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Purification and Biochemical Properties of Acid Phosphatase from Rohu Fish Liver

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ABSTRACT

The aim of the study was to isolate and purify high molecular weight acid phosphatase from Rohu fish liver. The purification processes included the enzyme precipitation by ammonium sulphate, chromatographic adsorption by CM-Cellulose, permeation chromatography on Sephadex G-100 gel and finally by affinity chromatography on Reactive Blue 4-Agarose column. The enzyme showed a purification to specific activity of 1.75 U/mg of protein with purification factor of 43 and the yield was about 0.15%. The molecular weight was found 50 kDa on SDS-Polyacrylamide gel elecrophoresis. The gelfiltration on calibrated Sephadex G-100 revealed molecular mass of 100 kDa indicating the dimeric nature of protein. The K_m for *p*-nitrophenyl phosphate was 0.25 mM and V_{max} was 1.1 µmol of substrate hydrolysed/min/mg of protein. The optimum pH for activity was 5.0. The enzyme had optimum temperature around 40°C. The enzyme exhibited broad range substrate specificity. *p*-NPP, phenyl phosphate, α-and β-naphthyl phosphate and β-glycero phosphate were found good substrates. Other substrates like phospho-amino acids, nucleoside phosphates and sugar phosphates were hydrolysed at reasonable rates. The enzyme was inhibited by phosphate, fluoride, vanadate and molybdate. Competitive type of inhibition was displayed with K_i values 2.6, 0.29, 0.035 and 0.02 mM, respectively. The enzyme was also competitively inhibited by tartrate (K_i, 0.69 mM) and concluded that it was recognized as tartrate sensitive acid phosphatase to distinguish it from tartrate resistant acid phosphatase class.

Key Words: Acid phosphatase; Fish liver; Purification; Characterization
Abbreviations used: EDTA, ethylenediamine tetraacetate; PAGE, polyacrylamide gel, electrophoresis; PMSF, phenyl-methylsulfonyl fluoride; SDS, sodium dodecyl sulphate

INTRODUCTION

Acid phosphatases (EC 3.1.3.2), which belong to hydrolases class, catalyse the hydrolysis of various phosphomonesters in acidic medium (pH 5-6) to release an inorganic phosphate (Vincent *et al.*, 1992; Miteva *et al.*, 2010). These are present in animals (Stubberud *et al.,* 2000; Siddiqua *et al.,* 2008; Sazmand *et al*., 2011), plants (Demir *et al*., 2004; Gonnety *et al.,* 2006; Kaida *et al.,* 2008; Tabaldi *et al.*, 2008) and lower organisms like protozoa (Amlabu *et al.,* 2009) and fungi (Leitao *et al.*, 2010). These enzymes are involved in many biological processes such as energy metabolism and signal transduction pathways (Kostrewa *et al.,* 1999; Shan, 2000). Five forms of acid phosphatases exist at structural level of genes, the erythrocytic, lysosomal, proststic, macrophagic and osteoclastics forms (Bull *et al.,* 2002) which are expressed in the cells to different extent (Moss *et al.,* 1995).

Acid phosphatases are frequently occur in multiple forms (Fujimoto *et al.,* 1984) and can be differentiated according to structural, catalytic, tissue distribution and localization (Suter *et al.,* 2001). In vertebrates, three types of acid phosphatase have been described based on molecular weight and its localization within the cell organellae (Saeed *et al.,* 1990; Naz *et al.*, 2001). High molecular weight acid phosphatase (80-200 kDa) is localized in the lysosomes and low molecular weight enzyme (10-30 kDa) is present in cytosol fraction of the cell while intermediate molecular weight acid phosphatase (30-50 k Da) was found in the mitochandria of some mammalian tissues such as kidney (Naz *et al.,* 2006). Further more, high molecular weight acid phosphatase forms are inhibited by fluoride and tartrate while low molecular weight enzyme forms are unaffected by either fluoride or by tartrate (Igarashi & Hollander, 1968; Hollander, 1971).

High molecular weight acid phosphatases have been isolated from several animal tissues such as human prostate (Van Etten & Saini, 1977) and human liver (Saini & Van Etten, 1978), Beside these, acid phosphatases from the liver of non-mammalian animals such as catfish and chicken etc., were purified (Szalewicz *et al.*, 1997). In our laboratory, attempt was made to purify and characterize 18 kDa acid

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phosphatase from the liver of Rohu fish, *Labeo rohita* (Siddiqua *et al.*, 2008). The present manuscript describes the purification and biochemical properties of high molecular weight acid phosphatase (100 kDa).

MATERIALS AND METHODS

Chemicals: *Labeo rohita* (common name Rohu) was captured from Indus River (Khyber Pakhtunkhwa, Pakistan). The liver was excised and kept in cold storage. The ionexchangers (SP- Sephadex C-50 & CM-Cellulose), Sephadex G-100, Reactive Blue 4- Agarose, *p*-nitrophenyl phosphate including other phosphate esters as substrates of the enzyme and SDS-protein markers Kit were purchased from Sigma Chemical Co., (USA). The chemicals for polyacrylamide gel-electrophoresis and chromatography were obtained from Acros (Belgium) and Fluka (Switzerland) Chemical Co., and the Mini PROTEAN II dual slab cell components and accessories including power supply for electrophoresis were obtained from Bio-Rad (USA).

Assay for acid phosphatase: Acid phosphatase activity was assayed at 37°C in 0.1 M acetate buffer, pH 5.0 using *p*-nitrophenyl phosphate as substrate as previously reported by Ramponi *et al.* (1989). One unit of enzyme is defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per min. under the assay conditions. Specific activity is expressed as enzyme units/mg of total protein.

Kinetic studies were carried out in acetate buffer, pH 5.0 as described (Siddiqua *et al.*, 2009). The K_m and K_i values were determined using double reciprocal plots. Six concentrations ranging from 0.1 mM to 4 mM *p*-nitrophenyl phosphate were used in absence and presence of two or three fixed concentrations of inhibitors to caculate the values of K_m, V_{max} and 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 was carried out under above assay conditions by measuring the release of inorganic phosphate after enzymic reaction according to method of Black and Jones (1983).

Optimum pH was determined in acetate and barbital buffers covering the range of pH 4-6 and pH 6- 6.7, respectively. Temperature optimal was determined in range of 0-70°C at interval of 10°C. The temperature stability was determined by incubating the enzyme at various temperatures indicated in the respective figure for 30 min. and the residual activity was assayed as described above. Likewise, the thermal inactivation of the enzyme was also examined at 40-70°C in time dependent manner. The pH stability was studied by incubating the enzyme in buffers of various pH ranging from pH 4 to 9 for 10 h period. The residual activity was assayed as usual.

Protein determination: Protein was measured by the biuret method according to Beisenherz *et al.* (1953). Effluent fractions from chromatographic procedures were monitored for the amount of protein by measuring the absorbance at 280 nm.

Fig. 1: (a) Elution profile from CM-Cellulose chromatography. The column was eluted with flow rate of 40 mL/h and 10 mL fractions were collected. The arrow indicates the start of linear gradient 0-0.5 M NaCl in buffer. (b) Gel chromatography on Sephadex G-100. The column was eluted with flow rate of 25 mL/h and 4ml fractions were collected. (c) Affinity chromatography on Reactive Blue 4- Agarose column; flow rate of 15 mL/ h and 3 mL fractions were collected. The arrow indicates the start of elution with 0.25 M NaCl in buffer. Ordinates: Protein at 280 nm ((•—•); acid phosphatase activity, U/mL (o····o)

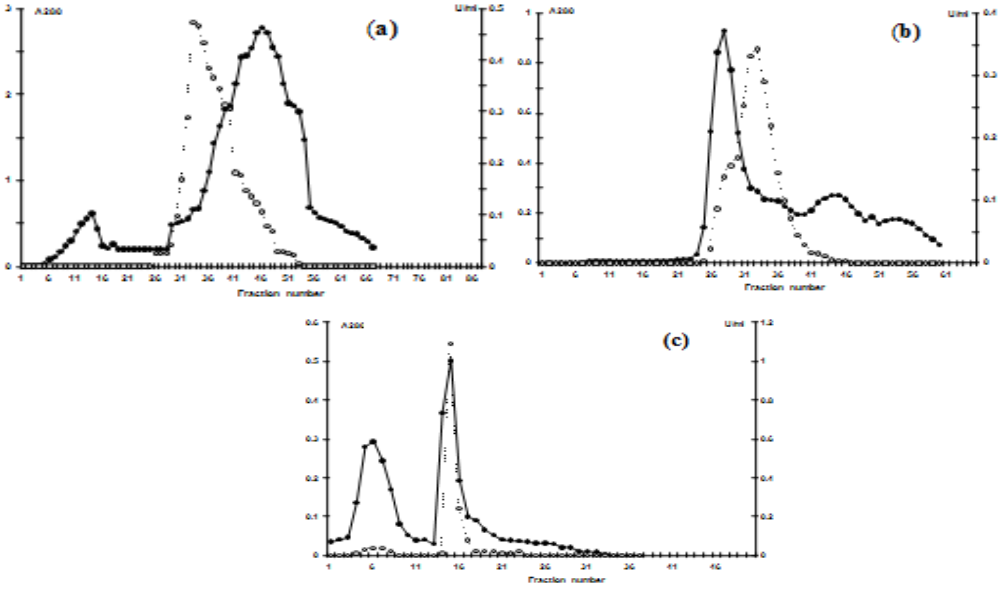


Table I: Purification of high molecular weight acid phosphatase from fish liver (1 kg)

	Volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Total proteins (mg)	Specific activity	Purification factor	Recovery %
Extract	3330	0.93	3100.23	29.2	97236	0.032	1	100
30% $(NH_4)_2SO_4$ saturation	3480	0.608	2115.84	28.4	98832	0.21	0.66	68.25
70% $(NH_4)_2SO_4$ saturation	505	2.08	1050.4	63.6	32118	0.033	1.03	33.88
SP-Sephadex C-50 (unbound)	895	0.158	141.41	6.1	5459.5	0.026	0.81	4.56
CM-Cellulose	179	0.3	53.7	4	716	0.075	2.34	1.732
Sephadex G-100	95	0.2	19	0.355	33.725	0.56	17.5	0.61
Reactive Blue	9	0.548	4.93	0.33	2.97	1.75	43	0.15

Table II: Effect of various midifiers on the high molecular weight acid phosphatase

Modifiers	Concentrations	Activity (%)
Tartrate	10 mM	6
Formaldehyde	0.2%	89
EDTA	10 mM	100
Triton X -100	1%	93
p-hydroxymercuribenzoate	0.1mM	90
$HgCl_2$	5 mM	0
$ZnCl_2$	5 mM	49
Cu_2SO_4	5 mM	5
$CaCl_2$	5 mM	97
$MgCl_2$	5 mM	82
$MnCl_2$	5 mM	105
Methyl alcohol	5 mM	97
Ethyl alcohol	5 mM	101
Glycerol	5 mM	103

Table III: Substrate specificity of high molecular weight acid phosphatase from fish liver

Substrate	Activity (%)
p-Nitrophenyl phosphate	100
Phenyl phosphate	80
Flavin mononucleotide	40
α-Naphthyl phosphate	78
β-Naphthyl phosphate	95
α-Glycero phosphate	15
β-Glycero phosphate	75
Phosphotyrosine	20
Phosphoserine	10
Phosphothreonine	13
Glucose-1-phosphate	12
Glucose-6-phosphate	18
ATP	35
AMP	45
UMP	36

Electrophoresis: SDS-PAGE was performed according to the method of Laemmli (1970). 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 Fig. 2.

Molecular weight determination: Apparent molecular weight of acid phosphatase was determined with gel chromatography on a column of Sephadex G-100 by a comparison of its elution volume to those of standard proteins. The proteins used were bovine serum albumin, carbonic anhydrase, cytochrome c and aprotinin.

Enzyme purification: Fish livers was brought into thawing state and was homogenized in blender and added 0.3 M acetate buffer pH 5.0 containing 1 mM EDTA, 0.1 mM PMSF, 2 mM β-mercaptoethanol at the rate of 1 g/3 mL. After homogenization, it was agitated for about 1 h and centrifuged at 5000 rpm (Rotor JA-14) for 30 min. Then it was filtered over glass wool. Ammonium sulphate was added to saturate the extract to the level of 30% (176 g/L of solution). Addition of salt was gradual with constant stirring and stirred further for 1 h. It was centrifuged at 5000 rpm. The precipitate was removed and to the supernatant further salt was added to form 70% saturation and stirred in cold place for 2 h. It was then centrifuged at 5000 rpm for ½ h discarding the supernatant and dissolved the precipitate in 0.01 M acetate buffer pH 5.0 containing 1 mM EDTA, 0.1 mM PMSF and 2 mM □- mercaptoethanol. It was then dialyzed against 20 vol. of 0.05 M acetate buffer pH 4.8 containing 1 mM EDTA, 0.1 mM PMSF and 2 mM □-mercaptoethanol over night .The dialyzed sample was centrifuged for 30 min at 8000 rpm. The clear supernatant was placed on SP-Sephadex C-50 column (33 x 8.5 cm), which was equilibrated and eluted with acetate buffer containing 1 mM EDTA, 0.1 mM PMSF and 2 mM □-mercaptoethanol with flow rate of 150 mL/h and 20 mL fractions were collected. The unbound high molecular acid phosphatase activity (HM-ACP) was pooled and concentrated by precipitation with 70% ammonium sulphate. The precipitate thus obtained was dissolved in acetate buffer, pH 5.9 containing same additives while bound proteins containing low molecular acid phosphatase activity (LM-ACP) was eluted with 0.3 M phosphate buffer, pH 5.5 to purify LM-ACP (Siddiqua *et al.,* 2008).

Eighty five mL of sample of HM-ACP after 70% $(NH_4)_2SO_4$ precipitation was dialyzed against 10 vol. of 0.01 M acetate buffer pH 5.9 containing same additives over night. The dialyzed sample was centrifuged and the clear supernatant was subjected to cation-exchange chromatography on CM-Cellulose (29 x 3 cm), which was previously equilibrated and eluted with dialyzing buffer. The bound enzyme was obtained with linear sodium chloride gradient 0-0.5 M (200+200 mL). This was followed by single step elution with buffer containing 0.5 M NaCl. Fractions containing activities were pooled together and concentrated by Amicon ultrafiltration with YM3 membrane. The concentrated sample (10 mL) was loaded on to the column Sephadex G-100 (85 x 2.5 cm) previously equilibrated and eluted with 0.01 M acetate buffer, pH 5.0

containing 0.1 M NaCl. The most active fractions were concentrated by ultrafiltration and dialysed against acetate buffer pH 5.0 containing same additives. The dialysed sample was applied on Reactive Blue 4- Agarose column (14 x 1.8 cm). After extensive washing, the innert proteins were removed. This was followed by washing the column with 0.25 M NaCl in buffer. The activity contained in two or three fractions were pooled and concentrated for PAGE analysis and biochemical properties.

RESULTS AND DISCUSSION

The summary of purification of acid phosphatase from Rohu fish is presented in Table I. Ammonium sulfate and SP- Sephadex C-50 chromatography steps were very effective in removing contaminating proteins. The elution profiles for CM-Cellulose, Sephadex G-100 and affinity chromato graphy on Reactive Blue 4 - Agarose column are presented in Fig. 1 (a-c). The enzyme was purified about 43-fold with a specific activity of 1.75U/mg/min when assayed with 4 mM *p*NPP in 0.1 M acetate buffer, pH 5.0. The SDS-PAGE of the final preparation showed a major band corresponding to 50 kDa (Fig. 2). However, some contaminating proteins were still found in the enzyme preparation. The molecular weight of native enzyme obtained by gel filtration was found 100 kDa, (Fig. 3) indicating that the native enzyme contained two subunits identical in molecular weight. These results are very similar to enzymes reported from human prostate (Van Etten & Saini, 1978) and human liver (Saini & Van Etten, 1978). The fish liver enzyme is also similar to these enzymes in that all are dimer with molecular weight of 100 kDa and subunit molecular mass of 50 kDa. The Km was 0.25 mM and a V_{max} was 1.1 µmol of substrate hydrolysed/min/mg of protein. The optimum pH for activity was 5.0 and pH stability was found between 4-6. The enzyme had optimum temperature around 40˚C and showed temperature stability at 50˚C. All the results are shown in Fig. 4. Phosphate, tartrate, fluoride, vanadate and molybdate were found inhibitors. Competitive type of inhibition was displayed as shown in Fig. 5 and their inhibition constants were calculated to be 2.6, 0.69, 0.29, 0.035 and 0.02 mM, respectively. The vanadate and molybdate seemed stronger inhibitors than phosphate, tartrate and fluoride. These results have in agreement with the findings for other phosphatases (Panara, 1985; Khan *et al*., 1997). It is possible to distinguish between different forms of acid phosphatase by means of different modulators. One of these is tartrate, which is strong inhibitor of acid phosphatases of high molecular weight, wheras low molecular weight acid phosphatase is unaffected by tartrate. So the differential inhibition together with the differences in molecular weight can be used to characterize these two forms of acid phosphatases. High molecular weight acid phosphatase from fish liver is recognized as tartrate sensitive enzyme, while purple acid phosphatases from plant and animal

Fig. 2: SDS-polyacrylamide gel electrophoresis of acid phosphatase

Lane 1 5 µL reduced enzyme
Lane 2 10 µL reduced enzyme
Lane 3 Standard proteins: Albumin (66 kDa), ovalbumin (45 kDa), glycerolaldehyde-3-phosphate dehydrogenase (36 kDa), carbonic; anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and lactalbumin (14.2 KDa)

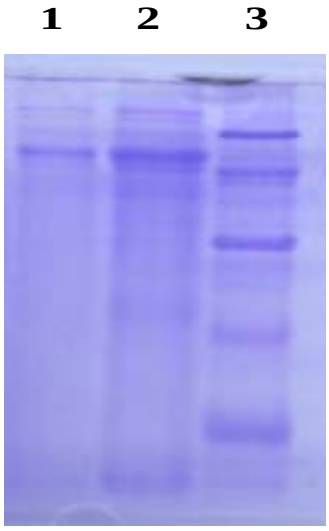
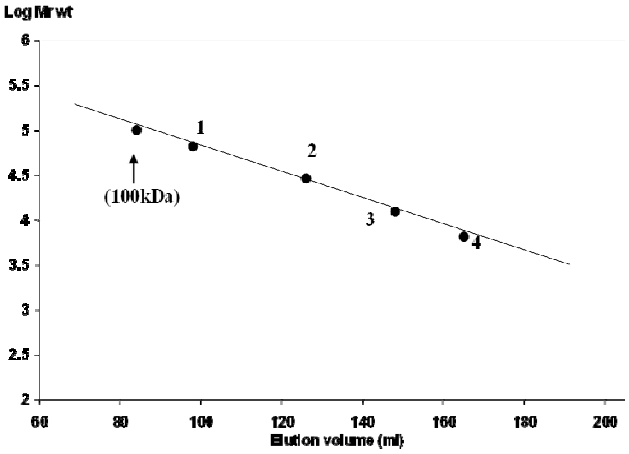


Fig. 3: Linear graph of log molecular weight versus elution volumes of standard proteins

The enzyme extract after 60% ammonium sulphate precipitation, was applied onto the column of Sephadex G-100 (1.8 x 85 cm) and eluted with 0.01 M acetate buffer, pH 5.1 containing 0.1M NaCl with flow rate of 40 mL/h and 3 mL fractions were collected. 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 280 nm for standard proteins (•) or by assay of enzyme activity at 405 nm for the 100 kDa enzyme sample. (1) Bovine serum albumin (M_r 66,000), V_e 98 mL; (2) Carbonic anhydrase (M_r 29,000), V_e 126 mL; (3) Cytochrome c (M_r 12,400), V_e 148 mL; (4) Aprotinin (M_r 6,500), V_e 165 mL; Acid phosphatase (100 kDa), V_e 84 mL; V_o 72 mL.



sources are reported to be tartrate resistant enzymes (Kaida *et al.,* 2008). Some other compounds were also tested as possible inhibitors or activators. The enzyme was not affected by Ca^{2+}, Mg^{2+} and Mn^{2+}, but was inhibited by Zn^{2+},

Fig. 4: (A) Optimum pH (B) pH stability (C) Optimum temperature (D) Temperature stability

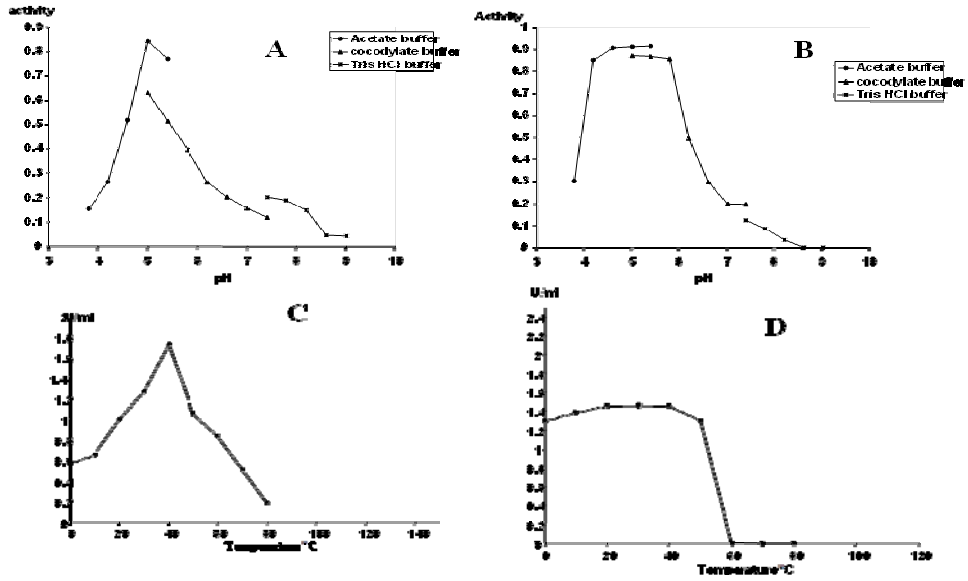
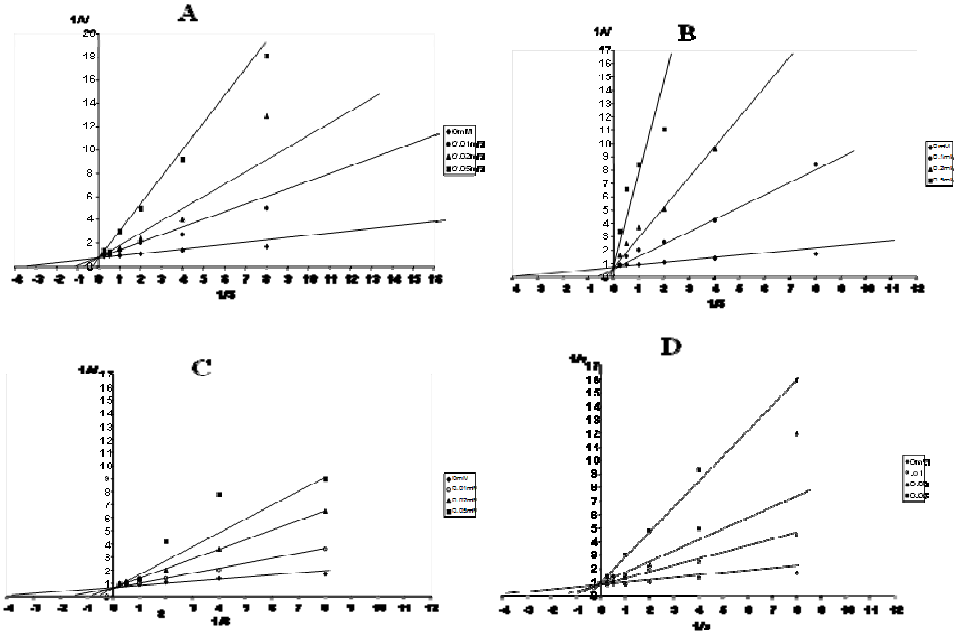


Fig. 5: Competitive inhibition of fish liver acid phosphatase. Lineweaver-Burk plots of 1/v versus 1/S
(A) molybdate (**B)** orthovanadate (**C**) sodium fluoride (**D)** phosphate



Cu^{2+} and Hg^{2+} indicating that divalent ions are not required for the activity of enzyme (Table II). There was no change in the enzyme activity in the presence of EDTA or Triton X-100. *p*-Hydroxymercuribezoate also produced no loss of activity. This suggests that a free-SH group was not essential enzyme action. The enzyme was not stimulated by phosphate acceptors such as methyl alcohol, ethyl alcohol and glycerol. Hence it possessed no phosphotransferase activity unlike LM-ACPases.

Table III shows substrate specificity of fish liver enzyme. Acid phosphatase hydrolyzed a large variety of phosphate esters. The hydrolysis rates indicate that *p*-NPP, phenyl phosphate, α-and β-naphthyl phosphate and β-glycero phosphate were found good substrates. Other substrates like phospho-amino acids, nucleoside phosphates and sugar phosphates were hydrolysed at reasonable rates. The broad substrate specificity have been reported for many high molecular weight acid phosphatases (Panara & Pascolini, 1989; Saeed *et al.,* 1998). Almost all mammalian and non-mammalian liver acid phosphatases are glycoproteins (Szalewicz *et al.*, 1997; Janska & Kubicz, 1985; Kubicz & Szalewicz, 1993). The enzyme from fish liver might be

glycoprotein. But it had failed to bind on concanavalin A-Sepharose 4B column during the purification. Perhaps this enzyme may exhibit structures with carbohydrate chains that weaken binding ability to Con A column.

CONCLUSION

In this study, 100 kDa acid phosphatase from liver of rohu fish was purified and biochemically characterized. Since the enzyme was found sensitive to tartrate inhibition, it may be recognized as a tartrate sensitive acid phosphatase class.

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