Enhancing Effect of Surfactant and Protein on Hydrolysis of Thymolphthalein Monophosphate by Purified Prostatic Acid Phosphatase

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Purified prostatic acid phosphatase catalyzes the hydrolysis of thymolphthalein monophosphate 10-fold faster if an optimal concentration of Brij 35 (a wetting agent) or protein (bovine serum albumin or human serum proteins) is present. Results of gel filtration, dialysis, and sucrose density-gradient centrifugation analysis suggest that the substrate must combine with detergent or protein before the enzyme can catalyze its hydrolysis.

Prostatic acid phosphatase [EC 3.1.3.2; orthophosphoric-monoester phosphohydrolase (acid optimum)] is one of the most important acid phosphomonoesterases used in clinical diagnosis (1–8) because the activity of this enzyme in the serum of prostatic cancer patients is usually increased (2–11). However, only 20–40% of patients with early disease (stage I–II) show an increased activity. Acid phosphatase activity in the serum of prostatic cancer patients changes after various types of therapy; orchiectomy (12–14), hormone therapy (13, 15–17), radiation therapy (18), and cryotherapy (19) usually decrease it. Resurgence of the disease can be detected by an increased activity of the enzyme.

Substrates used to measure serum acid phosphatase in prostatic cancer patients—including phenylphosphate (20–22), p-nitrophenylphosphate (23), β-glycerophosphate (24–26), phenolphthalein diphosphate (27, 28), and α-naphthyl-phosphate (29, 30)—are not specific for prostatic acid phosphatase. Moreover, acid phosphatase activity originating from erythrocytes, the spleen, or the liver can interfere with the results of this test (31). Various inhibitors such as L-tartrate and formaldehyde (31, 32), were introduced in attempts to make the test more specific. In 1971, a new substrate, thymolphthalein monophosphate, was introduced by Roy et al. (33, 34). It seems to eliminate the need for L-tartrate or any other inhibitors, because erythrocyte acid phosphatase does not act on this substrate. This paper reports the conditions necessary for maximal enzymatic activity on thymolphthalein monophosphate by purified prostatic acid phosphatase.

Materials and Methods

Chemicals: Bovine serum albumin, disodium p-nitrophenylphosphate, thymolphthalein monophosphate (magnesium salt), and Brij 35 (a surfactant, the polyoxyethylene ether of lauryl alcohol; concentration 300 g/liter) were obtained from Sigma Chemical Co., St. Louis, Mo. 63178. All other chemicals were analytical grade, from J. T. Baker Chemical Co., Phillipsburg, N. J. 08865.

Purification of prostatic acid phosphatase: Prostatic fluid, obtained from patients by prostatic massage, was pooled to give 4–6 ml. After adding 5 ml of 0.05 mol/liter tris(hydroxymethyl)aminomethane/0.1 mol/liter KCl buffer (pH 6.5), the solution was centrifuged (20 min, 5000 × g, 4 °C). The slightly turbid supernate was applied to a 100 × 2 cm column containing Sephadex G-100 which had been equilibrated with the same buffer. Elution was at 4 °C, with the same buffer, at a flow rate of 30 ml/h. Ten-milliliter fractions were collected. Protein appeared in two peaks, the first of which contained 85–87% of the protein and only 5–9% of the enzyme activity and the second of which contained 91–95% of the total activity and only 13–15% of the protein. The contents of the tubes corresponding to the second peak were pooled and used for further studies. Electrophoresis of this material on polyacrylamide gel showed it to contain only one protein, which co-migrated with the enzyme activity. Details of the purification of this enzyme appear elsewhere (35).

Gel filtration chromatography: A 40 × 1 cm column of Sephadex G-50 was packed and equilibrated in 0.1 mol/liter citrate buffer pH 6.0. One-milliliter samples were applied to the column and eluted with the same buffer. Two-milliliter fractions were collected at a flow rate of 0.5 ml/min. The position and con-

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centration of thymolphthalein monophosphate were determined by adding excess purified prostatic acid phosphatase (approximately 2000 Sigma units) and Brij 35. Elution of Brij 35 was determined by addition of prostatic acid phosphatase and thymolphthalein monophosphate. Hydrolysis of thymolphthalein monophosphate occurred only in the presence of Brij 35. The amount of hydrolyzed thymolphthalein indicates the concentration of Brij 35. Protein was determined by the method of Lowry et al. (37).

Sucrose density-gradient centrifugation: This was done as described by Martin and Ames (36), with a Beckman L2-65B ultracentrifuge and a SW-41 rotor. Sucrose gradients (50–300 g/liter) with a total volume of 11.3 ml were made up in 11.5-ml centrifuge tubes and 0.2-ml samples were layered on top. After a 22-h centrifugation at 5 °C, 0.5-ml fractions were aspirated from the tops of the tubes.

Measurement of activity of acid phosphatase: When p-nitrophenylphosphate (disodium salt) was used as the substrate, the method from Sigma Bulletin 104 was adapted (38).

When thymolphthalein monophosphate (magnesium salt) was used as the substrate, the procedure was that of Roy et al. (34). One milliliter of a substrate solution (2.2 mmol of thymolphthalein monophosphate per liter) in citrate buffer (0.1 mol/liter, pH 5.0) containing 5 g of Brij 35 per liter was preincubated at 37 °C for 2 min. Prostatic acid phosphatase, 0.2 ml, was added and the incubation was continued for 30 min, then stopped by adding 5.0 ml of NaOH (50 mmol/liter). The absorbance was measured at 590 nm.

Results

Effect of Brij 35 and bovine serum albumin on enzyme activity: Concentrations of Brij 35 and bovine serum albumin optimal for hydrolysis of thymolphthalein monophosphate by purified prostatic acid phosphatase were determined by varying the concentration of Brij 35 or bovine serum albumin between 0–10.0 g/liter, using 2.2 mmol/liter thymolphthalein monophosphate and 30 ng of enzyme. In the presence of increasing concentrations of Brij 35 the enzyme more rapidly hydrolyzed thymolphthalein monophosphate up to a maximum concentration of 5.0 g of Brij 35 per liter (Figure 1).

Bovine serum albumin alone also facilitated acid phosphatase hydrolysis of thymolphthalein monophosphate, with maximal activity at 7.0 g of bovine serum albumin per liter (Figure 2), but such enhancement of acid phosphatase activity by optimal concentrations of normal female serum or bovine serum albumin was diminished if Brij 35 was also present (Figure 1).

Hydrolysis of thymolphthalein monophosphate was directly proportional to enzyme concentration (5.4–350.0 μg/liter) in the presence of 5.0 g of Brij 35 per liter. If Brij 35 was omitted, the enzyme activity was decreased by 90% (Figure 3). Results were similar when 5.0 g of bovine serum albumin per liter was used (Figure 4).

Interaction of thymolphthalein monophosphate with Brij 35 and bovine serum albumin: These data suggest that thymolphthalein monophosphate must combine with Brij 35 or albumin before hydrolysis is catalyzed by prostatic acid phosphatase. We therefore investigated by gel filtration, dialysis, and sucrose density-gradient centrifugation whether Brij 35 or bovine serum albumin form complexes with thymolphthalein monophosphate. Using the chromatographic conditions described in Methods, we eluted thymolphthalein monophosphate (1.1 mmol/liter, chromatographed alone) from a column containing...
Association of thymolphthalein monophosphate with Brij 35 or bovine serum albumin can also be shown by dialysis experiments. Fifteen milliliters of 1.1 mmol/liter thymolphthalein monophosphate in 0.1 mol/liter citrate buffer was dialyzed against 1000 ml of the same buffer at 4 °C for 72 h. Equal amounts of thymolphthalein monophosphate in the presence of 5.0 g of Brij 35 per liter or 5.0 g of bovine serum albumin per liter (final concentrations) were dialyzed under similar conditions. Buffer was changed four times during three days before the concentration of thymolphthalein monophosphate in the dialysis bags was determined. Dialysis was continued for three more days, after which the concentration of thymolphthalein monophosphate was again determined. Dialysis was continued for three more days before final assay. In the presence of bovine serum albumin or Brij 35, thymolphthalein monophosphate dialyzes much more slowly (Table 1).

Dialyzed and undialyzed samples of thymolphthalein monophosphate, incubated with 5.0 g of...
bovine serum albumin per liter, and bovine serum albumin alone were analyzed by sucrose density-gradient centrifugation. The position of protein and thymolphthalein monophosphate from the dialyzed mixture coincided with the position of the marker, bovine serum albumin. In contrast, thymolphthalein monophosphate separated into two bands when the undialyzed bovine serum albumin/thymolphthalein monophosphate mixture was centrifuged. One peak of thymolphthalein monophosphate is at the top of the gradient, the other coincides with the protein (Figure 7).

**Discussion**

Roy et al. (33, 34) described a new substrate for prostatic acid phosphatase. Prostatic acid phosphatase is more specific for thymolphthalein monophosphate. Consequently, one does not need to use L-tartrate or formaldehyde to inhibit the activity of acid phosphatase derived from erythrocytes. Brij 35 was introduced into the reaction mixture by Roy et al. because it stabilized the substrate in the buffer solution. Our findings with highly purified prostatic acid phosphatase suggest that the enzyme cannot hydrolyze the thymolphthalein monophosphate when this substrate is in solution by itself. As Brij 35 (or bovine serum albumin) concentration is increased in the reaction mixture, prostatic acid phosphatase is increasingly able to hydrolyze the substrate. The purified enzyme has maximal reactivity at a final concentration of 5.0 g of Brij 35 per liter. Protein (bovine serum albumin, human serum proteins) can substitute for Brij 35, but when their combined concentration is greater than 5.0 g/liter, the activity of the enzyme is decreased. The activity of the purified enzyme alone with thymolphthalein monophosphate is about a tenth of the maximal activity in the presence of Brij 35 or bovine serum albumin. Chromatography and dialysis experiments suggest that protein or Brij 35 is required to present thymolphthalein monophosphate to the enzyme in an hydrolyzable form.

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**References**

38. Sigma Bulletin No. 104 (1961), Sigma Chemical Co., St. Louis, Mo. 63178.