Optimizing the o-Phenylenediamine Assay for Horseradish Peroxidase: Effects of Phosphate and pH, Substrate and Enzyme Concentrations, and Stopping Reagents

J. H. Bovalrd, T. T. Ngo, and H. M. Lenhoff

Horseradish peroxidase, assayed with o-phenylenediamine, is irreversibly inactivated when incubated in phosphate buffer, 100 mmol/L, at pH 5. The inactivation depends on both duration of incubation and phosphate concentration. Phosphate was the most potent inactivator and citrate the least poten of a series of buffers tested. The inactivation is not attributable to ionic strength per se or to Na\(^+\) or K\(^+\). The observed inactivation did not occur at high concentrations (2500 mmol/L, 0.1 g/L) of enzyme; however, this "protective" effect could not be reproduced by adding bovine serum albumin or a surfactant (Tween 20) to lower concentrations of enzyme. The inactivation was independent of commercial source of the enzyme or the kind of chromogenic assay used. On the basis of this information, we optimized the assay so that it gave eightfold greater absorbance values than those reported by others. The improved assay was sensitive to as little as 0.4 pmol/L (16 ng/L) of peroxidase, and was linear over the range of 0.4 to 5 pmol/L (16–200 ng/L).

Additional Keyphrases: enzyme activity • variation, source of

Although horseradish peroxidase (HRP; EC 1.11.1.7)\(^2\) is widely used as an auxiliary enzyme for oxidases and as a marker in enzyme-labeled immunoassays, its stability at different pH values and in different buffers has not been systematically investigated. We find that HRP activity diminishes rapidly when HRP is stored at 25 °C in 100 mmol/L phosphate buffer, pH 5. We describe here the effects of pH and phosphate buffer concentration on the stability of HRP, and the conditions for optimizing the assay of HRP with o-phenylenediamine (OPD).

Materials and Methods

Reagents. We used the following reagents as obtained from the manufacturers: HRP (Sigma Chemical Co., St. Louis, MO 63178; Millipore Corp., Freehold, NJ 07728; and Boehringer, Mannheim, F.R.G.); sodium phosphate, dibasic (Malnickrodt, Inc., St. Louis, MO 63147); sodium phosphate, monobasic (Fisher Scientific Co., Fair Lawn, NJ 07410); OPD, 3-dimethylaminobenzonic acid (DMAB), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) (Aldrich Chemical Co., Milwaukee, WI 53223); and H\(_2\)O\(_2\) (Matheson, Coleman and Bell, Norwood, OH 45712). All other reagents were of analytical grade.

With OPD as substrate, we measured HRP activity by use of a substrate solution containing, per liter, 3.7 mmol of OPD and 3.2 mmol of H\(_2\)O\(_2\) in 100 mmol/L sodium phosphate buffer, pH 5. The enzyme was incubated in different buffer concentrations. At the end of each incubation period, we initiated the reaction by adding 80 \(\mu\)L of HRP solution (62.5 pmol/L) to 1 mL of substrate solution and measured the absorbance at 435 nm after 15 min vs a blank containing only the substrate solution. In an alternative assay, the substrate solution contained, per liter, 0.2 mmol of MBTH, 10 mmol of DMAB, and 0.33 mmol of H\(_2\)O\(_2\) in 100 mmol/L sodium phosphate buffer, pH 7.1. At the end of the incubation period we added 100 \(\mu\)L of a 1 mmol/L solution of HRP to 3 mL of substrate solution and measured the absorbance at 590 nm at 15 min vs a blank containing only the substrate solution. All assays were done at 25 °C unless otherwise indicated, and with an Acta III spectrophotometer (Beckman Instruments Inc., Fullerton, CA 92634).

Results

Effect of pH. The optimum pH for HRP when MBTH and DMAB are the chromogens is pH 7 (1), whereas that reported for HRP with OPD as the chromogenic substrate is pH 5 (2–5). Using OPD and without incubating the enzyme in phosphate for an extended time before the assay, we also obtained an optimum of pH 5.

HRP incubated in high and low concentrations of citrate buffers over a wide range of pH values remains fairly active. On the other hand, not only is HRP that has been incubated in high and low concentrations of phosphate much less active at a low pH, but also the degree of activity at the different pH values is a function of phosphate concentration (Figure 1). HRP incubated in low concentrations of phosphate buffer has more activity over a wider pH range, with a broad optimum around 6, whereas, incubated at high concentrations of phosphate, the enzyme is less active from pH 5 through 9 and has a narrower range of stability around pH 6. At pH 5, the pH used in most assays of HRP when OPD is the chromogen, the enzyme is nearly 1.5-fold more active in low concentrations of phosphate than in high concentrations (Figure 1).

Effect of phosphate concentration and time of incubation. HRP incubated for 1, 2, or 3 h in phosphate (20 to 100 mmol/L) at pH 5 showed decreasing amounts of activity with increasing phosphate and with longer incubation times. Figure 2 shows these results for experiments carried out at 30 °C. The same pattern of loss of activity was shown at 25 °C, but slightly less activity was lost than at 30 °C.

Effect of ionic strength and of Na\(^+\) or K\(^+\). To determine whether the decrease in enzyme activity observed at higher concentrations of phosphate was owing to an increase in ionic strength and not to phosphate per se, we tested various combinations of NaCl and phosphate. As Table 1 shows, HRP was
least active in high concentrations of phosphate (100 mmol/L), but a high NaCl concentration (90 mmol/L) had no substantial effect on enzyme activity unless a high concentration of phosphate was also present. Furthermore, phosphate buffers prepared from either Na\(^+\) or K\(^+\) salts gave virtually identical results. Hence, phosphate ion itself is evidently responsible for the decrease in activity of HRP at pH 5.

**Effect of buffers other than phosphate.** HRP incubated for as long as 3 h in pH 5, 10 mmol/L buffers—arsenate, citrate, succinate, acetate, or phthalate—was relatively stable. At buffer concentrations of 100 mmol/L, however, the HRP was more active in these buffers, after a 3-h incubation, than in phosphate, and most active in citrate (Figure 3).

To determine whether the phosphate-induced decrease in activity was due to an irreversible inactivation of the enzyme, we incubated HRP in 100 mmol/L phosphate, pH 5, for 3 h and then diluted with citrate buffer, 10 mmol/L, pH 5, for various periods (5 min to 3 h) before assaying. In no case was enzyme activity restored. Hence, the observed inactivation appears irreversible.

We obtained further evidence for the irreversibility by incubating a 100 pmol/L (4 \(\mu\)g/L) concentration of enzyme in 100 mmol/L phosphate, pH 5, at 25 °C for 4 h, then dialyzing the enzyme overnight in 4 L of 10 mmol/L phosphate, pH 5, at 4 °C. Neither this procedure nor diluting the inactivated enzyme fivefold with distilled water restored its activity.

**Effect of EDTA.** To test whether the observed relative stability of HRP to citrate buffer, pH 5, is ascribable to the chelating effects of citrate, we incubated HRP in EDTA and phosphate, pH 5, for as long as 3 h. Including 50 mmol of EDTA per liter of low phosphate (10 mmol/L) and high phosphate (100 mmol/L) enhanced the inactivation by 25% and 18%, respectively, over that of low and high phosphate alone. Hence, the "stabilizing" effect of citrate probably is not a result of its chelating properties.

**Effect of HRP concentration.** To determine whether HRP activity would be protected by using concentrations of HRP higher than 62.5 pmol/L, we performed the experiment summarized in Figure 4. Before the assay, the enzyme preparations were incubated at either 10 or 100 mmol/L phosphate, pH 5. As expected, only the lower concentrations of HRP incubated in high-phosphate buffer were inactivated.

To determine whether the protective effect of a high concentration of HRP was due to a general protection of the HRP by protein or other polymeric reagents, we added either bovine serum albumin (10 g/L) or the surfactant, Tween 20 (0.9 mL/L), to the low–HRP, 100 mmol/L phosphate incubation mixture; neither of these protected.

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**Table 1. Effect of Ionic Strength on HRP Activity**

<table>
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<tr>
<th>Conc, mmol/L</th>
<th>Phosphate</th>
<th>% enzyme activity after incubation for 5 min</th>
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<th>3 h</th>
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<td>90</td>
<td>100</td>
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*Percentage activity as compared with that after 5-min incubation of enzyme (62.5 pmol/L) with OPD substrate.
Effect of different sources of HRP. HRP from three different suppliers (Sigma, Worthington, and Boehringer-Mannheim) were all consistently inactivated after a 3-h incubation at 25 °C in 100 mmol/L phosphate buffer at pH 5. Enzyme obtained from Sigma and Boehringer-Mannheim was only slightly inactivated at 10 mmol/L phosphate; the HRP from Worthington-Millipore was somewhat more sensitive to phosphate.

**Fig. 4.** Enzyme activity vs incubation interval for three concentrations of HRP exposed to two concentrations of phosphate, pH 5. Three different concentrations of enzyme—2500 (●), 25 (■), and 0.25 (△) mmol/L—were incubated in 100 (closed symbols) or 20 (open symbols) mmol/L phosphate for various intervals. Immediately after each incubation period, the mixtures were diluted to 82.5 pmol of enzyme per liter. Calculations as in Fig. 2.

Effect of phosphate and pH on assay with other chromogenic substrates. To determine whether the inactivation of HRP is related to the use of OPD as the chromogen, we also performed the assay with MBTH/DMAB substrate (1) at both pH 7 and pH 5. As with OPD, HRP was inactivated only at high phosphate concentration and pH 5.

**Effect of OPD and enzyme concentration: optimized assay.** By systematically varying the concentrations of OPD and H2O2, we obtained the highest absorbance value for the chromophore at 435 nm by using 100 mmol/L OPD and 20 mmol/L H2O2. These absorbance values for a 10- to 20-min incubation are about eightfold those obtained by other investigators (2-6) using the OPD assay for HRP. The conditions giving those values are those of our optimized assay.

Because most of those other assays involved suboptimal concentrations of OPD and H2O2, we considered it necessary to determine whether the absorbance obtained under our optimal concentrations of substrates was directly related to HRP concentration over a wide range of enzyme concentrations. Absorbance was linearly related to concentration over the range of 0.4 to 5 pmol of HRP per liter (Figure 5).

The absorbance values (Figure 5) were obtained after a 30-min incubation; during this period the autooxidation (as opposed to oxidation by HRP) of OPD to form chromophore corresponded to only about 0.01 A. After longer incubations, however, the absorbance from autooxidation of OPD was significant (e.g., 0.12 A at 4 h and 0.3 A after 7 h).

**Effect of “stopping” reagent.** In all our assays for HRP, the absorbance was measured directly as the chromophore was formed without stopping the reaction. If stopping reagents are used, the spectrum of the chromophore changes. For example, sulfuric acid slightly shifts and lowers the absorption of the spectrum (Figure 6, Curve II), whereas azide lowers the absorption of the spectrum significantly (Figure 6, Curve III). Curve I of Figure 6, serving as a control, shows the absorption spectrum of the chromophore when the final reaction mixture is diluted with acetate buffer at pH 5. Hence, if sulfuric acid is used as a stopping agent, the absorbance of the acidified chromophore should be measured near 490 nm.

**Discussion.** When used as an auxiliary enzyme, HRP is generally in excess. Thus, inactivation of the enzyme by phosphate may...
be minor. However, if the solution is prepared and stored in phosphate buffer for some time before use, it could be substantial.

At present we cannot explain the mechanism of this inactivation. Nonetheless, these observations should prove useful to those using HRP in clinical assays, particularly enzyme immunoassays in which HRP is the label.

References


L-Phenylalanine Inhibition of Human Alkaline Phosphatases with p-Nitrophenyl Phosphate as Substrate

T. Komoda, S. Hokari, M. Sonoda, Y. Sakagishi, and T. Tamura

With p-nitrophenyl phosphate as the substrate, there reportedly is no organ-specific inhibition of alkaline phosphatase (EC 3.1.3.1) activity by L-phenylalanine. However, we found that at pH 10.0, with p-nitrophenyl phosphate as the substrate, L-phenylalanine obviously inhibits the alkaline phosphatase isoenzyme from human placenta, whereas there is little if any inhibition of the isoenzyme from human intestine. Because of the differing effects of substrates (p-nitrophenyl phosphate and phenyl phosphate) and their enzymic products (p-nitrophenol and phenol) for L-phenylalanine action on the placental alkaline phosphatase isoenzyme, we suggest that the isoenzyme–inhibitor–substrate complex and the effect of released phosphate on L-phenylalanine inhibition of the isoenzyme activity differ from each other.

Additional Keyphrases: enzyme activity • isoenzymes • effect of pH on enzyme activity

Reportedly, placental and intestinal alkaline phosphatase (EC 3.1.3.1) activities are specifically inhibited by L-phenylalanine when phenyl phosphate is used as the substrate (1, 2). However, Keiding (3) reported that L-phenylalanine did not inhibit the activity of the intestinal isoenzyme when p-nitrophenyl phosphate was used as substrate. Moreover, L-phenylalanine inhibition of the phosphatase activity with phenyl phosphate or β-glycerophosphate as the substrate is said to be highly dependent on pH (2, 4).

In this study, we investigated the relationship between the p-nitrophenyl phosphate affinity for the respective alkaline phosphatases and the interaction of the isoenzymes with L-phenylalanine.

Materials and Methods

Materials. Disodium p-nitrophenyl phosphate, disodium phenyl phosphate, p-nitrophenol, and phenol were from Wako Pure Chemicals Co., Osaka 541, Japan; AH-Sepharose, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and o-carboxyphenyl phosphate were from Sigma Chemical Co., St. Louis, MO 63178; L-homoarginine was from ICN Pharmaceuticals Inc., Cleveland, OH 44128. All other chemicals were of analytical grade, from Wako Pure Chemicals Co.

Enzyme purification. Alkaline phosphatase preparations were purified from human tissue homogenates of term placenta and adult intestinal mucosa. The procedures for purification of intestinal enzyme were as described by Komoda and Sakagishi (5, 6). Alkaline phosphatase from term placenta was purified by the method of Doellgast and Fishman (7), with further purification by o-carboxyphenylphosphate/AH-Sepharose 4B and concanavalin A-Sepharose 4B affinity chromatographies (8). The specific activity (kU/g of protein) of the preparations were: adult intestine, 1140; term placenta, 841.

Enzyme assays and protein determinations. The reaction mixture contained 1 mmol of disodium p-nitrophenyl phosphate or disodium phenyl phosphate, 1 mmol of MgCl₂, and 3 mL of Triton X-100 per liter of carbonate buffer, 50 mmol/L, pH 10.0 at 30 °C (9).

The units (U) of activity are defined as micromoles of substrate hydrolyzed per minute, given a p-nitrophenolate molar absorptivity of 1.87 X 10⁴ at 405 nm, or as determined by the method of Kind and King (10). Inorganic phosphate was determined by the method of Komoda and Sakagishi (11).

Mixtures of the enzyme and some compounds were adjusted to pH 10.0 with 0.1 mol/L NaOH, and each aliquot was then assayed for phosphatase activity. The percentage of enzyme activity remaining after addition of L-phenylalanine (5 mmol/L) was determined as described earlier (7).

The effects of pH on the phosphatase activities were measured in 0.1 mol/L Tris HCl in the 8.5–9.0 pH range and in 0.1