An Optimized Continuous-Monitoring Procedure for Semiautomated Determination of Serum Acid Phosphatase Activity

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A continuous-monitoring method for measuring acid phosphatase activity with α-naphthyl phosphate as the substrate was critically evaluated and modified. Using partially purified prostatic acid phosphatase, we show that certain conditions for the assay must be satisfied to ensure linearity. These conditions include maintaining the pH between 5.6 and 5.9 and the addition of detergent to sustain linearity. The results obtained with α-naphthyl phosphate have been compared with those obtained by using p-nitrophenyl phosphate as substrate. When used with an automatic rate analyzer, the modified method is as sensitive but more reproducible.

One of the shortcomings of measuring acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) is that most of the assay procedures for the enzyme are “end-point” determinations that entail long incubation, stopping the reaction, and measuring the product (1-3). This is both time consuming and subject to analytical error. Although many substrates can be hydrolyzed by acid phosphatase, only α-naphthyl phosphate is suitable for continuous monitoring (4), the basis of this methodology being diazotization of the product, α-naphthol, with a diazo compound based on 2-amino-5-chlorotoluene as described by Hillmann (5). However, the published procedure has some deficiencies—linearity is not maintained and incubation for as long as 30 min is required—that make it unsuitable for use on automated rate analyzers.

Here, partly purified acid phosphatase from prostatic tissue has been used in making modifications to the procedure so that the rate of the reaction is linear and reagent conditions and stability are optimal. In addition, the procedure has been adapted to an automatic rate analyzer and the results obtained have been compared with a manual method in which p-nitrophenyl phosphate is used as the substrate.

Materials and Methods

Reagents and equipment. α-Naphthyl phosphate and p-nitrophenyl phosphate were obtained from Calbiochem and α-naphthol and Fast Red TR salt (4-chloro-o-toluidine diazotate) from the Sigma Chemical Co. Triton X-405 and X-100 surfactants were obtained from Technicon Instruments Corp. All other chemicals were reagent grade or the highest purity available.

Citrate buffer was used throughout this study because it is widely used in procedures for acid phosphatase determinations and reportedly enhances prostatic acid phosphatase activity (6). A Fast Red TR concentration of 1 mmol/litre was used throughout this investigation. When p-nitrophenol phosphate was used as the substrate, we used the method of Berger and Rudolph (7).

All manual assays with α-naphthyl phosphate were done with a Unicam SP1700 Spectrophotometer; the automated assays were done with a Beckman Enzyme Activity Analyzer System TR, with use of the AP channel. All assays were done at 37 °C. A unit of activity is defined as the hydrolysis of 1 μmol of substrate per minute.

Enzyme preparation. The enzyme was extracted from 30 g of frozen prostatic gland by homogenizing at 4 °C in four volumes of 10 mmol/litre tris(hydroxymethyl)aminomethane/citrate buffer, pH 3.7, containing Triton X-100 (50 ml/litre). The extract was centrifuged (4000 × g, 15 min) and the supernate collected and adjusted to pH 6.0 with tris(hydroxymethyl)aminomethane (1 mol/litre). This supernate was then placed on a 28 × 2.5 cm column containing cellulose phosphate equilibrated with 10 mmol/litre tris/citrate buffer, pH 6.0. The equilibration buffer was the eluting agent. Fractions were assayed by the method of Berger and Rudolph (7); those containing acid phosphatase activity were pooled and dialyzed vs. 17.5 mmol/litre sodium phosphate buffer, pH 7.0. The nondialyzable portion was loaded on a DEAE-Sephadex column (30.0 cm × 3.0 cm) and eluted with a linear gradient comprising 300 ml of 17.5 mmol/litre sodium phosphate buffer, pH 7.0, and 300 ml of 0.13 mol/litre sodium phosphate buffer, pH 5.8. The fractions containing the highest specific
activities were pooled, dialyzed vs. 20 mmol/litre sodium citrate, pH 5.0, and stored frozen. The final specific activity was 800 units of p-nitrophenyl phosphate hydrolyzed per milligram of protein. Protein concentration was determined by the spectrophotometric method described by Layne (8). Acid phosphatase control material was prepared by using, when required, partly purified enzyme diluted with human serum. This was adjusted to pH 6.0 before being stored frozen.

Results

Analytical Variables

pH optimum. To determine the pH optimum of purified acid phosphatase, we varied substrate concentration with pH. The $V_{max}$ values were determined from double reciprocal plots. These $V_{max}$ values were then corrected using the extinction coefficient for the naphthol/TR salt complex. This correction is necessary because the absorptivity for the complex varies as the pH changes from 2.6 to 6.7 (Figure 1). The resulting values are plotted in Figure 2a and indicate that there is a broad velocity maximum between 5.0 and 6.5.

While these experiments were being performed, we noticed that there was a significant lag period between adding the enzyme and the rate becoming linear. This was first evident on using purified enzyme, presumably because of the greater rates at non-optimum pH as compared to serum samples. However, careful examination of the rates obtained on using serum also showed this lag period. This lag for the purified enzyme is plotted in Figure 2b and demonstrates that it is significant until pH 5.5 is reached. To ensure that saturating substrate concentrations were maintained, we used 5.0 mmol/litre $\alpha$-naphthyl phosphate, a concentration more than 20 times the $K_m$ value at any pH, which should ensure that the substrate is not limiting the reaction.

The reason for this lag period is shown in Figure 3, which depicts the formation of the complex between $\alpha$-naphthol and Fast Red TR at various pH values. The time required for the complex to form decreases with increasing pH, and this pattern parallels the decrease in the lag when the enzyme or serum is assayed.

Effect of detergent. Another problem associated with the assay system was that the naphthol/Fast Red TR complex became insoluble during the reaction, causing turbidity in the assay solution with a resulting decrease in absorbance. The decrease in activity was most noticeable when enzyme or sera of high activity were used or if the assay was prolonged. Addition of a non-ionic detergent such as Triton X-405 or X-100
The increase in absorbance with time was monitored at 405 nm in the presence (A) and absence (B) of Triton X-405 (50 mg/100 ml) prevents the naphthol/Fast Red TR complex from becoming insoluble. With the detergent present the assay was linear until the substrate concentration became limiting (Figure 4).

**Substrate concentration.** We determined the $K_m$ value for $\alpha$-naphthyl phosphate by using a double reciprocal plot (Figure 5). We found it to be $1.2 \times 10^{-4}$ mol/litre, in agreement with published values (9). Thus, to ensure optimum assay conditions a concentration of 5 mmol of $\alpha$-naphthyl phosphate per liter is recommended, and this should give a rate at least 97% of the theoretical maximum velocity.

**Recommended Assay Conditions**

From our results, we recommend that if $\alpha$-naphthyl phosphate is used as the substrate for acid phosphatase the following conditions should be used: 50 mmol/litre sodium citrate, pH 5.7; 0.5 g of Triton X-405 per litre; 5 mmol/litre $\alpha$-naphthyl phosphate; 1 mmol/litre Fast Red TR salt; vol.

**Fraction of sample, 0.067 (1/15). For measurement of tartrate-stable activity, we used a tartrate concentration of 16.6 mmol/litre (7).**

**Substrate Storage**

We investigated the storage of the complete assay mixture, to establish a convenient method of storing this solution over an extended period of time. The reaction mixture was stored at either 4 °C or frozen at -20 °C and used in assays during 10 days. The mixture stored at 4 °C was unstable and lost its activity rapidly. Even after two days, this assay mixture developed a high background color. In contrast, the mixture stored at -20 °C for 10 days gave a result 95% that of the initial value. Moreover, the mixture could be used again later if refrozen immediately after use.

**Table 1. Comparison of $\alpha$-Naphthyl Phosphate Method and $p$-Nitrophenyl Phosphate Method for Acid Phosphatase Activity of Serum**

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<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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<td>CV, %</td>
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* Using $\alpha$-naphthyl phosphate as the substrate and a Beckman Enzyme Activity Analyzer System TR.
* Using $p$-nitrophenyl phosphate as the substrate and the manual method of Berger and Rudolph (7).
Reproducibility and Automation of the Assay

To test the reproducibility of the described procedure, we adopted the scheme of Barnett and Youden (10) in which three acid phosphatase activities were assayed on 20 consecutive days. The assays with α-naphthyl phosphate as the substrate were done with a Beckman TR rate analyzer and results compared with the manual method of Berger and Rudolph (7), for which p-nitrophenyl phosphate is the substrate. Table 1 shows the results, for both total and tartrate-stable acid phosphatase activity. As can be seen from the coefficient of variation, the method with α-naphthyl phosphatase is more reproducible. In addition, it should be noted that when α-naphthyl phosphate is the substrate the activity is about 1.8-fold greater than for p-nitrophenyl phosphate.

Discussion

The variation in methods for assaying the activity of acid phosphatase in biological fluids has usually been in the type of phosphate ester used as the substrate—e.g., p-nitrophenyl phosphate, phenyl phosphate, thymolphthalein monophosphate, and α-naphthyl phosphate. The only other notable variables have been the pH and choice of buffer used in these methods. Yet one of the deficiencies of the assay procedures for acid phosphatase has been the inability to continuously monitor the reaction rate, primarily owing to the low extinction coefficient of those substrates used to measure this enzymic activity. It was only when Hillmann (5) published a procedure involving the addition of a TR salt to complex the α-naphthol released from the enzymic hydrolysis of α-naphthyl phosphate that a continuous monitoring procedure for the enzyme became a possibility. Various authors have modified the procedure [e.g., Fabiny-Byrd and Ertingshausen (4)], yet this particular method has not been widely accepted by clinical laboratories, perhaps because there is a lag period and the reaction mixture is unstable.

Our results indicate that the rate-limiting step for this assay is the formation of the complex between the reaction product (α-naphthol) and the Fast Red TR salt. Complex formation rate is pH dependent and decreases with increasing pH; above pH 5.5 it is negligible. It is this step that causes a lag to be observed in many of the procedures in which pH values are used that are different from those we recommend. In addition, some commercial kits in which α-naphthyl phosphate is the substrate use a pH of 5.0, making them susceptible to problems with lag phases.

Thus, to ensure that the lag is not a problem in the assay procedure, a pH >5.5 should be used. This is an acceptable pH for this enzyme because it is within the broad pH optimum for the enzyme when α-naphthyl phosphate is the substrate.

Other problems encountered with this assay procedure were characterized by low results and nonreproducibility. Followed spectrophotometrically, these assays were found to deviate from linearity, because the α-naphthol/Fast Red TR salt complex becomes insoluble and so causes turbidity in the assay solution. We overcame the problem by adding a nonionic detergent, which kept the complex in solution. With the modifications we propose, the assay procedure is reproducible and gives more consistent results than does a manual method in which p-nitrophenyl phosphate is the substrate. This better reproducibility is probably the result of two features of our assay: the greater initial reaction velocity allows a greater absolute margin of error, and the assay can be used on an automatic analyzer, which helps to minimize analytical variation.

An important feature in any assay procedure for acid phosphatase is that tartrate inhibition is used as a specific test to detect the prostatic fraction in the serum (11, 12). This is also applicable to the method described because tartrate does not interfere with the formation of the α-naphthol/Fast Red TR complex. In addition, this substrate is also recommended because it shows low relative sensitivity to erythrocytic and platelet acid phosphatase as compared with many other substrates (5).

References