New strategies for enzyme stabilization involving molecular evolution and immobilization in mesoporous materials

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Heme containing peroxidase, for example manganese peroxidase (MnP), is easily inactivated by the hydrogen peroxide (H$_2$O$_2$) presented in the reaction. Here we extremely increased the H$_2$O$_2$ stability of MnP by molecular evolution and immobilization in mesoporous materials. A mutant MnP library containing three randomized amino acid residues located in the entry site of H$_2$O$_2$-binding pocket of MnP was evolved on a 384-well plate using SIMPLEX (single-molecule PCR-linked in vitro expression). The screening of more than 10$^4$ samples independently expressed for improved H$_2$O$_2$ stability led to four positive mutants, the H$_2$O$_2$ stability of which was nine times higher than that of the wild-type. Immobilized MnP mutant in mesoporous material (FSM) showed the high H$_2$O$_2$ stability, more than 50 folds than wild type MnP. But the stability of immobilized wild type MnP was not improved so much as that for immobilized mutant MnP. [DOI: 10.1380/ejssnt.2005.207]

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I. INTRODUCTION

In recent years, periodic mesoporous materials with uniform pore diameters of 2~30 nm have been synthesized [1–10]. Because the pore diameters of these materials approximate those of enzymes molecules, their application as enzyme supports has been suggested. We showed that the surface characteristics of mesoporous materials and matching of the size of protein molecules and the pore diameter of mesoporous materials were essential for the protein stabilization [11–14]. We also reported that manganese peroxidase (MnP) was successfully stabilized in a mesoporous material (FSM), when the mesopore size of FSM was nearly the same as the diameter of the enzyme [15]. MnP catalyzes the oxidation of Mn$^{2+}$ to Mn$^{3+}$ utilizing hydrogen peroxide (H$_2$O$_2$) and forms a complex with an organic acid. The Mn$^{3+}$-chelate complex is a highly reactive non-specific oxidant capable of oxidizing a variety of environmental pollutants [16–18]. However, MnP is very sensitive to inactivation by H$_2$O$_2$ or thermal treatment. An attempt to increase the thermo-stability of MnP has been reported [19], but one to increase the resistance to H$_2$O$_2$ has not been reported.

On the other hand, cell-free protein synthesis systems allow the rapid production of proteins directly from plasmid or polymerase chain reaction (PCR)-amplified DNA templates. Another striking advantage of the system is easy modification of the composition of reaction mixtures according to the requirements for the synthesis of each protein. However, heme-containing proteins have not been successful in functional expression. We have succeeded to synthesize a functional MnP using a modified E.coli in vitro protein synthesis system with various disulfide-forming catalysts (protein disulfide isomerase, PDI) and chaperones [22].

Recently, Nakano, et al. developed single-step single-

molecule PCR (SM-PCR) with a homo-primer for amplification from a single-molecule DNA template and proposed a novel protein library construction system named SIMPLEX (single-molecule-PCR-linked in vitro expression) [20]. Correspondence of a genotype (SM-PCR product) with a phenotype (in vitro synthesized protein) has made it possible to screen a large diversity of mutant enzymes and to increase the enzymatic function efficiently. We have increased the H$_2$O$_2$ stability by SIMPLEX technology exclusively on 384-well plates. The high-throughput construction of mutant MnPs and screening in the presence of H$_2$O$_2$ resulted in the finding of mutants with improved stability against H$_2$O$_2$ [21, 22].

In this paper, we show the excellent enzyme stabilizing strategies involving the molecular evolution with SIMPLEX and the immobilization in mesoporous materials.

II. EXPERIMENTAL

A. Mesoporous materials (FSM) preparation

Mesoporous materials (FSM) with pore diameters of about 7 nm were prepared from kanemite using hexadecyltrimethylammonium chloride and 1, 3, 5-trisopropylbenzene (TIPB) in the molar ratio of TIPB/surfactant = 3 as described by Inagaki, et al. [4].

B. 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 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.
C. In vitro coupled transcription/translation

The E. coli S30 extract without a reducing agent used for the cell-free protein synthesis was prepared [23]. The plasmid, pET23b/gcmnp, was amplified from the T7 promoter to the T7 terminator and used as cell-free expression template at a final concentration of 50 mg/ml. Then, 10 mM heme was added to a 40 ml transcription/translation reaction mixture [56.4 mM Tris-acetate, pH 7.4, 1.2 mM ATP, 1 mM each GTP, CTP and UTP, 40 mM creatine phosphate, 0.7 mM each of 20 kinds of amino acids, 4.1% (w/w) polyethylene glycol 6000, 35 mg/ml folinic acid, 0.2 mg/ml E. coli tRNAs, 36 mM ammonium acetate, 10 mM Mg(OAc)$_2$, 100 mM KOAc, 0.15 mg/ml creatine kinase, 10 mM/ml rifampicin, 25 units T7 RNA polymerase (TaKaRa, Japan), 10 mM heme and 28.3% (v/v) S30 extract], followed by incubation at 25°C for 180 min. According to need, the molecular chaperones and PDIs were added at the following concentrations: DnaK (Calbiochem, Germany) 1.0 mM, DnaJ (Stress-Gen Biotechnologies, Canada) 1.25 mM, GrpE (kindly provided by Mr M. Sakurai of Nagoya University) 0.4 mM, GSH 1.0 mM, GSSG 0.1 mM, fungal PDI 0.5 mM and bovine liver PDI (TaKaRa, Japan) 0.5 mM. Fungal PDI from Humicola insolens KASI was produced in a heterologous protein production system using Bacillus brevis 31-0K as a host [24].

D. Screening of the library for H$_2$O$_2$-resistant activity of MnP

The homo-tailed templates were diluted to one molecule/ml in 0.1% blue dextran 2000/TE buffer. The mixture for SM-PCR was comprised of one molecule of template, 0.02 units of LA Taq polymerase (TaKaRa, Japan), 0.2 mM each dNTP and 0.5 mM of the primer at 7 ml/well in a 384-well plate. The reaction was allowed to proceed at 94°C for 2 min, followed by 65 cycles of 96°C for 10 s, 62°C for 5 s and 72°C for 10 s. A 3 ml portion of the amplified SM-PCR product (containing ~500 ng) was directly transferred to 37 ml of the transcription/translation reaction mixture, followed by incubation for 3 h at 25°C. A 1.5 ml volume of the synthesized reaction mixture was added to 100 ml of MnP assay buffer comprising 5 mM ABTS [2, 2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma, USA], 2 mM oxalate, 0.1 mM MnSO$_4$, 50 mM sodium succinate, pH 4.5 and 1.0 mM H$_2$O$_2$ and the absorbance was measured at 415 nm after 10 min. All screening steps were carried out in 384-wellplates.

E. Immobilized enzyme preparation

MnP was immobilized by adding 100 mg mesoporous materials to 5 ml of 1 mg/ml MnP solution. The mixture was rotated for 16 h at 4°C and then centrifuged and resultant precipitate was washed with deionized water several times and then stored at 4°C under dark conditions.

F. H$_2$O$_2$ stability and dependency studies

MnP solution or immobilized MnP were incubated in the presence of 0-3.0 mM H$_2$O$_2$ at 37°C for 60 min, and then were diluted to 0.1 mM H$_2$O$_2$. MnP activity (Mn$^{2+}$ to Mn$^{3+}$) was measured using the general peroxidase substrate ABTS [25]. The concentrations used were 0.5 mM ABTS, 2 mM oxalate, 0.1 mM MnSO$_4$, 0.1 mM H$_2$O$_2$ and 25 mM sodium succinate, pH 4.5, and measured at 405 nm for 5 min.
Enzyme mixtures containing various concentrations of \( \text{H}_2\text{O}_2 \) (0.006-10 mM) were used for \( \text{H}_2\text{O}_2 \) dependency studies. MnP activity was measured as described above. All data are the mean values for at least four samples.

### III. RESULTS AND DISCUSSION

#### A. Enzyme immobilization in mesoporous materials

The synthesis and crystal structure of mesoporous materials were shown in Fig. 1. On the surface of micelle, silica molecules were self-assembled, after the removal of surfactant, the mesoporous materials could be obtained. Nano size controlled uniform pore (2-dimensional hexagonal structure) was formed. The general procedure for the enzyme immobilization of mesoporous materials was shown in Fig. 2. First, the axis of enzyme molecule and its iso-electric point (pI) was searched or measured.

The pore diameter of mesoporous materials was controlled to nearly the same as enzyme molecule. By gently mixing in pH region under pI, the enzyme molecule was immobilized in the pore of mesoporous materials. Hydrogen bond between the surface amino residues of enzymes and silanol residues of mesoporous material is formed during immobilization and then enzyme is excellently stabilized.

#### B. MnP synthesis by an E.coli cell-free protein synthesis system

In the preparation of the E.coli S30 extract for MnP synthesis, a reducing agent, dithiothreitol (DTT), was omitted to maintain oxidizing conditions for the formation of the disulfide bridges of the protein, because functional MnP was not formed in the presence of a strong reducing agent [22]. To enhance the yield of functional MnP, reduced and oxidized glutathione (GSH and GSSG), PDIs and chaperones were added to the in vitro expression mixture. PDI was added with GSH/GSSG to activate its own active site. The addition of only chaperones (DnaK/J, GroES/EL and GrpE) hardly changed the MnP activity. When fungal PDI was present in the reaction mixture, the MnP activity increased up to 4-fold compared with the case of a control mixture without PDI or chaperones, but bovine PDI had no such effect. Although MnP activity was high when both chaperones and fungal PDI were added, it was suggested that this was mainly an effect of fungal PDI. Although an E.coli disulfide-forming catalyst, DsbA might be included in the S30 extract, it dose not seem effective in producing active MnP. It is considered that fungal PDI, which has both isomerase and chaperone activities [26] and facilitates the folding of denatured and reduced SH-proteins, played an important role in MnP folding to the native form with the correct disulfide formation in this cell-free system.
FIG. 3: Schematic procedure of molecular evolution using single-molecule PCR-linked in vitro expression (SIMPLEX). A portion of the PCR product was used as a template for the cell-free expression, and resulting a mutated protein library was constructed. Subsequently the protein library was screened with desirable condition.

C. Design of mutant MnP library

The three-dimensional structure of MnP isozyme 1 (PDB: 1MNP) has been well characterized by X-ray crystallography [27]. In previous work, we constructed a model of MnP isozyme 2 by the homology modeling method using 1MNP as the backbone for the starting structure [21]. A structural model of the H$_2$O$_2$-binding pocket of MnP is shown in Fig. 4(A). Amino acid residues located near His46 and Arg42, which are conserved in H$_2$O$_2$-binding residues [27], were picked as candidates for substitution for the library. Conserved amino acid residues in various peroxidases were excluded as candidates for substitution. To bring about a minute conformational change around the entry site of the H$_2$O$_2$-binding pocket, three amino acid residues, i.e. A79, N81 and I83, were selected for replacement with all kinds of amino acid residues (A79X, N81X and I83X). The theoretical number of mutated MnP with different sequences was 8000.

D. Screening of the MnP library

We have thus constructed a mutant MnP library containing randomized A79, N81 and I83 by SIMPLEX and screened more than $10^4$ wells by measuring MnP activity in the presence of 1.0mM H$_2$O$_2$, where the wild-type gave no detectable. The whole process is illustrated in Fig. 3. 15 clones showed improved H$_2$O$_2$ stability and their whole sequences were confirmed. The clones were classified into four types of sequences according to amino acid substitution. The amino acid substitutions and the H$_2$O$_2$ stability of the four types of clones are shown in Table I.

TABLE I: H$_2$O$_2$ Stability and Amino acid substitutions of mutants.*

| clones | amino acid No. | H$_2$O$_2$ stability* |
|--------|----------------|-----------------------|
|        | 79 81 83       | 0.1 0.5 1.0 (mM)      |
| wild-type | A N I   | 1.0 1.0 1.0          |
| clone 1 | E S L     | 9.0 6.6 3.9          |
| clone 4 | S L L     | 7.2 6.5 4.4          |
| clone 6 | S S L     | 5.3 4.6 3.2          |
| clone 8 | E L L     | 7.0 3.5 1.8          |

Also other clones were five times more stable than the wild-type. Furthermore, even in the presence of higher concentrations of H$_2$O$_2$ of 0.5 and 1.0 mM, all of the four types of clones showed significantly higher stability than the wild-type. These four clones have a common substitution of I83L. In addition, A79 was changed for either E or S and N81 for S or L. Since these three amino acid residues were converged from a large variety in the library as a result of selection, this high-throughput screening system is likely to work well. Fine tuning of the local arrangement in the H$_2$O$_2$ binding pocket seems to be effective for improving the H$_2$O$_2$ stability of MnP.

E. H$_2$O$_2$ stability of MnP mutant immobilized in FSM

The structural model of H$_2$O$_2$ binding pocket, MnP molecule, in which H$_2$O$_2$ was directly supported by His46 and Arg42, and image model of immobilized recombinant MnP in mesoporous material was shown in Fig. 4. The improved oxidative stability of the immobilized native
FIG. 4: Structural model of H$_2$O$_2$-binding pocket (left), MnP molecule (middle) and image model of immobilized MnP mutant in mesoporous material (right). In the left figure, the residues of H$_2$O$_2$-binding site were His46 and Arg42 (green) in the distal heme pocket. The three amino acid residues (orange) around the entry site of H$_2$O$_2$-binding site pocket were selected and replaced with all kinds of amino acid residues.

FIG. 5: Synergetic effect of H$_2$O$_2$ stability. The H$_2$O$_2$ stability of wild type enzymes (A) and mutant enzymes (B) in non treated (blue) or immobilized in mesoporous materials (red) conditions. Enzyme mixtures were incubated in the presence of various concentrations of H$_2$O$_2$ and residual activity was measured.

MnP and the immobilized H$_2$O$_2$ resistant MnP mutant was shown in Fig. 5. The mutant MnP, evolved by SIMPLEX at the entry site of H$_2$O$_2$ binding pocket to stable amino acd residue showed the improved stability for H$_2$O$_2$ compared with native MnP. Immobilized H$_2$O$_2$ resistant mutant MnP in mesoporous material showed the excellent H$_2$O$_2$ stability (more than 50 fold than wild type MnP). But the stability of immobilized wild type MnP was not improved so much as that for immobilized mutant MnP.

The unstable amino acids located in outer surface region would be protected by immobilization in suitable mesoporous materials. But the unstable amino acids facing H$_2$O$_2$ binding pocket were not protected by mesoporous materials.

IV. CONCLUSIONS

To construct a mutant MnP library, SIMPLEX, which includes single-molecule PCR followed by cell-free protein synthesis, was used. Compared with conventional in vivo colony-based methods [28], this system is extremely rapid and efficient. For example, neither a transformation nor a cultivation step is necessary, and therefore replica plates, which are essential in cell-based screening for linking a genotype with a phenotype, also are not necessary. Instead, after using a portion of the SM-PCR product for protein synthesis, the remainder on a 384-well PCR plate can be frozen for long-term storage in a stable state. More importantly, the total time required for protein expression...
and screening was short, ~8 h: 4 h for SMPCR, 3 h for cell-free protein synthesis and 1 h for the screening of the protein library.

Our cell-free system involving fungal PDI would be applicable to the screening of other heme-containing proteins. Furthermore, the system can be widely applied for various kinds of screening of proteins with desired properties. The inactivation pathway of MnP by excess H₂O₂ has already been studied, suggesting that compound III formed in heme bleaching causes irreversible MnP inactivation [29, 30]. However, our results suggest that MnP instability against small amounts of H₂O₂ is closely related to the susceptibility to a conformational change around the active site, which eventually causes subsequent inactivation or denaturation.

We suggested that 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 converting the function of the protein efficiently. In this study, we constructed models of MnP isozyme 2 and predicted oxidizable and/or solvent accessible and/or conformationally unstable amino acid residues around the H₂O₂-binding pocket. This suggested that amino acid residues around the pocket critically affected the H₂O₂ 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 found the effectiveness of the combination of molecular evolution of critical residues around the functional region and the immobilization in a suitable mesoporous material. Immobilized mutant MnP in mesoporous material showed the excellent H₂O₂ stability (more than 50 fold than wild type MnP). But the stability of immobilized wild type MnP was not improved so much as that for mutant MnP. Unstable amino acids located in outer surface region would be protected by immobilization in suitable mesoporous materials. But unstable amino acids around the H₂O₂ binding pocket were not protected by mesoporous materials. Mutant MnP would show the synergistic effect by immobilization, because the unstable amino acids in H₂O₂ binding pocket were changed to stable amino acids. This new strategies involving enzyme evolution and immobilization in mesoporous materials would be applicable for the excellent stabilization of other enzymes which have unstable binding pocket.

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