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Purification, characterization, and bioinformatics studies of phosphatidic acid phosphohydrolase from *Lagenaria siceraria*

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ABSTRACT

Phosphatidic acid phosphohydrolase (PAP), EC 3.1.3.4, is the penultimate step in the Kennedy pathway of triacylglycerol (TAG) synthesis leading to the formation of diacylglycerol (DAG), which is a key intermediate in TAG synthesis. We partially purified a soluble PAP from mid maturing seeds of bottle gourd, *Lagenaria siceraria*. The steps include both anionic and cationic ion exchanger columns. Catalytic characterization of the partially purified PAP revealed that the optimum pH and temperature for activity were at 5.5˚C and 45˚C. Under optimum assay condition using dioleoyl phosphatidic acid (DPA) as the substrate, the V_{max} and K_m were 0.36 ηkat/mg of protein and 200 µM, respectively. For the synthetic sub-K_m were 33.0 nkat/mg of protein and 140 µM, respectively. The activity was neither inhibited nor enhanced by the presence of Mg^{2+} at a concentration range of 0 to 10 mM. Two major protein bands at 42-kDa and 27-kDa were visible in SDS-PAGE after partial purification. Bioinformatics analysis of trypsinized protein fractions containing PAP activity showed peptide sequences with sequence homology to various phosphate metabolizing enzymes including cucumber and castor bean purple acid phosphatase, polyphosphate kinase, fructose biphosphate aldolase, and enolase from various dicotyledonous plants including rice, corn, grape, and *Arabidopsis lyrata*.

Keywords: Phosphatidic Acid Phosphohydrolase; *Lagenaria siceraria*; Bioinformatics; TAG Biosynthesis

1. INTRODUCTION

Phosphatidic acid phosphohydrolase (PAP, 3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4) hydrolyzes the phosphomonoester bond present in phosphatidate (PtdOH) yielding diacylglycerol (DAG) and P_i. **Figure 1** schematically describes the reaction catalyzed by PAP.

In the cytoplasmic membranes of plant seed tissue that accumulate storage triglycerides (TAG), fatty acyl groups are added sequentially by specific acyltransferase enzymes to the *sn*-1, *sn*-2, and *sn*-3 positions of glycerol-3-phosphate (G3P) to form TAG. This pathway was discovered in 1950s and commonly referred to as the Kennedy or G3P pathway [1]. A key step in the formation of TAG is the dephosphorylation of the *sn*-3 position of phosphatidic acid (PtdOH), which is formed by the action of two specific acyltransferases, namely, glycerol-3-phosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT). The formation of DAG is therefore the penultimate step in Kennedy pathway. The DAG is not only crucial for *de novo* synthesis of TAG, but is also important for the synthesis of phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) [2,3]. PAP is present not only in microbes and plants but also in animals; recent studies have shown that human lipin 1 is PAP [3]. The lipin 1 deficiency in mouse prevents normal adipose tissue development, which results in lypodystrophy, a disease marked by the loss of body fat and insulin resistance, conversely, overexpression of lipin 1 promotes obesity and insulin sensitivity [4-6]. The fact that lipin 1 is a PAP provides a scientific basis for how it regulates body fat metabolism in mam-

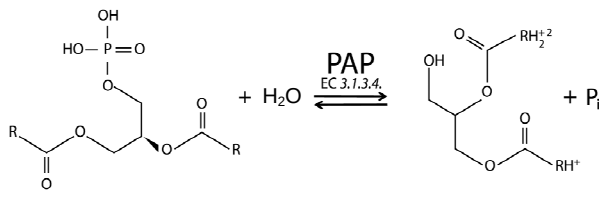

Figure 1. The schematic representation of enzymatic reaction catalyzed by phosphatidic acid phosphohydrolase (PAP). Phosphatidic acid (PtdOH) yields DAG and P_i when phosphoester linkage is hydrolyzed by PAP.

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malian cells. Therefore, PAP activity may represent a target of pharmaceutical research for the control of body fat synthesis in humans.

In the biosynthesis of TAG, PtdCho, and PtdEtn in developing seeds of oleaginous plants, the formation of DAG from PtdOH by the removal of inorganic orthophosphate (P_i) from the latter is considered a crucial step [7]. Moreover, PAP has been reported as being a possible rate limiting step in TAG biosynthesis [8]. Therefore, if PAP does not function optimally, it could create a bottleneck in TAG, PtdCho, and PtdEtn biosynthesis. The kinetic and other physico-chemical data of this key biocatalyst from oleaginous plants is deemed useful for further study of plant fatty acid synthesis systems and for the development of novel and alternative oil sources. Such studies may provide insights into processes for further modification, enhancement, and control the total fatty acyl composition of TAG in oil-producing plants. Of particular interest to plant biochemists are the gene sequences that code for PAP, which may be useful for applications in genetic engineering of oleaginous crops to boost the oil content and at the same time in- corporate novel fatty acids in the seeds.

As an oleaginous crop, bottle gourd (*L. siceraria*) is an understudied plant whose kernel is about 50.3% (weight per weight) oil [9]. The mature kernels of *L. siceraria* contain four major triacylglycerols (TAGs) which include trilinolein (LLL), palmitoyldilinoleoyl glycerol (PLL), oleoyldilinoleoyl glycerol (OLL) and dioleoyllinoleoyl glycerol (OOL) [10]. *L. siceraria* PAP genes could play an important role in determining the fatty acid composition of seed TAG. In this study, we characterized *L. siceraria* PAP as detected in the crude supernatant derived from developing cotyledons by measuring activity, conducting kinetics experiments, and determining pH optima, temperature optima, and effects of minerals and other reagents of this key biocatalyst. This is the first documented reporting of PAP activity in *L. siceraria*.

2. MATERIALS AND METHODS

2.1. Extraction of PAP from *L. siceraria* Cotyledons

Asian bottle gourds belonging to Indian subcontinent and more specifically to Bengal region were grown in New Orleans and mid maturity level seeds were obtained from about 6 weeks old fruits. A 16.3 g of cotyledons were extracted from 107 g of seeds using a scalpel, washed in deionized water and then soaked in 30 ml of extraction medium containing 25 mM imidazole, pH 7.0, 150 mM sodium chloride, 5 mM magnesium chloride, and 500 µM phenylmethylsulfonylfluoride (PMSF). The cotyledons were disrupted using a ground glass tissue homogenizer in an ice bath and then centrifuged at 18,000 × g using Sorvall SS 34 rotor. The resulting pellet was discarded while the supernatant (28 ml) was dialyzed overnight against 25 mM imidazole, pH 7.0 and 1 mM magnesium chloride at 4 ºC with three times buffer exchange. The dialyzed sample was centrifuged as before to remove the precipitated materials because the activity remained in the supernatant. The dialyzed supernatant after centrifugation still looked turbid; nonetheless, the material was applied to an ion-exchange column to facilitate purification of PAP.

2.2. Purification of *L. siceraria* PAP

Step 1. UNOsphere™ Q anion exchange chromatography: A 35 ml (2.5 × 7.1 cm) Kontes Flex-Column® was packed with hydrated UNOsphere™ Q (Bio-Rad Laboratories, Inc. Hercules, CA) and equilibrated in 25 mM imidazole, pH 7.0, 1 mM magnesium chloride buffer. A total of 177 mg protein in 30 ml imidazole buffer was applied to the column at a flow rate of 3.0 ml per min using BioLogic LP™ chromatographic workstation at room temperature. The bound proteins were eluted using a series of discontinuous sodium chloride gradient at a 5% interval starting from 5% to 30% and then at 10% interval from 30% to 50%. Finally, the column was washed with 100% sodium chloride in imidazole buffer. An automated fraction collector was used holding the fraction size to 6.0 ml. Fractions were assayed for PAP activity using dioleoyl phosphatidic acid and general purpose acid phosphatase activity using *ρ*-nitrophenylphosphate (*ρ*NPP).

Step 2. UNOsphere™ S cation exchange chromatography: The active fractions from the previous step (fractions 15 through 23) were pooled and dialyzed against 50 mM sodium acetate, pH 5.0, and 1 mM magnesium chloride and loaded onto a 5 ml S column (1.5 × 2.8 cm) Kontes Flex-Column® at the flow rate of 1.0 ml per min. The bound proteins were eluted using a series of discontinuous sodium chloride gradients in acetate buffer at the same flow rate at a 5% interval starting from 5% to 30% and then at 10% interval from 30% to 50% sodium chloride. Finally, the column was eluted with 100% sodium chloride in acetate buffer. The column eluate was fractionated for 5 min each at the flow rate of 1.0 ml per min and assayed for both PAP and *ρ*NPP-based acid phosphatase activity.

Step 3. UNOsphere™ Q cation exchange chromatography: The active fractions from the previous step (fractions 26 through 31) totaling 30 ml were pooled and dialyzed against 25 mM imidazole, pH 7.4 buffer supplemented with 1 mM magnesium chloride and loaded onto a 1.0 ml cartridge column pre-packed with Q cation exchange resin, which was equilibrated in the same buffer at the flow rate of 1.0 ml per min. The bound proteins were eluted using a series of discontinuous sodium chloride

gradients in imidazole buffer at the same flow rate at a 5% interval starting from 5% to 30% and then at 10% interval from 30% to 50% sodium chloride. Finally, the column was eluted with 100% sodium chloride in imidazole buffer. The column eluate was fractionated for 5 min each and assayed for both PAP and acid phosphatase activity.

2.3. Gel Electrophoresis

Electrophoresis of the purified PAP enzyme was performed using Xcell II, Mini-Cell and 4% - 12% Novex™ NuPage Bis-Tris gels with MOPS as running buffer. (Life Technologies, Grand Island, NY). Successful separation was achieved at a constant 200 V and 70 min run. For gel calibration, prestained and multicolored molecular weight markers (4 to 250-kDa) were used as standards. The separated protein bands were visualized with Pierce (Thermoscientific, Rockford, IL) silver staining kit.

2.4. Measurement of Inorganic Orthophosphate (P_i) Released by PAP

To measure nmole level of P_i released by *L. siceraria* PAP from the substrate, dioleoyl phosphatidic acid (1,2-dioleoyl-*sn*-glycero-3-phosphate, sodium salt, Avanti polar lipids, Inc.), we used ammonium molybdate-based acetone-molybdate-acid (AMA) method [11]. The reaction volume was fixed at 1.0 mL to which was added 50 mM sodium acetate, pH 5.5, 25 to 50 µL of samples containing *L. siceraria* PAP enzyme followed by 50 µL of 10 mM dioleoyl phosphatidic acid to start the enzymatic reaction. Experiments were conducted in a 45˚C water bath for up to 30 min. To stop the reaction 2 mL freshly prepared AMA reagent was added. After 30 sec, 0.1 mL of citric acid (1.0 M) was added to each tube to fix the color. To remove turbidity we centrifuged the reaction mix in an Eppendorf 5415C at 13,000 × g for 7 min. The clarified reaction mix was read at 355 nm after blanking the spectrophotometer with the appropriate control run at zero time. PAP activity was expressed as nkat/ml (nmoles ortho-phosphate liberated per sec).

2.5. Acid Phosphatase Activity Measurement

A 5 to 20 µL of PAP enzyme samples were incubated in 50 mM acetate buffer, pH 5.5 with 1.25 mmoles of *ρ*-nitrophenylphosphate (*ρ*NPP) in a final volume of 1.0 mL at 45˚C for 1 to 2 min. The reaction was terminated adding 0.1 mL 1.0 N sodium hydroxide to the reaction mix and the liberated *ρ*-nitrophenol was measured spectrophotometrically at 400 nm [12].

2.6. Tryptic Digestion of the Purified PAP Enzyme

A 200 µL of the enzyme from UNOsphere™ Q column (step 4) was concentrated in a Centricon-10™ (Amicon™ centrifugal filter devices, Millipore Corporation, Billerica, MA) concentrator and in the process the buffer was exchanged to protease digestion buffer (50 mM ammonium carbonate, pH 8.0). The protein concentration of this preparation was 11 mg/mL.

The tryptic digests were prepared using Thermo Scientific in-solution tryptic digestion and guanidination kit. Briefly, 5 to 10 µg of protein was added to 15 µL digestion buffer containing 5.6 mM DTT in a total volume of 27 µL. The mixture placed in a microcentrifuge tube was incubated at 95˚C for 5 min. To alkylate the proteins, 3 µL of 100 mM iodoacetamide was added and kept in the dark at room temperature. A 2 µL aliquot of activated trypsin (100 ηg/µL) was added to each tube and incubated at 37˚C for 3 hr. To achieve complete digestion the incubation was continued further for 2 hr after the addition of 1 µL of trypsin. The digests were guanidilated to convert lysine to homoarginine by mixing the contents with 10 µL of 30% ammonium hydroxide and 6 µl of guanidination reagent (50 mg O-methylisourea hemisulfate in 51 µL ultrapure water) and incubated at 65˚C for 12 min. The reaction was stopped by the addition of 3 µL of trifluoro acetic acid (TFA) and stored at –20˚C before sequence analysis.

2.7. Peptide Separation, Sequencing and Similarity Search for Bioinformatics Studies

The tryptic digests from the active step 4 (**Table 1**) *Lagenaria siceraria* PAP were analyzed via LC/MS/MS, using an Agilent 1200 LC system, an Agilent Chip-cube interface, and an Agilent 6520 Q-TOF tandem mass spectrometer (Agilent Technologies, Santa Clara, CA). Chromatographic separation of the peptides was accomplished using a Chip consisting of a 40 nL enrichment column and a 43 mm analytical column packed with C18, 5 µm beads with 300Å pores. One µL aliquots of the sample were transferred to the enrichment column via a capillary pump operating at a flow of 4 µL/min. The nano pump was operated at a flow rate of 600 nL/min. An initial gradient (Solvent A: 100% water, 0.1% formic acid; Solvent B: 90% acetonitrile (ACN), 10% water and 0.1% formic acid) of 97% A was changed to 60% Solvent A at 12 min, 20% at 13 min, and held till 15 min. A post run time of 3 min was employed for column equilibration.

The MS source was operated at 300˚C with 5 L/min N_2 flow and a fragmentor voltage of 175V. N_2 was used as the collision gas with collision energy varied as a function of mass and charge using a slope of 3.7 V/100 Da and an offset of 2.5 V. Both quad and Time-of-Flight (TOF) were operated in positive ion mode. Reference compounds of 322.048121 Da and 1221.990637 Da were continually leaked into the source for mass calibration.

An initial MS scan was performed from m/z 300 to 1600 and up to three multiply charged ions were automatically selected for MS/MS analysis. Following the initial run, a second injection was made excluding ions previously targeted for MS/MS analysis. LC chromatograms and mass spectra were examined using Mass-Hunter software (Version B.0301; Agilent Technologies, Santa Clara, CA). Data files were transferred to an Agilent workstation equipped with Spectrum Mill software (Agilent Technologies, Santa Clara, CA). The raw MS/MS data files were extracted, sequenced, and searched against the National Center for Biotechnology Information (NCBI) non redundant protein library.

3. RESEULTS

3.1. Purification

We employed a regimen of both anion and cation exchange chromatography to purify *L. siceraria* PAP from developing cotyledons. The results are shown in **Table 1**. Judging from the binding of PAP to both anion and cation exchange column at pH 7.0 and pH 5.0 respectively, we conclude that the isoelectric point of the biocatalyst lies closer to pH 7.0 than pH 5.0.

It is worth noting here that the flow through of Q column had turbidity and very little PAP activity associated with it. This also indicates that microsomal fraction of the preparation had a negligible PAP activity. This bolsters our notion that we have identified a soluble PAP activity in the cotyledon extract of *Lagenaria siceraria*. **Figure 2** (panel A) shows the elution profile of first Q column where 6 protein peaks were visible. These were eluted by discontinuous salt gradient starting from 50 mM through 300 mM salt concentration. The first peak, which was eluted with 5% salt had PAP activity, whereas, the second and third peak showed both PAP and *ρ*NPP activity. Only the first peak was pooled, dialyzed, and applied to the second ion exchange S column. The PAP activity which bound to the cation exchange column at pH 5.0 was eluted at 300 mM and 400 mM salt concentration (**Figure 2** panel B). The PAP activity was bound to the third ion exchange column and eluted with 100 mM sodium chloride in imidazole buffer (**Figure 2** panel C). Of the three protein peaks eluted in the last column all the PAP activity was associated with the second protein peak.

The partially purified PAP from the third ion exchange column (UNOsphere™ Q) was visualized by SDS-PAGE and shown in **Figure 3**. Two major protein bands one at 42-kDa and the other at 27-kDa were observed. These two protein bands are tentative candidates for *L. siceraria* PAP. There were however several minor contaminating protein bands which co-purified with PAP.

There was a rapid loss of enzymatic activity as the preparation underwent ion exchange chromatography. By the time the second UNOsphere™ Q column was completed, the purified preparation had lost 99% of the PAP activity (**Table 1**). Thus, no further purification was attempted.

3.2. Biochemical Characterization of *L. siceraria* PAP

To characterize the catalytic properties of *L. siceraria* PAP, we used the active preparations from the third step of purification (**Table 1**), which is the S cation exchange column, because the activity dropped precipitously in the next step of purification, *i.e.*, the second Q anion exchange column. To establish the rate linearity of the enzyme reaction we added increasing amounts of enzyme while holding dioleoyl phosphatidic acid concentration (500 μM) and the time of incubation (60 min) fixed. The inorganic orthophosphate liberated by PAP are shown in **Figure 4**. The catalytic rate was shown to increase linearly as a function of enzyme concentration (6 to 30 μg of protein per ml) when assayed at 45˚C in sodium acetate buffer at pH 5.5.

The pH and temperature optima were empirically determined to be 5.5˚C and 45˚C, respectively (**Table 2**). Performing the enzyme assay at 45˚C and pH 5.5 while using dioleoyl phosphatidic acid as the substrate we calculated the V_{max} and K_m to be 0.36 ηkat per mg and 200 μM, respectively. When the same protein sample was used to determine the kinetic parameters of *L. siceraria* PAP for the synthetic acid phosphatase substrate, *ρ*NPP,

Table 1. Purification of *Lagenaria siceraria* phosphatidic acid phosphohydrolase from developing cotyledon.

Step	Volume	Total activity*	Total protein	Specific activity	Purification (fold)	Yield (%)
	(mL)	(ηkat)	(mg)	(nkat/mg)		
1) Crude extract	30	106.0	177.0	0.59	1.0	100.0
2) UNOsphere Q column	50	36.0	33.0	1.10	1.9	18.6
3) UNOsphere S column	30	11.2	3.5	3.20	5.4	10.6
4) UNOsphere Q column	20	1.1	1.3	0.84	1.4	0.6

*PAP activity determined using dioleoyl phosphatidic acid as the substrate.

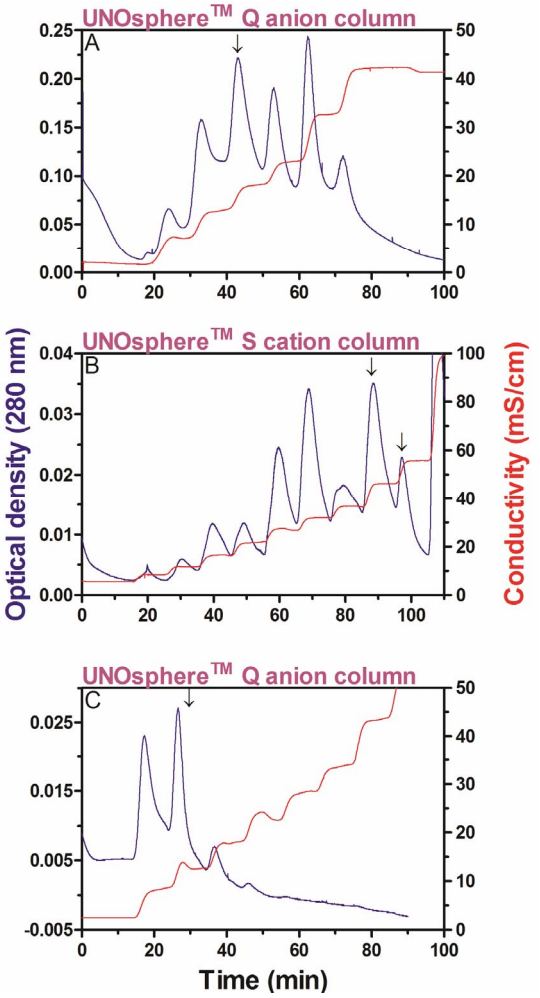

Figure 2. Chromatographic elution profile of developing cotyledon proteins from *L. siceraria* as generated by UNOsphere™ Q (Panel A), UNOsphere™ S (Panel B), and UNOsphere™ Q columns (Panel C). The solid line represents A_{280} and the dotted line represents conductivity (mS/cm). See methods section for details of ion-exchange chromatographies. The arrow shows the position where the PAP activity was eluted in the discontinuous salt gradient.

the V_{max} and K_m was found to be 33 ηkat per mg and 140 µM (**Table 2**).

We also have explored the role of Mg^{2+} in *L. siceraria* PAPactivity. Enzyme activity was unaffected by magnesium concentrations from 0 - 10 mM.

3.3. Bioinformatics with *L. siceraria* PAP

Against the backdrop of declining PAP activity after step 4 (UNOsphere™ Q anion exchange column) of the purification regimen (**Table 1**), the active fractions were pooled and tryptic digestion of the pooled protein performed to aid in sequencing peptides potentially emanating from highly purified PAP. We expected some de-

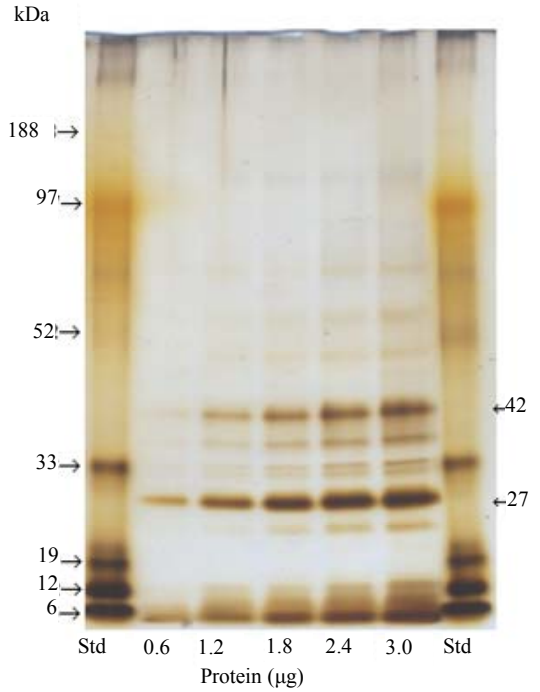

Figure 3. SDS-PAGE profile of silver-stained proteins from step 3 purified *L. siceraria* cotyledon PAP derived from the second A_{280} peak (marked by arrow) of **Figure 2** panel C. The gel was loaded with increasing amounts of proteins (0.6 to 3 µg per lane) to visualize the contaminating proteins.

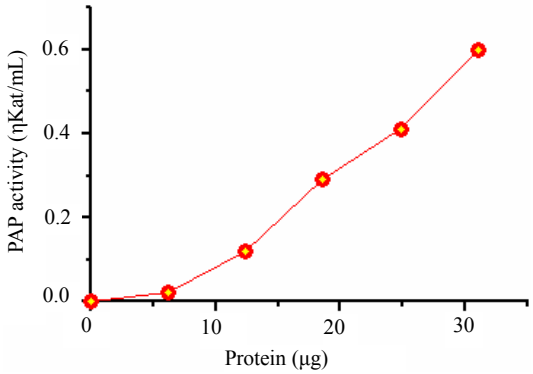

Figure 4. The rate linearity of *in vitro L. siceraria* PAP activity as a function of protein concentration.

Table 2. Kinetic properties of *L. siceraria* PAP.

Parameters	Values
pH optimum	5.5
Temperature optimum	45˚C
V_{max} for dioleoyl phosphatidic acid	0.36 ηkat per mg
K_m for dioleoyl phosphatidic acid	200 µM
V_{max} for *ρ*-nitrophenylphosphate	33 ηkat per mg
K_m for *ρ*-nitrophenylphosphate	140 µM

gree of contamination from non-PAP candidate peptides but sought to identify possible PAP sequences by performing sequence similarity searches against available plant sequence databases. Therefore, the peptides from GC-Mass Spectroscopy were searched against the NCBI non redundant protein library. Altogether there were about 128 proteins that showed varying degrees of identity to the trypsinized seed extract peptides. Our sample contained multiple peptides that matched with 13% - 16% of the total sequence of methionine synthase (EC 2.1.1.13) from a diversified group of plants such as potato, grape, castor bean, soybean, beet, *Arabidopsis lyrata*, etc., and 14% sequence identity to glutamate dehydrogenase (EC 1.4.1.2) of castor oil plant.

A few peptides from PAP tryptic digest however showed sequence homology to a diversified groups of phosphate metabolism enzymes such as enolase or phosphopyruvate hydratase (EC 4.2.1.11), fructose-biphosphate aldolase (EC 4.1.2.13), gamma-glutamyltransferase (EC 2.3.2.2), hydrogen dehydrogenase (EC 1.12.1.3), PEP-dependent phosphotransferase system (EC 2.7.3.9), and polyphosphate kinase (2.7.4.1). These *L. siceraria* peptides are listed in **Table 3**. Also noteworthy are *L. siceraria* peptide sequences similar to purple acid phosphatase (EC 3.1.3.2).

4. DISCUSSION

L. siceraria or bottle gourd is a dual-purpose crop both used as vegetables and folk medicine in India [13-15]. The oil content of the mature seeds is very high; as such, *L. siceraria* and other cucurbits could serve as model systems for understanding the metabolic pathway of triacylglycerol (TAG) formation in plant seeds. Furthermore, multiple genetic resources for cucurbit genomics are now available (http://www.icugi.org/cgi-bin/ICuGI/misc/project.cgi), which will make the task of locating the genes for Kennedy pathway enzymes less arduous.

Phosphatidic acid phosphohydrolase (PAP) is considered to be a key enzyme in the regulation of lipid synthesis [16]. This key biocatalyst has not been identified in oleaginous cucurbits. Ours is an attempt to identify and characterize the seed PAP from *L. siceraria*, an important member of the cucurbitaceae family.

We were able to follow the PAP activity in a purification regimen that employed both anion and cation exchange chromatography. However, the activity declined rapidly after the second Q anion exchange column (Step 4). One possibility merits mentioning here, which is the milieu in which TAG is synthesized. The conditions used for binding and elution of the PAP from the ion exchange column may have separated a required co-factor or subjected the biocatalyst to misfolding or distortion of the active site or substrate-binding domain.

The biochemistry, subcellular targeting, and putative physiological roles of PAP are complex and not yet completely understood. PAP activities are found in both the

Table 3. Peptides from proteins purified from *L. siceraria* seed that showed sequence homology to various phosphate metabolism enzymes.

Peptides obtained from *L. siceraria* cotyledons	Identity	Accession number
1) KFGIIGDLGQTFNSLSTLKH	Purple acid phosphatase (EC 3.1.3.2) from *Cucumis sativus*	A1BQL4
2) KSAPVYITVGDGGNQEGLAGRF	Purple acid phosphatase (EC 3.1.3.2) from *Ricinus communis*	B9RP16
3) KTAARDPFVVAIKQ	Polyphosphate kinase (EC 2.7.4.1) from *Thiomonas intermedia*	D5X1T9
4) RGNPTVEVDVILSDGTLARA	Enolase (EC 4.2.1.11) from *Ricinus communis*	B9R9N6
5) KSFASEYPIVSIEDPFDQDDWEHYSKL	Enolase (EC 4.2.1.11) from *Ricinus communis*	B9R9N6
6) KVNQIGSVTESIEAVKM	Enolase (EC 4.2.1.11) from *Oryza sativa*	A1YQJ3
7) RSGETEDTFIADLSVGLATGQIKT	Enolase (EC 4.2.1.11) from *Gossypium hirsutum*	A8IMP0
8) RALQQSTIKK	Fructose-biphosphate aldolase (EC 4.1.2.13) from *Oryza sativa*	B8B4J4
9) RLASINVENVESNRRA	Fructose-biphosphate aldolase (EC 4.1.2.13) from *Arabidopsis lyrata*	D7LJ40
10) KEGGVLPGIKV	Fructose-biphosphate aldolase (EC 4.1.2.13) from *Vitis vinifera*	A5B118
11) RTVPAAVPAVVFLSGGQSEEEATLNLNAMNKL	Fructose-biphosphate aldolase (EC 4.1.2.13) from *Zea mays*	B4FWP0
12) KVAPEVVAEYTVRA	Fructose-biphosphate aldolase (EC 4.1.2.13) from *Ricinus communis*	B9SRH4
13) KCAEVTERV	Fructose-biphosphate aldolase (EC 4.1.2.13) from *Physcomitrella patens*	A9S0F9
14) KYYEAGARF	Fructose-biphosphate aldolase (EC 4.1.2.13) from *Toxoplasma gondii*	B6KMB6
15) RFVLKDQTQVDYPRL	Phosphotransferase system EIIC (EC 2.7.3.9) from *Pantoea* sp	E0LSS4

cytosolic and membrane fractions of yeast [6,17] and plants [18-21]. Type II PAPs are Mg^{2+}-independent integral membrane proteins. Type I PAPs are Mg^{2+}-dependent and are often found distributed between cytosolic and membrane fractions. Type II PAPs seem to play no role in bulk cellular lipid metabolism, but are likely involved in signal transduction pathways [17,22,23]. Type I enzymes on the other hand, have been implicated in a direct role in phospholipid and triacylglycerol synthesis in yeast [6] and phospholipid and galactolipid synthesis in plants [18-20]. Ample evidence from yeast, plant, and mammalian systems suggests that type I PAP acts as both a metabolic enzyme and a transcriptional regulator [20, 24,25]. In yeast, both roles appear to rely on phosphatidic acid phosphohydrolase enzymatic activity, as mutations of critical catalytic residues render the enzyme unable to complement various mutant phenotypes [26].

The PAP characterized in this report most closely resembles the properties described by Pearce *et al*. [17] who partially purified a PAP activity from *Persea americana* (avocado) fruit. The enzyme was shown to be about 49-kDa by SDS-PAGE analysis. This protein was peripherally associated with the microsomal fraction, and could be dissociated from membranes using high salt and partially purified through the use of surfactants. The avocado enzyme did not require Mg^{2+} for activity, but was slightly stimulated by addition of 1 mM $MgCl_2$ [17]. The *L*. *siceraria* PAP preparation reported in this communication is unique in that it was found to be cytosolic and completely insensitive to magnesium. This is the first time that such a PAP was reported, and as such, the protein present in our preparations may represent a new class of PAP enzyme.

Unfortunately, neither the peptide nor gene sequence corresponding to the 49-kDa avocado protein has been reported; therefore, we cannot compare it to any of our peptide sequences.

However, the sequence similarity search with peptides generated from partially purified *L*. *siceraria* PAP has provided a plethora of information. Clearly, some of the peptides generated from the active fractions of the last UNOsphere™ Q column were not engendered from PAP; however, a significant number of peptides showed homology to phosphate metabolizing enzymes such as polyphosphate kinase (EC 2.7.4.1), enolase or 2-phosphoglyceratedehydratase (EC 4.2.1.11), fructose-biphosphate aldolase (EC 4.1.2.13), and purple acid phosphatase (EC 3.1.3.2) (**Table 3**). It is coincidental that both phosphatidic acid phosphohydrolase and purple acid phosphatase hydrolyze phosphomonoester bonds and it is possible that their catalytic sites may be structurally similar. However, we expect the substrate-binding domain to be markedly different in these two phosphomonoesterases. When the *Momordica charantia* seed transcriptome was analyzed for conjugated fatty acid metabolism-related genes, the 50 most abundant transcript reads in the non-normalized cDNA library contained enolase [27]. We also noticed several peptides in purified *L*. *siceraria* PAP that showed homology to enolase from castor bean, rice, and cotton (**Table 3**).

Literature is not replete with PAP from cucurbits; therefore, enzymatic characterization and sequence information of this important biocatalyst of lipid synthesis may stimulate further research in oleaginous plants belonging to the family Cucurbitaceae. To our knowledge this is the first documented reporting of any PAP from cucurbitaceae. The bioinformatics studies of PAP and other co-purified proteins from *L*. *siceraria* developing seeds have shown sequence similarities with a few phosphate metabolizing enzymes including purple acid phosphatase. It has not escaped our attention that phosphatidic acid phosphohydrolase or PAP (EC 3.1.3.4) is also capable of degrading *ρ*-nitrophenylphosphate, which is a general purpose acid phosphatase. Therefore, the PAP may share sequence homology with purple acid phosphatase. The final confirmation will come from cloning PAP genes from other well-studied oleaginous plants such as *Momordica charantia* and *Vernicia fordii* and overexpression in heterologous host such as *Pichia pastoris*. Now that cucurbit genome sequences are becoming available (http://www.icugi.org/cgi-bin/ICuGI/misc/project.cgi), the bioinformatics work vis-à-vis phosphatidic acid phosphohydrolase may yield a plethora of information regarding the conservation of PAP types between *L*. *siceraria* PAP and the Cucurbitaceae family, and higher plants in general.

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