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ISOLATION, PURIFICATION, STABILIZATION, AND CHARACTERIZATION OF PHOSPHOLIPASE D PREPARATIONS FROM *Raphanus sativus longipinnatus*[a]

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Raphanus sativus longipinnatus *was studied as a local cultivated source of phospholipase D. Methods for the isolation, purification, and biochemical characterization of this enzyme and for the preparation of its stabilized form capable of synthesizing phospholipids in anhydrous systems were described.*

Keywords: phospholipase D, *Raphanus sativus longipinnatus*, isolation, purification, stabilization, properties.

Phospholipases are often used for hydrolysis and synthesis of phospholipids [1] and the analysis and mild modification of biomembranes [2]. Phospholipase D (PLD, EC-3.1.4.4) catalyzes the hydrolysis and trans-alkylation of phospholipids [1, 3]. PLD was extensively studied in the past decade as a polyfunctional enzyme that participates in biosynthesis, membrane homeostasis of phospholipids, and cellular regulation and signaling [4, 5].

PLD from seeds of cotton *Gossipium hirsutum* [6], Central Asian radish *Raphanus sativus* [7], and seeds of soy *Glycine max* [8] was previously isolated, purified to a homogeneous state, and characterized. Herein we present results from a study of the properties of PLD isolated from the Oriental daikon radish *Raphanus sativus longipinnatus* that was recently introduced for culture in Uzbekistan and is one of the best plant sources for obtaining this enzyme [9]. PLD from *R. sativus longipinnatus* that was encapsulated in a matrix of regenerated silk was also prepared for the synthesis of phospholipids in organic solvents [10].

PLD was isolated by homogenization of *R. sativus longipinnatus* in the appropriate buffer, centrifugation of the homogenate, and two successive precipitations of the supernatant by cold acetone (1:2). The resulting precipitate was dissolved in doubly distilled H_2O and lyophilized to afford crude (unpurified) PLD preparation that did not contain other lipolytic and proteolytic enzymes and could be used for practical purposes [9]. The functional properties of PLD from *R. sativus longipinnatus* are given below. These include the lysolecithin hydrolysis kinetics compared with analogous PLD preparations from cabbage leaves and Central Asian radish root (Fig. 1*a*); the effects of one of the reaction products (lysophosphatidic acid), which is a PLD initiator, in addition to Mg^{2+} on Ca^{2+}-activated lysolecithin hydrolysis (Fig. 1*b*); a determination of the optimal pH values with respect to the specific activity and induction period (Fig. 2*a*); and the effect of lysolecithin concentration on PLD activity and length of the induction period.

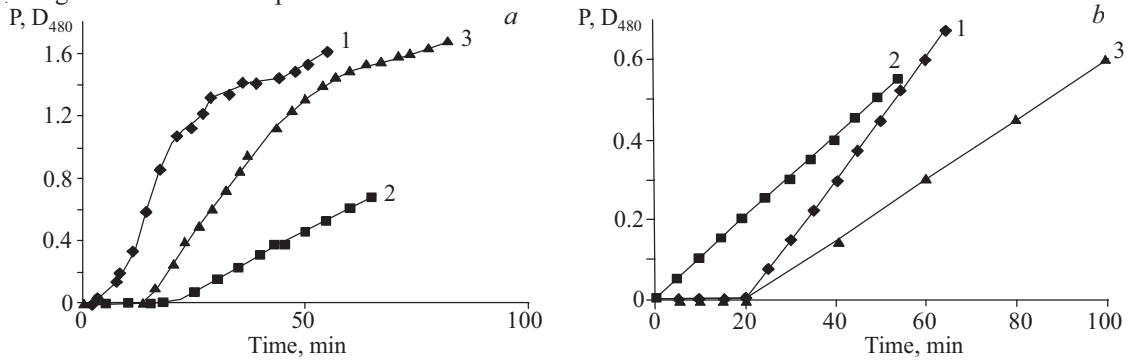

Fig. 1. Hydrolysis kinetics of lysolecithin by PLD from *Raphanus sativus longipinnatus* (PLD-1) compared with similar preparations from other sources (*a*) and under various reaction conditions (*b*). *R. sativus longipinnatus* (1), *R. sativus* (2), Savoy cabbage (3) (*a*); PLD-1 + $CaCl_2$ (20 mM) (1); (1) + lysophosphatidic acid (0.2 mM) (2); (1) + $MgCl_2$ (20 mM) (3) (*b*). P = lysophosphatidic acid, the reaction product; D_{480} = optical density at 480 nm.

[a] Materials presented at the 9th International Symposium on the Chemistry of Natural Compounds (SCNC, People's Republic of China, Urumqi, Oct. 16–19, 2011).

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TABLE 1. Effect of Temperature on Specific Activity and Induction Period of PLD Reaction at Various Ca^{2+} Concentrations and pH

Temperature, °C	Specific activity, μM/min·mg/lag-period, min			
	pH 5.70			pH 5.30
	Ca^{2+} – 5 mM	Ca^{2+} – 10 mM	Ca^{2+} – 20 mM	Ca^{2+} – 10 mM
25	0.040/31	0.040/16	0.040/11	0.040/24
30	0.044/15	0.041/16	0.050/8	0.047/14.5
38	0.0165/18	0.035/8	0.033/4	0.035/10.5
45	0.067/15	0.067/11	0.067/8.5	0.067/11.5
52	0.143/14.3	0.211/8.3	0.120/6	0.080/8

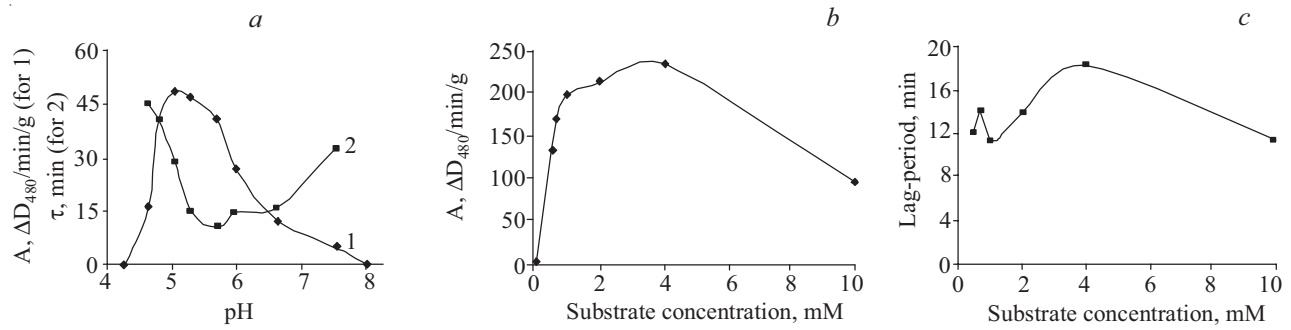

Fig. 2. Specific activity (A) of PLD from *R. sativus longipinnatus* and induction period (τ) as functions of pH at 30°C (*a*) and lysolecithin concentration at 30°C (*b*); lag-period (min) as a function of substrate concentration (mM) (*c*).

Figure 1*a* shows that lysolecithin hydrolysis by PLD from various sources has a characteristic feature. This is an induction period (τ) of different lengths that, like the PLD specific activity, is affected by various conditions. Therefore, the lag-period is an additional characteristic of PLD during hydrolysis of substrates in a micellar system. Figure 2*a* shows that the optimum pH of the crude preparation with respect to activity was 4.8–5.7; with respect to the induction period, 5.3–6.6. The optimum lysolecithin concentration with respect to both specific activity and lag-period was 4 mM (Fig. 2*b*).

Table 1 presents the temperature dependence of *R. sativus longipinnatus* PLD specific activity and lag-period at various Ca^{2+} concentrations and pH values.

Table 1 shows that the induction period decreased with increasing Ca^{2+} concentration and temperature. The lowest τ = 4 min was attained for pH 5.7, Ca^{2+} 20 mM, and 38°C; the highest A = 0.211 U/mg·min, for pH 5.7, Ca^{2+} 10 mM, and 52°C. At 45°C for the studied pH values and Ca^{2+} concentrations, A = 0.067 U/mg·min.

Purified PLD was obtained from the crude preparation by single-stage hydrophobic chromatography over Octyl-Sepharose CL-4B. PLD from *R. sativus* was prepared by this same method for comparison. The molecular weight of PLD from *R. sativus longipinnatus* was 75 kDa; from *R. sativus*, 82 kDa. These enzymes were capable of hydrolyzing and *trans*-phosphatidylating phospholipids. The specific activity of purified PLD from *R. sativus longipinnatus* for egg-yolk lysolecithin was ~1000 U/mg; from *R. sativus*, 410 U/mg.

The crude enzyme preparation was immobilized in fibroin hydrogel obtained from silk production wastes [10] in order to use PLD from *R. sativus longipinnatus* to synthesize phospholipids in anhydrous organic solvents. Immobilized PLD hydrolyzed phospholipids and synthesized phosphatidylmethanol, phosphatidylethanol, and other phospholipids from egg-yolk lecithin and the corresponding alcohols even in the absence of reaction initiators that are usually required in aqueous reaction systems [3].

Thus, *R. sativus longipinnatus* introduced for culture in Uzbekistan represents a significant source for biotechnological production and use of PLD in the food industry and pharmacy. Nanomatrix PLD preparations of prolonged activity that carried out biocatalysis in organic solvents were prepared from wastes from silk production.

EXPERIMENTAL

Isolation and Purification of PLD. *R. sativus longipinnatus* and *R. sativus* were purchased at a market. The cleaned roots were ground and homogenized for 15 min at 14,000 rpm and 0–4°C in *Tris*-HCl buffer (0.01 M) at pH 9.0 (1:0.3). The

homogenate was extracted for 2 h with stirring and centrifuged for 20 min at 12,000 rpm. The supernatant was precipitated by cold Me_2CO with vigorous stirring and decanted. The precipitate was centrifuged for 10 min at 6,000 rpm. The Me_2CO precipitate I was dissolved in the minimum volume of distilled H_2O and centrifuged at 18,000 rpm. The acetone precipitation procedure was repeated with the obtained supernatant. The Me_2CO precipitate II was homogenized in H_2O and centrifuged. The supernatant was lyophilized to afford crude PLD preparation from *R. sativus longipinnatus* that was used to prepare purified PLD by single-stage hydrophobic chromatography over Octyl-Sepharose CL-4B using PIPES (30 mM) containing $CaCl_2$ (50 mM) at pH 6.25 as the adsorption buffer (PIPES-Ca) and diluted (3×) (PIPES-Ca, 1:3) in addition to PIPES (10 mM) containing EDTA (0.5 mM) (PIPES-EDTA, 1:3) as the desorption solutions.

Immobilization of PLD in Regenerated Fibroin Matrix [10]**.** A solution of regenerated silk fibroin was prepared by dissolution of degummed fibers in $CaCl_2$ solution (5 mM) at 98°C for 1 h with stirring. Salt was completely removed from the solution by dialysis for 3 d at room temperature. The silk fibroin concentration was regulated by slow evaporation of the solution in a stream of air from a fan or in a Buchi Rotavapor-R-114. The natural gelation process during storage of the mixture of regenerated fibroin with a solution of PLD preparation was used for the immobilization. The gel was lyophilized using an IlShin sublimation chamber.

Electron Microscopy of the Produced Samples. The morphology of the samples was studied using Philips models 535M and XL30S FEG (Netherlands) scanning electron microscopes. Samples were coated with gold using a Hammer V (USA) sputterer in vacuo and an Ar atmosphere. We also used transmission electron microscopy. The sample adsorbed on a grid substrate was treated with a drop of phosphotungstic acid (1%, 30 sec), absorbed by filter paper, and dried in air. The sample was studied on a CM 20 electron microscope (Philips, Netherlands).

Electrophoresis in PAAG. The purity of the column chromatography fractions was monitored and the molecular weights of the purified proteins were determined by the Laemmli method [11] using polyacrylamide gels (10, 12.5, and 15%), SDS (0.1%), and mercaptoethanol (1 mM).

PLD activity was determined by spectrophotometry from the increase of optical density (formation of a reaction product) at 480 nm [3] of the lysolecithin dispersion as the micellar substrate under various experimental conditions. TLC analysis of reaction products on silica gel plates (Sigma Chem. Co.) using $CHCl_3$:MeOH:NH_4OH (25%) (65:35:5) and $CHCl_3$:MeOH:H_2O:NH_4OH (25%) (65:30:4:2) was performed in parallel. Spots were detected using molybdenum blue (Sigma Co.).

Determination of Protein and Other Spectrophotometric Analyses. Protein concentration was determined by the Bradford and Lowry methods [12] and the biuret method using bovine serum albumin as the standard protein. SmartSpec 3000 (Bio-Rad) and Uvikon-942 (Kontron Instruments) spectrophotometers were used to analyze protein and enzyme activity.

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