The Low and High Molecular Weight Acid Phosphatases in Sheep Liver

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Summary: Subcellular Localization of high and low Mr acid phosphatases was reported in the liver of sheep. Both enzymes were isolated by gel filtration on Sephadex G-100. Biochemical properties such as optimal pH, molecular weight determination, effect of inhibitors and some modifier substances on both enzymes were also described. Two isoenzymes of low Mr acid phosphatase namely Ac Pnase A and B were purified to specific activity of 27 and 23 U/mg of protein respectively. The isoenzymes were homogenous on SDS-PAGE, moved as single bands of Mr 18,000 and showed isoelectric points of 6.0 and 5.8 respectively. Both isoenzymes catalysed the hydrolysis of p-nitro phenyl phosphate and phenyl phosphate, phosphotyrosine and FMP but at different rates. Ac Pnase B showed higher $k_a$ than Ac Pnase A toward O. phosphotyrosine and FMP. Ac Pnase B was effectively activated by purine compounds whereas Ac Pnase A was not. No differences in sensitivity to inhibitors or modifier substances between Ac Pnase A and B were observed.

Introduction

The existence of at least two classes of acid phosphatases in the liver of vertebrates has been reported [1-4]. The differences among these two classes are based on molecular weight, substrate requirement, sensitivity to inhibitors and subcellular localization [5-7]. These can be distinguished on the basis of molecular size. The high molecular weight forms (Mr. 100,000) seem to be lysosomal non specific acid phosphohydrolases whereas low molecular weight form (Mr 20,000) is cytosolic in origin and displays a very restricted substrate specificity than that of high molecular weight acid phosphatases. The enzymes have been studied in liver of mammals [8-10]. Low Mr acid phosphatase has been purified and characterized from bovine liver [6-11] human liver [4,9] bovine brain [12], human placenta [13], avian pectoral muscle [14,15] and from many other sources [16]. Purification of low Mr enzymes from rat liver has been reported and is composed of two
different isoenzymes which can be isolated on the basis of isoelectric point, molecular weight and certain other properties [17,18]. This paper describes the subcellular localization, isolation of high and low Mr acid phosphatases from sheep liver and comparison of some molecular and kinetic properties between them in order to suggest their different physiological functions.

Results and Discussion

The distribution of total acid phosphatase activity in sheep liver homogenate and cellular fractions is shown in Table-1. Acid phosphatase activity is mainly associated with mitochondrial lysosomal, microsomal and soluble fractions of sheep liver as reported for rat liver [2] and chicken liver [7], 65% of the total activity was recovered in these cell particles while 35% of the enzyme activity was seen in soluble fraction. The elution profiles from Sephadex G-100 column of acid phosphatases extracted from total homogenate, mitochondrial-lysosomal, microsomal and soluble fractions are shown in Fig. 1. The total homogenate contains both the high and low molecular weight acid phosphatases. The extracts obtained from mitochondrial-lysosomes and

Table-1: Distribution of acid phosphatase activity in sheep liver cell fractions.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total Activity (U)</th>
<th>Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Homogenate</td>
<td>167.9</td>
<td>100</td>
</tr>
<tr>
<td>Nuclei</td>
<td>7.2</td>
<td>4.3</td>
</tr>
<tr>
<td>Mitochondrial Lysosomes</td>
<td>54.9</td>
<td>32.9</td>
</tr>
<tr>
<td>Microsomal</td>
<td>48.7</td>
<td>29.2</td>
</tr>
<tr>
<td>Soluble Fraction</td>
<td>56.4</td>
<td>33.8</td>
</tr>
</tbody>
</table>

Fig. 1: Elution profiles on Sephadex G-100 of extracts obtained from sheep liver cell fractions. A total homogenate; B, Mitochondrial lysosomal fraction; C, Soluble fraction; D, Microsomal fraction.
micromes contain only high molecular weight acid phosphatases while soluble fraction has low molecular weight enzyme. These findings suggest that high molecular weight enzymes are associated with cell particles while low molecular weight acid phosphatases are cytosol in nature.

The low molecular weight acid phosphatase was purified by salt fractionation, ion exchange chromatography, gel filtration procedures and finally by chromatofocusing technique using polybuffer exchanger Mono PHR column to resolve two isoenzymes at the same time. A summary of a purification procedure from 250g of fresh liver is reported in Table-2. Ac Pase A and Ac Pase B were purified about 700-800 fold from liver extract and have specific activities of 27 and 23 U/mg of protein respectively with overall yield being 0.6% in each case. Poor recovery perhaps may be due to denaturation of enzymes in polybuffer exchanger

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (ml)</th>
<th>Activity (U/ml)</th>
<th>Specific Total Activity (U/mg)</th>
<th>Yield</th>
<th>P.F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Enzymed</td>
<td>664</td>
<td>1.56</td>
<td>50</td>
<td>0.031</td>
<td>1036</td>
</tr>
<tr>
<td>2. Ammonium Sulphate (30% saturation)</td>
<td>664</td>
<td>1.130</td>
<td>30</td>
<td>0.038</td>
<td>710</td>
</tr>
<tr>
<td>3. Ammonium Sulphate (60% saturation)</td>
<td>120 l 3.89</td>
<td>105</td>
<td>0.038</td>
<td>467</td>
<td>45</td>
</tr>
<tr>
<td>4. Sephadex C-50 (Low Mr Ac Pase)</td>
<td>10 l 9.0</td>
<td>146</td>
<td>0.62</td>
<td>90</td>
<td>8.6</td>
</tr>
<tr>
<td>5. Sephadex G-75</td>
<td>7 l 11.8</td>
<td>2.35</td>
<td>5.02</td>
<td>82.6</td>
<td>79</td>
</tr>
</tbody>
</table>

Mono PHR column. The homogeneity was checked on SDS-PAGE. Single bands were detected with same electrophoretic mobility for both isoenzymes (Fig. 2). The molecular weights calculated from graph of the log of molecular weight of standard proteins versus the distance of migration were 18,000.

Activity of high molecular weight acid phosphatase towards number of physiological and non-physiological substrates was investigated. The enzyme hydrolysed p-nitrophenyl phosphate and phenyl phosphate at significant rates. Marked activity was also observed with FMN, β-glycerophosphate, phosphoenolpyruvate and phosphotyroxine. A number of hexose phosphate, ribulose-5-phosphate, ATP, ADP and many other nucleotides were also hydrolysed but at slower rates. No activity towards O-phosphoserine and O-phosphothreonine was detected. In contrast, low Mr Ac Pase did not possess broad specificity towards large variety of these phosphorylated compounds. The hydrolysis rates indicated that p-nitrophenyl phosphate, phenyl phosphate and FMN were found to be good substrates while α-glycerophosphate was hydrolysed at relatively slower rate. The enzyme also hydrolysed O-phosphotyrosine but not O-phosphoserine or O-phosphothreonine suggesting that the enzyme may act as phosphotyrosyl protein phosphatase as many low Mr acid phosphatases displayed similar activity [11,13,19,20]. The kinetic constants of two low molecular weight isoenzymes, Ac Pase A and Ac Pase B are reported in Table-3. Ac Pase B isoenzyme showed Km values for p-nitrophenyl phosphate, L-phospho-tyrosine and FMN 5.10 and 3 times greater than that of Ac Pase A isoenzyme.

Fig. 2: SDS-polyacrylamide gel electrophoresis of purified low Mr Ac Pase A and B. Lane 1, the standard proteins used were: phosphorylase-b (Mr = 97, 400); bovine serum albumin (Mr = 66,200); ovalbumin (Mr = 42,700); carbonic anhydrase (Mr = 31,000); Soyabean inhibitor (Mr 21,500); lysozyme (Mr = 14,400). Lane 2,3 and 4 increasing amount of Ac Pase A. Lane 5,6 and 7 increasing amount of Ac Pase B.
Table-3: The kinetic constants of Low Mr acid phosphatases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ac Pase A</th>
<th>Ac Pase B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>Vmax</td>
</tr>
<tr>
<td>p-nitrophenyl phosphate</td>
<td>0.05</td>
<td>32</td>
</tr>
<tr>
<td>Phenyl phosphatase</td>
<td>0.2</td>
<td>26</td>
</tr>
<tr>
<td>Phospho-tyrosine</td>
<td>0.46</td>
<td>20</td>
</tr>
<tr>
<td>FMN</td>
<td>0.20</td>
<td>23</td>
</tr>
</tbody>
</table>

Table-4: Effect of various purine compounds on the activity of low Mr acid phosphatases

<table>
<thead>
<tr>
<th>Purine and Compounds added</th>
<th>Ac Pase A (%)</th>
<th>Ac Pase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Guanosine</td>
<td>97</td>
<td>198</td>
</tr>
<tr>
<td>'5-GMP</td>
<td>104</td>
<td>229</td>
</tr>
<tr>
<td>c-GMP</td>
<td>95</td>
<td>195</td>
</tr>
<tr>
<td>6-Pyrimidinocarbosulfone</td>
<td>92</td>
<td>180</td>
</tr>
</tbody>
</table>

The activity was determined by incubation of the enzyme at 37°C and pH 5.5 in the presence of 4mM p-nitrophenyl phosphate and 2mM purine compound. The activity was expressed as percent of that in the no addition run.

respectively, but Km values for phenyl phosphate for two isoenzymes were found same. High Mr Ac Pase exhibited optimal pH range 4.5 to 5.0 while low Mr Ac Pase A and B isoenzymes had slightly higher optimal pH about 4.5 to 5.8. Since purine compounds are known to activate some low Mr Ac Pases, [15,17,21,22] their effect on both isoenzymic activities were examined in Table-4. Ac Pase B was effectively activated by Guanosine, '5-GMP, c-GMP and 6-ethylmercaptopurine. On the other hand Ac Pase A was not activated by these purine compounds. High Mr acid phosphatase was strongly inhibited by fluoride and L(+)-tartrate and found insensitive to formaldehyde while low Mr Ac Pases showed opposite behaviour. Both isoenzymes were unaffected by fluoride and tartrate and deactivated by formaldehyde (Table-5).

Phosphate, vanadate and molybdate were found to be effective inhibitors for both low and high Mr acid phosphatases but strong inhibition was observed in high Mr acid phosphatase case than in low Mr Ac Pases. Phosphate proved less powerful inhibitor than vanadate and molybdate. Millimolar concentrations of phosphate was needed to inhibit the enzymes effectively whereas micromolar concentration range of vanadate and molybdate was required to abolish the activity of the enzyme. Zn++, Ca++ and Mg++ ions had little or no inhibitory effect on both kinds of acid phosphatases. Table-6 summarizes the effect of various modifiers on the activity of acid phosphatases. Low Mr Ac Pases were stimulated in the presence of glycerol, methanol, ethanol and acetone. These activations were also appeared in many other low Mr Ac Pases which reflect a phosphotransferase activity [4,17,23] while high Mr acid phosphatase was not affected by these modifiers and thus possess phosphoryl transfer activity.

The high and low Mr acid phosphatases have been isolated and characterized in human [3-4], bovine [9] and rat liver [24]. Similar activities were also studied in amphibia [10,25] fish [26] and birds [27].

This paper for the first time reports the presence of high and low Mr acid phosphatases in sheep liver. Cell fractionation data and preliminary gel filtration experiments on sheep liver indicated that high-Mr acid phosphatases were localized in the cell particles such as lysosomes, mitochondria and microsomes etc. While low Mr acid phosphatase was present in the cytosol which is in agreement with preceding reports concerning the acid phosphatases in chicken liver [7] and rat liver [2].
Fig. 3: Elution profile of acid phosphatase activities on Sp-Sephadex C-50 (A) and on Sephadex G-75 column (B).
Low Mr Ac Pases isoenzymes are the most important enzymes among acid phosphatases because of its molecular and biochemical properties. Low Mr Ac Pase from sheep liver for the first time purified using unusual technique in a final step of purification which is based on ion exchange property combined with isoelectric focusing of proteins called chromatofocusing. Mono PHR column (Pharmacia) served the purpose. This chromatofocusing not only offered purification of the enzyme but also its resolution into two isoenzymes, Ac Pase A and Ac Pase B. Baxter and Sueter first reported the existence of two distinct forms of low Mr acid phosphatases in avian pectoral muscle. Recently two low Mr isoenzymes namely Ac P1 and AcP2 were purified from rat liver [18]. Both forms differed in several properties including pl, molecular weight, kinetic constants and purine activation. Two isoenzymes of low Mr acid phosphatase were found to exist in kidney, brain and erythrocytes of rat suggesting that two isoenzymes may be distributed in a wide variety of mammalian tissues.

The final preparations of low Mr Ac Pase A and B in sheep liver showed specific activity of 27 and 23 U/mg of protein which is relatively greater than the enzymes from skeletal muscle of frog [23] but much lower than human [4] and rat liver [18]. Previous studies [9,28] also showed that these isoenzymes were monomeric protein of 18,000 molecular weight as determined by SDS-PAGE. The possibility of small difference in molecular weights between these two isoenzymes Ac Pase A and B can not be excluded. The exact values can only be determined from their amino acid composition or sequences. However two isoenzymes differed from each other by their isoelectric points. As Ac Pase A was eluted slightly at higher pH than that of Ac Pase B when polybuffer was applied to Mono PHR Column. Thus Ac Pase A exhibited pl 6.0 whereas Ac Pase B showed pl 5.8 which are more or less similar to the values obtained for the isoenzymes purified from rat liver [17].

Both isoenzymes had quite similar pH optima. Moreover Ac Pase B was activated by purine compounds whereas Ac Pase A was insensitive to these compounds which are consistent with results reported earlier [17,18].

The low Mr acid phosphatases form sheep liver showed relatively strict substrate specificity similar to that reported for other low-Mr acid phosphatases isolated from different sources [4,12,13,18,20]. It is observed that low Mr acid phosphatases catalysed the hydrolysis of arylphosphates efficiently [4,6], on other hand aliphatic phosphatases were poor substrates with unique exception of FMN. Taga and Van Eeten [4] also noted that FMN is hydrolysed by human liver enzyme with vmax three times greater than that of p-nitrophenyl phosphate. Although the physiological significance of this high specificity for FMN is not clear. It is suggested that enzyme has a role in modifying the permeability of FMN so as to facilitate its passage through cellular membrane. Both isoenzymes Ac Pase A and B from sheep liver catalysed the hydrolysis of FMN at 72% of the vmax for p-nitrophenyl phosphate. Similar results have been reported for enzymes isolated from liver of Cyprinus carpio [29], Sea urchin and embryos [37] and rat liver [17]. Phospho-tyrosine was also hydrolysed by these both isoenzymes at same rate exhibiting 62% of vmax for p-nitrophenyl phosphate suggesting that in vivo the enzymes may act as phospho-tyrosyl protein phosphatases.

The attempt on purification of high-Mr acid phosphatase was not made. However its characterization was done with partial purified enzymes after simple gel chromatography in order to make comparison with low-Mr Ac Pases. The result obtained on calibrated Sephadex G-150 column gave an apparent molecular weight of 100,000 which is very similar to that determined for frog liver [31]. This enzyme could be a dimer. Subunit composition may be studied by SDS-PAGE provided enzyme is in purified form. The dimeric nature of the enzyme had already been reported for Zn++ -dependent acid phosphatases from chicken liver and monomer unit had Mr 50,000-52,000 [32]. High Mr acid phosphatase exhibited different behaviour. Fluoride and Li(+) tetrato were found potent inhibitors while activity of low Mr acid phosphatases were unaffected by these inhibitors. Phosphate, vanadate and molybdate inhibited both enzymes. Their inhibitory action was more pronounced in high-Mr acid phosphatases than that in low-Mr Ac Pases. These results are in accord with the findings of chicken liver [27]. Glycerol, methanol, ethanol and acetone increased the rate of
Fig. 4: Chromatofocusing of low Mr acid phosphatase and its resolution into two forms. The enzyme after Sephadex G-75 was loaded onto a polybuffer exchanger Mono PHER column (0.5 x 20 cm) equilibrated with solvent A and eluted with solvent B. Solvent A - 25 mM Bis- Tris iminodiacetic acid pH 7.1, 1mM EDTA and 1mM DTT. Solvent B - 1:10 diluted polybuffer 74 (pH 4.0), 1mM EDTA, 1mM 2-mercaptoethanol. Back pressure, 420 Psi; Flow rate, 1ml/min and Fractions, 1 ml each; Absorbance at 280 nm (-).

hydrolysis of p-nitrophenyl phosphate by low-Mr Ac Pases. This behaviour reflects a phosphotransferase activity [4,23] that catalyses transfer of phosphate group from p-nitrophenyl phosphate to alcohols as acceptors. In the presence of glycerol in the incubation mixture, the rate of phosphate transfer was greatly increased involving the formation of phosphorylated enzyme intermediate in which the removal of phosphate is rate limiting step in the reaction [33]. This effect has been documented for low-Mr Ac Pases from various sources [4,9]. No activation was observed for high Mr acid phosphatases possessing not such phosphotransferase activity.

Experimental

Sheep liver were obtained locally; p-nitrophenyl phosphate was purchased from Merck. Phenyl phosphate, β-glycerophosphate and α-D-glucose-1-phosphate from Serva while other phosphorylated substrates were the most highly purified available from Sigma Chemical Co., Sp-Sephadex C-50, Sephadex G-75, Sephadex G-100 and Sephadex G-150 were obtained from Pharmacia. All other reagents were of the purest commercially available grade.

Subcellar localization of acid phosphatases

Sheep liver was obtained immediately from slaughter house and rinsed in 0.25M cold sucrose. After two changes of sucrose solution, the livers were transferred to 0.1M Tris-HCl buffer pH 7.4 containing 0.25M sucrose, 1mM EDTA and 1mM PMSF. The tissues were cut into small pieces and homogenized with 10 full strokes in potter Elvejem homogenizer. The homogenate, filtered through gauze was adjusted to 4ml of buffer pH 7.4/g of original tissues. The cellular fractionations were carried out as reported by Araujo et al. [2] to give nuclear (650 g pellet), mitochondrial lysosomal
(10,000g pellet), microsomal (100,000g pellet) and soluble fractions. Acid phosphatases from total homogenate and particulate fractions were extracted by homogenizing in homogenizer driven at maximum speed in 2 ml of 0.3M acetate buffer pH 6.0 per gram of original tissues. Homogenates were stirred at 4°C for 1-2h, then 0.2 vol of cold 
CCl₄ was added and vigorously stirred for 10 min. Then it was centrifuged at 45,000g for 1h to get clear supernatant containing enzyme activities. Aliquots(1-1.5 ml) of extract from total homogenate, particulate fractions and soluble fraction were placed on Sephadex G-100 column (0.9 x 60 cm) and eluted with 0.1M NaCl in 0.01M acetate buffer pH 5.0 at flow rate of 15ml/h. Fractions (1ml) were collected and assayed for protein and enzyme activity.

Biochemical assays

Protein was estimated colorimetrically by the biuret method [34] with bovine serum albumin as a standard protein. Effluent fractions from column chromatographic procedures were monitored spectrophotometrically for the relative amount of protein by measuring the absorbance of the solution at 280 nm.

Acid phosphatase activity was assayed at 37°C in 0.1M sodium acetate buffer pH 5.5 containing 4mM p-nitrophenyl phosphate as substrate [35], 1mM EDTA and 50-100µl of enzyme solution in a final volume of 1.0 ml. After 5 min the reaction was stopped by the addition of 1 ml, 0.1M KOH and the absorbance was read at 405 nm. One unit of enzyme was defined as µmol of p-nitrophenol formed from substrate per min, taking a value of 1.8x10⁴ m⁻¹ cm⁻¹ as the molar extinction coefficient for p-nitrophenol.

Specific activity was expressed as units per mg of protein.

Substrate specificity studies were carried out by the determination of inorganic phosphate [36].

Optimal pH, the effects of metal ions and some modifier substances on the activity were assayed as given previously [37]. Kinetic parameters (Km and vₘₐₓ) were determined by Lineweaver Burke [38] plot using 6-8 substrate concentrations ranging from 0.025mM to 1.6mM. The ionic strength was maintained at 0.15M by adding NaCl[39].

SDS polyacrylamide gel electrophoresis was carried out by the method of Leammli [40].

The estimation of apparent molecular weight of high molecular weight acid phosphatase was obtained on calibrated column of Sephadex G-150 (0.9 x 60 cm) with the following protein markers. Alcohol dehydrogenase (80,000), bovine albumin (66,000). Carboxic anhydrase (29,000) and Aprotinin (6,500). Blue dextran was used to estimate void volume. Buffer and elution procedure were same as described above.

Purification of low Mr Ac Pases

All operations were carried out at 4°C. Acid phosphatase from sheep liver was purified as follows:

Sheep liver (250 g) was minced and immediately homogenized with 3 ml of 0.3M acetate buffer pH 5.0 containing 1mM EDTA, 0.1mM PMSF per gram of liver in waring blender for 4 min with 30 sec intervals. The homogenate was agitated for 1-2 h and centrifuged at 3840xg for 20 min in a Beckman centrifuge J-21 with rotor J-14. The supernatant was saved for next step.

Solid ammonium sulphate was added to 30% saturation with gradual additions. After 15 min, the precipitate was discarded by centrifugation at 3840xg for 20 min. and supernatant was brought to 60% saturation with ammonium sulphate and left to stand for 1h at 4°C. The precipitate obtained by centrifugation at 3840xg for 20 min was redissolved in 100ml of 0.01M acetate buffer pH 5.1 containing 1mM EDTA, and 0.1mM PMSF, 1mM 2-mercaptoethanol and dialysed overnight against the same buffer.

The dialysate was centrifuged at 10,000xg for 30 min and supernatant was poured onto a Sephadex C-50 column (3x30 cm) previously equilibrated with dialysis buffer.
The column was washed with same buffer until effluent optical density at 280 nm was less than 0.1. The enzyme was eluted as single peak with 0.3M phosphate buffer pH 5.5 containing 1mM EDTA, 0.1mM PMSF and 1mM 2-mercaptoethanol. Fractions (14 ml) were collected at a flow rate of 80ml/h. The high molecular weight acid phosphatase (lysosomal) was eluted during column washing while low molecular weight acid phosphatase (cytosol in origin) was eluted by phosphate buffer (Fig. 3A). The most active fractions from both enzymes peaks were pooled separately and precipitated with 70% saturation ammonium sulphate. The pellets were collected following the centrifugation at 3480xg for 20 min and redissolved in small volume of 0.01M acetate buffer pH 5.1, containing 1mM EDTA, 0.1mM PMSF and 1mM 2-mercaptoethanol individually.

Low molecular weight enzyme solution was applied in batches of five to Sephadex G-75 column (0.9 x 60 cm) equilibrated with 0.01M acetate buffer pH 5.1 containing 0.1M (NH₄)₂SO₄, 1mM EDTA, 0.1mM PMSF and 1mM 2-mercaptoethanol and eluted with same buffer at flow rate of 20 ml/h and 1 ml fractions were collected. Typical elution pattern is shown in Fig. 3B. The active fractions were collected and concentrated by ultrafiltration using Amicon YM₁ membrane.

The sample was dialysed against 500ml of 25mM bis-Trisminodiacetic acid buffer pH 7.1 containing 1mM EDTA and 1mM DTT. The dialysed solution was applied to a Mono PR column (0.5 x 20 cm) equilibrated with dialysing buffer and the enzyme was eluted with 1:10 diluted polybuffer 74 (pH 4.0) containing 1mM EDTA and 1mM mercaptoethanol. The pH of polybuffer was adjusted with saturated iminodiacetic acid solution. The two enzyme activity peaks were obtained and the enzymes designated as AcPase A and AcPase B, eluted at pH 6.0 and 5.8 respectively (Fig. 4).

The high Mr enzyme after Sp-Sephadex C-50 step was chromatographed in a Sephadex G-150 column (0.9x60 cm) equilibrated and eluted with buffer as described earlier. The active fractions were pooled and concentrated by ultrafiltration and used for biochemical analysis.

References