Catalytic Activities of Glass-Encapsulated Horseradish Peroxidase at Extreme pHs and Temperatures

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A high substrate specificity, regio- and enantioselectivity, and absence of bi-products are characteristics of an enzyme-catalyzed reaction. Some enzymatic reactions are very useful in organic syntheses.[1] However, biotechnological applications of these reactions often require stabilization of enzymes in a hostile environment such as organic solvent, extreme pHs, and high temperatures. Useful techniques to overcome the problems include entrapment in reverse micelles[2] and immobilization of the enzyme.[3]

Alternatively an enzyme can be stabilized in a silicate glass by using the sol-gel technique although the technique has a broader applicability in optically based molecular sensors. The method, improved by Ellerby *et al*.,[4] uses a mild condition to encapsulate a protein in the native state. Since the resulting glass is transparent, one can monitor structural changes in slow motion of the encapsulated protein by optical absorption if the protein is colored.

Horseradish peroxidase (HRP) is a commercially important enzyme and catalyzes many reactions of synthetic interest.[1] Wu *et al*.[5] encapsulated a few heme proteins in a silicate glass and compared their catalytic properties and optical absorption with those in aqueous buffer under normal conditions (room temperature and pH 7). In this study, we showed that HRP encapsulated in xerogels was stable enough to retain its full catalytic activity at extreme pHs and temperatures.

Results and Discussion

Encapsulation in glass did not significantly alter the optical absorption spectrum of HRP as shown in Figure 1A. The Soret band was slightly blue shifted from 402 nm for HRP in aqueous solution (*c*) to 399 nm for that in glass (*a*). Small blue shifts are also seen in the visible bands. The spectra indicate that the heme is in a 5-coordinate high-spin state. Upon lowering the pH to 2, the histidine residue (proximal heme ligand) of the native HRP in solution was protonated and replaced by a water molecule, as evidenced by a drastic change in the absorption spectrum (*d* compared with *c*).[6] The enzyme was unfolded at low pH (*d*). However, the encapsulated HRP suffered only a minor perturbation and the ligation state of the heme was largely intact (*b* compared with *a*).

We measured the catalytic activity of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) oxidation[7] by HRP in the presence of hydrogen peroxide. As shown in Figure 1B, the activity of native HRP in solution (*squares*) was maximal at pH 5.5 and, as expected from the absorption spectrum, decreased sharply when pH was lowered. Encapsulated enzyme (*circles*), on the other hand, was fully active even at pH 2. At high pH, both solubilized and encapsulated enzymes lost their catalytic activity, presumably due to involvement of protons in the reaction.

Encapsulation stabilized the enzyme at high temperatures as shown in Figure 2A. At 80 °C, HRP in solution underwent thermal denaturation as demonstrated by a large change in

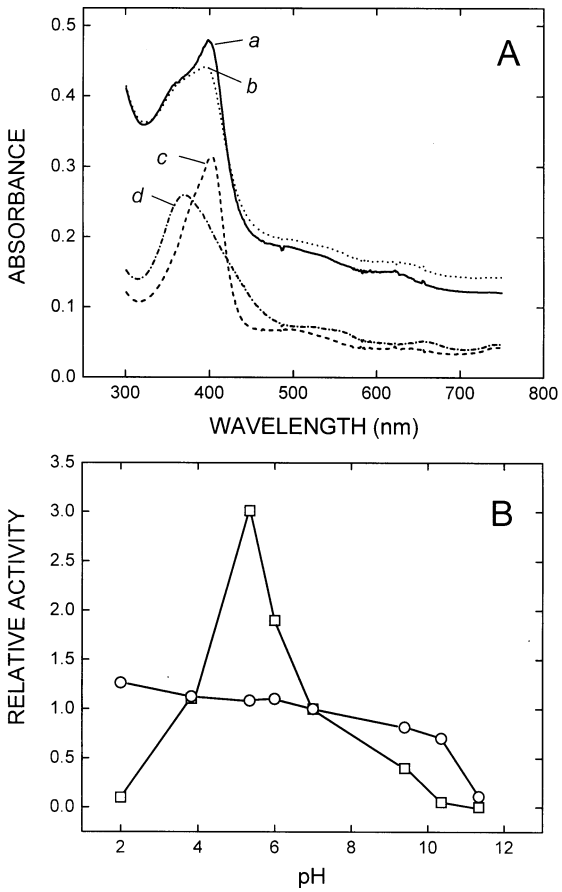

Figure 1. (A) Absorption spectra of HRP encapsulated in silica glass (*a*, *b*) and in aqueous solution (*c*, *d*) at pH 7 (*a*, *c*) and pH 2 (*b*, *d*). The spectra were obtained in a cuvette with 1 cm pathlength using a diode array spectrophotometer. The HRP concentration was 5 μM in 100 mM phosphate buffer. (B) Catalytic activities of HRP in solution (*squares*) and encapsulated HRP (*circles*) as a function of pH. The activity at pH 7 was set to 1.

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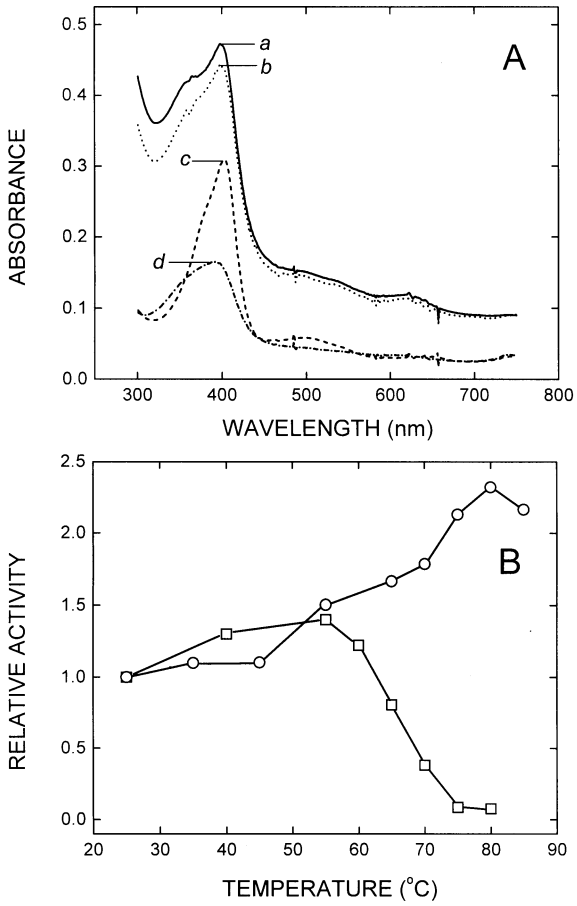

Figure 2. (A) Absorption spectra of HRP encapsulated in silica glass (*a*, *b*) and in aqueous solution (*c*, *d*) at 25 °C (*a*, *c*) and 80 °C (*b*, *d*). The HRP concentration was 5 μM in 100 mM phosphate buffer (pH 7). Other conditions were the same as those in Figure 1A. (B) Catalytic activities of HRP in solution (*squares*) and encapsulated HRP (*circles*) as a function of temperature. The activity at 25 °C was set to 1.

the absorption spectrum (*c* and *d*). There was little change in the absorption spectrum of HRP encapsulated in glass when the temperature was raised to 80 °C (*a* and *b*).

The catalytic activity of HRP in solution decreased sharply above 60°C due to thermal denaturation (Figure 2B, *squares*).[8] Encapsulated HRP, however, had a higher activity at a higher temperature up to 80 °C (*circles*). A small structural change around the substrate binding site or supply of thermal energy at high temperature may have enhanced the catalytic activity.

In conclusion, encapsulation of HRP in glass stabilized the enzyme at extreme pHs and temperatures so that its catalytic activity was retained or enhanced. The sol-gel technique can also be applied to organic reactions that need to be executed by enzymes at a low pH or a high temperature.

Experimental Section

Encapsulation of HRP in silicate glass. The method developed by Ellerby *et al.*[4] was used with minor modifications. We mixed 7.5 mL $Si(OCH_3)_4$ (tetramethylorthosilicate or TMOS) in 2.3 mL water and 0.2 mL HCl (40 mM), and sonicated the mixture for 15 min. The resulting solution was added to the same volume of 10 mM phosphate buffer (pH 7.0) to neutralize the pH. An aliquot was transferred into a disposable polystyrene cuvette (pathlength 4 mm) and 10-20 μL HRP (~10 μM final concentration) was mixed in immediately. The cuvette was sealed with parafilm and the solution was aged at 4 °C for 2 weeks, during which the silanol group (Si-OH, which is formed upon hydrolysis of $Si\text{-}OCH_3$ by HCl) reacted with the methoxy group ($Si\text{-}OCH_3$) to form siloxane (Si-O-Si). Air bubbles should be removed thoroughly to make a smooth and transparent glass. The resulting aged gel was allowed for another 2 weeks to prepare a xerogel after slow evaporation of the solvents (water and methanol).[9] The final volume shrank to 1/8 of the initial volume and therefore HRP was proportionally concentrated.

Activity measurements. HRP (10 nM) was mixed with ABTS (0.5 mM) in 100 mM phosphate buffer and the reaction was started by adding 10 mM hydrogen peroxide.[7] Increase in the absorbance at 418 nm due to oxidation of ABTS was monitored to obtain the reaction rate. Activity of encapsulated HRP was measured by using the same concentration (in terms of enzyme) of finely powdered HRP glass. The reaction mixture was incubated at a given temperature for 15 min before activity measurement.

Absorption spectroscopy. The absorption spectrum of HRP in solution was obtained in a quartz cuvette (1 cm pathlength) using a Hewlett-Packard photodiode array spectrophotometer. The spectrum of HRP xerogel was obtained by immersing the xerogel slide in a cuvette filled with an appropriate buffer. The buffer was stirred with a small magnetic bar. Pathlength of the xerogel was ~2 mm but encapsulated HRP was proportionally concentrated due to shrinkage during drying process.

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