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Identification of an Mg^{2+}-Independent Soluble Phosphatidate Phosphatase in Cottonseed (*Gossypium hirsutum* L.)

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Abstract

Cotton (*Gossypium hirsutum* L.) provides a major source of oil for food and feed industries, but little was known about the enzymes in the oil biosynthesis pathway in cottonseed. We are interested in a better understanding of enzymatic components for oil accumulation in cottonseed. The objective of this study was to identify one key enzyme in oil biosynthesis pathway: phosphatidic acid phosphatase (PAP, 3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4). PAP hydrolyzes the phosphomonoester bond in phosphatidate yielding diacylglycerol and P_i. PAPs are generally categorized into Mg^{2+}-dependent soluble PAP and Mg^{2+}-independent membrane-associated PAP. Cottonseed from 25 - 30 days post anthesis was used for the study. The results showed that an Mg^{2+}-independent soluble PAP activity was identified from the cottonseed. While the microsomal fraction of the extract provided only 9% of the PAP activity, 69% of the PAP activity was associated with the cytosol. The PAP activity correlated well with enzyme concentration and incubation time. The pH and temperature optima of the enzyme were pH 5 and 55˚C, respectively. Under optimized assay conditions, the V_{max} and K_m values of cottonseed PAP for dioleoyl phosphatidic acid as the substrate were 2.8 nkat/mg of protein and 539 μM, respectively. Inclusion of the detergent Triton X-100 (0% - 0.5%) or magnesium chloride (1 mM) in the reaction mix did not alter activity to a significant degree. This is the first report of a PAP activity in the seeds of *Gossipium hirsutum.* This study should provide a basis for purification and characterization of this important enzyme from cottonseed in the future.

Keywords

Cottonseed, EC 3.1.3.4, Phosphatidate Phosphohydrolase, Phosphatidic Acid Phosphatase, *Gossypium hirsutum*

1. Introduction

Cotton plant provides two economically important products, fiber as the major product and cottonseed as the minor product. However, cotton plant produces more seed in terms of quantity than fiber with a seed/fiber ratio of 1.5 - 1.7. Cottonseed typically accounts for 15% - 25% of the crop value. Approximately 95% of the cotton crop grown world-wide is derived from *Gossypium hirsutum* (short staple cotton varieties) and about 4% is derived from *Gossypium barbadense* (long staple cotton varieties). 5.6 million tons of cottonseed was produced in the United States in 2012 [1]. Among them, approximately, three million tons of cottonseed was used to feed dairy cows and 2.5 million tons of cottonseed was used for oil production, which generated 1.3 million tons of cottonseed meal and 0.4 million tons of vegetable oil. The amount of oil in the seed varies from 20% to 24% (dry-weight basis) [1].

Oil is the most valuable product of the cottonseed and used primarily as food or food ingredient. Cottonseed oil is rich in linoleic acid, a polyunsaturated fatty acid (~54%) and relatively low in oleic acid, a monosaturated fatty acid (~16%) [2]. This composition of fatty acids allows the oil to be useful for some particular applications, such as for salad dressings and cooking oil. However, the high ratio of polyunsaturates to mononsaturates results in reduced oxidative stability compared with other cooking oils.

Currently, much needs to be learned about the enzymatic mechanism of the oil biosynthesis in cottonseed. According to the Kennedy or G3P pathway [3], acyltransferases including diacylglycerol transferases [4] [5], add fatty acyl groups sequentially to the sn-1, sn-2 and sn-3 positions of glycerol-3-phosphate (G3P) to form triacylglycerol (TAG). One of the key step in TAG biosynthesis is the dephosphorylation of the sn-3 position of phosphatidate (PtdOH) catalyzed by phosphatidic acid phosphatase (PAP or lipins) to produce diacylglycerol (DAG) and inorganic phosphate (P_i) (**Figure 1**) [6]. PtdOH is synthesized by the actions of glycerophosphate acyltransferase (GPAT) and lysophosphatidic acid acyltransferase (LPAAT). DAG formation is thought to be the penultimate key step in Kennedy pathway because it is a critical metabolite for the synthesis of TAG, phosphatidylethanolamine (PtdEtn), and phosphatidylcholine (PtdCho)

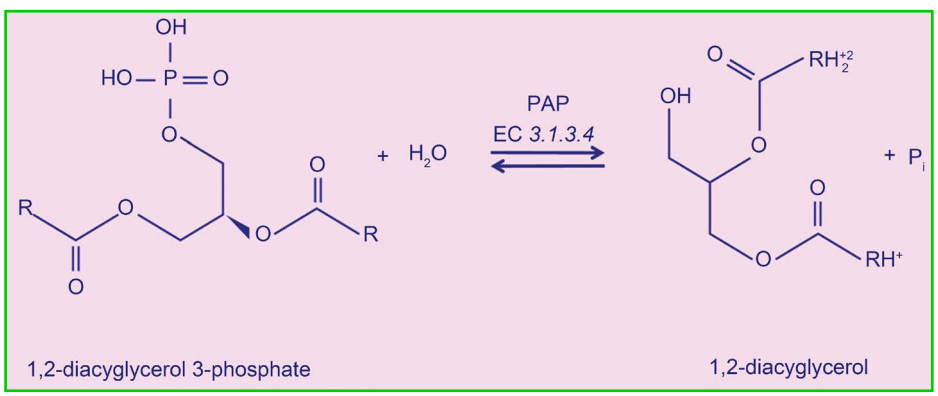

Figure 1. The schematic representation of enzymatic reaction catalyzed by PAP. The enzyme hydrolyzes the phosphoester linkage of PtdOH and generates DAG and P_i.

[6] [7] [8].

Towards better understanding the enzymatic steps responsible for oil accumulation in cottonseed, we initially focused our studies on PAP (3-sn-phosphatidate phosphohydrolase, EC 3.1.3.4) that dephosphorylates phosphatidic acid (PA, also called PtdOH) to generate DAG and P_i. PAP family enzymes were classified as either soluble PAP [9] or membrane-bound PAP [10]. Based on the requirement of Mg^{2+} for activity, PAP could also be divided into 2 classes: Mg^{2+}-dependent and Mg^{2+}-independent PAP [11]. Typically, soluble PAP is Mg^{2+}-dependent; whereas membrane-bound PAP is Mg^{2+}-independent. We recently identified and characterized a soluble PAP in bitter melon cotyledons [12] [13]. We report here the identification and partial characterization of PAP from cottonseed as a soluble and Mg^{2+}-independent enzyme.

2. Materials and Methods

2.1. Plant Material

Cotton (*Gossypium hirsutum* var. Coker 312) seeds were collected at the mid-maturity stage, approximately 25 - 30 days post anthesis. The seed coat was removed before preparation of enzyme extracts.

2.2. Preparation of Cottonseed Extracts

All operations were carried out at 4˚C. The seeds free of seed coat weighing 91 g were homogenized in 100 mL extraction buffer (50 mM NaOAc, pH 5.0, 150 mM NaCl and 10 mM $MgCl_2$) using polytron tissuemizer (Tekmar Tissumizer MarkII, Cincinnati, OH) at low, medium and high speed for 30 s each. The homogenate was cooled down on ice for 1 min between bursts. The homogenate was centrifuged at 10,000 g for 15 min and the resulting supernatant was centrifuged at 20,000 g for 30 min at 4˚C (Sorvall RC2B, Miami, FL). This supernatant was ultracentrifuged at 105,000 g for 45 min at 4˚C (Sorvall Discovery 100SE, Hitachi Ltd, Tokyo, Japan) and the resulting supernatant (cytosol) and the pellet (microsomal membranes) were collected. The 105,000 g supernatant was dialyzed to remove Pi from the seed extract against imidazole buffer (25 mM imidazole buffer, pH 6.5, 1 mM $MgCl_2$) with three 500 mL buffer changes. The dialyzed supernatant became cloudy after dialysis, which was removed by centrifugation at 20,000 g for 30 min at 4˚C. The final supernatant as cytosol and 105,000 g pellet as microsomal membranes were used for determining subcellular distribution of PAP activity. The protein content of the supernatant was determined by Bicinchoninic acid (BCA) method (Thermo Scientific, Rockford, IL).

2.3. PAP Activity Assay

PAP activity was determined by P_i-release assay. This activity assay was described in details for bitter melon PAP [12]. The Pi-release assay followed the ammonium molybdate-acetone-acid (AMA) method [14]. For standard assay except otherwise noted below, a 50 µL of enzyme extract was added to 900 µL of 50 mM imidazole buffer, pH 5 in a 55˚C water bath. The enzymatic reaction was initiated by the addition of 50 µL of

PtdOH/DPA (dioleoyl-phosphatidic acid or 1,2-dioleoyl-sn-glycero-3-phosphate, sodium salt, Avanti Polar Lipids, Inc., Alabaster, Alabama), incubated for 5 min and terminated by 2 mL of AMA reagent. Citric acid (0.1 mL, 1.0 M) was added to each tube 30 s later to fix the color followed by centrifugation at 13,000 g for 7 min (Eppendorf 5415C, Westbury, NY). The absorbance at 355 nm was measured after blanking the spectrophotometer with the appropriate control, which was stopped at zero time. The PAP activity was expressed as nanokatals per milliliter (nkat/mL, nmoles orthophosphate released per sec). One International Unit (IU) is equivalent to 16.67 nkat.

To determine the pH optimum of cottonseed PAP, the reaction mixtures were incubated at 55˚C with different buffer pH using 25 mM glycine-HCl (pH 2 - 3), 50 mM sodium acetate (pH 4 - 5), and 25 mM imidazole (pH 6 - 7). To measure the optimum temperature, the samples were incubated with substrate between 20˚C and 80˚C in 25 mM imidazole, pH 5. The Mg^{2+} dependency was determined by the phosphatase assay with or without 1 mM $MgCl_2$ in the assay mixtures. The general procedures for PAP characterization were similar to those used for soluble starch synthases [15].

2.4. Kinetics of PAP

The K_m and V_{max} values for cottonseed PAP using the P_i-release assay were determined at 55˚C and pH 5 as mentioned above. The concentration of DPA ranged from 0 to 500 μM. WindowChem's software Enzyme Kinetics version 1.1 (Fairfield, CA) was used to compute the K_m and V_{max} values.

3. Results and Discussion

3.1. Subcellular Distribution of PAP Activity in Cottonseed

PAP family enzymes were classified as either soluble PAP or membrane-bound PAP [9]. Differential centrifugation was used to separate the cytosolic and microsomal membrane fractions for determining the subcellular localization of PAP activity in cottonseed. The seed extract free of seed coat was successively centrifuged at 10,000 g, 20,000 g and 105,000 g. The final pellet and supernatant after ultracentrifugation are generally regarded as the microsomal membranes and the cytosol, respectively [16]. PAP activity in the 10,000 g, 20,000 g and 105,000 g pellet was 6.6%, 12.5% and 9.3% of the total PAP activity (**Figure 2**). PAP activity in the 105,000 g pellet represented the PAP activity associated with the microsomal membranes, whereas those activities in the 10,000 g pellet and 20,000 g pellet might be due to some seeds not completely homogenized and/or the pellet being contaminated with the cytosol. Following dialysis and centrifugation of the supernatant, the final supernatant contained 69% of the total activity (**Figure 2**). These subcellular distributions of PAP activity clearly demonstrated that the great majority of PAP activity in cottonseed was soluble and localized in the cytosol. We recognized that this conclusion is only based on cottonseed collected 25 - 30 days post anthesis. It is possible that the ratio of PAP activity distribution could be different in different stages of cottonseeds. Most likely, more membrane-bound PAP could be detected in older seeds.

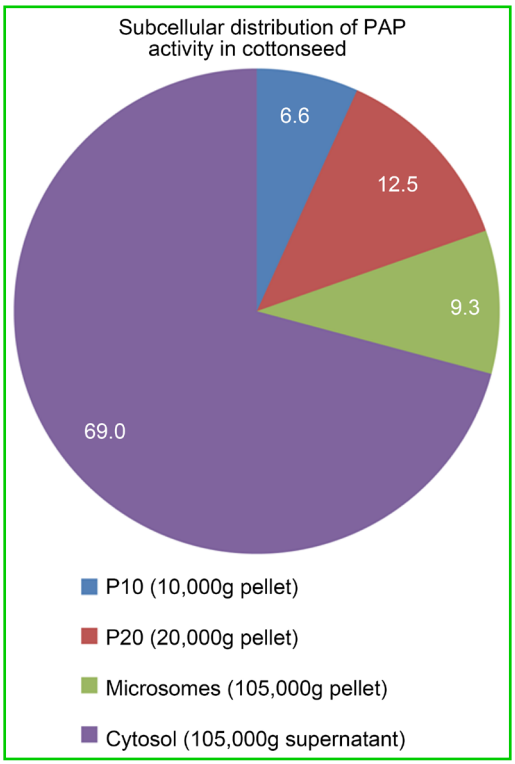

Figure 2. Subcellualr distribution of PAP activity in cottonseed. Cottonseed kernels free of seed coat collected from 25 - 30 days post anthesis were homogenized and the extract was successively centrifuged at 10,000 g, 20,000 g and 105,000 g. The final pellet after ultracentrifugation is regarded as the microsomal membranes, and the 105,000 g supernatant as cytosol. All fractions were analyzed for PAP activity. The data presented are the mean of two assays for each sample.

3.2. Linearity of PAP Assays

The linearity between the incubation time and PAP activity was observed in 50 mM imidazole buffer containing 0.3 mM $MgCl_2$, pH 5 (**Figure 3**). The linearity between PAP activity and reaction time was much better when the enzymatic assays were performed after incubation within 6 min with $R^2 = 0.9663$ (**Figure 3(a)**) than that incubation extended to 10 min with $R^2 = 0.8466$ (**Figure 3(b)**). Similar linearity of enzymatic reaction was observed in responding to the amount of enzymes used in the assay with $R^2 = 0.9828$ (**Figure 4**). These assay results suggest that the established assays are suitable for characterization of PAP from cottonseed.

3.3. The Temperature and Buffer pH Optima of PAP

To determine the temperature optimum, PAP activity was assayed under a series of reaction temperature ranged from 22˚C to 82˚C with 5˚C interval. Curve-fitting of PAP activity vs. reaction temperature showed that the temperature optimum was at 55˚C (**Figure 5**). Similarly, to determine the buffer pH optimum, PAP activity was assayed using a series of buffer pH ranged from pH 2 to pH 7 with an interval of 1 pH unit (**Figure 6**). The pH optimum of PAP was observed at pH 5 under the assay conditions using 5 μL of the enzyme and 500 μM dioleoyl-phosphatidic acid (DPA) in 50 mM

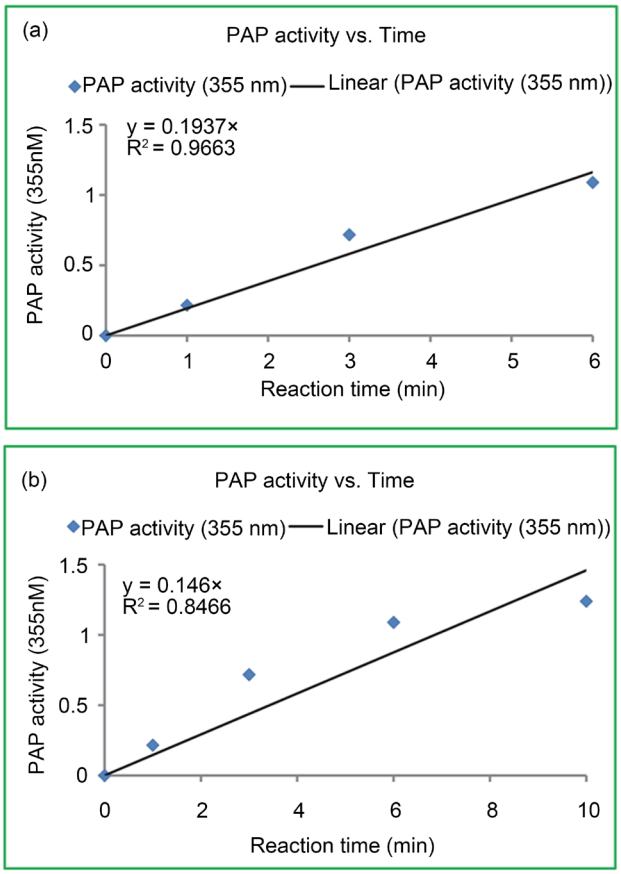

Figure 3. Linearity of PAP activity in a time-course study. PAP activity was assayed using aliquots of the protein extracts. The assay was performed at pH 5 and 55˚C for various times with 500 μM DPA and 0.3 mM $MgCl_2$. Aliquots of the enzymatic reactions were withdrawn for measurement at the indicated time points. (a) Curve-fitting of PAP activity vs. reaction time within 6 min. (b) Curve-fitting of PAP activity vs. reaction time extended to 10 min. The data presented are the mean of two assays for each sample.

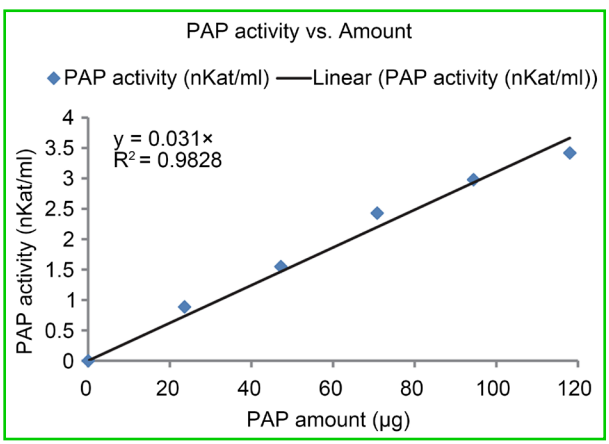

Figure 4. Linearity of PAP activity in a dosage study. PAP activity was assayed using different aliquots of the protein extracts. The assay was performed at pH 5 and 55˚C using various amounts of the PAP preparation with 500 μM DPA and 0.3 mM $MgCl_2$. The data presented are the mean of two assays for each sample.

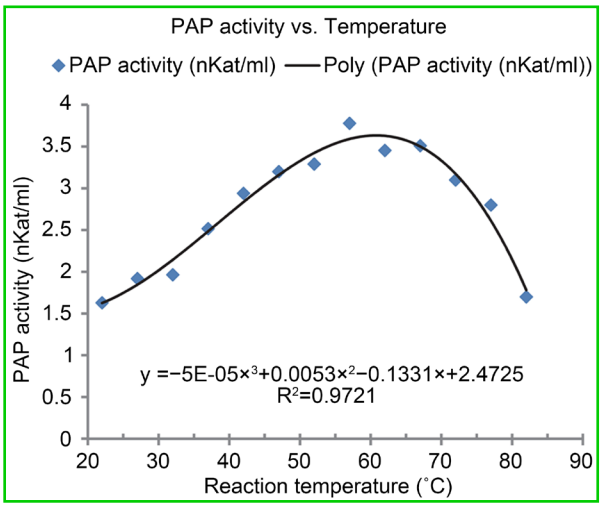

Figure 5. Temperature optimum of PAP activity. PAP assay was performed at pH 5 under various reaction temperature for 5 min using 5 µL of the PAP preparation with 500 µM DPA and 0.3 mM $MgCl_2$. The data points presented are the mean of two assays for each sample. The temperature optimum was calculated after curve-fitting of PAP activity vs. reaction temperature.

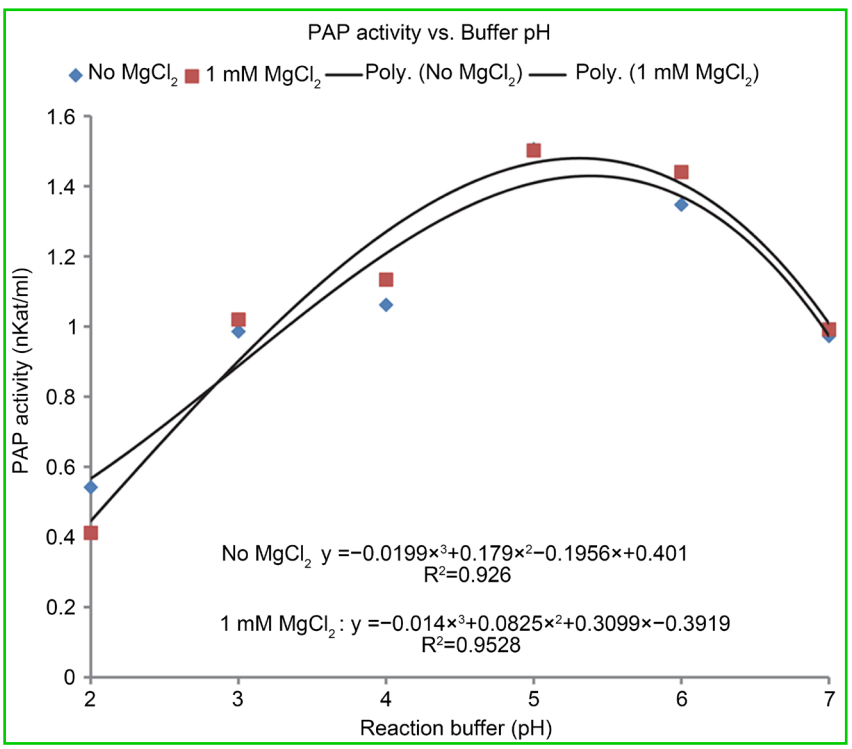

Figure 6. Buffer pH optimum of PAP and Mg^{2+} effect on PAP activity. The assay was performed at 55˚C for 5 min using 5 µL of the PAP preparation with 500 µM DPA and with or without 1 mM $MgCl_2$. The data presented are the mean of two assays for each sample. The temperature optimum was calculated after curve-fitting of PAP activity vs. reaction temperature.

imidazole buffer containing 0.3 mM $MgCl_2$ (**Figure 6**). These optimal pH and temperature values are similar to those of bitter melon extract [12].

3.4. Mg^{2+}-Independent Activity of PAP

Based on the requirement of Mg^{2+} for activity, PAP family enzymes including lipins and lipid phosphate phosphatases (LPPs) are divided into 2 classes: Mg^{2+}-dependent PAP and Mg^{2+}-independent PAP [11]. It is known that yeast and invertebrates have a single lipin ortholog, but plants have two PAP/lipin genes and mammals have three lipin genes [6]. The lipin family members of PAP are soluble enzymes and require Mg^{2+} for their activity. LPPs also exhibit PAP activity but they are structurally unrelated to lipin proteins. LPPs are localized to the plasma membrane and do not require Mg^{2+} for their activity. Our results showed that PAP activity was not affected or minimally affected by 1 mM $MgCl_2$ in the assay mixtures (**Figure 6**). The effects of ion chelaters EDTA and EGTA on PAP activity were measured. However, both chelators did not have significant effects on PAP activity (data not shown). This result further confirmed that the soluble PAP activity was Mg^{2+}-independent in cottonseed extract. These assay results confirm the previous observations from crude extract of bitter melon and partially purified bottle gourd PAP that these PAPs are Mg^{2+}-independent enzyme [12] [17]. The overall results suggest that a new class of PAP exists in cottonseed which is a soluble and Mg^{2+}-independent enzyme.

3.5. Kinetic Parameters of PAP

The kinetic parameters of PAP were determined using DPA as the substrate under the optimized assay conditions (pH 5, 55˚C and 0.3 mM $MgCl_2$). The enzyme gave a typical sigmoidal curve for the substrate (**Figure 7**). The K_m and V_{max} values of PAP for DPA were calculated to be 539 μM and 2.8 nkat/mg of protein, respectively (**Table 1**). Triton X-100 did not have significantly effects on PAP activity (**Table 1**).

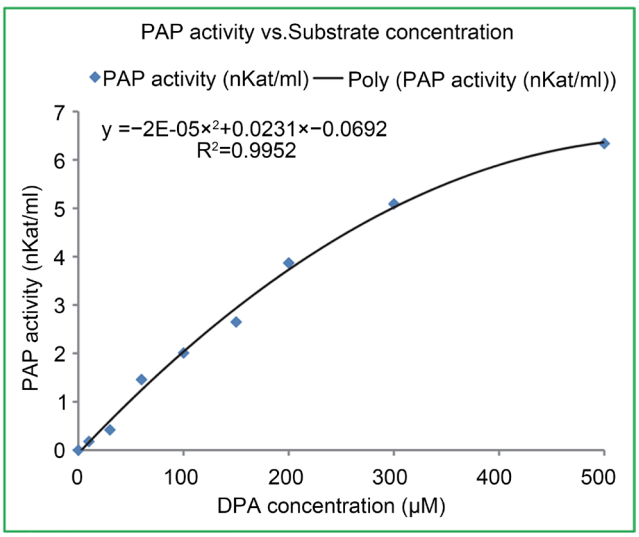

Figure 7. Kinetcis of PAP enzyme activity. PAP activity vs. substrate concentration. PAP assay was performed at pH 5 and 55˚C for 5 min using various concentrations of DPA. The data points presented are the mean of two assays for each sample. WindowChem's software Enzyme Kinetics version 1.1 (Fairfield, CA) was used to compute the K_m and V_{max} values.

Table 1. Summary of PAP from developing cottonseed.

Parameters	
Substrate	Dioleoyl phosphatidic acid
pH optimum	5.0
Temperature optimum	55˚C
Mg^{2+} dependency for activity	No effect
Triton X-100	No effect
K_m (μM)	539 ± 131.7
V_{max} (nkat/mg of protein)	2.8 ± 0.27

4. Conclusion

Phosphatidic acid phosphatases (PAPs) catalyze the dephosphorylation of phosphatidic acid to diacylglycerol, the penultimate step in TAG synthesis. PAPs are widely present in plants, animals, microbes and human. PAPs are typically categorized into two subfamilies: Mg^{2+}-dependent soluble PAP and Mg^{2+}-independent membrane-associated PAP. In this study, we provided evidence for the existence of a new class of PAP enzyme in cottonseed. This class of PAP is soluble and Mg^{2+}-independent. Although this study reports preliminary characterization of PAP in cottonseed, it should provide a basis for purification and characterization of this important enzyme in the future.

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Abbreviations

AMA: Acetone-molybdate-acid; BCA: Bicinchoninic acid; DAG: Diacylglycerol; DPA: Ioleoyl-phosphatidic acid or 1,2-dioleoyl-sn-glycero-3-phosphate; G3P: Glycerol-3-phosphate; GPAT: Glycerophosphate acyltransferase; LPAAT: Lysophosphatidic acid acyltransferase; LPP: Lipid phosphate phosphatase; PA: Phosphatidic acid; PAP: Phosphatidic acid phosphatase; P_i: Inorganic phosphate; PtdCho: Phosphatidylcholine; PtdEtn, Phosphatidylethanolamine; PtdOH: Phosphatidate or phosphatidic acid; TAG: Ttriacylglycerol.