Biochemical Properties of Human Prostatic Acid Phosphatase

K. W. Lam, Olivia Li, C. Y. Li, and L. T. Yam

The electrophoretic pattern (in polyacrylamide gel) for acid phosphatases in the prostate gland was compared with that for other tissues. Isoenzyme 2 predominates in the prostate. The isoenzyme was isolated from the prostate and its biochemical properties were compared with those of acid phosphatases isolated from spleen. Isoenzyme 2 has a molecular weight of about 100,000. Its optimum pH is between 5 and 7, unlike other lysosomal enzymes. Its substrate specificity is not very much different from those of the most active isoenzymes of acid phosphatase in other tissues. Our results contraindicate the use of a specific substrate in the analysis of prostatic acid phosphatases. Determination of the isoenzyme pattern is a new approach in the specific analysis of prostatic acid phosphatases.

Additional Keyphrases: isoenzymes • diagnostic aid: prostatic cancer • enzyme patterns in various tissues • substrate specificity • organ specificity • DEAE-cellulose chromatography • enzyme molecular weight • polyacrylamide-gel electrophoresis

Increased plasma acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) activity associated with prostatic cancer was first described by Gutman et al. (1). Since then, many attempts have been made to establish specific methods for the analysis of prostatic acid phosphatase activity in plasma. Fishman and Lerner (2) suggested that tartrate sensitivity was a specific criterion for prostatic acid phosphatase. A recent study by Li et al. (3) on isoenzymes of acid phosphatases in leukocytes showed that four of five acid phosphatase isoenzymes separated by acrylamide gel electrophoresis were sensitive to tartrate inhibition, indicating that tartrate sensitivity is not a specific criterion for prostatic acid phosphatase.

Babson et al. (4) and Woodard (5) reviewed the usefulness of substrate specificity for the diagnosis of prostatic cancer, and Babson et al. proposed that α-naphthylphosphate has the same organ specificity as does glycerophosphate. The use of this substrate for prostatic acid phosphatase was criticized by Bruhn and Keller (6) and Amador et al. (7). Recently, Roy et al. (8) claimed that thymolphthalein monophosphate is a much better substrate for prostatic acid phosphatase than is naphthylphosphate.

In the meantime, multiple acid phosphatases have been demonstrated in different tissues by various workers. For example, Smith and Whitby (9) could separate prostatic acid phosphatase into 20 bands by starch-gel electrophoresis. Such reports on the multiplicity of acid phosphatases have revealed the complexity of this enzyme; much more investigation is needed in order to establish a reliable diagnostic procedure for prostatic cancer based on acid phosphatase activity. It is hoped that such a procedure will in turn lead to a better understanding of the biochemical properties of purified prostatic acid phosphatase and its relationship to other acid phosphatase isoenzymes.

We recently observed that one of the acid phosphatases in the prostate gland differs from any of the acid phosphatases of most other tissues. The purification and biochemical characterization of this acid phosphatase from human prostate are described in this report, and its unique biochemical characteristics are discussed with regard to possible applications in clinical diagnosis of prostatic cancer.

Experimental Procedures

Tissue Preparations

Human tissues used in electrophoresis studies were obtained from three refrigerated autopsy cases (Figure 1) within 4 h of death. One gram of wet tissue was homogenized in 4 ml of "Triton-100X" (5 g/100 ml). The homogenate was frozen and thawed five times, and insoluble matter was removed by centrifugation at 20,000 × g for 10 min and discarded. Equal quantities of each extract (0.1 ml) were used in all electrophoresis experiments.

Electrophoresis

Separation of acid phosphatase isoenzymes by electrophoresis in 7.5% acrylamide gel at pH 4.0 has been described in our previous publication (10). The enzymatically active bands in the gel column were stained with α-naphthylphosphate coupled to Fast
Garnet GBC, as described previously (10). Protein bands were stained with Aniline Blue-Black (2 g/liter of 10-fold diluted acetic acid) (Figure 2).

Sucrose Density-Gradient Sedimentation

The molecular weight of the purified prostatic acid phosphatase was estimated by a sucrose density-gradient sedimentation procedure (11). A preparation of purified acid phosphatase (4 U) was mixed with 0.5 mg of hemoglobin and layered on top of a sucrose density gradient (50 to 250 g/liter in a total volume of 4.6 ml) and centrifuged (Spinco Centrifuge, Rotor 50SW, 30,000 rpm) for 16 h. After this centrifugation, the gradient was fractionated by puncturing the bottom of the centrifuge tube, and taking 5 μl of each fraction for acid phosphatase analysis, with p-nitrophenylphosphate as a substrate. Samples of 0.1 ml were diluted to 0.5 ml with water, and analyzed for hemoglobin by measuring the absorbance at 410 nm.

Enzyme Activity Analysis

The substrate to be tested was incubated with a known amount of enzyme at 37°C for 30 min. We have found that the activity was linear with time during the first hour. Citrate buffer (0.1 mol/liter, pH 6.0) was used for routine analyses (Tables 1–3, and Figure 3). Because citrate interferes with phosphatase analysis by the method of Fiske–SubbaRow, acetate buffer (0.1 mol/liter, pH 6.0) was used in cases when glycerol phosphate and adenosine monophosphate were used as substrates. For the determination of the pH optimum (Figure 4), citrate buffer (0.1 mol/liter) was used below pH 6.5, Tris–citrate1 buffer (0.1 mol/liter) above pH 6.5. At the end of incubation, an alkali reagent was added to stop the enzymatic reaction and develop the color of the hydrolyzed substrate.

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1 Nonstandard abbreviations used: Tris, tri(hydroxymethyl)-aminomethane; DEAE, diethylaminoethyl; CM, carboxymethyl.

All assays were done on 1 ml of reaction mixture. The composition of the alkali reagent used to stop the reaction varied, depending on the type of substrate used in the incubation: In the case of p-nitrophenylphosphate, it was stopped by adding 2 ml of 0.2 molar NaOH, and the color measured at 410 nm (12). In the case of α-naphthylphosphate, it was stopped by adding 1 ml of a mixture consisting of Fast Red ITR salt (2 g/liter), lauryl sulfate (40 g/liter), sodium acetate (100 mmol/liter) and barbital (pH 8.0, 10 mmol/liter); the color was measured at 540 nm (13). In the case of thymolphthalein phosphate, the reaction was stopped by adding 20 ml of a mixture of Na₂CO₃ and NaOH (each 50 mmol/liter); the color was measured at 590 nm (8). In the case of adenosine monophosphate and β-glycerophosphate, trichloroacetic acid (100 g/liter) was used to stop the reaction, and hydrolysis was calculated from the results of phosphate analysis (14).

Purification of Prostatic Acid Phosphatase

Because isoenzyme 2 is the most active acid phosphatase in the prostate, we directed our efforts at purifying this isoenzyme. The purification procedure was carried out at 0°C. Fresh prostate (2 g) obtained from patients with benign hypertrophy was frozen at −70°C, then pulverized by an “Auto-pulverizer”
The pH of the supernatant fluid was adjusted to 4.0 with citric acid, the pellet discarded, and ammonium sulfate was added to the supernatant fluid to 55% saturation at 0°C (351 mg/ml). The precipitate was removed by centrifugation and discarded. The ammonium sulfate concentration in the supernatant fluid was increased to 75% saturation by adding crystalline ammonium sulfate (141 mg/ml). Most of the acid phosphatase activity was recovered in the precipitate that then appeared.

This second precipitate was dissolved in 4 ml of distilled water and dialyzed against 4 liters of Tris sulfate (10 mmol/liter, pH 8.0) for 4 h. The dialyzed sample was absorbed on a 1 × 10 cm column of DEAE-cellulose (Whatman DE-52). The column was equilibrated to the Tris buffer. This same buffer was the solvent for the salt solutions used to elute the column. The column was first eluted with 50 ml of NaCl (60 mmol/liter), then with 100 ml of NaCl (100 mmol/liter). The active fractions were then eluted by NaCl (200 mmol/liter), combined, dialyzed against the Tris buffer, and reabsorbed on another 1 × 10 cm DEAE-cellulose column. This second column was eluted by a linear concentration gradient of NaCl (increasing from 60 to 500 millimolar NaCl in 200 ml of eluate). Most of the activity was found in fractions collected when NaCl concentration was 100 mmol/liter.

After re-chromatography on a second DEAE-cellulose column, the active fraction appeared to be quite homogeneous. When the acrylamide gel column was stained for protein with Aniline Blue-Black, we saw only a single band, in the same location as the activity band (Figure 1E). Protein and activity accounted for in each purification step are summarized in Table 1. The electrophoretic analysis of acid phosphatase in different stages of purification is shown in Figure 1.

Results

Tissue Specificity of Acid Phosphatase Isoenzymes

We have recently used acrylamide-gel electrophoresis to compare the electrophoretic profiles of 20 different human tissues. The results were identical for the three different subjects. The electrophoretic profile for one of these is shown in Figure 2.

Prostatic extract was the only sample having un-

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Table 1. Activity and Protein Accounted for at Each Step of the Purification Procedure

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein, mg</th>
<th>Total activity ( \mu \text{moles} \times \text{min}^{-1} )</th>
<th>Specific activity ( \mu \text{moles} \times \text{min}^{-1} \times \text{mg}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract</td>
<td>14.0</td>
<td>820</td>
<td>58</td>
</tr>
<tr>
<td>2. 0-45% ( \text{NH}_4 )\text{SO}_4</td>
<td>2.0</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>3. 45-55% ( \text{NH}_4 )\text{SO}_4</td>
<td>2.5</td>
<td>130</td>
<td>52</td>
</tr>
<tr>
<td>4. 55-75% ( \text{NH}_4 )\text{SO}_4</td>
<td>1.6</td>
<td>360</td>
<td>225</td>
</tr>
<tr>
<td>5. First DEAE column</td>
<td>0.4</td>
<td>152</td>
<td>380</td>
</tr>
<tr>
<td>6. Second DEAE column</td>
<td>0.2</td>
<td>80</td>
<td>400</td>
</tr>
</tbody>
</table>

\( \text{a} \) Micromoles of substrate hydrolyzed per minute.  
\( \text{b} \) Activity per milligram of protein.

Table 2. Kinetic Properties of Purified Isoenzyme 2 from Prostate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m ), mmol/liter</th>
<th>( V_{max} ) ( \mu \text{moles} \times \text{min}^{-1} \times \text{mg}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \rho )-Nitrophenol phosphate</td>
<td>0.045</td>
<td>66.6</td>
</tr>
<tr>
<td>( \alpha )-Naphthol phosphate</td>
<td>0.100</td>
<td>143.0</td>
</tr>
<tr>
<td>Thymolphthalein phosphate</td>
<td>0.222</td>
<td>60.0</td>
</tr>
<tr>
<td>Adenosine 5-monophosphate</td>
<td>0.370</td>
<td>50.0</td>
</tr>
<tr>
<td>( \beta )-Glycerophosphate</td>
<td>10.000</td>
<td>5.7</td>
</tr>
</tbody>
</table>

The activity was assayed for 10 different concentrations of each substrate. The \( K_m \) and \( V_{max} \) values were calculated by plotting 1/V vs. 1/S.

Table 3. Relative Activities of Acid Phosphatase Isoenzymes Isolated from Spleen Tissue

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \rho )-Nitrophenol phosphate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Thymolphthalein phosphate</td>
<td>0.0</td>
<td>1.1</td>
<td>3.1</td>
<td>1.6</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Isoenzymes 2, 3, 4, and 5 were extracted from splenic tissue by freezing and thawing in water and were separated by DEAE-cellulose chromatography as described earlier (3). Isoenzyme 1 was extracted from splenic tissue by Triton, after exhaustive removal of all of the other isoenzymes by water extraction. Enzyme activity was assayed as described in the text.
usually high activity in the position of isoenzyme 2 (Figure 2A). Pancreas (Figure 2B) also had a distinct isoenzyme 2, but the activity was very low relative to that of the other isoenzymes. Spleen extract had some diffuse activity in the location of isoenzyme 2 (Figure 2C); we saw no distinct band. Our previous report showed that granulocytes also showed distinct isoenzyme 2 (3). Negligible isoenzyme 2 activity was observed in all the other tissues examined, isoenzymes 1 and 3 being the most active acid phosphatases in most tissues. Isoenzyme 4 activity was minor in the prostate and in several other tissues. The electrophoretic profile of semen showed only isoenzyme 2 (Figure 1F); the pattern was identical to that for the purified enzyme shown in Figure 1D.

**Molecular Weight of Purified Acid Phosphatase Isoenzyme 2**

When prostatic acid phosphatase isoenzyme 2 was mixed with hemoglobin and subjected to centrifugation, acid phosphatase activity migrated slightly faster than hemoglobin (Figure 3). The molecular weight of isoenzyme 2, calculated according to the method of Martin and Ames (11), was 100,000. This value is similar to the molecular weight reported for the prostatic acid phosphatase purified by Ostrowski and Rybarska (15).

**Biochemical Properties of Isoenzyme 2**

The pH optimum for purified prostatic acid phosphatase varied, depending on the substrate used. In general, optimal activity for all substrates was observed between pH 5.5 and 7.0 (Figure 4), a range higher than that for most lysosomal acid phosphatases, which have an optimum between pH 3 and 5 (3, 15). Roy et al. (8) also indicated that the pH optimum of prostatic acid phosphatase was between 5.8–6.4.

The kinetic data on purified prostatic acid phosphatase, with different substrates, are summarized in Table 2. The $V_{\text{max}}$ for each substrate was obtained by extrapolating to a substrate concentration of infinity ([1/S] = 0). These extrapolated values are higher than the velocities shown in Figure 3, in which the activity was obtained from assays under specified substrate concentration. Purified prostatic acid phosphatase (isoenzyme 2) was inhibited by thymolphthalein phosphate at concentrations above 0.2 mmol/liter. The $V_{\text{max}}$ values for the hydrolysis of thymolphthalein phosphate shown in Table 1 were obtained by extrapolating the data, with use of substrate concentrations of less than 0.2 mmol/liter. Substrate inhibition caused by thymolphthalein phosphate could be minimized by adding bovine serum albumin (5 mg/ml of assay medium). When purified isoenzyme 2 was mixed with a normal serum sample, thymolphthalein phosphate was inhibitory in concentrations greater than 2 mmol/liter. The results obtained with added albumin were similar to those described by Roy et al. (8), who first described the relationship between thymolphthalein phosphate (or p-nitrophenylphosphate) concentration and enzyme activity, with use of crude prostatic extract or serum. Purified prostatic acid phosphatase was not inhibited when p-nitrophenyl or naphthylphosphate were used as substrates.

The activity of purified isoenzyme 2 was compared with that of other acid phosphatase isoenzymes isolated from spleen extract. The spleen sample was obtained from a patient with leukemic reticuloendotheliosis. This sample was chosen because it contains acid phosphatase isoenzyme 2 as well as the others (17). Isoenzymes 1, 2, 3, 4, and 5 were separated from each other on a DEAE-cellulose column, as described previously (3). We assayed the activity of known amounts of each isoenzyme, with use of different substrates under identical experimental conditions. The rate of hydrolysis for $p$-nitrophenylphosphate was arbitrarily taken as 1. Table 3 shows the rate of hydrolysis of thymolphthalein phosphate relative to that of $p$-nitrophenylphosphate. Thymolphthalein phosphate was a good substrate for isoenzyme 2. However, it was also hydrolyzed rapidly by isoenzyme 3, which is a major acid phosphatase isoenzyme in most tissues.

**Discussion**

The electrophoretic procedure used in this investigation has demonstrated the distinctive features of extracellular acid phosphatase isoenzyme 2 secreted by the prostate, as compared to other acid phosphatase isoenzymes inside the lysosome. This procedure is much better than the starch-gel procedure (9), which yields a continuous smear of activity bands.

Because only isoenzyme 2 was present in semen, this isoenzyme evidently has a specific extracellular function. The other isoenzymes in prostatic tissue (1 and 4) remain inside the tissue cells, as do the lysosomal acid phosphatases in other tissues. The existence of a specific extracellular function for isoenzyme 2 was also suggested by its pH optimum of 6.5, which is the physiological acidity of prostatic fluid (18). In contrast, lysosomal acid phosphatases are most active between pH 3 and 5 (3, 16).

The present data on the electrophoretic characteristics of prostatic acid phosphatase provide a new approach to the specific diagnosis of prostatic cancer. The activity of isoenzyme 2 in the spleen, granulocyte, and pancreas is very low compared to that of isoenzymes 1 and 3—not high enough to present a problem for our approach. (The findings concerning the profile of acid phosphatase in the plasma of normal and pathologic subjects will be presented in a future publication.)

The biochemical data from the purified prostatic acid phosphatase show that activity is maximal with naphthylphosphate as the substrate (Table 2). Our previous data (3) showed that naphthylphosphate is also a good substrate for isoenzymes 1, 3, and 4, which are the major isoenzymes in all tissues. We
have now examined the specificity of thymolphthalein phosphate. This substrate is also acted upon by isoenzymes 3 and 4, which are the major acid phosphatase isoenzymes in other tissues (Table 3).

Purification procedures for prostatic acid phosphatase have been described by Davidson and Fishman (19) and by Ostrowski and Rybarska (15). We found that the ammonium sulfate fractionation procedure described by Davidson and Fishman (19) yielded a preparation containing both isoenzymes 2 and 4. DEAE-cellulose chromatography was introduced in our present investigation to separate isoenzyme 2 from all of the other acid phosphatases. The DEAE-cellulose used in our investigation was equilibrated in a pH 8.0 buffer, and the elution buffer was also adjusted to pH 8.0. At this pH, isoenzyme 2 has a higher affinity for this ion-exchange resin and a higher salt concentration is required to elute it from the column. In this way, we obtained a resolution very much better than that obtained by Ostrowski and Rybarska (15) with a pH 6.0 buffer. The CM-cellulose chromatography step described by Ostrowski did not improve the specific activity of our preparations. Thus, the purification procedure was simplified solely by our method.

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References