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PURIFICATION AND CHARACTERIZATION OF ACID PHOSPHATASE FROM SORGHUM (*SORGHUM VULGARE*) SEEDS

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An acid phosphatase from sorghum (*Sorghum vulgare*) seeds was purified 90-fold by ion exchange chromatography on DEAE-Cellulose and gel filtration on Sephadex G-75 with enzyme recovery of 65 %. The purified enzyme preparation exhibited pH optimum at 4.9 and optimum temperature of 55°. Km value with *p*-nitrophenyl phosphate was 0.36 mM. The first order rate constant for the phosphorylation reaction (k_2) was 3.1 x 10^{-5} sec^{-1} and 3.6 x 10^5 sec^{-1} for p.nitrophenyl phosphate and phenyl phosphate respectively. Mercury, zinc and molybdate inhibited the enzyme strongly. The inhibition by inorganic phosphate was competitive while the fuoride was non-competitive. The kinetic studies revealed the inhibition by fluoride of mixed type and the dissociation constant (K_i) is important parameter of the reaction EI $\rightleftharpoons$ E+I.

Key words: An acid phosphatase from sorghum (*Sorghum vulgare*)

INTRODUCTION

Phosphatases are commonly found in both plant and animal tissues [1-2] Acid phosphatases (3.1.3.2) have an important role in phosphorous metabolism during the early phases of seedling growth [3]. Several studies have been reported on the function of such enzymes in germinating seeds of legumes as well as cereals [4-7]. Acid phosphatases from maize scutellum, sweet potato, yam and peanut seeds have been purified and characterized [8-11].

The present paper deals with the purification and evaluation of certain properties of acid phosphatase. Such data may serve guideline for additional studies related to the enzymes of rainfed area crops, developmental changes in their activity level and their regulation during seed maturation and seedling growth.

MATERIALS AND METHODS

The seeds of high yielding and approved variety of sorghum (Giza) were collected from the Agriculture Research Station, Dera Ismail Khan and finely ground. The powder was extracted with 0.01M Tris-HCl buffer pH 7.0 (0.5g powder/10ml buffer) at room temperature. The mixture was then centrifuged at 8000g for 10 min. The resulting supernatant was used for enzyme purification.

Acid phosphatase assay: The assay for acid phosphatase has been described previously [12]. The enzyme (0.2ml) was incubated with 1 ml of *p*-nitrophenyl phosphate (2.5mM) in 0.1M acetate buffer pH 4.9 for 30 min. at room temperature and the reaction was terminated by the addition of 5ml of 1N NaOH. The *p*-nitrophenol released was determined by measuring the absorption at 405 nm. Each unit (U) of enzyme was defined as the amount of enzyme required to release 1 nano mole of p.nitrophenol/min under the above assay conditions. The molar extinction coefficient of 17600$M^{-1}cm^{-1}$ was used for *p*-nitrophenol and specific activity (S.A) was expressed as 1 nano mole of *p*-nitrophenol released/min/mg of protein. To study substrate specificity with different phosphate esters, the activity was assayed by measuring the amount of inorganic phosphate (Pi) liberated according to the method of Fiske and Subbarow [13].

Protein determination: Protein concentration was determined by the method of Lowry *et al.* [14]., while optical density at 280 nm was taken for the column eluates.

RESULTS

Purification of the enzyme: The enzyme extract was dialysed against 0.01M Tris-HCl buffer (pH 7.0) and applied to a column (2x20 cm) of DEAE-Cellulose equilibrated with 0.01M Tris-HCl buffer (pH 7.0). After washing with a small amount of the same buffer, the column was eluted by a linear gradient with 0.0 to 0.5 M NaCl (total volume 150ml). The enzyme was eluted as a single peak between 0.1M and 0.2M NaCl (Fig. 1). The active fractions were pooled, concentrated under vacuum and further purified on a column of Sephadex G-75 (2x40 cm) previously

equilibrated with 0.01M Tris-HCl buffer at pH 7.0. The enzyme was eluted as single peak in void volume (Fig. 2) More active fractions were pooled, dialysed against distilled water and freeze dried. The results are given in Table 1. The final preparation had a specific activity of 2194 U with 90 fold purification.

Catalytic properties of the enzyme: The acid phosphatase showed a linear relationship between the initial reaction rate and enzyme concentration, a linear rate of hydrolysis over 75 min; an optimum activity around pH 4.9 (Fig. 3), and an optimum temperature near 55° (Fig. 4) The enzyme was found to be stable between pH 4.5 to 8.5 when incubated for 24 hr at 25°. The enzyme activity showed maximum temperature stability upto 25° on incubation for 30 min at the temperatures ranging from 15° to 70° (Fig. 5). The thermal inactivation of the enzyme was examined at 40, 50, 60 and 70° as the temperature higher than 25° strongly affected the enzyme. Figure 6 shows the enzyme was inactivated by only 25 % when heated at 40° for 1 hr whereas 60 % of its activity was lost at 50°. At 60° the decrease in activity was 96 % and the enzyme was completely denatured after 10 min of incubation at 70°.

Substrate specificity: The activity of the enzyme with various substrates was compared. As shown in Table 2, the highest activity was observed with phenyl phosphate followed by p-nitrophenyl phosphate, α-Glycerophosphate, β-glycerophosphate and adenine 3,5 cyclic phosphate were hydrolysed to a small extent. No hydrolytic activity for ATP and sodium pyrophosphate was observed.

Action of effectors: As shown in Table 3, Na^{+}, K^{+}, Ba^{+2}, Pb^{+2} and Triton X-100 had no effect on the activity of acid phosphatase, while Mn^{+2}, Mg^{+2}, Ca^{+2} and Cd^{+2} had little effect as an inhibitor. Mercury, zinc and molybdates inhibited the enzyme very strongly while the tarta-

Table 1. Purification of acid phosphatase from sorghum seeds.

Purification Steps	S.A (U)	P.F.	% Recovery
Crude extract	24.38	1	100
DEAE-Cellulose	73.14	3.0	72
Sephadex G-75	2194.2	90	65

Table 2. Substrate specificity of acid phosphatase.

Substrates	% Activity
p.nitrophenyl phosphate	10
Phenyl phosphate	107
α.glycerophosphate	14
β.glycerophosphate	16
Adinine 3,5 cyclic phosphate	10
ATP	0
Sodium pyrophosphate	0

Table 3. Effect of various reagents on the acid phosphatase activitiy.

Reagents	Final concentration (mM)	% Activity
None	–	100
NaCl	25	101
KCl	25	106
$MgCl_2$	25	90
$MnCl_2$	25	75
$HgCl_2$	25	32
$BaCl_2$	25	101
$ZnCl_2$	25	33
$CaCl_2$	25	87
$CdCl_2$	25	86
$PbSO_4$	25	100
$(NH_4)_6Mo_7O_{24}$	25	15
$NaMoO_4$	25	21
EDTA	25	285
DTNB	5	98
Triton X-100	0.25 %	94
Tartarate	25	161

Activity was measured under standard conditions except that various reagents were added as indicated above and activities are expressed as % activity relative to that obtained without addition.

Table 4. Estimation of inhibitor constants Ki and KI for sorghum seeds acid phosphatase.

K_i (mM)		K_I (mM)
I/V versus I	Slope versus I	Intercept versus I
0:39*	0.34	0.44

*The mean value of three determinations from three lines is reported.

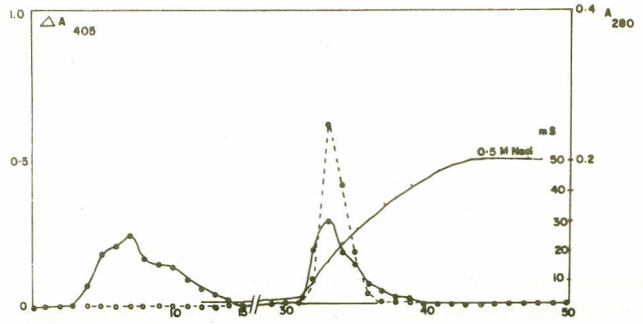

Fig. 1. DEAE-Cellulose chromatography of sorghum seed acid phosphatase at pH 7.0. Flow rate: 10ml/10min and 8ml fractions were collected. Ordinates: Protein at 280nm (●–●), activity at 405nm (o– – –o) and NaCl concentration (●—●—●).

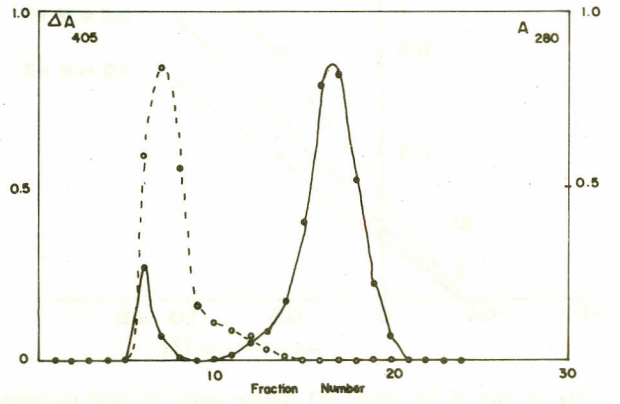

Fig. 2. Gel filtration on Sephadex G-75 of sorghum seed acid phosphatase:- 3ml sample after DEAE-Cellulose chromatography was applied to the column (2x40cm).

Flow rate: 5ml/5min and 5 ml fractions were collected.

Ordinates: Protein at 280nm (●–●) and activity at 405nm (o– · · ·–o).

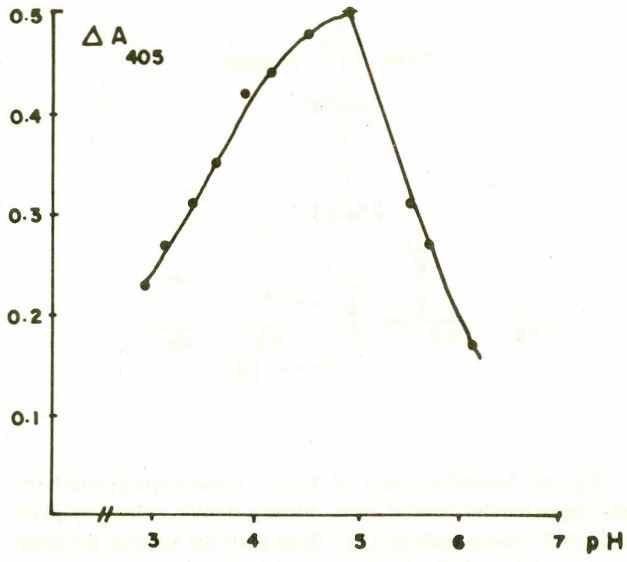

Fig. 3. pH optimum of acid phosphatase activity.

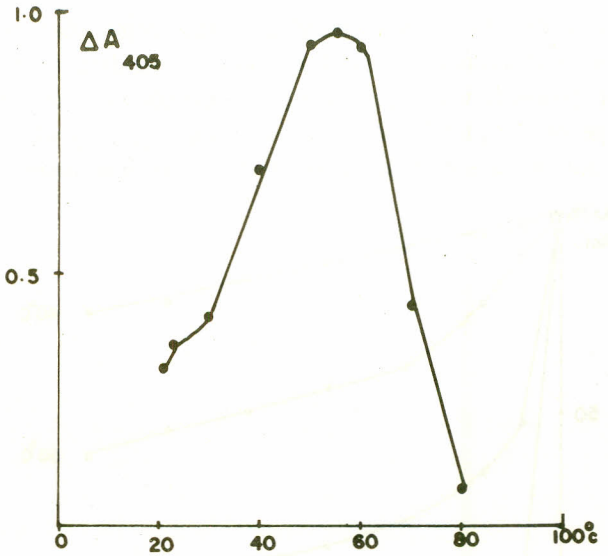

Fig. 4. Temperature optimum of acid phosphatase activity.

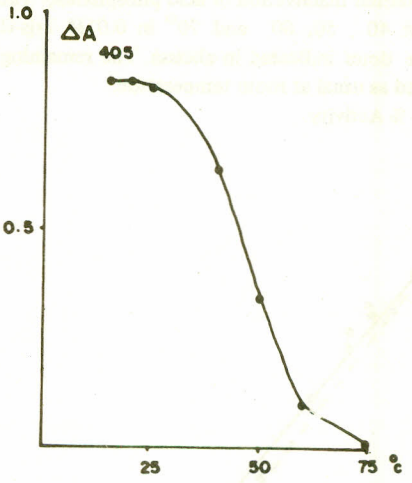

Fig. 5. Effect of incubation temperature on the acid phosphatase enzyme.

rate and EDTA activated the enzyme. The activity was also not affected by DTNB.

Kinetic measurements of the enzyme: Hydrolysis of p-nitrophenyl phosphate or phenyl phosphate by acid phosphatase proceeded according to the following mechanism as suggested by Kosar and Rahman [15].

$$E + S \underset{k\text{-}1}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_2} ES^{*} \xrightarrow{k_3} E + P_2$$

Where $k\text{–}1/k_1$ or K_s is the dissociation constant of the enzyme-substrate complex, k_2 and k_3 are the first-order

rate constants of phosphorylation and dephosphorylation reactions respectively. The slowest or rate determining step proceeds with k_3. The k_2 was calculated from the slope of the plot between time and log $(A_\infty - A_t)$ where A_t and A_∞ are enzyme activities at various time intervals

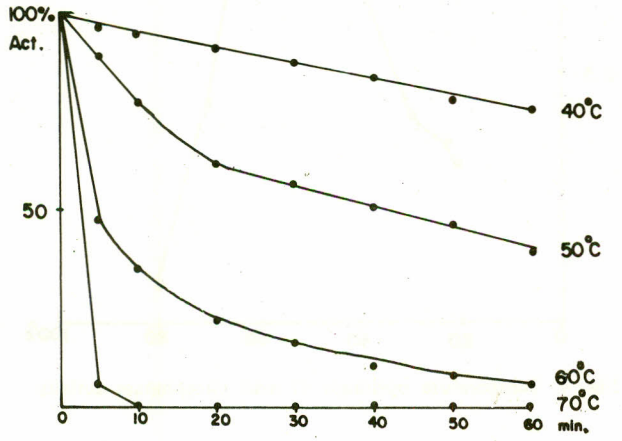

Fig. 6. Thermal inactivation of acid phosphatase: The enzymes were heated at 40 , 50, 60 and 70° in 0.01M Tris-HCl buffer pH 7.0 for the times indicated in abcissa. The remaining activities were determined as usual at room temperature.

Ordinate: % Activity.

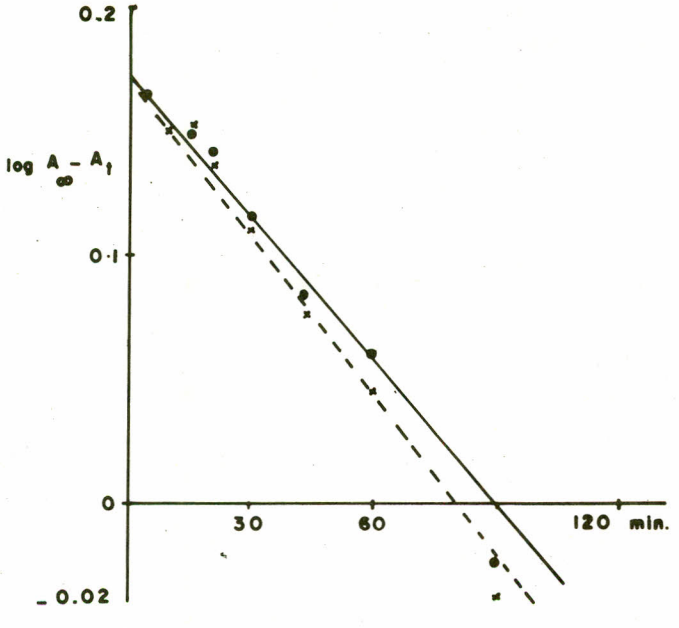

Fig. 7. Time dependent acid phosphatase catalysed hydrolysis of p nitrophenyl phosphate (●—●) and phenyl phosphate (*- - - *- - - *) under standard conditions described. Successive measurements were made at time t = 0,5,10,20,30,45,60,90,120 and t = 240 min.

K_2 for p.nitrophenyl phosphate = 1.9 x 10^{-3} min^{-1} or 3.1×10^{-5} Sec^{-1}.

K_2 for prenyl phosphate: 2.2×10^{-3} min^{-1} or 3.6×10^{-5} Sec^{-1}.

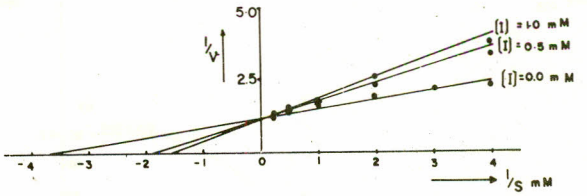

Fig. 8. Inhibition of acid phosphatase by sodium phosphate: Lineweaver-Burk plots of 1/v versus 1/S in the absence or presence of 0.5mM and 1mM Na_3PO_4. The lines were plotted from statistical calculation by employing the method of least squares. Each point is the mean value of at least three experiments.

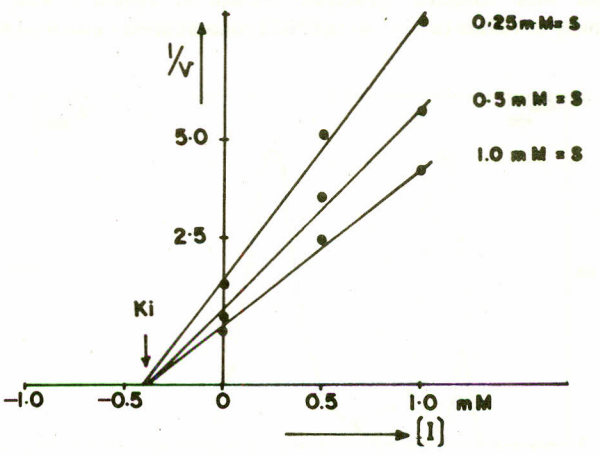

Fig. 9. Plot of 1/v versus [1] (Dixon plots) for acid phosphatase of sorghum seed at three substrate concentrations; 0.25mM, 0.5mM and 1mM in the presence of three concentrations of NaF (0mM, 0.5mM and 1.0mM) for the determination of K_i. All the lines are approximately found to intersect on the abcissa near to each other. The mean value of $-K_i$ is reported in table 4.

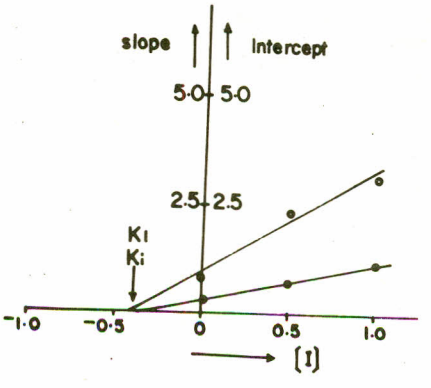

Fig. 10. Secondary plots of slopes (closed circle) and intercepts (open circle) derived from primary double reciprocal plots, against NaF concentrations [1]. These plots are used in the determination of K_i and K_I for fluoride inhibition of acid phosphatase of sorghum seed.

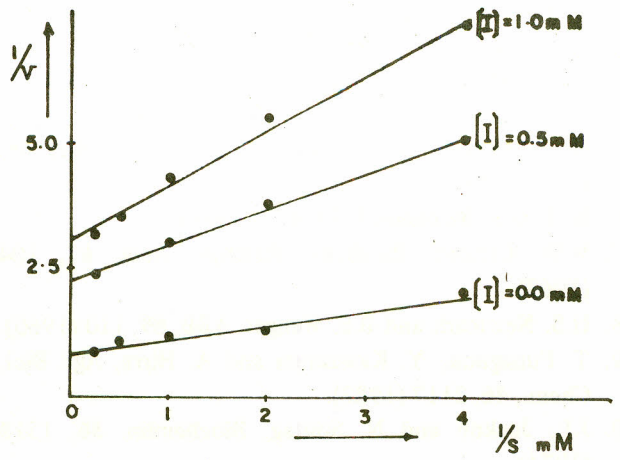

Fig. 11. Primary double reciprocal plots or Lineweaver-Burk plots (1/v vs 1/S) of fluoride inhibition of acid phosphatase of sorghum seed in the absence or presence of 0.5mM and 1mM NaF. Each point is taken as mean value of at least three experiments. Lines are plotted from statistical calulations employing the least squares method of linear regression analysis to determine the slope and intercept for each concentration of inhibitor for secondary plots.

(Fig.7) found to be 3.1 x 10^{-5} Sec $^{-1}$ for *p*-nitrophenyl phosphate and 3.6 x 10^{-5} Sec^{-1} for phenyl phosphate. The K_m value of 0.36mM was obtained from the Lineweaver-Burk plot [16] (Fig. 8). The addition of inorganic phosphate (P_i) increased the apparent Km value of the enzyme (Fig. 8). The inhibition by Pi was of pure competitive type and Ki was 0.40mM. The inhibition by F was non-competitive. The estimate of Ki was made by using the method of Dixon [17] (Fig. 9). Ki value was also found to be 0.34 mM as calculated from the plot between slopes against inhibitor concentrations (Fig. 10) whereas slopes were calculated from primary double reciprocal plots (Fig. 11). Similarly the K_I value of 0.44mM was obtained by using the method of Cleland [18] on plotting (Fig. 10) inhibitor concentrations V. intercepts which were calculated from primary double reciprocal plots as given in Fig. 11 and Table 4.

DISCUSSION

Phosphatases are widely distributed in plant tissues and seeds playing very important roles. To characterize various properties prior purification is needed. A three-fold purification was achieved on DEAE-Cellulose column giving 72 % enzyme recovery (Table 1). A further thirty fold purification was done on Sephadex G-75 with overall recovery of 65 %. The enzyme can be further purified; however, this level of prufication is sufficiently high for the study undertaken.

The enzyme showed highest activity at pH 4.9. The result is comparable to the reported values of pH 5.0 for peanut seeds [11] and pH 5.2 to 5.5 for lupine seedling [19]. The enzyme was relatively heat labile and retained almost full activity at 25° in contrast to heat sensitivity of peanut seeds acid phosphatase which is stable at 40°. The temperature optimum was found to be 55°.

Phenyl phosphate was found as good substrate as *p*-nitrophenyl phosphate while glycerophosphates and other nucleotides proved poor substrates. This finding is quite dissimilar to the report of Rossi *et al.* [8] in which acid phosphatase from scutellum of germinating maize seed showed highest activity towards β-glycerophosphate, though phenyl phsophate and p-nitrophenyl phsophate were also hydrolysed at significant rates. The difference may be due to inherent variation in the source. Km value of the enzyme was 0.36 mM. This value is comparable with the reported value of acid phosphatse from Zizania Latifolia parasite *Ustilago esculenta* [20].

The first-order rate constants of phosphorylation reaction for *p*-nitrophenyl phosphate and phenyl phosphate were found to be 3.1 x 10^{-5} Sec^{-1} and 3.6x10^{-5} Sec^{-1} respectively giving no significant preference for substrates. Regarding the metal ion requirement the ions are not necessary for the activity of the enzyme. Peanut acid phosphatase was peculiar in that it was activated by Ca^{+2} and Mg^{+2} ions which have usually no action on plant acid phosphatases [21-22]. The enzyme was not affected by incubation with DTNB, suggesting that enzyme is not SH-dependent. Mercury, zinc, molybdate, phosphate and fluroide acted as inhibitors. Inorganic phosphate was found to be a competitive inhibitor with Ki value of 0.4 mM, whereas fluoride (a non competitive inhibitor) with Ki and K_I value 0.39 mM and 0.44 mM respectively gave a higher dissociation constant (K_I) of the reaction ESI $\rightleftharpoons$ Es+I than that of Ki of reaction EI $\rightleftharpoons$ E+I. This observation clearly shows that inhibitor binds preferentially to the enzyme surface and has a higher affinity for the enzyme alone compared to the enzyme – substrate complex.

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