



Document heading doi: 10.1016/S1995-7645(14)60150-4

Phospholipase C from *Pseudomonas aeruginosa* and *Bacillus cereus*; characterization of catalytic activity

Nooran Sherif Elleboudy, Mohammad Mabrouk Aboulwafa*, Nadia Abdel-Haleem Hassouna

Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

ARTICLE INFO

Article history:

Received 30 May 2014

Received in revised form 15 June 2014

Accepted 25 July 2014

Available online 20 November 2014

Keywords:

Phospholipase C

*Pseudomonas aeruginosa**Bacillus cereus*

Catalytic activity

Substrate specificity

ABSTRACT

Objective: To study characteristics of phospholipases C (PLCs), their importance for producing microorganisms as well as the potential of their use for industrial purposes. **Methods:** PLC from *Bacillus cereus* (*B. cereus*) D101 was selected as an example of Gram-positive PLCs and PLC from *Pseudomonas aeruginosa* (*P. aeruginosa*) D183 of Gram-negative ones. Enzymes were partially purified by ammonium sulfate precipitation followed by membrane dialysis. Partially purified preparations were used to study effect of different factors on activities as well as in substrate specificity tests which were conducted using a turbidimetric assay method. **Results:** Maximum activity was at pH 7 and 8 and 40 °C for *P. aeruginosa* PLC, and pH 8–10 and 37 °C for *B. cereus* PLC. Both PLCs were inhibited by Pi at 5 mM or higher, whereas, PLC from *B. cereus* only was inhibited by EDTA. Activity of *P. aeruginosa* PLC was not affected by removing Zn²⁺ ions from reaction mixture or their replacement with Ca²⁺, Ba²⁺, Mg²⁺ or Mn²⁺ ions. Vis-à-vis, activity of *B. cereus* PLC was found to be metal ion dependent. PLCs from both isolates were relatively thermostable and showed maximum affinity toward phosphatidylcholine. Sphingomyelin and phosphatidylethanolamine were not good substrates and phosphatidylinositol, phosphatidylserine, phosphatidylglycerol and cardiolipin could be considered non-substrates. **Conclusions:** Human body physiological conditions could favor activity of *P. aeruginosa* and *B. cereus* PLCs. These enzymes may participate in phosphate scavenging and virulence of producing isolates but not in autolysis. PLCs from both isolates are potential candidates for industrial use.

1. Introduction

Phospholipases are a heterogeneous group of enzymes that share the ability to hydrolyze one or more ester linkage in glycerophospholipids. Phospholipase C (PLC) hydrolyzes the phosphodiester bond in the phospholipid backbone[1]. There are sundry functions for phospholipases in prokaryotic biology, including their noteworthy contributions to microbial virulence[2]. The most important class of phospholipases that have shown to play a significant role

in pathogenesis is PLC[3]. PLC can be classified into Gram-positive PLCs and Gram-negative PLCs[4-5]. All Gram-positive PLCs, prototyped by those of Clostridia, Bacillus and Listeria, have molecular masses ranging from 29–43 kDa[4]. Gram-negative PLCs is a superfamily of PLCs with molecular weights of 50–80 kDa and prototyped by *Pseudomonas aeruginosa* (*P. aeruginosa*) PLC[6].

There are numerous industrial applications for phospholipases. Lipopan FTM, by Novozymes A/S, a phospholipase A from *Fusarium oxysporum* is in the market for baking application[7]. Hydrolysis of milk phospholipids with phospholipases before renneting process significantly increases cheese yield, possibly due to fat retention[8]. PLC mediated oil degumming is a well established process step in the physical refining of vegetable oil[9]. This article

*Corresponding author: Mohammad Mabrouk Aboulwafa, PhD, Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Al Khalifa Al Maamoun St., Abbassia, Cairo, Egypt. Postal code: 11566.

Tel: (202)24025824

Fax: (202)24051107

E-mail: maboulwafa@yahoo.com

deals with different parameters that may affect the catalytic activity of PLC produced by two different clinical isolates as well as the substrate specificity of the two produced enzymes.

2. Materials and methods

2.1. Chemicals

All chemicals were supplied, unless otherwise stated, by El-Nasr Chemicals (Adwic), Egypt. Phospholipid substrates, *p*-nitrophenylphosphorylcholine (NPPC) and bovine serum albumin (BSA) fraction V were products of Sigma–Aldrich Co., St. Louis, MO, USA.

2.2. Bacterial strains and maintenance

Isolates D101 and D183 were obtained through screening of 230 clinical isolates for phospholipase production. Both isolates were recovered from pus specimens obtained from patients of El–Demenrdash Hospital, Cairo, Egypt^[10].

2.3. Enzyme production

Cells from 1–day–old cultures on nutrient agar (Difco) slants were harvested in sterile normal saline. The final count in the cell suspension was adjusted to about 2×10^8 CFU/mL turbidimetrically at 640 nm with reference to calibration curves constructed for each isolate between turbidity of the bacterial suspension and the bacterial count determined using the viable count technique. The growth medium used for the production of PLC from *P. aeruginosa* D183 was phosphate–starved tris minimal medium [100 mM Tris–HCl (pH 7.2), 11 mM glucose, 5 mM NH_4Cl , 0.01 mM KH_2PO_4 , 0.5 mM K_2SO_4 , 0.1 mM CaCl_2 , 10 mM MgCl_2] and from *B. cereus* D101 the same medium was used but supplemented with 0.05% sodium cholate. Incubation was done at 37 °C in a platform orbital shaker at 225 rpm for 48 h.

2.4. Partial purification of PLC

This was carried out as described by Wang^[11]. Cells were removed from 500 mL of culture by centrifugation at $3\ 220 \times g$ for 20 min. Solid ammonium sulfate was added very slowly, while stirring at 4 °C, to cell–free supernatant to 70% saturation (472 g/L), and the mixture was stirred slowly at 4 °C overnight. The resulting precipitate was pelleted at $20\ 000 \times g$ for 20 min at 4 °C using Beckman J2–HS cooling centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). Formed pellet was suspended in 25 mL of 10 mM Tris–HCl,

pH 7.2. Suspension was loaded in dialysis tubing (molecular weight cutoff, 6–8000 Da) and dialyzed for 48 h at 4 °C against the same buffer, which was replaced by fresh buffer after 24 h. Protein concentrations were measured by method of Lowry^[12] using BSA as a standard.

2.5. Enzyme assays

2.5.1. Chromogenic assay

PLC activity was measured by the method of Kurioka^[13], adapted to a microtiter system by Berka^[14]. Four hundred units (One unit of PLC activity was defined as the amount of enzyme that released one nM of *p*-nitrophenol by hydrolysis of NPPC per min at 37 °C^[14]) of the partially purified PLC were added to 90 μL of NPPC reagent in a microtiter test plate. NPPC reagent contained 250 mM tris (hydroxymethyl)–aminomethane–hydrochloride buffer (pH 7.2), 60% glycerol (wt/wt), 1.0 μM ZnCl_2 , and 10 mM NPPC. Plates were then incubated at 37 °C for 17 h before the absorbance at 405 nm was measured with MicroReader 4 plus microplate reader (Hyperion. Inc., USA). A yellow color was developed in positive cases. Blank containing 10 μL of the clear growth supernatant fluid and 90 μL of NPPC reagent lacking chromogenic substrate, and control containing 10 μL uninoculated culture medium and 90 μL NPPC reagent were treated similarly.

2.5.2. Turbidometric assay

Activity of PLC against different phospholipid substrates was measured turbidimetrically as stated by Geoffroy^[15]. Reaction mixtures containing 200 μL phospholipid substrate solutions (phospholipid substrate, 3.6 g; sodium cholate, 2.4 g; ZnSO_4 , 1 mM final concentration; distilled water, 100 mL) and 700 μL normal saline were incubated with 100 μL of partially purified enzyme preparations at 37 °C for 17 h. At the end of incubation, enzymatic activity was estimated turbidimetrically at 510 nm using an Ultrospec 2000 spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Positive reaction was indicated by an increase in turbidity of solution. Measurements were done against a blank containing normal saline instead of substrate solution. For each phospholipid solution, a reaction mixture without enzyme addition was treated similarly to serve as control. Relative activities of PLC toward different phospholipid substrates were determined in comparison to its activity toward the universal substrate; egg–yolk phosphatidylcholine. Turbidity produced by a constant amount of enzyme when reacted with test phospholipid substrate was compared to that produced by the same amount of enzyme when reacted with egg–yolk

phosphatidylcholine at the same concentration^[16].

2.6. Characterization of catalytic activity

To determine the effect of pH on enzyme, activity was measured at various pH values (pH 5–11) and to determine the effect of temperature, activity was measured at various temperatures (20–50 °C). However, for studying metal requirement, NPPC reagent lacking zinc chloride was used and several bivalent cations were added at concentrations 0.1–10 mM. Relative enzyme activity was expressed as percentage of the original activity. The same was done for ethylenediaminetetraacetic acid disodium.

2.6.1. Thermostability testing

Thermostability testing was determined by incubating partially purified enzyme preparations at 40–90 °C for 15 min using ATC 401, Nyxtechnik thermal cycler (Nyxtechnik, San Diego, CA, USA) then cooling immediately in an ice bath. Residual enzyme activities were then measured.

2.6.2. Determination of substrate specificity of PLC

Determination of substrate specificity of PLC was carried out by testing the ability of PLC from the two isolates D183 and D101 to hydrolyze different phospholipid substrates. Tested phospholipids were phosphatidylcholine (PC) from soybean, PC from egg yolk, phosphatidylethanolamine (PE) from *Escherich coli* (*E. coli*), PE from egg yolk,

phosphatidylinositol (PI) from soy bean, phosphatidylserine (PS) from bovine brain, phosphatidylglycerol (PG) from egg yolk, Sphingomyelin (SM) from bovine brain and cardiolipin from bovine heart. PLC activity was measured turbidimetrically as described. Partially purified PLC preparations were incubated with test phospholipid substrate solutions at 37 °C for 17 h before enzymatic activity was estimated turbidimetrically at 510 nm.

3. Results

3.1. Partial purification of PLC from *P. aeruginosa* D183 and *B. cereus* D101 culture supernatants

The different parameters measured for the partially purified enzyme preparations as well as the growth supernatants of the selected two isolates are summarized in Table 1.

3.2. Effect of different factors on catalytic activity of PLC from *P. aeruginosa* D183 and *Bacillus cereus* (*B. cereus*) D101

3.2.1. Effect of pH

For *P. aeruginosa* PLC, optimum pH values for activity were 7 and 8. The activity decreased sharply at higher pH values and became negligible at pH 11. However, for *B. cereus* PLC, activity was detected at all pH values tested, although

Table 1

Different parameters measured for the partial purified PLC preparations from *P. aeruginosa* D183 and *B. cereus* D101 in comparison to growth supernatants.

Isolate	Fraction	Volume (mL)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)	Recovery (%)
<i>P. aeruginosa</i> D183	Growth supernatant	480	141.12	40 680 000	288 265.30	100.00
	Partially purified preparation	25	16.30	29 687 500	1 821 319.01	72.98
<i>B. cereus</i> D101	Growth supernatant	480	58.79	24 312 000	413 539.71	100.00
	Partially purified preparation	25	18.55	4 460 000	240 431.27	18.35

Table 2

Activity of PLC toward different phospholipid substrates.

Phospholipid substrate	Relative PLC activity*	
	<i>P. aeruginosa</i> D183 PLC	<i>B. cereus</i> D101 PLC
Phosphatidylcholine from egg yolk	100.00%	100.00%
Phosphatidylcholine from soy bean	73.00%	72.00%
Phosphatidylethanolamine from <i>E. coli</i>	14.00%	12.00%
Phosphatidylethanolamine from egg yolk	7.62%	12.00%
Phosphatidylinositol from soy bean	3.38%	3.42%
Phosphatidylserine from bovine brain	4.47%	3.21%
Phosphatidylglycerol from egg yolk	3.42%	11.58%
Sphingomyelin from bovine brain	39.69%	41.05%
Cardiolipin from bovine heart	2.64%	5.00%

*Relative activity was calculated as percentage of activity towards the universal substrate; egg yolk phosphatidylcholine (100% activity).

extremely low at pH 5. Optimum pH values for *B. cereus* PLC activity were 8, 9 and 10 (Figure 1).

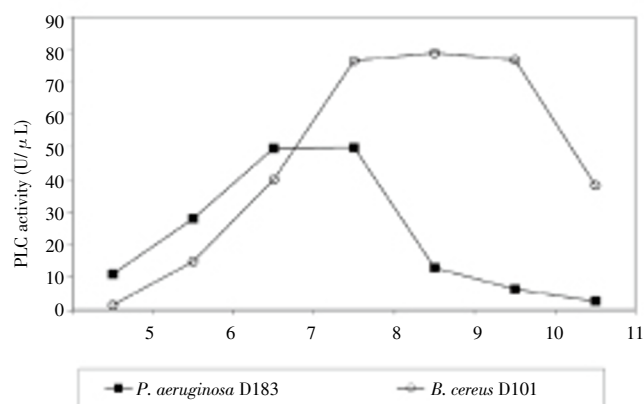


Figure 1. Effect of pH on activity of PLC from *P. aeruginosa* D183 and *B. cereus* D101.

3.2.2. Effect of temperature

For *P. aeruginosa* D183 PLC, no activity was detected below 30 °C and a sharp decrease was detected above 40 °C; however for *B. cereus* D101, various levels of activity were detected at all tested temperatures (Figure 2). Maximum activity was obtained at 4 °C for PLC from *P. aeruginosa* D183 and 37 °C for PLC from *B. cereus* D101.

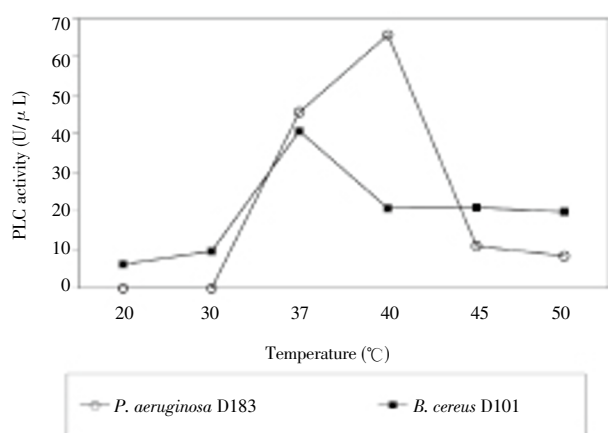


Figure 2. Effect of temperature on activity of PLC from *P. aeruginosa* D183 and *B. cereus* D101.

3.2.3. Effect of inorganic phosphate (Pi)

A range of concentrations; 0.05, 0.1, 0.2, 0.5, 1, 5, 10, 50, 100 mmol/L of potassium monobasic phosphate was tested. Slight stimulation of activity was detected at low Pi concentrations; 0.05–1 mmol/L for *P. aeruginosa* PLC and 0.05–0.2 mmol/L for *B. cereus* PLC (Figure 3) followed by an inhibition until activity was completely abolished at 100 and 50 mmol/L for *P. aeruginosa* and *B. cereus* PLCs respectively.

3.2.4. Effect of metal ions

NPPC reagent free from ZnCl₂ was used and activity was measured before and after the addition of various metal chlorides. Tested metal chlorides were barium chloride, calcium chloride, cobalt chloride hexahydrate, lithium chloride, magnesium chloride heptahydrate, manganous chloride tetrahydrate, nickel chloride hexahydrate, and zinc chloride. Each metal chloride was tested at concentrations; 0.1, 0.5, 1, 5, 10 mM.

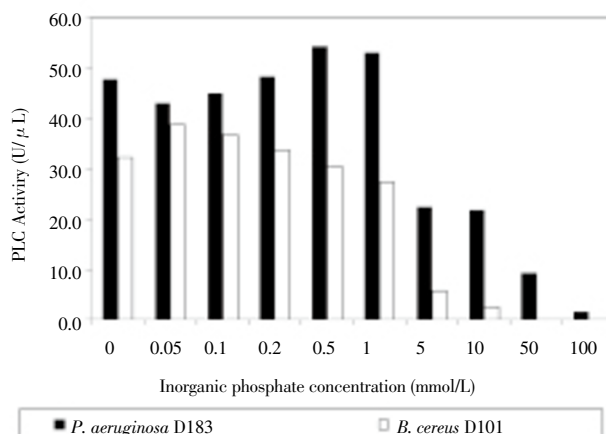


Figure 3. Effect of inorganic phosphate (K₂HPO₄) on activity of PLC from *P. aeruginosa* D183 and *B. cereus* D101.

3.2.5. Effect of metal ions on the catalytic activity of PLC from P. aeruginosa D183

The activity of PLC from *P. aeruginosa* D183 was not affected by the removal of Zn²⁺, as comparable activities were obtained when NPPC reagent with and without ZnCl₂ were used (Figure 4A).

3.2.6. Effect of metal ions on the catalytic activity of PLC from B. cereus D101

When NPPC reagent free from ZnCl₂ was used, activity of PLC from *B. cereus* (35 U/μL) dropped to almost zero (0.8 U/μL). Activity was restored, at various degrees, by addition of all tested metal ions. MnCl₂ was found to cause maximum enhancement (approximately 160% at concentration 10 mmol/L). Zinc ions inhibited PLC activity at concentrations starting from 1 mmol/L and nearly abolished it at 10 mmol/L (Figure 4B).

3.2.7. Effect of EDTA

EDTA did not remarkably affect activity of *P. aeruginosa* D183 PLC at concentrations 0.1, 0.5, 1, 5, and 10 mmol/L. However, it showed an 80% inhibition of PLC from *B. cereus* D101 starting from 0.5 mmol/L and higher concentrations

(Figure 5).

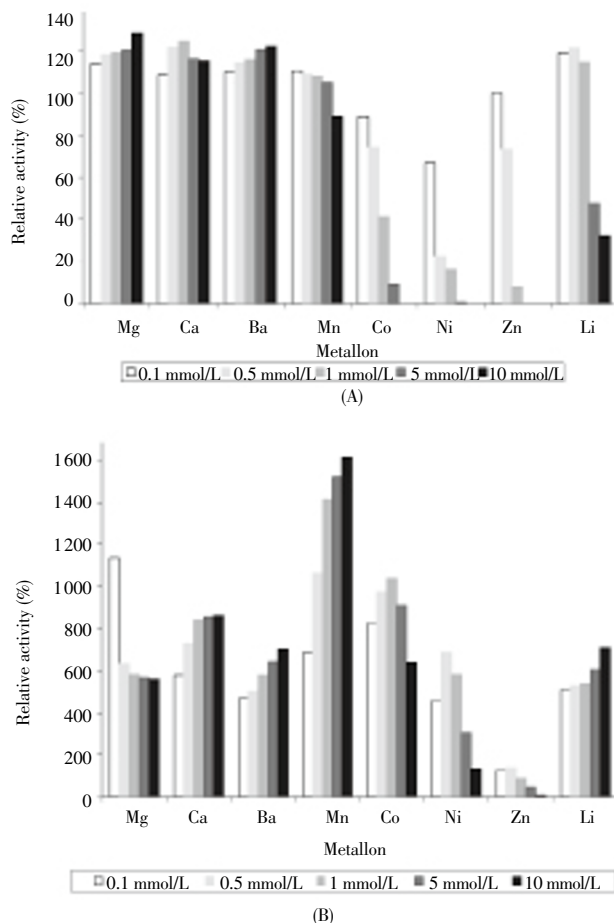


Figure 4. Effect of some metal ions on activity of PLC from *P. aeruginosa* D183 (A) and *B. cereus* D101(B).

Relative activity was calculated as percentage of activity in absence of metal ions where $47.6 \text{ U}/\mu\text{L}$ was considered as 100% activity.

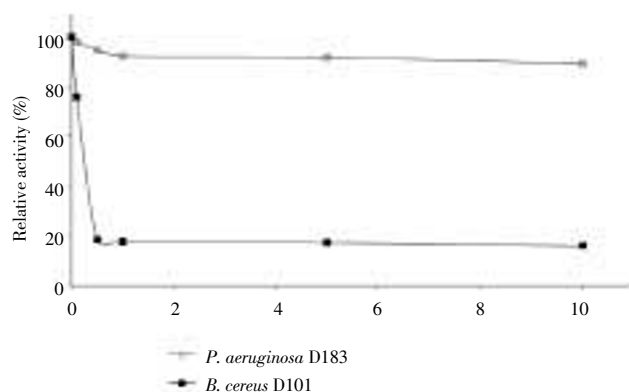


Figure 5. Effect of EDTA on activity of PLC from *P. aeruginosa* D183 and *B. cereus* D101.

Relative activity was calculated as percentage of activity in the absence of EDTA where $45.5 \text{ U}/\mu\text{L}$ and $37.7 \text{ U}/\mu\text{L}$ were considered as 100% activity for *P. aeruginosa* D183 and

B. cereus D101, respectively.

3.2.8. Thermostability testing.

Enzyme preparations from the two test isolates retained approximately 50% of their activities after incubation at 55°C , and lost their activities at 70°C (*P. aeruginosa* PLC) and 80°C (*B. cereus* PLC) (Figure 6).

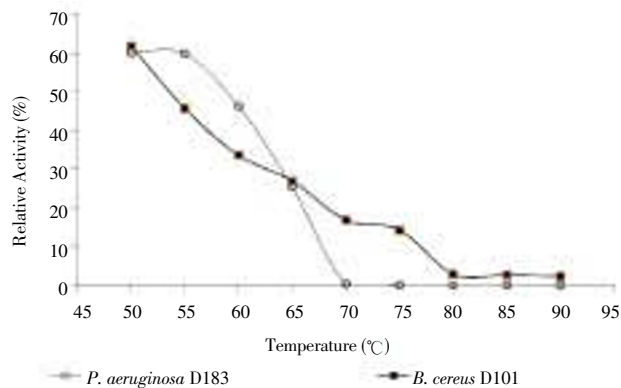


Figure 6. Effect of temperature on stability of PLC from *P. aeruginosa* D183 and *B. cereus* D101.

Residual activity was expressed as percentage of the initial activity before heat treatment where $43.1 \text{ U}/\mu\text{L}$ and $35.8 \text{ U}/\mu\text{L}$ were considered as 100% activity for *P. aeruginosa* D183 and *B. cereus* D101 respectively.

3.2.9. Activity of PLC on different phospholipid substrates

PLC from *P. aeruginosa* D183 hydrolyzed phospholipids in the following order: egg yolk PC, soy bean PC, SM, PE from *E. coli*, PE from egg yolk. The enzyme showed very low affinity toward PG, PS, PI and cardiolipin (Table 2). PLC from *B. cereus* D101 hydrolyzed phospholipids in the following order: egg yolk PC, soy bean PC, SM, PE from *E. coli* or from egg yolk, then PG. The enzyme showed very low affinity toward PI, PS and cardiolipin.

4. Discussion

PLCs from the two test isolates were found to have optimum activities at an alkaline pH and at 37 and 40°C . Similar results were recorded by other researchers; Sugimori^[16] recorded that maximal activity of partially purified PLC from a strain of *Pseudomonas* sp. was 7.2 and between 40 and 60°C , Hergenrother^[17] reported that optimal pH for activity of *B. cereus* PLC was 8. Vis-à-vis, Durban^[18] reported that the highest activity of PLC in the culture supernatants of 12 strains of *B. cereus* against NPPC was at an acidic pH between 3.5 and 6 at 20 to 60°C . Results obtained in the present study show that human body's physiological

conditions could favor catalytic activity of *P. aeruginosa* PLC, hence virulence of this isolate. Secretion and accumulation of PLC in food could contribute to the hazards associated with *B. cereus* food poisoning.

Finding out that PLC production is dependent on the level of inorganic phosphate in the medium^[10], drew some interest to study the effect of inorganic phosphate on activity of PLC. Results have shown that Pi has an inhibitory effect on activity of PLC from both isolates at concentrations 5 mM and higher. This may confirm its role in securing phosphate supplies to producing isolates as their activities are inhibited in Pi levels. Hansen^[19] reported similar results and could determine crystal structure of the complex formed between PLC from *B. cereus* and inorganic phosphate. In this complex, which was obtained at 2.1° resolution, the phospho moiety binds to the three Zn ions at the catalytic site of PLC, where one of the non-bridging oxygens on the phosphate replaced the bridging water molecule between Zn1 and Zn3, while the other oxygen displaced one of the waters coordinated to Zn2. Determining the structure of the phosphate inhibitor complex provided additional evidence for the crucial role of Zn ions in the catalytic process.

Activity of PLC from *P. aeruginosa* D183 was not affected by removal of ZnCl₂ proving that PLC from this isolate has no requirement for zinc as a cofactor. This observation was confirmed by the results showing that enzyme activity was not affected by addition of EDTA (up to 10 mM) to the reaction medium. This property is quite different from those of PLC produced by Gram-positive bacteria, which contain essential zinc ions^[13,15]. Nevertheless, ZnCl₂ had an inhibitory effect on PLC activity from this isolate at concentrations 1 mM and higher. Tawara^[20] indicated that Zn²⁺ competitively inhibited PLC activity against NPPC with an apparent K_i of 0.3 mmol/L, and hemolytic activity of PLC was remarkably decreased in the presence of Zn²⁺.

Activity of PLC from *P. aeruginosa* D183 was not significantly enhanced by divalent metal ions, in contrast with PLCs of various bacteria, which have been reported to require Ca²⁺, Mg²⁺ or Zn²⁺. The property of PLC from *P. aeruginosa* is similar to that of phosphatidylinositol-specific phospholipase C (PI-PLC) in that there is no requirement of metal ions for activity^[21].

B. cereus PLC is a tri-Zn enzyme with two tight binding and one loose binding sites^[17,22]. This explains why PLC from *B. cereus* D101 lost its catalytic activity when tested in absence of ZnCl₂ or when EDTA was added. The tested divalent cations, in addition to Li⁺, could replace Zn,

restore and increase enzyme activity. The inactivation was accompanied by the removal of one zinc atom from the enzyme and could be reversed by the addition of divalent cations. Prolonged exposure to o-phenanthroline removed the second zinc atom also and produced an enzyme species which was reactivated by Zn²⁺ only. These results are consistent with the view that PLC from *B. cereus* is a metalloenzyme^[17].

One of the interesting features about PLC is its remarkable stability^[21,23]. Enzyme preparations retained 50% of their activities after exposure, for 15 min, to 55 °C. This noticeable thermal stability makes the enzyme candidate for industrial application. Phospholipase A from various sources is also a very stable enzyme^[24]. Stability may arise from extensive disulphide cross-linking in the enzyme. Possibly the metalloenzyme character of PLC from *B. cereus* accounts for the high stability of this enzyme.

PC was the best substrate for *P. aeruginosa* D183 and *B. cereus* D101 PLCs. SM and PE, regardless of source, were not such good substrates and PI, PS, PG and cardiolipin could all be considered non-substrates. This specificity range comes in agreement with that found by other investigators^[21,23]. This substrate specificity reflects apparent affinity for phospholipids having quaternary ammonium side groups^[25]. Interestingly, these phospholipids are major components of eukaryotic cell membranes and are almost never found in prokaryotes^[26]. This selective ability may explain why the invading organism can lyse host cells without damaging its own membrane. These findings also support the hypothesis that PLC functions as part of a phosphate-scavenger system where it degrades host phospholipids producing phosphomonoesters that are acted upon by alkaline phosphatase releasing inorganic phosphates. They also suggest that the enzyme is not involved in autolysis of the bacterial cells.

PC, toward which PLC from the test isolates has shown maximum affinity, constitutes 70% of lung surfactants^[27], thus degradation of PC of lung surfactant by PLC from *P. aeruginosa* in cystic fibrosis patients could be involved in induction of the characteristic alveolar epithelial injury and therefore enhance bacterial colonization.

Conflict of interest statement

We declare that we have no conflict of interest

References

- [1] Aloulou A, Ben Ali Y, Bezzine S, Gargouri Y, Gelb MH. Phospholipases: An overview. In: Sandoval, G editor. *Lipases and phospholipases. Methods in molecular biology*. New York: Humana Press; 2012, p. 63–85.
- [2] Elleboudy N, Aboulwafa M, Hassouna N. Phospholipases C from *Pseudomonas aeruginosa* and *Bacillus cereus* isolates, chromosome-mediated enzymes with roles in virulence. *Turk J Biol* 2013; **37**: 433–442.
- [3] Ramrakhiani L, Chand S. Recent progress on phospholipases: different sources, assay methods, industrial potential and pathogenicity. *Appl Biochem Biotechnol* 2011; **164**(7): 991–1022.
- [4] Alouf JE. Membrane damaging and cytotoxic phospholipases. In: Alouf JE, Popoff MR, editors. *The comprehensive sourcebook of bacterial protein toxins*. Amsterdam: Elsevier; 2006, p. 516–534.
- [5] Vasil M. *Pseudomonas aeruginosa* Phospholipases and Phospholipids. In: Ramos JL, Roger C, editors. *Pseudomonas*. Vol 4. Netherlands: Springerlink; 2006, p. 69–97.
- [6] Stonehouse MJ, Cota-Gomez A, Parker SK, Martin WE, Hankin JA, Murphy RC, et al. A novel class of microbial phosphocholine-specific phospholipases C. *Molec Microbiol* 2002; **46**(3): 661–676.
- [7] De Maria L, Vind J, Oxenboll KM, Svendsen A, Patkar S. Phospholipases and their industrial applications. *App Microbiol Biotechnol* 2007; **74**: 290–300.
- [8] Lilbaek HM, Broe ML, Hoier E, Fatum TM, Ipsen R, Sorensen NK. Improving the yield of mozzarella cheese by phospholipase treatment of milk. *J Dairy Sci* 2006; **89**: 4114–4125.
- [9] Yang JG, Wang YH, Yang B, Mainda G, Guo Y. Degumming of vegetable oil by a new microbial lipase. *Food Technol Biotechnol* 2006; **44**: 10–104.
- [10] Elleboudy NS, Aboulwafa MM, Hassouna NAH. Characterization of phospholipase C productivity by *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus* isolates. *J Am Sci* 2011; **7**(8): 545–566.
- [11] Wang CG, Chen MK, Chen T. Improved purification and some properties of a novel phospholipase C from *Bacillus mycoides* strain 970. *Afr J Microbiol Res* 2010; **4**(7): 523–526.
- [12] Lowry H, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biological Chem* 1951; **193**: 265–275.
- [13] Kurioka S, Matsuda M. Phospholipase C assay using *p*-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. *Anal Biochem* 1976; **75**: 281–289.
- [14] Berka RM, Gray GL, Vasil ML. Studies of phospholipase C (Heat-labile hemolysin) in *Pseudomonas aeruginosa*. *Infect Immun* 1981; **34**(3): 1071–1074.
- [15] Geoffroy C, Raveneau J, Beretti JL, Lecroisey A, Vazquez-Boland JA, Alouf JE, et al. Purification and characterization of an extracellular 29-kilodalton phospholipase C from *listeria monocytogenes*. *Infect Immun* 1991; **59**: 2382–2388.
- [16] Sugimori D, Nakamura M. Partial purification and some properties of a phospholipase C from *Pseudomonas* sp. strain KS3.2. *Bioscience Biotechnol Biochem* 2006; **70**: 535–537.
- [17] Hergenrother PJ, Martin SF. Phosphatidylcholine-preferring phospholipase C from *B. cereus*. Function, structure, and mechanism. *Top Curr Chem* 2000; **211**: 132–167.
- [18] Durban M, Silbersack J, Schweder T, Schauer F, Bornscheuer U. High level expression of a recombinant phospholipase C from *Bacillus cereus* in *Bacillus subtilis*. *App Microbiol Biotechnol* 2007; **74**(3): 634–639.
- [19] Hansen S, Hansen L, Hough E. Crystal structures of phosphate, iodide and iodate-inhibited phospholipase C from *Bacillus cereus* and structural investigations of the binding of reaction products and a substrate analogue. *J Molec Biol* 1992; **225**(2): 543–549.
- [20] Tawara S, Matsumoto Y, Matsumoto S, Maki K, Koyama Y, Goto S. Effect of metal ions on phospholipase C (Heat-labile hemolysin) from *Pseudomonas aeruginosa*. *J Infect Chemother* 1997; **3**: 20–26.
- [21] Bora L. Characterization of novel phospholipase C from *Bacillus licheniformis* MTCC 7445 and its application in degumming of vegetable oils. *Appl Biochem Microbiol* 2013; **49**(6): 555–561.
- [22] Epperson JD, Ming LJ. Cobalt(II) and copper(II) binding of *Bacillus cereus* trinuclear phospholipase C: a novel 1H NMR spectrum of a 'Tri-Cu(II)' center in protein. *J Inorganic Biochem* 2001; **87**: 149–156.
- [23] Mo S, Kim J, Cho K. A novel extracellular phospholipase C purified from a marine bacterium, *Pseudoalteromonas* sp. J937. *Biotechnol Lett* 2009; **31**: 89–94.
- [24] Ray S, Jackson E, Tatulian SA. Unusual thermal stability of human secreted phospholipase A2 enzymes. *Biophysical J* 2010; **98**(3): 86a–87a.
- [25] Fitzsimmons L, Hampel KJ, Wargo MJ. Cellular choline and glycine betaine pools impact osmoprotection and phospholipase c production in *Pseudomonas aeruginosa*. *J Bacteriol* 2012; **194**(17): 4718–4726.
- [26] Ohvo-Rekila H, Ramstedt B, Leppimaki P, Slotte JP. Cholesterol interactions with phospholipids in membranes. *Prog Lipid Res* 2002; **41**: 66–97.
- [27] Postle, AD, Henderson NG, Koster G, Clark HW, Hunt AN. Analysis of lung surfactant phosphatidylcholine metabolism in transgenic mice using stable isotopes. *Chem Phys Lipids* 2011; **164**(6): 549–555.