Research Report

New Strategies for Enzyme Stabilization Involving Protein Engineering and Immobilization in Mesoporous Materials

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Abstract

The manganese peroxidase (MnP) produced by Phanerochaete chrysosporium, which catalyzes the oxidation of Mn^{2+} to Mn^{3+}, is easily inactivated by the H_2O_2 present in the reaction. We attempted to increase the H_2O_2 resistance by the conformational stabilization around the H_2O_2-binding pocket. Based on its structural model, the engineering of oxidizable Met residues located near the pocket to non-oxidizable Leu residues showed a significant improvement. The immobilized MnP mutant (IMnP; M67L/M237L/M273L) in mesoporous material (FSM-16) showed a high H_2O_2 stability, more than 100 fold greater than wild type MnP. However, the stability of the immobilized wild type MnP was not improved as much as that for the immobilized IMnP.

This new strategy involving protein engineering and immobilization in mesoporous materials would be applicable for other enzymes which have an unstable binding pocket.

Keywords Protein engineering, Mesoporous materials, Hydrogen peroxide resistant

1. Introduction

In recent years, many support matrices and coupling chemistries have been developed and made commercially available for use in protein immobilization. However, some useful enzymes are unstable and are not ideal for immobilization on a commercially available support.

Periodic mesoporous materials with uniform pore diameters of 10~300 Å have been synthesized[1)]. Because the pore diameters of these materials approximate those of enzymes molecules, their application as enzyme supports has been suggested. We previously reported[2, 3)] that horseradish peroxidase immobilized in mesoporous materials with suitable mesopore sizes had the best thermal stability and highest peak activity in an organic solvent. We also reported that MnP was successfully stabilized in a mesoporous material (FSM-16), when the mesopore size of FSM-16 was nearly the same as the diameter of the enzyme. MnP catalyzes the oxidation of Mn^{2+} to Mn^{3+} utilizing H_2O_2 and forms a complex with an organic acid. The Mn^{3+}-chelate complex is a highly reactive non-specific oxidant[4)] capable of oxidizing a variety of environmental pollutants[5)]. This powerful oxidant has been proposed to be important for the degradation of sterically bulky compounds that are unable to gain access to the active site of a peroxidase. This mediated system of degradation is potentially valuable for some applications, such as in the pulp and paper industries, the degradation of synthetic polymers and the degradation of environmental pollutants. However, MnP is very sensitive to inactivation by H_2O_2 or thermal treatment. An attempt to increase the thermo-stability of MnP has been reported[6)], but one to increase the resistance to H_2O_2 has not yet been reported. The inactivation pathway by H_2O_2 of MnP has been studied and the formation of compound III results in heme bleaching and irreversible inactivation[7)]. One of the primary causes of protein instability is its susceptibility to conformational change around the active site and subsequent inactivation or denaturation. These phenomena have been especially reported for proteins containing methionine, cysteine and tryptophan residues, by oxidation in or around the active site[8)].

In this paper, we show the excellent enzyme stabilizing strategies involving protein engineering and immobilization in mesoporous materials.

We have already constructed a two-stage reaction system for pulp bleaching involving native MnP[9)]. Our ultimate goal is to produce a more resistant enzyme, which would be suitable for use in the presence of a high concentration of H_2O_2 for commercial applications.

2. Materials and methods

2. 1 FSM-16 preparation.

FSM-16 materials with pore diameters of 69Å were prepared from kanemite using hexadecyl-trimethylammonium chloride and 1, 3, 5-tri-isopropylbenzene (TIPB) in the molar ratio of TIPB/surfactant = 3 as described by Inagaki et al.[1)].

2. 2 Structural modeling of MnP

We constructed models of MnP isozyme 2 and mutants by the homology modeling method using the LOOK&SEGMOD module of GeneMine. Crystal structure files for the MnP isozyme 1 were obtained from the Brookhaven Protein Data Bank (1MNP), and used as the backbone of our starting structure. The addition of the necessary hydrogen atoms to the structure was performed by means of the biopolymer module of Insight II 97.0. Simulations were performed with the Discover 3.0.0. program (Molecular Simulations Inc., USA) with energy minimization and molecular dynamic calculations. Nonbonded parameters were obtained by the Cell Multipole method under the Esff force field. The molecular dynamic calculations were performed with the NVT ensemble at 296K.

2. 3 Construction of an E. coli expression vector and production

In the plasmid pET21b(+)-MnP, the MnP gene under the control of the T7 promoter, possesses an C-terminal six-His tag, and the ampicillin marker. pET21b(+)-MnP and mutants were transformed into the E. coli strain BL21(DE3)LysS (Novagen), and then grown at 37°C in Luria broth to an absorbance of 0.6 at 600nm. IPTG was added to a final concentration of 0.1mM, followed by harvesting by centrifugation. The cell pellets were resuspended in lysis buffer (20mM Tris-HCl, pH8.0, 10mM DTT,

1mM EDTA), and then frozen overnight. After thawing, 1mM AEBSF and 0.5% TritonX-100 were added, followed by sonication for 20min. After centrifugation, the pellets containing the inclusion bodies were resuspended overnight in solubilization buffer (8M urea, 50mM Tris-HCl, pH8.0, 2mM EDTA, 1mM DTT).

2. 4 Immobilized enzyme preparation

MnP was immobilized by adding 100mg mesoporous materials to 5ml of MnP solution. The mixture was rotated for 16h and then centrifuged and the resultant precipitate was washed with deionized water and then stored at 4°C under dark conditions.

2. 5 H_2O_2 stability and dependency studies

MnP solution was incubated in the presence of 0-3.0 mM H_2O_2 at 37°C for 60min, and then diluted to 0.1mM H_2O_2. The MnP activity (Mn^{2+} to Mn^{3+}) was measured using the general peroxidase substrate ABTS. The used concentrations were 0.5mM ABTS, 2mM oxalate, 0.1mM $MnSO_4$, 0.1mM H_2O_2, and 25mM sodium succinate, pH4.5, and measured at 405nm for 5min.

Enzyme mixtures containing various concentrations of H_2O_2 (0.006-10 mM) were used for the H_2O_2 dependency studies. The MnP activity was measured as described above. All data are the mean values of at least four samples.

3. Results and discussion

3. 1 Engineering the methionine residues of MnP

Chemical oxidation of a methionine residue to a sulfoxide derivative has been reported for some proteins[7, 8]. H_2O_2 treatment leads to the inactivation of enzymes that is correlated directly with the production of methionine sulfoxide. MnP has seven methionines in its molecule. **Figure 1** shows the positions of the engineered Met of the structural model of the cloned MnP. Among them, Met 67, 237 and 273 were located within 12Å from the center of the active site, and Met 94, 223 and 346 were located on the surface of the MnP molecule. Predictions based on the homologous exchange data of amino acids suggest that Leu or Val is the most suitable for the substitution of a Met. Four of them are the components of the helix structure, and Lue is one of the most suitable for the form action of a helix structure, so we chose Lue for the substitution of Met.

We engineered two mutants; one was engineered at the inner three methionines (IMnP; M67L/M237L/M273L), which would prevent the conformational changes near the active site caused by chemical oxidation, and the other was engineered at the outer three residues, whose side chains were directed toward the outer region (OMnP; M94L/M223L/M346L). Also, the side chain of one more, Met265, was buried in the molecule, so we did not substitute this position.

IMnP and the wild-type were incubated at 37°C in the presence of 0-3 mM H_2O_2 for 60min. The residual activities are shown in **Fig. 2**. IMnP

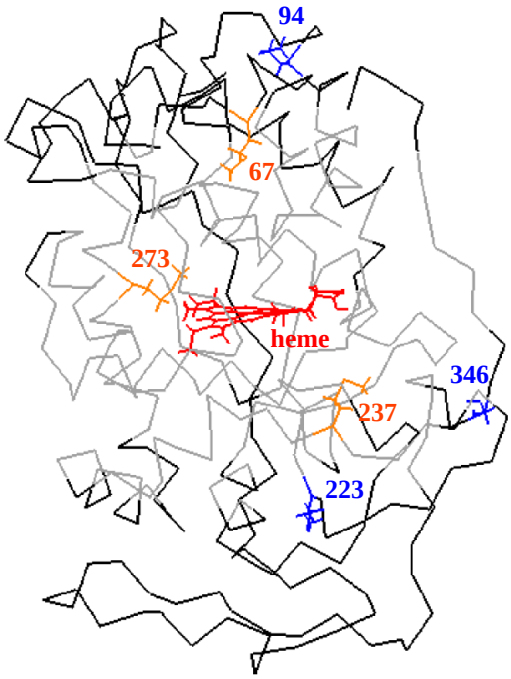

Fig. 1 Structural mapping of the substituted methionine residues.
Substitution of three methionine residues (67, 237 and 273; orange) located within a radius of 12Å (gray) from the heme, the resulting enzymes being designated as IMnP. Another three methionine residues (94, 223 and 346; blue), whose side-chains were located on the surface of the molecule, were also substituted with leucine, the resulting enzymes being designated as OMnP.

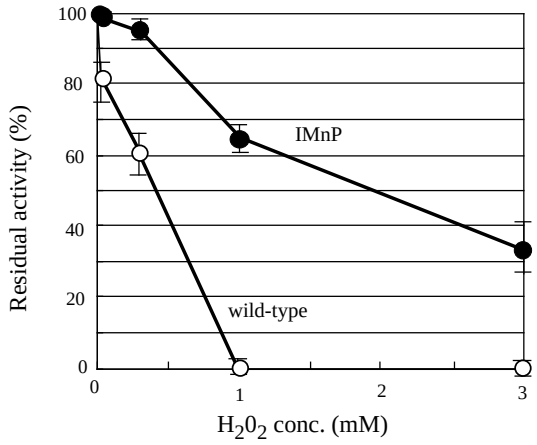

Fig. 2 Oxidative stability of IMnP.
Enzyme mixtures were incubated in the presence of various concentrations of H_2O_2 at 37°C for 60min, and then the residual activity of MnP was measured. ○ , wild-type; ●, IMnP (substitution of inner methionine residues; M67L, M237L and M273L). All data are the mean values for at least four samples.

retained more than 60% of its initial activity at the concentration of 1mM H_2O_2 and more than 30% at a concentration of 3mM H_2O_2, while wild-type was completely inactivated at the concentration of 1mM H_2O_2 .

In order to produce a more resistant enzyme for commercial applications, we evaluated the H_2O_2 resistance of MnP by measuring the MnP activity in the presence of various concentrations of H_2O_2. The H_2O_2 dependency of IMnP and OMnP are shown in **Table 1**. IMnP showed excellent H_2O_2 resistance, being 50% stable with 1.13mM H_2O_2 for 5min. The wild-type was 50% stable with 0.18mM H_2O_2 , so that H_2O_2 dependency of IMnP was 6.3 fold higher than that of the wild-type. OMnP showed almost the same dependency with the wild-type. We concluded that the engineering of the inner Met was a critical method for improving both the stability and the dependency to H_2O_2 , because of the protection of the oxidative environment around the active site. The engineering of the surface Met (OMnP) was not effective.

To determine which Met is most critical for the H_2O_2 resistance, we engineered three single mutants

Table 1 Improvement of the H_2O_2 resistance of various mutants.

Clone No.	Amino acid No. [1] 67	81	94	223	237	273	346	H_2O_2 dependency H_2O_2(mM) [2]	mutant/wild-type [3]	H_2O_2 stability [4] residual activity
wild-type	M	N	M	M	M	M	M	0.18 ± 0.1	1.0	0
OMnP	-	-	L	L	-	-	L	0.22 ± 0.1	1.2	6.8 ± 4.2
IMnP	L	-	-	-	L	L	-	1.13 ± 0.1	6.3	64.2 ± 4.4
M273L	-	-	-	-	-	L	-	0.74 ± 0.2	4.1	-
M237L	-	-	-	-	L	-	-	0.18 ± 0.1	1.0	-
M67L	L	-	-	-	-	-	-	0.25 ± 0.1	1.4	-

1) The amino acid substitutions in each mutant are shown.
2) The H_2O_2 concentrations which exhibited 50% relative activity are shown.
3) Mutant/wild-type ratio of H_2O_2 concentrations of 2).
4) % residual activity after treatment for 60min at 37°C in the presence of 1mM H_2O_2 .

(M67L, M237L and M273L). These mutants were reacted in the presence of various concentrations of H_2O_2 (Table 1). Among the mutants, only the M273L showed high H_2O_2 resistance, i.e., 4.1 fold higher than that of the wild-type. Met273 is the nearest residue to the active site. It is located about 4.3Å from the heme edge and faces the active site pocket, therefore this residue is solvent-accessible and easily oxidized. M67 and M237 are located 6.6Å and 12Å from the heme edge. These residues are near the active site, but are buried in the MnP molecule, so they are not solvent-accessible. Our data suggested that the conversion of the oxidizable methionine residue around the pocket to the non-oxidizable residue was critical for improvement of the oxidative resistance.

3. 2 H_2O_2 stability of MnP mutant immobilized in FSM-16

The structural model of the H_2O_2 binding pocket, the MnP molecule (left panel) , in which H_2O_2 was directly supported by His46 and Arg42, and image model of the immobilized recombinant MnP in mesoporous material (FSM-16, right panel) are shown in **Fig. 3**. The improved oxidative stability of the free MnP mutant (a) and the immobilized MnP mutant (b) are shown in **Fig. 4**. The mutant (IMnP), that engineered the oxidizable Met residues near the H_2O_2 binding pocket to the non-oxidizable Leu residue showed an improved stability for H_2O_2 . Immobilized IMnP in the mesoporous material showed an excellent H_2O_2 stability (more than 100 fold higher than the wild type MnP). However the stability of the immobilized wild type MnP was not improved as much as that for the immobilized IMnP.

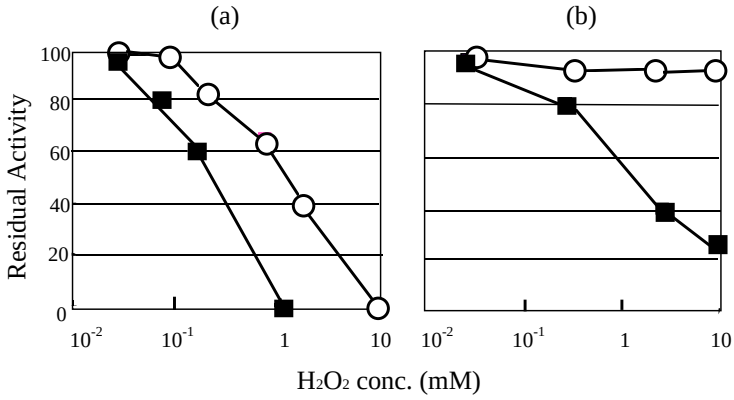

Fig. 4 The oxidative stability of non-treated enzymes (a) and immobilized enzymes in mesoporous materials (b). Enzyme mixtures were incubated in the presence of various concentrations of H_2O_2 and residual activity was measured.
■ : wild-type MnP; ○ : recombinant MnP (IMnP)

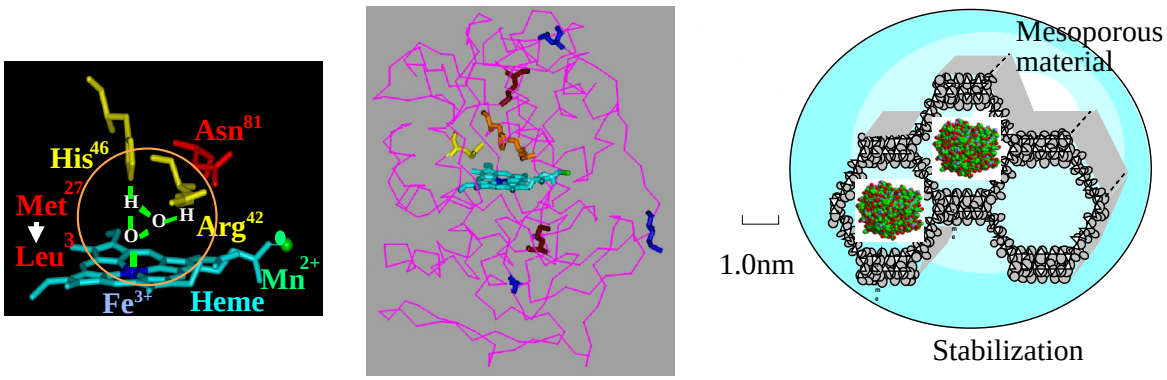

Fig. 3 Structural model of H_2O_2-binding pocket (left), MnP molecule (middle) and image model of immobilized MnP mutant in mesoporous material (FSM-16).
In the left panel, the residues of H_2O_2-binding site are His46 and Arg42 (green) in the distal heme pocket. The residues around the pocket are 78 (blue), 79 (orange), 80 (pink), 81 (red), and others (light pink), and heme (yellow), with CPK (Corey-Pouling-Keltun) space filling.

The unstable amino acids located in the outer surface region would be protected by immobilization in suitable mesoporous materials. However, the oxidizable amino acids facing the H_2O_2 binding pocket were not protected by the mesoporous materials. IMnP would have excellent oxidative stability by immobilization, because the oxidizable Met residues near the H_2O_2 binding pocket were changed to non-oxidizable Leu. The combination of protein engineering and immobilization in suitable mesoporous materials shoud be important for the best enzyme stability.

4. Conclusion

We suggested that the prediction of critical restricted mutations in the functional region, using a structural model generated with a computer-driven model-building system or an X-ray crystal structure study, is an effective method for efficiently converting the function of the protein[10]. In this study, we constructed models of the MnP isozyme 2 and predicted the oxidizable and/or solvent accessible and/or conformationally unstable amino acid residues around the H_2O_2-binding pocket. All the characterized mutants are shown in Table 1. We investigated the effects of engineering the oxidizable Met facing the pocket to non-oxidizable residues (IMnP, M273L), also the conformational stabilization around the pocket was proved to be very effective for improving the H_2O_2 resistance.

In addition, the engineering of an oxidizable Met located on the surface is not very effective. This suggested that amino acid residues around the pocket critically affected the H_2O_2 resistance. Moreover, fine-tuning for accommodation by the pocket of MnP would require a random mutation process such as the directed evolution method.

We also determined the effectiveness of the combination of the site-directed mutagenesis of critical residues around the functional region and the immobilization in a suitable mesoporous material. This new strategy involving protein engineering and immobilization in mesoporous materials would be applicable for the excellent stabilization of other enzymes which have an unstable binding pocket, especially for the H_2O_2-binding pocket-containing proteins for improvement of the H_2O_2 resistance. The present results will be useful for industrial applications, such as in the pulp and paper industries and the degradation of environment pollutants.

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