

# Identification of a soluble phosphatidate phosphohydrolase in the developing cotyledons of *Momordica charantia*

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## ABSTRACT

Phosphatidate phosphatase (EC 3.1.3.4), PAP, catalyzes the dephosphorylation of phosphatidate (PtdOH) to form diacylglycerol. In eukaryotes, PAP driven reaction is the committed step in the synthesis of triacylglycerol. A  $Mg^{2+}$  independent PAP activity was identified in the soluble extract of *Momordica charantia* cotyledons undergoing maturation. While the microsomal fraction of the extract gave only 10% of the PAP activity, the remaining 90% of the activity was associated with the soluble fraction. At pH 3.0, the soluble PAP was bound to S column and eluted with glycine-HCl buffer containing high salt. The pH and temperature optima of the PAP activity were 6.0 and 53°C, respectively. Under optimum assay condition, the  $V_{max}$  and  $K_m$  for dioleoyl phosphatidic acid were 1.89  $\eta$ kat/mg of protein and 142  $\mu$ M, respectively. For the synthetic substrate,  $\rho$ -nitrophenylphosphate,  $\rho$ -NPP, the  $V_{max}$  and  $K_m$  were 10.4  $\eta$ kat/mg of protein and 107  $\mu$ M, respectively. The inclusion of  $Mg^{2+}$  and  $\beta$ -mercaptoethanol into the reaction mix did not change the enzyme activity nor did the addition of N-ethylmaleimide and phenylglyoxal, which indicates that cysteine and arginine are not involved in catalysis of PtdOH. The addition of  $Mg^{2+}$  up to 10 mM also did not change the level of PAP activity. Triton X-100, however, inhibited the activity. This is the first documented case of an *in vitro* PAP activity in the developing cotyledons of *Momordica charantia*. The PAP described here could serve as a model for lipin-1 or lipin-2 in humans. Mutations in these genes lead to acute myoglobinuria in human infants.

**Keywords:** PAP; EC 3.1.3.4; Phosphatidic Acid Phosphatase; *Momordica Charantia*

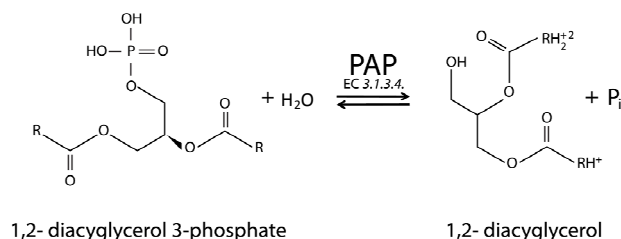
## 1. INTRODUCTION

Phosphatidic acid phosphatase (PAP, 3-sn-phosphatidate

phosphohydrolase, EC 3.1.3.4) hydrolyzes the phospho-monoester bond present in 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 (oil), 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 triacylglycerol (TAG). This pathway was discovered in the 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 PtdOH, which is formed by the action of two specific acyltransferases, namely, glycerophosphate 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 TAG formation, it 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 lipodystrophy, a disease that eventuates in the loss of body fat and insulin resistance, conversely, lipin 1 promotes obesity and insulin sensitivity [4-6]. The discovery of lipin 1 being a PAP, the enzyme is linked to the regulation of body fat metabolism in mammalian cells. Therefore, it follows that the activity of PAP will be probed by pharmaceutical researchers for the control of body fat metabolism 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 critical 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 biocata-

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**Figure 1.** The schematic representation of enzymatic reaction catalyzed by phosphatidic acid phosphohydrolase (PAP). PtdOH yields DAG and P<sub>i</sub> when phosphoester linkage is hydrolyzed by PAP.

lyst 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. This may provide an insight into further modify, enhance, and control the total fatty acyl composition of triglycerides in oil-producing plants if so desired. Of particular interest to plant biochemists are the gene sequences that codes for PAP, which may be useful for applications in genetic engineering of oleaginous crops.

In this study, we characterized *Momordica charantia* PAP as detected in the crude supernatant derived from developing cotyledons by measuring activity, conducting kinetics experiments, and determining pH optima, temperature optima, effects of minerals and other reagents. In mature seed of *Momordica charantia*, a high percentage of oil consists of  $\alpha$ -eleostearic acid [9] which gives the oil its properties as a drying oil. Therefore, bitter gourd PAP genes could play an important role in determining high content of this industrially useful and unusual fatty acid in storage oils. This is the first documented reporting of PAP activity in *Momordica charantia*.

## 2. MATERIALS AND METHODS

### 2.1. Source of PAP

*Momordica charantia* (bitter melon) fruits at mid maturity level, which is ideal for human consumption, was purchased at an oriental grocery in New Orleans, LA, USA. The cultivar is widely grown in India and the fruits were each about 6 inches in length on average.

### 2.2. Extraction of Soluble PAP from *Momordica charantia* Developing Cotyledons

The seeds (18.0 gm) of mid-level maturity were obtained from the seed cavity of *Momordica charantia* fruit and washed with 0.9% ice cold saline solution. The outer coverings of the seeds were removed at room temperature using scalpel and the cotyledons removed manually. All subsequent operations were carried out at 4°C.

The extraction buffer (5.0 ml of 50 mM acetate, pH 5.0, 150 mM sodium chloride and 10 mM MgCl<sub>2</sub>) was added to the seeds and homogenized using Tekmar Tis-

sumizer MarkII (Cincinnati, OH) at low, medium and high speed for 30 sec each. The homogenate was cooled on ice for 1 min in between bursts. This was followed by a centrifugation using Sorvall RC2B (Miami, FL) centrifuge at 20,000 × g for 30 min at 4°C. The pellet containing unbroken cotyledon was discarded. The resulting supernatant was dialyzed overnight against 50 mM imidazole, pH 6.0 containing 1 mM MgCl<sub>2</sub> with three 500 ml buffer changes to remove any inorganic molecules originating from the seed tissue. The dialyzed supernatant became cloudy after dialysis, which was removed by centrifugation as above. The pellet was discarded and the supernatant was used for PAP activity measurement. The protein content of the supernatant as determined by Bicinchoninic acid (BCA) method (Pierce, Rockford, IL) was 4.27 mg/mL and the total protein was 18 mg in 4.2 mL volume. The specific activity of the preparation was 1.17 ηkat/mg of protein when the assay was performed at 53°C and pH 6.0.

### 2.3. Ultracentrifugation Study of the Crude Extract

To determine whether the PAP derived from the developing cotyledons of *M. charantia* was a soluble protein, an ultracentrifugation experiment was conducted with the 18,000 × g supernatant fraction of the crude cotyledon preparation. The sample (4.5 ml) was subjected to ultracentrifugation (Sorvall discovery 100SE, Kendro, Newton, CT) at 105,000 × g for 1 hr. The pellet was resuspended in 4.0 mL 50 mM sodium acetate, pH 5.0, 1 mM MgCl<sub>2</sub>, buffer. Both the supernatant and resuspended pellet from ultracentrifugation study was assayed for PAP activity.

### 2.4. Binding of Soluble Proteins from Cotyledon Extract to Ion-Exchange Column

The supernatant from cotyledon extract of *M. charantia* was dialyzed against 25 mM glycine-HCl buffer, pH 3.0, overnight with three times buffer exchange, 500 ml each. The column chromatography was performed using Bio-Logic LP™ (Bio Rad, CA) chromatographic workstation at ambient 25°C. The dialyzed protein was applied to a 20 ml UNOsphere™ S column (2.5 × 4.0 cm) equilibrated with the glycine-HCl buffer at the flow rate of 3.0 ml per min. The column was then washed with the equilibration buffer followed by a stepwise sodium chloride gradient in Glycine-HCl buffer ranging from 0.2 to 0.4 M at 0.05 M increment and then with 0.5 and 1.0 M sodium chloride in the glycine-HCl buffer.

### 2.5. Measurement of P<sub>i</sub> Released from PtdOH by PAP Activity

To measure nmole level of P<sub>i</sub> released by *Momordica*

*charantia* PAP from the substrate, dioleoyl-phosphatidic acid (1,2-dioleoyl-sn-glycero-3-phosphate, sodium salt, Avanti polar lipids, Inc. Alabaster, Alabama) we used ammonium molybdate-acetone-acid (AMA) method [10]. Briefly, a 50  $\mu$ L aliquot of supernatant containing PAP enzyme was added to 900  $\mu$ L of 50 mM imidazole buffer, pH 6.0, which was kept at 53°C in a water bath. The enzymatic reaction was started by the addition of 50  $\mu$ L of substrate followed by 2 mL AMA reagent to stop the reaction at the end of enzyme assay that was typically 30 min. After 30 sec, 0.1 mL of citric acid (1.0 M) was added to each tube to fix the color. To get a clear solution, we centrifuged in an Eppendorf 5415C (Westbury, NY) at 13,000 rpm for 7 min. The absorbance was read at 355 nm after blanking the spectrophotometer with the appropriate control, which was stopped at zero time. The PAP activity was expressed as  $\eta$ kat/ml ( $\eta$ moles orthophosphate released per sec).

## 2.6. Phosphatase Assay

A 10  $\mu$ L aliquot of PAP enzyme was added to 940  $\mu$ L of buffer (50 mM acetate, pH 5.0) and incubated with 1.25 mmole of *p*-nitrophenylphosphate (*p*NPP) in a final volume of 1.0 mL at 55°C for 2 min. The reaction was terminated using 0.1 mL 1.0 N NaOH and the liberated *p*-nitrophenol was measured spectrophotometrically at 400 nm [11].

## 2.7. Determination of pH Profile

To measure both the PAP and phosphatase activity, the choice of buffers were 25 mM glycine-HCl (pH 1.5 - 3.0), 50 mM sodium acetate (pH 3.5 - 5.5), and 25 mM imidazole (pH 6 - 9). The dialyzed seed extract was incubated in various buffers as described before to determine the pH optima of *Momordica charantia* PAP.

## 2.8. Determination of Michaelis Constant ( $K_m$ ) and $V_{max}$

The  $K_m$  and  $V_{max}$  for *Momordica charantia* PAP were determined at 53°C and pH 6.0 using the enzyme assay mentioned earlier. The concentration of dioleoyl-phosphatidic acid (DPA) ranged from 0 to 500  $\mu$ M, whereas the phosphatase assay was performed at pH 5.0 and 53°C with 0 to 1250  $\mu$ M *p*NPP as the substrate. Window-Chem's (Fairfield, CA) software Enzyme Kinetics version 1.1 was used to compute both the  $K_m$  and the  $V_{max}$  values.

## 3. RESULTS

### 3.1. *Momordica charantia* PAP Is a Soluble Enzyme

Both the ultracentrifugal study and chromatographic be-

havior of PAP in ion-exchange column have pointed out that the cucurbit PAP is a soluble enzyme. In ultracentrifugal study, the bulk of the activity (87.2%) remained in the soluble fraction. Also, the protein was bound to the S column at pH 3.0 and eluted at 0.5 M sodium chloride concentration in glycine-HCl buffer, pH 3.0 (Figure 2).

### 3.2. The pH Optima of *Momordica charantia* PAP

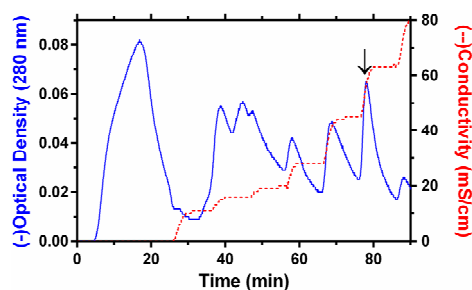
The pH versus activity profile of PAP for both the natural and synthetic substrates is shown in Figure 3. When DPA was used as substrate at 37°C, the maximum activity was observed at pH 6.0 (Figure 3, panel A). However, a substantial amount of activity was seen at pH 5.0 and 7.0. Approximately 79% of the activity was found at pH 5.0 and 24% activity at pH 7.0. However, when *p*NPP was used as the substrate at 53°C, a bi-hump profile was observed (Figure 3, panel B). A minor peak was observed at pH 3.0 that constituted about 34% of activity; the major activity peak was observed at pH 5.0.

### 3.3. The Temperature Optima of *Momordica charantia* PAP

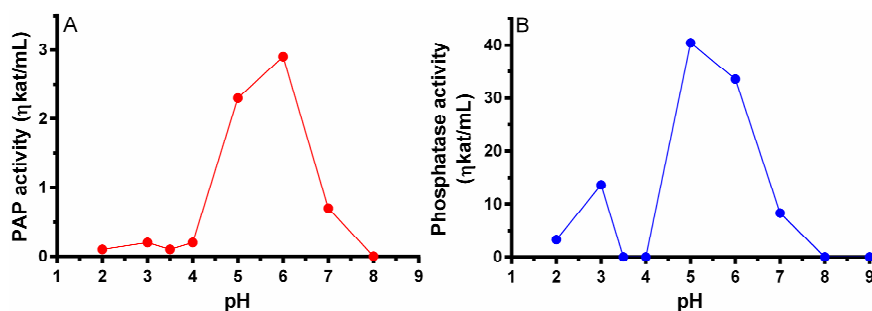
The temperature dependence of PAP activity of *Momordica charantia* crude supernatant is shown in Figure 4, panel A. The optimum temperature for activity was found to be 53°C. At 50°C, the activity dropped only about 7% and at 62°C, about 35% drop in activity was observed. Looking at the temperature versus activity profile, one could discern that *Momordica charantia* PAP is a thermostable biocatalyst. The associated phosphatase activity also showed a similar temperature optima profile when *p*NPP was used as the substrate (Figure 4, panel B).

### 3.4. The Kinetic Properties of *Momordica charantia* PAP

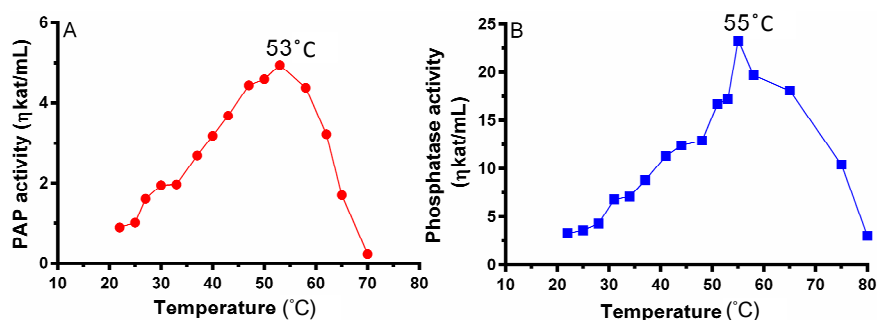
In these experiments we challenged the substrate, DPA,



**Figure 2.** The cation exchange chromatography (UNOSphere™ S) of *M. charantia* soluble cotyledon extract. Both the optical density profile at 280 nm and conductivity of the column eluate are shown. The arrow marks the position where PAP activity was eluted by 0.5 M sodium chloride in glycine-HCl buffer.



**Figure 3.** The pH profile of the *Momordica charantia* PAP enzyme catalyzing PtdOH (Panel A), and  $\rho$ NPP (Panel B).



**Figure 4.** The temperature versus activity profile of the *Momordica charantia* PAP enzyme catalyzing PtdOH (Panel A) and  $\rho$ NPP (Panel B).

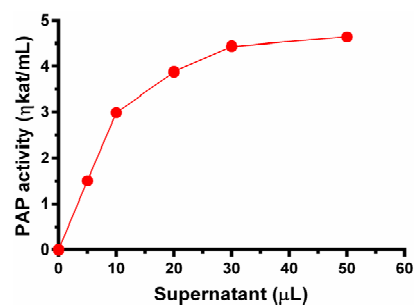
with the dialyzed supernatant that contained PAP at pH 6.0 and 53°C in order to assess whether the phosphohydrolase reaction exhibits the hallmark of enzymatic reaction, viz. a sigmoidal curve for the reaction as a function of enzyme concentration and the rate linearity curve for time dependence. The amount of supernatant extract containing PAP enzyme was varied between 0 to 50  $\mu$ L corresponding to 0 to 210  $\mu$ g of protein and the activity was measured. In this case, a sigmoidal curve was obtained (Figure 5). In another set of experiments the incubation time of reaction was varied between 0 to 20 min. In this case, the rate linearity of the enzymatic reaction was observed (Figure 6).

### 3.5. Michaelis Constant ( $K_m$ ) and $V_{max}$ Determination for *Momordica charantia* PAP

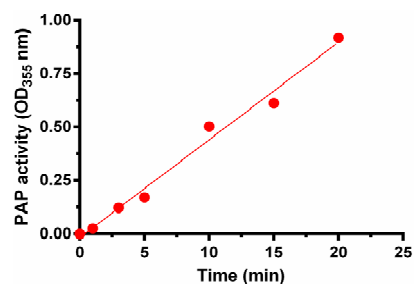
When both the natural and artificial substrates were used to drive the enzymatic reaction, the enzyme gave a typical sigmoidal curve for both the substrates (Figure 7, panel A and panel B). The  $K_m$  and  $V_{max}$  for DPA were 142  $\mu$ M and 1.89  $\eta$ kat/mg of protein, respectively. The kinetic parameters for  $\rho$ NPP driven assay were computed to be 107  $\mu$ M and 10.4  $\eta$ kat/mg of protein, respectively.

### 3.6. The Effect of Non-Ionic Detergent Triton X-100 on *M. charantia* PAP Activity

Triton X-100/phosphatidic acid mixed micelles was used

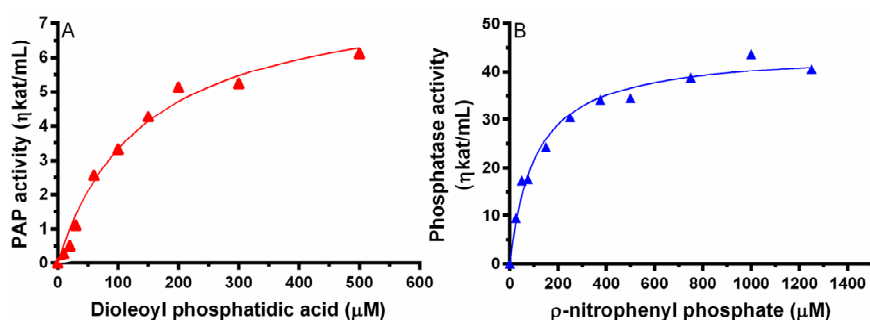


**Figure 5.** Phosphohydrolase activity of the *Momordica charantia* PAP as a function of enzyme concentration.



**Figure 6.** Phosphohydrolase activity of the *Momordica charantia* PAP exhibiting the time course dependence of  $P_i$  release when fixed volume of the enzyme was assayed under experimental condition.





**Figure 7.** The PAP enzyme activity as a function of substrate concentration. Panel A shows the hydrolysis of PtdOH and panel B shows the hydrolysis of  $\rho$ NPP as a function of the concentration of substrate.

to perform kinetic studies with the membrane-bound PAP from yeast [12]. To investigate whether the non-ionic detergent would facilitate the soluble PAP from *Momordica charantia* in the same way, we performed PAP activity in presence of non-ionic detergent Triton X-100. The results are shown in **Figure 8**. The enzyme could tolerate a low concentration of Triton X-100; however at concentration ranging from 0.3% to 0.5%, the activity was decreased about 30% - 60%. The accessibility of the substrate, DPA, to the active site of the enzyme was not facilitated by the presence of the detergent.

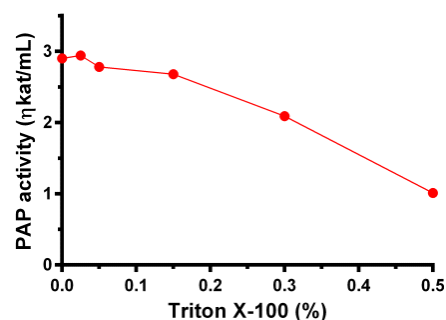
### 3.7. Reaction of *Momordica charantia* PAP to Various Protein Sensitive Chemicals

The dialyzed crude supernatant from *Momordica charantia* cotyledons were treated with cysteine modifier (N-ethylmaleimide, NEM), thiol protectant ( $\beta$ -mercaptoethanol,  $\beta$ -ME), and arginine modifier (phenylglyoxal) to investigate whether these had any effect on catalytic rate of the *Momordica charantia* PAP. All three reagents had no effect on PAP activity (**Table 1**).

## 4. DISCUSSION

This is the first reporting of any phosphatidic acid phosphohydrolase (EC 3.1.3.4) activity in *Momordica charantia*, which was chosen for study because the mature seeds of the plant make TAG that contains  $\alpha$ -eleostearic acid as the acyl chains in all three positions [13]. Maturing seeds of *Momordica charantia* have all the enzymes to assemble storage triglycerides (TAG); therefore, it is a perfect candidate for biochemical dissection. When the supernatant of cotyledon extracts from mid-maturity level seeds of this plant were examined for PAP activity in an *in vitro* assay, we detected quantifiable phosphohydrolase activity in the supernatant fraction, which was characterized and reported in this communication.

In this study, the soluble nature of *Momordica charantia* PAP was established by two methods, namely, the ultracentrifugation study and ion-exchange chromatog-



**Figure 8.** *Momordica charantia* PAP enzyme activity measured in presence of varying concentration of Triton X-100.

**Table 1.** Effect of various reactive agents on the PAP activity.

Additives	Concentration	$\eta$ Kat/mL
None		1.93
$\beta$ -mercaptoethanol	1 mM	1.95
N-ethylmaleimide	10 mM	1.98
Phenylglyoxal	25 mM	2.02
Cyclohexanedione	16 mM	1.99

raphy. In particular, the binding of the PAP to S column and its subsequent elution from the column at 0.5 M sodium chloride in glycine-HCl buffer give credence to the notion that this protein is not a component of any membrane. There are reports of two kinds of PAP in oleaginous plants and yeasts, namely, the soluble [14] and membrane-bound PAP [15]. Based on the dependence of the biocatalyst to  $Mg^{2+}$  for activity, the enzyme could also be divided into 2 classes, namely Mg-dependent [6] and Mg-independent PAP [16].

Even though the biochemical evidence for PAP activity had been published over the years, the primary sequence of the biocatalyst and the nucleotide sequence of the gene coding for the protein was only discovered in 2006 from the yeast *Saccharomyces cerevisiae* [6]. The coded enzyme derived from PAH1 gene was reported to be associated with both the supernatant and membrane

fractions of the cell; however, its association with membranes is now considered peripheral in nature [17]. The yeast PAP was reported to be specific for PtdOH and exhibited its phosphohydrolytic activity based on the active site motif DXDX(T/V) within a halo acid dehalogenase (HAD) domain. Furthermore, the yeast PAH1 gene showed sequence homology to mammalian lipins encoded by *Lipin1*, *Lipin2*, and *Lipin3* [4].

We have provided preliminary evidence for no participation of Arg and Cys residues in the active site of *Momordica charantia* PAP (Table 1). However, further research is needed to verify these findings.

The roles PAP play in lipid metabolism and cell physiology were documented through studies employing mutants defective in PAH1 encoded protein. The yeast containing the defective PAH1 gene showed elevated levels of PtdOH and decreased levels of both DAG and TAG [6, 18]. In addition, the contents of the major phospholipids PC, PE, and PI, and fatty acids were affected by the deletion mutation in PAH1 gene [6,18]. Therefore, PAP probably plays a role in the regulation of overall lipid synthesis.

The effect of non-ionic detergent Triton X-100 on *Momordica charantia* PAP, which was found not to be associated with internal membranes of the seed, was inhibitory. Triton X-100 forms a mixed micelle with the lipid substrate PtdOH, providing a membrane mimic for catalysis [12,15]. However, in the case of *Momordica charantia* PAP, the enzyme is not associated with membrane; therefore, instead of stabilizing the substrate, it probably disturbs either the substrate binding site or the catalytic site of the *Momordica charantia* PAP.

A thorough study on the kinetic, biochemical properties and active site mapping of the *Momordica charantia* PAP awaits its purification.

## 5. CONCLUSION

The developing seeds of *Momordica charantia*, the bitter gourd, were investigated for soluble phosphatidic acid phosphohydrolyase (EC 3.1.3.4) activity using an *in vitro* assay method. We found detectable PAP activity in the supernatant fraction of the seed extract. The enzyme activity was characterized for time course, concentration dependency, pH optima, temperature optima, and susceptibility to Arg and Cys modification. The enzyme activity did not appear to be dependent on  $Mg^{2+}$  and the  $V_{max}$  and  $K_m$  were estimated to be 1.89  $\eta$ kat per mg of protein and 142  $\mu$ M, respectively, when assayed at pH 6.0 and 53°C, respectively. The enzyme was readily inactivated by non-ionic detergent Triton X-100, which indicates that *Momordica charantia* PAP is not a membrane bound enzyme. This PAP could serve as a model for human lipodystrophy, which is characterized by loss

of body fat, fatty liver, hypertriglyceridemia, and insulin resistance.

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