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A CHEMICAL MODIFICATION TO MAKE HORSERADISH PEROXIDASE SOLUBLE AND ACTIVE IN BENZENE

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SUMMARY: Horseradish peroxidase was modified with 2,4-bis(O-methoxypolyethylene glycol)-6-chloro-s-triazine. The modified peroxidase, in which 60% of the amino groups were coupled with polyethylene glycol, had 70% of the enzymic activity in aqueous solution and was found to be soluble in benzene. Since the modified peroxidase in benzene had an absorption spectrum similar to that of unmodified peroxidase in aqueous solution, the prosthetic group, protohaemin IX, remained with the apoprotein even in benzene. The modified peroxidase in benzene had 21% of the enzymic activity relative to that of unmodified enzyme in aqueous solution.

Although there are various enzymes that function in hydrophobic environments such as plasma membrane in vivo, little information about their reactions have been accumulated, due to the difficulty in performing these reactions in vitro. In order to understand these reactions, it would be advantageous to make enzymes soluble and active in organic solvents by chemical modifications. These modified enzymes may also be very useful in industrial processes.

Horseradish peroxidase is a glycoprotein with a molecular weight of 40,000 (1) and has one mole of protohaemin IX as the prosthetic group. This enzyme is a good candidate for this modification, because its enzymic activity was reported not to be affected by the modification of amino groups(2). As this enzyme was haemoprotein, it was expected to give spectrophotometric information in the visible region even in benzene, a typical organic solvent.

Polyethylene glycol has been used in our laboratory as a modifier of enzymes, mainly to suppress immunoreactivity(3,4,5). In this study we have exploited the amphipathic nature of this interesting polymer to modify

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peroxidase in aqueous solution and have successfully made it soluble and active in an organic solvent.

MATERIALS AND METHODS

Horseradish peroxidase(100 units/mg) was obtained from Miles Laboratories Ltd.(Good Wood, South Africa). Monomethoxypolyethylene glycol with an average molecular weight of 5,000 was obtained from Polyscience Inc.(Warrington, PA). Other reagents were of analytical grade.

2,4-Bis(O-methoxypolyethylene glycol)-6-chloro-s-triazine(activated PEG_2) was synthesized by the method of Nishimura et al.(5) from monomethoxypolyethylene glycol and cyanuric chloride.

The modification of peroxidase was carried out as follows. To 10 mg of peroxidase in 3 ml of 0.4 M borate buffer(pH 10.0) was added 1 g of activated PEG_2, and the reaction mixture was kept at 37°C for 1 hr under stirring. After the addition of 100 ml of cold phosphate buffered saline(PBS)(pH 7.0) to stop the reaction, the sample was ultrafiltrated with Amicon Diaflo PM-30 membrane(Lexington, Mass.) to remove free activated PEG_2. The degree of modification of peroxidase with activated PEG_2 was determined by measuring the non-reacted amino groups of the peroxidase molecule with trinitrobenzene sulfonate(6).

A modified peroxidase preparation in the dry state was dissolved in benzene. The protein concentration in benzene was determined by the Biuret method after completely removing the solvent by evaporation.

Substrates of peroxidase in benzene were prepared as follows. Hydrogen peroxide in benzene was prepared by vigorously mixing an aqueous solution of hydrogen peroxide and benzene, followed by removing the aqueous layer after settling. The concentration of hydrogen peroxide in the benzene layer was determined from the standard curve constructed by unmodified peroxidase in aqueous solution. The effect of benzene in this determination was corrected by adding the same volume of benzene to the control sample. o-Phenylenediamine, which is a hydrogen donor, was dissolved in benzene to give a 100 mM solution.

RESULTS AND DISCUSSION

Horseradish peroxidase was modified and 60% of the total amino groups were coupled with polyethylene glycol. The modified peroxidase retained 70% of the enzymic activity in aqueous solution, PBS(pH 7.0), when 6.3 mM hydrogen peroxide and 100 mM o-phenylenediamine were used as substrates.

In contrast to the absolute insolubility of the unmodified enzyme, the modified peroxidase was quite soluble in benzene. Fig. 1 shows the absorption spectrum of modified peroxidase in benzene(curve A) together with the spectra of modified peroxidase in aqueous solution (curve B) and unmodified peroxidase in aqueous solution (curve C). As is seen in curve A, a strong Soret band with an absorption peak at 403 nm was observed. Its molar extinction coefficient, 9.9×10^4 $M^{-1}cm^{-1}$, was in good agreement with that obtained with unmodified enzyme in aqueous solution, 9.1×10^4 $M^{-1}cm^{-1}$. The strong absorption in the ultraviolet region below 370 nm is attributed to the

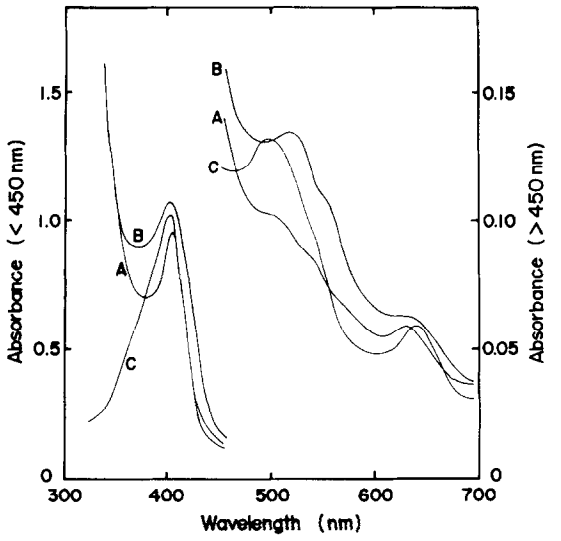

Fig. 1. Absorption spectra of peroxidase. Curve A; 0.38 mg/ml modified peroxidase in benzene. Curve B; 0.48 mg/ml modified peroxidase in PBS(pH 7.0). Curve C; 0.45 mg/ml unmodified peroxidase in PBS(pH 7.0).

triazine ring in modified peroxidase. This spectrum strongly suggests that haemin is not liberated from the apoprotein of modified peroxidase. This conclusion was confirmed by thin-layer chromatography(data not shown).

The absorption spectrum of peroxidase is changed when one of the substrates, hydrogen peroxide, is added to the enzyme in aqueous solution. A similar spectral change was also observed in the case of modified peroxidase in benzene; four bands with shoulders appear with the peak positions at 417, 545, 583 and 670 nm. Modified peroxidase, therefore, interacts with hydrogen peroxide and probably forms an enzyme-H_2O_2 complex, and this would suggest the possibility that the modified enzyme should retain the enzymic activity even in benzene.

To check this possibility, the reaction mixture(2.5 ml) contained 190 ng of modified peroxidase, 79 μM hydrogen peroxide and 16 mM o-phenylenediamine in benzene. During the reaction at 25°C, the absorption maximum at 490 nm was observed to increase with incubation time(data not shown). This was never observed when unmodified enzyme or haemin was used in stead of modified peroxidase. This result suggests that modified peroxidase still retained the enzymic activity even in benzene.

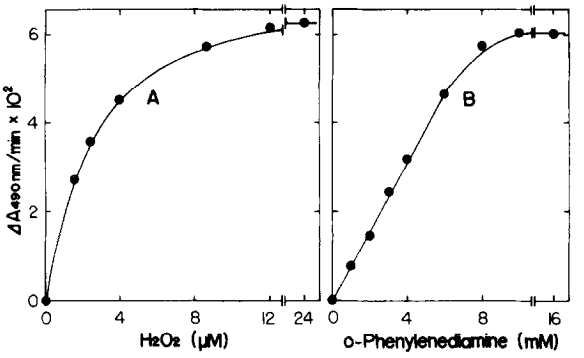

Fig. 2. The reaction velocity(ΔA_{490} nm/min x 10^2) of modified peroxidase in benzene at 25°C with varied concentrations of hydrogen peroxide(curve A) and o-phenylenediamine(curve B). Curve A; 16 mM o-phenylenediame and 83 ng/ml modified peroxidase were used. Curve B; 79 μM hydrogen peroxide and 78 ng/ml modified peroxidase were used.

The changes in reaction velocity with changes in the concentration of either hydrogen peroxide(curve A) or o-phenylenediamine(curve B) are showed in Fig. 2. As the structure of the product was not identified, the reaction velocity was expressed as the change of absorbance with time. The reaction velocity increased with increasing concentrations of each substrate, and reached a constant level with 12 μM and 10 mM of hydrogen peroxide and o-phenylenediamine, respectively. It is noteworthy that the modified peroxidase retained strong affinity towards the substrates, especially towards hydrogen peroxide.

Table 1. ACTIVITY OF MODIFIED PEROXIDASE IN BENZENE RELATIVE TO UNMODIFIED PEROXIDASE IN PBS(pH 7.0)

Sample	ΔA/min	Protein Conc. μg/ml	ΔA·ml/μg·min	Relative Activity (%)
unmodified peroxidase in water	0.077	0.030	2.57	100
modified peroxidase in benzene	0.055	0.098	0.56	21

The concentrations of substrates in the reaction system(2.5 ml), PBS(pH 7.0) or benzene, were 79 μM hydrogen peroxide and 16 mM o-phenylenediamine. To 0.5 ml of the reaction mixture removed at a given time were added 1.5 ml of ethanol and 0.5 ml of benzene or PBS(pH 7.0). Sulfuric acid was added to 0.125 N to stop the reaction.

The next experiment was conducted to compare the activity of the modified peroxidase in benzene and that of unmodified peroxidase in aqueous solution (Table 1). The absorption spectrum of the reaction product in benzene was different from that in aqueous solution, but this was not due to a difference in the product formed in these two different solvents, because the spectra with the peaks at 463 nm became comparable after adjusting the final composition of the reaction mixture as follows; bezene:water:ethanol = 1:1:3. Concentrations of two substrates were fixed at 79 μM for hydrogen peroxide and 16 mM for o-phenylenediamine. They are both higher than the concentrations required to obtain the maximum activity. The enzymic activity of the modified peroxidase in benzene was as much as 21% of the activity of the unmodified enzyme in aqueous solution.

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