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Research Article
Isolation and Characterization of three Thermostable Acid Phosphatase isozymes from *Cassia occidentalis* (Coffee Senna) Seeds

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Abstract

Background and Objective: Acid phosphatase, an enzyme that acts to liberate phosphate under acidic conditions, is ubiquitous being detected from bacteria to complex eukaryotic cells. This work was undertaken to isolate and purify acid phosphatase activity. **Materials and Methods:** Three acid phosphatase isozymes ACP40, ACP60 and ACP80 were isolated and partially purified from the *cassia occidentalis* seeds crude extract by fractional precipitation using salting-out techniques. Kinetics parameters like Km and Vmax were calculated using p-Nitrophenyl Phosphate (p-NPP) as a substrate. The enzyme's stability was studied with respect to storage at the room, temperature optima and denaturing agent. **Results:** The three enzymes shared a common pH optimum at 5.6 however with a wide range of temperature optima. Cu^{++} and Zn^{++}were inhibited the enzymes, whereas Mn^{++}, Mg^{++} and Ca^{++} stimulated the enzymes. However, they exhibited different degrees of stability against storage temperature and durability against denaturing agents like urea. **Conclusion:** The variations in some biochemical parameters between the ACP's isozymes may indicate variability in their biological functions. In addition to these, the ability to cope with the high concentration of nitrogen as well as high temperatures may make these enzymes qualified for the use in biotechnology.

Key words: *Cassia occidentalis*, acid phosphatase, thermostable, isozymes, characterization, biotechnology

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cassia occidentalis is a legume tropical annual short-lived plant, that has been transferred to the genus *senna* and thereafter widely accepted as *senna occidentalis*[1]. The plant is considered an edible and used by some locals as a substitute for coffee[2]. Phosphorus is an important nutritional element required in large quantities to maintain essential plant metabolic activities, deficiency of which leads to impairment of plant photosynthesis, respiration and eventually to retardation of growth[3,4]. Uptake and availability of phosphorus in its inorganic form required action of several hydrolytic enzymes among which is a phosphatase, which is widespread in the biological system ranging from bacteria to humans [5]this ubiquitous distribution emphasizes on the importance of this enzymes[6]. The enzyme catalyzes the hydrolysis of a broad range of phosphate monoester substrates[7] and is being classified into acid and alkaline phosphatase according to their pH optima below or above pH 7 respectively[4]. Plant acid phosphatase works in a pH below 7.0 and could exist intracellularly (vascular) and as extracellular protein (excreted)[8]. Though of the extensive available data on these enzymes their physiological roles are still not very clear which is attributed to the vast heterogeneity and lack of substrate specificity[9]. In industry and biotechnology acid phosphatase is being routinely used in waste remediation[10], food processing[6,11] and animal feeds[12]. Though of the great importance of the nitrogenous fertilizers for plant growth and fruit yields, their irrational unscientific use may result in adverse effect may lead to growth impairment and sometimes plant death. These unfavorable effects may partly be due to inhibition of hydrolase enzymes such as acid phosphatase[13], therefore it is of great importance, beside characterization of these enzymes, to know their favorable working conditions (optimum catalytic conditions pH, temperature, etc) it might be of interest to know how these enzymes perform in the presence of high concentrations of nitrogenous fertilizers such as urea. Therefore this work aimed to isolate and purify the three acid phosphatases from the seeds of *Cassia occidentalis*.

MATERIALS AND METHODS

Study area: This work was undertaken at the laboratory of glycobiology and proteomics, Africa City of Technology, Khartoum, Sudan, in the duration between April to Nov 2019.

Sample collection: *Cassia occidentalis* good quality seeds were collected from the Medicinal and Aromatic Plants Research Institute, Khartoum. Sudan. The seed was washed three times with distilled water to remove any foreign materials then processed for extraction as well be explained later.

Chemicals: The chemicals and reagents for the enzyme assay were purchased from Biosystem, SD fine Chem. All other materials and chemicals were either of analytical grade or highest grade available. The equipment and apparatus from Jenway(UK), Bibby scientific (UK) and Hettick (Germany).

Extraction: Twenty gram *Cassia occidentalis* seeds were soaked in warm water for 24 hrs, seed softened cotyledons were mixed with 300 mL 50mM Tris-HCl buffer pH 7.8 in a blender, The extract was stirred for 3 hat 4°C. The extract was filtered through cheesecloth. The turbid filtrate was then centrifuged at 6000 rpm for 45 min at room temperature, clear supernatant was collected and termed fraction A (FrA). This FrA was subjected to successive solid$(NH4)_2SO_4$(AS) at40, 60 and 80% saturation. The resultant precipitant protein at 40, 60 and 80% saturation of AS was dissolved in a small amount of 50 mM Tris-HCl pH 7.8 and dialyzed against the same buffer with several changes till free of AS. Enzyme activity was assayed as previously shown[14]. These solutions were then termed as Fr40, Fr60 and Fr80 according to the concentrations of $(NH4)_2SO_4$ used [15]. The enzyme isoform detected in each of the obtained AS saturation precipitants were denoted henceforth ACP40, ACP60 and ACP80, respectively.

Enzyme assay: The enzyme (ACP40, ACP60 and ACP80)assay was performed using the chromogenic pseudosubstrate ρ. nitrophenyl phosphate as a substrate. The reaction mixture was incubated at 37°C for 10 min, then terminated by the addition of 1 mL of 2 M sodium carbonate. The canary yellow color produced which corresponds to the amount of released p-NPP was measured photometrically at 405 nm. One unit of the enzyme was defined as the amount of enzyme required to hydrolyze 1 μM of substrate ρ-nitrophenyl phosphate(ρ-NP) per min. Specific activity was defined as Units of enzyme activity per mg protein. Enzyme activity was assayed as previously shown[16].

Protein content determination: Protein concentration was determined by Bradford method[17]using bovine serum albumin (BSA) as the standard.

Protein purification: The enzymatically active protein fractions obtained after AS saturations (ACP 40, 60 and 80) were applied to the DEAE- cellulose column that was previously equilibrated with 20 mM Tris-HCl buffer pH 7.8,

Protein effluent was recycled at least three times. The column was washed till reading at OD_{280nm} dropped to less than 0.01. Bound protein was eluted using a gradient of NaCl (0.2M, 0.4M and 0.6M) prepared in 20mM Tris-HCl buffer pH 7.8[14].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli system[18]. Protein was loaded in 10% gel and run for 1.5 hrs. The gel was stained by coomassie brilliant blue G250.

Effect of pH on ACPs catalyzed reaction: The suitably diluted enzyme solution was incubated with 250 µL of buffers of different pHs ranging from pH 3.6 to pH 6.0 for 10 min. Then 250 µL, 8 mM of ρ-NP was added. The reaction was arrested after a suitable period by the addition of 1.775 mL of 2 M sodium carbonate. The released ρ-nitrophenol was monitored spectrophotometrically at 405 nm.

Effect of temperature on ACPs catalyzed reaction: The suitably diluted enzyme solution was incubated with 250 µL of 0.2 M sodium acetate buffer pH 5.6, at different temperatures ranging from 25 to 70°C for 10 min, then 250 µL of the substrate was added. The reaction mixture was incubated for a suitable period and then arrested by the addition of 1.775 mL 2 M sodium carbonate. The released ρ-nitrophenol was monitored spectrophotometrically at 405 nm.

Effect of substrate concentration on enzyme activity: For studying the effect of p-NP concentration on enzyme-catalyzed activity, 50 µL of the enzyme was added to 200 µL 200 mM sodium acetate buffer pH 5.6 containing various concentrations of substrate ranging from 1 to 6 mM. K_m and V_{max} values were determined from Lineweaver-Burk plot[19].

Effect of metal ions: Enzyme solutions were initially exhaustively dialyzed overnight against 30 mM EDTA. Followed by exhaustive dialysis against double distilled deionized water to completely remove EDTA. 5 mM solutions of metal ions Mn^{++}, Mg^{++}, Ca^{++}, Cu^{++} and Zn^{++} were prepared from their corresponding salts. The assay was done by incubating 10 µL of each metal ion solution with 30 µL of the enzyme for a suitable period under standard conditions of pH and temperature. The substrate was added and the reaction mixture was re-incubated for a suitable period. The reaction was arrested by the addition of 1.775 mL 2 M sodium carbonate and enzyme activity was assayed as previously shown.

ACPs thermal stability under their optimum temperature: This experiment was performed by incubating the enzymes at their optimum temperature for up 2 hrs. Aliquots were removed every 30min and the residual activity was calculated.

Enzyme stability at room temperature for a month: To investigate the effect of room temperature storage for a month on the ACPs activity. The enzyme extract was left at room temperature, under sterile condition, for a month. Aliquots were removed every three days and the enzyme was assayed as shown by Khan *et al.*[20].

Effect of urea on ACPs stability: The activity of the enzyme at different concentrations of urea ranging (1-5 M) was performed. In brief: ACP aliquots were incubated with different concentrations of urea (1-5 M) for 30 min. Followed by the addition of substrate and buffer and residual activity of the enzyme was calculated as previously shown. Enzyme without urea served as 100% control[21]. Another experiment was performed by incubating the enzyme in 5 M urea for 24 hrs.

RESULTS AND DISCUSSION

The purification strategy of the acid phosphatase activity detected in the crude extract of *Cassia occidentalis* seeds started with soaking the good quality seeds in boiled water for overnight. The seeds coat of the imbibed seeds were then carefully removed to get the softened creamy yellow cotyledon. The outer coat removal was essential to avoid the interference of dark brown color which results from the seeds coat. As depicted in Table 1 starting from 20 g seeds resulted

Table 1: Partial Purification chart of ACP isoforms extracted from *cassia occidentalis* seeds

Protein extract	Total volume (mL)	Protein content (mg mL^{-1})	Total protein (mg)	Unit activity (U min^{-1})	Total unit (U)	Specific activity (U mg^{-1})	Purification (Fold)	Yield (%)
Crude	300	0.858	257.447	52.921	15876.30	61.679	1.000	100.000
Fr40	120	0.591	70.970	15.600	1872.00	26.395	0.427	11.791
Fr60	25	0.753	18.847	29.775	744.375	39.541	0.641	4.688
Fr80	30	0.778	23.340	29.775	893.258	38.271	0.620	5.626
Fr40-DEAE-Cellulose	17	0.323	2.936	2.639	22.021	17.528	0.283	0.138
Fr60-DEAE-Cellulose	16	0.807	6.038	6.291	42.541	14.019	0.226	0.267
Fr80-DEAE-Cellulose	13	1.377	8.978	10.093	66.291	14.605	0.236	0.416

in 0.257.4 g of soluble protein, which accounts for around 13% of the total weight of the seed. To the obtained 300 mL crude extract sequential addition of solid Ammonium Sulfate (AS) at 40, 60 and 80% saturation resulted in the fractional separation of crude extract protein. As a rule, large molecular weight proteins would often get precipitated at a low concentration of AS and vice versa[22]. Upon solubilization of obtained AS precipitant protein and subsequent ACP analysis, the enzyme activity was detected in the three AS fractions i.e., Fr40, Fr60 and Fr80, which might highlight the presence of this enzyme in multiform, which named them henceforth as ACP40, ACP60 and ACP80 according to the concentration of AS at which protein is precipitated. The presence of ACPs in isoforms is very common[23-26]. After the fractionation stage, the enzyme was, collectively, purified by almost two-times and 22.1% yield. The SDS-PAGE pattern for the purification stages, which are described in detail in the methodology section, is shown in Fig. 1, in which clear segregation of the crude extract protein was seen between the different ammonium sulfate precipitated fractions.

Effect of pH on ACPs catalyzed reaction: The three isoforms in the three fractions showed similar pH optima at 5.6 after which a sharp drop in ACP activities was evident (Fig. 2). This pH optimum was largely consistent with other pH optima reported in the literature for other acid phosphatases which all fall between 5-6[16,23,27,28].

Effect of temperature: When ACP40 and 60 exhibited similar temperature optima range from 45 to 55°C. ACP80 showed a temperature optima range from 35 to 50°C which may indicate that ACP80 is distinctly different from ACP40 and ACP60. These results are again in good accordance with other reports on plant acid phosphatases[28,29]. At 70°C all isozymes lost more than 75% of their activity (Fig. 3). A temperature optimum at 55°C has been reported for a germinating *Vigna mungo* L. seedling[30] and 55°C for peanut seedlings[16]. Interestingly, four cytoplasmic acid phosphatase isoforms, purified from mature soybean seeds showed a remarkable high-temperature optimum[31] at 80°C. Interestingly, after 10 min of incubation at up to an hour at 60°C, the three isoforms lost around 60% of their activity, however, at 70°C, the isozymes lost about 70% of their original activity. These results indicate the high stability of the ACP. In animal feeds industry, according to Wyss and his colleagues only those enzymes which could withstand the high temperature between 60 to 80°C are appropriate to be used in animal feeds supplement[32]. Therefore, the ACP of the current study may deem appropriate to be used in this industry.

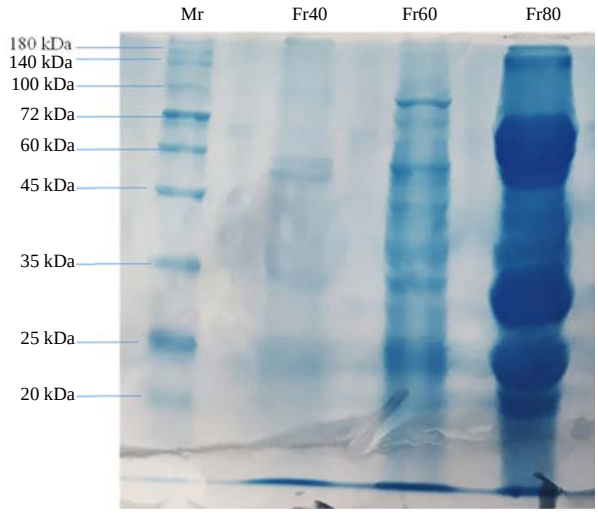

Fig. 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for Fr40, 60 and 80. Lane1: Molecular weight protein markers. Lane 2,3 and 4 are Fr40, Fr60 and Fr80 respectively

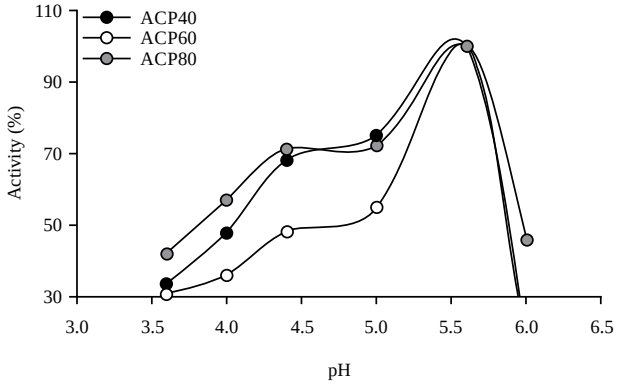

Fig. 2: Effect of pH on ACP40, ACP60 and ACP80 catalyzed reaction

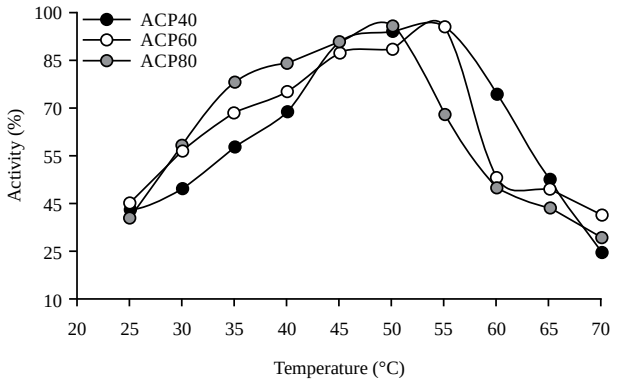

Fig. 3: Effect of temperature on ACPs catalyzed reaction incubated for 10 min

Thermal stability of ACP at its optimum temperature for 3 hrs: To study the enzyme stability at its optimal temperature the ACP isoforms were incubated at their optimum temperature for up to 2 hrs. Enzyme aliquots were removed every 30 min and the assay was performed. The enzyme was stable up to 30 min with minor loss in its activity after which enzyme started rapidly losing activity. ACP60 was more stable to the incubation for a long time, whereasACP80 was the one to get largely affected with a loss of about 90% of its activity after 2 hrs. (Fig. 4). The decrease in activity with time was probably due to the denaturation of the enzyme secondary and tertiary structures.

ACP stability at room temperature for one month: At 3 days intervals, aliquots of the enzyme were removed and its activity was assayed. ACP60 and ACP80 were fairly stable up to 6 days and lost about 25% of their activity. However, ACP40 was very sensitive for room temperature storage where the enzyme lost about 50% of its activity just within the first 3 days. Until day 15th, there was no appreciable loss inactivity after which enzyme activity started again to decrease till it lost about 70% of activity by a month (Fig. 5). ACP isoforms 60 and 80 were however more stable in the first three days of incubation with a loss of about 20% of the original activity.

Effect of urea on ACPs Activity: Acid phosphatase is one of the several enzymes that act as a good indicator of soil quality due to its role in the cycling of elements such as carbon, nitrogen and phosphorous[33]. Since urea is being used routinely as a nitrogen source in many fertilizers, unscientific use of urea may lead to inactivation of many important soil enzymes among which is acid phosphatase[13]. Therefore it was tempting to study the effect of urea on ACPs investigated in this study. The ACPs were incubated at different concentrations of urea for 2 hrs as well as at 5 M urea for 24 hrs. Results showed that the increase in urea concentration adversely affects the enzyme activity. ACP40 was the most stable isoform lost only about 35% of its total activity when incubated in up to 5M urea for 2 hrs followed by ACP60 and finally ACP80. The pattern of ACPs urea inactivation is parallel to the thermal inactivation of these isozymes which might highlight a similar mechanism of denaturation[34]. After 24 hrs of incubation at 5M urea, the three isoforms lost about 80% of their total activity (Fig. 6).

Effect of metal ions: The partially purified ACP isoforms presented in this work seemed to be manganese dependent enzymes. More than 2 folds increment in both ACP40 and

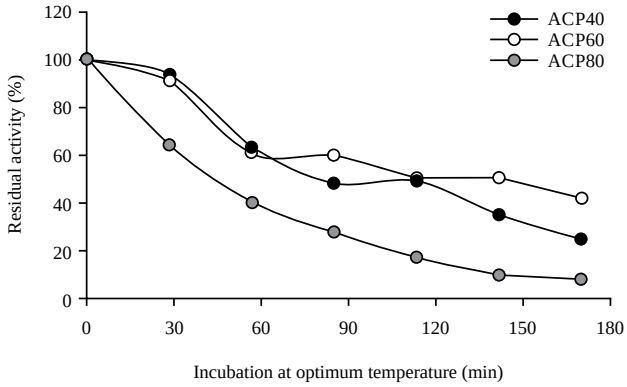

Fig. 4: Effect of incubation time at optimum temperature for up to 2 hrs on ACP40, ACP60 and ACP80 activity

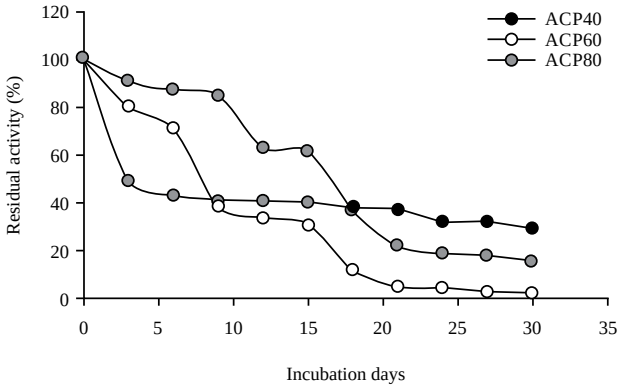

Fig. 5: Effect of room temperature incubation for one month on ACP40, ACP60 and ACP80

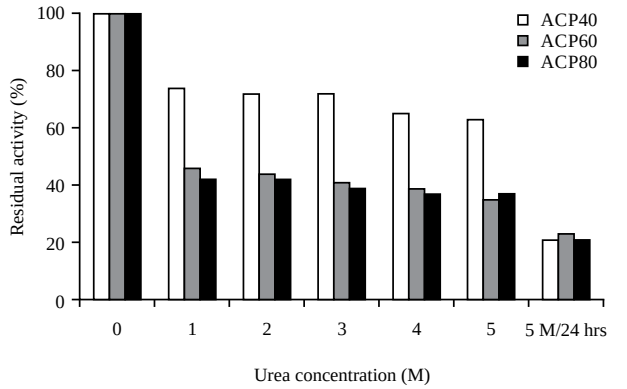

Fig. 6: Effect of urea on ACPs incubated at varying concentrations for 2 hrs and at 5 M for 24 hrs

ACP60 activities in the presence of Mn metal were noticed when compared to the control experiment which runs the absence of Mn. Whereas around 1.3 times increase was seen in the case of ACP80. This activity enhancement was also noticed, however to varying extent, in the case of Ca and Mg.

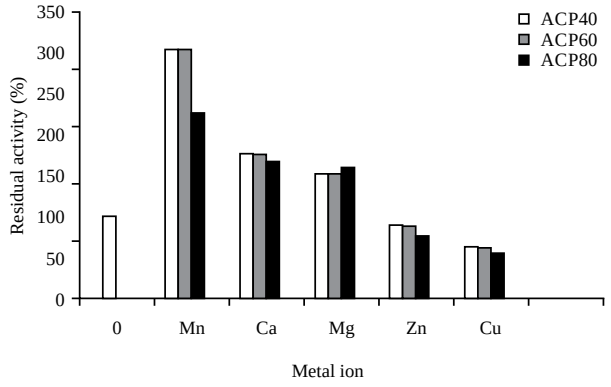

Fig. 7: Effect of metal ions on ACPs catalyzed reaction

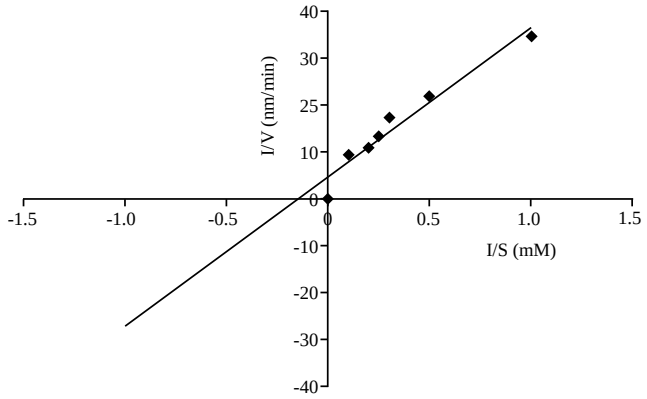

Fig. 8: ACP60 km and Vmax from Lineweaver-Burk plot

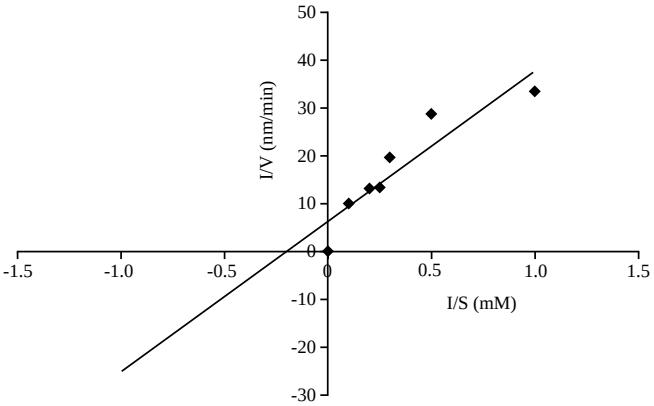

Fig. 9: ACP80 Km and Vmax from Lineweaver-Burk plot

On the other hand, Cu acted inhibitory to the three ACPs (Fig. 7). Another study showed that the enzyme from germinating *vignaradita* seeds was not affected by Mn^{++}, Mg^{++} and Ca^{++} while Cu was inhibitory[35], while Gadgil *et al.*[29] reported that $Ca^{++}$was potent activator for the enzyme from germinating horse gram. The inhibition of the enzyme by Zn^{++} has been previously reported in pea[36], tobacco [37]and cotyledon of *Erythrina indica*[14].

Effect of substrate concentration on enzyme activity: Considering the specific activities for the varying ACP isoforms, ACP40 was the least in activity as compared to ACP60 and 80, therefore, the kinetic studies using the double reciprocal plot were performed for these two enzymes. Using ρ-nitrophenyl phosphate at varying concentrations ranging from 1 to 6mM and further calculation of the activity after incubation at a suitable time interval both ACP isoforms 60 and 80 exhibited high Km values 6.6 mM and 5 mM, respectively (Fig. 8 and 9). Km value signifies the affinity of the interaction of the enzyme with the substrate, lower the value the higher the affinity between the enzyme and its substrate. Whereas Vmax points to the reaction when the enzyme is fully saturated by the substrate. The obtained values of the current study were higher than that of Arabidopsis and *Macrotyloma uniflorum* purple acid phosphatases using ρ-NP as a substrate[29,38], very low to the one obtained by Al-Omair[4] for *Vigna aconitifolia*. However, comparable to the enzyme purified from *Erythrina indica*[14].

Fractions exhibiting ACP60 activity obtained after DEAE-cellulose treatment were pooled. Aliquots containing approximately 50 µL (10 µg) of protein were assayed for ACP60 activity in a total volume of 0.5 mL. The mixture was incubated at 37°C for a suitable time in the presence of increasing concentrations of pNP from 1 to 6 mM. The double-reciprocal (Lineweaver-Burk plot) was drawn and Km and Vmax values were calculated.

Fractions exhibiting ACP80 activity obtained after DEAE-cellulose treatment were pooled. Aliquots containing approximately 50 µL (10 µg) of protein were assayed for ACP80 activity in a total volume of 0.5 mL. The mixture was incubated at 37°C for a suitable time in the presence of increasing concentrations of p-NPX from 1 to 6 mM. The double-reciprocal (Lineweaver-Burk plot) was drawn and Km and Vmax values were calculated.

CONCLUSION

This study isolated and purified three isozymes from *Cassia occidentalis* (Coffee Senna) seeds. Though the three enzymes seem to share major chemical characters like pH (around pH 5.5) and temperature optima (up to 55°C), they, however, exhibited different degrees of stability against storage temperature and durability against denaturing agents

like urea. Based on these results, it can be concluded that the enzyme of the current study may seem appropriate to be employed in plant fertilizers to facilitate the liberation and mobilization of phosphate in the soil and animal feeds industry.

SIGNIFICANCE STATEMENT

This study revealed, for the first time, the existence of three ACP isozymes in the crude protein extract of *Cassia occidentalis* seeds. These enzymes exhibited high thermostability and could, therefore, be beneficial for the applications in plant and animal biotechnology. This study will help the plant fertilizer industry by providing efficient and easily available acid phosphatases.

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