Production and Characterization of Phospholipases C from some *Bacillus thuringiensis* Isolates Recovered from Egyptian Soil

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Abstract: Two hundred and thirty one isolates, with the characteristic morphology of Genus *Bacillus,* were recovered from 100 soil samples collected from 7 different Egyptian governorates, and were screened for phospholipase C (PLC) production by egg-yolk plate method. Sixty isolates have shown very high PLC production and were further assessed using chromogenic assay method. The highest five producers, identified by 16S rRNA gene sequencing as *Bacillus thuringiensis*, were selected and their PLCs were purified to homogeneity using ammonium sulfate precipitation and Sephadex G-75 gel filtration chromatography. PLCs had molecular masses of 28.5 kDa as indicated by SDS-PAGE. The characteristics of the studied five PLCs were having maximal activities at 35-45°C and pH 7.2. The enzymes could retain more than half of their maximum activities at 30-60°C and pH 7-8. Equivalent activities were recorded at low water tension. PLC from *B. thuringiensis* KT159186 was relatively thermostable with a maximum activity at 40°C. The half-inactivation temperature was above 50°C, which compared favorably to that of other enzymes. Activity at the wide temperature range (20-80°C) was high (about 50% of maximum),. This PLC could tolerate pH as high as 12 with only 30% loss of activity. Specificity pattern of PLC from the same isolate showed equivalent activities toward phosphatidylcholine and phosphatidylinositol in addition to marked activity toward phosphatidylethanolamine, which makes it a typical non-specific PLC for industrial purposes. In conclusion, these characteristics of PLC from the test isolate make it attractive for various industrial applications.

Keywords: Phospholipase C, *Bacillus thuringiensis*, Purification, Chracterization.

INTRODUCTION

Phospholipase C (PLC) (EC 3.1.4.3) hydrolyzes the phosphodiester bond in the phospholipid backbone to yield 1, 2-diacylglycerol (DAG) and a phosphomonoester depending on the specific phospholipid species involved [1]. These enzymes function in various roles, ranging from the digestion of nutrients to the formation of bioactive molecules [2]. PLCs can be obtained from strains of *B. cereus and B. thuringiensis* as well as from *Clostridium welchii* and *C. perfringens*. These enzymes have rather different structures, substrate specificities and requirements [3]. The PLC most often used is the enzyme from *Bacillus* species. It is secreted outside the cell and the activity can be isolated with a rather simple procedure [4].

Bacillus PLC is a 28.5 kDa enzyme whose structure has the three important features, the Zinc (Zn) centre consisting of three Zn ions that are all pentacoordinate; the choline binding site which is the substrate binding cleft that accommodates the choline headgroup and is lined with Glu4, Asp55, and Tyr56; and the lipid binding site [5]. In *B. cereus* group species, the transcriptional regulator PlcR (Phospholipase C Regulator) controls PLC production [6, 7]. The transcription of *plcR* starts shortly before the onset of the stationary phase t_0 and reaches a plateau two hours later (t_2) while being dependent on the growth medium [8]. *plcR* transcription is autoinduced, and is repressed by the sporulation factor Spo0A [7]

PLC has multiple industrial applications including for the production of emulsifiers [9], novel lecithins [10] and other structural phospholipids with nutritional values [11-13]. Also, in cheese making [14], for improving the softness of bread [15, 16] and in the production of ceramide [17, 18] for cosmetic as well as pharmaceutical purposes. The use of PLCs in the degumming of vegetable oils may be a major contributor to putting PLCs in the hot spot. Utilizig PLC for degumming offers, in addition to the advantages of enzymatic processes over chemical ones, specific advantages for PLC whose degumming products are readily water-soluble organophosphates and oil-soluble DAG [19]. The first marketed PLC is Purifine®, a product from Verenium Corp. (San Diego, CA, USA) which is available for industrial degumming purposes either for edible oil industry or for the synthesis of biodiesel [20].

Special characteristics of microbial enzymes include their capability and appreciable activity under abnormal conditions, mainly of temperature and pH [21]. The quality of thermostability in enzymes promotes the breakdown and digestion of raw materials; also the higher reaction temperature enhances the penetration

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of enzymes [22]. Therefore, The hydrolytic enzymes are well sought after, being active at higher temperatures as well as retaining stability over a prolonged period of processing at a range of temperatures. The high temperature enzymes also help in enhancing the mass-transfer and reduction of the substrate viscosity during the progress of hydrolysis of substrates or raw materials in industrial processes [23].

In the present study, we screened *Bacillus* soil isolates for production of PLC and selected the five highest producers. PLCs from those five isolates were purified to homogeneity and characterized regarding properties of industrial importance.

MATERIALS AND METHODS

Chemicals

All chemicals were supplied by (unless otherwise indicated) El-Nasr Chemicals (ADWIC), Egypt. Bovine serum albumin (BSA), diacylglycerol (DAG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), p-nitrophenylphosphorylcholine (NPPC), and Sephadex G-75 were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Acrylamide was a product of Aldrich Co., St. Louis, MO, USA, while ammonium persulphate and bis-acrylamide were products of Acros Organics, Geel, Belgium. The protein molecular weight marker was manufactured by Jena Bioscience (Jena, Germany).

Bacterial Strains, Isolation and Maintenance

Isolates B4, B70, B100, B11c, and B58c were selected from 231 *Bacillus* sp. isolates screened for PLC production. The isolates were recovered from 100 soil samples collected from 7 different Egyptian governorates. For bacterial isolation purposes, soil samples were treated in such a way to recover spore forming organisms. Accordingly, the samples were heated at 80°C for 40 min in a water bath. The treated suspensions were serially diluted in sterile distilled water, and the dilutions from 10^{-1} to 10^{-6} were plated on nutrient agar medium. The plates were incubated at 37°C for 24 h [24, 25]. Isolated colonies were picked up, purified and those with the microscopical characteristics of Genus *Bacillus* were screened for PLC production. PLC producers were maintained onto nutrient agar slants kept at 4°C subcultured every month. Glycerol stocking [26] and lyophilization [27] were used for the long term preservation of some selected isolates.

Isolate Identification

Five isolates proved to have promising PLC productivities were selected and identified by 16S rRNA gene sequencing [28]. DNA extraction and PCR amplification of 16S rRNA genes of the test isolates were carried out at Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams Universty, Cairo, Egypt. PCR products were sequenced at Bioneer Company, Germany. The sequence assembly and DNA analysis were carried out using Vector NTI® software (http://www.lifetechnologies.com/eg/en/home/life-science/cloning/vector-nti-software.html) and the final sequences were aligned against sequences in Genbank database using NCBI BLAST® (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/; [29].

Growth Conditions for PLC Screening and Production

Cells from 1-day-old cultures on nutrient agar slants were harvested in sterile normal saline. The cell suspension was adjusted turbidimetrically at 640 nm to a count of about 1×10^7 CFU/ml using a reference calibration curve constructed between turbidity of the bacterial suspension and bacterial count determined by viable count technique. The growth media used in the present study are listed below. The solid medium used for primary screening the isolates was egg-yolk nutrient agar prepared by aseptically beating up one egg-yolk with 15 ml sterile saline using sterile magnet and a stirrer, then mixing with 85 ml sterile molten nutrient agar (60˚C) and pouring in Petri dishes, 20 ml each [30]. In secondary screening experiments, 50 ml Erlenmeyer flasks containing 9 ml of phosphate starved-tris minimal medium (PS-TMM; 100 mM Tris-HCl {pH 7.2}, 11 mM glucose, 5 mM NH_4Cl, 0.1 mM KH_2PO_4, 0.5 mM K_2SO_4, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$) supplemented with 0.5% BSA (Sigma-Aldrich Co., St. Louis, MO, USA) were used [31]. In purification experiments, 1L flasks containing, each containing 250 ml of Luria Bertani broth were employed. For enzyme production in flasks, incubation was done at 30˚C and 200 rpm for 24 h in a rotatory shaker (Newbrunswick).

Enzyme Assays

Egg-Yolk Plate Method

Primary screening was done using the egg-yolk plate method where isolates were spot inoculated on the surface of egg-yolk agar plates and incubation was done 37ºC for 24h. PLC activity (Pz) was expressed in

terms of the ratio between the diameter of the colony to the diameter of the zone of precipitation [32]. The isolates were classified according to the method mentioned previously where a Pz of 1.0 was evaluated as negative (-), 0.99–0.9 as weak (+), 0.89–0.8 as mild (++), 0.79–0.7 as relatively strong (+++) and <0.69 as very strong (++++) activity.

Chromogenic Assay

PLC assay was performed by the method of [33]. In secondary screening experiments, at the end of the incubation period, cells were removed from 1 ml culture by centrifugation at 10,000 rpm for 10 min, and 10 µl of the clear supernatant fluid was added to 90 µl of p-nitrophenylphosphorylcholine (NPPC) reagent in a microtiter test plate. In characterization experiments, 10 µl of purified enzyme preparations were used instead. The NPPC reagent contained 10 mM NPPC, 60% sorbitol (w/v), 1.0 µM $ZnCl_2$ in 250 mM Tris buffer (pH 7.2) (Oxford, India), and. The plates were then incubated at 37°C for the predetermined time period before the absorbance at λ405 nm was measured with Sinnowa ER504 plus microplate reader (Sinnowa, China). A yellow color was developed in positive cases. A blank containing 10 µl of either the clear growth supernatant fluid or the purified enzyme preparation and 90 µl of NPPC reagent that lacked the chromogenic substrate.

Turbidometric Assay

Activity of PLC against different phospholipid substrates was measured turbidimetrically as mentioned previously [34]. Reaction mixtures containing 200 µl phospholipid substrate solution under test (phospholipid substrate, 3.6 g; sodium cholate, 2.4 g; $ZnSO_4$, 16.14 mg; distilled water, 100 ml) and 700 µl normal saline were incubated with 100 µl of enzyme preparations at 37°C for 17 h. At the end of incubation, enzymatic activity was estimated turbidimetrically at 510 nm. 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 a control. Assays were conducted in triplicate.

The relative activities of PLC toward the different phospholipid substrates were determined in comparison to its activity toward the universal substrate; egg-yolk PC. The turbidity produced by a constant amount of the enzyme when reacted with the test phospholipid substrate was compared to that produced by the same amount of the enzyme when reacted with egg-yolk PC at the same concentration [35, 36].

Purification

Ammonium Sulfate Precipitation

This was carried out as described previously [37]. Cells were removed from 500 ml of culture by centrifugation at 10,000 rpm for 15 min. Solid ammonium sulfate was added very slowly, while stirring at 4°C, to the cell-free supernatant (472 g/liter), and the mixture was kept stirred slowly at 4°C overnight. The resulting precipitate was pelleted at 18,000 rpm for 20 min at 4°C using Beckman J2-HS cooling centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). The formed pellet was suspended in 8 ml of 10 mM tris-hydrochloride, pH 7.2.

Membrane Dialysis

The enzyme preparation obtained was loaded in dialysis tubing (molecular weight cutoff, 13,000 Da) and dialyzed for 48 h in the cold against 500 ml 10 mM tris-hydrochloride, pH 7.2. The buffer was replaced once by 500 ml fresh buffer after 24 h.

Sephadex G-75 gel Chromatography

The dialyzed partially purified enzyme preparation of the test isolate were loaded on Sephadex G-75 gel exclusion column (2.5 × 30 cm) previously equilibrated with Tris-HCl (0.2 M, pH 7.8). PLC was eluted with the same buffer at a flow rate of 0.5 ml/min and a fraction volume of 4 ml. Fractions were collected and analyzed for total protein (OD_{280}) and for PLC activity using the chromogenic assay. The fraction showing peak activity and the ones showing not less than half this peak were pooled.

Protein Assay

Protein concentration was measured by the method of Lowry [38] using bovine serum albumin as a standard.

Determination of Molecular Weight

SDS-PAGE was carried out a ccording to [39] to determine the purity and molecular weight of PLC. Aliquots from pooled purified fractions obtained from gel filtration of the dialyzed partially purified enzyme preparations from different test isolates were run on a SDS-PAGE against the standard protein markers consisting of β-galactosidase from *E. coli* (116.0 kDa), phosphorylase b from rabbit muscle (97.4 kDa), bovine

serum albumin (66.2 kDa), alcohol dehydrogenase from yeast (37.6 kDa), carbonic anhydrase from bovine erythrocytes (28.5 kDa), myoglobin from horse skeletal muscle (18.4 kDa) and lysozyme from chicken egg white (14.0 kDa).

Characterization of PLCs

This was done for by testing purified enzyme preparations for testing their potential industrial importance. All tests were conducted in triplicate.

Thermotolerance

Aliquots (20µl containing approximately 1µg protein) of the test enzyme preparations were incubated at different temperatures ranging from 40 - 75°C for 15, 30 and 60 min in ATC 401, Nyxtechnik thermal cycler (Nyxtechnik, San Diego, CA, USA) then cooled immediately in an ice bath. Residual activities were determined and compared to the corresponding values before heat treatment.

Enzyme Activity at Different Temperatures

This was carried out by conducting the chromogenic assay at different reaction temperatures ranging from 20 to 85°C and expressing it as relative activity compared to enzyme activity at 37°C [37].

pH Tolerance

Aliquots (20µl containing approximately 1µg protein) of the test enzyme preparations were pre-incubated with equal volumes of buffers at varying pH levels (3.0 - 12.0 ± 0.2). Pre-incubation was done at 4°C for 15 and 60 min. For pH 3.0, 4.0, and 5.0, the test enzyme aliquots were incubated with 10 mM citrate buffer, for pH 6.0, 7.2, 8.0, and 9.0, 10 mM Tris-HCl buffer was utilized while for pH 10.0, 11.0, and 12.0, bicarbonate buffer was used. In all cases, the pH of the buffer was adjusted to the required values. After treatment, the enzymes were readjusted to pH 7.2 and PLC activity estimated. Residual were determined and compared to the corresponding values before heat treatment [40].

Enzyme Activity at Different pH Values

This was conducted by assaying PLC activity using a set of NPPC reagents solutions of different pH values (5.0 to 10.0 ± 0.2). Relative PLC activity was calculated as a percentage of the enzyme activity at pH 7.2 [37].

Activity at Low Water Tension

High NaCl concentrations were used to decrease water activity (a_w) in solution, thus PLC activity was measured in the presence of NaCl at final concentrations ranging from 50 to 500 mM NaCl in the reaction mixture [41].

Substrate Specificity

The ability of PLC from the test isolates to hydrolyze different phospholipid substrates was assessed turbidimetrically and the results were expressed as percentages compared to the corresponding activity against the universal substrate; PC from egg yolk [42].

RESULTS

Recovery of Isolates

A total of 350 isolates were recovered from 100 soil samples collected from different locations over 7 Egyptian governorates. Gram staining revealed that 231 isolates had the characteristic morphology of *Bacillus* species and were selected to be screened for PLC production.

Screening for PLC Production

PLC activities of the selected isolates were detected on egg yolk nutrient agar plates (Figure **1**). A total of 101 isolates were found to produce PLCs at different levels (Table **1**). Very strong producers (Pz <0.69) were further screened using NPPC enzyme assay and the isolates B4, B70, B100, B11c and B58c showed maximal production and specific productivities of PLC. These isolates were selected for completing the present study and were identified by 16S rRNA sequencing as *B. thuringiensis*. Sequences were deposited in GenBank with accession numbers stated in Table **2**.

Purification of PLC

PLC was purified by gel filtration of the dialyzed partially purified enzyme preparations obtained from ammonium sulfate precipitation followed by pooling of the activity peaks (Figure **2** and Table **3**). Enzyme preparations were purified to homogeneity as indicated by the observation of single bands on SDS-PAGE, with molecular weight 28.5 kDa (Figure **3**).

Characterization of PLCs

Thermotolerance

As shown in Figure **4**, PLCs from the test isolate was affected by heat treatment, an effect dependent on both exposure time and temperature.

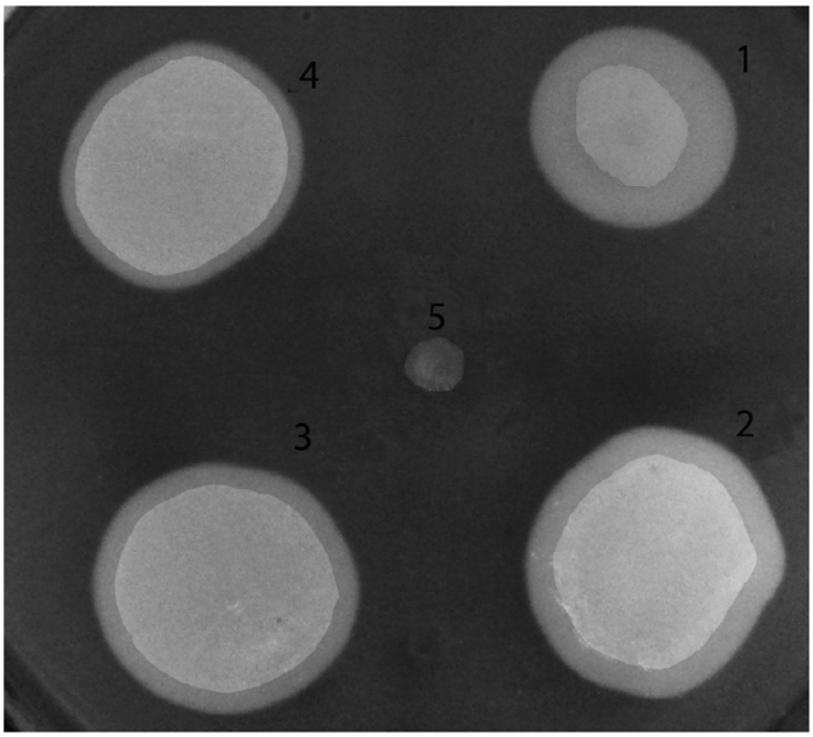

Figure 1: Photo of an Egg Yolk Plate showing PLC Production by 5 Different *Bacillus* Isolates depending on Pz ratio (the ratio between colony diameter to precipitation zone diameter) [32], where 1 is a Strong Producer (Pz<0.69), 2 is a Relatively Strong Producer (Pz=0.79–0.7), 3 is a Mild Producer (Pz=0.89–0.8), 4 is a Weak Producer (Pz=0.99–0.9) and 5 is a Non-PLC Producer(Pz=1.0).

Table 1: The Level of PLC Production by the Recovered *Bacillus* Isolates

Level of Phospholipase Production	Number of *Bacillus* Isolates
Very strong (++++) (Pz <0.69)	60
Relatively strong (+++) (Pz=0.79–0.7)	16
Mild (++) (Pz=0.89–0.8)	16
Weak (+) (Pz=0.99–0.9)	9
	∑ 101

Table 2: Parameters Describing Degrees of Homology for BLAST Alignment of16S rRNA Sequences Used for Identification of the Five Identified Isolates

Isolate Code	Identification	Score[a]	Query Coverage[b]	Expect Value[c]	Identities[d]	Gaps[e]	Strain Access Code
B4	*B. thuringiensis*	1428	99%	0.00	777/779 (99%)	0/779 (0%)	KT159185
B70	*B. thuringiensis*	1163	100%	0.00	618/622 (99%)	1/622 (0%)	KJ934598
B100	*B. thuringiensis*	2407	98%	0.00	1316/1322 (99%)	1/1322 (0%)	KT159186
B11c	*B. thuringiensis*	1604	100%	0.00	844/847 (99%)	1/847 (0%)	KJ934596
B58c	*B. thuringiensis*	1150	100%	0.00	632/639 (99%)	4/639 (0%)	KJ934597

[a]Score is a numerical value that describes the overall quality of an alignment. Higher numbers correspond to higher similarity.
[b]Query coverage is the percent of the query length that is included in the aligned segments.
[c]Expect value describes the random background noise. The lower the E-value, or the closer it is to zero, the more "significant" the match is.
[d]Identities refer to degree of resemblance between the two aligned sequences.
[e]Gaps refer to number of mismatch in nucleotides between query and subject (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs)

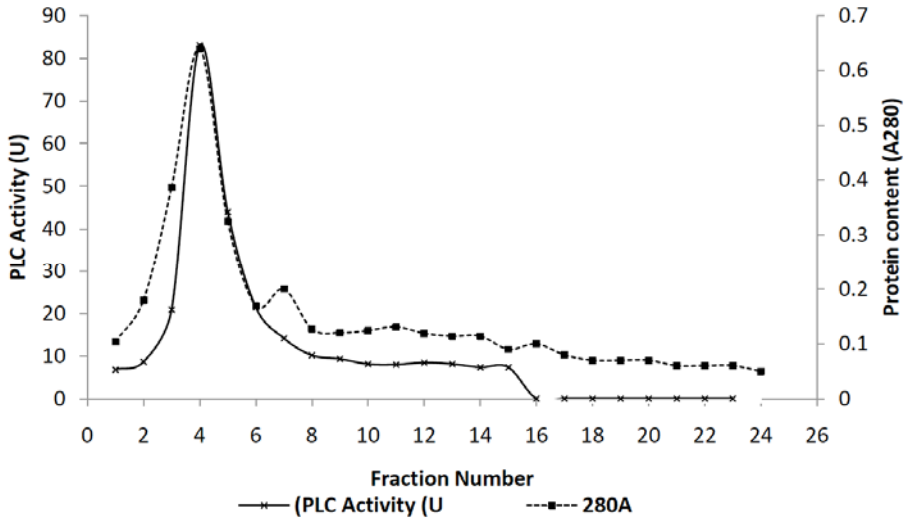

Figure 2: Gel-filtration of PLC from *B. thuringiensis* KT159186. Elution was done with 10 mM tris buffer (pH7.2) at a flow rate of 0.5 ml/min and fraction volume of 4 ml.

Table 3: Purification of PLC from *B. thuringiensis* KT159186

Step	Total Activity (U)	Protein Content (mg)	Specific Activity (U/mg)	Purification (fold)
Cell Free Supernatant	12431	1347	9.24	1
Ammonium Sulfate Precipitate	6695	28.17	238	26
Sephadex G-75 Fraction	1062	1.02	1042	113

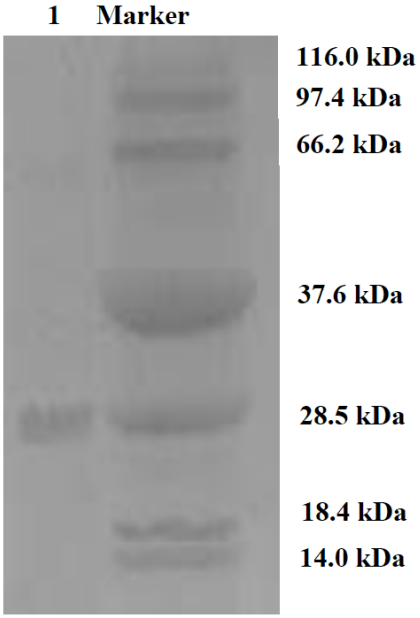

Figure 3: SDS-PAGE. Stacking gel was 5% polyacrylamide in pH 6.8 Tris-HCl buffer.

Separation gel is 12% polyacrylamide in pH 8.8 Tris-HCl buffer.The samples was treated with the reducing agent 2-mercaptoethanol and denatured by a thermal cycler at 100°C for 5 min before electrophoresis. Protein bands were stained by Coomassie brilliant blue R-250. Lane 1: active fraction from Sephadex G-75 active fraction for *B. thuringiensis* KT159186.

When exposed to a temperature 40°C, PLC from *B. thuringiensis* KT159186 was hardly affected by heat treatment for up to an exposure time of 60 min Heat treatment at 50°C for the mentioned exposure times showed that PLC from *B.* thuringiensis KT159186 was stable as it retained more than 80% of its activity even after 60 min of treatment. When treated at 60 and 70°C, PLC was drastically affected. Activity dropped to less than 15% after 60 min of exposure.

Enzyme Activity at Different Temperatures

Maximal PLC activity occurred at temperatures 37, 40 and 45°C (Figure **4**). PLCs from *B. thuringiensis* KT159186 showed activity at 20°C and was able to retain more than half of its activity at a temperature as high as 80°C.

pH Tolerance

As shown in Figure **5**, PLCs from the test isolate lost more than 50% of its activity when pre-incubated with buffers at the acidic side of the pH scale (3-5) for the two test periods 30 and 60 min. On the contrary, PLC was able to retain about 80% or more of its activity when pre-incubated with buffers at the alkaline side of the pH scale (8-11) for the two tested incubation

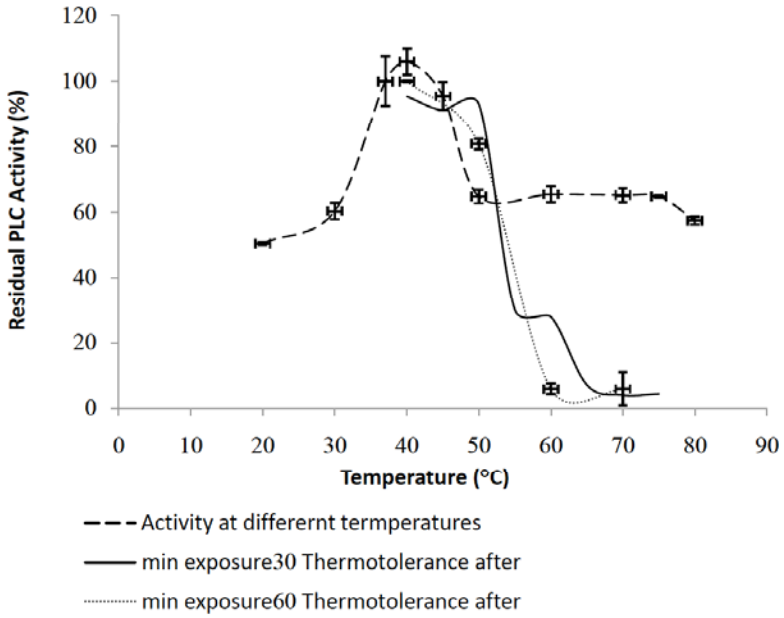

Figure 4: Effect of temperature on the activity and stability of the purified PLC preparation from *B. thuringiensis* KT159186. Results are the average of three independent experiments and error bars represent the standard deviation. For the effect of temperature on activity residual PLC activity was calculated as a percentage of the activity at 37°C (111.79 U), while for the effect of temperature on stability residual activity was calculated as a percentage of the activity of untreated preparations.

periods. PLCs of most isolates could preserve about 60% of their activities when pre-incubated with a pH as high as 12 for 15 min and 50% when the pre-incubation was extended to 60 min. At pH 6, the enzyme activities of the test isolate were slightly affected when pre-incubated for 30 and 60 min. In conclusion, the neutral pH value could be considered optimum for enzyme stability and the acidic pHs have a more detrimental effect on enzyme activity than alkaline pH values.

Enzyme Activity at Different pH Values

Maximum PLC activity was recorded at pH 7.2. PLC from the selected isolate was able to retain more than 75% of its activity at pH 8 and 25% at pH 9 (Figure **5**).

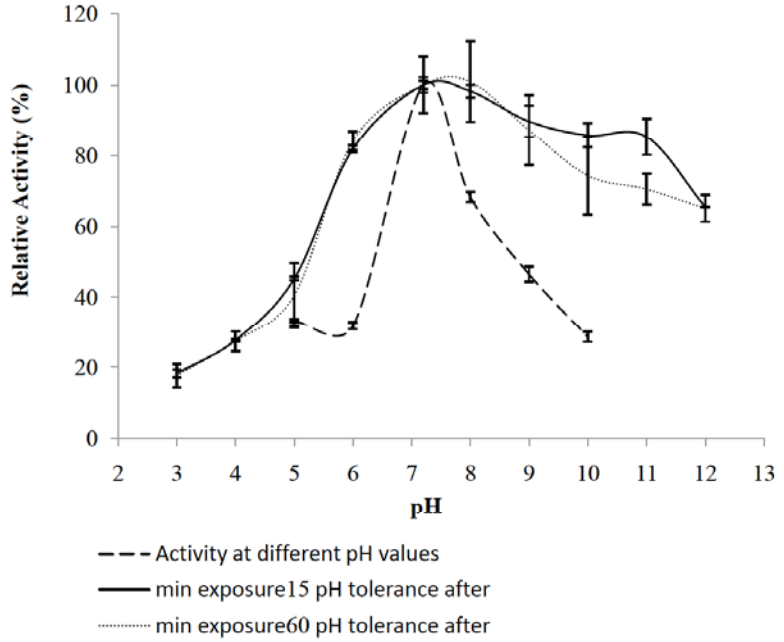

Figure 5: Effect of pH on activity and pH tolerance of PLC from *B. thuringiensis* KT159186. Results are the average of three independent experiments and error bars represent the standard deviation. For the effect of pH on activity residual PLC activity was calculated as a percentage of the activity at pH 7.2 (100.12 U), while for the effect of pH on stability residual activity was calculated as a percentage of the activity of preparations treated at pH 7.2.

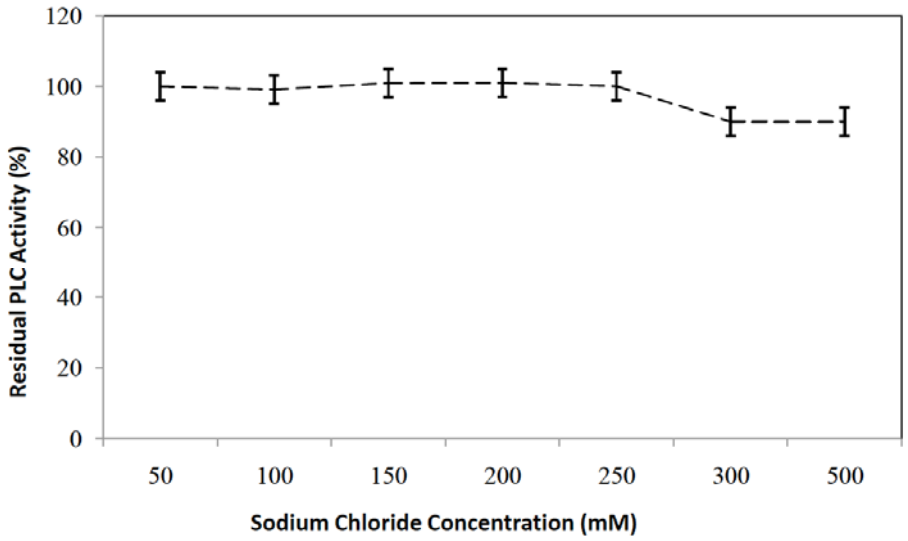

Figure 6: Effect of salinity on activity of PLC from *B. thuringiensis* KT159186. Residual PLC activity was calculated as a percentage of the activity in absence of NaCl. Results are the average of three independent experiments and error bars represent the standard deviation.

Activity at Low Water content

The effect of decreasing water tension on enzyme activity was evaluated by measuring PLC activity in the presence of sodium chloride at different concentrations. As shown in Figure **6**, PLC from *B. thuringiensis* KT159186 was marginally affected by water tension and could be considered resistant to saline stress at the test concentrations.

Substrate Specificity

PLC from *B. thuringiensis* KT159186 hydrolyzed phospholipids in the following order: phosphatidylcholine (100%), phosphatidylinositol, phosphatidylethanolamine, phosphatidylglycerol (phosphatidic acid). PLC was almost as active toward phosphatidylinositol as it was toward the universal substrate, phosphatidylcholine (Table **4**).

DISCUSSION

Applications of PLC

With a global annual turnover of over 3.3 billion dollars, the industrial enzyme sector has become of substantial economic importance [43]. There are several reasons why a study on PLC production is desirable. The first concerns the aim to reduce the amount of chemical reagents to be used in the synthetic steps as part of the general approach toward “green processes” rather than chemical ones. Phospholipids are non-crystalline materials difficult to purify and of limited stability. Their purification often requires several steps [44]. Due to their ability to form aggregates with a wide variety of compounds of different classes their separation from unreacted material is not always an easy task [45]. This fact is of particular concern when the destination of phospholipids is in the food, cosmetics, and pharmaceutical fields. Enzymatic catalysis is expected to simplify the purification procedure. Moreover, due to their intrinsic selectivity and specificity it is expected that fewer by-products will be formed. Another consideration associated with phospholipid purification is that, in case of incomplete reaction, possible impurities in the final product will constitute a much more acceptable problem if the starting materials are compounds which are already GRAS (Generally Regarded As Safe) as natural phospholipids are, than in the case of residual chemical reagents [4]. There is also the possibility to envisage a third approach to the preparation of new or less abundant phospholipids which is the combined chemo-enzymatic synthesis of phospholipids [44].

PLCs have a wide range of industrial applications in a variety of fields. The most prominent application is in the degumming of edible oils. The PLC, Purifine®, has been in the market since 2008 [20]. In addition to safety considerations, PLC degumming is also more efficient than its chemical counterpart. Another booming application for PLCs is their use in the refining of vegetable oils prior to biodiesel separation. The energy crisis is the most serious problem facing the modern world [46]. Biodiesel production offers a glimpse of hope and the use of enzymatic purification processes make it even more promising. Aside from being

greener, PLC induced degumming improves operating efficiency by yielding less phosphorus. In some cases, the phosphorus content in the oil is reduced to virtually zero [47]. Thus, making the downstream processing more reliable. Besides, PLCs work by reacting with the phospholipids releasing both the oil in the gum and the oil-component of the phospholipids, allowing a higher percentage of oil to be converted into biodiesel [48].

The safety advantage enzymatic processes have over chemical processes rendered PLC induced fat stabilization more preferable in dairy product industries. PLCs are not only safer to include in edible products, but they also produce cheese and cream with better palatability and stability [14]. Synthesis of functional foods can be added to the industrial applications of PLC. These food products have high nutritional capacity, yet are not abundant in basic dietary components. Enzymatic modification offers a solution. PLCs are used to convert more abundant, low priced phospholipids of lower nutritional value to rare, expensive phospholipids that provide better health benefits [49].

Vis-a-vis, the industrial usage of PLCs is somewhat limited by their relatively low availabilities and high costs. Therefore, the potential of PLC enzymes appears of great interest in synthesis, particularly on an industrial scale [4].

Screening Studies

The first task for an industrial microbiologist is to find a suitable microorganism for use in the desired process [50]. A total of 100 soil samples were and treated by the method of [25] which involves heating the soil samples at 80°C for 40 min. This method was adopted to allow the selective recovery of thermostable micro-organisms for the hunt of *Bacillus* sp. *Bacillus* species continue to be dominant bacterial workhorses in microbial fermentations. This may be attributed to many reasons. First, many *Bacillus* species, are on the Food and Drug Administration's GRAS list. Second is the capacity of selected *Bacillus* strains to produce and secrete large quantities of extracellular enzymes. Finally, the ability of different species to ferment in the acid, neutral, and alkaline pH ranges [51].

The recovered spore-forming isolates, 350 in number, were Gram stained and examined microscopically to exclude the isolates that did not show the characteristic morphology of *Bacillus* species. A total of 231 isolates showed the large Gram-positive bacilli, in short-to-long chains characteristic of the genus *Bacillus* [52]. The discrepancy might have belonged to genus *Sporosarcina*. *Sporosarcina urea* is widely distributed in soil [53].

The production costs of enzymes are tightly associated with the productivity of enzyme producing microbial strains [54]. Thus, it was crucial to search for micro-organisms with high enzyme productivities. The screening process was conducted in two steps. First, primary screening which was used to screen all 231 recovered *Bacillus* isolates followed by secondary screening the highest producers as revealed in step one.

Primary screening for PLC production was performed by the egg-yolk agar plate method described by [55]. Since egg yolk contains large amounts of phospholipids, predominantly PC and PE, it was incorporated into a nutrient agar-based medium. When grown on this medium, PLC-positive isolates produce a distinct, well-defined, dense white zone of precipitation around the growth. This white zone is probably due to the formation of calcium complex with the 1,2 diacylglycerol released by the action of PLC on the phospholipids present in the egg yolk [56]. Although overall strain growth rates on egg-yolk nutrient agar medium are similar, a large variation in PLC activity has been found in the tested *Bacillus* isolates, ranging from very strong phospholipase producers to isolates that produced none.

When subjected to primary screening for PLC production, about 44% of the screened isolates were able to produce PLC. Of the positive isolates, 60% were very strong producers, 16% were relatively strong producers, and the same percentage was for mild producers. Weak producers constituted 5% of the PLC producers.

Egg yolk agar plate is the most predominantly used method for screening isolates for PLC production. It has the advantages of being sensitive, affordable and very suitable for screening large numbers of isolates. On the other hand, disadvantages include the need for strict aseptic techniques during the inclusion of egg yolk, and careful result interpretation as PLAs result in the formation of clear zones around the colonies, while PLCs give cloudy ones. Finally, egg yolk contains large amounts of phospholipids and trace amounts of triglycerides [57]. Thus, it contains substrates for both phospholipases (phospholipids) and lipases (triglycerides), the egg yolk based assay is not very

specific, and therefore its use should be limited to screens only [58].

Of the 101 PLC producing isolates obtained in the primary screening, 60 were considered very strong PLC producers. Therefore, these isolates were subjected to a more quantitative secondary screening test for characterizing their PLC productivities using the chromogenic assay in which NPPC was used as a substrate for the enzyme. Not all the very strong PLC producing isolates obtained from primary screening exhibited the same high PLC productivities when assayed by the chromogenic assay method. This may be attributed to the fact that the formation of opacity on egg-yolk plates may be caused by another enzyme [59].

The lack of complete specificity of the egg yolk reaction in no way affects the reliability of the method for screening purposes. Though a false positive result is possible, it is highly unlikely that a false negative one would be. Cultures possessing phospholipase activity may certainly be depended upon to give the egg-yolk reaction. With its simplicity and its great sensitivity, the reaction should still serve in screening a large number of organisms for phospholipase activity. Its value will be enhanced if organisms apparently possessing phospholipase activity are then reexamined, as was done here, by a more specific chemical method [59].

The use of NPPC as an artificial substrate to detect PLC activity is very common. NPPC is cleaved by PLC to phosphocholine and p-nitrophenol. The optical density of the yellow compound p-nitrophenol (p-NP) is determined colorimetrically at 405 nm. Since the rate of hydrolysis of NPPC by PLC is very low in aqueous media, 60% sorbitol was added to the reagent to improve the hydrolytic rate. Such an effect of sorbitol may be related to an improvement in the characteristics of the reaction medium; a hydrophobic environment surrounding NPPC and PLC is suspected to exist as a result of hydration of sorbitol added to the reaction medium. The reaction mixture also included $ZnCl_2$, since the maximal catalytic activity of PLC is obtained in the presence of 10^{-4} M $ZnCl_2$ as this divalent metal cation, at the specified concentration, increases the V_{max} of the catalytic reaction [60].

The isolates that gave the maximum PLC production, as well as maximum specific productivities, were selected for further studies were subject to enzyme characterization and were identified using 16S rRNA gene sequencing as *B. thuringiensis*. Sequences were deposited in GenBank and assigned accession numbers.

Enzymatic Properties

PLC was purified to apparent homogeneity by ammonium sulphate precipitation followed by gel filtration chromatography using Sephadex G-75 columns. Single protein bands were seen on the SDS-PAGE, indicating the PLCs had been purified to apparent homogeneity by this purification process. The molecular mass of the purified PLC from all the test isolates was estimated to be 28.5 kDa. The same molecular weight was obtained for PLC from *B. cereus* group isolates by investigated multiple researchers [61-64]. Bora [65] purified and characterized a novel PLC from *B. licheniformis* with a somewhat higher molecular weight of 33 kDa. On the contrary, Wang *et al.* [37] purified a PLC from *B. mycoides* and estimated its molecular weight as 75.1 kDa. A molecular weight that is much higher than that of other *Bacillus* species PLCs, but similar to that of *Pseudomonas* species PLCs [66].

While *B. cereus* group PLC is a single-domain protein consisting of approximately 250 amino acids, and does not display hemolytic, lethal or dermonecrotic activity, the two-domain PLC of *Clostridium perfringens* has a molecular weight of 43 kDa [67] and is typically composed of 370 amino acids. The presence of the C-terminal domain (approximately 120 amino acids) can be correlated with the ability of the enzymatic domain to cause hemolysis, but not necessarily with the ability of the enzyme activity. It only plays a role in alpha-toxin activity [68].

The objective of this study was selecting a PLC that possesses industrially desirable characteristics. Nigam [69] summarizes the special characteristics of enzymes exploited for their commercial interest and industrial applications in the following properties; thermotolerance, tolerance to a varied range of pH, stability of enzyme activity over a range of temperature and pH. Accordingly, PLCs from the selected five isolates were tested for these characteristics in addition to substrate specificity and activity at low water tension.

The major reason behind the dormancy of biocatalysts for many applications is the high fluctuations in operating environment i.e. exposure to temperature and pH farther away from optimum conditions for the enzymes used. The high temperature and pH variability and hence instability of enzymes,

leading to low shelf life is a major concern for commercial viability. The low free energy difference between the native and denatured structure of enzymes makes them fragile molecules to deal with and limits their unlimited applications [70]. Thus, enzymes that show relative stability over temperature and pH ranges are highly desirable from the industrial view point.

Studying the thermotolerance of PLC revealed the remarkable thermostability of PLCs. Outstandingly, PLC from *B. thuringiensis* KT159186 could preserve all its activity after exposure to a temperature of 50°C, and one third of it after exposure to 60°C. Such decreases in catalytic activity at elevated temperatures are, of course, common with enzymes and usually arise from thermal denaturation. The reason for this noticeable stability of PLC is not clear. Phospholipases A from various sources, including venom and mammalian PLAs, are also very stable enzymes [71]. However, in this case, the stability may arise from the extensive disulphide cross-linking in the enzyme. Possibly the metalloenzyme character of PLC accounts for the high stability of this enzyme [72].

Seo and Rhee [63] cloned the phospholipase c (plc) gene from *Bacillus cereus* into the pPICZC vector and integrated into the genome of *Pichia pastoris*. The PLC when expressed in *P. pastoris* was fused to the α-factor secretion signal peptide of *Saccharomyces cerevisiae* and secreted into a culture medium. This was done in an attempt to improve the catalytic properties of *B. cereus* PLC. However, the recombinant PLC had an optimum activity at 80°C and much lower activities at lower temperatures.

To investigate stability of the biocatalysts when exposed to extremes of pH, PLC was incubated, for 30 and 60 min, with buffer solutions at pH values 3 to 12 before assaying their activities. PLC could retain most of its catalytic power at a pH as high as 11. On the other hand, the enzymes were immensely affected by acidic pH values below 6. Similar results were recorded by Wang *et al.* [37] who purified and studied the properties of a novel PLC from *Bacillus mycoides* reporting that PLC was stable over the broad pH range of pH 5 - 9.5. pH tolerance does not seem to be a characteristic specific for PLC from *Bacillus* species. It has been recorded for PLCs from other bacterial sources. Mo *et al.* [73] found out that a PLC produced by the marine bacterium *Pseudoalteromonas* was stable over the same pH range. PLC from *Pseudomonas* sp. also showed more stability when exposed to pH values at the alkaline side of the pH scale rather than the acidic side [35].

Stability of biocatalytic activity over temperature and pH is another industrially important trait. A biocatalyst that can maintain its activity against the rise in temperature and change in pH during the reaction is advantageous [74]. PLC from *B. thuringiensis* KT159186 showed exceptional stability by retaining more than 60% of its maximal activity at a reaction temperature as high as 80°C. The optimum pH for PLC activity was recorded to be 7.2 with less than 25% loss in activity at pH 8. Activity dropped as we moved away on the pH scale. Similar results were recorded by other researchers; Wang *et al.* [37] found the maximum activity of PLC from *B. mycoides* to be at pH 7.0 - 7.5. Hergenrother and Martin [5] reported that the optimal pH for activity of *Bacillus cereus* PLC was 8, while Otnaess *et al.* [75] proved that the variation of pH from 7.2 to 8.3 affected neither activity nor substrate specificity of purified PLC from *Bacillus cereus* and the highest activity occurred at 37 to 60°C. *Vis-à-vis*, Durban *et al.* [64] stated that the highest activity of PLC in the culture supernatants of 12 strains of *Bacillus cereus* against p-nitrophenylphosphorylcholine was at an acidic pH between 3.5 and 6 at 20 to 60°C.

An interesting and potentially useful consequence of the ability of enzymes to function at low water activity may be in extending their range of function to non-aqueous environments [76]. This is especially important in case of PLCs as their main industrial application is in the degradation of phospholipids in vegetable oils either for food purposes or for biodiesel production. Water molecules are known to play a critical role in biological functions of proteins by binding to the surface and incorporating into the interior of protein molecules [77]. Water has a tendency to form ordered cages around hydrophobic groups on the protein surface [78].

When water activity is perturbed by extreme temperatures, high salinity, or other extreme conditions, normally structured liquid water may become limiting to enzymes, with deleterious consequences to enzyme structure and/or function. For example, at high salinity, water is sequestered in hydrated ionic structures, limiting the availability of free water molecules for protein hydration. Salt ions are known to disrupt the local water structure, diminishing the number of intermolecular hydrogen bonds [79]. High salt concentrations critically affect the solubility, binding, stability, and crystallization of proteins [80].

They also alter the interactions between proteins and protein subunits in solution and perturb the electrostatic interactions between charged amino acids with significant consequences for protein structure and function [81]. As salt ions increases, water is removed from hydrophobic regions of protein surfaces, until proteins are no longer sufficiently hydrated [82].

Based on the argument above, high salt concentrations were used to study the ability of PLC to function at low water tension. Sodium chloride concentrations as high as 500 mM had minimal effects on the catalytic powers of the test PLCs. This indicates their ability to catalyze phospholipid hydrolysis at less aqueous environments and the potential of their use for oil degumming.

Since phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and phosphatidylglycerol (phosphatidic acid) are the phospholipid components of vegetable oils [83-85]. Therefore, they are the target of enzymatic action in oil degumming processes either for purification or biodiesel production purposes. The affinities of PLCs from the test isolates toward these substrates were evaluated. PLC showed maximum activities toward the universal substrate, phosphatidylcholine, which is advisable as it comprises the major component of oil phospholipids (about 42% of total oil phospholipids) [86]. Second came phosphatidylinositol then phosphatidylethanolamine. This finding is also desirable as PE and PI collectively constitute approximately 50% of oil phospholipids [86]. Minimal affinity was shown toward phosphatidylglycerol which accounts for less than 10% of the phospholipids content of vegetable oils. Thus, PLC from *B. thuringiensis* KT159186 could be considered a non-specific PLC with affinity toward more than one phospholipid substrate [87].

These results come in agreement with those recorded by Hergenrother and Martin [62]. In this study, the authors used a chromogenic assay to quantify inorganic phosphate released by the action of alkaline phosphatase on the head groups resulting from PLC activity. They found that substrates for the PLC from *Bacillus cereus* were in the order of phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylserine (PS). Similarly, in a study of naturally occurring phosphatidylcholines, PE, and PS having acyl side chains ranging in length from 12 to 18 carbon atoms each, PLC from *Bacillus cereus* group exhibited activities toward PE and PS that were approximately 25% that of PC [5]. In study of the hemolytic properties of *Bacillus cereus* PLC and sphingomylenase, Beecher and Wong [88] also reported that *Bacillus cereus* PLC has an affinity for phosphatidylinositol (PI) in addition to PC.

It is worth noting that the substrate specificity of the enzyme reflects the apparent affinity of the protein for phospholipids having quaternary ammonium side groups [89]. Interestingly, these phospholipids are major components of eukaryotic cell membranes and are almost never found in prokaryotes [90]. In contrast to mammalian cells in which the most common phospholipids are PC, PS, PI, and PE, prokaryotic membranes are composed of cardiolipin (a double phospholipid joined at the phosphate by glycerol) as a major phospholipid, along with PE and phosphatidylglycerol [91]. This selective ability may explain why the invading organism can lyse the 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. Moreover, the facts that bacterial phospholipids are probably not hydrolyzed by the enzyme, and that phospholipase production is considered growth associated, supported the suggestion that the enzyme is not involved in autolysis of the bacterial cells [92].

ABBREVIATIONS

NPPC = p-nitrophenyphosphorylcholine

PC = phosphatidylcholine

PE = phosphatidylethanolamine

PG = phosphatidylglycerol

PI = phosphatidylinositol

PLC = phospholipase C

PS = phosphatidylserine

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