the recombinant PoPLC-δ3B protein (Fig. 11A). The results also showed the differences that PoPLC-δ3B demand higher concentration of U-73122 to inhibit inherent activity of the recombinant protein unlike PoPLC-δ1s (Kim *et al*., 2013) and PoPLC-δ4 (Bak *et al*., 2013),

To identify the regulatory properties of PoPLC-δ3B, 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-δ3B and several cofactors was respectively confirmed from the assay of PIP2 hydrolysis with varied concentrations (0-1000 μM). The recombinant PoPLC-δ3B was activated by sphingosine as the concentration increases, on the contrary, the enzymatic activities were reduced in the presence of more than 200 μM spermine and 100 μM 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.

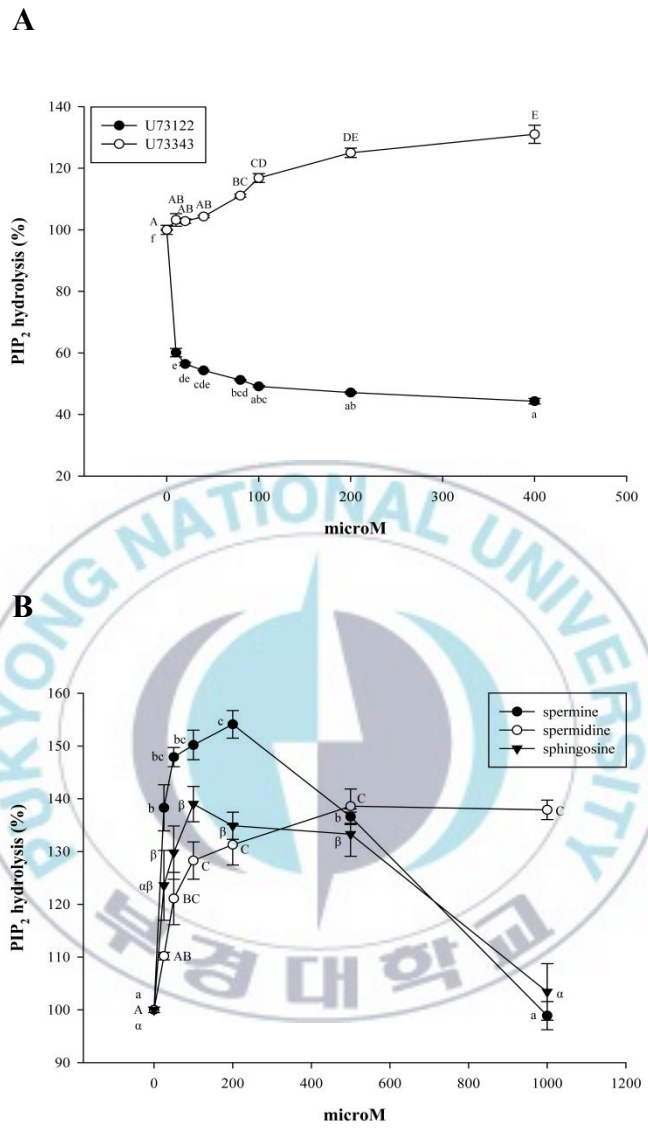

Fig. 11. The effect of inhibitor and activator on the activity of PoPLC-δ3B. The activity of recombinant PoPLC-δ3B was assayed with inhibitor (U73122) or structure analog of inhibitor (U73343) (A), and multifarious cofactor (spermine, spermidine or D-sphingosine) (B). The results are expressed as means ± 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-δ3B to phospholipid were observed by the lipid binding assay using PIP strip as described above materials and methods. PoPLC-δ3B indicated strong binding affinity to PS followed by $PI(5)P_2$, PA and $PI(4)P_2$ but low binding affinity to PE, followed by $PI(3,4,5)P_2$, PI and PC (Fig. 12A and B). Although the recombinant PoPLC-δ3B protein has weak affinity to $PI(4,5)P_2$ is the preferred substrate for human PLC-δ (Nagano et al., 1999; Coward *et al*., 2007), PoPLC-δ3B has similar binding properties with *P. olivaceus* PLC-δ4 (Bak *et al*., 2013) as well as *Mus musculus* PLC-δ1 (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-δ3A and PLC-δ3B. Even though two paralogous PLC-δ3 genes having high similarity for sequence homology were derived from the same ancestor, the expressed PoPLC-δ3A protein has non-activity. Therefore, we surmise that PoPLC-δ3A 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.

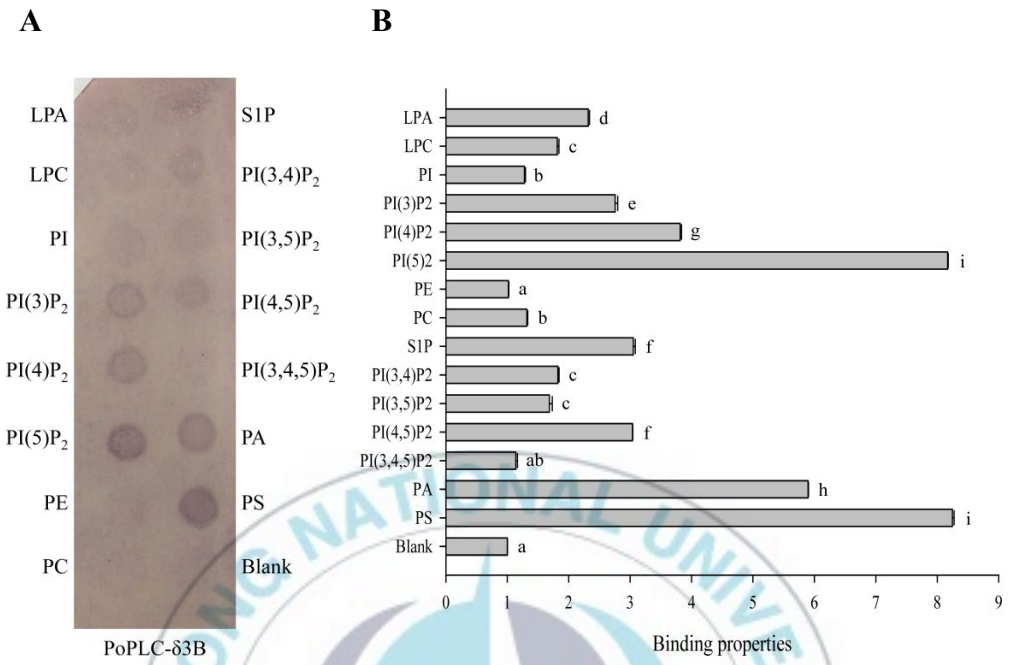

Fig. 12. Binding properties of PoPLC-δ3B on phospholipid. (A) The phospholipid binding assay was used to detect protein-lipid interactions (PIP strip: P-6001). PIP strips contain an array of acidic phospholipids, including lysophosphatidic acid (LPA); lysophosphocholine (LPC); phosphatidylinositol (PtdIns); PtdIns(3)P_2; PtdIns(4)P_2; PtdIns(5)P_2; phosphatidyl-ethanolamine (PE); phosphatidylcholine (PC); sphingosine-1-phosphate (S1P); PtdIns(3,4)P_2; PtdIns(3,5)P_2; PtdIns(4,5)P_2; PtdIns(3,4,5)P_3; phosphatidic acid (PA); and phosphatidylserine (PS). PoPLC-δ3B 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 ± SEM (n = 3). Values with different letters indicate statistical difference ($P < 0.05$).

References

Ahn, S.J., Kim, N.Y., Jeon, S.J., Sung, J.H., Je, J.E., Seo, J.S., Kim, M.S., Kim, J.K., Chung, J.K., Lee, H.H., (2008), Molecular cloning, tissue distribution and enzymatic characterization of cathepsin X from olive flounder (*Paralichthys olivaceus*). Comparative Biochemistry and Physiology Part B, 151, 203–212.

Ahn, S.J., Bak, H.J., Park, J.H., Kim, S.A., Kim, N.Y., Lee, J.Y., Sung, J.H., Jeon, S.J., Chung, J.K., Lee, H.H., (2013), Olive flounder (*Paralichthys olivaceus*) cystatin B: cloning, tissue distribution, expression and inhibitory profile of piscine cystatin B. Comparative Biochemistry and Physiology Part B, 165, 211–218.

Asano, M., Tamiya-Koizumi, K., Homma Y, Takenawa, T., Nimura, Y., Kojima, K., Yoshida, S., (1994), Purification and Characterization of Nuclear Phospholipase C Specific for Phosphoinositides. The Journal of Biological, Chemistry, 269, 12360-12366.

Bak, H.J., Kim, M.-S., Kim, N.Y., Lee, A.R., Park, J.H., Lee, J.Y., Kim, B.S., Ahn, S.J., Lee, H.H., Chung, J.K., (2013), Expression analysis and enzymatic characterization of phospholipase Cδ4 from olive flounder (*Paralichthys olivaceus*). Comparative Biochemistry and Physiology Part B, 166, 215-224.

Chao, C.P., Laulederkind, S.J., Ballou, L.R., (1994), Sphingosine-mediated phosphatidylinositol metabolism and calcium mobilization. J Biol Chem, 269, 5849–5856.

Cifuentes, M.E., Honkanen, L., Rebecchi, M.J., (1993), Proteolytic fragments of

phosphoinositide specific phospholipase C-delta 1. Catalytic and membrane binding properties. J Biol Chem, 268, 11586–11593.

Coward, K., Owen, H., Tunwell, R., Swann, K., Parrington, J., (2007) Phospholipid binding properties and functional characterization of a sea urchin phospholipase Cdelta in urchin and mouse eggs. Biochem Biophys Res Commun, 357, 964–970.

Ghosh, S., Pawelczyk, T., Lowenstein, J.M., (1997), Phospholipase C Isoforms δ1 and δ3 from Human Fibroblasts. Protein Expression and Purification, 9, 262-278.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., Mathieu, C., (2001), An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods, 25, 386–401.

Goñi, F.M., Alonso, A., (2006), Biophysics of sphingolipids I. Membrane properties of sphingosine, ceramides and other simple sphingolipids. Biochim Biophys Acta 1758, 1902–1921.

Guan, Z.B., Shui, Y., Zhang, S.Q., (2007), Two related ligands of the TNF family, BAFF and APRIL, in rabbit: molecular cloning, 3D modeling, and tissue distribution. Cytokine, 39, 192–200.

Katan, M., (1998), Families of phosphoinositide-specific phospholipase C: structure and function. Biochim Biophys Acta, 1436, 5–17.

Kim, M.S., Seo, J.S., Choi, G.E., Lim, S.U., Chung, J.K., Lee, H.H., (2004), Molecular cloning and expression analysis of phospholipase Cδ from mud loach, *Misgurnus mizolepis*. Comparative Biochemistry and Physiology Part B,

139, 681–693.

Kim, M.S., Seo, J.S., Ahn, S.J., Kim, N.Y., Je, J.E., Sung, J.H., Lee, H.H., Chung, J.K., (2008), Duplication of phospholipase C-δ gene family in fish genomes, Genomics, 92, 366-371.

Kim, N.Y., Ahn, S.J., Kim, M.S., Seo, J.S., Kim, B.S., Bak, H.J., Lee, J.Y., Park, M.A., Park, J.H., Lee, H.H., Chung, J.K., (2013), PLC-δ1-Lf, a novel N-terminal extended phospholipase C-δ1, Gene, 528, 170-177.

Kim, N.Y., Kim, M.S., Ahn, S.J., Seo, J.S., Bak, H.J., Kim, B.S., Jo, H.I., Jang, H.Y., Jo, H.S., Lee, H.H., Chung, J.K., (2013), Functional analysis of duplicated genes and N-terminal splice variant of phospholipase C-δ1 in *Paralichthys olivaceus*. Comparative Biochemistry and Physiology Part B, 165, 201-210.

Kumar, S., Nei, M., Dudley, J., Tamura, K., (2008), MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in Bioinformatics, 9, 299-306.

Laemmli, U.K., (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685.

Liu, N., Fukami, K., Yu, H., Takenawa, T. (1996), A new phospholipase C delta 4 is induced at S-phase of the cell cycle and appears in the nucleus. J. Biol. Chem, 271, 355-360.

Livak, K.J., Schmittgen, T.D., (2001), Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25, 402–408.

Nagano, K., Fukami, K., Minagawa, T., Watanave, Y., Ozaki, C., Takenawa, T., (1999), A novel phospholipase C delta4 (PLCdelta4) splice variant as a negative regulator of PLC. J. Biol. Chem, 274, 2872-2879.

Pawelczyk, T., Matecki, A., (1997), Expression, purification and kinetic properties of human recombinant phospholipase C δ3. Acta Biochimica Polonica, 44, 221-230.

Pawelczyk. T., Matecki, A., (1998), Localization of phospholipase C delta3 in the cell and regulation of its activity by phospholipids and calcium. Eur J Biochem, 257, 169-177.

Pawelczyk T., Matecki, A., (1999), Phospholipase C-δ3 binds with high specificity to phosphatidylinositol 4,5-bisphosphate and phosphatidic acid in bilayer membranes. Eur J Biochem, 262, 291-298.

Razzini, G., Brancaccio, A., Lemmon, M.A., Guarnieri, S., Falasca, M., (2000), The role of the pleckstrin homology domain in membrane targeting and activation of phospholipase Cβ1. J Biol Chem, 275, 14873–14881.

Rebecchi, M.J., Pentyala, S.N., (2000), Structure, Function, and Control of Phosphoinositide-Specific Phospholipase C. Physiological Reviews, 80, 1291-1335

Rhee, S.G., Bae, Y.S., (1997), Regulation of phosphoinositide-specific phospholipase C isozymes. J Biol Chem, 272, 15045–15048.

Runkel, F., Hintze, M., Griesing, S., Michels, M., Blanck, B., Fukami, K., Guénet, J.L., Franz, T., (2012), Alopecia in a viable phospholipase C delta 1 and phospholipase C delta 3 double mutant. PLoS One, 7, e39203.

Tabor, C.W., Tabor, H., (1984), Polyamines. Annu Rev Biochem, 53, 749–790.

Thompson, J.D., Higgins, D.G., Gibson, T.J., (1994), CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res, 22, 4673–4680.

Towbin, H., Gordon, J., (1984), Immunoblotting and dot immunobinding-current status and outlook. J Immunol Methods, 72, 313–340.

Wilsher, N.E., Court, W.J., Ruddle, R., Newbatt, Y.M., Aherne, W., Sheldrake, P.W., Jones, N.P., Katan, M., Eccles, S.A., Raynaud, F.I., (2007), The phosphoinositide-specific phospholipase C inhibitor U73122 (1-(6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) spontaneously forms conjugates with common components of cell culture medium. Drug Metab Dispos, 35, 1017–1022.

Yamamoto, T., Takeuchi, H., Kanematsu, T., Allen, V., Yagisawa, H., Kikkawa, U., Watanabe, Y., Nakasima, A., Katan, M., Hirata, M., (1999), Involvement of EF hand motifs in the $Ca^{(2+)}$-dependent binding of the pleckstrin homology domain to phosphoinositides. Eur J Biochem, 265, 481–490.

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

이 진 영

부경대학교 대학원 생물공학과

요 약

인지질가수분해효소 C-δ3 (PLC-δ3)는 원형질막에 존재하며, PIP_2 를 가수분해하여 2 차 전령체인 IP_3 와 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-δ1 와 PLC-δ3 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, 787 개의 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 PoPLC-δ3B 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 $PI(4,5)P_2$ 외에 PS, $PI(5)_2$ 등에 높은 감수성을 나타내었다. PoPLC-δ3A 가 활성을 가지지 못한 것에 대해, PoPLC-δ3A 가 non-processed pseudo gene 으로 존재하거나 PoPLC-δ3B 보다 더 극적인 조건하에 활성을 가질 것이라는 가설을 세웠다. 이 연구는 최초로 해양생물 유래의 PLC-δ3 에 대해 특성분석을 하였으며, 세포 신호전달에 관련해 분자생물학적 기초자료를 제공한다.

Acknowledgement

석사과정을 한마디로 요약하면, 실패와 작은 성공의 연속 끝에 끊임없이 제 자신을 돌이켜 보며 반성하는 시간이었던 것 같습니다. 또한, 이 글을 쓰기까지 3년 가까이의 석사과정 동안 정말 많은 사람을 만나고, 동시에 많은 일을 겪었던 것 같습니다. 더 열심히 하지 못한 것에 대한 후회가 남지만 동시에 제 인생의 초석을 닦을 수 있게 된 뜻 깊은 시간이었습니다.

우선 제가 어떤 결정을 내리든 항상 응원해주시고 아낌없이 제 뒷바라지를 해주신 저희 부모님께 먼저 감사의 인사를 드리고 싶습니다. 마찬가지로 언제나 절 믿어주신 할머니와 우리 모든 가족 분들 그리고 모자란 오빠지만 항상 도와주고 제가 신경 못쓰는 만큼 부모님께 더 잘해주는 누나 같은 동생 하영이에게 감사합니다. 많이 부족한 제자지만 학업 외에도 인생에 대해서 가르침을 선사해주신 지도교수인 이형호 교수님, 언제든 항상 저희를 먼저 생각해주시고 배려해주신 정준기 교수님 감사합니다. 그리고 어느 때고 느닷없이 찾아가도 도움을 주시기만 한 박남규 교수님, 몇 번 찾아 뵙지는 못했지만 그때마다 학문적으로 큰 가르침을 주신 공인수 교수님, 학사 과정 중 제가 정신차리고 열심히 공부 할 수 있는 계기를 주신 김중균 교수님, 이어 홍용기 교수님, 김성구 교수님, 정귀택 교수님께도 감사의 말씀 올립니다. 그리고 언제고 든든하게 저희를 이끌어 주신 김무상 박사님, 무지했던 절 이만큼이나 오게 만들어주신 안상중 박사님, 그리고 물심양면으로 저희를 지원해주시는 황지연 박사님, 양질의 음식을 사주시는 경훈 선배님, 수진 선배님, 강은 선배님, 그리고 같은 실험실 소속이 아님에도 불구하고 오랫동안 절 아껴주신 고혜진 박사님, 막바지에 제 실험결과가 한층 가치 있을 수 있게 도와주신 김찬희 박사님, 또 학사과정부터 지금까지 여러모로 지도해준 나영이 누나께 감사 드립니다. 그리고 욕 잘하는 목청 큰 시집간 아람이, 실험실의 영원한 롤모델 혜진이, 가족처럼 아껴주신 희성이형, 영림이형, 희영이, 혜인이, 성환이, 아는 것 하나 없이 욕밖에 안하는데도 잘 따라준 1호 제자이자 동생들 종득이, 기덕이, 다빈이, 예진이, 그리고 석사과정 동안 여러모로 많이 도와준 수경이누나, 지숙이누나, 종민이형, 정기형, 경환이, 규유, 건아, 귀요미 동현이, 창한이, 현이, 지영이, 다솜이, 쓸데없이 간접흡연 하면서 제 얘길 잘 들어준 태영이, 혜빈이, 늦은 새벽에도 혼자 있는 저의 곁을 지켜준 뚱이, 너희 만나서 너무 즐거웠고 고마웠습니다. 같은 과는 아니지만 같이 석사과정을 하며 여러모로 많은 도움을 주신 현호형, 짧은 만남에도 우리 실험실을 택해서 와준 현경이, 선빈이, 효정이, 태연이, 은빈이, 조연이 그리고 애들이랑 같이 앞으로 실험실을 이끌어 가게 될 진현이랑 소희에게 응원의 말을 전합니다. 수지랑 준형이, 지혜에게, 진현이랑 소희를 잘 부탁한다는 말과 함께 무사히 석사과정을 마칠 수 있게 응원합니다. 그리고 실험실 가족 중 가장 오랜 시간 동안 부족한 저 때문에 많이 고생한 세환이 그리고 특히 주현이에게 고맙단 말과 함께 어딜 가서든 지금의 모습 잃지 않으며 좋은 일만 가득하길 바랍니다.

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

주영이형, 창현이형, 동은이형, 혜령이누나, 저 인간 만들어주셔서 고맙습니다. 그리고 우리 32기 동기들 유열이, 창한이, 동민이, 미리, 수희, 특히 머나먼 타국까지 같이 가서 고생하고 또 같이 석사과정을 함께 할 수 있어서 좋았던 하빈이에게도 감사의 말을 전합니다. 10년 동안 모비딕스에 발을 끊을 수 없게 해준 후배들, 준우, 태영이, 재성이, 상욱이, 선승이, 단비, 창우 및 군대 안가는 지민이, 동백가디언 동율이, 짜바리 철희, 얼른 군대 가야 될 형우, 사서 고생하는 규준이, 어디서든 지금처럼 열심히 해서 좋은 결과 있길 바랍니다. 최근 활동기가 된 광민이, 영민이, 경준이, 채은이, 윤영이, 하림이, 예린이 다시 한 번 축하하고 멋진 무대 보여주길 기대합니다. 지금은 떠난 모비딕스의 수호자 구름이, 장군이, 동아리를 지켜줘서 고맙습니다. 그리고 후배임에도 불구하고 오랜 친구처럼 대해준 술친구, 담배친구인 혜림이와 승훈이한테 고맙습니다. 어릴 적부터 멀리에서도 항상 응원해주고 오랜 시간 함께해준 한빈이와 인창이, 동기, 창미, 끝으로 바쁜척하느라 제대로 챙겨주지도 못하는데 항상 제 옆을 지켜주고 자신감을 잃지 않게 해주며, 이 논문이 나오도록 마지막까지 도와준 저의 소중한 인연인 가을이에게 진심으로 고맙고 사랑한단 말을 전하고 싶습니다.

감사의 말을 끝으로 이 짧은 글에 이름을 언급하는 것으로, 혹은 제 짧은 기억력으로 언급하지 못한 모든 분들께 제가 느끼는 고마움을 다 표현하진 못하겠지만, 분명 모든 한 분, 한 분이 지금의 저를 만들어주셨을 것이라 생각하며, 그 모든 분들께 감사하며 이 소중한 인연이 오래 갈 수 있도록 은혜에 보답 하며 살아 가겠습니다.

다들 행복하시고 항상 건강하세요.

다시 한 번 감사합니다.