Zn^{++}- Dependent Acid Phosphatase from Chicken's Liver:- Isolation, Purification and Biochemical Properties

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Summary: Zn^{++}-dependent acid phosphatase from chicken's liver was purified to almost homogeneity by heat treatment at 60°C, cation exchange chromatography on CM-Cellulose at pH 5.5, gel filtration on Sephadex G-150 and affinity chromatography on Sepharose-4B tartramic acid affinity gel with specific activity of 4U/mg and recovery of 8%. Purification achieved was 50 fold. The purified enzyme migrated as single band. The enzyme has molecular weight of 100,000 and is a dimer with two apparent identical subunits of 48,000 – 50,000 as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The enzyme requires Zn^{++} for catalytic activity. The maximum enzyme activity was obtained in the presence of 5 – 8 mM $ZnCl_2$ at pH 6.0. Higher concentrations of Zn^{++} were found to be inhibitory. As compared to Zn^{++} activation, Mn^{++} and Co^{++} showed 32% and 16% activation respectively. The enzyme had optimum pH 5.5 – 7.5, optimum temperature 55°C and was stable at 40°C. The enzyme showed restricted specificity. p-nitrophenylphosphate was found to be a better substrate while phenylphosphate, ß-glycerophosphate and FMN were also hydrolyzed at a significant rate. Other substrates like phosphoserine, ATP and phosphoenol pyruvate etc. were not hydrolyzed. NaF, a strong inhibitor of high molecular weight acid phosphatase, had no effect on Zn^{++}- dependent acid phosphatase activity. However, the enzyme was inhibited by phosphate, tartrate, EDTA and ATP. The Km value against p-nitrophenylphosphate as a substrate, was found to be 1mM at pH 6.0. Phosphate and tartrate were competitive inhibitors with Ki 0.5mM and 6mM respectively. ATP was noncompetitive inhibitor (Ki = 2.3mM) while EDTA was found to be uncompetitive inhibitor (Ki = 2.5mM).

Introduction

There is a particular class of acid phosphatase, Zn^{++}-dependent aicd phosphatase (Zn^{++}-AcPase) which requires Zn^{++} to hydrolyze the substrate p-nitrophenyl phosphate (p-NPP) in an acid environment, has been detected in several animal tissues and species [1-10]. This exists in two major forms differing in molecular weight and tissues distribution .[11]. High molecular weight (HMW) Zn^{++}-AcPase has a molecular weight of about 100 kDa. and has been found in liver [4-9] and kidney while low molecular weight (LMW) Zn^{++}-AcPase is of about 57-62 kDa. and has been found in brain, heart, skeletal muscles, erythrocytes, lungs, spleen and stomach [7-9]. Small intestine contains significant quantities of both forms [11-13].

LMW enzymes have been purified from bovine brain. The molecular weight was about 62 kDa. by gel filtration and 31 kDa. by SDS-PAGE and was found to be a dimeric protein. Panara and Angiolillo has purified HMW - Zn^{++}AcPase from liver of bovine [4] and human [8]. Tsuda *et al.*, reported that bovine liver contains a LMW - Zn^{++}-AcPase as a minor component in addition to HMW - Zn^{++}-AcPase and purified two forms of Zn^{++}AcPase [13]. The apparent molecular weight of these two forms were estimated to be about 100 kDa. and 62 kDa. The LMW- Zn^{++}-AcPase catalyzed the hydrolysis of *myo*-inositol-1-phosphate in the presence of Mg^{++} at pH 7.4 but HMW- Zn^{++}-AcPase did not [13]. Further, LMW- Zn^{++}-AcPase was recognized by polyclonal antibodies developed against Zn^{++}-AcPase of bovine brain but HMW-Zn^{++}-AcPase was not recognized.

This paper describes the new procedure for the purification of HMW- Zn^{++}-AcPase from chicken's liver and was characterized with respect to molecular

The authors feel great pleasure to dedicate this research paper to Dr. Atta-ur-Rahman. He is a well renowned scientist of Pakistan. He developed many programmes in strengthening of Science both at University level and in R&D organizations. He led the nation in particular direction in the field of science and proved that our nation cannot develop without I.T and biotechnology. He gave very much initiatives to the scientists to adopt goal oriented research. Dr. Atta-ur-Rahman has contributed greatly to the science particularly in the field of Natural Products. For this reason, we dedicate this research paper to Prof. Dr. Atta-ur-Rahman on his 60th birthday.
Prof. Ahmad Saeed

weight, matal ions dependence, inhibition, pH optima, temperature sensitivity, substrate specificity and kinetic properties.

Results and Discussion

Enzyme Purification

HMW-Zn^{++}-AcPase activity is mainly associated with soluble fraction of liver of frog, carp and rat in which 80 –90 % activity is present [6].

Very simple purification procedure was developed in which affinity chromatography step was found to be critical for enzyme purification. Purification procedure consists of heat treatment at 60°C followed by sequential chromatography on CM-Cellulose, Sephadex G-150 and affinity chromatography on Sepharose 4B-tartaric acid amide gel. A summary of purification steps from 50gm of chicken's liver is reported in Table-1.

Zn^{++}-AcPase is tartrate inhibitable enzyme like rat and human liver acid phosphatase isoenzymes

Table 1: Final Purification Scheme for Zn^{++}-AcPase from 50gm of Chicken's liver

Steps	Vol. (ml)	Act. (U/ml)	T.Act. (U)	Prot. (mg/ml)	T.Prot. (mg)	S.A (U/mg)	P.F	Rec. %
Extract	120	1.60	192	18	2160	0.088	1	100
Heat treatment	100	1.50	150	7.1	710	0.21	2.4	78
Dialysis and Centrifugation	100	1.75	175	7.2	720	0.243	2.7	91
CM-Cellulose Chromatography at pH 5.5	90	1.27	114.3	2.8	252	0.45	5.1	59.5
Conc. with 70% $(NH_4)_2SO_4$	20	3.25	65	7.6	152	0.42	4.8	33.8
Sephadex G-150	130	0.27	35.8	0.24	31.8	1.12	12.8	18.6
AffinityChromatography	Peak I							
	75	0.12	9.1	0.03	2.25	4.06	46.2	4.7
	Peak II							
	75	0.09	6.75	0.02	1.5	4.5	51.1	3.5

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

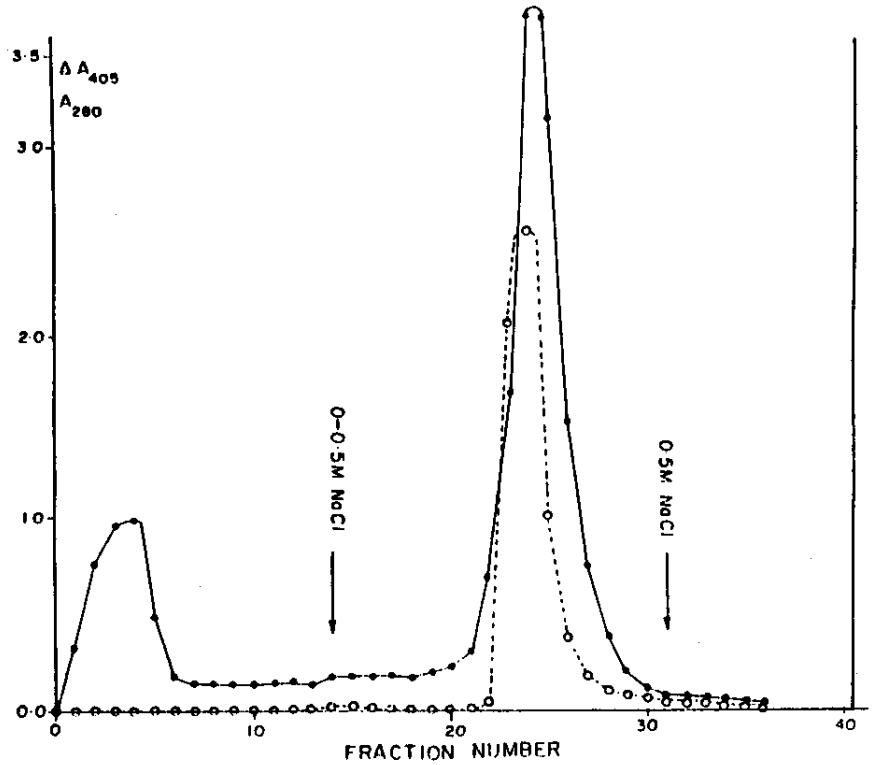

Fig.1. CM- cellulose chromatography.
100ml sample (175U) was applied, Column (3x10cm), flow rate 50ml/h, each fraction 20-22ml. Arrows indicate starting points of gradients. Ordinates: Protein at 280nm (•—•), activity at 405nm (o····o).

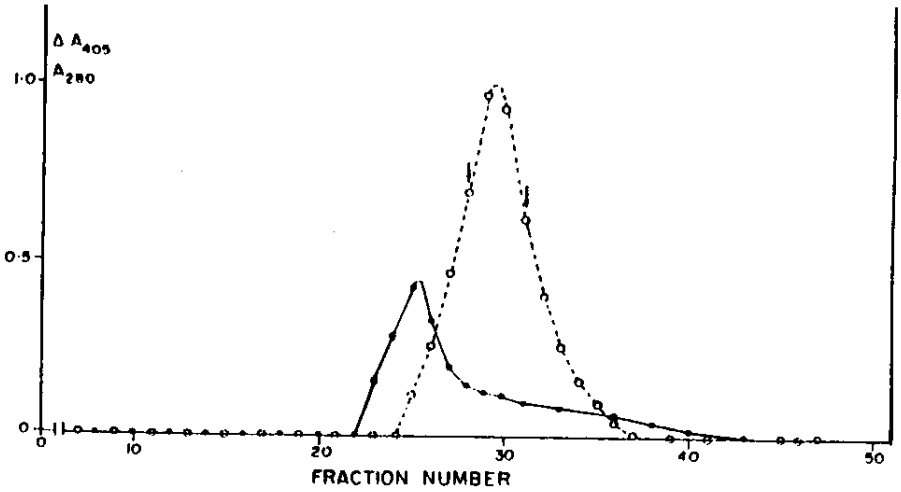

Fig.2. Gel chromatography on Sephadex G-150.
20ml sample (65U) was applied, column (1.8x70cm), flow rate 20-22 ml/h, each fraction 15ml. Ordinates: Protein at 280nm (•—•). activity at 405nm (o····o).

[14] which can be purified by affinity chromatography as prostate acid phosphatase was purified on tartramic acid affinity gel [15]. In this chromatography whole of enzyme was immobilized by column and much of impurities were eluted on washing the column. Non specific protein binding occurred and this impurity could be eluted by increasing the ionic strength to 60mM NaCl. In this non specific elution, Zn^{++}-AcPase activity (peak I) was eluted as shown in Fig. 3. Similar results were also reported in chicken's liver lysosomal acid phosphatase enzyme purification [16]. Specific elution with phosphate as a competitive inhibitor gave peak II enzyme (Fig. 3).

Peak I and II were purified to specific activity of 4U/mg each with overall recovery of 4%. 50 fold purification was achieved. S.A is too low as compared to other enzymes reported [4,8]. The homogeneity of both enzymes was checked on 12% SDS-PAGE. Peak II enzyme showed single band corresponding to 50kDa. while peak I enzyme showed major band corresponding to 48kDa. along with some other faint bands as impurities as shown in Fig.4. The molecular weight of native enzyme obtained by gel filtration on Sephadex G-100 was estimated 100 kDa. (Fig. 5). This enzyme indicates that enzyme is a dimer with subunit molecular weight of 50kDa. These molecular weights are in good agreement with bovine liver enzyme [13], frog liver enzyme [6] and human liver [8]. It seems to be an HMW Zn^{++}-AcPase type.

Enzyme Properties

The purified enzyme remains active for 1-2 weeks if stored at –4°C in 0.01M acetate buffer pH 5.1 containing 20mM NaH_2PO_4. The enzyme was found to be stable at 40°C and was inactivated at high temperature in a time dependent manner. It was not significantly inactivated at 50°C upto 30min. but inactivated with a half life of 40min. at 60°C and of about 10min. at 65°C. At 70°C enzyme was appeared to be completely inactivated after 10min. as shown in Fig.6.

HMW-Zn^{++}-AcPase exhibited optimal pH ranging from 5.5 – 7.5. Below pH 5.5 and above 7.5, the activity was declined gradually. Fig.7 shows that enzyme had optimum temperature 55°C.

HMW-Zn^{++}-AcPase showed no p-nitrophenyl phosphate hydrolyzing activity in the absence of metal ions; significant p-NPP hydrolysis was observed only in the presence of divalent cations such as Zn^{++}, Mn^{++} and Co^{++}. Zn^{++} was the most effective cation for activating the catalytic activity of enzyme. Analysis of effect of $ZnCl_2$ at various concentrations on p-NPP hydrolysis indicated that 5 – 8mM $ZnCl_2$ was necessary for maximal hydrolysis as shown in Fig. 8. However high concentrations of Zn^{++} ions were found to be inhibitory. Mn^{++} could substitute for Zn^{++} but gave only 32% activation. Similarly Co^{++} gave 16% activation. Mg^{++} and other divalent cations such as Ca^{++} and Ba^{++} etc. showed no

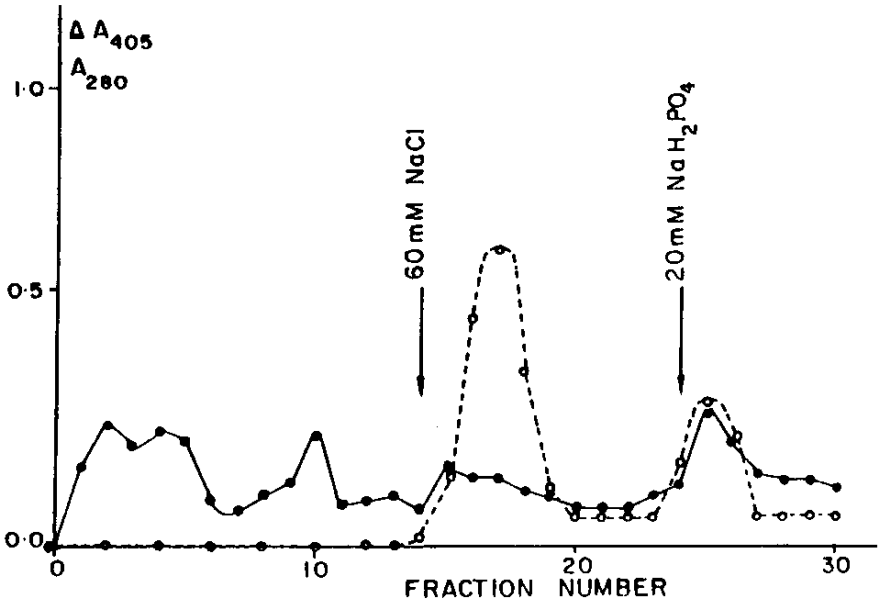

Fig.3. Affinity chromatography on Sepharose 4B- tartaric amide gel.
13ml sample (35U) was applied, column (1x7cm), flow rate 12-15ml/h, each fraction 10ml. Arrows indicate starting points of gradients. Ordinates: Protein at 280nm (•—•), activity at 405nm (o····o).

Fig.4. SDS-Polyacrylamide gel electrophoresis of high molecular weight Zn^{++}-dependent acid phosphatase.
Lane 1Standard proteins used: Albumin Bovine plasma(66,000), Albumin egg (45,000), Trypsinogen (24,000), β-lactoglobulin (18,400), Lysozyme (14,300).
Lane 2 High Mr Zn^{++} - dependent acid phosphatase (peak 1) of tartrate affinity column.
Lane 3 High Mr Zn^{++} - dependent acid phosphatase (peak 2) of tartrate affinity column.

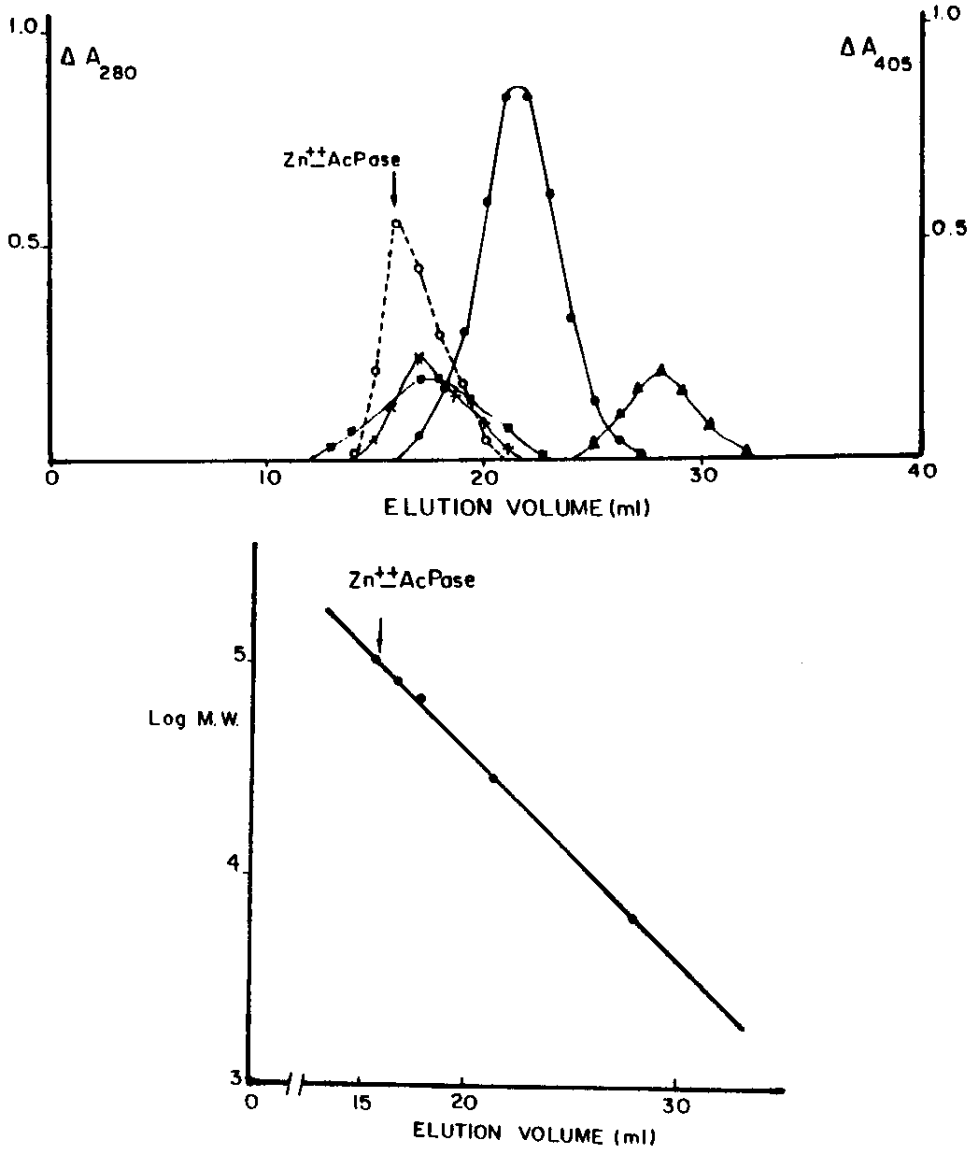

Fig.5 (a) Elution pattern of standard proteins on Sephadex G-100.
The column (60x0.9 cm) was equilibrated and eluted with 0.01M acetate buffer pH 6 containing .1 M sodium chloride at flow rate of 20ml/h and 1ml fractions were collected; Alcohol dehydrogenase (80,000), Ve 17.0ml (•—•); Albumin (66,000), Ve 18ml (•—•); carbonic anhydrase (29,000), Ve 21.5ml (•—•); Aprotinin (6,500), Ve 28.0ml (▾—▾); Zn^{++}dependent AcPase, Ve 16ml (o---o).
(b) Linear graph of log M.W. versus elution volumes of standard proteins.

effect. These results have good agreement with that of bovine liver [4], frog liver [6] and human liver [8].

Among physiological and non-physiological substrates tested, HMW-Zn^{++}-AcPase showed narrow range substrate specificity. Chicken's liver HMW-Zn^{++}-AcPase efficiently hydrolyzed p-NNP. Phenyl phosphate and β-glycerophosphate were hydrolyzed at significant rate while FMN, glucose-6-phosphate, AMP and other phosphorylated amino acids were hydrolyzed at slower rate (Table-2). Similar results were reported for HMW-Zn^{++}-AcPase of bovine liver [4].

Effect of various inhibitors on the activity of the enzyme was tested by adding NaF, tartrate,

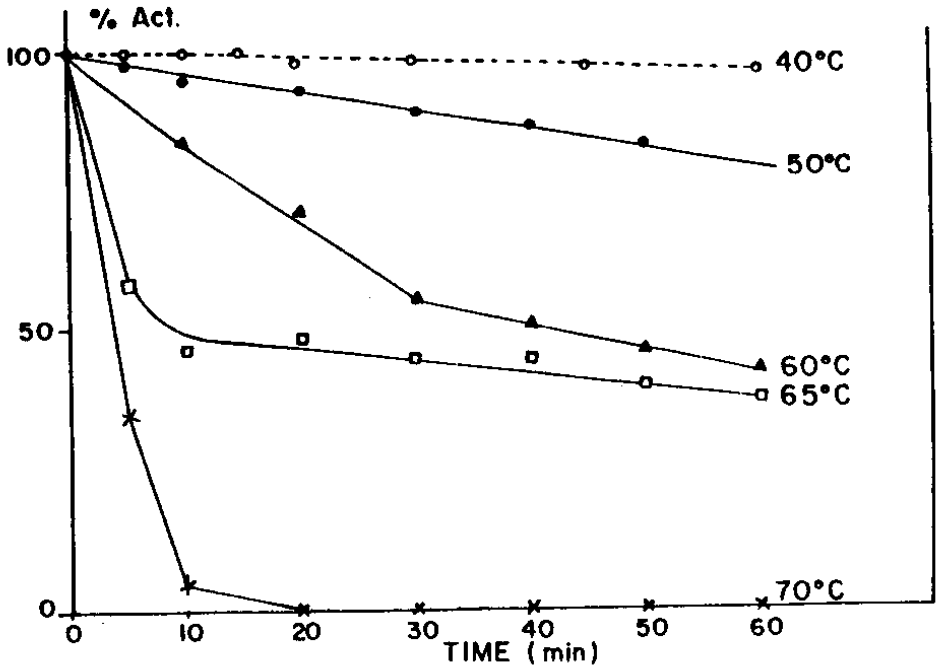

Fig.6 Temperature stability of Zn^{++}- dependent acid phosphatase activity as a function of time.

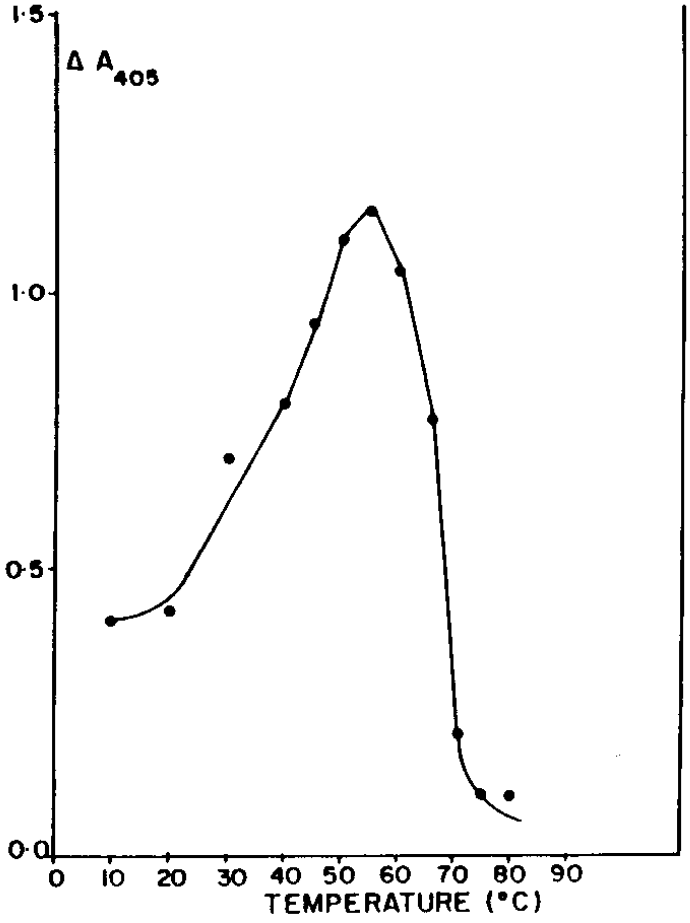

Fig.7 Optimum temperature of Zn^{++}- dependent acid phosphatase activity.

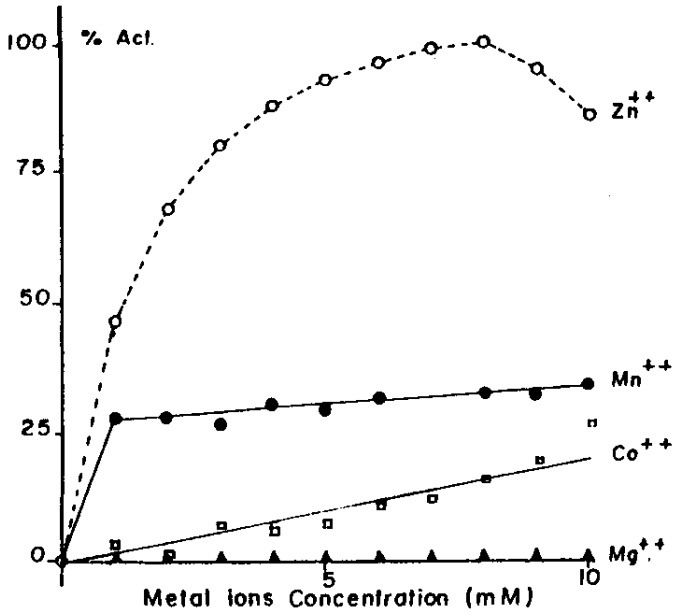

Fig.8 Influence of metal ions as activators on the hydrolysis of p-nitrophenyl phosphate. The activity was determined in the presence of metal ions at concentrations in abcissa. The maximum activity was expressed by taking the highest activity in any fraction as 100% and the other activities were expressed as a percentage of the maximum activity.

Table 2: Substrate Specificity of Zn^{++}-AcPase

Substrates	Activity (ΔA_{660})	% Activity
p-nitrophenyl phosphate	0.199	100
Phenyl phosphate	0.179	65
ß-glycero phosphate	0.183	67
FMN	0.113	32
Glucose-1-phosphate	0.197	25
Glucose-6-phosphate	0.079	15
Phosphoserine	0.085	18
Phospho threonine	0.049	0
AMP	0.073	12
ATP	0.051	1
α-glycero phosphate	0.063	7
Phosphoenol pyruvate	0.047	0

EDTA and Na_3PO_4 at concentrations ranging from 0-10 mM.

Fig. 9 indicates that EDTA was a strong inhibitor. At the concentration of 5 mM of EDTA, activity was zero while relative activity with tartrate and Na_3PO_4 was 60% and 27% respectively at the concentration being 5mM. Tartrate was found to be slight inhibitor while EDTA and Na_3PO_4 seemed strong inhibitors. NaF had no effect on Zn^{++}-AcPase activity which is the strong inhibitor of high molecular weight acid phosphatase.

Michaelis-Menten constant, Km for p-NPP as substrate determined at pH 6.0 was found to be 1mM.

Inhibitions produced by Na_3PO_4 and tartrate are shown in Fig. 10 and Fig. 11. The plots suggest competitive type of inhibitions. The inhibitor constants (Ki) for Na_3PO_4 and tartrate were found to be 0.5mM and 6mM respectively. Inhibition produced by ATP is shown in Fig. 12. Non competitive type of inhibition was observed with Ki 2.3 mM while inhibition produced by EDTA was found to be uncompetitive (Fig. 13). Inhibitiors constants (Ki) amounted to be 2.5mM. These results seem to be very similar to that of bovine liver [4] and human liver [8].

Methanol, glycerol, ethanol and acetone that enhance the activity of LMW-AcPase, had no stimulatory effect on HMW-Zn^{++}-AcPase. Similar results are reported in various mammalian tissues [7].

Experimental

Materials

Substrates and molecular weight markers were purchased from Sigma Chemical Co; Sepharose 4B-tartaric acid amide gel was a gift from Chemsitry Department, Gomal University, D.I.Khan. Sephadex G-150 was purchased from Pharmacia and CM-Cellulose from Whatman Biosystem Ltd. All other reagents used were of analytical grade mostly from BDH and Aldrich Chemical Company.

2.3 mM while inhibition produced by EDTA was found to be uncompetitive (Fig. 13). Inhibitiors constants (Ki) amounted to be 2.5mM. These results seem to be very similar to that of bovine liver [4] and human liver [8].

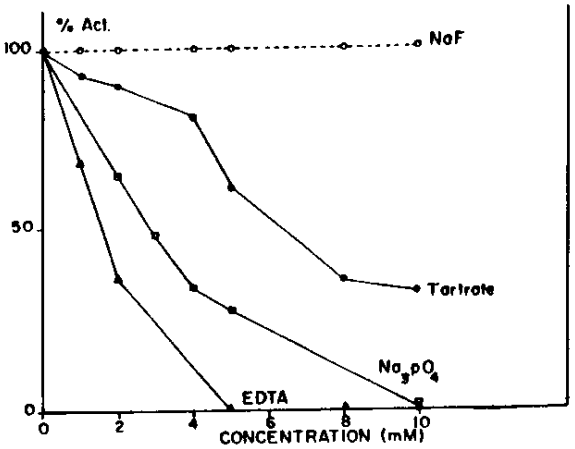

Fig.9 Effect of inhibitors on Zn^{++} - dependent acid phosphatase activity.

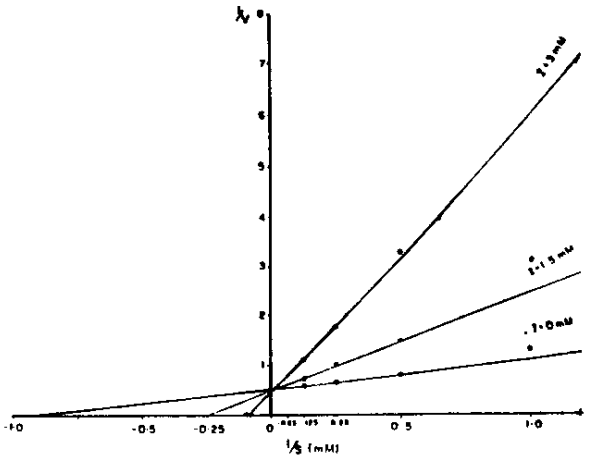

Fig.10 Inhibition of Zn^{++} - dependent acid phosphatase by sodium phosphate. Lineweaver-Burk plots of 1/v V/s 1/S in the absence or presence of 1.5mM and 3mM Na_3PO_4

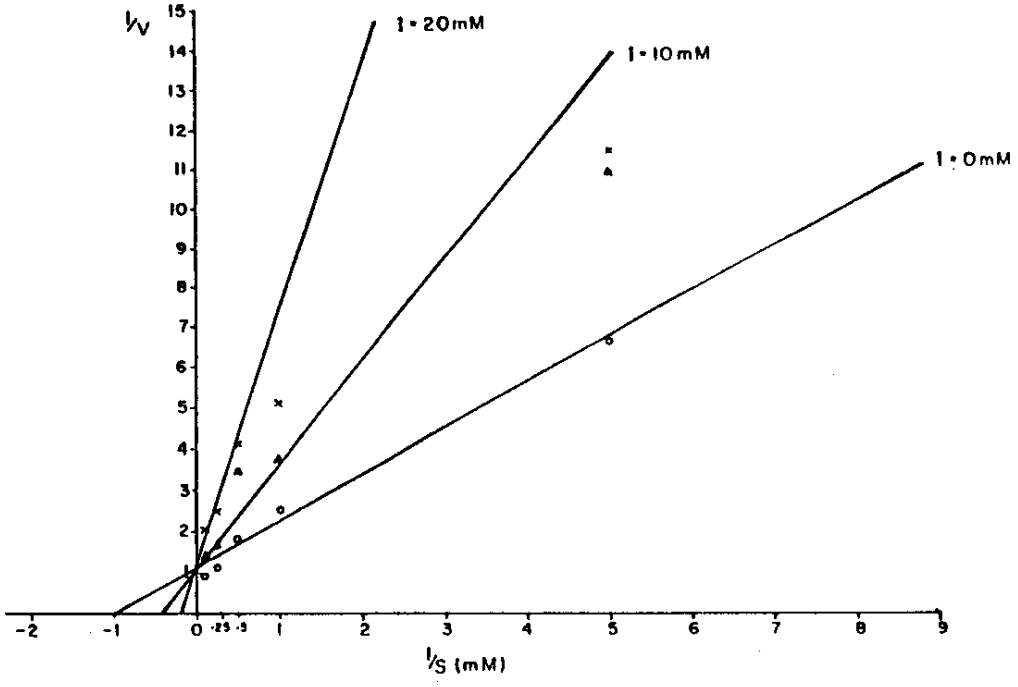

Fig.11 Inhibition of Zn^{++}-dependent acid phosphatase by tartrate. Lineweaver- Burk plots of 1/v V/s 1/S in the absence or presence of 10mM and 20mM tratrate.

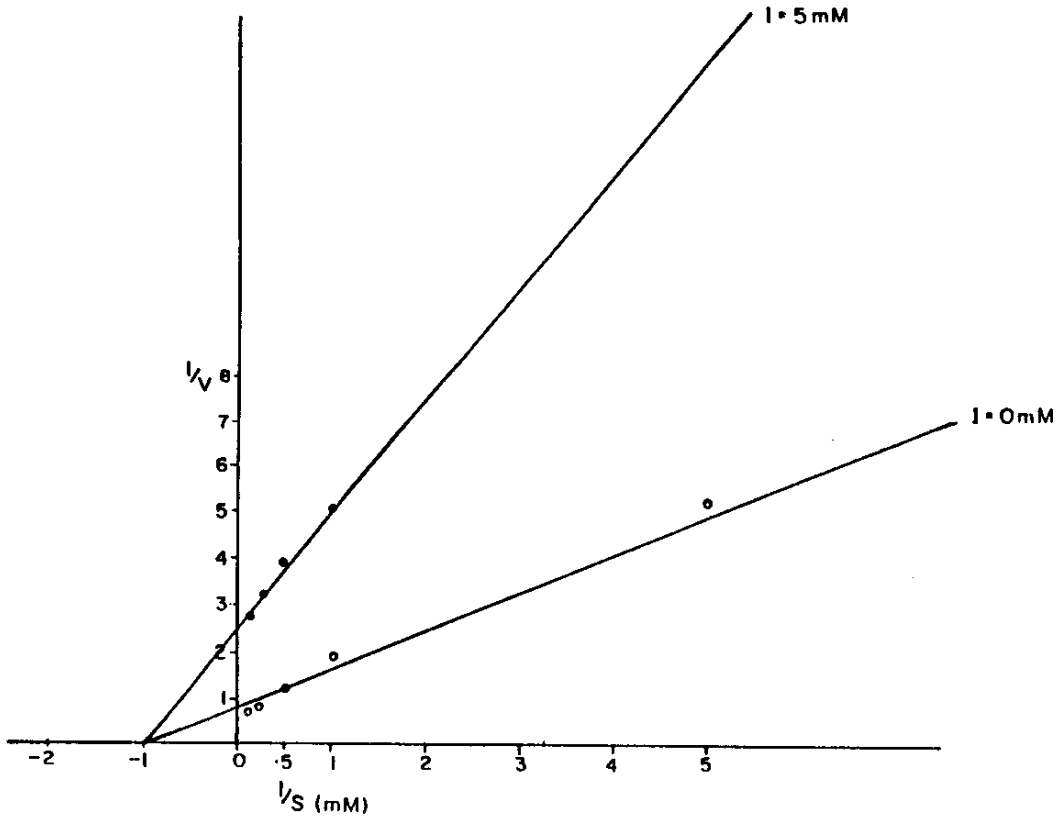

Fig.12 Inhibition of Zn^{++}- dependent acid phosphatase by ATP. Lineweaver-Burk plots of 1/v V/s 1/S in the absence or presence of 5mM ATP.

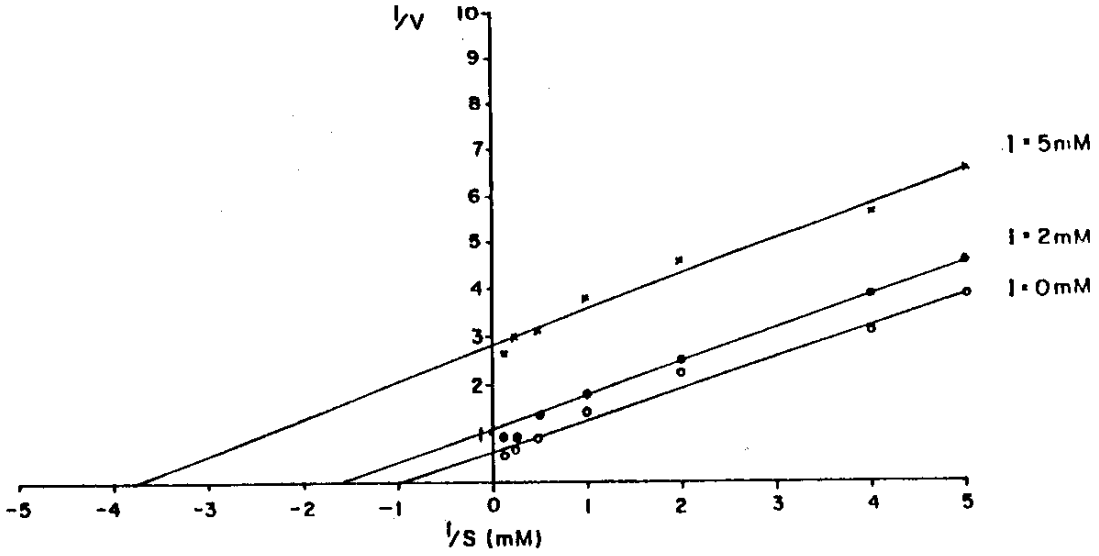

Fig.13 Inhibition of Zn^{++}-dependent acid phosphatase by EDTA. Lineweaver-Burk plots of 1/v V/s 1/S in the absence or presence of 2mM and 5mM EDTA.

Biochemical Assays

Protein concentration was determined by Lowry method [17]. Effluent functions from column chromatographic procedures were monitored spectrophotometrically for the relative amount of protein by measuring the absorbance of solutions at 280nm.

The Zn^{++}-AcPase activity was determined as described by Panara [10] at 37°C using 4mM p-nitrophenyl phosphate in 125 mM acetate buffer pH 6.0, containing 5mM $ZnCl_2$ and 10mM NaF and 50-100 µl of enzyme solution in a final volume of 1ml. After five min. reaction was stopped by addition of 4ml of 0.1N KOH and the absorbance of yellow colour due to phenolate ions produced was read at 405nm (ϵ = 1.8 x $10^4 M^{-1}cm^{-1}$). Units of enzyme activity are expressed as µ moles of p-nitrophenol released per minute. Specific activity is expressed as units per mg of protein.

In substrate specificity studies, the enzyme activity was estimated by liberation of inorganic phosphate (Pi). The liberated Pi was determined by method of Black and Jones [18].

The pH activity curves, obtained in pH range 4-9, the effect of metal ions and modifier substances were assayed as reported by Panara [4].

Km was determined graphically (Lineweaver-Burk plot) using substrate concentrations ranging from 0.2-8 x Km. The inhibition constants for the inhibitors were calculated at two fixed concentrations of inhibitors at various concentrations of p-nitrophenyl phosphate as substrate. The molecular weight of Zn^{++}-AcPase was determined by gel filtration on a Sephadex G-100 column (60x0.9cm) which was previously calibrated with the following standard proteins:

Aprotinin (6,500), Carbonic anhydrase (29,000), Albumin (66,000) and Alcohol dehydrogenase (80,000).

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [19].

Enzyme Purification

Step I: Isolation of high molecular weight Zn^{++}-AcPase

The fresh chicken's liver was washed with cold 10mM Tris HCl buffer pH 7.4, containing 0.25 M sucrose and 1mM EDTA and cut into small pieces. The pieces were homogenized gently in blender containing small volume of 10mM Tris HCl buffer pH 7.4, containing 1mM EDTA, 2mM β-mercapto-ethanol and 0.1mM PMSF. The homogenate was adjusted to 3ml buffer per gram of original tissues. The homogenate was centrifuged at 3840 x g for 30 min. and supernatant was collected.

Step II: Heat Treatment

The supernatant was heated at 60°C for 10-15 min. and was centrifuged at 3840 x g for 30min. Pellet was discarded and supernatant was collected. The sample was dialyzed against 0.01M acetate buffer pH 5.5 containing 0.1mM PMSF, 2mM β-mercaptoethanol overnight with several changes of buffer.

Step III: CM-Cellulose Chromatography at pH 5.5

Dialyzed sample was centrifuged at 3840 x g for 30 min. and clear supernatant was applied to CM-Cellulose column (3 x 10cm) equilibrated with dialyzing buffer. The column was washed with same buffer to remove unbound proteins. Linear gradient from 0-0.5M NaCl in same buffer (total 300 ml) was applied to elute bound enzyme as shown in Fig.1.The most active fractions were pooled and precipitated by adding solid ammonium sulphate to 70% saturation. The precipitate was collected by centrifugation at 10,000 x g for 30 min. and dissolved in small amount of 0.01M acetate buffer pH 6.0 containing 1mM EDTA and 0.1 M PMSF.

Step IV: Gel Chromatography on Sephadex G-150

The enzyme was applied to Sephadex G-150 column (1.8 x 70cm), equilibrated and eluted with 0.01M acetate buffer pH 6.0 containing 1mM EDTA, 2mM β-mercaptoethanol and 0.1M NaCl. The elution profile is shown in Fig. 2. The active fractions were pooled and the enzyme was concentrated in dialyzing tube over which sucrose was sprinkled for 4 to 5 h.

Step V: Affinity Chromatography on Tartramic Acid Affinity Gel

The concentrated sample was dialyzed against 1L of 0.01 M acetate buffer pH 5.1 containing 0.1mM PMSF, 2mM β-mercaptoethanol overnight and centrifuged at 3840 x g for 30 min. to remove precipitate if formed. The clear solution was applied to tartramic acid affinity column (1 x 7cm). The column was washed with dialyzing buffer to remove

protein impurities. The column was then washed with same buffer containing 60mM NaCl to remove further impurities. Finally column was washed with 20mM of NaH_2PO_4 in acetate buffer pH 6.0. Active fractions from two peaks were pooled separately. The elution profile is shown in Fig. 3. The major purification steps are summarized in Table-1. The homogeneity of these two peaks were checked on 12% SDS-PAGE.

Conclusions

Thus Zn^{++}-dependent acid phosphatase from chicken's liver seems to very similar to that of human liver, frog liver and bovine liver with respect to molecular weight, Zn^{++}-stimulation, inhibition with EDTA, ATP, PO_4 and tartrate, pH optima, thermal inactivation and substrate specificity.

This enzyme is also similar to that reported for high molecular weight acid phosphatases from mammalian liver [20-22], prostatic [15] and placental tissues [23]. These enzymes, however, were not stimulated by Zn^{++}, showed an optimal pH in the more acidic region (pH 4.5-7.5) and were strongly inactivated by NaF.

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