High Molecular Weight Acid Phosphatase of Chicken Liver: Separation and Characterization

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Summary: High molecular weight acid phosphatase was purified from chicken liver with a specific activity of 21 U/ mg and a recovery of 1 %. Purification was achieved 550 fold. The enzyme was seemed to be homogeneous on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight was estimated to be 100 killo daltons (kDa) by gel filtration on Sephadex G-100 and 50 kDa by SDS-PAGE. K_m against *para*-nitrophenyl phosphate and phenyl phosphate at pH 5.5 were found to be 0.11mM and 0.15mM respectively, while V_{max} for these two substrates were 22.9 $Umin^{-1}mg^{-1}$ and 19.7 $Umin^{-1}mg^{-1}$ of protein respectively. The enzyme was strongly inhibited by fluoride, tartrate, phosphate, vanadate and molybdate and were found competitive inhibitors. *Para*-nitrophenyl phosphate, phenyl phosphate and α-naphthyl phosphate were found good substrates. Other substrates were also hydrolyzed but at slower rates. No activation was observed with purine basis, glycerol or methanol etc. and thus not possessing phosphotransferase activity..

Introduction

Acid phosphatases (3.1.3.2) are a group of genetically distinct enzymes capable of hydrolyzing phosphate esters in acid medium [1]. Four forms of acid phosphatases exist at the structural level of genes, the erythrocytic form, lysosomal, prostatic and macrophagic form [2] which are expressed in the cells to different extent. The erytrocytic and macrophagic forms are distinguished from the prostatic and lysosomal enzymes in resisting inhibition by L-tartrate.

In vertebrates, three large classes of acid phosphatase, differing in molecular weight have been reported [3 - 4]. A high molecular weight (Mr ≥ 100,000) acid phosphatases are localized in lysosomes and microsomes[5 - 10], intermediate molecular weight (Mr=35,000-50,000) enzymes are localized in mitochondria of some mammalian tissues[11 - 14] and low molecular weight (Mr ≤ 20,000) enzymes are localized in the cytosolic fraction of the cell [15 - 22]. In addition to differences in molecular size and localization within the cells, high and low molecular weight forms also differ with respect to substrate specificity [15 – 16, 20] and sensitivity to inhibitiors [23-28,5-6, 9] .

None of these enzymes need metal ions for their activity. An other class of acid phosphatases called zinc-dependent acid phosphatases, requires zinc ion for their activity. This also exists in two forms, differing in molecular weight [29] which have been isolated and characterized.

In human liver, lysosomal high molecular weight acid phosphatase (Mr = 93000) form was isolated and characterized [8]. The enzymes from livers of carp, catfish and frog have also been purified [30-32] to focus on elucidating the glycoprotein nature.

In this paper we attempted to purify high molecular weight acid phosphatase from chicken's liver using a different procedure to that reported for chicken liver [33] to study its molecular and kinetic properties.

Results and Discussion

The enzyme was relatively stable at room temperature and activity decreased after 10-15 h but the enzyme remained active if stored at low temperature ranging form 0-4 °C for 1-2 weeks at pH 5-6. The enzyme had optimum pH 4.5-5.5 and optimum temperature 50 °C. Thermal stability showed that enzyme was not inactivated at 40°C for 1h but inactivated is some time dependent manner

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with a half life of 15 min at 50 °C and of about 2 min at 60 °C. At 70 °C, the activity was lost completely. The inactivation was slightly variable depending upon the enzyme at different stages of its purification.

Elution profile of acid phosphatases (chicken's liver extract after ammonium sulphate fractionation) from Sephadex G-75 chromatography indicated the separation of high and low molecular weight acid phosphatases in the ratio of 30 : 70 % (Fig. 1). These results are more or less compatible with those obtained from other tissues such as bovine liver and stomach [25] and bovine spleen and porcine kidney [15]. Bovine liver, rat liver, rat kidney and other mammalian sources also contain intermediate molecular weight acid phosphatases in lesser than 10 % of total acid phosphatase activity [25]. In all tissues, low molecular weight acid phophatase is predominant of the three activities with exception of porcine kidney, rat liver and bovine spleen where high molecular weight acid phosphatase exists in larger quantites. Therefore, low molecular weight acid phosphatases from various sources have been purified and extensively characterized as compared to high molecular weight acid phosphatases.

The molecular weight of low and high molecule weight acid phosphatases were estimated on a calibrated Sephadex G-100 column (Fig. 2 and 3) and found to be 100 kDa for high molecular weight enzyme and 18 kDa for low molecular weight acid phosphatase. The extract also contain Zinc-dependent acid *para*-nitrophenyl phosphatase which had molecular weight 110 kDa different from high molecular weight acid phosphatase. These values are very much similar to those obtained in the liver of *Esox lucius* [29] but are in consistent with those of Panara [34] who isolated two acid phosphatase forms from chicken liver of 89 kDa and 21 kDa. Szalewicz et al.[33] also reported high and low molecular weight acid phosphatases from chicken liver to be of 102 kDa and 27 kDa respectively. Thus it is presumed that our 100 kDa enzyme is the same as the 102 kDa enzyme purified by Szalewicz et al [33]or 89 kDa enzyme reported by Panara [34]. We also believe that low molecular weight acid phosphatase has molecular weight of 18 kDa as similar enzyme exists in several other mammalian tissues from which it has been purified homogeneously [35 - 40]. It is not clear that these discrepancies might be due to methods of extraction used or other experimental conditions maintained such as pH, ionic strength and temperature etc.

High molecular weight acid phosphatase from chicken liver was purified to homogeneity by salting out the enzyme from the extract at 30-60 %

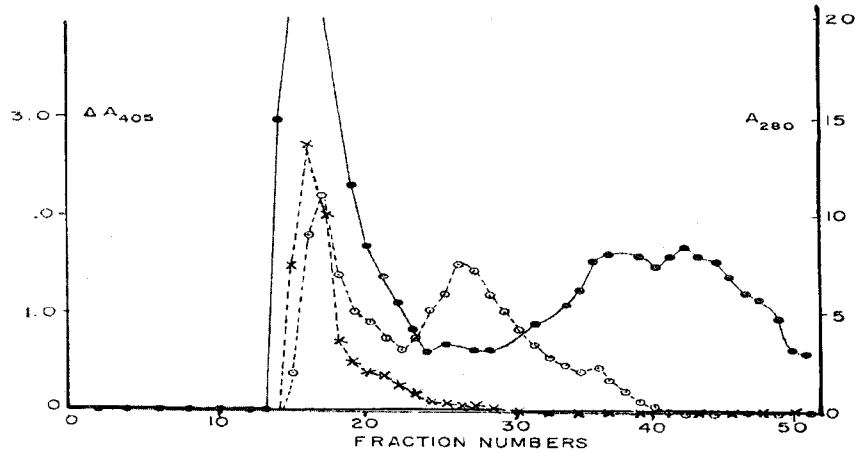

Fig. 1. Separation of high and low molecular weight acid phosphatases on Sephadex G-75. 10 ml sample was applied onto the column(1.8x85 cm) and eluted as for Sephadex G-100 column described in material and method.Acid phosphatase activity at 405 nm (o····o);Zn-dependent acid *para*-nitrophenyl phosphatase activity(x····x); Protein at 280nm (•—•)

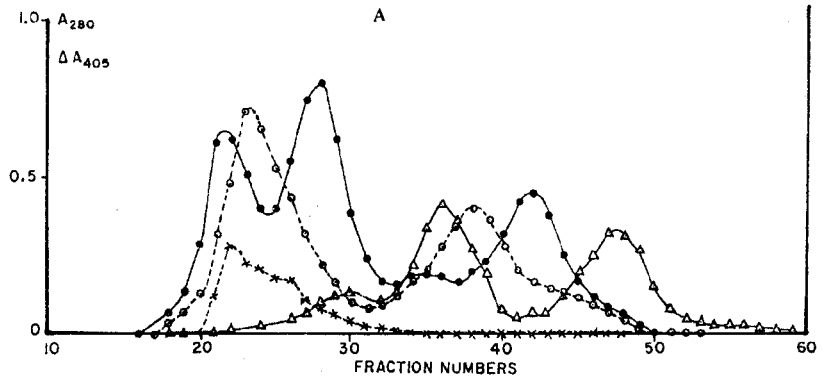

Fig. 2. Elution pattern of standard proteins on Sephadex G-100.
The column (1.8 x 85cm) was equilibrated and eluted with 0.01M acetate buffer pH 5 containing 0.1 M sodium chloride at flow rate of 20ml/h and 3.5ml fractions were collected;Vo 72ml, Blue dextran 2000:Bovine serum albumin (66,000),Ve 98ml (•—•); carbonic anhydrase (29,000), Ve 126ml(Δ....Δ) ;cytochrome C(12,400),Ve 148 ml(•—•);Aprotinin (6,500),Ve 165 ml(Δ....Δ);Zn-dependent Acid phosphatase,Ve 81ml(x....x); High molecular weight acid phosphatase,Ve 84ml (o····o); Low molecular weight acid phosphatase,Ve 134ml(o····o)

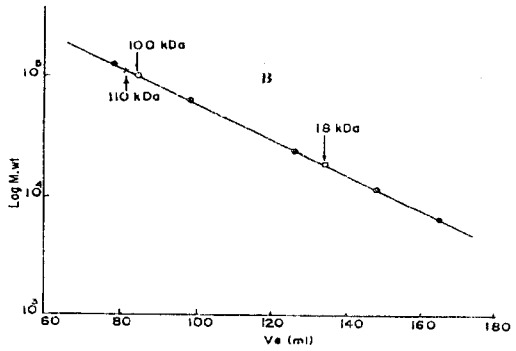

Fig. 3. Linear graph of log M.wt. versus elution volumes of standard proteins.

of ammonium sulphate saturation , cation exchange chromatography on CM-cellulose, gel filtration on Sephadex G-100 followed by Reactive Blue 4-Agarose column chromatography. Their elution profiles are shown in Figure. 4 and 6 and Table- 1 reports a summary of the purification procedure. The enzyme had specific activity 21U/ fc mg of protein. The value is more or less similar to that reported for chicken liver by Szalewicz et al. [33]. SDS-PAGE is shown in Figure. 7 Single protein band corresponding to a molecular weight of 50 kDa was obtained. The molecular weight of native enzyme obtained by gel filtration on Sephadex G-100 was estimated 100 kDa indicating that enzyme is a dimmer with subunit of moleculalr weight of 50

Table- 1. Purification of High Molecular weight acid phosphatase from chicken liver

	Volume (ml)	Total Activity (U)	Total Protein[b] (mg)	Specific Activity (U/ mg)	Purification Factor	Recovery %
Extract[1]	270	361.8	9720	0.037	1	100
30 % $(NH_4)_2SO_4$	278	264.1	8618	0.030	1	73
60 % $(NH_4)_2SO_4$	83	181.8	5312	0.034	1	50.2
Dialysis and Centrifugation	69	138	1966.5	0.07	1.89	38.1
CM-Cellulose	12	13.8	63.6	0.22	5.86	3.8
Sephadex G-100	41	13.5	11.22	1.20	32.43	3.7
Reactive blue-Agarose	9	3.7	0.18	20.55	555.4	1.0

[1]Starting from 65g of chicken liver [b]Protein concentration was determined by Biuret method.

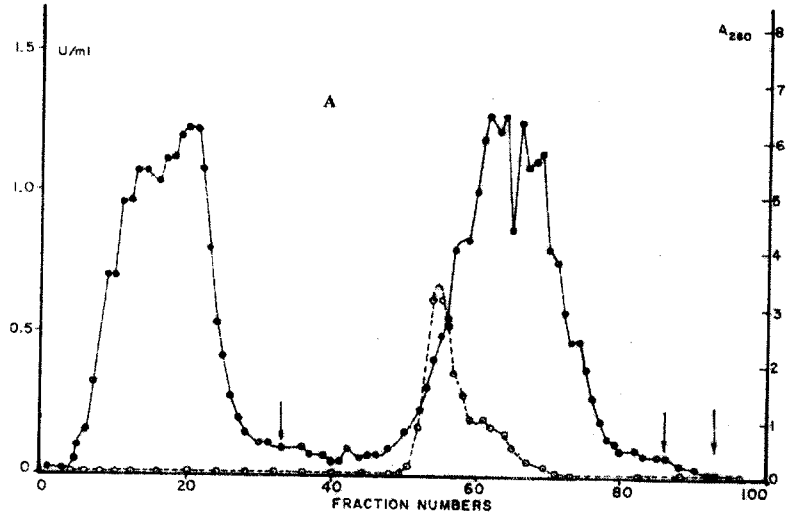

Fig. 4. Elution profile of high molecular weight acid phosphatase from CM-Cellulose column. Fracitions of 5 ml were collected at flow rate of 80/ ml. Arrow indicates the starting point of linear gradient.

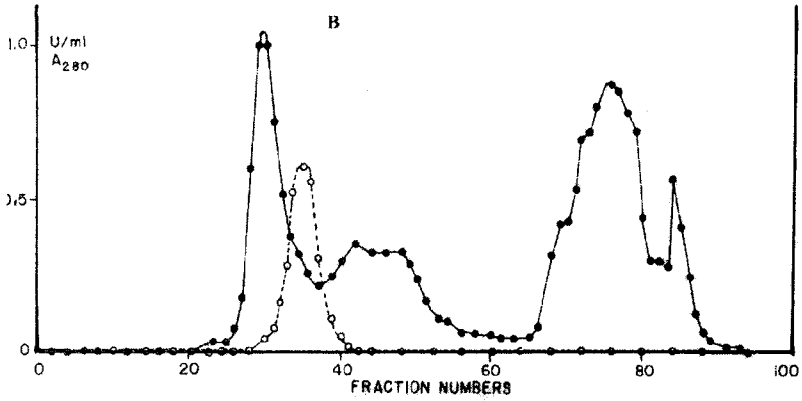

Fig. 5. Elution profile from Sephadex G-100 column(2.8X85 cm);Flow rate,30ml; 4 ml fractions were collected.

kDa. Similar results were obtained with prostatic acid phosphatases [41-42] and rat and human liver enzymes [43]. K_m and V_{max} values of high molecular weight were determined graphically from Lineweaver-Burk plots under standard assay conditions. K_m and V_{max} for *para*-nitrophenyl phosphate were found to be 0.11 mM and 22.9 U $min^{-1}mg^{-1}$ protein respectively, while for phenyl phosphate, the value amounted to 0.15 mM and 19.7 U $min^{-1}mg^{-1}$ protein. V_{max} values were found to be almost same for both substrates suggesting good substrates for this enzyme.

Activity of high molecular weight acid phosphatase towards number of phosphate monoester was investigated. The enzyme hydrolyzed *para*-nitrophenyl phosphate, phenyl phosphate and α-napthyl phosphate at significatant rate. Marked activity was observed with β-glycerophosphate, flavin mononucleotide (FMN), adenosine monophosphate (AMP) and phosphotyrosine. Other substrates e.g. α-glycerophosphate, hexose phosphates and ATP were also hydrolyzed but at slower rates. No activity towards phosphoserine, phosphothreonine and ADP was detected (Table- 2). Thus the enzyme showed broad substrate specificity and it hydrolyzed aryl phosphates much more efficiently than aliphatic phosphates (except β-glycerophosphate). This nonspecificity of enzyme

is similar to high molecular weight acid phosphatases from rat liver [6], rabbit kidney [9], bovine kidney [25] and human liver [8]. On the other hand low molecular weight acid phosphatases (14-18 kDa enzyme) have very restricted substrate specificity in that these efficiently hydrolyse p.nitrophenyl phosphate and flavin mononucleotide only [7].These two enzymes can also be distinguished from each other by their rates of hydrolysis of α-napthyl phosphate and β-glycerophosphate. High molecular weight acid phosphatases hydrolyse these two substrates much more effieiently whereas low molecular weight acid phosphatases hydrolyse these at negligible rates.

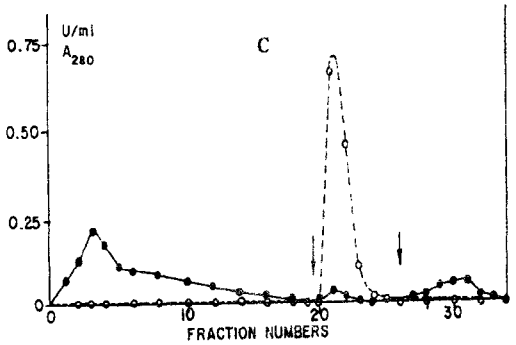

Fig. 6. Elution profile from Reactive Blue 4-Agarose column (1 x 8 cm);Flow rate,9ml; 3 ml fractions were collected. Arrows indicate the point of application of salt elutions with. 0.25M and 0.5M NaCl in same buffer respectively. Acid phosphatase activity U/ml (o····o); Protein at 280nm (•—•).

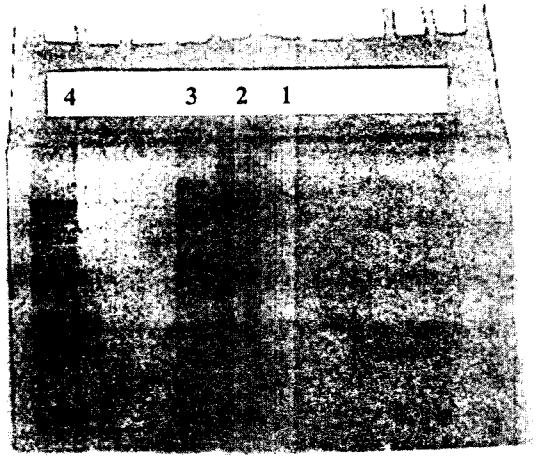

Fig. 7. SDS-Polyacrylamide gel electrophoresis of high molecular weight acid phosphatase from chicken liver. Lane 1 – 3µl of enzyme. Lane 2 - 20 µl of enzyme. Lane 3- The standard proteins used from top to bottom were albumin bovine (66,000 Da), oval albumin (45,000 Da), carbonic anhydrase (29,000 Da), trypsin inhibitor (20,000 Da), lactalbumin (14,200 Da). Lane 4- 10µl of enzyme.

Table- 2. Substrate specificity of High Molecular weight acid phosphatase from chicken liver

Substrate	Activity %
p-Nitrophenyl phosphate	100
Phenyl phosphate	78.3
Flavin mononucleotide	42
L-Naphthyl phosphate	85
L-Glycero phosphate	15
B-Glycero phosphate	59
Phosphotyrosine	25
Phosphoserine	3.2
Phosphothreonine	3.8
Glucose-1-phosphate	10
Glucose-6-phosphate	14
ATP	13
ADP	2
AMP	45

The incubation mixture consisted of 0.1 M acetate buffer pH 5.5, 4 mM 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 of 10% TCA and liberated inorganic phosphate was estimated by Black and Jones method. The enzyme activity was expressed as a percent of that of the same enzyme towards para-nitrophenyl phosphate as 100.

Table- 3 summarizes the effect of various compounds on the acid phosphatase activity. High molecular weight acid phosphatase was strongly inhibited by fluoride and tartrate and insensitive to formaldehyde and parahydroxymercuribenzoate, thus distinguishing from low molecular weight acid phosphatases [19-20] where these compounds behave oppositely. Phosphate, vanadate and molybdate were found to be good inhibitors but their inhibitory action was more pronounced than in low molecular weight acid phosphatases. Phosphate

Table 3. Effect of various Inhibitors on the High Molecular weight acid phosphatase

Inhibitor	Concentration	Activity %
None	-	100
Fluoride	10mM	3
Tartrate	10 mM	5
Phosphate	1 mM	42
	2 mM	20
	5 mM	12
Vanadate	0.1 mM	3
Molybdate	0.1 mM	4
Formaldehyde	0.2 %	94.5
p-Hydroxymecuri-benzoate	0.01 mM	87
Triton X-100	1 %	89
EDTA	10 mM	96
Adenine	1 mM	101
Guanine	1 mM	82.8
Adenosine	1 mM	99
6-ethylmercaptopurine	1 mM	109
	1 mM	96
	10 %	100
cGMP	10 %	97
Glycerol	10 %	86
Methanol	10 %	91
Ethanol	5 mM	40
Acetone	5 mM	5
$ZnCl_2$	5 mM	0
$CuSO_4$	5 mM	0
Hg_2Cl_2		
$AgNO_3$		

The activity was determined by incubating the enzyme in a final mixture volume of 1 ml containing 4 mM p-nitrophenyl phosphate, 0.1 M acetate buffer pH 5.5 in the presence and absence of the indicated additions. After a 5 minute incubation at 37 °C, the reaction was stopped by the addition of 1 ml of 0.1N KOH and the absorbance at 405 nm was measured. The control activity without inhibitor was taken as 100 % and the other activities were expressed as a percentage of this activity. Values are the means of triplicate determination.

showed less powerful inhibitor than vanadata and molybdate. Millimolar concentrations was needed to inhibit the enzyme whereas micromolar concentration range was required to abolish the activity. These results are in accord with the finding of human liver high molecular weight acid phosphatase [8] and chicken liver enzyme [34]. There results are confirmed by their inhibition constant (K_i) of these inhibitors which are presented in Table- 4. Metal ions such Ag^+, Cu^{++}, Zn^{++} and Hg^{++} deactivated the enzyme while EDTA and Triton X-100 had little or no effect on the enzyme activity. No activation was observed with purine bases or its derivatives such as adenosine, 6-ethyl mercaptopurine and cGMP etc. and no activation was found with glycerol, methanol, ethanol and acetone for high molecular weight acid phosphatase and thus not possessing phosphotransferase activity.

Table- 4. Ki values of inhibitors of high molecular weight acid phosphatase

Inhibtor	Mode of inhibition	Ki
Phosphate	Competitive	0.35 mM
L(+)Tartrate	Competitive	0.69 μM
Vanadate	Competitive	19 μM
Molybdate	Competitive	10 μM
Fluoride	Competitive	0.47 mM

Straight lines were drawn by applying Least Square Rule. Each point was the average of at least three readings.

Experimental

Materials

Chicken liver were obtained from a local poultry shop. *Para*-nitrophenyl phosphate and phenyl phosphate was purchased from Merck. Bovine serum albumin, SDS molecular weight markers, Sephadex G-75 , Sephadex G-100 and Reactive Blue 4-Agarose were supplied by Sigma Chemical Co.; The materials for SDS-polyacrylamide gel electrophoresis were from Bio-Rad and Acros Chemical Co.; CM-Cellulose was obtained from Whatman Biosystem Ltd All other reagents were of the highest purity commercially available.

Methods

Acid phosphatase activity was measured as described by [44] using 4 mM *para*-nitrophenyl phosphate in 0.1M acetate buffer pH 5.5 in a final volume of 1 ml. the reaction was stopped after 5 minute incubation at 37°C by the addition of 4 ml of 0.1 N KOH and the absorbance was measured at 405 nm. Zinc-dependent acid *para*-nitrophenyl phosphatase activity was determined as described by Panara [29] using 4 mM *para*-nitrophenyl phosphate as substrate in 125 mM acetate buffer pH 6.0 containing 5 mM $ZnCl_2$ and 10 mM NaF as an inhibitor of high molecular weight acid phosphatase in order to exclude the activity of high molecular weight acid phosphatase. One unit of enzyme is defined as the amount of the enzyme that produces 1μ mol of *para*-nitrophenol/ min. Specific activity was expressed as enzyme units per mg of total protein.

The activity against other phosphate monoesters was determined by estimation of inorganic phosphate. The liberated phosphate was determined according to Black and Jones method [45] Km values were determined by measuring the initial rates of reactions at different concentrations of substrate ranging from 0.1 mM to 1.6 mM in the absence and presence of one or two fixed concentration of inhibitors. Lineweaver-Burk plots were used to determine the value of Ki.

Optimum temperature for enzyme catalysed reaction was determined in range of 0-70 °C at intervals of 10 °C by incubating the enzyme in the presence of acetate buffer pH 5.0 for 5 min.

Thermal stability was studied by incubating the enzyme solution at temperatures ranging from 40 to 70 °C in the presence of 0.1M acetate buffer pH 5.0 for various intervals of time. The reaction was stopped by immersion in the ice-cold bath and residual activity was assayed as usual.

Optimal pH was determined in 0.1M acetate buffer (pH 4.0-6.0), 0.1M barbital buffer (pH 6.0-6.7) and 0.1M Tris-HCl buffer (pH 7.0-8.0).

Protein concentration was determined by the Biuret method [46]. For column effluents, the relative protein concentration was estimated from absorbance at 280nm.

SDS-PAGE was carried out on 12.5 % polyacrylamide gel by method of Laemmli [47]. Proteins in gel were stained with coomassive blue.

Apparent molecular weights of acid phosphatases were estimated on a calibrated Sephadex G-100 column (1.8 x 85cm) by comparison of their elution volumes to those of standard proteins. Chicken liver extract was salted out with ammonium sulphate (30-60 % saturation) and placed on Sephadex G-100 column which was previously equilibrated and eluted with 0.01 M acetate buffer pH 5.5 containing 0.1 M NaCl at flow rate of 20 ml/ h. Fractions of 3.5ml were collected for protein and enzyme activity.

Enzyme Purification

All operations were carried out at 4°C. High molecular weight acid phosphatase from chicken's livers was purified as follows.

Step- 1. Fresh chicken livers were washed with cold 10 mM Tris-HCl buffer pH 7.4 containing 0.25 M sucrose and 1 mM ethylenediamine tetraacetate (EDTA) and were cut into 1-2 cm pieces. Then, it was homogenized in a Waring belender with 3 volumes of 0.3M sodium acetate buffer pH 5.0 containing 1 mM EDTA, 1 mM β-mercaptoethanol and 0.1mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was stirred for 2-3 h at 4 °C and centrifuged at 5000xg for 30 min. The supernatant was collected and the pellet was discarded.

Step- 2. Solid ammonium sulphate was added to the supernatant to give 30 % saturation. After stirring gently, it was centrifuged at 5000xg for 30 min. The supernatant was brought to 60 % saturation with solid ammonium sulphate. The resulting mixture was allowed to stir for 1-2 h and then centrifuged at 5000xg for 1h. The precipitate, thus obtained was dissolved in a reasonable volume of 0.01 M acetate buffer pH 6.0 containing 1 mM EDTA, 1 mM β-mercaptoethanol and 0.1 mM PMSF. The suspension was stirred for 1-2 h to extract the enzyme and again centrifuged at 10,000xg for 1h. The clear supernatant was dialysed against 5L of same buffer with 2-3 replacements of fresh buffer. The dialysate was centrifuged at 10,000xg to get clear supernatant.

Step- 3. The clear supernatant solution was applied to a CM-Cellulose column (2 x 28cm) which was previously equilibrated with dialyzing buffer. The column was washed with same buffer until absorbance at 280 nm was less than 0.1.The bound acid phosphatase activity was eluted by 0-0.5 M NaCl linear gradient in same buffer (total volume of 300ml). The most active fractions were pooled and the enzyme was precipitated by adding ammonium sulphate to 70 % saturation. The precipitate was collected by centrifugation at 10,000xg for 1h and dissolved in about 10 ml of 0.01M acetate buffer pH 5. 0 containing 1mM EDTA, 0.1mM PMSF and 1 mM β-mercaptoethanol.

Step- 4. A Sephadex G-100 column (2.8 x 85cm) was packed and equilibrated with 0.01 M acetate buffer pH 5.0 containing same additives and 0.1M NaCl. 12ml sample was applied to a column and eluted with equilibration buffer. The active fractions were pooled and concentrated to 10 ml by ultrafiltration using YM3 membrane at 2-3 atmospheric pressure of nitrogen gas.

Step- 5. The enzyme after gel filtration was dialyzed against 1L of 0.1 M acetate buffer pH 5.0 containing 1 mM EDTA and 1 mM β-mercaptoethanol. The sample was applied to Reactive Blue 4-Agarose column (1 x 8cm) equilibrated with same buffer. The column was washed with buffer to eliminate unbound proteins. The pure enzyme was eluted by 0.25 M NaCl in the same buffer (single step). The active fractions were pooled and used for further analysis.

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