130 kDa Acid Phosphatase from the Liver of *Labeo Rohita*: Isolation, Purification and some Kinetic Properties

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Summary: An isoenzyme of high molecular weight acid phosphatase (HM-ACP) from the liver of fish Rohu (*Labeo Rohita*) was isolated and purified to homogeneity. The enzyme had specific activity of 14.96 U/mg and a recovery of about 4 %. The purification procedure included ammonium sulphate precipitation and series of chromatographic separations on SP-Sephadex C-50, CM-Cellulose and Sephacryl HR-200 columns. Nearly 500-folds purification was achieved. The molecular weight was estimated to be 120-130 kDa by polyacrylamide gel electrophoresis (PAGE) of native enzyme and 130 kDa by gel filtration on calibrated Sephadex G-100 column. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced & non - reduced conditions showed a band corresponding to 66 kDa confirming the dimeric nature of enzyme. *Para* - nitrophenyl phosphate and flavin mononucleotide were hydrolyzed effectively by the enzyme and found to be good substrates. Optimum temperature for the enzyme was 50°C and temperature stability was 0°-50°C. Similarly optimum pH for the enzyme was 5.4 and pH stability was 4.8-6.0. The K_m for the p-nitrophenyl phosphate was estimated to be 0.15 mM. The enzyme was competitively inhibited by the phosphate, vanadate, molybdate, tartrate, fluoride and pyridoxal-5′-PO_4 while pyridoxamine-5′-PO_4 showed poor inhibition. Metal ions such as Ag^+, Cu^{++}, Zn^{++} showed strong inhibition on the enzyme activity while other divalent ions like Mg^{++}, Mn^{++} and Co^{++} were found to be poor inhibitors. Modifiers like EDTA, methanol, ethanol, acetone and glycerol had no effect on the enzyme's activity.

Introduction

Acid phosphatases are present in a variety of plants, animal tissues and microorganisms [1-3]. These occur in multiple forms which differ in molecular mass, pI value, substrate specificity, susceptibility to inhibitors or carbohydrate content [4-6]. Despite their wide spread distribution and abundance in nature, the physiological significance of acid phosphatases is not known with certainity but it may be involved in many biological systems which are linked to metabolism of phosphorylated compounds, metabolic regulation and signal transduction pathways [7-8].

Mammalian liver contains at least two acid phosphatase forms (molecular weight 90-107 kDa and 14-30 kDa) which can be separated by gel chromatography [9-12]. These can be distinguished from each other by their localization within cells. High molecular weight forms appear to be lysosomal in origin and the low molecular weight forms a cytosolic in nature [13]. Beside the differences in size, high molecular weight forms are strongly inhibited by tartrate and fluoride whereas the low molecular weight acid phosphatases (LM-ACP) are insensitive to these [14]. The HM-ACP was purified from human liver to homogeneity and extensively characterized [11,15].The enzymes from liver of carp, catfish and frog had also been purified and found to be glycoprotein in nature [16-18]. In recent years, other acid phosphatases were isolated from boar seminal plasma, spider venum, desert locus, earthworm and fresh water snails [19-23].

Studying acid phosphatase is difficult due to their multiforms, occurrence, relative non-specificity, small quantity and instability in dilute solution [24]. In our earlier study, during the purification of HM-ACP (100kDa) isoenzyme from fish liver, a small shoulder in the ascending part of the enzyme activity peak was observed in gel chromatography on Sephadex G-100 column, pointing to the existence of 130 kDa isoenzyme [25]. These two isoenzymes

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separated by Ultragel AcA 44 column, have been reported in plants too [26].

Until now there have been no reports concerning the isolation and characterization of 130 kDa isoenzyme

In this paper, we isolated HM-ACP (130 kDa) isoenzyme from fish liver using our own purification procedure and partially characterized. Further study may help us in understanding the role of these isoenzymes in cell function.

Results and Discussion

HM-ACP was purified 500-fold by series of chromatography on SP-Sephadex C-50, CM-Cellulose and Sephacryl HR-200 columns with a specific activity of 15 U/mg of protein and recovery of 4 % with respect to starting material. The elution profiles are shown in Figs. 1 and 2 and the summary of purification scheme is represented in Table-1. The homogeneity of the enzyme was checked on SDS-PAGE. Single band was detected. The molecular weight of denatured enzyme on SDS-PAGE (12 % gel) was found to be 66 kDa under reducing and non-reducing conditions (Fig. 3). The molecular weight of native enzyme obtained by gel filtration on Sephadex G-100 column was esimaed 130 kDa (Fig. 4). This indicates that fish liver HM-ACP isoenzyme (130 kDa) is a dimer, consisted of almost two equivalent subunits. This molecular weight value is similar to molecular weight reported for acid phosphatases from livers of carp (122 kDa) and frog (140 kDa) and rice plants (130 kDa) [16,18,26]. Nearly all of the known mammalian acid phosphatases are heterogeneous glycoproteins and non-mammalian acid phosphatases from liver of carp, catfish, frog and chicken are also gycoproteins [27]. The enzyme from fish liver might be glycoprotein but its purification by affinity chromatography on Con A column failed which may exhibit structures weakening their Con A binding abilities with carbohydrate chains [25]. The optimum temperature for the enzyme was 50° C and temperature stability was 0-50°C (Fig. 5). The enzyme retained maximum activity (87.5 %) after heating at 50°C for 30 min. Temperature higher than 50°C strongly affected the enzyme activity. 80 % inactivation was observed at 60°C on heating for 10 min. The enzyme was completely denatured after 3-5 min of incubation at 70°C (Fig. 6). The effect of pH indicated that maximum activity was achieved at pH

Table-1: Purification scheme for 130 kDa acid phosphatase from fish Liver.

	Volume (ml)	Total activity (U)	Total Protein[b] (mg)	Specific activity (U/mg)	Purification factor	Recovery %
Extract[a]	1270	1485.9	53640	0.028	1	100
30 % - 80 % $(NH_4)_2SO_4$ saturation	336	1414.5	24060	0.059	2.1	95.2
SP-Sephadex C-50	50	350	2200	0.153	5.5	23.6
CM - Cellulose	11.5	95.4	24.87	3.84	137.1	6.4
Sephacryl HR-200 chromatography	13.5	57.1	3.159	14.96	534.3	3.8

[a] Starting from 400 gm of fish liver.
[b] Protein concentration was determined by Biuret method.

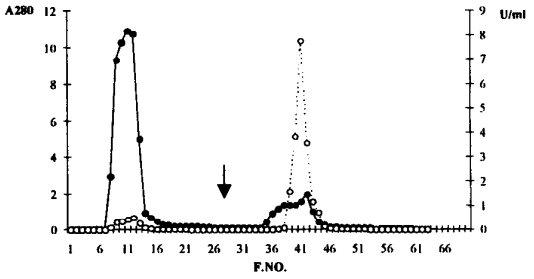


Fig. 1: Elution profile from CM-Cellulose chromatography with flow rate of 30ml/hr and10ml fractions were collected with a flow rate. The arrow indicates the start of linear gradient 0-0.5 M NaCl in buffer. Ordinates: Protein at 280nm ((•—•); acid phosphatase activity, U/ml(o····o).

5.4 with pH stability over the range of 4.8-6.0. The apparent K_m for *para* -nitrophenyl phosphate was estimated to be 0.15mM. As reported for mammalian tissues, the fluoride and tartrate are the potent inhibitors of high molecular weight acid phosphatases while these enzymes are insensitive to formaldehyde [15, 28]. Fish liver HM-ACP was also found to be insensitive to formaldehyde, but was strongly inhibited by fluoride, tartrate, phosphate, orthovanadate, molybdate and pyridoxal-5'-PO_4. These inhibitors were found pure competitive (Fig. 7) and their K_I values were estimated to be 55 μM, 40 µM, 0.19 µM, 0.5 µM, 0.11µM and 15 µM respectively. Phosphate, vanadate and molybdate are powerful inhibitors than the others. These results are

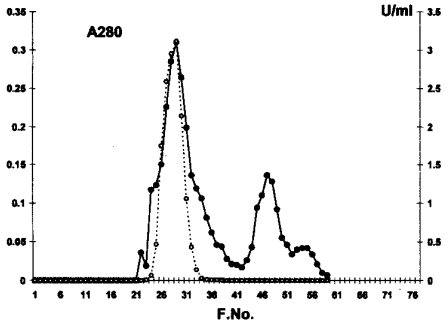


Fig. 2: Sephacryl HR-200 chromatography. Flow rate of 15 ml/h; 2.5 ml fractions were collected. Ordinates: Protein at 280nm ((•—•); acid phosphatase activity, U/ml(o····o).

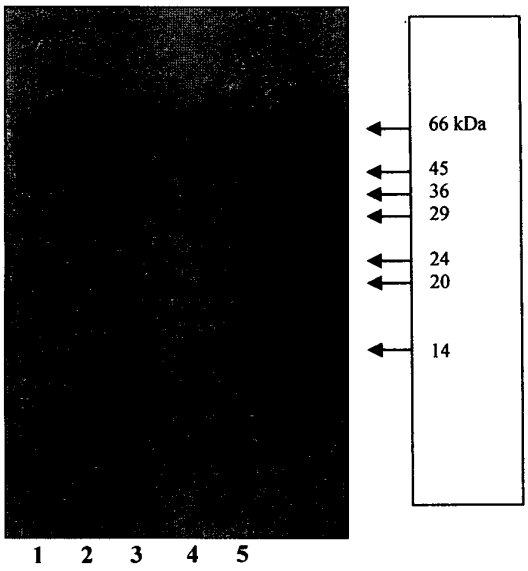


Fig. 3: SDS-polyacrylamide gel electrophoresis of HM-ACP enzyme. Lane 1 & 2, 10µl reduced and non-reduced enzyme (corresponding to 66 kDa) , Lane 3 & 4, 3µl reduced and non reduced enzyme, Lane 5 Standard proteins: Albumin (66 kDa) , ovalbumin (45 kDa) , glycerolaldehyde-3-phosphate dehydrogenase (36 kDa) , carbonic anhydrase (29 kDa) , trypsinogen (24 kDa) , trypsin inhibitor (20.1 kDa) and lactalbumin (14.2 kDa).

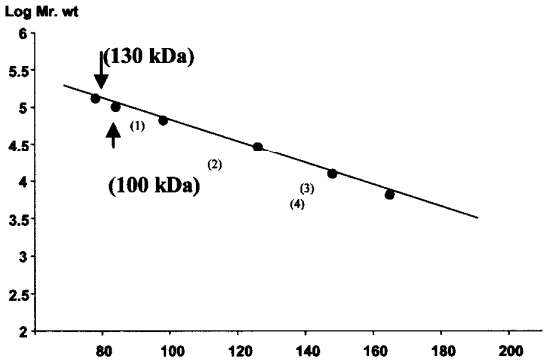


Fig. 4: Iinear graph of log molecular weight versus elution volumes of standard proteins. 3-5mg of each protein in about 3-4ml of buffer was applied onto the column of Sephadex G-100 and eluted as described in material and methods. Blue dextrin 2000 (average M_r $2x10^6$) was used to measure void volume (V_o) of the column and elution volume (V_e) was determined from the absorbance at 280nm for standard proteins (•) or by assay of enzyme activity at 405nm for the 100 kDa and 130 k Da enzyme samples. (1) Bovine serum albumin (M_r 66,000) , V_e 98ml; (2) Carbonic anhydrase (M_r 29,000) , V_e 126ml; (3) Cytochrome c (M_r 12,400) , V_e 148ml; (4) Aprotinin (M_r 6,500) , V_e 165ml; HM-ACP (100 kDa) , V_e 84ml; HM-ACP (130 kDa) , V_e 78ml; V_o 72ml.

in accord with the finding of human liver and wheat germ high molecular weight acid phosphatases [15, 29] , human prostate acid phosphatase [30] and 100 kDa chicken liver enzyme [31], while pyridoxamine-5'-PO_4 showed poor inhibition. Metal ions such as Hg^{++}, Cu^{++}, Zn^{++} expressed inhibitory effect on the enzyme. Similar results were also obtained with purple acid phosphatase from starved tomato [32] and acid phosphatase from rice plants [26]. Other divalent ions such as Mg^{++}, Mn^{++}, Co^{++} were found ineffective on the enzyme activity. There was no change in the enzyme activity in the presence of EDTA and Triton X-100, while *p*-hydroxy mercuribenzoate showed slight inhibition as observed in high molecular weight (100 kDa) acid phosphatase form chicken liver [33].

The relative hydrolytic rates on the different phosphate esters are shown in Table-2. HM-ACP

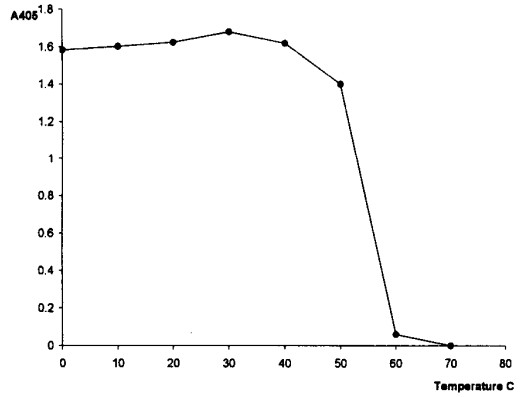


Fig. 5: Effect of incubation temperature on the acid phosphatase enzyme.

Table-2: Substrate Specificity of fish liver acid phosphatase.

Substrate	K_m (mM)	V_{max} ($\mu mol^{-1}mg^{-1}min^{-1}$)
para-nitro phenyl phosphate	0.150	15.9
Flavin mononucleotide	0.185	14.8
β-naphthyl phosphate	0.256	13.7
α-naphthyl phosphate	0.345	13.7
Phenyl phosphate	0.333	12.1
β-glycero phosphate	0.555	10.7

The incubation mixture consisted of 0.1M acetate buffer pH 5.5, 4mM substrate and reasonable amount of enzyme in a final volume of 0.5 ml. After incubation at 37°C for 6-10 min, the reaction was stopped by adding 0.2 ml

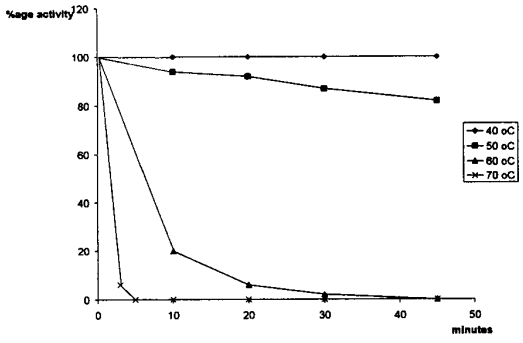


Fig. 6: Thermal inactivation of acid phosphatase. The enzyme was heated at 40°C, 50°C, 60°C and 70°C in 0.1 M acetate buffer pH 5.5 for times indicated in abcessa. The remaining activities were determined as usual. Ordinate: %age activity.

showed broad substrate specificity. The values of K_m and V_{max} indicate that *para*-nitrophenyl phosphate, flavin mononucleotide, phenyl phosphate, α- and β-naphthyl phosphates and β-glycerophosphate are good substrates. Other substrates, including phosphoamino acids, nucleoside phosphates and sugar phosphates were hydrolyzed at reasonable rates.

The effect of acetone, ethanol, methanol and glycerol on the activity of acid phosphatases with *para*-nitrophenyl phosphate as a substrate was determined. Acetone, methanol and glycerol at 10 % concentration caused no activation of the enzyme, thus showing no phosphotransfer ability. Activation was appeared in many low molecular weight acid phosphatases [28, 34] which reflect phosphor-transferase activity.

Thus 130 kDa isoenzyme from fish liver showed non-specific enzyme similar to 100 kDa acid phosphatase isoenzyme in biochemical and kinetic properties.

Experimental

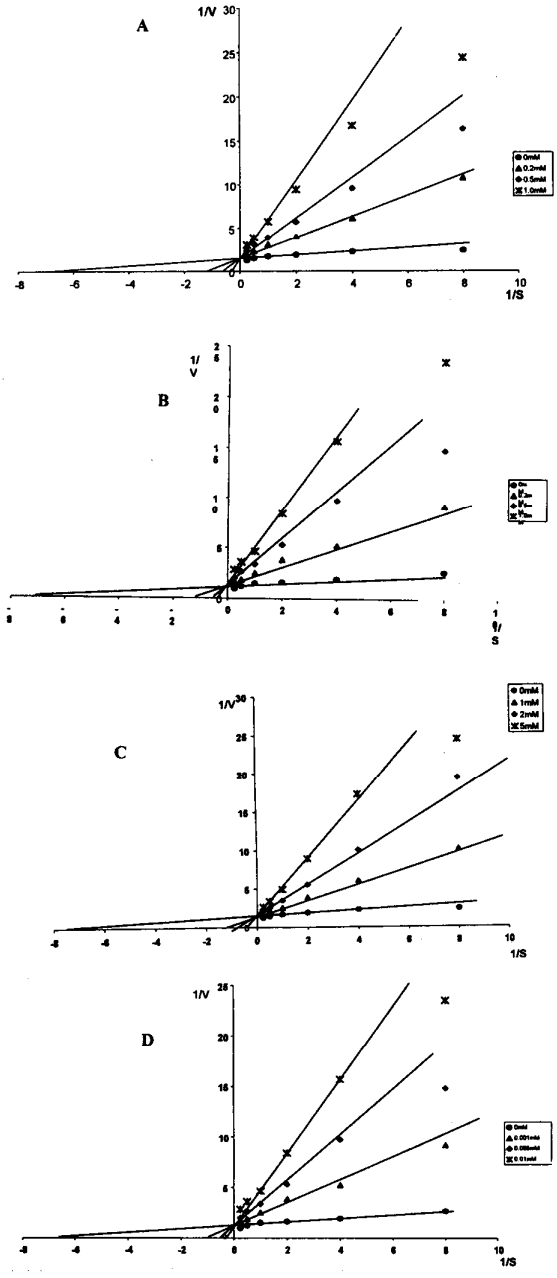
Chemicals

L. rohita (common name Rohu) was captured from Indus river (N.W.F.P. Pakistan) and the liver was excised immediately. SP-Sephadex C-50, Sephacryl HR-200, p-nitrophenyl phosphate, α-naphthyl phosphate, β-glycerol phosphate, β-naphthyl phosphate, flavinmononucleotide phosphate (FMN) , phenyl phosphate, bovine serum albumin, SDS molecular weight markers were purchased from Merck, Sigma Chemical Co. & Fluka Chemical Co., CM- Cellulose from Whatman Biosystem:, the material for polyacrylamide gel-electrophoresis were obtained from Acros Chemical Co. All other chemicals were of highest purity analytical grade.

Assay for Acid Phosphatase

Acid phosphatase activity was assayed by measuring production of *p*-nitrophenol as previously reported by Asma Saeed et al [33]. Reaction mixture contained 900 µl of 0.1M acetate buffer pH 5.5 containing 4 mM p-nitrophenyl phosphate as substrate and 100 µl enzyme sample. It was incubated at 37°C for 5 min and the reaction was stopped by the addition of 1.0 ml of 0.1M NaOH. Amount of p-nitrophenol formed was determined spectrophotometrically at 405 nm. The non-enzymatic hydrolysis of p-nitrophenyl phosphate was corrected by measuring the control without added enzyme. To correct absorbance due to colour of crude

enzyme extract, a control was employed in which NaOH was added prior to the addition of raw enzyme. One unit of enzyme activity is defined as μmol of p-nitrophenol liberated /min. Specific activity is expressed as units/mg of protein.



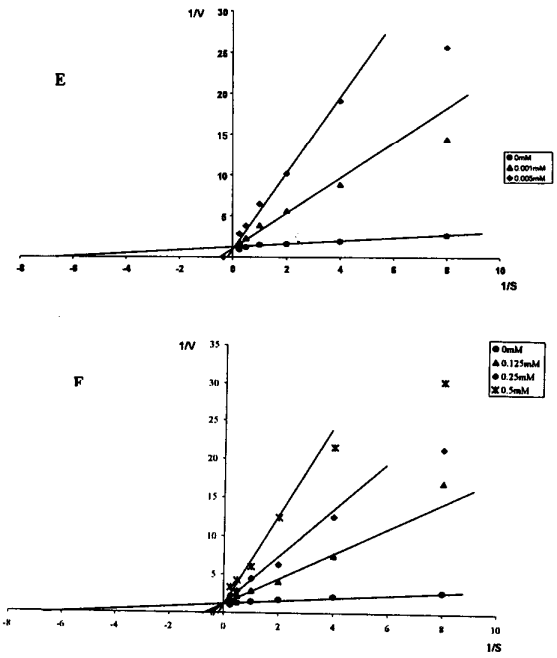


Fig. 7: Competitive inhibition of fish liver acid phosphatase. Lineweaver-Burk plots of 1/v versus 1/S. A. sodium fluoride; B. tartrate; C. phosphate; D. orthovanadate; E. molybdate; F. pyridoxal-5′-phosphate.

K_m values were determined by measuring the p-nitrophenol produced at different concentrations of substrate ranging from 0.1mM to 4 mM in absence and presence of two or three fixed concentrations of inhibitors. Lineweaver-Burk plots were used to arrive at the values of K_I. Straight lines were drawn by applying least square rule. Each point was the average of at least three readings.

Substrate specificity study towards number of substrates such as phenyl phosphate, flavin mononucleotide, α- and β-naphthyl phosphates and β-glycerophosphate was carried out by measuring the release of inorganic phosphate (P_i) after enzymic reaction by Black & Jones method [35]. The amount of P_i released was calculated from standard curve using KH_2PO_3. K_m and V_{max} values against these substrates were determined graphically (Lineweaver-Burk plots) with substrate concentrations ranging from 0.1 mM to 4 mM.

Optimum pH was determined in 0.1 M acetate buffer (pH 4-6) and 0.1 M barbital buffer (6-6.7).Temperature optimal was determined in range of 0-70°C at interval of 10°C. The temperature stability was studied by incubating the enzyme solutions at temperatures ranging from 0 to 70°C in the presence of 0.1 M acetate buffer pH 5.5 for 30 min. The inactivation reaction was stopped by immersion in ice bath. After 1 h, the residual activity was assayed as described above. Likewise, the thermal inactivation of the enzyme was also examined at 40°C, 50°C, 60°C and 70°C in time dependent manner. The pH stability was determined by incubating the enzyme in 0.1 M acetate buffer (pH 4-6) and 0.1 M barbital buffer (6-9) for 10 h period .The residual activity was assayed as usual.

Protein Determination

Protein was measured by Biuret method according to Beisenherz *et al.,* [36] utilizing bovine serum albumin to establish a standard curve. For column effluents, relative protein concentration was estimated from the absorbance at 280 nm.

Electrophoresis

SDS-PAGE was carried out by the method of Laemmli [37] under reduced and non-reduced conditions. The samples of acid phosphatase were prepared in sample buffer with and without reduction by β-mercaptoethanol and heated at 95 °C for 5 min. The enzyme purity was checked in 12 % acryl amide mini-slab gel. The molecular weight estimate was made using standard size marker proteins as indicated in its figure. The correlation coefficients of plots of log of the molecular weight versus mobility of protein band were between 0.991-0.993.

Gel Filtration and Apparent Molecular Weight Determination

Fish liver extract was salted out with ammonium sulphate (30-60 % saturation) and placed on Sephadex G-100 column (1.8 x 85 cm) , which was previously equilibrated and eluted with 0.01 M acetate buffer pH 5.0 containing 1mM β-mercaptoethanol and 0.1 M NaCl at flow rate of 30 ml/h. Fractions (about 3.5 ml each) were collected for assays of protein and enzymes activities. Apparent molecular weights of HM-ACP and LM-ACP were estimated on a calibrated Sephadex G-100 column by a comparison of their elution volumes to those of standard proteins. The proteins used were bovine serum albumin, carbonic anhydrase, cytochrome c and aprotinin.

Enzyme purification

Frozen fish livers (400 gm) was brought into thawing state and was homogenized in blender and added 0.3M acetate buffer pH 5.5 containing 1mM EDTA, 0.1mM PMSF, 2mM β-mercaptoethanol at the rate of 1Kg/3 L. After homogenization, it was agitated for 1-2 hours and centrifuged at 8000 rpm (Rotor JA-14) for 30 minutes. Then it was filtered over glass wool. Solid $(NH_4)_2SO_4$ was added to 1270 ml of extract to 30 % saturation (176g /l). Addition of salt was gradual with constant stirring and stirred further for 1 hour. It was centrifuged at 8000 rpm. The supernatant was collected and precipitate was discarded. Solid $(NH_4)_2SO_4$ was added to supernatant to form 80 % saturation and stirred in cold place for 2 h. It was then centrifuged at 8000 rpm for ½ h discarding the supernatant and dissolved the precipitate in 0.05M acetate buffer pH 4.8 containing 1mM EDTA, 0.1mM PMSF and 2mM β-mercaptoethanol. It was then dialyzed against 20 vol. of 0.05M acetate buffer pH 4.8 containing 1mM EDTA, 0.1mM PMSF and 2mM β- mercaptoethanol over night .The dialyzed sample was centrifuged for 30 minutes at 8000 rpm. The clear supernatant was subjected to cation-exchange chromatography on SP-Sephadex C-50 column (33 x 8.5cm) which was previously equilibrated with 0.05M acetate buffer pH 4.8 containing 1mM EDTA, 0.1mM PMSF and 2mM β-mercaptoethanol. The column was washed with same buffer. The unbound high molecular acid phosphatase activity (HM-ACP) was pooled and concentrated by precipitation with 70 % $(NH_4)_2SO_4$. The precipitate thus obtained was collected by centrifugation and dissolved in 0.01M acetate buffer, pH 5.9 containing 1mM EDTA, 0.1mM PMSF and 2mM β-mercaptoethanol.

50 ml of sample of HM-ACP after 70 % $(NH_4)_2SO_4$ precipitation was dialyzed against 10 vol. of 0.01M acetate buffer pH 5.9 containing 1mM EDTA, 0.1mM PMSF, 2mM β-mercaptoethanol over night. The dialyzed sample was centrifuged and the clear supernatant was subjected to cation-exchange chromatography on CM-Cellulose (32.5 x 3 cm) which was previously equilibrated with 0.01M acetate buffer pH 5.9 containing 1mM EDTA,

0.1mM PMSF, 2mM β-mercaptoethanol. The column was washed with same buffer. The bound enzyme was eluted with gradient 0-0.15M NaCl in the same buffer. This was followed by single step elution with 0.5M NaCl in the same buffer. The most active fractions were collected and concentrated by Amicon ultrafiltration with YM3 membrane. The concentrated sample was applied to Sephacryl HR-200 column (86 x 1.8 cm) previously equilibrated and eluted with 0.05M Tris HCl buffer pH 7.0 containing 1mM EDTA, 0.1M NaCl, 0.01mM PMSF. The fractions containing reasonable activity were pooled and concentrated by ultrafiltration for further study.

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