High Stability in Organic Solvent of Heme Proteins Immobilized in the Interlayers of Magadiite Nanoparticles

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Enzymatic activities of heme proteins, myoglobin (Mb) and hemoglobin (Hb), immobilized in the interlayers of magadiite nanoparticles under mild condition, toward the oxidation of *o*-phenylenediamine (OPD) in an organic solvent were firstly reported. The immobilized heme proteins showed higher stability than that of free Mb and Hb in organic toluene solution.

Enzymes have great potential for the use in organic synthetic chemistry due to their high efficiencies and specific catalytic characters. Since the pioneering work of Zarks and Klibanov,[1] organic solvents have been used extensively in enzymatic syntheses and considerable progresses have been achieved in this field. The advantages for the use of organic solvents as media for enzymatic reactions are obvious, such as increased thermal stability[2] and lower inhibition effects[3] due to a reduced product concentration at the enzyme resulting from the normally better solubility of the product in the surrounding organic phase than in the microenvironment of the hydrophilic enzymatic surface. Owing to these obvious merits, an increasing interest on enzymatic catalysis in nonaqueous media has undergone rapid expansion, particularly over the past decades. However, drastic reductions of catalytic efficiency for enzymes in organic media can occur, which is attributed to the phenomena such as aggregation or irreversible conformational changes. It confined the applications of enzyme in industrial chemistry in a large scale.

To effectively utilize enzymatic catalysis, different approaches to the construction of biocatalytic systems based on organic solvents have been successfully developed,[4] such as surface modification of enzyme with surfactant and polymer, reversed micelles and immobilization of enzyme. While enzyme immobilization seems to be the most promising approach to prevent enzyme denaturation not only in aqueous solvents but also in organic media,[5] immobilization may greatly increase the stability of enzymes, be separated from products and reused easily. In order to improve the enzymatic catalytic efficiency in organic solvents, it is the key to increase the contact probability between enzyme and substrate. Nanomaterials provide larger specific surface areas, which are good candidates of support matrixes for enzyme immobilization. Enzymes immobilized on ordered surfaces, such as layered inorganic materials[6] and phosphate bilayer membranes,[7] have been reported. Hamachi realized the functional conversion of Mb bound to synthetic bilayer membranes from an oxygen storage protein to a redox enzyme. The advantages for the layered inorganic materials as support matrixes are obvious in aqueous solvent to preserve biological activity of enzyme. So far, it is unknown for enzymes immobilized in the interlayer region of layered materials whether the enzymatic activity is still preserved in organic media. In this paper, we combined the advantages of layered materials with nanomaterials, firstly report the catalytic activities of two kinds of water-soluble heme proteins, Mb and Hb, immobilized in the interlayers of magadiite nanoparticles toward the oxidation of *o*-phenylenediamine (OPD) in organic solvents. We have found that the stability or durability of immobilized heme proteins were higher than that of free Mb and Hb.

Stable layered silicate sol consisting of 10–100-nm diameter particles of tetrabutylammonium hydroxide (TBAOH) intercalated magadiite (TBA-magadiite) was successfully obtained by intercalation method.[8a] Mb or Hb-intercalated magadiite (Mb- or Hb-magadiite) nanocomposites were achieved by dripping TBA-magadiite suspensions (2.0 mM) into Mb (60 μM) or Hb (15 μM) (pH was controlled at 7 by K_2HPO_4) solutions, respectively, and stirring for 1 h at room temperature. Drying of Mb- or Hb-magadiite was achieved by lyophilizing the samples.

Catalytic activities and kinetic constants of Mb and Hb were measured by spectrophotometric handles. The addition of *tert*-butyl hydroperoxide (10 mM) to the reaction system containing 1.0 mg free or immobilized heme proteins and *o*-phenylenediamine (2.5 mM) in toluene solvent resulted in the formation of 2,3-diaminophenazine, as observed in the absorption spectra. It has found that the intensity of the absorbance at 496 nm increases along with the increased reaction time. The kinetics of enzymatic reaction was studied by the initial rate method and the steady-state assumption. The Michaelis–Menten constants K_m and the maximum rate V_{max} were obtained from Lineweaver–Burk plots and the transformation constant K_{cat} was obtained from $V_{max} = K_{cat} \times [E]_0$, where $[E]_0$ is the initial concentration of enzyme. The K_{cat} values represent the catalytic activities.

The characterization of novel heme proteins, Mb and Hb, immobilized in the interlayers of magadiite was described in another paper.[8b] SEM and HRTEM images showed that the composites of about 30-nm diameters with a certain aggregation were formed. UV–vis and FTIR spectra showed that no visible denaturation occurred. Immobilized Mb and Hb retained their activity and could be readily accessible for substrates in aqueous solvent. The effect of organic solvent on the activity of Mb and Hb immobilized in the interlayers of magadiite nanoparticles was studied here. Catalytic oxidation of *o*-phenylenediamine by the free and immobilized proteins with the absorbance at 496 nm in toluene solvent was shown in Figure 1. The immobilized Mb and Hb in the galleries of layered magadiite nanoparticles exhibited higher stability than that of the free Mb and Hb. The formation amount of 2,3-diaminophenazine for immobilized Mb and Hb increased 60.0 and 113.0% compared to that of free Mb and Hb after 30 min, respectively. When the immobilized Mb and Hb were stored in toluene solvent for 30 days under stirring condition, the formation amount of product for im-

Table 1. Kinetic constants of free and bound heme proteins in toluene solvent

Heme Proteins	K_m/mM	V_{max}/μM min^{-1} mg^{-1}	$K_{cat} * 10^3$/min^{-1}	$(K_{cat}/K_m) * 10^3$/min^{-1} mM^{-1}
Free Mb	0.54	0.78	6.61	12.24
Bound Mb	0.28	0.42	3.56	12.71
Free Hb	3.23	0.72	24.49	7.58
Bound Hb	1.75	0.48	16.33	9.33

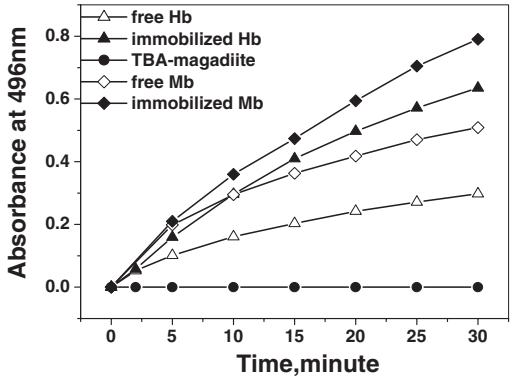

Figure 1. *tert*-Butyl hydroperoxide activated peroxidase activity for oxidation of *o*-phenylenediamine to 2,3-diaminophenazine with the absorbance at 496 nm at different interval times for free and immobilized Mb and Hb. ([*tert*-butyl hydroperoxide] = 10 mM, [*o*-phenylenediamine] = 2.5 mM, [Mb] = [Hb] = 1 mg).

mobilized heme proteins decreased 71.1% (Mb) and 68.9% (Hb), respectively, while the enzymatic activities for the free heme proteins were almost totally lost under the same conditions. These results suggest that there may exist facile diffusion of the reactants through the layered support and facile access to the active sites of the immobilized proteins in the organic media. The higher stability of the immobilized Mb and Hb composites demonstrates that the layered magadiite nanoparticle matrix could efficiently prevent the conformational changes of Mb and Hb in the organic solvent.

Comparison of the values of the kinetic constants based on the Michaelis–Menten equation for the free and the bound Mb and Hb is represented in Table 1. It is evident from the table that the free Mb and Hb have larger values of K_m and V_{max} than that of bound Mb and Hb, about twofold in K_m and V_{max} values of bound Mb and Hb. The transformation constants (K_{cat}) decrease accordingly and the catalytic specific constants (K_{cat}/K_m) of bound Mb and Hb are improved compared to that of free Mb and Hb. The decrease in V_{max} values was similar to that from other methods for protein immobilization,[9] indicating that the mass transport limitations still existed in the composites. The decrease in K_m values indicated that the affinities of immobilized Mb and Hb to substrate were stronger than that of free heme proteins, similar to the results in aqueous solvent. It suggested that the substrate molecules had no difficulty to access the active sites, which might result from the more open framework of the matrix than that of the other supports. Both decreases in K_m and V_{max} values might be due to the existence of product inhibition. This could be due to the restricted environments imposed by the rigid matrix. The reductions in V_{max} and K_{cat} for the immobilized Mb and Hb indicated that the catalytic activities were lower than that of the free Mb and Hb. Although the catalytic activity for the immobilized Mb and Hb was low, a larger amount of products were obtained after 30 min, which indicated that it was still an efficient method to protect enzyme from harming by organic solvents.

In conclusion, Mb and Hb immobilized in the interlayers of magadiite nanoparticles showed relatively lower initial or intrinsic activity and higher stability or durability than that of free Mb and Hb, which demonstrated that the layered magadiite nanoparticle matrix could efficiently prevent the conformational changes of Mb and Hb in the organic solvent. The encouraging results would be useful for and applicable to industrial processes and other applications, especially some environmentally benign enzymatic reactions.

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