Biochemical Characterization of a Phospholipase A_2 from *Photobacterium damselae* subsp. *piscicida*

Po-Yuan Hsu[a], Kuo-Kau Lee[a], Pei-Shan Lee[b], Chih-Chuang Hu[a], Cheng-Hui Lin[a], and Ping-Chung Liu[a,*]

[a] Department of Aquaculture, National Taiwan Ocean University, 2, Pei-Ning Road, Keelung, Taiwan. E-mail: c0034@mail.ntou.edu.tw
[b] Eastern Marine Biology Research Center, Fisheries Research Institute, 22, Wuquan Road, Chenggong Township, Taitung, Taiwan

* Author for correspondence and reprint requests

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Photobacterium damselae subsp. *piscicida* (*Phdp*) is the causative agent of fish photobacteriosis (pasteurellosis) in cultured cobia (*Rachycentron canadum*) in Taiwan. A component was purified from the extracellular products (ECP) of the bacterium strain 9205 by fast protein liquid chromatography (FPLC) and identified as a phospholipase. An N-terminal sequence of 10 amino acid residues, QDQPNLDPGK, was determined by mass spectroscopy (MS) and found to be identical with that of another *Phdp* phospholipase (GenBank accession no. BAB85814) at positions 21 to 30. The corresponding gene sequence of the phospholipase (GenBank accession no. AB071137) was employed to design primers for amplification of the sequence by the polymerase chain reaction (PCR). The PCR products were transformed into *Escherichia coli*, and a recombinant protein product was obtained which was purified as a His-tag fusion protein by Ni-metal affinity chromatography. A single 43-kDa band was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Phosphatidylcholine was degraded by this protein to lysophosphatidylcholine and a fatty acid. These products were characterized by thin-layer (TLC) and gas chromatography (GC), respectively, allowing the identification of the protein as a phospholipase A_2. The recombinant protein had maximum enzymatic activity between pH 4 and 7, and at 40 °C. The activity was inhibited by Zn^{2+} and Cu^{2+}, activated by Ca^{2+} and Mg^{2+}, and completely inactivated by dexamethasone and *p*-bromophenacyl bromide. A rabbit antiserum against the recombinant protein neutralized the phospholipase A_2 activity in the ECP of *Phdp* strain 9205 and the recombinant protein itself. The recombinant protein was toxic to cobia of about 5 g weight with an LD_{50} value between 2 and 4 μg protein/g fish. The results revealed phospholipase A_2 as a fish toxin in the ECP of *Phdp* strain 9205.

Key words: Photobacterium damselae subsp. *piscicida*, *Rachycentron canadum*, Phospholipase A_2

Introduction

Fish photobacteriosis is a bacterial fish disease commonly called "pasteurellosis" or "pseudotuberculosis" due to infection by halophilic *Photobacterium damselae* subsp. *piscicida* (*Phdp*) (formerly *Pasteurella piscicida*). The signs of the disease in the chronic form are typical whitish tubercles in the internal organs which consist of bacterial accumulations (Magarinos *et al*., 1996). It is an important disease in aquaculture, especially in the intensive culture of different fish species in the Mediterranean Sea, Atlantic Ocean, and in Japan (Daly, 1999).

The classification of phospholipases (PLs) gives a general overview of phospholipases A_1, A_2, B, C, D; PLs are an important group of enzymes that hydrolyze phospholipids and release a variety of products, like lysophospholipids, free fatty acids, diacylglycerols, choline, phosphate, and phosphatidates, depending on the site of hydrolysis (Molly and Wadstrom, 1978). Phospholipase A (PLA) has been studied exhaustively in snake venoms and can be found in most Gram-negative bacteria (Snijder and Dijkstra, 2000). Phospholipases A_1 (PLA_1) and A_2 (PLA_2) have a specific capacity to hydrolyze the carboxylic ester bond at the *sn*-1 and *sn*-2 position, respectively, of a phospholipid. The action of PLA_1 and PLA_2 results in the accumulation of free fatty acid and 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively (Istivan and Coloe, 2006).

PLs are considered to be virulence factors for bacterial species which cause disparate disease

syndromes, from infections causing massive tissue destruction, such as gas gangrene, skin and lung infections by *Pseudomonas aeruginosa*, and food-borne listeriosis (Schmiel and Miller, 1999). There are many studies concerning the role played by PLs in these bacterial diseases. PLA has been reported as a potent virulence factor of *Vibrio vulnificus*, inducing cytotoxicity and lethality (Koo *et al*., 2007); *Legionella* PLA may act as a powerful agent in the mediation of pathogenicity due to destruction of lung surfactant and epithelial cells (Flieger *et al*., 2000); PLA activity contributes to the cell-associated hemolytic activity of *Campylobacter coli* and may play some role in its virulence (Grant *et al*., 1997).

In a previous study, a PL activity was found among the extracellular products (ECP) of *Phdp* strain 9205 (Hu, 2005); however, it was difficult to purify and obtain in larger quantity for further studies. In the present study, we purified the enzyme and determined its N-terminal sequence by mass spectroscopic sequencing to identify its similarity to the PL from *Phdp* (GenBank accession no. BAB85814). Therefore, the corresponding gene sequence of the PL from *Phdp* (Naka *et al*., 2007) was cloned and expressed in *Escherichia coli*. The recombinant PL was purified, characterized, and found to be a toxin of the bacterium in cobia (*Rachycentron canadum*).

Materials and Methods

Bacterium and extracellular products (ECP)

Photobacterium damselae subsp. *piscicida* (*Phdp*) strain 9205, originally isolated from diseased cobia (*Rachycentron canadum*) in Taiwan, was used in this study (Liu *et al*., 2003). A stock culture of strain 9205 was grown on brain heart infusion agar (Difco, Detroit MI, USA) containing 2% NaCl (BHIA2) for 24 h at 28 °C. The ECP were harvested by a procedure previously described (Lee and Ellis, 1990). Total protein was measured by the method of Bradford (1976) with bovine serum albumin as the standard. PL activity of the ECP was detected by placing 20-μl samples of ECP in wells cut in agarose [1% in phosphate buffered saline (PBS), pH 7.2] that contained 0.2% egg yolk. The plates were incubated in a humidified chamber for 24 h at 25 °C. The diameter of the lytic halo of each well was measured (Titball and Munn, 1981).

Purification of extracellular PL

According to previous studies (Hu, 2005; Liu *et al*., 2011), purification of the enzyme was achieved by fast protein liquid chromatography (FPLC) (Pharmacia, Uppsala, Sweden) with anion exchange columns (Q Sepharose high-performance and Resource Q; Pharmacia) equilibrated with 20 mM tris(hydroxymethyl) methylamine (Tris buffer, pH 7.0). Fractions were eluted with a sodium chloride gradient (0–1.0 M NaCl) at a rate of 1 ml/min. The fractions possessing PL activity were pooled and dialyzed against 20 mM Tris buffer, pH 7.0, without loss of activity.

PL zymogram and determination of sequence

At each purification step, electrophoresis of the fractions was performed on native-polyacrylamide gel electrophoresis (PAGE) 10–15 gradient Phastgels (Pharmacia, Wauwatosa, WI, USA). After electrophoresis, the Phastgels were overlaid with gels (1% agarose, 100 mM Tris, pH 8.0, 0.9% NaCl) containing 5% egg yolk suspension for a PL zymogram (egg yolk to PBS ratio, 1:1, v/v) (Fiore *et al*., 1997). Then, the band exhibiting PL activity was excised and subjected to sodium dodecyl sulfate (SDS)-PAGE (Novex 10% Bis-Tris Gel; Invitrogen, Carlsbad, CA, USA), and thereafter transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was used for N-terminal sequencing by automated Edman degradation in an Applied BioSystems (Foster, AL, USA) Procise 494A protein sequecncer. Mass spectroscopic analysis of the purified enzyme was performed using the QSTAR XL system (Applied BioSystems).

Purification of recombinant PL from Escherichia coli

The identified PL sequence was compared to the information in GenBank and translated into a DNA sequence to design primers for polymerase chain reaction (PCR). The PCR product was excised and ligated into the pGEM-T Easy Vector (Promega, Madison, WI, USA), and transformed into *E. coli* JM109. The PL gene was digested with *Nde* I and *Sal* I, subcloned into the pET25a (+) vector, and transformed into *E. coli* BL21 (DE3) pLysS (Novagen, Madison, WI, USA). A single colony was inoculated into 3 ml Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract,

1% NaCl) containing 50 μg/ml ampicillin, and cultured for 16 h at 37 °C.

One ml of precultured *E. coli* suspension was inoculated into 600 ml of LB medium, and the bacteria were grown for 2 h to reach an OD600 of 0.6. The expression of the protein was induced by adding isopropyl-2-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the culture was incubated for 4 h at 37 °C under vigorous shaking. Bacterial cells were harvested by centrifugation, and the resulting pellet was resuspended in 20 mM Tris-HCl (pH 8.0). The bacterial cell suspension was sonicated; the resulting pellet was resuspended in 6 M urea and dissolved for 2 h at 4 °C. The suspension was then centrifuged and filtered through a 0.22-μm (Millipore, Dublin, Ireland) syringe filter unit to remove undissolved material.

A Ni-metal chelating affinity chromatography column packed with 5 ml Ni-NTA resin and precharged with 50 mM $NiSO_4$ was applied for protein purification. The column was equilibrated with binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9). The samples were loaded onto the column and washed with 10 volumes of washing buffer (0.5 M NaCl, 20 mM Tris-HCl, 50 mM imidazole, pH 7.9). The protein was then eluted with eluting buffer (0.5 M NaCl, 20 mM Tris-HCl, 200 mM imidazole, pH 7.9). The protocol of the purification followed the standard operating procedures manual of Novagen (Ni-NTA His-Bind$^{\circ,R}$resins). SDS-PAGE and silver stain reagent (Amersham, London, England) were used to check the purity. The apparent molecular mass of the purified protein was determined by comparison with the PageRuler™ Prestained Protein Ladder #SM0671 (Fermentas, Vilnius, Lithuania). Native-PAGE was employed in each purification step, and PL zymograms were established as described above.

Thin-layer chromatography (TLC) and gas chromatography (GC)

To determine the specificity of the PL activity of the purified protein, L-α-phosphatidylcholine (L-α-PC) (P5394; Sigma, St. Louis, MO, USA), L-α-lysophosphatidylcholine (L-α-LPC) (L-4129; Sigma), and the reaction products were separated by TLC. One hundred μg of L-α-PC were mixed with 1 mg/ml PLA and then incubated at 37 °C for 16 h. The reaction was terminated by addition of 1 ml chloroform/methanol (2:1, v/v). After vortexing, the mixtures were centrifuged, and the chloroform layer was collected evaporated using a rotary evaporator; the resulting residue was redissolved in 20 μl chloroform. The samples were spotted on a silica gel-60 F_{254} (Merck, Darmstadt, Germany) plate which was developed with chloroform/methanol/acetic acid/water (55:17:6.5:2.5) to detect L-α-LPC or L-α-PC. After development, the plate was sprayed with 50% aqueous sulfuric acid and heated at 115 °C for 30 min to visualize L-α-LPC or L-α-PC spots (Hayashi and Kishimura, 1996). The free fatty acids were absorbed on Sep-pak® silica cartridges (25 mm × 10 mm i.d.; Water Associates, Milford, MA, USA) as described by Bitman *et al.* (1984). Then, neutral and polar lipids were eluted by diethyl ether/*n*-hexane (1:1) and chloroform/methanol (1:1), respectively. The preparation of fatty acid methyl esters followed the method described by Metcalfe and Schmitz (1961). Fatty acid analysis was performed using gas-liquid chromatography (Trace GC2000; ThermoFinnigan, Austin, TX, USA) and column chromatography (Restek's capillary column, 30 m × 0.28 mm i.d., 0.25 μm film thickness, Stabilwax; H & P Co., Bellefonte, PA, USA). Fatty acids were identified by comparison of the retention time of the sample compared to those of standards of fatty acid methyl esters (GLC-68A; Nu-Chek-Prep, Elysian, MN, USA).

Rabbit antiserum against the recombinant protein

The recombinant protein (140 μg protein/ml) was treated with 3% formalin at 4 °C for 48 h, and then dialyzed against three changes of distilled water for 24 h at 4 °C, prior to the immunization of a New Zealand white rabbit weighing 2 kg. Two ml of the dialyzed preparation were emulsified in an equal volume of Freund's complete adjuvant (Sigma), and injected into the rabbit subcutaneously. The rabbit was boosted with the same antigen emulsified in Freund's incomplete adjuvant three times at two-week intervals. The antiserum against the recombinant protein was obtained two weeks later after a final booster. Aliquots of 1 ml of antiserum were stored at –70 °C.

Western blotting

Eighteen μl of the recombinant protein (1 μg/μl), pre-mixed with 6 μl of sample buffer (0.06 M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 4%

β-mercaptoethanol, 0.0025% bromophenol buffer) and 3 μl of dithiothreitol (10 mM), were boiled for 15 min, then subjected to SDS-PAGE and transferred to a PVDF membrane. For Western blot analysis, the blotted membrane was immersed in a blocking solution [5% (w/v) fat-free milk, 0.2% Tween 20, 0.02% sodium azide] for 2 h and then washed with 100 mM Tris-base, 1.5 M NaCl, pH 7.9, four times, 5 min each. The membranes were incubated with the first antibody (rabbit antiserum to the purified recombinant protein) in blocking solution for 2 h. After thorough washing for four times, the membrane was incubated with the second antibody (goat antibody to rabbit immunoglobulins was coupled to alkaline phosphatase for 1 h prior to the incubation). After thorough washing for four times, alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-base, pH 7.9) containing BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (KPL, Gaithersburg, MD, USA) was used for colour development. One hundred ml of 20 mM EDTA were used as a stop solution.

PLA_2 activity assay

The Cayman Chemical Secretory PLA_2 Assay kit (Ann Arbor, MI, USA) (Reynolds *et al.*, 1992) was employed for the PLA_2 assay, and a bee venom PLA_2 was used as a standard. The substrate used for the assay was the 1,2-dithio analogue of diheptanoyl phosphatidylcholine (PC). Free thiols released in PLA_2 catalyzed the hydrolysis of the thioester bond at the *sn*-2 position which was detected using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB). One unit (U) of the enzymatic activity corresponds to the hydrolysis of 1 μM of diheptanoyl thio-PC per minute at 25 °C.

Effects of temperature, pH, and divalent cations on PL activity

For determination of the optimal pH value of the reaction, the purified PL preparation was added to buffers with pH values adjusted from 3 to 11; then the substrate was added, and the assay carried out as described above. For determination of the thermostability of the enzyme, aliquots of the purified PL were incubated separately for 30 min at 4 °C, 10 °C, and then in 10-degree intervals up to 90 °C, respectively, then directly cooled on ice prior to the PL assay, carried out as described above. For both pH and temperature dependence, the highest PL activity was used as a control (100% of relative activity), as previously described (Liu *et al.*, 2011).

For determination of the effect of divalent cations, 300 μl of the PL preparation were added to an equal volume of buffer containing 20 mM divalent cations to give 10 mM final concentration. The mixture was incubated for 30 min at 37 °C, and then assayed as described above. The activity of the sample without added cations was used as a control (100% of relative activity).

For determination of the effect of inhibitors, 300 μl of the sample preparation were added to an equal volume of buffer containing either EDTA (final concentration 10 mM) or dexamethasone (DEX, specific PLA_2 inhibitor; Sigma, D1756), to give 1 or 10 μM (Piltch *et al.*, 1989), or *p*-bromophenacyl bromide (BPB, specific secretory PLA_2 inhibitor; Sigma, D38308), to give 10 or 100 μM (Seibert *et al.*, 2006); then the mixture was assayed as above. The activity of the sample without added inhibitor was used as a control (100% activity).

Immuno-neutralization tests of PLA_2 activity

One hundred μl rabbit antiserum against the recombinant protein were incubated with an equal volume of ECP (0.7 mg protein/ml) or the recombinant protein (0.44 mg protein/ml) at 37 °C for 60 min prior to the assay of the PL activity. The activity of the recombinant protein incubated with PBS only was used as a control (100% activity).

Lethality of the recombinant protein to cobia

Cobia weighing (5 ± 0.5) g were purchased from a fish farm and held in tanks (2500 l) supplied with aerated 3% salinity seawater at 25–28 °C. The LD_{50} tests, with batches of six fish per dose (doubling dilution), were conducted by intraperitoneal (i.p.) injection of 0.1 ml sample in different doses into the fish (Trevors and Lusty, 1985). Sterile PBS was injected into control fish. Mortalities were recorded daily for 5 d. Experiments with the fish were performed according to "The Ethical Guideline for Using Vertebrates as Experimental Animals in Taiwan" and had been approved by the "Ethical Committee for Using Vertebrates as Experimental Animals of National Taiwan Ocean University".

Results

Purification of extracellular PL

Total protein concentration of ECP was 0.831 mg/ml. ECP exhibited strong degrading activity against egg yolk (15–20 mm diameter of lytic halo). ECP of *Phdp* were eluted as three major peaks in the FPLC on a Q Sepharose high-performance column (FPLC-HP); fractions 111–117 ml exhibited PL activity (Fig. 1). After pooling and dialysis of fractions possessing PL activity, they eluted as five major protein peaks in the FPLC on a Resource Q column, with PL activity eluting between 24 and 26 ml (Fig. 2).

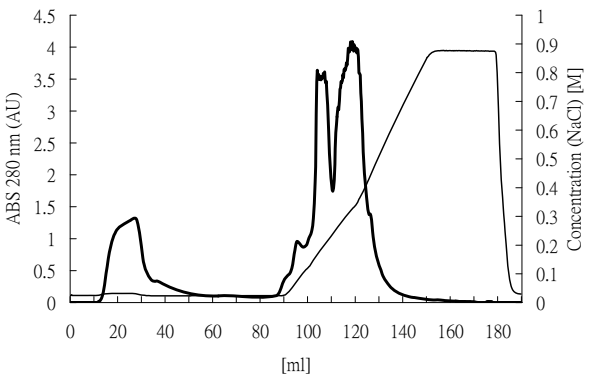

Fig. 1. Profile of FPLC-anion exchange chromatography (FPLC-HP) of ECP. The sample was applied to a Q Sepharose high-performance column and eluted with a step gradient of 0 to 1 M NaCl in Tris buffer (pH 7.0) at the rate of 1 ml/min. Fractions 111–117 ml exhibited phospholipase activity.

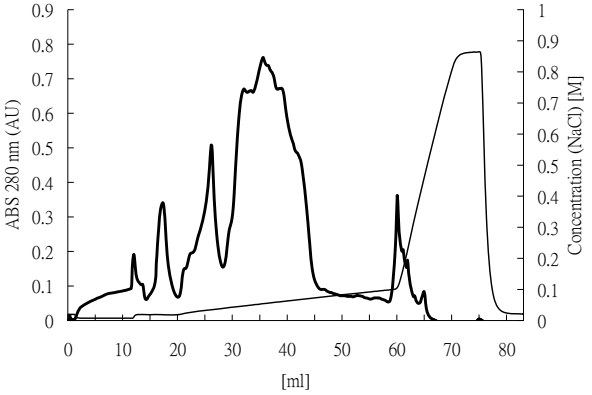

Fig. 2. Profile of FPLC-anion exchange chromatography (FPLC-Resource Q) of the pooled fractions 111–117 ml from FPLC-HP. Elution was performed as in Fig. 1. Fractions 24–26 ml exhibited phospholipase activity.

PL zymogram and determination of sequence

Protein preparations of each purification step were subjected to native-PAGE, and one single band was visualized in the final purified fraction (Fig. 3). The band was excised, subjected to SDS-PAGE, and then transferred to a PVDF membrane for mass spectroscopic N-terminal sequencing. A sequence of 10 amino acid residues, QDQPNLDPGK, was determined and found to be the same as in *Phdp* PL (GenBank accession no. BAB85814) at positions 21–30, C-terminal to a predicted signal peptide sequence at positions 1–20. The gene of this *Phdp* PL (GenBank accession no. AB071137) was used for expression in an *Escherichia coli* system. The gene encoding this PL was obtained by PCR amplification. The forward primer (5-CATATGAAAAAGTCAGTACTTACATTGACG-3) began from the initiation codon and added an *Nde* I site at the 3' end of the gene, and the reverse primer (5-TTAATGATGATGATGATGATGGAAGTTAAATTGTGAAGATAC-3) contained the sequence for six histidine residues (His tag) and ended before the stop codon. The sequence of the PCR product was identical with that of GenBank accession no. AB071137.

Purification of recombinant PL from E. coli

Bacterial cells from 600 ml culture were disrupted by sonication, and 75 mg of total protein were obtained. After refolding of the inclusion

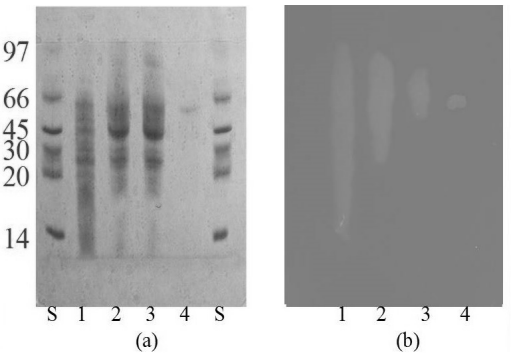

Fig. 3. (a) Protein profile and (b) zymogram of phospholipase activity in samples from each step of the purification of ECP. (a) Native-PAGE 10-15 gradient Phastgel stained with Coomassie Brilliant Blue. (b) Zymogram of overlay of (a). Lane S, low-molecular weight calibration kit (14.4 to 97 kDa; Amersham); lane 1, ECP (1 μg); lane 2, Q sepharose (0.8 μg); lane 3, Resource Q (0.8 μg); lane 4, purified protein (0.1 μg).

body, the specific activity increased 2.85-fold, while recovery was 62.5%. The enzyme was finally purified by metal affinity chromatography, and 1 mg enzyme was obtained. The specific activity of the purified protein was 2.08 U/mg (Table I), and was increased 8.125-fold over that of the cell homogenate. The recombinant protein exhibited a single band on SDS-PAGE after silver staining, and its apparent molecular mass was about 43 kDa (Fig. 4).

Classification of PL

To define the specificity of the recombinant PL, L-*α*-PC was used as the substrate. PLA would produce L-*α*-LPC and a fatty acid from L-*α*-PC. As shown in the TLC chromatogram of the reaction mixture (Fig. 5), a spot was visualized at *Rf* 0.25, the same as that of standard L-*α*-LPC, together with a fatty acid. The free fatty acids released from L-*α*-PC by the enzyme were separated into neutral and polar lipids, and further analysed by GC. As shown in Table II, four peaks of the esters methyl myristate (25.5%), methyl palmitoleate (31.5%), methyl oleate (18%), and methyl linoleate (25%), appearing between 5 and 18 min, were detected in the polar lipids. However, no peak was detected in the neutral lipids.

Western blotting

The rabbit antiserum against the recombinant protein recognized both a component in the ECP of *Phdp* and the recombinant protein as a single polypeptide band at 43 kDa (Fig. 6). This confirmed that the sequence encoding the 43-kDa protein had been precisely cloned from the *Phdp* strain 9205 and furthermore, that the rabbit antiserum to the recombinant protein was monospecific.

Table I. Purification of the recombinant protein.

Purification step	Total protein [mg]	Specific activity [U/mg]	Total activity [U]	Yield (%)	Purification fold
Cell homogenate	75	0.256	19.2	100	1
Inclusion body refolded	16.5	0.73	12	62.5	2.85
Ni-NTA affinity chromatography	1	2.08	2.08	10.8	8.125

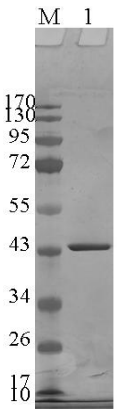

Fig. 4. SDS-PAGE and silver stain of recombinant protein purified by metal affinity chromatography. The apparent molecular mass of the purified protein was determined by comparison with a prestained protein ladder. Lane M, marker (from 10 to 170 kDa); lane 1, purified recombinant protein (1 μg).

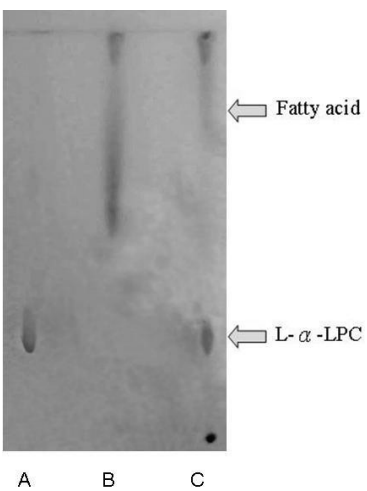

Fig. 5. Thin-layer chromatogram of the reaction products of the recombinant PL. Reaction mixtures containing 100 μg L-*α*-PC and 1 mg/ml PLA_2 (see Materials and Methods for details) were incubated for 16 h. Lane A, standard of L-*α*-LPC; lane B, standard of L-*α*-PC; lane C, L-*α*-PC incubated with the recombinant protein.

Effects of temperature, pH, and divalent cations on recombinant PL

The recombinant PL was active in the temperature range of 4 to 40 °C, but activity decreased at 50 °C (residual activity 64%) (Fig. 7). The recombinant protein showed maximal enzymatic activity at pH 7, with more than 80% of the activity at pH 6 and 8 (Fig. 8). The enzymatic activity of the recombinant protein was activated by Ca^{2+} and Mg^{2+} and inactivated by Zn^{2+} and Cu^{2+} (Table III).

PLA_2 inhibitors

The PL activity of the recombinant protein was almost completely (> 97%) inhibited by 1 μM dexamethasone (DEX; specific PLA_2 inhibitor) and 10 μM *p*-bromophenacyl bromide (BPB; specific inhibitor of secretory PLA_2) (data not shown).

Immuno-neutralization and fish lethality of the recombinant protein

In the immuno-neutralization test, the majority of PLA_2 activity of the ECP was inhibited by the rabbit antiserum against the recombinant protein, while that of the purified protein was completely inhibited.

The purified recombinant protein from *Phdp*, injected intraperitoneally, was lethal to cobia (*Rachycentron canadum*) with an LD_{50} value between 2 and 4 mg protein/g fish. No mortality was observed in the controls injected with sterile PBS (Table IV).

Table II. Fatty acid esters released from L-α-phosphatidylcholine by recombinant protein.

Fatty acid ester	Content (%)[a]
14:0 (methyl myristate)	25.5
16:1 (methyl palmitoleate)	31.5
18:1 (methyl oleate)	18
18:2 (methyl linoleate)	25

[a] Percentage of total fatty acids.

Discussion

Cobia (*Rachycentron canadum*) is an important fish in aquaculture widely cultured in sea cages in Taiwan, either for domestic consumption or for

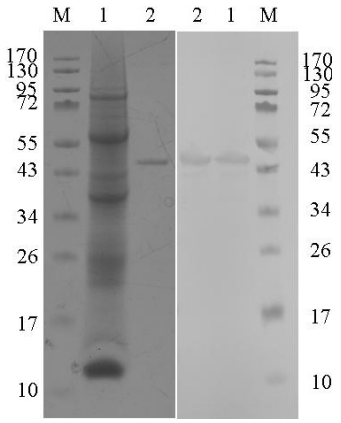

Fig. 6. Western blotting analysis of recombinant protein. Rabbit antiserum to the recombinant protein was used as 1st antibody and goat antibody to rabbit immunoglobulins as 2nd antibody. Lane M, marker (from 10 to 170 kDa); lane 1, ECP of *Phdp* (5 μg); lane 2, recombinant protein (1 μg).

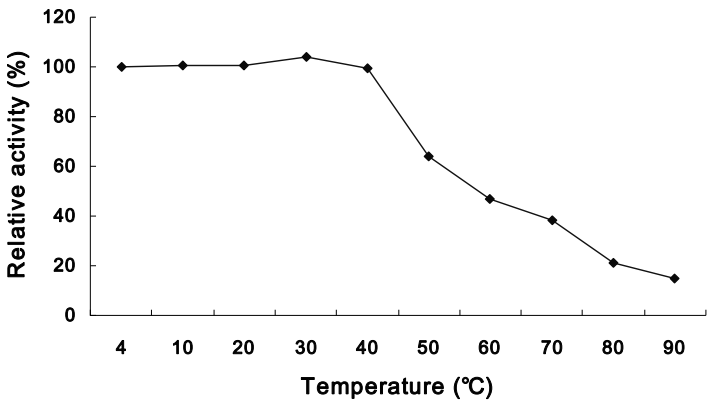

Fig. 7. Stability of the phospholipase activity of the purified recombinant protein as function of temperature. See Materials and Methods for details.

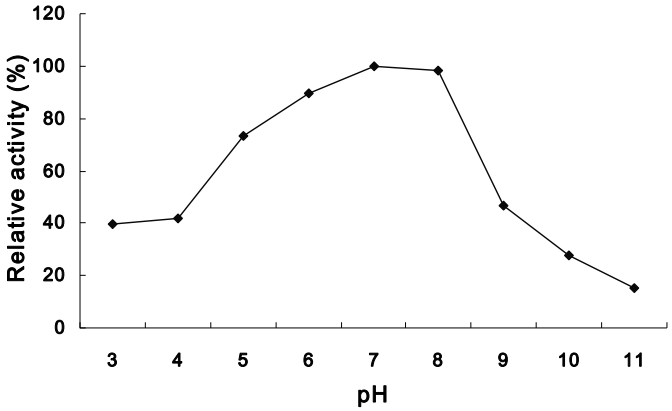

Fig. 8. Phospholipase activity of the recombinant protein as a function of the pH value.

export, mainly to Japan (Su *et al*., 2000). Three dominant bacterial diseases, caused by *Vibrio* sp., *Streptococcus* sp., and *Photobacterium damselae* subsp. *piscicida* (*Phdp*), have been identified in cobia in Taiwan (Ku and Lu, 2000; Liu *et al*., 2003, 2004a, b). Of these, *Phdp* was found to be the most important pathogen (Ku and Lu, 2000).

In previous studies, phospholipase (PL) and protease activities were found in the extracellular products (ECP) of *Phdp* (Hu, 2005; Liu *et al*., 2011). The extracellular protease was purified and characterized as a 34.3-kDa toxic metalloprotease (Liu *et al*., 2011), however, the extracellular PL required further characterization. PL has been suggested to be an important toxin in the pathogenesis of some bacterial species causing different disease syndromes such as massive tissue destruction related to gas gangrene of skin and lung infection caused by *Pseudomonas aeruginosa* (Schmiel and Miller, 1999). In addition, a phospholipase A_2 (PLA_2) [also termed glycerophospholipid: cholesterol acyltransferase (GCAT)] was identified as a component highly toxic to Atlantic salmon in ECP of *Aeromonas salmonicida* for Atlantic salmon (Lee and Ellis, 1990).

Both secreted and membrane-bound PLA_2 activities have been described in bacteria, fungi, and protozoa. These PL produced by various pathogens have been suggested to be important virulence factors (Istivan and Coloe, 2006). Some bacteria produce both types of PL, while others produce only one type (Schmiel and Miller, 1999; Snijder and Dijkstra, 2000). Two PL activities with different molecular masses were confirmed in ECP of *Phdp* (Hu, 2005). The one with the lower mass was confirmed as a phospholipase C (Hsu *et al*., unpublished results), while the one with the higher mass studied here was found similar to that reported by Naka *et al*. (2007). These authors reported the hemolytic activity of a PL from the ECP of *Phdp*, but did not characterize this PL further.

In recent studies, PLA has been extracted from the pancreas of pig, cattle, and human, and has been expressed heterologously and produced in larger quantity in prokaryotic systems (de Geus *et*

Table III. Effect of divalent metal ions on the PLA_2 activity of the recombinant protein.

Treatment (10 mM)	Relative enzymatic activity (% of control)
None	100
$ZnCl_2$	38
$MgCl_2$	126
$CuCl_2$	10
$CaCl_2$	122

Table IV. Lethality tests of the recombinant protein for cobia [(5 ± 0.5) g; 6 fish per dose)] by intraperitoneal injection of a 0.1-ml sample.

Dose (μg protein/g fish)	Mortality	LD_{50} (μg protein/g fish)
PBS	0/6	2–4
0.5	1/6	
1	2/6	
2	2/6	
4	4/6	
8	5/6	

al., 1987; Deng *et al*., 1990; Han *et al*., 1997). In the present study, we purified a 43-kDa PL from ECP of *Phdp* (Figs. 1–3) and revealed by N-terminal sequencening that it was identical with the PL from another strain of *Phdp* (GenBank accession no. BAB85814). Primers were designed for the corresponding gene sequence of our strain (GenBank accession no. AB071137) for cloning and expression in an *Escherichia coli* system (Fig. 4). The recombinant protein was purified and characterized as a PL.

N-terminal sequencing indicated that the PL gene coded for a protein of 405 amino acid residues containing a 20 amino acid signal, similar to that reported by Naka *et al*. (2007). As demonstrated by Mergulhão *et al*. (2005), the type II secretion system of bacteria mediates a two-step process for the extracellular secretion of proteins by periplasmic translocation. The signal peptide of recombinant proteins is usually cleaved during secretion across the cytoplasmic membrane. According to Istivan and Coloe (2006), an outer-membrane PLA (OMPLA) gene of *Escherichia coli* encodes a 31-kDa protein of 269 amino acids containing a 20 amino acid signal sequence. It is likely that the export of the PL of *Phdp* strain 9205 is also mediated by the type II secretion system.

Phospholipase C (PLC) is an enzyme well studied which acts on phospholipids to produce the phospho head group and diacylglycerol (DAG). PLA_2 catalyzes the selective hydrolysis of the *sn*-2-acyl group in 1,2-diacyl-*sn*-glycero-3-phospholipids and releases fatty acids; it occurs in both intracellular and secreted forms (Lee *et al*., 2002). In this study, L-α-LPC and fatty acids were detected by TLC as the products of L-α-PC hydrolysis by the recombinant protein (Fig. 5), indicating that the protein is a member of the PLA family. By GC, four different fatty acids as their methyl esters, *i.e.* myristate, palmitoleate, oleate, and linoleate were detected (Table II), confirming that the recombinant protein was PLA_2 rather than PLC. In a Western blot, the rabbit antiserum against the recombinant protein specifically recognized the PL in ECP of *Phdp* (Fig. 6), confirming that the gene cloned and expressed in the *Escherichia coli* system came, indeed, from *Phdp* strain 9205.

The recombinant protein maintained its activity upon incubation for 30 min between 4 and 40 °C, but was inactivated at higher temperatures (Fig. 7). It had a maximal activity at pH 7.0 (Fig. 8). Thus, the protein is a heat-labile neutral PL. In previous studies, divalent metal ions, such as calcium and magnesium, were reported as cofactors of prokaryotic PLs (De-Maria *et al*., 2007). The calcium ion is related to pathogenesis in most microorganisms (Istivan and Coloe, 2006). PLA from *Vibrio mimicus* was activated by 25% by 1 mM Ca^{2+} (Lee *et al*., 2002), while we found that the recombinant protein was activated by 22% and 26% by 10 mM Ca^{2+} and Mg^{2+}, respectively (Table III).

The activities of PLA_2 from many sources can be inactivated by a specific inhibitor of secretory PLA_2, *p*-bromophenacyl bromide (BPB), strongly suggesting that a histidine is conserved in the active site (Sajal, 2003; Tischfield, 1997). Park and Kim (2003) reported that a specific PLA_2 inhibitor, dexamethasone (DEX), effectively inhibits the activation of prophenoloxidase (proPO) *in vivo* in the hemolymph of *Spodoptera exigua*. The PL activity of our recombinant protein was inhibited by both BPB and DEX.

The antiserum against the recombinant protein neutralized the PL activity of the recombinant protein and most, but not all of the activity in ECP indicating that some other PL activities may be present in ECP, including PLC (Hu, 2005). However, further studies are required to identify these activities.

The toxicity of PLs has been linked to the cytotoxic activity resulting from the accumulation of membrane-destabilizing products, or by the extensive destruction of membrane phospholipids (Istivan and Coloe, 2006). Koo *et al*. (2007) identified PLA activity as an important factor in the cytotoxicity and lethality caused by *Vibrio vulnificus*. In the present study (Table IV), the purified recombinant protein (LD_{50} value between 2 and 4 μg protein/g fish) was found more virulent than the metalloprotease from the same strain (LD_{50} 6.8 μg protein/g fish) (Liu *et al*., 2011). As the LD_{50} value of the recombinant protein was similar to that of the total ECP (LD_{50} 3.25 μg protein/g fish), it must be considered as an important virulence factor in ECP of the bacterium.

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