Enzyme Immunoassay for Tartrate-Resistant Acid Phosphatase

Kwok-Wal Lam, Mark Siemens, Tseih Sun, Chin-Yang Li, and Lung T. Yam

An immunochemical method for quantitative analysis of the tartrate-resistant acid phosphatase (EC 3.1.3.2), band 5, is presented. This method involves precipitation of the enzyme from the serum by the antibody specific to band 5 and by sheep anti-rabbit immunoglobulin, followed by analysis of the enzyme activity in the precipitate. The precipitation procedure eliminates the interferences of the tartrate-sensitive phosphatase of all tissues, of the tartrate-resistant phosphatase of erythrocytes, and of unknown substances that interfere with the colorimetric method. We compare the present method with previously described colorimetric and electrophoretic methods.

**Additional Keyphrases:** isoenzymes • enzyme activity • cancer • pediatric chemistry

Acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] has been known generally as a marker enzyme for the diagnosis of prostatic cancer for decades. Extensive effort has been directed to establish analytical methods specific to the prostatic phosphatase. Abdul-Fadil and King (1) observed that the prostatic acid phosphatase was inhibited by tartrate, while the acid phosphatases of erythrocytes and of normal serum were not. Since then, the tartrate-sensitive fraction of serum acid phosphatase has been called the "prostatic fraction" by some clinical chemists. The tartrate-resistant fraction of serum phosphatase usually is ignored in clinical laboratory reports. In recent years the immunochemical methods designed for the prostatic acid phosphatase do not measure the tartrate-resistant fraction at all.

Our interest in the tartrate-resistant acid phosphatase began in the recognition of its diagnostic value for leukemic reticuloendotheliosis (haire cell leukemia) (2). Electrophoresis on an acidic acrylamide gel column showed that the enzyme in the leukemic cells occurs as a band (5) that is distinctly separated from the tartrate-sensitive enzymes (bands 0, 1, 2, 3, and 4) of tissue cells. Acid phosphatase band 5 is absent or exists in trace amounts in normal human tissues (3) and in serum of normal adults (4). A histochemical study of the occurrence of band 5 revealed strong enzyme activity in hairy cells (2), Gaucher's cells, and osteoclastic bone tumor (5). Increases of band 5 in the serum were seen in Gaucher's disease (6-9), in the serum of normal children during physiologic bone growth (4, 5), and in malignancies metastasized to bone (10, 11). The fact that acid phosphatase band 5 is increased in pathologic sera prompted our interest in the quantitative analysis of band 5 acid phosphatase.

Previous data showed that the tartrate-resistant acid phosphatase in hairy cells, Gaucher's cells, osteoclasts, and serum from children are antigenically identical (12).

Therefore, the specific antiserum produced for band 5 is useful for the analysis of tartrate-resistant acid phosphatase in all of the pathologic serum specimens described above. We describe a simple enzyme immunoassay procedure and compare it with electrophoretic and the classic colorimetric methods.

**Materials and Methods**

**Antigen and antibody:** Acid phosphatase band 5b was purified from the spleen of a patient with hairy cell leukemia. The primary antiserum was produced by injecting the purified acid phosphatase into a rabbit as described previously (12). The antiserum was stored at -45 °C and diluted with saline before use. The secondary antiserum was sheep anti-rabbit immunoglobulin, provided to us by New England Nuclear, Boston, MA 02118.

**Colorimetric analysis:** The sample was incubated at 37 °C with p-nitrophenyl phosphate (4 mmol/L) in acetate buffer (0.1 mol/L, pH 5.5) in a final volume of 1 mL. The enzyme activity, mU, is defined as nanomoles of substrate hydrolyzed per minute under these assay conditions (13).

**Electrophoresis:** The purified enzyme was mixed with the sample gel containing 4 mg of bovine serum albumin and placed on top of the spacer gel as described elsewhere (13). When serum samples were analyzed, albumin was not added to the sample gel. The gels were stained for enzyme activity, with use of 1-naphthyl phosphate as substrate (13).

**Enzyme immunoassay:** Briefly, the optimized assay procedure is as follows:

1. Mix 0.2 mL of sample (serum or pure enzyme) with 0.2 mL of 0.1 mol/L Tris phosphate buffer (pH 7.0), 0.4 mL of primary antiserum (500-fold dilution), and 1.0 mL of secondary antiserum (sheep anti-rabbit immunoglobulin), and let stand at room temperature for 15 min.

2. Separate the antigen–antibody complex by centrifugation at 1000 × g for 10 min; discard the supernatant fraction.

3. Resuspend the precipitate in 2.0 mL of a solution of citrate (0.1 mol/L, pH 5) and albumin (1 g/L) by vortex-mixing for 30 s. Recover the precipitate by centrifuging at 1000 × g for 20 min; discard the supernatant fraction.

4. Repeat step 3.

5. Suspend the precipitate in 1.0 mL of assay medium for colorimetric analysis of acid phosphatase activity as described earlier. Incubate the assay mixture at 37 °C for 30 min. Stop the reaction by adding 2 mL of 0.1 mol/L NaOH, then measure the absorbance of the yellow color at 410 nm.

**Results**

The antigen formed an insoluble precipitate with the primary and secondary antibodies within 15 min. Only 42% of the enzyme activity was recovered in the precipitate. Pre-incubation of the antigen with the primary antibody before the addition of the secondary antibody did not improve antigen–antibody binding (Table 1), nor did prolonging the incubation improve the recovery of enzyme activity. There was no detectable enzyme activity in the supernatant fraction, indicating that the antigen was completely precipitated by the antibody. The low recovery of the enzyme activity in the precipitate indicated that the antigen–antibody complex had

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Table 1. Effect of Incubation Time on Antigen–Antibody Binding

<table>
<thead>
<tr>
<th>Incubation time, min</th>
<th>Activity in antigen–antibody complex, mU</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
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<tr>
<td>Experiment 1</td>
<td></td>
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<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>0</td>
<td>10</td>
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<tr>
<td>0</td>
<td>30</td>
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<tr>
<td>0</td>
<td>60</td>
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<tr>
<td>Experiment 2</td>
<td></td>
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<td>0</td>
<td>10</td>
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<td>10</td>
<td>10</td>
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<tr>
<td>30</td>
<td>10</td>
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<tr>
<td>60</td>
<td>10</td>
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<tr>
<td>120</td>
<td>10</td>
</tr>
<tr>
<td>240</td>
<td>10</td>
</tr>
<tr>
<td>Overnight</td>
<td>10</td>
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*Experiment 1: One incubation of enzyme with both antisera. Experiment 2: The enzyme was incubated first with the primary antiserum, then incubated again with the second antiserum.*

less enzyme activity than the free antigens. The enzyme activity in the precipitate was not improved by increased substrate concentration.

The recovery of enzyme activity is about the same between pH 5.5 and 7.5 (Figure 1), decreasing as the pH of the incubation medium increased above pH 7.5 or below pH 5.5. The recovery of enzyme activity was about the same even when the primary antibody was diluted as much as 1000-fold; greater dilution of the primary antiserum decreased recovery.

The percentage of enzyme activity recovered was about the same when the enzyme activity added during incubation was less than 7.42 mU (Table 2). Recovery was less at greater enzyme activities, indicating saturation of the antibody. There was no binding of the antibody to the other types of acid phosphatase isolated from the prostate (bands 2 and 4), leukocytes (band 3), platelets (band 3), or erythrocytes. The enzyme activity in the antigen–antibody is linearly proportional to the incubation time in step 5 (Figure 2).

Table 2. Specific Recovery of Band 5b in the Enzyme Immunoassay

<table>
<thead>
<tr>
<th>Enzyme incubated with antibody, mU</th>
<th>n</th>
<th>Acty recovered in the precipitate, mU</th>
<th>% recovery, mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.47</td>
<td>4</td>
<td>1.04 ± 0.08</td>
<td>42.1 ± 3.3</td>
</tr>
<tr>
<td>7.42</td>
<td>6</td>
<td>3.13 ± 0.03</td>
<td>42.2 ± 1.2</td>
</tr>
<tr>
<td>17.32</td>
<td>5</td>
<td>6.16 ± 0.03</td>
<td>35.6 ± 1.0</td>
</tr>
<tr>
<td>24.73</td>
<td>4</td>
<td>7.37 ± 0.02</td>
<td>29.8 ± 0.7</td>
</tr>
<tr>
<td>37.10</td>
<td>5</td>
<td>8.12 ± 0.03</td>
<td>21.9 ± 1.2</td>
</tr>
</tbody>
</table>

The highly purified enzyme preparation (spec. acty. >100 kU/g) is labile at 4 °C, as determined colorimetrically, with about half of the original activity being lost after standing at 0–4 °C for three days (Table 3). However, as determined by enzyme immunoassay, the same enzyme preparation was quite stable throughout the eight days of testing (Table 3); apparently, the aged enzyme was reactivated upon binding to the antibody.

The enzyme immunoassay gave lower values than the colorimetric method and the electrophoretic method, unless one corrects for the fact that recovery in the precipitate is 42%. The comparison of three analytical methods for serum samples of normal children and adults is summarized in Table 4; little change was noted for samples analyzed on two different days. The three methods gave similar results for sera obtained from prostatic cancer patients with metastasis to bone (diagnosis based on bone scan).

Discussion

At least four types of acid phosphatase have been thoroughly studied in many laboratories: (a) the enzyme secreted by the prostate, (b) the enzyme confined to the lysosome of all tissues, (c) the erythrocytic enzyme, and (d) band 5 enzyme, found in hairy cells, Gaucher's cells, and osteoclasts. The prostatic and lysosomal enzymes are tartrate sensitive, whereas the erythrocytic and band 5 enzymes are resistant. Our previous immunodiffusion data (12, 14) indicated that the antiserum prepared for band 5b of hairy cells binds to the tartrate-resistant band 5a and 5b isolated from normal serum and from cases of Gaucher's spleen and osteoclastic tumor,
but does not bind the tartrate-resistant enzymes or the erythrocytic enzymes. We obtained the same results with this enzyme immunoassay. The immunochemical method described probably measures the activity of both bands, 5a and 5b. However, the increase in tartrate-resistant acid phosphatase in serum is caused by an increase of band 5b only. We have not observed increases of band 5a in any pathologic serum specimens. Therefore, the method presented here is satisfactory for estimating the increase of band 5b in Gaucher’s disease, and in osteoclastic lesions among cancer patients.

One of the unexpected observations of the above method is the short incubation time for precipitating the enzyme. We have used similar immunochemical procedures to detect other hydrolases, for which much longer incubation times are required. Use of this short incubation time avoids the long waiting times required in radioimmunoassay, immunodiffusion, and immunoelectrophoresis.

The stability of the enzyme is another surprising observation. The purified enzyme loses its activity upon storage at 0–4 °C, as measured by colorimetry. However, the inactivated enzyme is re-activated after precipitation with the antibody. Inactivation during storage of a diluted enzyme solution is a common problem for many enzymes, owing to denaturation of the quaternary structure. It is quite possible that binding the aged enzyme to the antibody converts the enzyme back to the active form. Also, because some of the enzyme in the serum may be partly inactivated, the immunochemical method could produce a higher activity than the analysis of whole serum by the colorimetric method. The ability of the antibody to re-activate the aged enzyme caused some confusion in our earlier study in calculating the percentage of enzyme recovered. If the purified enzyme is partly inactivated, the enzyme activity in the antigen–antibody complex exceeds the activity of the freshly prepared enzyme. The average recovery in this study, based on freshly prepared enzyme, is 42%.

Four methods are available now for the analysis of the tartrate-resistant acid phosphatase band 5: colorimetric, column-chromotographic, electrophoretic, and immunochemical methods. The colorimetric method is the easiest way to estimate tartrate-resistant acid phosphatase activity. The major problem of the colorimetric method, regardless of substrate used, was seen among serum specimens obtained from prostatic cancer patients, in whom serum acid phosphatase often exceeds 500 U/L and increases of band 5 due to malignancies metastasized to bone vary between 10 to 100 U/L. Interference from residual activity of prostatic acid phosphatase is, therefore, a serious obstacle to obtaining accurate estimation of band 5 activity in the serum of prostatic cancer patients. This is not as serious a problem for serum in which the tartrate-sensitive fraction is not great.

The electrophoretic method (13) distinguishes band 5a and 5b, eliminates interference from the erythrocytic phosphatase and the tartrate-sensitive enzymes, and is quite satisfactory for qualitative purposes. However, quantitative estimation of band 5 by electrophoresis is difficult and requires an expensive densitometer. Moreover, for samples with extremely high band 2 activity (>1000 U/L), excessive hydrolase in the band 2 position rapidly affects the staining solution, resulting in poor staining in the band 5 position.

Ion-exchange column chromatography is appropriate for isolating each enzyme in large quantities, but is too tedious for quantitative analysis. A unique characteristic of band 5 is its high affinity on a cation-exchange resin. However, some of the band 5 in serum interacts with serum protein and is eluted out of the column at a low salt concentration, so that the activity recovered in the fraction eluted by a high salt concentration does not represent total band 5 activity in the serum.

The immunochemical method is much simpler than the column-chromatographic and electrophoretic methods, and is more sensitive than any of the other methods. Separation
of acid phosphatase from the serum proteins by precipitation with the antibody eliminates the contamination by other acid phosphatases, and also the background absorbance of the serum at 410 nm. The small amount of enzyme in a large volume of serum is concentrated in the immunochemical precipitation step, giving an intense yellow color in the final colorimetric analysis, with a low blank value. The linear relationship between quantity of substrate hydrolyzed and incubation time, up to 2 h (Figure 2), allows one to use long incubation times for samples having low enzyme activity.

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References


