Purification and Characterization of 29 kDa Acid Phosphatase from Germinating Melon Seeds

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Summary: Not much progress on the purification and characterization of low molecular weight acid phosphatases from plants has been made as yet. In the current study a low molecular weight acid phosphatase from seedling of melon was purified about 114-fold with specific activity of 45 U/ mg of protein and a recovery of 3 %. The enzyme was found to be homogeneous and showed a single band corresponding to 29 kDa on SDS-polyacrylamide gel electrophoresis. The K_m for *p*-nitrophenyl phosphate was found to be 0.175 mM and V_{max} was 42 µmol of substrate hydrolyzed /min/mg of protein at pH 5.5 and at 37° C. The enzyme showed its optimum activity at pH 5.0 and 50° C. The enzyme was thermostable and it retained 70 % activity for 45 min at 60° C. The pH stability was 4.8-6.0. Phosphate, vanadate, molybdate and fluoride acted as strong inhibitors. Metal ions such as Zn^{+2}, Cu^{+2}, Ag^{+1} and Hg^{+2} deactivated the enzyme while other divalent ions such as Ca^{+2} and Mg^{+2} had no effect.

Key Words: Acid phosphatase; Melon seeds; Purification; Characterization; Seedlings; Isoenzyme.

Introduction

Acid phosphatase is one of the most important enzymes for living organisms. It imparts a most important role inside the body of organism for various biochemical processes. Plant acid phosphatases are plentiful in plant tissues in numerous forms which hydrolyze the phosphomonoesters to release inorganic phosphates (Pi) in acid medium (pH 4-6). Acid phosphatases are also found in bacteria, fungi and animals [1]. They also differ in their molecular mass, substrate specificity, sensitivity to inhibitors and some kinetic properties [2-4].

Many roles of phosphatases have been described. Their main function is to release inorganic phosphate from phosphate esters in the soil and in the plants [5,6]. These are also involved in the transport and recycling of Pi [7]. A number of plant acid phosphatases have been isolated and characterized from different plant organs such as leaves, roots, seeds and tubers [8-14]. Many studies have also been devoted to the purification of acid phosphatases from cotyledons of germinating seeds and various seedlings [11-12, 15-17]. All the acid phosphatases mentioned above and some others have high molecular weights in range of 45-240 kDa. Not much progress on the purification of low molecular weight acid phosphatases from plants has been made [18,19].

The present work deals with the purification of low molecular weight acid phosphatase from germinating seeds of melon and its characterization with respect to kinetic parameters, pH dependence, optimum temperature, thermal stability, substrate specificity, molecular weight and the effect of metal ions and other substances on its activity. This is an isoform of the enzyme (29 kDa) which has been previously reported from *vigna radiata* seedlings [20].

Results and Discussion

A summary of purification of acid phosphatase from 400g of melon seedlings is given in Table-1 and the elution profiles of various chromatographic procedures are shown in Fig. 1 (A-D). One hundred and fifteen times purification was achieved to specific activity of 45 U/mg of total protein and recovery of 3 %. The homogeneity of the enzyme was checked on 12 % SDS-PAGE. A single band was detected and molecular weight of 29 kDa was obtained (Fig. 2). The molecular weight of native enzyme was determined by gel filtration on Sephadex G-100 column. Three isoenzyme peaks were detected when extract salted out from 80 % ammonium sulphate saturation was placed on a calibrated Sephadex G -100 column. Fig. 3 shows elution profile of three isoenzymes. The molecular weights of three enzyme peaks were calculated from a linear graph of log molecular weight versus elution volumes of the three isoenzyme peaks. Molecular weights of isoenzyme I, II, and III were found to be 36kDa, 29kDa and 18kDa respectively (Fig. 4).

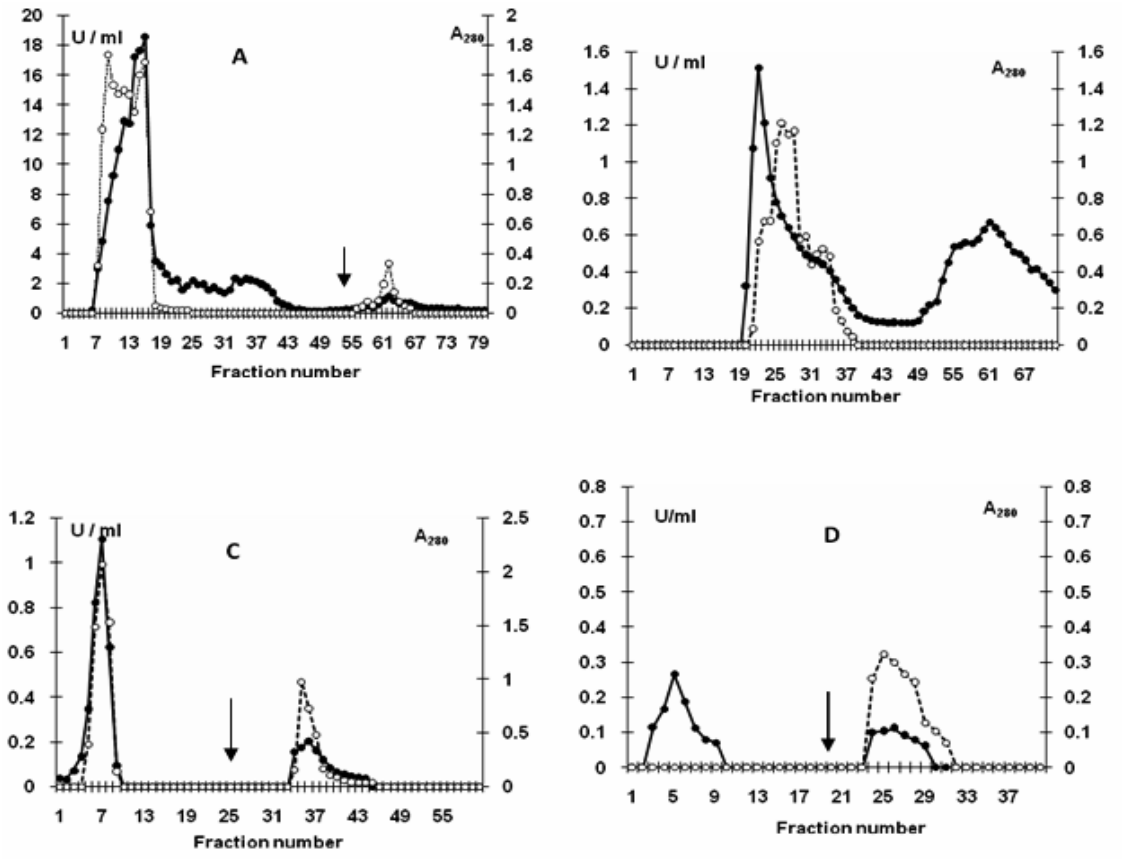

Fig. 1: (A). CM-Cellulose chromatography. The column was eluted with flow rate of 27mL/h and each fraction of 10 mL was collected. Arrow indicates the starting point of linear gradient. Ordinates: protein at 280 nm (•—•); acid phosphatase activity U/mL (o····o).
(B). Elution profile of Sephadex G-75: The column was eluted with flow rate of 60 mL/h and each fraction of 10 mL was collected. Ordinates: protein at 280 nm (•—•); acid phosphatase activity, U/mL (o····o).
(C). Elution profile from Reactive Blue 4-Agarose chromatography.
Flow rate of column was 27 mL /h and 3 mL of fractions were collected. The arrow indicates the start Linear gradient 0 – 0.25 M NaCl in buffer. Ordinates: protein at 280 nm (•—•); acid phosphatase activity U/mL (o····o).
(D). Concanavaline A Sepharose 4-B chromatography.
The column was eluted with flow rate of 10 mL/h and each fraction of 3 mL was collected. The arrow indicates the start of linear gradient 0-10 % methyl- α-D-mannopyranoside. Ordinates: protein at 280 nm (•—•); acid phosphatase activity U/mL (o····o).

Table-1: Various steps involved in purification of 29 kDa acid phosphatase from 400g melon seedlings.

Steps	Vol mL	Act. U/mL	T. act. (U)	Prot. mg/mL	T. prot. (mg)	S.A	P.F	Rec. %
Extract	**1570**	**0.175**	**247.75**	**0.444**	**697.08**	**0.394**	**1.00**	**100.00**
80% $(NH_4)_2SO_4$ Precipitation	**85**	**0.50**	**42.50**	**1.20**	**102.00**	**0.41**	**1.05**	**17.15**
CM–Cellulose (Unbound)	**45**	**0.80**	**36.00**	**1.00**	**45.00**	**0.80**	**2.00**	**14.50**
Sephadex G –75 chromatography	**24**	**1.40**	**33.60**	**0.60**	**14.40**	**2.30**	**5.83**	**13.56**
Reactive Blue Sapharose 4 B affinity chromatography	**15**	**1.00**	**15.00**	**0.10**	**1.50**	**10.00**	**25.38**	**6.00**
Concanavalin A affinity chromatography	**8**	**0.90**	**7.20**	**0.020**	**0.16**	**45.00**	**114.20**	**2.90**

Vol.Volume; Act. Activity; T. act. Total activity; Prot. Protein; T. prot. Total protein; S.A. Specific activity; P.F. Purification factor; Rec. Recovery.

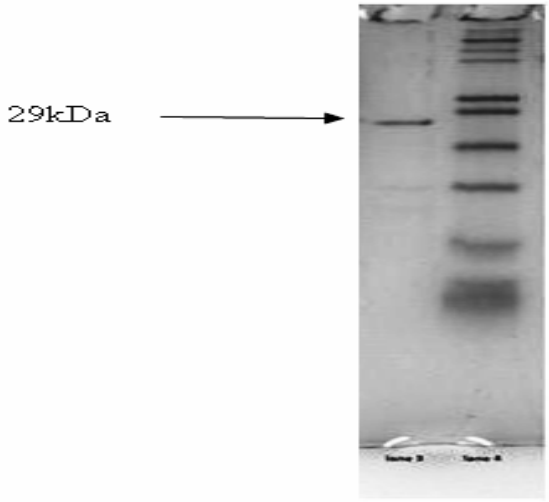

Fig. 2: SDS-polyacrylamide Gel electrophoreses of acid phosphatase of melon seedlings.
Lane 1. Enzyme after Concanavaline A Sepharose 4 -B chromatography.
Lane 2. Markers : β -galactosidase 116.3 kDa, Phosphorylase B 97.4 kDa, BSA 66.3 kDa, Glutamic dehydrogenase 55.4 kDa, Lactate dehydrogenase 36.5 kDa, Carbonic anhydrase 31 kDa, Trypsin inhibitor 21.5 kDa, Lysozyme 14.4 kDa, Aprotinin 6.0 kDa, Unresolved insulin chain B 3.5 kDa and chain A 2.5 kDa.

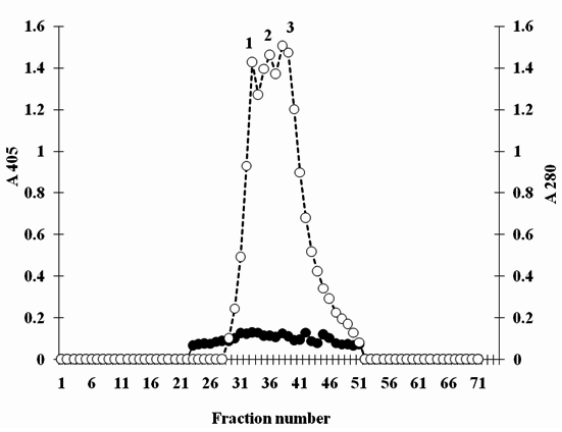

Fig. 3: Elution profile of acid phosphatases from melon seedlings on Sephadex G-100 column. Elution volumes of three acid phosphatase peaks were 99 mL, 108 mL and 114 mL, respectively. Ordinates: protein at 280 nm (•—•); acid phosphatase activity, ΔA_{405} (o····o).

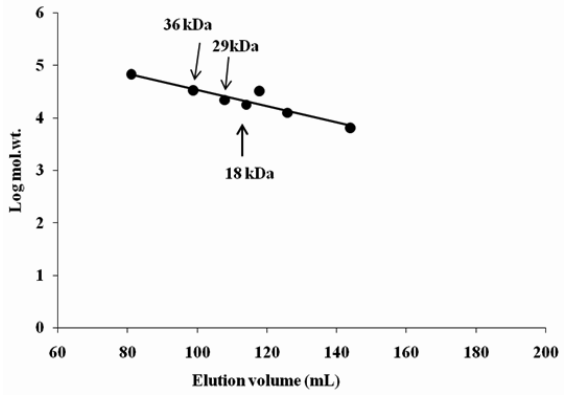

Fig. 4: Estimation of molecular weight acid phosphatase on calibrated Sephadex G-100 column.
Log molecular weight versus elution volumes.
3-5 mg of each standard protein in about 4 mL of buffer was applied onto the column of Sephadex G-100 and eluted as described in material and methods. Elution volume (Ve) was determined from the absorbance at 280 nm for standard proteins. Serum albumin (Mr 66 kDa), Ve 81 mL; Lysozyme (Mr 14.4 kDa), Ve 118 mL; Cytochrome (Mr 12.4 kDa), Ve 126 mL; Aprotinin (Mr 6.5 kDa), Ve 144 mL. Unknown protein Peak 1 (Mr 36 kDa), Ve 99 mL; Peak 2 (Mr 29 kDa), Ve 108 mL; Peak 3 (Mr 18 kDa), Ve 114 mL.

The enzyme had optimum pH at 5.0 (Fig. 5) which is consistent with the optimum pH of acid phosphatases from caster bean seed [12] and germinating seed of *vigna sinensis* [21] and lower than that of acid phosphatases from *vigna aconitifolia* (pH 5.4) [22] and *vigna radiata* seedlings (pH 5.7) [16]. Moreover, the obtained pH 5.0 for acid phosphatase from melon seedlings was higher than the value of pH 4.75 obtained for the enzyme from (*Agaricus bisporus*) commercial mushroom [23]. An optimum temperature of 50°C was found for the 29 kDa enzyme (Fig. 6) similar to the value reported for acid phosphatase isolated from garlic seedlings [17] and higher than those described for other plant acid phosphatases such as caster beam seeds (45° C) [12], barley roots (30-35° C) [9], cotton seeds (37°C) [16] and lower than temperature optima for isoforms of soybean acid phosphatases (80° C) [24]. The enzyme seemed to be stable at pH 4-7 (Fig. 7) after incubating at 37°C for 24 h in pH 3.6-9. The enzyme retained its activity at 50° C for incubation of 45min and had lost 30 % of its activity at 60° C. At 70° C, the loss of activity was more than 90 % (Fig. 8). The

K_m for *p*-nitrophenyl phosphate was found to be 0.175 mM and V_{max} was 42 µmol of substrate hydrolysed/min/mg of protein at pH 5.5 and at 37° C. The effect of various compounds on the enzyme activity is shown in Table-2. The enzyme was inactivated by metal ions such as Zn^{+2}, Cu^{+2}, $Ag^{+1}$and Hg^{+2} but not affected by $Ca^{+2}$and Mg^{+2}. There was also no change in the activity in the presence of EDTA, *p*-hydroxyl-mercuri benzoate, citrate and tartrate.. However, the enzyme was slightly activated by Triton X-100.

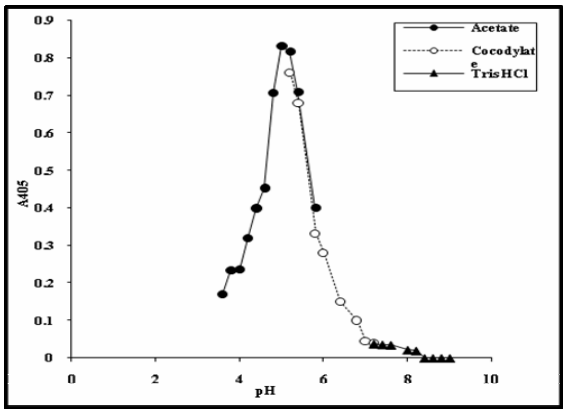

Fig. 5: Optimum pH of acid phosphatase of melon seedlings.

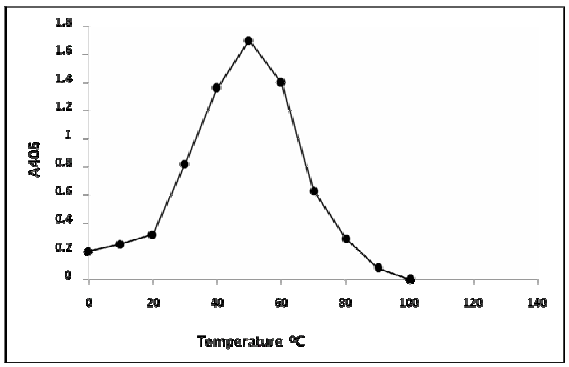

Fig. 6: Optimum temperature of acid phosphatase of melon seedlings.

Table-2: Effect of different metal ions on the acid phosphatase activity from melon seedlings.

Inhibitor	Concentration (mM)	%age Activity
H_2O	–	100.00
$ZnCl_2$	5	2.00
Cu_2SO_4	5	1.00
$CaCl_2$	5	104.00
$HgCl_2$	5	17.50
$MgCl_2$	5	103.80
Tartrate	5	101
Citrate	5	98
$AgNO_3$	5	12.00
EDTA	10	103
p-hydroxyl-mercuribenzoate	1%	97
Triton X-100	1%	126.00

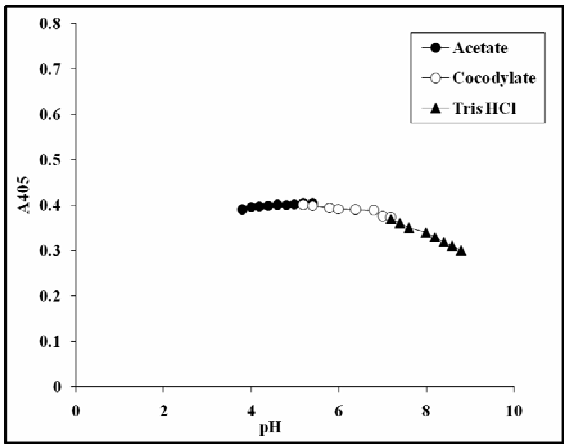

Fig. 7: pH stability of acid phosphatase of melon seedlings.

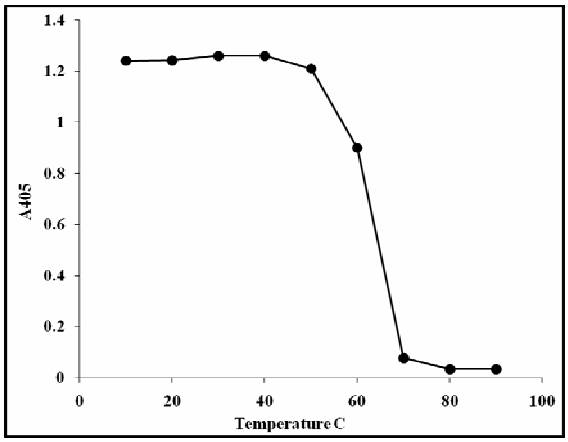

Fig. 8: Temperature stability of acid phosphatase of melon seedlings.

The enzyme was inhibited by phosphate, vanadate, molybdate and fluoride. The patterns of inhibition and the inhibition constants are presented in Table-3. Competitive inhibition by Pi, common in other acid phosphatases [25] suggests an important role of end product inhibition. Vanadate is the most effective and producing competitive inhibition with K_i of 2 µM. The same results were observed in enzymes from cucumber, radish and rocket salad [26]. Mixed type inhibition by molybdate was found in enzymes from germinating soybean [15]. Non-competitive inhibition by fluoride was also in accord with results displayed by acid phosphatase from rice plant [27]. Table-4 shows the substrate specificity of the enzyme. Hydrolysis rates of para-nitrophenyl

phosphate, phenyl phosphate, α- and β-naphthyl phosphate and β-glycerophosphphate were significant indicating they were good substrates, while the hydrolysis rates of FMN, α– glycrophosphate, phospho amino acids, sugar phosphate and ATPase were slower. The AMP and ribose-5-phosphate were not hydrolysed.

Table-3: Types of inhibition and inhibition constants of acid phosphatase from melon seedlings.

Inhibitor	Types of inhibition	K_i
Phosphate	Competitive inhibition	0.532 mM
Vanadate	Competitive inhibition	2.0 µM
Molybdate	Mixed inhibition	5.0 µM
Fluoride	Non-competitive inhibition	0.37 mM

Table-4: Substrate specificity of acid phosphatase from melon seedlings.

Substrate	% Activity
p-nitrophenyl phosphate	100
Phenyl phosphate	87
Flavin mononucleotide	32
α-Naphthyl phosphate	75
α-Glycero phosphate	38
Phosphothreonine	12
Glucose-1-phosphate	23
Glucose-6-phosphate	32
Adenosine triphosphate	18
Adenosine monophosphate	0
Ribose phosphate	0

Experimental

Material

Fresh samples of melon seedlings were collected from Punjab seeds corporation Lahore. Flavin mono nucleotide (FMN), *p*-nitrophenyl phosphate, phenyl phosphate, β-glycerophossphate, o-phosphotyrosine, protein markers, acrylamide and bisacrylamide were from Acros Chemical Co, and Sephadex G-75, CM -Cellulose, Reactive Blue 4-Agarose and Concanavaline A Sepharose 4-B were purchased from Sigma-Aldrich chemicals.All other chemicals used were of analytical grade, mostly from BDH and Aldrich Chemical Company.

Methods

Enzyme Assay

Acid phosphatase activity was determined according to the method of Panara et al.(1990) [9], using *p*-nitrophenyl phosphate as substrate. *p*-Nitrophenol obtained as the result of hydrolysis of substrate, was converted into phenolate ions (yellow color) at an alkaline pH. The absorbance was measured at 405 nm.

The enzyme assay medium contained 900 µL of 4 mM of substrate in 0.1 M acetate buffer, pH 5.5 containing 1mM EDTA and 0.1mL of enzyme. The mixture was incubated for 5 minutes at 37º C. After incubation, 4mL of 0.1M KOH was added yielding yellow color which was determined by using a UV/VIS Spectrophotometer. One blank sample was also prepared in which 0.1mL water was used instead of enzyme.

The molar extinction coefficient of 18000 M^{-1} cm^{-1} was used for *p*-nitrophenol. The specific activity was expressed as µmol of *p*-nitrophenol released / minutes / mg of protein.

Substrate specificity studies were carried out by determining the release of inorganic phosphate as the result of hydrolysis of various substrates. Inorganic phosphate was determined by Black and Jones method [28]. The incubation mixture consisted of 450 µL buffer containing 4 mM of substrate and 50 µL enzyme solution, was incubated at 37º C for 5 minutes to release Pi from enzymatic reaction. This hydrolytic reaction was stopped by addition of 200 µL of 10% TCA. The color was developed with molybdate reaction which was as follows: The 500 µL mixture (composed of 200 µL of 2 % ammonium molybdate and 300 µL of 14 % ascorbic acid in 50 % trichloroacetic acid) was added to the above mixture (700 µL) followed by the addition of 1 mL solution containing 2 % trisodium citrate and 2 % sodium arsinate in 2 % acetic acid to make the total volume of 2200 µL. The color was developed for 30 minutes and absorption was determined at 700 nm. The enzyme activity was expressed as a percent of *p*-nitro phenyl phosphate.

To study the effect of various compounds such as inhibitors and activators on acid phosphatase, the activities were determined under standard assay conditions as described above [9] in the presence of these compounds at the desired concentrations. The control activity without these compounds was taken as 100% and other activities were expressed as percentage of control activity.

K_m values were plotted (Line weaver-Burk plots) with 6 substrate concentrations ranging from 0.25-16 mM in the absence and presence of inhibitors. The inhibition constant (K_i) for the inhibitors were calculated at 2 or 3 fixed concentrations of inhibitors.

Protein Determination

Protein concentration was determind by the Lowery method [29] or by measuring the absorption at 280 nm.

SDS- polyacrylamide gel electrophoreses

Protein preparation was subjectd to electrophoresis in SDS- polyacrylamide gel (12 %) (Laemmli, 1970) [30] and protein was visualised by Coomassie blue staining.

Apparent molecular weight determination

The apparent molecular weight of the acid phosphatase was estimated on calibrated Sephadex G-100 column (1.8 x 85cm) by comparing its elution volumes with that of a standard protein. The extract of melon seedlings were salted out with ammonium sulphate 80 % saturation and placed on Sephadex G-100 column which had been previously equilibrated and eluted with 0.01M acetate buffer pH 5.5, containing 0.1M NaCl at flow rate of 30 mL /h. Each fraction of 3 mL was collected for and analyzed for protein and enzyme activity.

Purification of enzyme

Extraction, isolation and purification of acid phosphatase from melon seedlings were carried out at 4^{o} C.

Fresh melon seedlings (400 gm) were grounded in 10mM Tris HCl buffer pH 7.4 and then homogenized. After homogenization the volume was adjusted with same buffer to 3mL per gram of seedlings and gently stirred for 1–2 hours. Then the whole contents were centrifuged in Beckman centrifuge J 2–21 (using rotor JA-14) at 7000 rpm for 30 minutes. The pellet was discarded and supernatant was collected. Then solid ammonium sulphate $(NH_4)_2SO_4$ was added to 80 % saturation with constant stirring. After 1 hour, the mixture was centrifuged at 7000 rpm. Then the pellet was collected and the supernatant was discarded. The precipitate thus obtained was dissolved in the 0.01M acetate buffer pH 5.9.

The dissolved precipitate was dialyzed in 2-3 liters of same buffer. The dialyzed sample was centrifuged at 7000 rpm for 30 min and clear supernatant was collected. Before loading the sample, the CM-Cellulose column (21 X 2.7 cm) was washed thoroughly with 0.01M acetate buffer pH 5.9 until pH of effluent and eluent became same. Then the sample (125 mL) was loaded. When the sample was completely absorbed, washing of column was carried out with same the buffer. Unbound protein was eluted during washing. Bound protein was eluted by applying linear gradient from 0-0.5 M NaCl in 0.01M acetate buffer pH 5.9 (250 + 250 mL), and the activity and protein of each fraction were determined. Unbound acid phosphatase from CM cellulose column was precipitated by the addition of ammonium sulphate to 70 % level. The precipitate was collected after centrifugation at 7000 rpm for 30 min and was dissolved in 0.01M acetate buffer pH 5.6 containing 1mM EDTA and 2mM β-mercaptoethanol. The sample was applied to a Sephadex G-75 column (4.5×85 cm) which had been previously equilibrated with acetate buffer having pH 5.6 containing the same additives and eluted with same buffer but containing 0.1M sodium chloride. The most active fractions were pooled, concentrated and dialyzed overnight against 1L of 0.01M acetate buffer pH 5.0 containing the same additives.

The dialyzed sample was applied to Reactive Blue 4-Agarose column (2.8× 14 cm). The column was washed with 0.01M acetate buffer having pH 5 containing 1mM EDTA and 2mM β-mercaptoethanol. The inert proteins were eluted. The bound enzyme was eluted by applying linear gradients 0-0.25 M NaCl. The most active fractions were dialyzed against 1L of 0.1M Tris HCl buffer pH 7. The dialyzed sample was applied to Concanavaline A Sepharose 4-B column (4 x 1.8 cm). The column was washed with 0.1 M Tris HCl pH 7.0. The inert protein was eluted. After washing the column, a linear gradient from 0-10 % methyl-α-D-mannopyranoside (25 mL buffer + 25 mL buffer containing 10 % methyl-α-D-mannopyranoside) was applied. The most active fractions of enzyme were pooled and concentrated by ultra filtration for biochemical analysis.

Conclusion

Acid phosphatase was purified from seedlings of melon to homogeneity with specific activity of 45 U/mg of protein and characterized. Since the enzyme was found insensitive to tartrate inhibition, it may be recognized as a tartrate resistant acid phosphatase.

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