Research Article

Isolation, purification and characterization of Phospholipase D from almond (*Amygdalus spinosissima*) from Ziarat, Balochistan

Mahgul Baloch¹, Ashif Sajjad^{1*}, Sabeena Rizwan², Shaista Anjum³, Nukhba Akbar², Irfan Hafeez⁴ and Khalid Mahmood¹

1. Institute of Biochemistry, University of Balochistan, Sariat Road-87300, Quetta, Pakistan

2. Department of Chemistry, Sardar Bahadur Khan, Women's University, Brewery Road, Quetta, Pakistan

3. Department of Botany, University of Balochistan, Sariat Road-87300, Quetta, Pakistan

4. PCSIR Laboratories Complex, Ferozepur Road, Lahore-54600, Pakistan

*Corresponding author's email: ashifsajjad@hotmail.com ; ashif.sajjad@um.uob.edu.pk

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Abstract

This study was carried out for the isolation, purification, and characterization of enzyme phospholipase D (PLD) from *Amygdalus spinosissima* Bunge (wild thorny almond). It was observed that the crude extract showed higher values of enzyme activity 1206 U than that of purified which was found to be 915 U. The optimum time for extraction was 1.5 hours, after which the activity started decreasing. Purification of enzyme was done by hydrophobic interaction chromatography with a specific effect of calcium using Octyl Sepharose. In the absence of calcium ion, the fraction eluted from the column showed high enzymatic activity. The highest specific activity (4575 Umg⁻¹), purification fold (53.2) and yield (75.8%) were obtained. Molecular weight was estimated as 62 kDa by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis. Highest activity of PLD was observed at 50°C and at 6 pH. Calcium ion stability of PLD activity was found at 40-90 mM. It is concluded from the present study that PLD has numerous industrial applications. It can be extracted and purified from *A. spinosissima* which is a cheap and easily available source at industrial scale.

Keywords: Activity; *Amygdalus spinosissima*; Isolation; Phospholipase D; Purification

Introduction

Almond, wild thorny almond (*A. spinosissima*) is one of the most important genera in the family Rosaceae. Iran is considered as the core centre of its diversity, from the southern to northern slopes the species of this genus are found. In the neighbouring countries like Afghanistan, Pakistan, Tajikistan, Turkey, Turkmenistan and Uzbekistan different

species of *Amygdalus* are also found [1]. In Pakistan *A. spinosissima* is found on the sloppy hill of cold and dry areas of Balochistan province, approximately at 600 to 1500 metre altitude [2].

Phospholipase D (PLD) is a universal enzyme that is broadly spread in prokaryotic and eukaryotic systems and performs several functions in cellular glycerol-phospholipids, membrane

degradation and reorganization [3]. It was first found in plants, afterward discovered in animals, fungi and bacteria. A novel gene phospholipase D was identified from a new strain of bacteria, *Bacillus cereus* ZY12 in recent years. The expression of gene is affected by different sources of carbon in this strain [4]. Phospholipase D breaks phosphodiester bonds of glycerol-phospholipids at the terminals and converts phosphoglycerides into phosphatidic acid and free hydrophilic head group [5]. Phospholipids are the initial and chief components of biological membranes that are cut by the enzymes, hydrolases (phospholipases).

Phospholipases comprises essential and complex class of enzymes that catalyse the phospholipid hydrolyses at ester or phosphodiester bond. Phospholipases are classified into A1, A2, B, C and D [3]. The hydrolysis of ester bond in *sn*-1 and *sn*-2 position is catalysed by phospholipase A1 and A2. Phospholipase B catalyses the hydrolysis of glycerol-phospholipids. Phospholipase C (PLC) hydrolyses is responsible for phosphodiester bond at the glycerol side and phospholipase D (PLD) catalyses the hydrolysis at the polar side [6]. In the presence of suitable acceptor alcohol, it catalyses the hydrolysis of transesterification reaction. Different studies reported that phospholipase D has fast and dramatic response to extra cellular stimuli due to which it is an important “signal activated” phospholipase and become an area of interest for researchers in molecular biology. Phospholipase D exists in different forms having difference in their regulation but mostly catalyse the hydrolysis of phosphatidylcholine. PLD plays various regulatory roles in different processes of plants, including programmed cell death, abscisic acid signalling, root growth, root hair patterning, freezing tolerance and other stress response [7]. Phospholipase D has been involved in a various cellular processes including alternation in cell motility and morphology, intracellular protein trafficking and

secretion. Recently it has been shown that phospholipase D plays a demanding role in neurodegenerative and traumatic disorders and central nervous system ischemia [8]. Membrane PLD has neuronal functions including axonal growth, formation of secretory vesicles, synaptogenesis, apoptosis and neurodegeneration [9]. Recent reports suggest that a common neurodegenerative disorder Alzheimer’s disease can be inhibited by PLD and is also used in the treatment of cancer [10].

PLD plays a significant role in metabolism of animals and plants and has been identified from several sources. The current study is aimed at the enzyme PLD isolation, purification and its characterization from *A. spinosissima* Bunge (wild thorny almond) and to report the presence of this enzyme in this specie. This wild plant species is selected for its importance in flora of Balochistan and is explored, to ensure utilization of local resources while monitoring sustainable biodiversity conservation and to the best of authors knowledge related to this specie no work has been published.

Materials and Methods

Material

Lecithin (phosphatidyl choline) from egg yolk, ammonium reineckate (ammonium tetrathiocyanodiammonochromate), PIPES, choline chloride, Octyle Sepharose, and all other chemicals of American Chemical Society (ACS) reagent grade were used.

Sample collection

A. spinosissima samples were collected from Ziarat district of Balochistan province, Pakistan. The seeds were washed with distilled water and then dried. The sample was crushed with the help of pestle and mortar using liquid nitrogen and stored at 20°C.

Extraction

PLD was extracted according to Tosi *et al*, [11]. The sample was weighted 5.0g and mixed in 50ml of 0.1M sodium acetate buffer (pH 5.6). The solution was then kept on shaker for 1.5 hours at 4°C. The

dissolved sample was transferred to centrifuge tubes and was centrifuged at 5000xg for 10 minutes in a refrigerated centrifuge machine. The supernatant was transferred to an Erlenmeyer flask and then was divided into two equal parts, one part for activity determination and the second part for purification of PLD.

Determination of PLD activity

By following Tosi *et al.* [11], the activity of PLD was determined according to Bergmeyer & Gavehn, [12]. Supernatant (10ml) was transferred in an Erlenmeyer flask. The percentage of egg yolk lecithin was (0.5%) which was prepared in ethyl ether solution. Lecithin solution (10ml) and 0.1M calcium chloride (0.1ml) was added to the supernatant. The solution was dissolved by shaking at 5°C for 180 minutes with shaker machine. Trichloroacetic acid (3M) was added about 1.0ml to the solution to terminate the hydrolysis step. Ethyl ether was removed by using vacuum rotary evaporator. The solution was centrifuged for 10 minutes at 14000xg. Supernatant 6ml was transferred to a conical centrifuge tube and 1.0ml of 3% ammonium reineckate (ammonium

tetrathiocyanodiammonochromate) in solution containing methanol was added and vortexed. Appearance of light violet, pink colour correspondent to choline reineckate was obtained. Then the obtained pink solution was centrifuged for 5 minutes at 5000xg. After discarding the supernatant, 3ml of distilled water was added and vortexed until the precipitates were suspended and then the solution was centrifuged at 5000xg for 10 minutes. The washing step was repeated again. After washing step, the supernatant was discarded and 3.0ml of acetone was added and vortexed till the precipitates were dissolved. Mixture was centrifuged at 5000xg for 5 minutes. Absorbance of the supernatant was measured against acetone blank at 520nm.

Standard choline curve

All procedures were repeated as in the method of extraction but choline standard

solution was used in a specific amount (0.1432 m.moles of choline per ml of solution) instead of enzyme (sample). Trichloroacetic acid was used in the standard solution to stop the reaction. By using the given expression, the activity of PLD was estimated. During the experiment, one unit of enzyme (PLD) activity was defined as the amount of enzyme which releases 1 μ mole of choline min⁻¹. The choline quantification was determined by the corresponding measured absorbance value by using a standard choline curve with the correlation coefficient R²=0.9904.

Purification of PLD

The part of the supernatant of extraction which was stored for purification process was mixed with equal amount of chilled acetone for precipitation of protein. To recover the protein, the precipitated protein was centrifuged for 20 minutes at 18000xg and then in acetate buffer 0.1M, pH 5.6 was used to dissolve the precipitates.

The precipitated protein was purified according to Lambrecht *et al.* [13] by Hydrophobic Affinity Chromatography with modifications to some extent. A mini-column by using a Pasteur pipette with length of 59mm and width of 15mm was prepared and filled with 5ml of Octyle-Sepharose. The column was washed with buffer A (50mM CaCl₂, 30mM PIPES pH 6.2). After washing, 5ml of crude plant extract was loaded on the column. Various fractions were collected by passing, buffer A 5ml for 6 times. While, buffer B (30mM CaCl₂, 10mM PIPES pH 6.2) was passed 5 times and 6 times buffer C (0.1M EDTA, 10mM PIPES pH 6.2) was passed. Eighteen fractions were collected, and absorbance of each fraction was checked at 280nm. By elution profile, the activity of phospholipase D was calculated in active fraction. The protein quantification in crude extract and active fraction was obtained by Bradford *et al.* [14] method.

Characterization of phospholipase D

The molecular weight of phospholipase D was determined by the process of SDS-PAGE. Proteins were separated by using 10

and/or 12.5% polyacrylamide resolving gel one dimensional SDS-PAGE with a discontinuous buffer system [15]. Wide range protein molecular weight marker ranging from (10 to 180 kDa) was used to determine the size of proteins. Gels were stained using Coomessie Brilliant Blue and/or silver staining by following the method of Heukeshoven & Dernick, [16].

Kinetic properties of extracted phospholipase D

Various factors that affect the activity of Phospholipase D were determined by following the procedures by Sirkhada *et al.* [17] and Khatoon *et al.* [18] with slight modifications where needed.

Effect of extraction time

The extraction time determination and its effect on PLD activity, extraction was performed on different time intervals with the difference of half hour (0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 hrs).

Effect of temperature

The temperature effect on PLD activity, the reaction mixture was incubated at a range of different temperatures (0-80°C) for 30 minutes (at each temperature) and for standard assay the sample ice bath was used to cooling purpose.

Effect of pH

To find out ideal pH for maximum PLD activity, different pH buffers were added (ranged from 4-10). The enzyme extraction was checked by using 4-5 sodium acetate buffer, PIPES buffer ranged from 6 to 7 and glycine-NaOH ranged from 8 to 10.

Effect of calcium ion

Phospholipase D belong to the plants are considered Ca^{+2} dependent therefore, different calcium chloride concentration ranged from (10mM to 120mM), the enzyme solution was treated in order to evaluate the maximum enzyme activity.

Results and discussion

The present study was conducted for the purpose of isolation, purification and characterization of phospholipase D from a new source *A. spinosissima* almond. As almonds are nutritive and are high source of lipids containing lipase and phospholipase.

Due to its applications, phospholipase D was selected.

Phospholipase D was extracted by a method in which acetate buffer (0.1M pH 5.6) was used. Sample was dissolved in 5g of buffer and homogenized by keeping the solution at a shaker machine for 90 minutes. Centrifugation was done at 5000xg for 10 minutes. Activity was determined by following Bregmayer & Gavihn, [12] method. By using standard choline solution, a standard curve was obtained. The observed activity was found to be 1206 U in crude sample and 915 U in purified sample. It is shown by the result that crude extract has high activity then that of purified.

At various time intervals with difference of 30 min (30 mins to 4 hours), extraction was done in order to check the effect of time on PLD activity. The (Fig. 1) shows that 1.5 hrs is the best time for high enzyme activity the obtained result is similar with that of a previously published study on soybean [11] and was not in agreement with the results of a published study on *Streptomyces recemochromogenes* which was 1 hr [19].

Hydrophobic affinity chromatography was used to purify PLD. A suitable binding material, Octyle sepharose was used in the column. By using calcium chloride and PIPES, the elution was done. The results obtained are shown in the (Fig. 2) which shows that the enzyme binds strongly with Octyl residue at high Ca^{+2} concentration (50 mM) in buffer A, because in this fraction no activity was noticed. The high peak at this portion was observed which shows that the majority of proteins are present in the crude. The proteins eluted because they didn't have any interaction with the medium. The proteins which were bounded loosely with the medium also eluted at low Ca^{+2} concentration in buffer B (i.e., 30 mM). EDTA instead of Ca^{+2} was used in buffer C, as a result PLD was eluted and showed a single peak which confirmed the activity in this fraction.

Quantity of protein was measured by Bradford quantification assay and at 595

nm. After purification step, the specific activity and recovery rates of PLD are shown in the (Table 1). The protein concentration in crude was found to be 14 mg and in purified sample was 0.2 mg. A 53.2 purification fold was obtained with recovery of 75.8%. From 5g of sample about 0.2 mg of purified PLD was obtained with specific activity of 4575 (U mg⁻¹) which are not exactly same but near to the previously published on PLD from *Streptomyces sanglieri* [20] and from Indian mustard seed [18].

SDS-PAGE was done for molecular mass determination. Among different gel concentration, 10% acrylamide gel presents clear bands. Lane 1 shows the single band of purified sample, Lane 2 shows the bands of molecular mass marker ranges from 10-180 kDa and Lane 3 represents the bands of crude sample as shown in (Fig. 3). Purified enzyme gave a single band with molecular mass calculated as 62 kDa which showed close similarity to the molecular mass of PLD from *Dacus carota* which was 60 kDa [19] and was 68 kDa in *Allium sativum* bulbs and *Brassica Juncea* seeds [7].

Effect of different factors (Temperature, pH, calcium ion) was shown by its kinetic study (Table 2). PLD activity at different temperature (10-80°C) was observed. 50°C

was found optimum temperature for PLD activity as shown in (Fig. 4). The activity of PLD decreased gradually when the temperature was increased from 50°C to 80°C. The enzyme does not lose its activity below 30°C and above 80°C which indicates that the enzyme is cold stable and thermostable. Same results were observed in publications by Liu *et al.* [22] from *Jatropha curcas* and by Khatoon *et al.* [16] from Indian mustard seed.

PLD activity was checked by treating it with various pH from 4-10 (4-5 sodium acetate buffer, PIPES buffer ranged from 6 to 7 and Glycine-NaOH ranged from 8 to 10) and 6 pH was found ideal (Fig. 5). Enzyme also showed activity to some extent when the pH was increased to 9. The results agree as that of with previously recorded with *Papaver somniferum* L. PLD [23], *Jatropha curcas* [22] and Indian mustard seed [16].

Enzyme was treated with different calcium ion concentration 10-120mM, among which 40mM was found best concentration of calcium ion for PLD activity. Enzyme showed notable activity from 50 to 90 mM (Fig. 6). The obtained results of the study were found to be almost the same as were recorded in effect of calcium ion on sunflower [24] and in plant PLD [25].

Figure 1. Effect of extraction time

Figure 2. Elution profile of PLD on Octyle-Sepharose column

Table 1. PLD purification from *A. spinosissima*

S. No.	Purification steps	Protein (mg)	Activity (U)	Specific Activity (Umg ⁻¹)	Purification fold	%age Yield
1	Crude extract	14	1206	86	1	100%
2	Purified sample	0.2	915	4575	53.2	75.8%

Figure 3. SDS- PAGE of *Amygdalus spinosissima*, Lane 1 represents the purified enzyme, Lane 2 protein Molecular weights markers and Lane 3 represents the crude fraction the gel visualized by coomassie blue staining. Rf analysis and molecular weight of Lanes by GelAnalyzer 2010a©

Table 2. Kinetic properties of *A. spinosissima*

Source of PLD	Specific activity (U/mg)	Mol Mass kDa	Optimum pH	pH Stability	Optimum Temp (°C)	Thermal stability (°C)	Ca+2 stability mM
<i>Amygdallus spinosissima</i> (Almond)	915	62	6	4-9	50	<30 >50	40-90

Figure 4. Temp effect on PLD activity of *Amygdalus spinosissima*

Figure 5. pH effect on PLD activity of *Amygdalus spinosissima*

Figure 6. Calcium ion effect on PLD activity of *Amygdalus spinosissima*

Conclusion

PLD has been purified successfully from crude extract of *A. spinosissima* with a specific activity of 4575 U.mg⁻¹ and yield of 75.8%. The purification procedure included hydrophobic interaction chromatography by which 53.2 folds of purification was achieved. The molecular weight of *A. spinosissima* PLD was estimated 62 kDa by SDS-PAGE. Enzyme activity was observed in various temperature ranges from 10 to 80°C among which 50°C was calculated optimum for PLD activity of *A. spinosissima*. pH for PLD activity in *A. spinosissima* was found acidic 6 among 4-10 pH range. Remarkable activity was shown in 40mM of calcium ion when the enzyme was treated with different calcium chloride concentration ranged from 10-120mM. It is concluded that *A. spinosissima* may be considered a new economical source for PLD and can be isolated at industrial scale.

Authors' contributions

Conceived and designed the experiments: M Baloch, A Sajjad & S Rizwan, Performed the experiments: M Baloch, S Rizwan, S Anjum & N Akbar, Analyzed the data: A Sajjad, S Rizwan & S Anjum, Contributed reagents/ materials/ analysis: K Mahmood & I Hafeez, Wrote the paper: M Baloch & A Sajjad.

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