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Peroxidase chemically attached on polymeric micelle and its reaction with phenolic compounds

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ABSTRACT

Horseradish peroxidase was chemically modified with comb-shaped polymaleic anhydride-*alt*-1-tetradecene (PMA-TD) in microemulsion systems to produce surface-active peroxidase that has capability to form micellar structures in aqueous solutions and can be concentrated at liquid/liquid interfaces without unfolding of the enzyme. For chemical modification oil-in-water (O/W) and water-in-oil (W/O) microemulsion systems composed of *n*-butyl acetate and a buffer solution were prepared because *n*-butyl acetate turned out to be less detrimental to the activity of peroxidase at high degree of modification compared to other organic solvents. The modification degree of amine groups on the surface of peroxidase by maleic anhydride groups on PMA-TD was reached at equilibrium after 1 h reaction at 0 °C, and 42% of amine groups were modified with 7-fold amount of PMA-TD to peroxidase (wt/wt). The activity of the PMA-TD-modified peroxidase measured with 2,4-dichlorophenol at pH 7.0 was increased by approximately 2-fold compared to native peroxidase. There was no significant shift in optimum pH after modification, and optimum pH measured with 2,4-dichlorophenol was observed at pH 7.0. For all six phenolic compounds tested, there was a significant increase in the reaction efficiency with the PMA-TD-modified peroxidase. The remarkable enhancement of the reaction efficiency by the modification was presumably because of micellar structures of PMA-TD that could concentrate hydrophobic phenolic oligomers into the core of the micelles. Overall, horseradish peroxidase chemically attached to the surface of PMA-TD micelles was found to be significantly effective for the oxidative polymerization of phenolic compounds.

1. Introduction

The surface of the enzyme molecules is composed of mainly hydrophilic residues because hydrophobic residues tend to be concentrated around the interior of enzyme molecules during folding, thereby leading to enzyme molecules soluble in aqueous solutions [1]. Accordingly, high enzymatic reaction rates were achieved with hydrophilic substrates soluble in aqueous solutions [2]. While the miscibility between enzymes and substrates cannot be overcome, low rates can be obtained for enzymatic reactions in which substrates are hydrophobic. To resolve the problem and perform enzymatic synthesis reactions, several strategies have been employed such as (1) using aqueous polar solvent mixtures in which substrates and products are miscible with native enzymes so that the system is a single phase and rates are not limited by inter-phase transport [3–5], (2) using biphasic aqueous-organic systems in which native enzymes and substrates are soluble in aqueous solutions and products are soluble in nonpolar organic solvents [6–8], (3) dissolving substrates in relatively nonpolar solvents in which products can be soluble and then add modified enzymes with polyethylene glycol (PEG) [9,10], surfactant [1,11], lipid [12] to solubilize enzymes into the solvents, (4) dissolving enzymes and substrates in reversed micellar systems, resulting in surfactant-induced positioning of the substrate at the interface [13], and (5) performing enzymatic reactions in aqueous surfactant solutions, eliminating the need for organic solvents [11].

In addition to the strategies mentioned above to overcome the miscibility problem in the enzymatic reactions, we have developed an artificial surface-active enzyme having hydrophilic heads (enzyme) and hydrophobic tails (polymer) concentrated at hydrophobic substrate/water interfaces similar to lipases that are concentrated at an oil/water interface and can hydrolyze lipid structures [14–16]. Nonetheless, most enzymes cannot catalyze their reactions at the interfaces because

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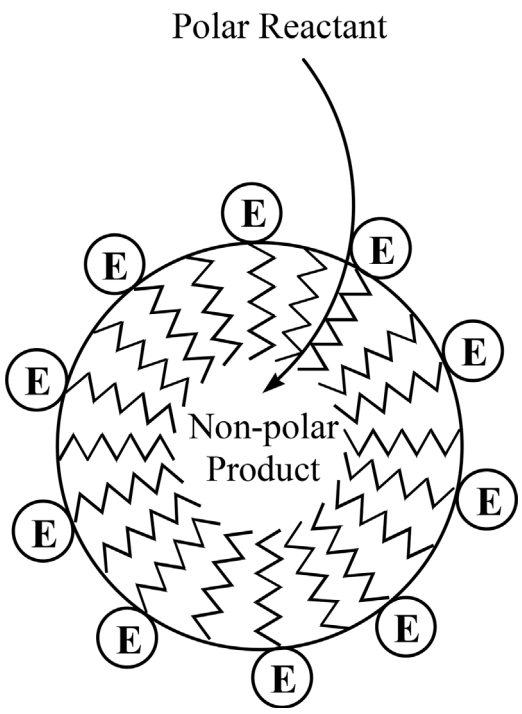

Fig. 1. Simplified concept of interfacial enzyme reactions with surface-active peroxidase in aqueous solutions.

enzymes tend to be soluble only in aqueous solutions and cannot be concentrated at the interfaces without unfolding [17,18]. Generally, proteins exposed to oil/water interfaces exhibited irreversible adsorption because of hydrophobic residues on the protein having a great affinity to the oil phase, resulting in unfolding of protein at the interfaces. Therefore, the most difficult problem is endowing delicate enzymes with suitable new properties by chemical modifications so that modified enzymes cannot lose their activities at the interfaces. To validate our concept of the surface-active enzyme, we developed a surface-active peroxidase, which has horseradish peroxidase as a hydrophilic head and comb-shaped polymaleic anhydride-*alt*-1-tetradecene (PMA-TD) as a hydrophobic tail, and studied its catalytic characteristics in oxidative polymerizations of phenolic compounds as a model system (Fig. 1).

Peroxidases catalyze coupling reactions of phenolic compounds through activation by hydrogen peroxide (H_2O_2), leading to generating phenoxyl radical intermediates that can form phenolic oligomers in aqueous solutions. Thus, peroxidases have been often used to remove phenolic pollutants such as bisphenol A and chlorophenols for bioremediation [19–21]. However, phenolic compounds are likely to be insoluble in aqueous solutions owing to their high hydrophobicity, which make them difficult to remove from soil. In addition, high molecular weight (MW) of phenolic products generated by enzymatic reactions are easily precipitated in aqueous solutions due to the increase in hydrophobicity, thereby hindering further polymerization reactions. To alleviate these problems, we chemically attached peroxidase on PMA-TD micelles in microemulsion systems, eventually improving solubility of phenolic compounds and enzyme accessibility to substrates through the localization of phenolic products [14]. The PMA-TD micelles were expected to solubilize and emulsify hydrophobic phenolic oligomers while the role of peroxidase was to shuttle the phenolic monomers from the aqueous phase into the micellar environment where further polymerization reactions might take place. Our present works focus on several factors for the enzyme modification such as organic solvents in microemulsion systems, the time of chemical modification, and the amount of the modifier. In addition, the catalytic behavior of the modified peroxidase with PMA-TD was determined in terms of reaction conditions such as pH, H_2O_2 content, and substrate types to evaluate the potential application of the modified peroxidase in contaminated soil washing.

2. Materials and methods

2.1. Materials

Horseradish peroxidase (264 units/mg, RZ of 3.30) was purchased from Biozyme Laboratories (South Wales, UK). One unit of peroxidase activity is defined as the amount of enzyme, which produces 1.0 mg of purpurogallin from pyrogallol in 20 s at 20 °C and pH 6.0. PMA-TD polymer was used as a modifier for the chemical modification of peroxidase and was supplied by Sigma-Aldrich Chemical Co. (St. Louis, MO). The phenolic compounds including phenol, *o*-chlorophenol, *m*-chlorophenol, *p*-chlorophenol, 2,4-dichlorophenol, and 2,6-dimethoxyphenol were obtained from Wako Chemical Ind. (Osaka, Japan). All chemicals were of the highest purity available and were used without further purification.

2.2. Chemical modification of peroxidase

The chemical modification of peroxidase was carried out in buffer-solvent microemulsion systems because PMA-TD was insoluble in aqueous solutions (Fig. 2). PMA-TD was dissolved in organic solvents including *n*-butyl acetate, and microemulsion systems were prepared by vigorous stirring for 30 min after addition of boric acid-borax buffer (pH 8.0) at 0 °C. Peroxidase prepared in the buffer solution was added slowly to the microemulsion with gentle mixing for 1 h at 0 °C. After the modification reaction, the reaction mixture was freeze-dried for 6 h and the excess PMA-TD was removed by extraction with *n*-butyl acetate and drying. The recovered modified peroxidase with PMA-TD was dissolved in a phosphate buffer solution (100 mM, pH 7.0) by mild sonication at 0 °C. The modification degree of amine groups on peroxidase was determined by a 2,4,6-trinitrobenzene sulfonic acid (TNBS) method [22,23]. Briefly, a mixture was prepared by adding 1 mL of 0.1% TNBS, 1 mL of 10 mM sodium sulfate, 4 mL of 500 mM borate buffer (pH 9.3), and 1 mL of PMA-TD-modified peroxidase solution. The mixture was continuously stirred with a shaker at 80 rpm for 30 min at 37 °C. The rate of color development at 420 nm proportional to the number of unmodified amine groups in the modified peroxidase with PMA-TD was measured in triplicates. The activity of peroxidase modified with PMA-TD in microemulsion systems was determined by a 4-aminoantipyrine (AAP) method [24,25]. Briefly, a mixture was prepared by adding 2500 μL of 10 mM phenol, 1650 μL of 100 mM phosphate buffer solution (pH 7.0), 250 μL of 48 mM 4-AAP, 500 μL of 2 mM H_2O_2, and 100 μL of 1 mg/mL peroxidase solution. All assay reactants were prepared in the phosphate buffer. The mixture was continuously stirred with a shaker at 80 rpm for 60 min at 25 °C. The rate of color development by antipyril quinoneimine synthesized was measured at 510 nm, which was proportional to the concentration of active peroxidase. Absorbance obtained from native and modified peroxidases was directly compared to calculate relative activity.

2.3. Polymerization reaction with phenolic compounds

A typical reaction mixture consisted of 7 mL of 100 mM phosphate buffer (pH 7.0), 12.5 mL of 0.8 mM phenolic compounds, and 0.5 mL of 1 mg/mL PMA-TD-modified peroxidase, and enzymatic reaction was initiated by adding 5 mL of 2 mM hydrogen peroxide. The mixture was incubated with constant shaking at 80 rpm for 1 h at 25 °C. To measure the concentration of remained phenolic compounds in the solution, the mixture was filtrated with 0.45 μm membrane filter and the filtrate was analyzed by HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a 25 cm × 4.6 mm fluofix 120N column (Wako Chemical Ind., Osaka, Japan) and a UV absorbance detector operated at 280 nm. The