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High Molecular Weight Acid Phosphatase from Chicken Liver: Isolation, Purification and Characterization

A Saeed*, A R Khan, Rubina Naz and Mukhtiar Hassan

Department of Chemistry, Gomal University, Dera Ismail Khan, Pakistan

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High molecular weight acid phosphatase from chicken liver was purified by salt fractionation, gel filtration on Sephadex G-150 and by affinity chromatography on Sepharose 4B-L-Tartramic amide column. The purified enzyme moved as a single band. The molecular weight was estimated to be approximately 100 k.Da. by gel filtration on Sephadex G-100 and approximately 50 k.Da. by SDS polyacrylamide gel electrophoresis indicating dimeric protein. K_m against *p*-nitrophenyl phosphate and phenyl phosphate at pH 5.0 and ionic strength 0.15 were found to be 0.08 mM and 0.12mM respectively, while the V_{max} for these two substrates were 275 $Umin^{-1}$ mg^{-1} and 250 $Umin^{-1}$ mg^{-1} of protein respectively. The enzyme was strongly inhibited by phosphate, tartrate, vanadate and molybdate. The ideal substrates for the enzyme were 4-ethyl phenyl phosphate, 4-trifluoromethy-phenyl phosphate, α-naphthyl phosphate and *o*-phosphotyrosine in addition to *p*-nitrophenyl phosphate and phenyl phosphate. Other substrates were also hydrolysed but at slower rates.

Key words: Chicken liver, Acid phosphatase.

Introduction

Several mammalian tissues have been shown to contain three types of acid phosphatase, based on differences in molecular weight, localization in cells, substrate specificity and sensitivity to inhibitors. One is known as high molecular weight (Mr ≥ 100 k.Da.) acid phosphatase localized in the lysosomal fraction of the cell and the other as low molecular weight Mr ≤ 20 k.Da.) acid phosphatase enzeme localized in the cytosol (De Araujo *et al* 1976). The existence of acid phosphatases of intermediate molecular weight (Mr ≅ 40 K.Da.) has been reported in mitochondrial fraction of some mammalian tissues such as kidney and liver (Heinrikson 1969; Galka *et al* 1980).

Substrate specificity of low Mr acid phosphatases is more restricted than that of high Mr acid phosphatases in that the former efficiently hydrolyse *p*-nitrophenyl phosphate and FMN only, while high Mr acid phosphatases catalyse hydrolysis of most phosphate esters. Low Mr acid phophatases are found to be inhibited by SH-blocking agents (Chaimovich and Nome 1970; Lawrence and Van Etten 1981; Taga and Van Etten 1982), but unaffected by either fluoride or tartrate which are strong inhibitors of high Mr acid phosphatases (Igarashi and Hollander 1968).

Acid phosphatase has been used to describe different enzymes of completely unrelated clinical and biological interest. Martland *et al* 1924 distinguished the phosphatase of red blood cells from that of bone based on the optimal activity in an acid medium. De Duve used acid phosphatase as a marker to identify lysosomes (De Duve *et al* 1958). This lysosomal enzyme was confined in subcellular granules for intracellular catabolic reactions. On the other hand, the remarkably great amount of acid phosphatase in prostate drew wide attention of clinician to associate acid phosphatase with prostate cancer. The prostatic and lysosomal acid phosphatases had same molecular size (100 k.Da.) and a broad substrate specificity towards all monophosphoesters (Lam *et al* 1973; Osrtowski 1980). However, the prostatic acid phosphatase is not confined in the lysosome. It is secreted into the prostatic fluid in high concentration for unknown physiological function.

In *Drosophila,* acid phosphatase has often been used as a tool for survey of genetic polymorphism (Mac Intyre 1966) Electrophoretic analyses have detected the electromorphs of an acid phosphatase specified by four alleles ($Acph^1$, $Acph^2$, $Acph^3$, and $Acph^4$) at the Acph locus in *Drosophila virilis* (Ohba 1977). $Acph^2$ form, isoenzyme from strain homozygous for $Acph^2$ allele has been purified to homogeneity (Narise 1984). The enzyme is considered to be a dimer composed of identical subunits which have molecular weight of 50k.Da.

However, the structure and molecular properties of high Mr acid phosphatases are remarkably obscure as compared with low Mr acid phosphatases.

*Author for correspondence

Mr, Molecular weight; FMN, Flavin mono nucleotide. EDTA, Ethylene diamine tetra acetic acid; SDS, Sodium dodecyl sulphate; PMSF, Phenylmethyl sulphonyl fluoride.

Low Mr acid phosphatases have been purified and characterized from bovine liver and heart, human liver, rat liver, bovine brain, human placenta, avian pectoral muscle and from other sources (Saeed *et al* 1990; Manao *et al* 1992). Subcellular localization of high and low Mr acid phosphatases in chicken liver have been studied so far (Panara and Mileti 1986) but serious attempt on purification is not yet made. This paper describes for the first time a development of purification procedure for high Mr acid phosphatase Lysosomal from chicken liver together with some of its molecular and kinetic properties and substrate specificity.

Experimental

Enzyme Assay. During chromatography and for other experiments high Mr acid phosphatase activity was assayed at 37°C in reaction mixture (0.5ml) containing 10 mM *p*-nitrophenyl phosphate as substrate in 0.1 M acetate buffer, 1mM EDTA, pH 5.0 (Ramponi *et al* 1989). The reaction was initiated by addition of enzyme and quenched after 5 min by addition of 0.5 ml of 1 N NaOH. The non enzymatic hydrolysis of *p*-nitrophenyl phosphate was corrected by measuring the control without adding enzyme. The amount of product *p*-nitrophenol was determined from the absorbance at 405 nm using a molar extinction coefficient of 18,000 $M^{-1}cm^{-1}$.

One unit of activity is defined as the amount of enzyme that is needed to hydrolyse 1μ mol of *p*-nitrophenyl phosphate per minute at 37°C. Specific activity is defined as the number of enzyme units per milligram protein.

The enzyme activity against other phosphate monoesters was determined under above conditions by estimation of inorganic phosphate (Pi). The enzymic reaction was quenched by addition of 0.2 ml of 10% trichloroacetic acid. The liberated Pi was determined according to Black and Jones method (1983). The color was developed with molybdate and ascorbic acid in the presence of arsenite and the absorbance at 700 nm was read. The amount of inorganic phosphate produced was calculated from standard curve constructed using KH_2PO_4 as a standard.

Inhibition studies were conducted at pH 5.0, 0.1M acetate buffer, 1 mM EDTA, I=0.15 M. The substrate concentrations were chosen to be 0.065 to 4 mM and the initial velocities were measured at two different inhibitor concentrations. K_m & V_{max} were calculated by linear regression analysis.

Protein Determination. Protein concentration was determined by Lowry method (Lowry *et al* 1951). In column effluents the relative concentration was estimated by absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis. SDS-Polyacrylamide gel electrophoresis was performed according to Laemmli (1970).

Purification of Enzyme.

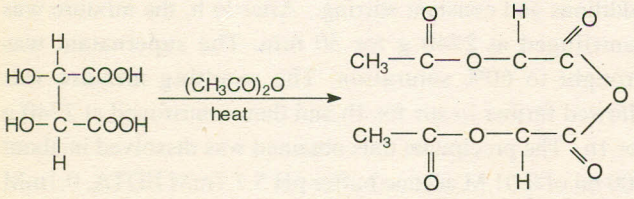
Step-1 Isolation of High Mr Acid Phosphatase. Chicken liver (1 kg) was homogenized in a Waring blender for 2 min with 30 sec intervals and 3 litre of 0.3 M acetate buffer pH 5.0, 1m MEDTA,0.1mM PMSF and 1mM β–mercaptoethanol was added followed by stirring for 1h. The homogenate was centrifuged at 2740g for 30 min. To the supernatant, solid $(NH_4)_2\ SO_4$ was added to 30% saturation with gradual additions and constant stirring. After ½ h, the mixture was centrifuged at 2740 g for 30 min. The supernatant was brought to 60% saturation. The resulting mixture was allowed further to stir for 1h and then centrifuged at 2740 g for 1h. The precipitate thus obtained was dissolved in about 500 ml of 0.01 M acetate buffer pH 5.1,1mM EDTA, 0.1mM PMSF and 1mM β-mercaptoethanol and the solution was dialysed against total volumes of 12-16 l of 0.01 M acetate buffer pH 5.1 containing the same additives over 24 h, with 2-3 replacements of fresh buffer. The dialysate was centrifuged at 10,000g for 1 h and supernatant was applied to a SP-Sephadex C-50 column (43x8cm≅2L) that was equilibrated with 0.01M acetate buffer pH 5.1 containing 1 mM EDTA, 0.1 mM PMSF and 1 mM β--mercaptoethanol. During the column washing, high Mr acid phosphatase (lysosomal) not bound to the column, was eluted. Fractions containing this activity were pooled. This enzyme constituted about 10 % of the total activity and was considered as starting material for further purification while low Mr acid phosphatases (cytosol in nature) bound to the column were eluted by single step with 0.3M NaH_2PO_4 adjusted to pH 5.5.

Step-2 Gel Filtration on Sephadex G-150. Non bound enzyme from SP-Sephadex C-50, was precipitated by adding solid ammonium sulphate to 70% saturation and collected by centrifugation at 10,000g for 1h. The precipitate was dissolved in 32 ml of 0.01 M acetate buffer pH 5.1, containing 1mM EDTA, 0.1 mM PMSF and 1 mM β-mercaptoethanol and applied in two batches to Sephadex G-150 (3.4 x 85cm) equilibrated with 0.01M acetate buffer pH 5.1, 1mM EDTA, 0.1mM PMSF, 1mM β-mercaptoethanol and 0.1 M NaCl and eluted with the same buffer at flow rate 40ml h^{-1} and 9 ml fractions were collected. Active fractions were pooled and the enzyme was concentrated to 12 ml by using UM-30-membrane at 10-20 psi-pressure.

Step-3 Tartramic Acid Affinity Gel Chromatography. High Mr acid phosphatase was further purified by affinity chro-

matography as prostate acid phosphatase, an example of high Mr acid phosphatase from prostate gland was purified on tartramic acid affinity gel (Van Etten and Saini 1978).

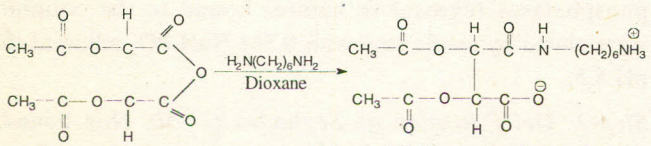
Preparation of Tartramic Acid Affinity Gel. (1) Diacetyl -2-tartaric anhydride was synthesized by method of Shriner and Furrow (1963). Acetic anhydride (250 ml; 2.6 mol) was placed in a 1 litre three-necked flask equipped with a reflux condenser, drying tube and magnetic stirrer.Sulfuric acid (1 ml) was added, followed by 65. 8g (0.44 mol) of L (+) tartaric acid. Some heat was evolved and after a short time crystals began to form. The reaction was heated to 90°C for 30 min.



Di acetyl L-tartaric anhydride

After cooling overnight, the crystalline product was collected on a Buchner funnel, washed with two 25 ml portions of anhydrous benzene, then placed in a vacuum desiccator.

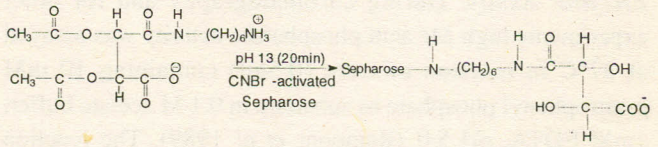
(2). In order to synthesize long chain monoamide derivative of L (+) tartaric acid, 8.7g of 1,6 diaminohexane in 1.2 liltre of anhydrous dioxane was placed in a 2 litre three-necked flask with an additional funnel and drying tube and solution was stirred rapidly with magnetic stirrer while a solution of 24g of diacetyl L-tartaric anhydride in 400 ml of anhydrous dioxane was added drop-wise over a 90 min period. A white precipitate appeared immediately.



di-O-acetyl-N-(6-amino hexyl) tartramic acid

After the addition was completed, the amide derivative (Zwitter ionic form) was isolated by filtration on a Buchner vacuum funnel, the white powder was washed with anhydrous diethyl ether, sucked dry on the filter funnel and then dried overnight in a vacuum oven at 45°C.

(3). Amide derivative of tartaric acid having attached amino alkyl spacer side chain was coupled to Sepharose 4B. 20 g amide derivative was dissolved in 150 ml of water; the pH raised to 13.2 with slow titration of 50% NaOH solution for 20 min to hydrolyze off the *O*-acetyl groups; the pH was readjusted to 10.5 with conc HCl (HCl: water, 1:1) and 100 ml of 0.25 M sodium bicarbonate buffer, as coupling buffer was added to the solution. At the same time, Sepharose 4B (200 ml) was washed twice with equal volumes of water and suspended in 150 ml of water. Two volumes of 2M Na_2CO_3 was added, stirred and then rapidly added a solution of cyanogen bromide (15 g) in 8 ml of fresh acetonitrile. After stirring for 2-3 min; the cyanogen bromide-activated Sepharose was poured on to Buchner funnel and washed successively with 2 litre 0.1 M sodium bicarbonate buffer pH 9.5, 2 litre of water and finally 2 litre of coupling buffer. The gel suspension was transferred to a beaker containing amide derivative dissolved in coupling buffer. This suspension was allowed to stand at 4°C for 12h with occasional gentle stirring.



After coupling the slurry was placed in a sintered glass funnel and washed with 1 litre of 1 M glycine in order to remove unbound amide derivative and then let stand in 1 M glycine for 6 h at 25°C and then 24 h at 4°C with occasional mixing in order to block any remaining reactive sites on the Sepharose. The slurry was washed with 2 litre each of 1 M glycine, 2 M urea and 0.1M sodium acetate buffer, pH 4.0, each solution contained 1 M NaCl and was at 4°C. The slurry was then washed with column buffer of 0.01 M acetate buffer pH 5.0, 1 mM EDTA, 0.1 mM PMSF and 1 mM β-mercaptoethanol and poured into 2.5 x 4 cm column and equilibrated with column buffer.

Affinity Chromatography. High molecular weight acid phosphatase was purified by L-tartramic acid affinity chromatographic procedure that was slightly modified as described by Ostanin *et al* (1994) for the purification of recombinant human prostate acid phosphatase (hPAP).

Sample after Sephadex G-150 column (Step 2) was dialysed against 2 litre of 0.01 M acetate buffer pH 5.0, 1 mM EDTA, 0.1 mM PMSF and 1 mM β -mercaptoethanol overnight and centrifuged at 10,000g for 20 min to remove precipitate if any formed. The clear solution was applied to the tartramic acid affinity coulmn at flow rate of 20 ml h^{-1}. The column was washed with column buffer to remove most of the protein impurities. The column was then washed with column buffer containing 60 mM NaCl to remove further impurities. Finally, pure enzyme was eluted with 20 mM of NaH_2PO_4 in acetate buffer pH 5.0. Two peaks having acid phosphatase activity were obtained. The active fractions from both peaks were pooled separately and concentrated by ultrafiltration.

Table 1
Purification of high Mr acid phosphatase from chicken liver

Steps	Vol (ml)	Act. (Uml^{-1})	Prot ($mg\ ml^{-1}$)	S A ($U\ mg^{-1}$)	T Act (U)	Rec (%)	P F
1. Non bound to SP-Sephadex C-50 Column	1900	0.135	1.38	0.097	256.5	100	1
2. Concentration by 70% Sat.$(NH_4)_2\ SO_4$ Precipitation and Centrifugation	32	7.53	69.72	0.108	240.9	93.9	1.1
3. Sephadex G-150	184	1.05	1.8	0.583	193.2	75.3	6.0
4. Ultrafiltration	12	15.0	23	0.652	180	70.1	6.7
5. Dialysis & Centrifugation	13	12	-	-	156	60.8	-
6. Tartramic Acid affinity Chromotagraphy							
Peak-I	4	27	11.2	2.410	108	42.1	24.8
Peak-II	0.5	6.3	0.025	252	3.2	1.2	2597.9

S A, Specific activity; T Act, Total activity; Rec, Recovery; P F, Purification factor.

Results and Discussion

High Mr acid phosphatase was purified to homogeneity from chicken liver. The major purification steps are summarized in Table 1. Elution profile for Sephadex G-150 is shown in Fig 1 and elution profile for tartramic acid affinity gel is shown in Fig 2. Purification was achieved 2600 fold approximately with recovery of 1 %. The homogeneous enzyme has a specific activity of 252 μ mol min^{-1} mg^{-1} at pH 5.0, at 37°C, using *p*-nitrophenyl phosphate as substrate. This specific activity is comparable to that of human prostate acid phosphatase and is far greater than that of canine prostate acid taphosphatase (Van Etten and Saini 1978) but the yield is too low owing to the loss of non specific elution of peak 1 (42.1%) by 0.01M acetate buffer pH 5.0 containing 60mM NaCl while specific activity is two times less than the specific activity of recombinant hPAP purified from yeast culture medium using tartramic acid-affinity chromatography technique (Duncan *et al* 1984; Ostanin *et al* 1994).

As shown in Fig 2 whole enzyme was immobilized by the column and much of the foreign proteins were eluted when column was washed with column buffer. Since 0.01M acetate buffer pH 5.0 was used, non specific protein binding occurred and this impurity could be eluted by increasing the ionic strength of buffer to 100mM with sodium chloride. Therefore, column was washed with buffer containing 60mM sodium chloride but most of the acid phosphatase activity (Peak 1) was eluted alongwith protein impurities. In case of acid phosphatase from prostate glands and recombinant hPAP purification, the activity was not eluted with buffer containing 60mM NaCl. The reaction was not still known why does lysosomal enzyme behave differently from prostatic acid phosphatase in this regard. The work is in progress to optimize conditions of buffer, pH and ionic strength.

However, it should be possible to achieve further purification of peak 1 subsequent to purification by the present method.

After the removal of impurities, the homogeneous enzyme (Peak II) was eluted from affinity column on application of single step-gradient or linear gradient of sodium phosphate (0-20mM). Phosphate is a competitive inhibitor of the enzyme and therefore competes with tartrate derivative for the active site of the enzyme; this competition leads to displacement of the enzyme from column.

Thus this affinity chromatographic purification procedure works very well with prostatic enzymes but it could be applied to other tartrate-inhibitable acid phosphatases of rat and human liver to obtain purified isoenzymes homogeneously (Saini and Van Etten 1978a).

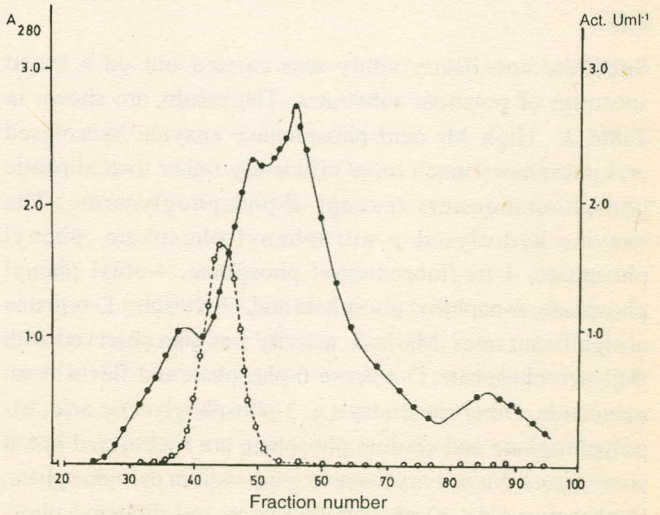


Fig 1. Elution profile of high Mr acid phosphatase from Sephadex G-150. Fractions (9 ml) were collected and measured for phosphatase activity (0- - -0, Uml^{-1}) and protein content (•—• , A_{280})

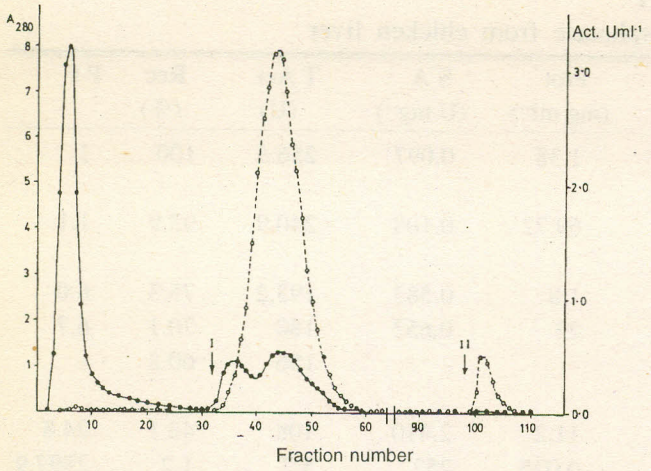


Fig 2. Purification of high Mr acid phosphatase by use of the Sepharose 4B-L tartramic acid amide column.
I arrow indicates elution with buffer containing 60mM NaCl.
II arrow indicates elution with buffer containing 20 mM NaH_2PO_4.
Fractions (4 ml) were collected. Activity(0——0, U ml^{-1}) and protein content (•—•, A_{280})

Homogeneity of the enzyme was checked on 12% SDS-Polyacrylamide gel electrophoresis and only a single band was detected by coomassie blue stain which corresponds to molecular weight of 50 k. Da. (Fig 3). The molecular weight of native enzyme obtained by gel filtration on Sephadex G-100 was estimated 98 k. Da (Fig 4). This indicates that the enzyme is a dimer with subunit molecular weight of 50 k. Da. These molecular weights are in good agreement with all prostatic acid phosphatases (Van Etten and Saini 1978; Ostanin *et al* 1994), acid phosphatase of *Drosophila virilis* (Narise 1984) and that of rat and human liver (Saini and Van Etten 1978a). These are all glycoproteins, composed of identical or very similar sub units of molecular weight of 50 k.Da.

Substrate specificity study was carried out on a broad spectrum of potential substrates. The results are shown in Table 2. High Mr acid phosphatase enzyme hydrolysed aryl phosphates much more efficiently rather than aliphatic phosphomonoesters (except β-phosphoglycerate).The enzyme hydrolysed *p*-nitrophenyl phosphate, phenyl phosphate, 4-tri-fluoromethyl phosphate, 4-ethyl phenyl phosphate, α-naphthyl phosphate and *O*-phospho L-tyrosine at significant rates. Marked activity was also observed with β-glycerophosphate, D-glucose-6-phosphate and flavin mono nucleotide. Other substrates e.g. 3-phosphoglyceric acid, tri-polyphosphate and choline phosphate are hydrolysed but at slower rates. No activity towards tetra-sodium pyrophosphate, O-phosphoserine, O-phosphothreonine and diphenyl phosphate was detected.

This nonspecificity of high Mr acid phosphatase is similar to HMW acid phosphatases from rat liver (Igarashi and

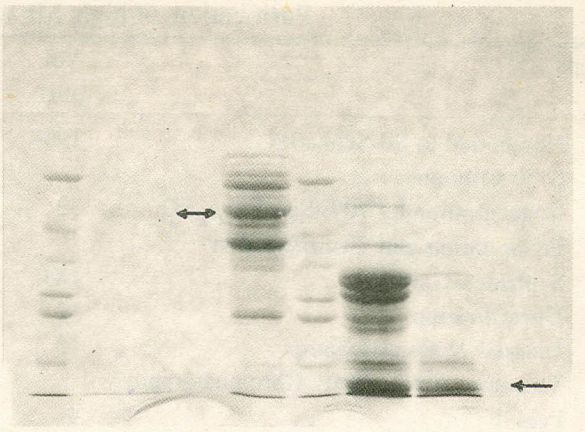


Fig 3. SDS- polyacrymide gel electrophoresis of high Mr acid phosphatase.
Lane1. The standard proteins used were Albumin bovine (Mr = 66 k.Da.) (Albumin egg(Mr = 45 k.Da.); Glyceraldehyde 3 phosphate (Mr = 36 k.Da.), Carbonic anhydrase (Mr =29 k.Da.) Trypsinogen (Mr = 24 k.Da); Trypsin inhibitor (Mr =20 k.Da.); Lactalbumin (Mr =14 k.Da.).
Lane 2. High Mr acid phosphatase from peak II of tartramic affinity column. (Mr =50 k.Da).
Lane 3. High Mr acid phosphatase activity (Peak 1) of tartrate affinity column shows various bands of protein impurities. Arrow with double head indicates the position of bands of high Mr acid phosphatase (Mr = 50 k.Da) in Lane 3 and 2.
Lane 4. Standard protein as in Lane 1.
Lane 5. After SP-Sephadex C-50 chromatography showing sample devoid of high Mr acid phosphatase but containing sharp band of low Mr acid phosphatase corresponding to Mr =18 k.Da.
Lane 6. Low Mr acid phosphatase band (Mr = 18 k.Da.) after SP-Sephadex C-50 followed by Sephadex G-75 chromatography as indicated by arrow.
Note:- Lanes 4,5 and 6 are related to low Mr acid phosphatase purification (unpublished results).

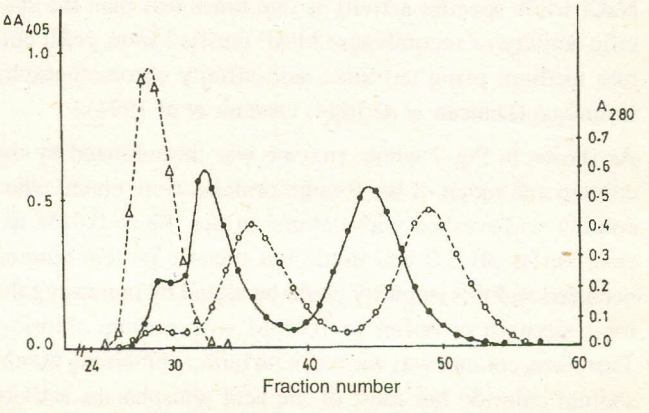


Fig 4. Elution pattern of standard proteins on Sephadex G -100 Column(1.7 x 80 cm) was equilibrated and eluted with0.01M acetate buffer pH 5.0, at flow rate of 20 ml h^{-1} and 2 ml fractions were collected. Elution volume (Ve) was determined by the absorbance at 260 nm for standard proteins or by assay of acid phosphatase activity at 405 nm. Albumin bovine (Mr = 66k. Da.) Ve. 65 ml (•—•) ; Albumin egg (Mr = 45 k.Da.) Ve.72 ml (O—O); Trypsin inhibitor (Mr = 20.1k.Da.)Ve.89 ml (•—•); Cytochrome (Mr =12.6 k.Da.) Ve. 96 ml (O——O). High molecular weight acid phosphatase, Ve.57 ml (Δ——Δ).

Table 2
Substrate specificity of homogeneous high molecular weight acid phosphatase

Substrates	% Activity
p- nitrophenyl phosphate	100
Phenyl phosphate	80
Flavin mono nucleotide	50
β -glycero phosphate	51
D -glucose -6 -phosphate	37
3 -phosphoglyceric acid	10
Tetra sodium pyrophosphate	4
Tripolyphosphate	12
4 -trifluoromethyl phenyl phosphate	71
4 -ethyl phenyl phosphate	93
4-phenyl butyl phosphate	61
α -naphthyl phosphate	80
O-phospho-L-serine	3
O-phospho-L-threonine	0
O-phospho-tyrosine	82
Choline-*O*-phosphate	15
Diphenyl phosphate	0
Bis (*p*-nitrophenyl)phosphate	0

Activity was determined in the presence of 4 mM substrate under condition mentioned in experimental procedure.

Table 3
K_m and V_{max} values for high Mr acid phosphatase at pH 5.0

Substrate	K_m(mM)	V_{max} ($Umin^{-1}mg^{-1}$ protein)
p-nitrophenyl phosphate	0.08	275
Phenyl phosphate	0.12	250

Table 4
Effect of inhibitors on the high Mr acid phosphatase activity

Competitive inhibitors	Ki (M)
Na_3PO_4	2.1×10^{-4}
Sodium potassium tartrate	7.4×10^{-7}
$Na_3 VO_4$	1.5×10^{-7}
$Na_2 MoO_4$	3.0×10^{-8}

Hollander 1968), rabbit kidney (Helwig *et al* 1978), bovine kindney (Fujimoto *et al* 1984) and human liver (Saini and Van Etten 1978b).

K_m and V_{max} values of high Mr acid phosphatase were determined graphically from Lineweaver-Burk plots under standard assay conditions and are reported in Table 3. K_m and V_{max} against *p*-nitrophenyl phosphate and phenyl phosphate were found to be almost the same suggesting good substrates for this enzyme.

Inorganic phosphate, tartrate, vanadate and molybdate inhibited the enzyme strongly. All inhibitors were purely competitive their Ki values are reported in Table 4. Phosphate proved less powerful inhibitor than tartrate, k vanadate and molybdate.

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