Prostatic Acid Phosphatase Assay with Self-Indicating Substrate 2,6-Dichloro-4-acetylphenyl Phosphate

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We characterized six self-indicating substrates, synthesized as the derivative compounds of acetylphenyl phosphate, for serum prostatic acid phosphatase (PAP) activity. One of the substrates, 2,6-dichloro-4-acetylphenyl phosphate (DCAPP), is superior to others in terms of stability, affinity, and low $K_m$ for PAP. The hydrolyzed product, 2,6-dichloro-4-acetylphenol (DCAP), has a maximum absorption at 334.2 nm, a $pK_a$ of 4.15, and a molar absorptivity at 340 nm of 21 490 L · mol$^{-1}$ · cm$^{-1}$ in citrate-HCl buffer, pH 5.4. PAP activity was assessed by subtracting tartaric acid-inhibited acid phosphatase activity from total acid phosphatase activity. Our assay system involving DCAPP is a unique kinetic method that shows good reproducibility, wide analytical dynamic range, and high specificity for PAP. Moreover, it is easily adaptable to automated analyzers because the product, DCAP, can be monitored at 340 nm.

Indexing Terms: enzyme kinetics/enzyme immunoassay

Many methods for the determination of serum acid phosphatase (ACP; EC 3.1.3.2) activity have been reported, but most of them were not adaptable to automated analyzers (1–3).4 Only the kinetic method of Hillmann and its modifications (4–6), which basically utilize 1-naphthyl phosphate (1-NA) and diazodye Fast Red TR, have been adapted to automated analyzers. Although 1-NA was superior to other substrates in terms of specificity, its color reaction was subject to interference by bilirubin (7). Another kinetic method involving a self-indicating substrate, 2,6-dichloro-4-nitrophenyl phosphate (DCNPP), has been reported (8). The hydrolyzed product of DCNPP, 2,6-dichloro-4-nitrophenol (DCNP), shows stronger 400-nm absorption at pH <9 than does 4-nitrophenol and can be monitored by automated analyzers at pH 5.4. However, this method also has several disadvantages; e.g., serum albumin quantitatively accelerates the rate of hydrolysis of DCNPP to DCNP (9), and hemoglobin, denatured in acid solution, influences spectrophotometric measurement at 405 or 415 nm.

To develop an assay that does not have these drawbacks, we focused on six synthesized derivative compounds of acetylphenyl phosphate (10), and found that 2,6-dichloro-4-acetylphenyl phosphate (DCAPP) had advantages of stability, affinity, and $K_m$ for prostatic acid phosphatase (PAP).

In this study, we describe the characteristics of a new assay and its application to automated analyzers.

Materials and Methods

Apparatus

Spectra of substrates and their products were analyzed with a Hitachi U-3200 spectrophotometer (Hitachi, Tokyo, Japan) and enzyme activity was measured with a Hitachi 7050 automated analyzer. The enzyme immunoassay (EIA) was done with an IB-500 analyzer (Toyobo, Tokyo, Japan).

Reagents

Substrates [DCAPP; 2,6-dichloro-4-propionylphenyl phosphate; 2,6-dichloro-4-(2-butyryl)phenyl phosphate; 2,6-dichloro-4-(1-butyryl)phenyl phosphate; 2,6-difluoro-4-acetylphenyl phosphate (DFAPP), 2,6-dibromo-4-acetylphenyl phosphate (DBAPP)] and their hydrolyzed products were obtained from Nitto Boseki (Fukushima, Japan). Bovine serum albumin and human PAP were obtained from Sigma Chemical Co. (St. Louis, MO). Assay kits for DCNPP, 1-NA, and PAP EIA were purchased from Ono (Osaka, Japan), Boehringer Mannheim (Tokyo, Japan), and Dainabot (Tokyo, Japan), respectively. All other reagents were analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan). In measurement of total ACP (T-ACP) activity, reagent 1 consists of citrate buffer (0.1 mol/L sodium citrate-HCl, pH 5.4, and 5.0 g/L bovine serum albumin, which gives a final concentration of 3.8 g/L in assay mixture) and reagent 2 contains substrate (6.0 mmol/L DCAPP in 0.01 mol/L sodium citrate-HCl, pH 3.0). In measurement of tartaric acid-inhibited ACP (TIAP) activity, citrate-HCl buffer containing 26 mmol/L L- (+)-tartaric acid was used instead of reagent 1. These reagents were stable for at least 1 year at 4°C.

Samples

PAP in serum was stabilized by adding 10 μL of 3.3 mol/L acetic acid per 1.0 mL of serum immediately after serum was separated from clotted blood. Pre-treated sera were stored at −20°C until use.

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4 Nonstandard abbreviations: ACP, acid phosphatase; PAP, prostatic acid phosphatase; T-ACP, total acid phosphatase; TIAP, tartaric acid-inhibited acid phosphatase; DCAPP, 2,6-dichloro-4-acetylphenyl phosphate; DFAPP, 2,6-difluoro-4-acetylphenyl phosphate; DBAPP, 2,6-dibromo-4-acetylphenyl phosphate; DCAP, 2,6-dichloro-4-acetylphenol; 1-NA, 1-naphthyl phosphate; DCNPP, 2,6-dichloro-4-nitrophenyl phosphate; DCNP, 2,6-dichloro-4-nitrophenol; and EIA, enzyme immunoassay.

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Tissue Extracts

To assess $K_m$ values of ACP from various organs, we prepared ACP from tissue extracts by homogenization and centrifugation (1400g, 10 min, 5°C) in 0.1 mol/L citrate-HCl buffer (pH 5.4) (11).

PAP Assay

In experiments comparing substrates, we pipetted 100 μL of sample, mixed it with 2 mL of buffer solution, incubated the mixture for 3 min to reach 37°C, and started monitoring the reaction rate at 340 nm immediately after adding 500 μL of substrate solution. The final pH of the mixture was 5.4. We used the Hitachi 7050 automated analyzer in other experiments, mixing 20 μL of sample with 400 μL of buffer solution and then adding 100 μL of substrate solution to start the reaction. We monitored the reaction in rate mode from 140 to 240 s at 340 nm and calculated the enzyme activity from ΔA/min. We estimated the PAP activity by subtracting TiAP activity from T-ACP activity; we investigated the inhibitory effects of L-tartaric acid on PAP activity in crude extract from various organs.

Characterization of Substrates and Their Products

To characterize the six synthesized substrates and their products, we determined their pH optimum, nonenzymatic hydrolysis, $K_m$, and molar extinction coefficient.

Nonenzymatic hydrolysis was assessed by measuring the 340-nm absorbance of the substrate solution (final concentration 1.15 mmol/L) at 37°C against a simple buffer solution.

$K_m$ was calculated by a Lineweaver–Burk plot. To obtain the $K_m$ of ACP from various organs, we used eight concentrations of DCAPP between 0.01 and 0.5 mmol/L and measured each point three times.

The apparent $e$ was determined by using the Hitachi U-3200 spectrophotometer and a Hitachi automated analyzer.

Interferences

Besides examining interferences of various compounds with PAP activity, we also investigated the effects of various abnormal sera on the spectrophotometry itself, not on the enzyme activity. The sera tested were hemolytic (n = 10; maximum concentration 1000 mg/L as hemoglobin), icteric (n = 13; maximum concentration 20 mg/L as total bilirubin), and lipemic (n = 5; turbid appearance). In this experiment we omitted substrate from reagent 2.

Comparison of Methods

To compare the present method (applied to automated analyzers) with the DCNPP method, the 1-NA method, and the PAP EIA method, we used several stabilized human sera. The enzymatic methods were run on the Hitachi 7050 analyzer and the PAP EIA method on the IB-500 analyzer according to each manufacturer’s instructions.

Results

Characterization of Substrates and Their Products

The optimum pH for DFAPP and DBAPP was 5.6 and 5.8, respectively; that for the other four substrates was 6.0.

Nonenzymatic hydrolysis accounted for between 0.5 × 10⁻⁸ and 3.8 × 10⁻⁹ A/min at pH 5.4.

The $K_m$ of the six substrates ranged from 0.147 to 0.233 mmol/L. DCAPP had the smallest $K_m$ value and the highest velocity in the PAP assay, and showed satisfactory stability as a substrate. Therefore, we chose DCAPP as the substrate for PAP activity measurement. The absorption curves of DCAPP and DCAP are shown in Fig. 1.

DCAP had a maximum absorption at 334.2 nm, a $pK_a$ of 4.15, and a molar absorptivity of 21 490 L·mol⁻¹·cm⁻¹ at 340 nm in citrate-HCl buffer (pH 5.4) containing 3.8 g/L bovine serum albumin. The $pK_a$ of DCAP was 4.6 in 0.1 mol/L citrate-HCl buffer, but the apparent $pK_a$ shifted to 4.15 and the $e$ increased from 15 200 to 21 490 L·mol⁻¹·cm⁻¹ when bovine serum albumin was added to the buffer solution at a final concentration of 3.8 g/L.

Optimization of Variables for PAP Assay

Buffer solution. After we had examined various buffer solutions for substrates, we selected citrate-HCl buffer because of its stabilizing effect on substrate, absorbance of DCAP, tartaric inhibition of DCAPP, and $pK_a$. The pH optimum for the PAP-catalyzed hydrolysis of DCAPP in citrate-HCl buffer was ~5.6, with activity ≥95% maximum over the range 4.8–6.2. We selected pH 5.4 because the ionized DCAP dissociates nearly 100% at that pH and autohydrolysis of the substrate was less marked at pH 5.4 than at 6.0. The buffer
concentration was set to 0.1 mol/L because PAP activity was maximum.

$K_m$ for human ACP in various organs. $K_m$ values for ACP from various human organs in 0.1 mol/L citrate-HCl buffer (pH 5.4) containing 3.8 g/L bovine serum albumin is summarized in Table 1. The $K_m$ of PAP (1.4 $\times$ 10^{-4} mol/L) was 0.1 that of other organ ACPs, and DCAPP showed stronger affinity for PAP than for other organ ACPs. The final concentration of DCAPP in the PAP assay was 1.15 $\times$ 10^{-3} mol/L, based on maximum velocity ($V_{max}$).

Evaluation of the Present Method by Automated Analyzers

Molar absorptivity ($e$). As described above, the apparent $e$ for DCAP was determined to be 21 490 L·mol^{-1}·cm^{-1} at 340 nm on the Hitachi U-3200 spectrophotometer. With the Hitachi 7050 automated analyzer, $e$ was 19 950 L·mol^{-1}·cm^{-1} at 340 nm, and the calculated K-factor was 1295.

Analytical range. The detection limit of the PAP assay was calculated according to Miller and Miller (12). The mean value obtained from 30 measurements of a blank (isotonic saline) was 0.83 nkat/L (SD 1.7 nkat/L), and the detection limit was 5.0 nkat/L. The upper limit of linearity with PAP was 2167 nkat/L at 37°C.

Precision. Within-run imprecision was evaluated with three different concentrations of serum samples assayed 20 times each. The means and CVs for low, medium, and high concentrations of PAP were 17.2, 150, and 415 nkat/L and 8.30%, 1.19%, and 0.48%, respectively. Between-run imprecision was evaluated with two stabilized pooled serum samples. The means and CVs were 173 and 723 nkat/L and 1.20% and 1.23% (n = 20), respectively.

Recovery. Analytical recovery was assessed with Precinorm-E control serum (151 nkat/L) with addition of three concentrated PAP solutions (92, 385, 992 nkat/L). The recovery was between 103% and 104%.

Interferences. Substances tested did not interfere with measured PAP activity. Spectrophotometrically, the interfering effects of abnormal sera tested were negligible at those concentrations (Table 2).

Table 1. $K_m$ for human ACP from various organs with DCAPP as substrate.

<table>
<thead>
<tr>
<th>Origin of enzyme</th>
<th>$K_m$ (10^{-3} mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>0.147</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.250</td>
</tr>
<tr>
<td>Liver</td>
<td>1.429</td>
</tr>
<tr>
<td>Heart</td>
<td>0.800</td>
</tr>
<tr>
<td>Lung</td>
<td>1.667</td>
</tr>
<tr>
<td>Bone</td>
<td>2.500</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>2.857</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>2.000</td>
</tr>
<tr>
<td>Platelets</td>
<td>1.667</td>
</tr>
</tbody>
</table>

Table 2. Results of interference study.

<table>
<thead>
<tr>
<th>Substances tested</th>
<th>Maximum conc with no interference, mmol/L (except as indicated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>2.5</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>50.0 (g/L)</td>
</tr>
<tr>
<td>Ditaurobilirubin</td>
<td>0.23</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.3</td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>1.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>25.0</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>4.5 (g/L)</td>
</tr>
<tr>
<td>Uric acid</td>
<td>1.2</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>50.0</td>
</tr>
<tr>
<td>FeCl$_2$</td>
<td>0.25</td>
</tr>
<tr>
<td>NaCl</td>
<td>600.0</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>35.0</td>
</tr>
<tr>
<td>EDTA-2Na</td>
<td>5.0</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>30.0</td>
</tr>
<tr>
<td>Sodium heparin</td>
<td>200.0 (mg/L)</td>
</tr>
</tbody>
</table>

Methods Comparison

Correlation coefficients between the present method (y) and the 1-NA method, the DCNPP method, and the PAP EIA method were 0.999 (y = 0.96x + 0.2, n = 98), 0.995 (y = 0.80x - 7.7, n = 98), and 0.986 (y = 0.39x + 1.1, n = 121), respectively.

Discussion

To overcome several disadvantages involved in conventional methods for PAP activity (1–8), we have developed a new assay and described its performance. DCAPP, a self-indicating synthetic substrate, has played a key role.

The $K_m$ of DCAPP for PAP was 0.147 mmol/L in 0.1 mol/L citrate-HCl buffer (pH 5.4), close to that of DCNPP (0.137 mmol/L) already reported (8). However, the $e$ of the hydrolyzed product, DCAP, was 38% greater than that of DCNP (21 490 vs 15 600 L·mol^{-1}·cm^{-1}) (8). Therefore, the DCAPP method is expected to be more precise than other methods even for low PAP activity. Moreover, our method is less susceptible to hemoglobin interference than the DCNPP method, and is applicable to automated analyzers because DCAP has a maximum absorption at 334.2 nm.

The pK$_a$ of DCAP was shifted from pH 4.5 to 4.15 by adding albumin to the buffer solution. This increases the $e$ of DCAP up to 15% in 0.1 mol/L citrate-HCl buffer (pH 5.4). The shift may be related to the acetyl radical (C=O bond) forming resonance structures with amino groups of serum proteins in acidic conditions.

Because some serum albumin increases the rate of DCNPP hydrolysis (9), we added albumin to the assay buffer to give a final concentration of 3.8 g/L. We did not see any significant DCAPP hydrolysis, and obtained good correlations with the three methods except for the DCNPP method, which showed the bias already reported (9). The citrate buffer containing albumin was stable for 1 year at 4°C without any stabilizer.

The $K_m$ value of PAP was 1.4 $\times$ 10^{-4} mol/L in 0.1 mol/L citrate-HCl buffer, whereas those of ACP from
blood cells (erythrocytes, leukocytes, and platelets) were 1.67–2.86 \times 10^{-3} \text{ mol/L} \text{ when using DCAPP as the substrate}. Since the \(K_m\) of PAP was 0.1 that of ACP from blood cells, the affinity of DCAPP for PAP turned out to be stronger than for ACP from blood cells (Table 1).

We selected 1.15 \times 10^{-3} \text{ mol/L} as the final substrate concentration at which the \(V_{max}\) was 89% with PAP. For PAP activity measurement, the substrate concentration should be >1.15 \times 10^{-3} \text{ mol/L}, but this will result in high blank value (>0.1 \text{ A}), as shown in Fig. 1.

In this study, we measured PAP activity by L-tartrate inhibition. The PAP activity was inhibited by 98% with \(\geq 20 \text{ mmol/L L-tartrate}\), whereas ACP from blood cells was inhibited by 6–20.1%. The inhibition rate of PAP in this study was in good agreement with that of the 1-NA method, whereas that of ACP from other organs was not (3). The inhibition of other organ enzymes ranged from 50.0% to 72.8%, but they were less than that of the enzyme from blood cells contaminated with serum. Consequently, the tartrate inhibitory method does not impair the specificity of the PAP activity measurement. The correlation between the present method and PAP EIA method (\(r = 0.986\)) validates the specificity.

In summary, for the measurement of PAP activity, our kinetic method involving the self-indicating substrate DCAPP showed satisfactory performance on automated analyzers. Moreover, the new method was free from albumin interference, unlike the DCNPP method.

References