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Isolation, Purification and Characterization of Phospholipase D from Karpolah Almond (*Prunus Dulcis*) of Ziarat Balochistan, Pakistan.

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Abstract:
Phospholipase D allows the release of phosphatidic acid on hydrolysis of phospholipids which is an important intermediate of different signaling reactions and pathways, including resistance response in plants and carcinogenicity in mammals. In this study Phospholipase D was isolated, purified, and characterized from Karpolah almond (*Prunus dulcis*) of District Ziarat, Balochistan. Phospholipase D activity was observed in crude extract, in acetone precipitates and in purified sample using Bergmeyer Gavehn technique with slight modifications. The results showed 1246±0.02, 1007±0.03 and 987±0.01 U of activity in crude extract, acetone precipitates and in purified sample, respectively. The maximum extraction time for the activity of PLD was observed as 60 minutes. Utilizing Octyle-Sepharose gel and Hydrophobic Interaction Chromatography, the enzyme was isolated with the aid of Ca^{+2} ion effect. In first fraction the enzyme was strongly bound with Octyl residue in the presence of 50mM of Ca^{+2}ion and hence no activity was observed. When the concentration of Ca^{+2} ion was lowered from 50 to 30mM, the loosely bounded proteins left the column in different fractions. Finally, by removing Ca^{+2}ion from the column using 0.1 M EDTA, Phospholipase D was eluted and displayed highest activity. The purification of enzyme was confirmed by SDS-PAGE, which showed a molecular weight of 54kDa. The optimum temperature for that purified enzyme was observed as 50°C and at optimum pH as 6. Phospholipase D showed a stability of 40-90mM for Ca^{+2} ion which confirms that this purified enzyme is Ca^{+2} ion dependent.

Keywords: PLD, Karpolah almond, *Prunus dulcis,* Characterization

Introduction:
In the body of living organisms almost all the chemical reactions are facilitated by enzymes. In essence, there are six different categories of enzymes, and hydrolases represent a significant category. These enzymes catalyze hydrolysis, or the breakdown of the substrate by adding a water molecule. Phospholipase D is one of many hydrolases that hydrolyzes phosphatidylcholine and other phosphatidic acid derivatives, including phosphatidylethanolamine, phosphatidylserine, and others. The activation of phospholipase D is thought to be crucial for the control of cellular processes. Recently, other Phospholipase D genes have also been cloned in eukaryotic cells, and multiple Phospholipase D activities have been identified (Liscovitch *et al.*, 2000).
Due to its membership in a superfamily of signaling enzymes, phospholipase D is most frequently found to create the lipid second messenger. Initially discovered in a plant (in the carrot), phospholipase D was later discovered and characterized in human, yeast, and bacterial tissues (Hanahan & Chaikoff, 1947).
One class of phospholipid to another is catalyzed by phospholipase D. Specifically, this is an interest in the industrial manufacture of low-abundance or synthetic phospholipids with pharmacological and nutraceutical effects (Borrelli & Trono, 2015).
Phospholipase D with thermostable properties have been identified in microorganism such as *Streptomyces olivochromogenes* (Simkhada *et al.*, 2009), and in many plant sources such as *Allium sativm* bulbs (Khatoon *et al.*, 2008) and in *Brassica juncea.* It was observed that Phospholipase D showed optimal activity between 60 and 70 degrees Celsius as well as over 85 degrees Celsius (Khatoon *et al.*, 2007).
Shewanella, a psychrophilic bacterium, is also found to have cold-active Phospholipase D. It was most active at 40 ºC, also active below 30 ºC, and gradually declined in activity at 60 ºC (Tsuruta *et al.*, 2007).
Numerous physiological cellular processes, including membrane remodeling, cytoskeletal dynamics, protein trafficking, cell proliferation in mammalian cells, and meiotic division and sporulation in yeast, are carried out by phospholipase D (Choi *et al.*, 2004).In plants isoform of Phospholipase D(ε) is responsible of root and biomass growth and also responsible for nitrogen signaling (Walch-Liu *et al.*, 2006).
In many diseases, Phospholipase D was suggested as a high therapeutic potential of inhibition such as Alzheimer's disease (Oliveira *et al.*, 2010) and stroke (Elvers *et al.*, 2010), cancer treatment (Buchanan *et al.*, 2005) and diabetes type II (Qin *et al.*, 2016).

Phospholipase D in *Escherichia coli* is utilized in food flavors and in emulsifiers. From lecithin (egg-yolk, baking), Phospholipase D is broadly used on enormous scale on modern level for the creation of Phosphatidyl subsidiaries (Li *et al.*, 2008; Zhang *et al.*, 2010). Bioactive Phospholipase D is used for the production of non-plentiful Phospholipids, which play an important role in supporting mental health. It is additionally utilized in the creation of Phospholipid-Nutrient derivative which shows higher fondness to their organic layer and can show cell reinforcement exercises (Borrelli & Trono, 2015).

Almonds (*Prunus dulcis L.*), locally known as "badam" are edible tree nuts with high nutritional values as it has high oil content (Ali, 2012). Multiple varieties, type of cultivation and climatic conditions usually determine the chemical composition of different almonds (Yada *et al.*, 2011).

For present work, samples of almond were collected from Ziarat, it has been called Karpolah Badam, as it looks similar to Karpolah plant (*Teucrium stocksianum*), which is utilized for the drug of Intestinal sickness fever (Sarangzai *et al.*, 2013).

Phospholipase D is an extremely critical protein, which has numerous modern and natural applications and because of its special purposes, it has become an area of interest for many researchers, they isolate and purify it from different plants and animal sources. Present work depends on the isolation, purification and characterization of Phospholipase D from (Karpolah) almond of Ziarat.

Materials and Methods

Sample Collection:

Karpolah almonds (*Prunus dulcis*) were collected from Ziarat, Balochistan. Nuts were washed with distilled water and dried later. Liquid nitrogen was used to crush 250 grams of nuts, which were later kept in a container at 20 ºC for further analysis.

Isolation of Phospholipase D:

Extraction of Phospholipase D was done using a technique with slight alteration (Tosi *et al.*, 2007). 5 gram nuts were combined with 50 mL of 0.1 M sodium acetate with a pH of 5.6. The mixture was shaken at 4℃ for 90 minutes. Mixture was then divided into two sections, one section was used for activity determination and the other for purification of PLD.

Activity determination of Phospholipase D by Bergmeyer and Gavehn method:

To determine the activity of PLD Bergmeyer and Gavehn method was applied with minor changes (Bergmeyer, 2012). 10 ml of 0.5% egg yolk lecithin solution (prepared in ethyl ether and 0.1 ml of O.1M CaCl2) was added into supernatant. For 1 hour and 30 minutes, this solution was then mixed on shaker machine at 4 ℃. As reaction was accomplished, to stop the further hydrolysis process, 1 mL of 3M Trichloroacetic acid was added into the solution. On completion of reaction, three layers were formed. Greater layer of ethyl ether was removed by vacuum rotating evaporator. Remaining solution was centrifuged for 10 minutes at 14000xg. 6 mL of supernatant was taken in a centrifuge conical tube and was mixed with 1 mL of 3% Ammonium reineckate (Ammonium Tetra Thiocyanodiammonochromate) which was prepared in methanol. Violet pink color was noticed due to formation of Choline reineckate. The solution was centrifuged for 8 minutes at 5000xg, supernatant was discarded and added 3 mL of distilled water into it, to wash it. Same procedure was repeated for 3 times, then the precipitates were dissolved in acetone. Solution was centrifuged for 10 minutes at 5000xg. Absorbance was then measured against acetone blank at 520 nm on UV/Vis spectrophotometer. The quantity of Choline was measured in using standard Choline Curve.

Standard Choline Curve:

An explicit measure of Choline was used in a standard arrangement (0.15 mmol/1 mL 0.1M acetic acid derivation cushion) rather than sample, all the strategy was same as in extraction of sample. The reaction was stopped by adding Trichloroacetic acid in standard arrangements. Based on following approach Phospholipase D activity was determined. One unit of PLD activity was defined as the amount of enzyme that releases 1 μmole of choline per minute.

Purification of Phospholipase D:

Equal volume of acetone(chilled) was added to the section of mixture that was reserved for protein purification, followed by centrifugation for 20 minutes at 18,000xg. The precipitates were then dissolved in 0.1M acetate buffer with a pH of 5.6.
With minor modification in the method of (Lambrecht & Ulbrich-Hofmann, 1992), the precipitates of protein were purified by using HAC (Hydrophobic Affinity Chromatography). A smaller segment was loaded up with 5 mL of Octyle-Sepharose gel. The column was washed with buffer A with the pH of 6.2 (50mM CaCl2, 30mM PIPES). Subsequently, 5 ml of crude extract was loaded on the column. Additionally, 5 ml of Buffer A was used six times to collect different sections. 5 ml of buffer B (30mM CaCl2, 10mM PIPES pH 6.2) and 5 ml of buffer C (0.1M EDTA, 10mM PIPES pH 6.2) were passed to get 18 portions. By using UV/Vis Spectrophotometer, absorbance of every fraction was observed at 280 nm.

Quantification of protein:

For the quantification of protein, Bradford method (1976) was used (Bradford, 1976). By using UV/Vis Spectrophotometer, at 595 nm the absorption of the solutions was observed. Bovine Serum Albumin was used as standard.

Characterization of Phospholipase D:

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) technique was used to calculate the molecular weight of the Phospholipase D (Laemmli, 1970). Isolation of protein were done by using one dimensional SDS-PAGE with

discontinuous buffer system. proteins were separated by one dimensional SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis). Proteins were separated by using 12.5% polyacrylamide. Wide range protein molecular weight marker ranging from (10 to 180 kDa) was used to determine the size of proteins. Gels were stained using Coomassie Brilliant Blue and silver staining.

Staining of Polyacrylamide gel:
By using the methods of (Heukeshoven & Dernick, 1985) Coomassie Brilliant Blue and silver staining, gels were stained and examined through Gel analyzer.

Kinetics/Properties of Phospholipase D:
With slight modifications in (Khatoon *et al.*, 2015) and (Simkhada *et al.*, 2007) methods, different factors were observed which included effect of extraction time, Calcium ion concentration, pH and temperature effect on the activity of Phospholipase D.

Effect of extraction time:
To determine the impact of extraction time on Phospholipase D action, the extraction of Phospholipase D was done at various time intervals (30, 60, 90 minutes, 2 hours, 2 hours and 30 minutes and 4 hours).

Effect of Calcium ion:
Approximately plant and microbial Phospholipase D is Ca^{+2} ion dependent, so the extracted PLD solution was treated with different concentration of $CaCl_2$ solution ranged from 10mM to 120mM.

Effect of pH:
For obtaining the ideal pH, PLD action was observed at various pH from 4-10 utilizing 4-5 sodium acetic acetate buffer, PIPES buffer ranged from 6 to 7 and glycine-NaOH ranged from 8 to 10.

Effect of temperature:
Temperature shows great effect on enzymatic reactions. At various temperatures (0-80°C) for 30 minutes each, PLD activity was measured by incubating the reaction mixture followed by rapid cooling with the help of an ice bath.

Results and Discussion:
The current investigation was mainly based on isolation, purification, and characterization of phospholipase D from Karpolah almond (*Prunus dulcis*) that was collected from Ziarat. With a high content and enzymatic activity, almonds are considered as good source of lipids and rich in nutrients. Activity of Phospholipase D was identified due to its significant impact and and potential applications. All observations were carried out in triplicates and standard deviation was determined using statistical method.

Phospholipase D was extracted from almond by extraction method. 5-gram sample was crushed and mixed with 50 ml of acetate buffer (0.1 M,5.6 pH). Later the sample was kept on a shaker machine for 180 minutes at 4 ^{0}C. Sequentially, sample was centrifuged for 10 minutes at 5000xg (Zhang *et al.*, 2010).

The activity of Phospholipase D in crude, acetone precipitates and in sample was determined by Bergmeyer and Gavehn (1974) method (Bergmeyer, 2012). With the help of Spectrophotometer, absorbance was observed at 280 nm. A standard curve was generated by using a standardized solution of choline. One unit (U) of enzyme activity was described as the total of enzyme that catalyzes the release of 1 μmol of choline per minute.

The activity of PLD was observed 1246±0.02 U in crude extract, 1007±0.03 U in acetone precipitates and 987±0.01 U in purified sample. The results indicated that crude extract had higher activity of PLD than acetone precipitates and purified sample.

Extraction was carried out multiple times with a 30-minute interval (30 minutes to 4 hours) to observe the impact of time on PLD activity. Effect of extraction time on the activity of Phospholipase D is shown in Fig 1. From result it was evaluated that 1 hour was the ideal time at which the Phospholipase D showed highest activity. These findings were in accordance with previous studies where PLD activity showed similar results in wound-induced accumulation of Jasmonic acid in Arabidopsis (Wang *et al.*, 2000) and PLD from *Streptomyces racemochromogenes* (Nakazawa *et al.*, 2010). However, another study that was conducted on wild thorny almonds *(A. spinosissima)* collected from the same area observed highest enzyme activity at 90 minutes (Baloch *et al.*, 2021).

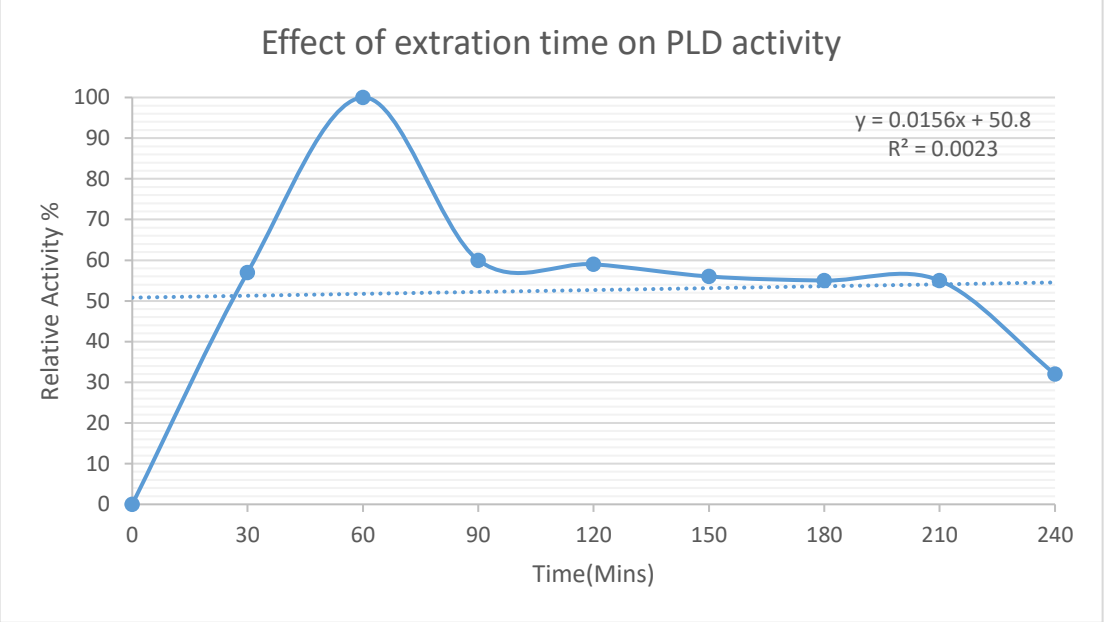

Fig.1. Effect of extraction time on the activity of PLD

Different chromatographic techniques are used for the purification of PLD. In the current study Hydrophobic affinity chromatographic technique was applied. Octyle-Sepharose was used in the column, as it is a suitable binding material. Elusion was completed using $CaCl_2$ and PIPES. Figure. 2 displayed that at 50mM concentration of $CaCl_2$ (Ca^{+2} ion) in buffer A, PLD was strongly bound to the residue of Octyle-Sepharose which showed that PLD expressed no activity in that fraction but the high peak at this point indicated that most of proteins which were present in the solution showed no interaction with the medium and eluted at this fraction. In buffer B the concentration of Ca^{+2} decreased from 50 to 30mM, most of loosely bound proteins were also eluted. In the last step of purification method, by using EDTA the Ca^{+2}ion was removed and finally Phospholipase D was also eluted from the column, at this fraction PLD showed maximum activity (highest peak), which confirmed its presence at this fraction. These results showed similarity with previous investigations of purified PLD from (Vignaunguiculata L. Walp) (Maarouf *et al.*, 2000), purification profile of PLD in Indian mustard seeds (Khatoon *et al.*, 2015) ,in thermally stable PLD in rice bran (Bhardwaj *et al.*, 2001), in PLD of *Papaver somniferum L* (Lerchner *et al.*, 2005).

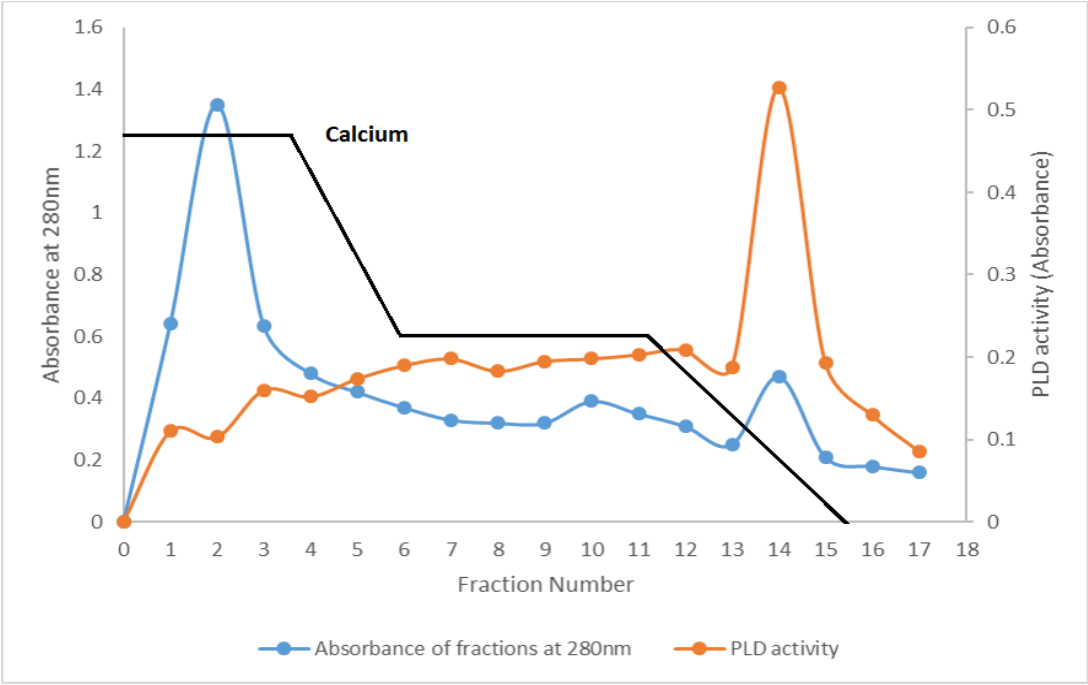

Fig.2. Elusion profile of Karpolah almond PLD on Octyle-Sepharose CL-4B column

The quantity of protein was determined by Bradford protein quantification method and observed the absorbance at 595nm using UV/Vis Spectrophotometer. Protein quantification assay was conducted in crude extract, acetone precipitate and in sample three times, the mean was evaluated, the result showed that the concentration of protein was in crude 19.2±0.04 mg, 5.7±0.02 mg in acetone precipitate and 0.2±0.01 mg in sample. The obtained results are in accordance with previous

observations, in PLD of *Streptomyces sanglieir* (Sugimori *et al.*, 2014) and in PLD of Indian Mustard seeds (Khatoon *et al.*, 2015). Specific activity, purification-fold and percentage yield was also determined. Results showed (Table.1) that crude extract contain more protein as compared to purified fraction , these results showed close resemblance to the previous observed findings of Phospholipase D in Indian mustard seeds (Khatoon *et al.*, 2015),*Streptomyces sanglieri* Phospholipase D (Sugimori *et al.*, 2014), (Nakazawa *et al.*, 2010) and in PLD of rice bran (Bhardwaj *et al.*, 2001).

Table.1. Activity and Purification fold of Phospholipase D

S. No	Purification Steps	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification-fold	% Yield
1	Crude Extract	19.2±0.04	1246±0.02	65	1	100%
2	Acetone Precipitation	5.7±0.02	1007±0.03	177	2.72	81%
3	Octyle-Sepharose	0.2±0.01	987±0.01	4935	76	79%

For the determination of molecular mass of purified protein (enzyme), according to the method of Laemmli 1970, SDS-PAGE method was applied. For obtaining good results, different concentrations of gels were used, among them 10% acrylamide gel showed more clear results (bands) as shown in Fig 3. Lane 1 represented the purified sample's band, lane 2 represented the bands of protein which were present in crude extract and lane 3 represented the proteins molecular mass markers which ranged from 14 to 212 KDa. By using software Gel Analyzer 2010a©, calculated the molecular mass of different proteins of crude and purified sample with reference to the molecular mass of protein markers. Comparing with protein markers the calculated molecular mass of purified enzyme (PLD) was 38 KDa. The obtained results were in agreement with the studies conducted before in plant source, in Peanut which molecular weight was 22 kDa (Sharma & Gupta, 2001) and in animal sources, in *Ochrobactrum sp. ASAG-PL1*whose molecular mass was 37 kDa (Hatanaka *et al.*, 2002) and PLD in Brown spider which molecular weight was 34.4 kDa (Vuitika *et al.*, 2013).

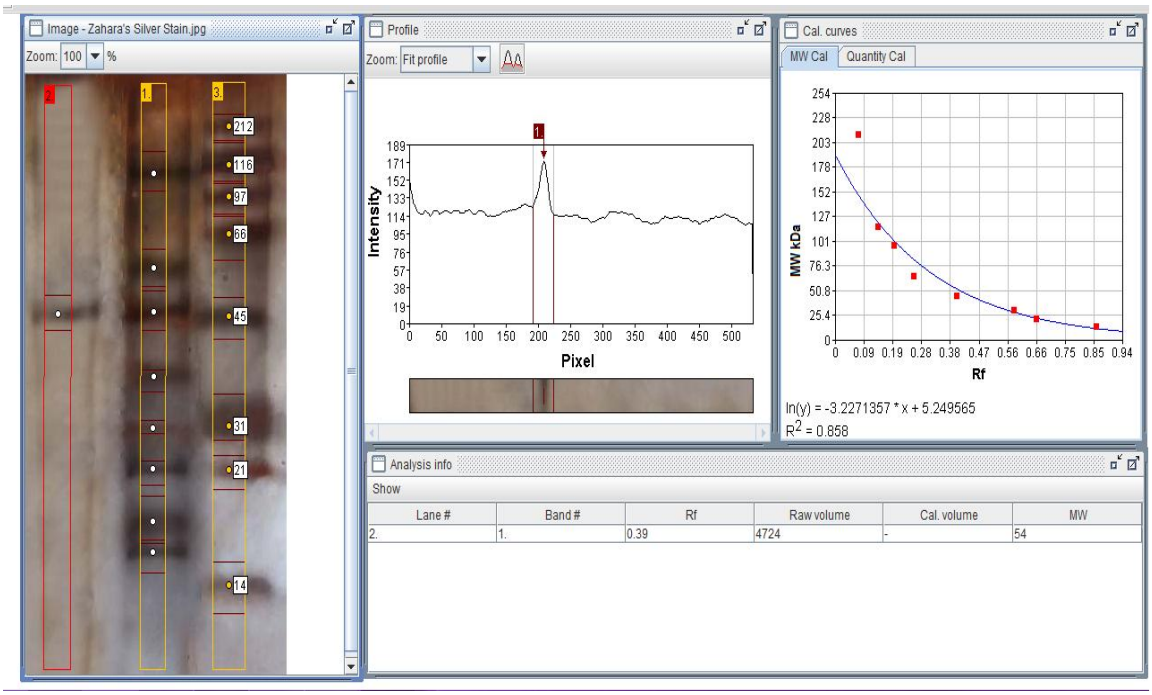

Figure 3. SDS-PAGE of *Prunus dulcis;* Lane 1 represents the purified enzyme, Lane 2 represents the crude extract and Lane 3 represents Amresco® Molecular mass marker of protein ranging from 14-212 kDa. 10% polyacrylamide gel visualized by silver staining. Rf analysis and molecular weight of Lanes by Gel Analyzer 2010a©

Kinetic studies on the activity of Phospholipase D showed that there are different factors which affect this enzyme activity which are pH, temperature and different concentration of Calcium ion.

For the hydrolytic activity of PLD, the activity of PLD was observed at different temperature ranged from 10-80^{0}C. The optimum temperature was found to be at 50 ^{0}C shown in Figure 4. When the temperature decreased from 50 to 80^{0}C the activity of PLD was gradually decreased. The results also indicated that this novel PLD was thermostable and cold stable enzyme because it did not loss activity below 30^{0}C as cold stable and above 80^{0}C act as thermostable enzyme. Same temperature effect was observed in the result of PLD in *Jatropha curcas* (Liu *et al.*, 2010), and in Indian mustard seed's PLD (Khatoon *et al.*, 2015).

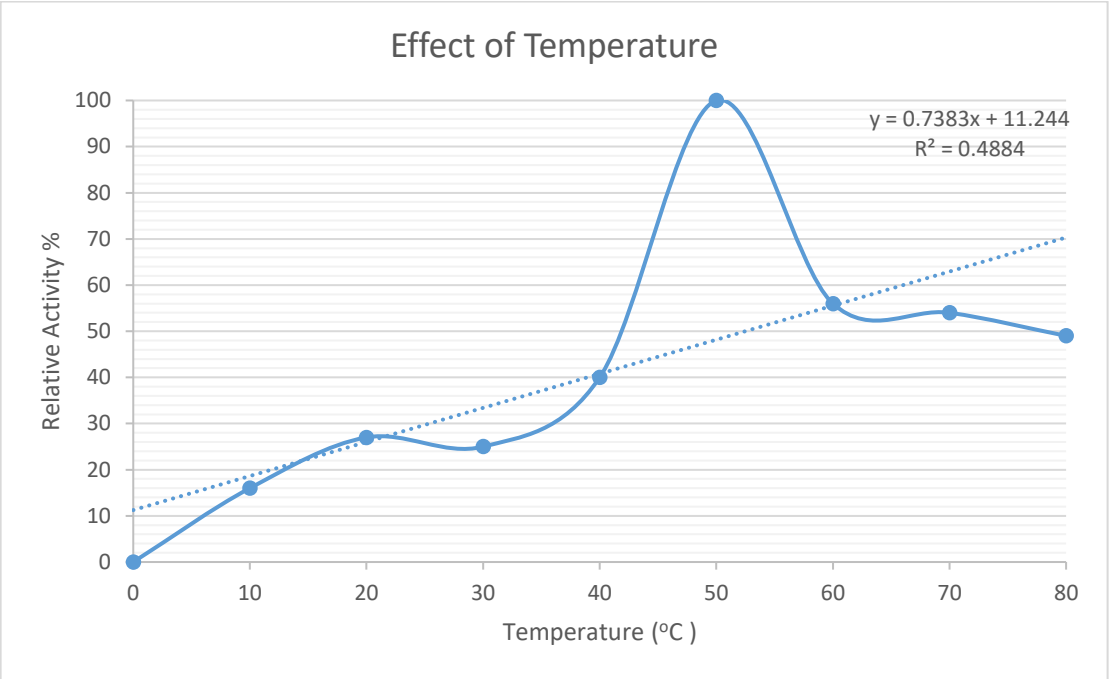

Fig.4. Effect of temperature on the activity of PLD of *Prunus dulcis*

At different pH ranged from 4-10 (4-5 sodium acetate buffer, 6-7 PIPES buffer and 8-10 Glysine-NaOH Buffer) the activity of PLD was determined. The optimum pH for Karpolah almond was 6. The enzyme showed its activity after 24 hours (kept the enzyme at different pH for 24 hours). The results showed that enzyme also showed its activity when the pH was increased from 6-9, which indicated that this novel enzyme was pH stable resemble to the results of PLD of *Papaver somniferum L.*(Lerchner *et al.*, 2005), PLD from *Jatropha curcas* (Liu *et al.*, 2010), PLD of mustard seeds (Khatoon *et al.*, 2015).

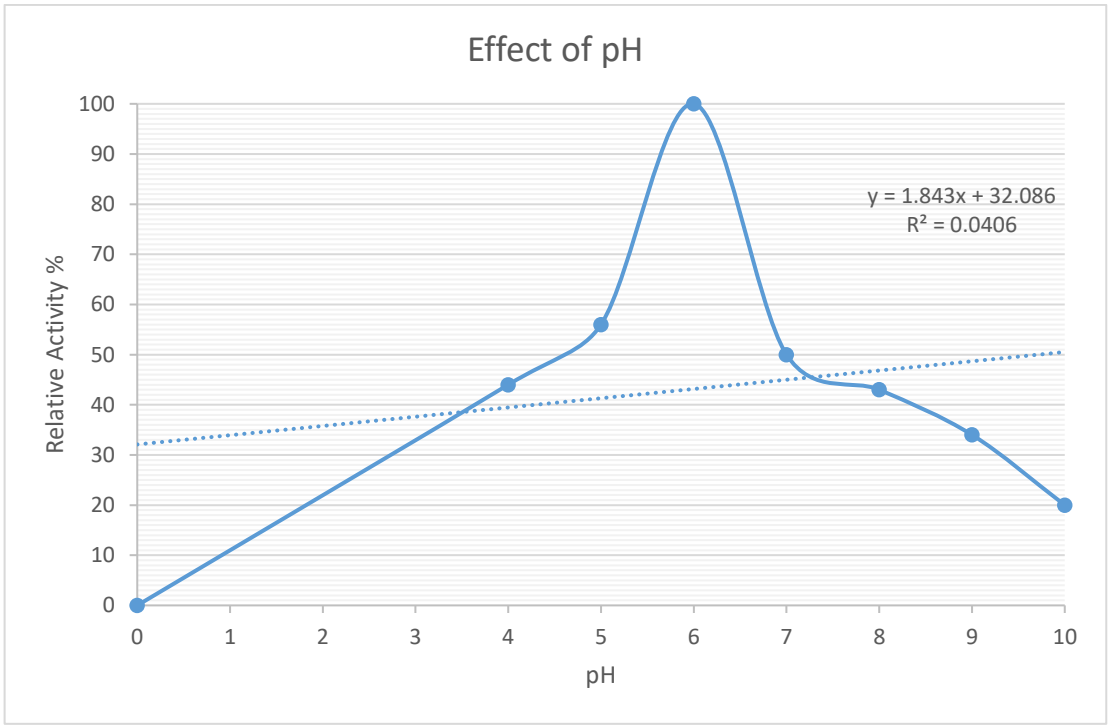

Fig.5. Effect of pH on the activity of PLD

As PLD is calcium ion dependent enzyme so enzyme was treated with different concentration of Ca^{+2} ion (mM). The results indicated that PLD showed maximum activity at 40mM, after 40mM from 50-90 mM. Enzyme also showed a noticeable activity which was then gradually decreased from 100-120mM. This result indicated that this enzyme showed remarkable activity from 40-90mM of Ca^{+2}ion even on 120mM as it was not completely deactivated (showed activity). Similar results were observed in previous studies, where in result of *Papaver somniferum L.*'s PLD (Lerchner *et al.*, 2005), Calcium ion effect in sunflower (Abdelkafi & Abousalham, 2011) and meta ion effect in the activity of plant PLD (Dreßler *et al.*, 2017).

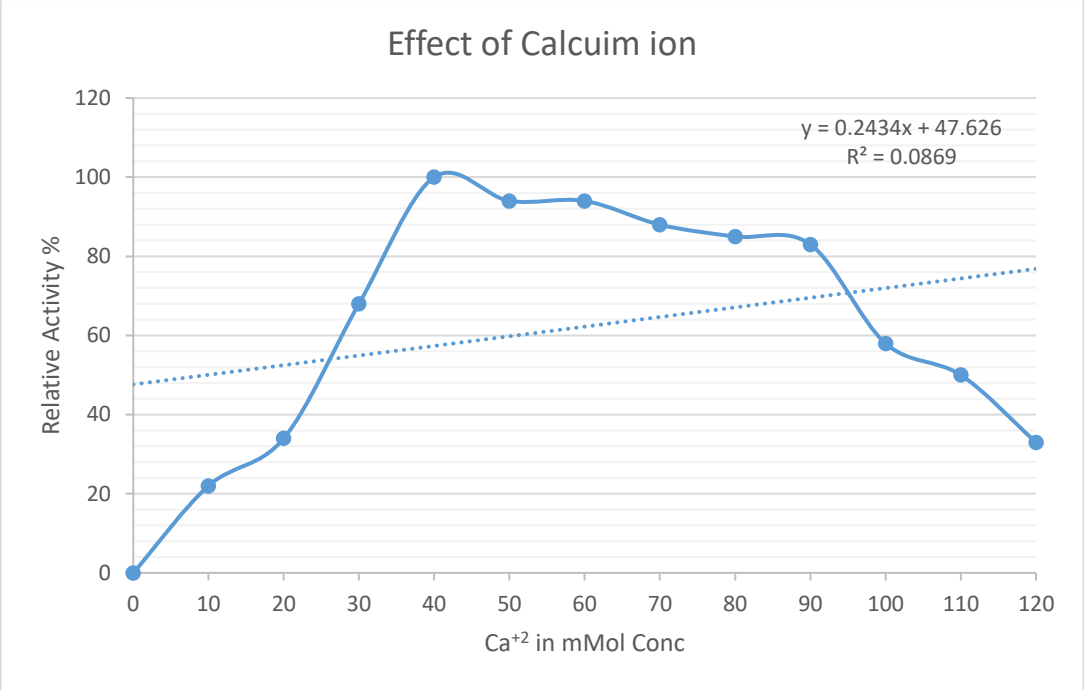

Fig.6. Effect of Ca^{+2}ion concentration on the activity of PLD

The specific activity of PLD was observed (Table. 2) as 4935 U/mg and its molecular weight was 38 kDa. The optimum pH of this novel PLD was 6 and it was also active at pH 9 and after that it lost its activity. Optimum temperature was 50^0C. Enzyme was active below 30^0C which showed that it is cold stable enzyme and also active above 50^0C-80^0C, which indicated that it is a thermostable enzyme too.

Table.2. Kinetic properties of PLD

Source of PLD	Specific activity (U/mg)	Molecular Mass	Optimum pH	Optimum Temperature (^{0}C)	pH stability	Thermal stability (^{0}C)	Ca^{+2} Stability mM
Almond	4935	38	6	50	4-9	< 30 > 50	40-90

Conclusion:

In the current study Karpolah almond (*Prunus dulcis)* was investigated and Phospholipase D was isolated, purified and characterized. Multiple observations concluded that *Prunus dulcis* showed remarkable activity of Phospholipase D. The activity reached at its peak at 1 hour. And after that a gradual decline was observed.

The purification-fold of PLD was 76, which indicated that for separation of Phospholipase D from other proteins separation techniques, the hydrophobic affinity chromatography (Wang *et al.*) was a suitable technique. Molecular weight of purified PLD was determined by software Gel Analyzer 2010a © which was 38 kDa on 1-D (one dimensional) SDS-PAGE.

At various temperature enzyme activity was noticed ranges from 10 to 80℃ among which 50℃ was calculated as optimum for PLD activity. In Karpolah almond pH for PLD activity was found slightly acidic with the pH of 6 among 4-10 pH range. A significant activity was observed in 40mM of calcium ion when the enzyme was treated with different calcium chloride concentration ranged from 10-120mM. It is concluded that for the good economical source of PLD, Karpolah almond (*Prunus dulcis)* must be considered and it must be isolated on industrial scale. As PLD has many industrial applications, and is used in cheese industries to enhance the cheese production and also used in pharmaceutical and nutraceutical industries.

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Conflict of Interest: The authors have no conflict of interest.

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