# Low Molecular Weight Zinc-Dependent Acid Phosphatase from Chicken's Heart

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**Summary:** $Zn^{++}$-dependent acid phosphatase from chicken's heart was partially purified by ammonium sulfate precipitation, heat treatment at 60 °C, CM-Cellulose chromatography and gel filtration on Sephadex G-100 to the specific activity of 0.8 $\mu$mol $min^{-1}$ $mg^{-1}$ at pH 6.0. A 34-folds purification was achieved with the recovery of 8 %. The enzyme had molecular weight 57 kDa as revealed by gel filtration and 29 kDa by SDS-polyacrylamide gel electrophoresis, indicating the dimeric nature of the enzyme. The enzyme was activated by $Zn^{++}$, $Co^{++}$ and $Mn^{++}$ and inhibited by phosphate, while EDTA, tartrate and flouride had little or no effect on the activity, which are the potent inhibitors of high molecular weight acid phosphatases.

## Introduction

Phosphatases belong to the hydrolase class and catalyze the hydrolysis of almost any phosphate monoesters. They play a very important role in biological systems, which are linked to processing involving energy metabolism, metabolic regulation and cellular signal transduction pathways [1]. Phosphatases are widely distributed in nature and differ greatly in their sizes, structures and mechanisms. They can be classified in several ways. In one classification, phosphatases have been divided into five classes, namely alkaline phosphatases, purple acid phosphatases, high molecular weight acid phosphatases, low molecular weight acid phosphatases and protein phosphatases.

In vertebrate tissues, at least two classes of acid phosphatases (E.C.3.1.3.2), differing in molecular weight and localization have within the cells have been described [2]. Another class of acid phosphatase, which requires $Zn^{++}$ for activity called $Zn^{++}$- dependent acid phosphatase has been reported [3].

Phosphatase also exists in two major forms, which have similar biochemical properties but vary in molecular weight and tissue distribution [4]. High molecular weight $Zn^{++}$- dependent acid phosphatase (~ 100 kDa) is expressed in the liver of animal tissues [3, 5], while low molecular weight $Zn^{++}$ - dependent acid phosphatase is about 57-62 kD and has been found in other tissues like brain, heart, skeletal muscle, erythrocytes, lungs, spleen and stomach [6, 7]. Small intestine contains significant quantities of both forms [8, 9].

Purification and biochemical properties of chicken's liver high having molecular weight $Zn^{++}$-dependent acid phosphatase were reported recently [10].

Contrarily, in this paper, we report the existence of low molecular weight $Zn^{++}$ - dependent acid phosphatase in the chicken's heart. The enzyme was purified using almost the similar procedure to that reported for liver enzyme and characterized with respect to molecular weight, metal ion dependence, inhibition, pH and temperature optima and kinetic properties.

### Results and Discussion

Low molecular weight $Zn^{++}$ - dependent acid phosphatase (57-63 kDa) was observed to be present in the chicken's heart. Purification procedure for this enzyme was the same as that for high molecular weight enzyme of chicken liver [10]. The major purification steps are summarized in Table-1. Elution profile for CM-Cellulose chromatography and Sephadex G-100 is shown in Figs. 1 and 2. A-34 fold purification was achieved with the recovery of 8 %. The enzyme had specific activity of 0.8 $\mu$ mol $min^{-1}$ $mg^{-1}$ at pH 6.0 at 37° C. SDS-polyacrylamide gel electrophoresis (12 %) showed almost a single band at 29 kDa with Coomassive Blue Staining (Fig. 3)

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Table 1- Partial purification scheme for 57 kDa $Zn^{++}$-dependent acid phosphatase from 50 gm of Chicken's heart

| Steps | Vol. (ml) | Act. (U/ml) | T.Act. (U) | Prot. (mg/ml) | S.A (U/mg) | P.F | Rec. % |
|---|---|---|---|---|---|---|---|
| Extract | 188 | 0.26 | 48.88 | 11 | 0.023 | 1 | 100 |
| 60% ammonium sulphate precipitation | 22 | 0.943 | 20.7 | 35 | 0.026 | 1.17 | 42.4 |
| Heat treatment at 60° C and dialysis | 30 | 0.22 | 6.6 | 5.34 | 0.041 | 2 | 13.4 |
| CM-Cellulose Chromatography at pH 5.5 | 3 | 1.53 | 4.6 | 11.7 | 0.131 | 5.7 | 9.4 |
| Sephadex G-100 | 17.5 | 0.23 | 4.02 | 0.29 | 0.795 | 34.57 | 8.2 |

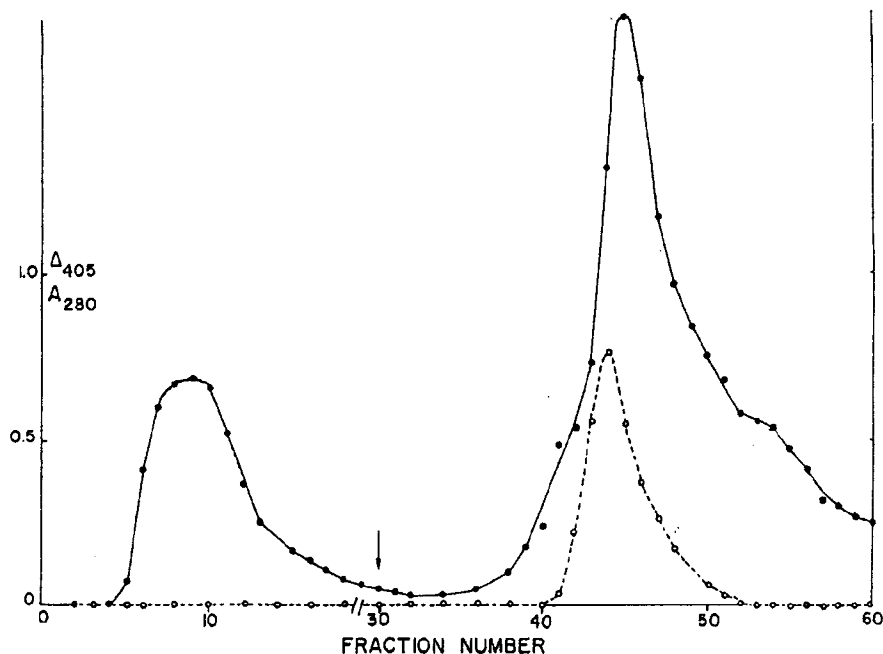


Fig. 1: CM-cellulose chromatography at pH 5.5.
30 ml sample (7U) was applid, Column (1.5x20 cm), flow rate 30 ml/h, each fraction 3-4 ml. Arrow indicates starting point of gradients. Ordinate: Protein at 280 nm (• •), activity at 405 nm (o......o).

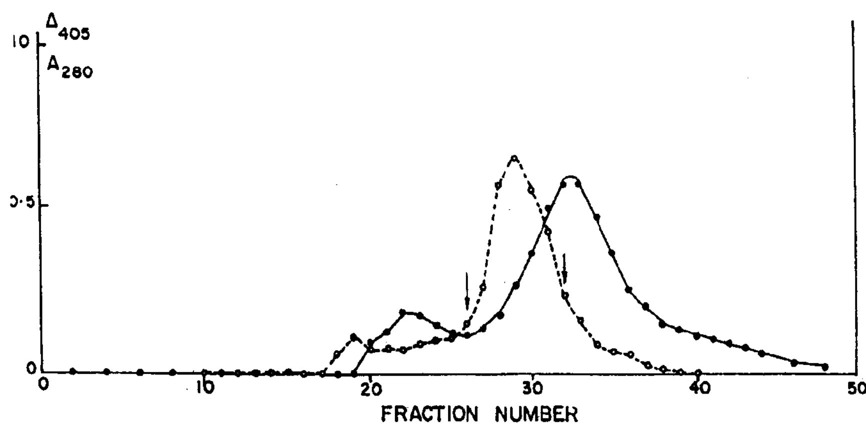


Fig. 2: Gel chromatography on Sephadex G-100.
3 ml sample (6U) was applied, column (1.8x85 cm), flow rate 20 ml/h, each fraction 3.5 ml. Ordinates: Protein at 280 nm (•—•) activity at 405 nm (o......o)

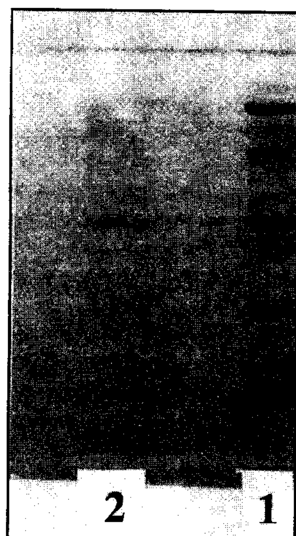


Fig.3 SDS-Polyacrylamide gel electrophoresis of $Zn^{++}$ - dependent acid phosphatase.

Lane 1: Standard proteins used from top to bottom were Albumin Bovine (66,000); Oval albumin (45,000); Carbonic anhydrase (29,000); Trypsin inhibitor (20,000); Lact albumin (14,200).

Lane 2 5 ul of $Zn^{++}$- dependent acid phosphatase.

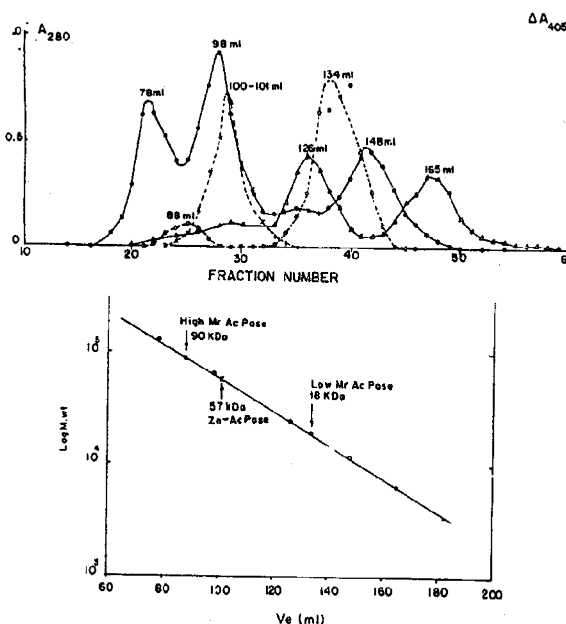


Fig. 4: (a) Elution pattern of standard proteins on Sephadex G-100.
The column (1.8x85 cm) was equilibrated and eluted with 0.01M acetate buffer pH 5 containing 0.1 M sodium chloride at flow rate of 20 ml/h and 3.5 ml fractions were collected; o 72 ml, Blue dextran 2000:Boine serum albumin (66,000), e 98 ml (•—•); carbonic anhydrase (29,000), Ve 126 ml (Δ—Δ); cytochrome C(12,400), Ve 148 ml (•—•); Aprotinin (6,500), Ve 165 ml (Δ—Δ) $Zn^{++}$ pendent Acid phosphatase, Ve 100 m (x....x); High Molecular weight acid phosphatase, Ve 88 ml (o.....o) lecular weight acid phosphatase, e 134 ml (o.....).
(b) Linear graph of log M.W. versus elution volumes of standard proteins.

after rechromatography on Sephadex G-100. The molecular weight of native enzyme obtained by gel filtration on Sephadex G-100 was estimated to be 57 kDa (Fig. 4), which indicated this enzyme to be a dimer. 57 kDa enzyme is also present in rat brain, testis, prostate and adrenal glands [6], which is distinguished from the 100 kDa enzyme isolated from the liver of various vertebrates [11-13]. High molecular weight acid phosphate and low molecular weight acid phosphate from chicken's heart displayed molecular weight values (Fig. 4 b) being similar to those previously reported for the high and low molecular weight enzymes isolated from other sources [13].

Chicken heart low molecular weight $Zn^{++}$ - dependent acid phosphatase activity showed maximum activity over pH of 4.5-6.0 (Fig. 5). The optimal temperature for activity was 65° C (Fig. 6). Km was found to be 0.6mM.

The low molecular weight $Zn^{++}$ - dependent acid phosphatase showed no p-nitrophenyl phosphate hydrolyzing activity in the absence of $Zn^{++}$ ion. The hydrolysis was observed in the presence of $Zn^{++}$, $Mn^{++}$ and $Co^{++}$. $Zn^{++}$ was the most effective cation for activating the catalytic activity of the enzyme (Fig. 7). 5mM $Zn^{++}$ concentration was required for maximum hydrolysis. Similar finding was obtained with high molecular weight liver enzyme. In comparison with $Zn^{++}$, $Mn^{++}$ and $Co^{++}$ showed 16 % and 12 % activation respectively. $Mg^{++}$ and other divalent ions such as $Ca^{++}$, $Fe^{++}$ a $Ni^{++}$ had little or no effect (Table 2). These results are in good agreement

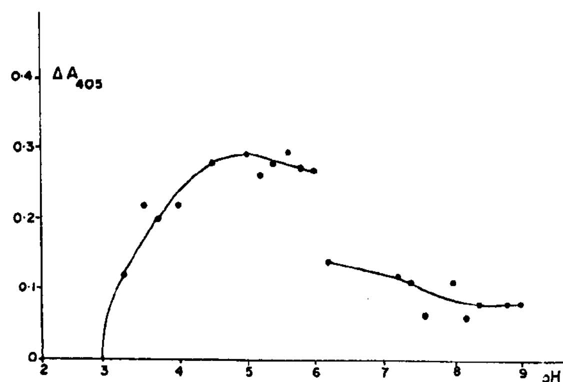


Fig. 5: Optimum pH of acid phosphatase from chicken heart.

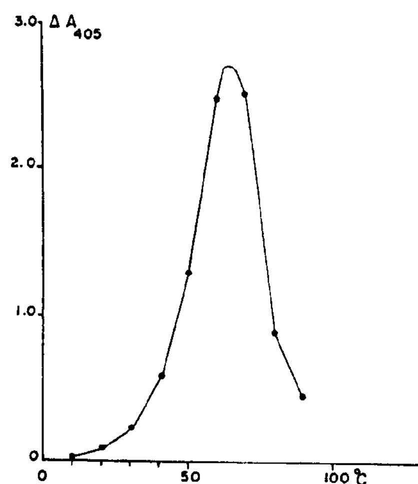


Fig. 6: Optimum temperature of $Zn^{++}$ -dependent acid phosphatase activity.

Table 2- Effect of metal ions as activators (5mM) on the p-nitrophenyl Phosphate hydrolysis

| Metal ion | Δ $A_{405}$ | Activity (%) |
|---|---|---|
| Water | 0.007 | 1.34 |
| $ZnCl_2$ | 0.521 | 100 |
| $MnCl_2$ | 0.083 | 16 |
| $CoCl_2$ | 0.062 | 12 |
| $MgCl_2$ | 0.005 | 1 |
| $CaCl_2$ | 0.00 | 0 |
| $NiCl_2$ | 0.00 | 0 |
| $FeSO_4$ | 0.0 | 0 |

with that of high molecular weight enzymes of chicken liver, bovine liver and human liver. In another experiment, the effect of divalent cations at a concentration of 5mM on $Zn^{++}$ - dependent acid phosphates activity is reported in Table 3, which showed that $Mn^{++}$ appreciably altered the $Zn^{++}$ dependent acid phosphatase activity whereas $Ba^{++}$, $Co^{++}$, $Ca^{++}$, $Mg^{++}$ and $Ni^{++}$ did not, while heavy

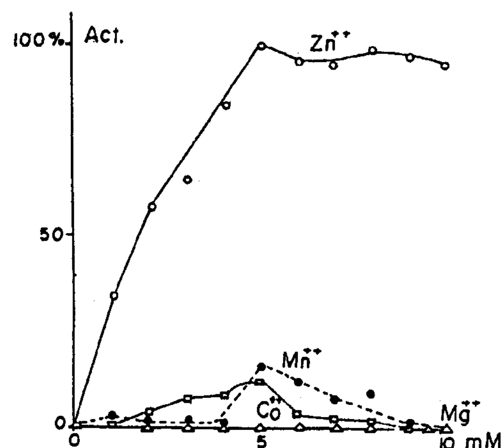


Fig. 7: Influence of metal ions as activators on the hydrolysis of p-nitrophenyl phosphate. The activity was determined in the presence of metal ions at concentrations in abscissa. The maximum activity was expressed by taking the highest activity in any fraction as 100% and the other activities were expressed as a percentage of the maximum activity.

Table 3- Effect of metal ions (5mM) on $Zn^{++}$-dependent acid phosphatase activity

| Divalent metal ion | Activity (%) |
|---|---|
| None | 100 |
| $MnCl_2$ | 37.11 |
| $BaCl_2$ | 100 |
| $CoCl_2$ | 90.9 |
| $CaCl_2$ | 100 |
| $MgCl_2$ | 81.03 |
| $NiCl_2$ | 85.5 |
| $FeCl_3$ | 0 |
| $FeSO_4$ | 0 |
| $HgCl_2$ | 14.9 |

Table 4- Effect of inhibitors on $Zn^{++}$-dependent acid phosphatase activity

| Concen-tration | Tartrate (%) Activity | EDTA (%) Activity | Phosphate (%) Activity | Fluoride (%) Activity |
|---|---|---|---|---|
| 0 mM | 100 | 100 | 100 | 100 |
| 1 mM | 100 | - | 98 | 100 |
| 2 mM | 100 | - | 77 | 94.3 |
| 3 mM | 90 | - | - | 95.5 |
| 4 mM | 87 | 98 | 68 | 98 |
| 5 mM | 80.6 | - | 65 | - |
| 6 mM | 79 | 96 | 60 | - |
| 8 mM | 77 | 95 | 52 | 100 |
| 10 mM | 69 | 93 | 37 | 93.3 |

metals such as $Fe^{++}$, $Cu^{++}$ and $Hg^{++}$ inhibited the enzyme strongly.

The effect of various inhibitors or modifiers on the activity of $Zn^{++}$ - dependent acid phosphates was tested by adding tartrate, fluoride, EDTA and $Na_3PO_4$ at concentrations ranging from 0 to 10mM. Table 4 shows that fluoride, tartrate and EDTA had little or no effect on the activity, which are potent inhibitors of high molecular weight acid phosphates. Phosphate indeed seemed to be a strong inhibitor. The inhibition of this enzyme was greater than that of liver enzyme [10]. The $Zn^{++}$ dependence, pH activity curve and modifiers sensitivity strongly suggested that the 57 kDa $Zn^{++}$ - dependent acid phosphatase displays biochemical properties very similar to those of 100 kDa enzymes but with a molecular weight, a = which is half that of liver enzyme. Both liver and heart enzymes are composed of two apparently identical subunits of about 50 kDa and 29 kDa respectively, reflecting molecular and structural analogies. Thus, purification of 57 kDa $Zn^{++}$ - dependent acid phosphatase is necessary to elucidate these points.

## Experimental

### *Material*

Chicken's heart was obtained from a local poultry shop. Para-nitrophenyl phosphate, molecular weight markers, CM-cellulose and Sephadex G-100 were purchased from Sigma Chemical Co. The material for SDS-polyacrylamide gel electrophoresis was obtained from Bio-Rad and Across Chemical Co. All other reagents were of the highest purity commercially available.

### *Enzyme Assays*

The $Zn^{++}$ - dependent acid phosphatase activity was determined as described by Panara [14] at 37 °C using 4mM p-nitrophenyl phosphate in 125 mM acetate buffer at pH 6.0, containing 5mM $ZnCl_2$ and 10 mM NaF and 50-100 $\mu$l of enzyme solution in a final volume of 1ml. After 5 minute, the reaction was stopped by the addition of 1 ml of 0.1 N KOH and the absorbance was measured at 405 nm ($\varepsilon$ = 1.8 x $10^4$ $M^{-1}cm^{-1}$). Units of activity were expressed as $\mu$ mol of p-nitrophenol released per minute. The specific activity was defined as units per mg of protein.

The activity over a pH range of 3.2-9.0, the temperature optima, determined at 10°C intervals from 10-90°C and the effect of metal ions were determined as reported earlier [11].

### *Protein Determination*

Protein concentration was estimated by the Biuret method [15]. For column effluents, the relative protein concentration was estimated from absorbance at 280 nm.

### *Molecular Weight Determination*

The molecular weight of $Zn^{++}$ - dependent acid phosphatase and acid phosphatases was determined by gel filtration on a Sephadex G-100 column (1.8 x 85 cm), which was previously calibrated with the following standard proteins: aprotinin (6,500), cytochrome C (12,400), carbonic anhydrase (29,000) and bovine serum albumin (66,000).

SDS polyacrylamide gel electrophoresis was carried out by the method of Laemmli [16].

### *Enzyme Purification*

Step 1: Isolation of low molecular weight $Zn^{++}$ - dependent acid phosphatase:

The fresh chicken's heart was washed with 10mM Tris-HCl buffer at pH 7.4, containing 0.25 M sucrose and 1 mM EDTA and was cut into small pieces. The pieces were homogenized gently in a blender containing small volume of 10 mM acetate buffer at pH 6.0 containing 1mM EDTA, 2mM $\beta$-mercaptoethanol and 0.1 mM PMSF. The homogenate was adjusted to 3ml buffer per gram of original tissue. The homogenate was centrifuged at 3840 x g for 30 minutes and the supernatant was collected and precipitated by 60 % saturation ammonium sulfate. The precipitate collected by centrifugation at 3840 x g was dissolved in extraction buffer.

### *Step 2: Heat Treatment*

The supernatant was heated at 60 °C for 2-5 minutes and centrifuged at 10,000 x g for 20 minutes. The pellet was discarded and the supernatant was collected. The sample was dialyzed against 0.01 M acetate buffer at pH 5.5, containing 0.1 mM PMSF, 2 mM $\beta$-mercaptoethanol overnight with 2-3 times changes of buffer.

*Step 3: CM-Cellulose Chromatography at pH 5.5*

Dialyzed sample was centrifuged at 3840 x g for 30 min. and the clear supernatant was applied to CM-Cellulose column (3 x 10cm) equilibrated with dialyzing buffer. The column was washed with the same buffer to remove unbound proteins. Linear gradient from 0-0.5M NaCl in the 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 sulfate to 70 % saturation. The precipitate was collected by centrifugation at 10,000 x g for 30 min. and dissolved in a small amount of 0.01M acetate buffer at pH 6.0 containing 1mM EDTA and 0.1 M PMSF.

*Step 4: Gel Chromatography on Sephadex G-100*

The enzyme was applied to Sephadex G-150 column (1.8 x 70cm), equilibrated and eluted with 0.01M acetate buffer at pH 6.0 containing 1mM EDTA, 2mM $\beta$-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.

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