Random Mutagenesis and Selection of Organic Solvent-Stable Haloperoxidase from *Streptomyces aureofaciens*

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Haloperoxidases are useful oxygenases involved in halogenation of a range of water-insoluble organic compounds and can be used without additional high-cost cofactors. In particular, organic solvent-stable haloperoxidases are desirable for enzymatic halogenations in the presence of organic solvents. In this study, we adopted a directed evolution approach by error-prone polymerase chain reaction to improve the organic solvent-stability of the homodimeric BPO-A1 haloperoxidase from Streptomyces aureofaciens. Among 1,000 mutant BPO-A1 haloperoxidases, an organic solvent-stable mutant OST48 with P123L and P241A mutations and a high active mutant OST959 with H53Y and G162R mutations were selected. The residual activity of mutant OST48 after incubation in 40% (v/v) 1-propanol for 1 h was 1.8-fold higher than that of wild-type BPO-A1. In addition, the OST48 mutant showed higher stability in methanol, ethanol, dimethyl sulfoxide, and N,N-dimethylformamide than wild-type BPO-A1 haloperoxidase. Moreover, after incubation at 80°C for 1 h, the residual activity of mutant OST959 was 4.6-fold higher than that of wild-type BPO-A1. Based on the evaluation of single amino acid-substituted mutant models, stabilization of the hydrophobic core derived from P123L mutation and increased numbers of hydrogen bonds derived from G162R mutation led to higher organic solvent-stability and thermostability, respectively. © 2015 American Institute of Chemical Engineers *Biotechnol. Prog.*, 31:917–924, 2015
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Introduction

Haloperoxidases catalyze the halogenation of organic compounds in the presence of halide ions and hydrogen peroxide.[1] Recently, these enzymes have attracted considerable attention because of their ability to halogenate a range of organic compounds, and the reaction is important in the industrial and pharmaceutical fields. However, the requirement of high-cost cofactors for the activity of most oxygenases hampers their industrial application. Haloperoxidases require the inexpensive hydrogen peroxide for oxidation reactions and are classified into three groups; heme-containing, vanadium-containing, and metal-free haloperoxidases.[2] These classes have different reaction mechanisms. Although heme- and vanadium-containing haloperoxidases have been well characterized, studies on metal-free haloperoxidases are limited. However, metal-free haloperoxidase BPO-A1 from *Streptomyces aureofaciens* has been relatively well studied[3,4]; its homodimeric structure has been elucidated.[5]

For industrial application of enzymes, organic solvent-stability and thermostability are desirable because enzyme reactions with organic solvents and/or elevated temperature may often be beneficial in many ways.[6,7] In particular, most haloperoxidase halogenation reactants are poorly soluble in an aqueous solution,[8] and water solubility is improved by addition of organic solvents to the reaction mixtures. Hence, the reaction rates and productivity could be improved using solvent-stable enzymes. In previous studies, we showed that disulfide bonding[9] and amino acid residues located at the surface of the protein molecule were important factors in organic solvent-stability.[10,11] Moreover, stabilization of the hydrophobic core and increased numbers of hydrophilic amino acid residues, salt bridges, and hydrogen bonds improved enzyme stability in the presence of organic solvents.[12] Reported mechanisms that improve thermostability include stabilization of the α-helix conformation,[13,14] oligomerization of the enzyme,[15,16] improvement of subunit interactions,[17,18] and enhancement of electrostatic interactions.[19,20] However, the mechanisms underlying organic solvent-stability and thermostability and their correlations are not completely understood.

Directed evolution is one of the approaches for improving enzyme functions, and involves the production of large mutant libraries using recombination, followed by high-throughput screening and selection of desirable mutants. Random mutagenesis via directed evolution has also been used to identify biocatalysts with high organic solvent-stability and thermostability in multiple studies. Accordingly, we generated lipases from *Pseudomonas aeruginosa* with

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ninefold to 11-fold longer half-lives in the presence of 25% (v/v) cyclohexane or *n*-decane than wild-type lipase by directed evolution.[10] Moreover, Zhang et al.[21] generated lipase from *Candida antarctica* and showed >20-fold increase in half-life at 70°C compared with wild-type lipase. Miyazaki et al.[22] also enhanced the half-life of psychrophilic protease subtilisin up to 500-fold at 60°C compared with wild-type subtilisin. In contrast with rational protein design approaches, details of functional domains and three-dimensional (3-D) structures of target enzymes are not required for the directed evolution approach,[23] which offers a promising approach for improving the organic solvent-stability of various enzymes.

Recently, we developed a stable BPO-A1 haloperoxidase using random mutagenesis and selected mutants with enhanced heat stability.[24] In this study, mutant BPO-A1 haloperoxidases were constructed using error-prone polymerase chain reaction (PCR), and high-throughput screening of organic solvent-stable haloperoxidases was performed. Further, mutant BPO-A1 haloperoxidases were purified, and their activity, organic solvent-stabilities, and thermostabilities were evaluated. Finally, single amino acid-substituted mutants were constructed using site-directed mutagenesis, and their properties were evaluated.

Materials and Methods

Organisms

Escherichia coli JM109 cells were used as the cloning host for recombinant DNA manipulations. *E. coli* Rosetta 2 (DE3) (Novagen, Madison, WI) cells were used for the production of wild-type and mutant BPO-A1 haloperoxidases. Transformed *E. coli* JM109 cells were cultivated on Luria–Bertani (LB) agar medium containing 30 mg/L kanamycin sulfate and 1.5% (w/v) agar. Transformed *E. coli* Rosetta 2 (DE3) cells were cultivated on LB agar medium containing 30 mg/L kanamycin sulfate, 17 mg/L chloramphenicol, and 1.5% (w/v) agar.

Plasmids

BPO-A1 haloperoxidase gene conjugated with the hexahistidine tag expressing plasmid pET42-b_BPO-A1-His was constructed as follows. BPO-A1 haloperoxidase gene was amplified using *S. aureofaciens* NBRC 12843 genomic DNA as a template with the primers 5′-CAC AGG CCT ATC TGC ACC ACC CGC GAC G-3′ and 5′-CTG GGC CTC GAG CTT GAG GAA TTC CAA CAG GTC CCG-3′ and LA *Taq* DNA polymerase (Takara Bio, Shiga, Japan). The amplified DNA fragment was digested using *Xho*I and *Stu*I restriction enzymes and was then ligated into the *Xho*I/*Psh*AI site of pET42-b (Novagen). The resulting plasmid was designated pET42-b_BPO-A1-His.

Construction of plasmid library of randomly mutated genes

Mutant BPO-A1 haloperoxidase genes were constructed using error-prone PCR. BPO-A1 haloperoxidase gene was amplified using pET42-b_BPO-A1-His as a template with the primers 5′-GAA GGA GAT ATA CAT ATG CCC ATC TGC-3′ and 5′-GGT GGT GGT GCT CGA GCT TG-3′ and the GeneMorph PCR mutagenesis kit (Stratagene, La Jolla, CA). The plasmid library of randomly mutated genes was obtained after digesting amplified DNA fragments with *Nde*I and *Xho*I and then ligating into the *Nde*I/*Xho*I site of pET42-b_BPO-A1-His.

Substitution of residues using site-directed mutagenesis

Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene) according to the supplier's protocol. Primers contained mutations in their centers, with 10–15 bases functioning as complementary template sequences on both sides. DNA fragments were digested with the restriction enzyme *Dpn*I, which digests parental methylated DNA. *Dpn*I-digested DNA was then transformed into competent *E. coli* JM109 cells, and transformants were cultured in LB agar media containing 30 mg/L kanamycin sulfate. Mutations were confirmed using nucleotide sequencing.

High-throughput screening of organic solvent-stable BPO-A1 haloperoxidases

E. coli Rosetta 2 (DE3) cells were transformed with mutant BPO-A1 haloperoxidase genes from the plasmid library and were cultured in LB agar media containing 30 mg/L kanamycin sulfate and 17 mg/L chloramphenicol. High-throughput screening of thermostable haloperoxidases was then performed using a 96-well plate-based cultivation and enzyme assay. Transformants were then cultivated in 100 μL of LB medium containing 30 mg/L kanamycin sulfate and 17 mg/L chloramphenicol. After 18 h at 37°C with rotary shaking at 150 rpm, 10 μL of culture was inoculated into 100 μL of fresh LB medium containing 30 mg/L kanamycin sulfate and 17 mg/L chloramphenicol. Transformants were then cultured for 135 min at 37°C with rotary shaking at 150 rpm, and 11 μL of 10 mM isopropyl-β-D(−)-thiogalactopyranoside was then added to induce the expression of mutant BPO-A1 haloperoxidases for the subsequent 7 h culture at 37°C with rotary shaking at 150 rpm. After induction, the cells were collected by centrifugation at 825*g* for 10 min and were resuspended in 100 μL 0.2 M Tris–H_2SO_4 (pH 8.3). Subsequently, cell suspensions were lysed at 80°C for 10 min, and debris were removed by centrifugation at 825*g* for 10 min at 4°C. Subsequently, 40 μL supernatant was mixed with 10 μL 1-propanol and incubated at 30°C with rotary shaking at 1,000 rpm for 15 min to inactivate mutant BPO-A1 haloperoxidases with low organic solvent-stability. The resulting mixtures were used for assays of haloperoxidase activity.

Haloperoxidase activity was assayed according to the reductive halogenation of monochlorodimedone (MCD) as previously described,[25] with some modifications. Enzyme solutions (10 μL) were mixed with a substrate mixture containing 150 μL 2 M sodium acetate buffer (pH 5.5), 6 μL 5 M NaBr, 3 μL 1 M NaN_3, 1 μL 2.64 M H_2O_2, 1 μL 14.4 mM MCD, and 129 μL deionized water. The enzyme and substrate mixture was incubated at 30°C for 3 min, and absorbance was recorded 18 times at 290 nm using a Multiskan GO microplate reader (Thermo Scientific, Rochester, NY). Enzyme activity was calculated as reductions in MCD absorbance.

Preparation of purified BPO-A1 haloperoxidases

Purified wild type and mutant BPO-A1 haloperoxidases were prepared from *E. coli* Rosetta 2 (DE3) transformants

cultivated in 500-mL baffled Erlenmeyer flasks containing 200 mL of LB medium supplemented with 30 mg/L kanamycin sulfate and 17 mg/L chloramphenicol. Transformants were initially grown at 37°C with shaking at 150 rpm. When the optical density at 600 nm reached approximately 0.75, isopropyl-β-D(−)-thiogalactopyranoside was added to a final concentration of 1 mM. The cells were then cultured for a further 6 h at 37°C with shaking and were then harvested by centrifugation at 3,000*g* for 10 min at 4°C.

The cells from 200 mL of culture medium were suspended in 10 mL 200 mM Tris–H_2SO_4 buffer (pH 8.3) and were intermittently disrupted using an ultrasonic disruptor (250DA; Branson Ultrasonics, Danbury, CT) at 52 W for 10 min in an ice bath. Disrupted cells were removed by centrifugation at 20,000*g* for 10 min at 4°C, and the supernatants were collected as soluble fractions.

Soluble fractions from *E. coli* transformants were incubated at 4°C for 30 min in 50% saturated ammonium sulfate solution with agitation. Precipitates were then collected by centrifugation at 20,000*g* for 10 min at 4°C and dissolved in 50 mL 50 mM sodium phosphate buffer (pH 7.4) containing 20 mM imidazole; they were purified using immobilized nickel ion affinity chromatography with a HisTrap HP column (GE Healthcare Bio-Sciences, Piscataway, NJ). Dissolved precipitates were applied to the HisTrap HP column, and adsorbed proteins were washed with 20 mL 50 mM sodium phosphate buffer (pH 7.4) containing 100 mM imidazole and were eluted with 10 mL 50 mM sodium phosphate buffer (pH 7.4) containing 300 mM imidazole. Finally, active fractions were pooled and desalted using a PD-10 column (GE Healthcare Bio-Sciences), and protein concentrations were evaluated using the Bradford method.[26]

Evaluation of organic solvent-stability of BPO-A1 haloperoxidases

Purified enzyme solutions (3 μg/L) were incubated for 1 h at 30°C with 1-propanol at concentrations ranging from 0% to 50% (v/v). Thereafter, the residual activity was measured as described later in the Assay of Purified BPO-A1 Haloperoxidase Activity section.

Separate purified enzyme solutions (3 μg/L) were incubated for 48 h at 30°C in 40% (v/v) methanol, ethanol, dimethyl sulfoxide (DMSO), or *N*,*N*-dimethylformamide (DMF). Subsequently, the residual activity was measured as described later in the Assay of Purified BPO-A1 Haloperoxidase Activity section.

Evaluation of thermostability of BPO-A1 haloperoxidases

Purified enzyme solutions (3 μg/L) were incubated for 1 h at temperatures ranging from 30°C to 80°C and were then cooled on ice. Thereafter, the residual activity was measured as described later in the Assay of Purified BPO-A1 Haloperoxidase Activity section.

Separate purified enzyme solutions (3 μg/L) were incubated for 10 min at temperatures ranging from 80°C to 87°C and were then cooled on ice. Subsequently, the residual activity was measured as described later in the Assay of Purified BPO-A1 Haloperoxidase Activity section, and the temperature at which the residual activity reached 50% (T_{50}) was calculated.

Assay of purified BPO-A1 haloperoxidase activity

Purified haloperoxidase activity was assayed as described in the High-Throughput Screening of Thermostable BPO-A1 Haloperoxidase section, with some modifications. Enzyme solutions (100 μL, 3 μg/L) were mixed with substrate mixture containing 1.5 mL 2 M sodium acetate buffer (pH 5.5), 60 μL 5 M NaBr, 30 μL 1 M NaN_3, 10 μL 2.64 M H_2O_2, 10 μL 14.4 mM MCD, and 1.29 mL deionized water. Enzyme–substrate mixtures were incubated at 25°C, and absorbance was continuously monitored at 290 nm using a spectrophotometer (UV-2500PC; Shimadzu, Kyoto, Japan). Enzyme activity was calculated as reductions in MCD absorbance. One unit of haloperoxidase activity was defined as the amount of enzyme that reduces 1 μmol of MCD per minute at 25°C.

Native polyacrylamide gel electrophoresis

Purified enzyme solutions (10 μL, 60 μg/mL) were mixed with 10 μL native polyacrylamide gel electrophoresis (PAGE) treatment solution containing 10% (w/v) glycerol and 31.25 mM Tris–HCl buffer (pH 6.8). Samples were run in a stacking gel comprising 4.0% (w/v) polyacrylamide and 0.2% (w/v) *N*,*N*′-methylenebisacrylamide, and then in a separating gel containing 14.8% (w/v) polyacrylamide and 0.2% (w/v) *N*,*N*′-methylenebisacrylamide under the conditions developed by Laemmli.[27] Proteins were stained with Coomassie brilliant blue R-250 and were analyzed using Gel-Pro Analyzer (Media Cybernetics, Rockville, MD).

Structural analysis of BPO-A1 haloperoxidases

Substituted amino acid residues in mutant BPO-A1 haloperoxidases were mapped onto the 3-D structure of wild-type BPO-A1 haloperoxidase (PDB 1A8Q).[3]

Three-dimensional structures were constructed, and solvent accessibilities of mutant and wild-type BPO-A1 haloperoxidases were calculated using the Discovery Studio developed by Accelrys (San Diego, CA). To construct the 3-D structure of BPO-A1 haloperoxidase, the peptide terminus of *S. aureofaciens* BPO-A1 haloperoxidase (PDB 1A8Q) was charged, and simulations were performed using the Chemistry of the HARvard Macromolecular Mechanics (CHARMM) force field.[28] The steepest descent method was used as the free energy minimization algorithm and was subsequently applied with a minimization RMS gradient of 1. Three-dimensional structures of mutant BPO-A1 haloperoxidases were estimated by replacing single amino acid residues (P123 and G162), and free-energy minimizations were performed. Solvent accessibilities were determined from the resulting 3-D structures of mutant BPO-A1 haloperoxidases.

Results

Screening of organic solvent-stable mutants of BPO-A1 haloperoxidases using directed evolution

Approximately 1,000 *E. coli* transformants with mutant BPO-A1 haloperoxidases were cultured on LB media in 96-well plates, and the cells were disrupted by heating at 80°C for 10 min. After centrifugation, supernatants, including mutant BPO-A1 haloperoxidases, were incubated at 30°C for 15 min in the presence of 1-propanol, and their residual

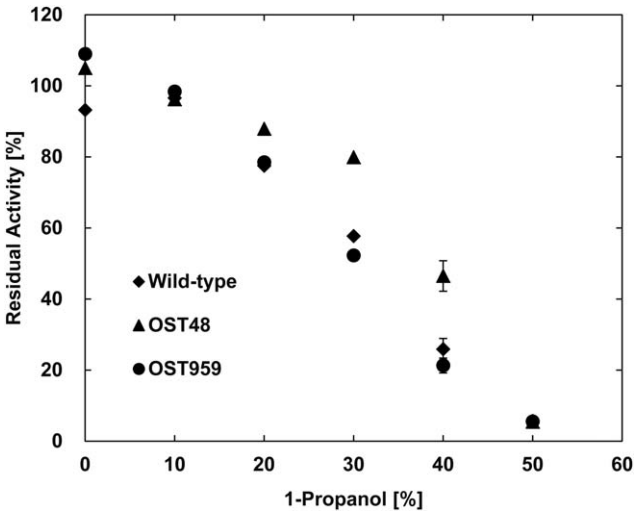

Figure 1. Stability of wild type and mutant BPO-A1 haloperoxidases in 1-propanol.

Wild type and mutant BPO-A1 haloperoxidases were incubated in various concentrations of 1-propanol for 1 h at 30°C, and the residual activities were measured at 25°C. Data from the experiments in 40% (v/v) 1-propanol are presented as means of two independent experiments.

haloperoxidase activities were evaluated. Approximately, 60 *E. coli* transformants showed higher residual activity than that of the wild-type transformant (data not shown). Among these, a candidate transformant with organic solvent-stable mutant BPO-A1 haloperoxidase named OST48 and another transformant with high active mutant BPO-A1 haloperoxidase named OST959.

Wild-type, OST48 mutant, and OST959 mutant BPO-A1 haloperoxidases were purified from supernatants of the disrupted cells using Ni affinity chromatography, and their specific activities were 56.4, 49.0, and 83.3 kU/g, respectively. Although the specific activity of the OST48 mutant BPO-A1 haloperoxidase was similar, the activity of the OST959 mutant haloperoxidase was about 1.5-fold greater than that of wild-type enzyme.

1-Propanol stability of mutant BPO-A1 haloperoxidases

To evaluate the organic solvent-stability of mutant BPO-A1 haloperoxidases, purified wild-type and mutant BPO-A1 haloperoxidases were incubated at 30°C for 1 h in various concentrations of 1-propanol, and their residual activities were measured (Figure 1). Residual activities of both mutant enzymes and wild-type BPO-A1 haloperoxidase decreased with increasing 1-propanol concentrations. In the presence of 10% 1-propanol, both mutant and wild-type BPO-A1 haloperoxidases showed similar residual activity. Conversely, in the presence of 20%–40% 1-propanol, the OST48 mutant BPO-A1 haloperoxidase showed higher residual activity than both wild-type and OST959 mutant BPO-A1 haloperoxidases. In the presence of 40% 1-propanol, the OST48 mutant BPO-A1 haloperoxidase showed 46.5% residual activity, which was 1.8-fold higher than that of wild-type BPO-A1 haloperoxidase. These data indicated that at least one mutation contributing to improve 1-propanol stability was inserted into the OST48 mutant BPO-A1 haloperoxidase.

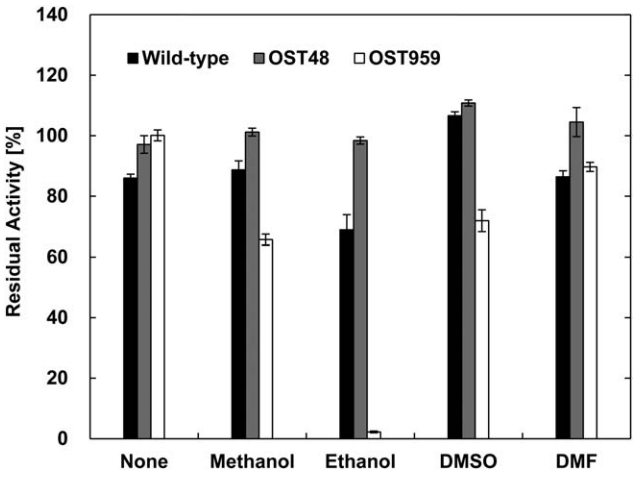

Figure 2. Organic solvent stability of wild type and mutant BPO-A1 haloperoxidases.

Wild type and mutant BPO-A1 haloperoxidases were incubated in various organic solvents at 40% (v/v) for 48 h at 30°C, and the residual activities were measured at 25°C. Data are presented as means of two independent experiments.

Stability of mutant BPO-A1 haloperoxidases in various organic solvents

To evaluate the organic solvent-stability of mutant BPO-A1 haloperoxidases, purified wild-type and mutant BPO-A1 haloperoxidases were incubated in various organic solvents at 40% (v/v) for 48 h at 30°C, and residual activities were measured (Figure 2). After incubation in the presence of methanol, ethanol, DMSO, or DMF, the OST48 mutant BPO-A1 haloperoxidase showed higher residual activity than wild-type BPO-A1. In contrast, the OST959 mutant BPO-A1 haloperoxidase showed lower or similar residual activity than wild-type BPO-A1.

Thermostability of mutant BPO-A1 haloperoxidases

To evaluate the thermostability of mutant BPO-A1 haloperoxidases, purified wild-type and mutant BPO-A1 haloperoxidases were incubated at elevated temperatures for 1 h, and their residual activities were measured (Figure 3). Both mutant and wild-type BPO-A1 haloperoxidases were stable below 70°C. However, wild-type BPO-A1 haloperoxidase showed 8.7% residual activity at 80°C and that of the OST48 mutant BPO-A1 haloperoxidase was similarly reduced. In contrast, the OST959 mutant BPO-A1 haloperoxidase showed 40.1% residual activity, which was 4.6-fold higher than that of wild-type BPO-A1. These data indicated that at least one mutation contributing to improve thermostability was inserted into the OST959 mutant BPO-A1 haloperoxidases.

Effect of 1-propanol on dimerization of BPO-A1 haloperoxidases

To evaluate the effects of 1-propanol on dimerization, purified wild-type and mutant BPO-A1 haloperoxidases were incubated in 40% (v/v) 1-propanol for 1 h at 30°C and were then analyzed using native PAGE (Figure 4). All haloperoxidases showed single protein bands before incubation in the presence of 1-propanol, whereas double protein bands were observed after incubation. Because high-molecular-weight proteins may have low mobility in native PAGE, protein

bands A and B were inferred as dimeric and monomeric forms, respectively. Relative intensity changes of the upper band A of wild-type, OST48, and OST959 mutant haloperoxidases from before to after incubation were 11.8%, 11.6%, and 4.9%, respectively. Thus, the OST48 mutant BPO-A1 haloperoxidase may have similar dimerization stability as wild-type BPO-A1 in the presence of 1-propanol. In contrast, the OST959 mutant BPO-A1 haloperoxidase may have lower dimerization stability than wild-type BPO-A1 in the presence of 1-propanol.

Effect of heat treatment on the dimerization of BPO-A1 haloperoxidases

To evaluate the relationship between thermostability and dimerization, purified wild-type and mutant BPO-A1 haloperoxidases were incubated at 80°C for 1 h, and dimerization was analyzed using native PAGE (Figure 5). Single protein bands were obtained from purified protein solutions of wild-type and mutant BPO-A1 haloperoxidases before incubation.

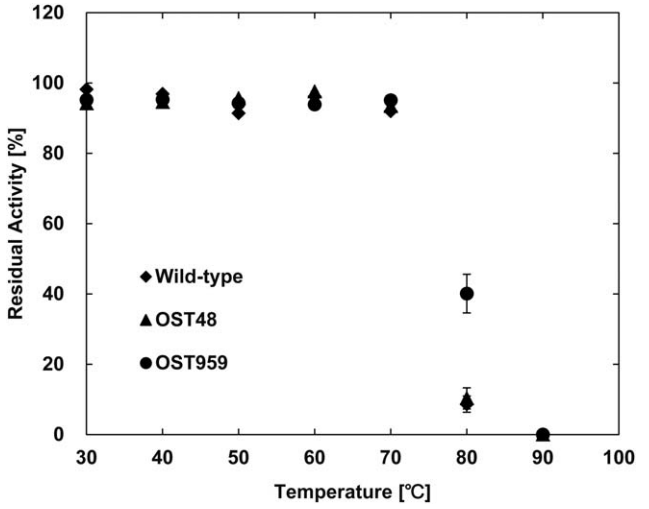

Figure 3. Thermostability of wild type and mutant BPO-A1 haloperoxidases.

Wild type and mutant BPO-A1 haloperoxidases were incubated at various temperatures for 1 h. Subsequently, the residual activities were determined at 25°C. Data are presented as means of two independent experiments performed at 80°C.

In contrast, double protein bands were obtained after incubation at 80°C for 1 h. Relative intensity changes of the upper band A of wild-type, OST48, and OST959 mutant haloperoxidases were 12.3%, 22.0%, and 60.3%, respectively. Hence, the higher thermostability of the OST959 mutant BPO-A1 haloperoxidase may reflect high dimerization stability of BPO-A1 haloperoxidase at high temperatures.

Analysis of 3-D structures of mutant BPO-A1 haloperoxidases

Plasmids containing the OST48 and OST959 mutant BPO-A1 haloperoxidase genes were extracted from their transformants, and nucleotide sequences were determined (Table 1). In the OST48 mutant BPO-A1 haloperoxidase, P123L and P241A mutations were found, and in the OST959 mutant, H53Y and G162R mutations were found. Thus, the substituted residues of mutant BPO-A1 haloperoxidases were mapped onto the 3-D structure of *S. aureofaciens* BPO-A1 haloperoxidase (PDB 1A8Q; Figure 6). Subsequently, the substituted amino acid residues 123 and 241 were located in coil regions, the substituted amino acid residue 53 was located in a sheet region, and the substituted amino acid residue 162 was located in a helix region. The high solvent accessibility of the substituted amino acid residues 241 and 162 indicated that these residues were located on the surface of the protein.

Construction of single amino acid-substituted mutants and evaluation of specific activity and stability

To evaluate the effects of amino acid substitutions in mutant BPO-A1 haloperoxidases on the specific activity and stability, the four mutant BPO-A1 haloperoxidases H53Y, P123L, G162R, and P241A were generated using site-directed mutagenesis. Mutants H53Y and G162R BPO-A1 haloperoxidases showed higher specific activities than wild-type BPO-A1, and mutants P123L, P241A, and G162R BPO-A1 haloperoxidases showed higher T_{50} than wild-type (Table 2). Furthermore, mutants P123L and H53Y BPO-A1 haloperoxidases showed higher 1-propanol stability, whereas mutants P241A and G162R BPO-A1 haloperoxidases showed lower 1-propanol stability than wild-type.

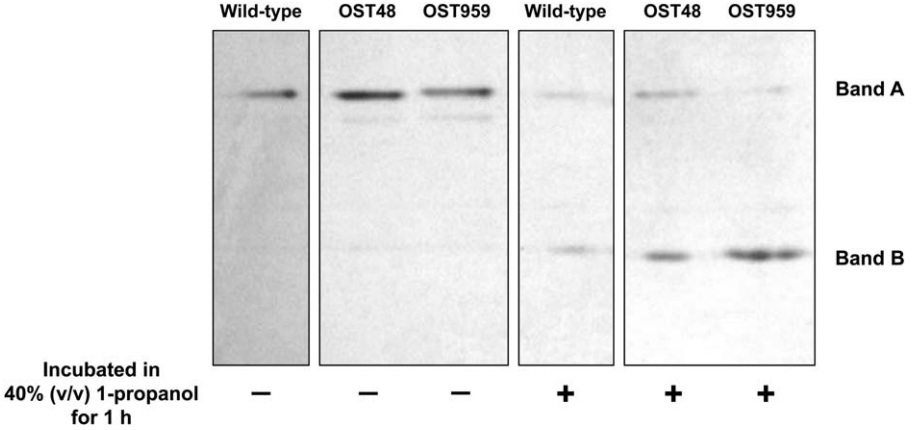

Figure 4. Effects of 1-propanol on dimerization of wild type and mutant BPO-A1 haloperoxidases.

Wild type and mutant BPO-A1 haloperoxidases were incubated in 40% (v/v) 1-propanol for 1 h, and the dimers and monomers of original and incubated enzymes were resolved using native PAGE.

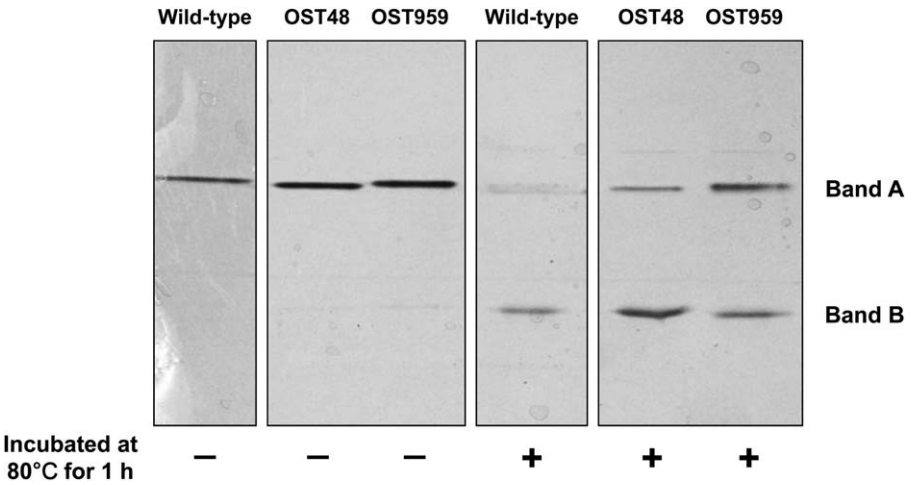

Figure 5. Effects of heat treatment on dimerization of wild type and mutant BPO-A1 haloperoxidases.

Wild type and mutant BPO-A1 haloperoxidases were incubated at 80°C for 1 h, and the dimers and monomers of original and incubated enzymes were resolved using native PAGE.

Table 1. Relevant Features of Substituted Amino Acid Residues in Mutant BPO-A1 Haloperoxidases

Mutants	Substituted Residues	Secondary Structure	Solvent Accessibility (%)
OST48	P123L	Coil	0
	P241A	Coil	42.2
OST959	H53Y	Sheet	4.88
	G162R	Helix	27.9

Modeling of 3-D structures of mutant BPO-A1 haloperoxidases

Because the 3-D structure of BPO-A1 haloperoxidase from *S. aureofaciens* has been determined (PDB 1A8Q), the structures of the present mutant and wild-type BPO-A1 haloperoxidases were constructed using the 1A8Q structure as a template for molecular mechanics analyses. Wild-type and mutant BPO-A1 haloperoxidases were then compared to identify key structural differences. Residue 123 and the surrounding residues of wild-type (A) and mutant P123L (B) BPO-A1 haloperoxidases are illustrated in Figure 7. Several hydrophobic amino acid residues, including Leu, Ile, and Ala, were found proximal to residue 123 in the BPO-A1 haloperoxidase. Thus, it was inferred that substitution of the native proline residue with leucine may improve the stability of the hydrophobic core, reflecting additional packing by the bulky leucine side-chain.

Residue 162 and the neighboring residues of wild type (C) and mutant G162R (D) haloperoxidases are shown in Figure 7. Two additional hydrogen bonds between R162 and the Cϵ and Nη of R162 and the oxygen atom attached to C at the carboxyl group of Q225 were formed by mutating the native glycine residue to arginine. These additional hydrogen bonds may have improved the structural stability of the BPO-A1 haloperoxidase.

Discussion

In this study, random mutagenesis of *S. aureofaciens* haloperoxidase was performed using error-prone PCR, and the two mutants with higher residual activity after incubation in

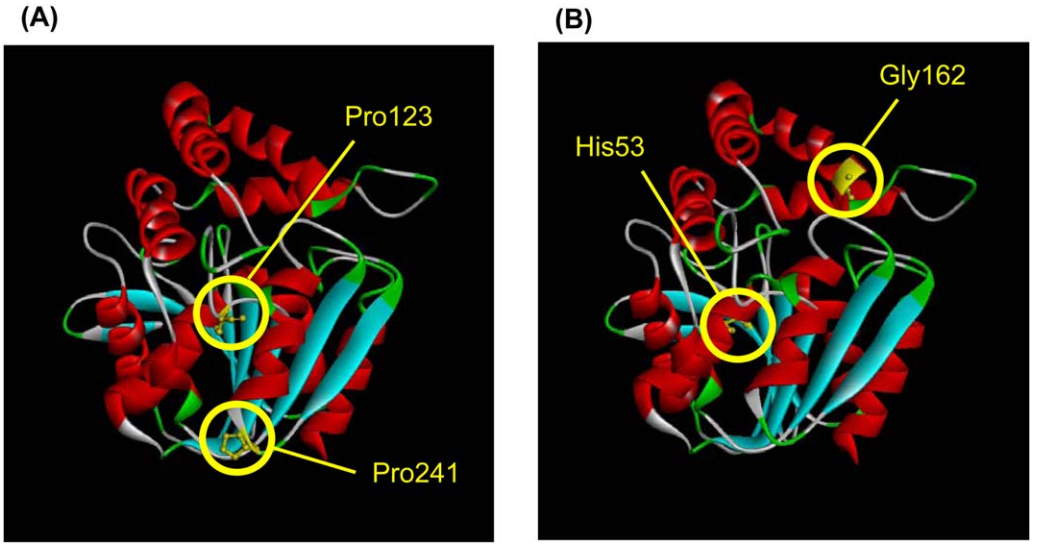

Figure 6. Positions of substituted amino acid residues in mutant BPO-A1 haloperoxidases.

Substituted residues in OST48 (A) and OST959 (B) mutants.

the presence of 1-propanol were identified. Although one of these had higher organic solvent-stability, the other had higher thermostability and specific activity. In addition, single amino acid-substituted mutants were constructed, and residues that were associated with organic solvent-stability, thermostability, and/or activity were identified.

The OST48 mutant BPO-A1 haloperoxidase had higher organic solvent-stability in 1-propanol, methanol, ethanol, DMSO, and DMF than wild-type BPO-A1 (Figures 1 and 2). Previous reports indicate that the enhanced interactions between the subunits improve enzyme stability.[17,18] In agreement, the present native PAGE analyses showed that the stability of the OST48 mutant dimer in 40% (v/v) 1-propanol was not improved compared with that of wild-type BPO-A1. Moreover, 3-D structure analyses indicated that the P123L substitution improved organic solvent-stability, reflecting packing of the leucine side-chain within the hydrophobic core (Figure 7). Accordingly, evaluations of the single amino acid mutant P123L (Table 2) showed that its residual activity in 40% (v/v) 1-propanol was 3.2-fold higher than that of wild-type BPO-A1. Previous reports indicate that this proline residue restricts the conformational flexibility of the folded polypeptide chain, leading to improved conformational stability.[29,30] However, the present P123L substitution improved organic solvent-stability in BPO-A1 haloperoxidase, and stabilization of the hydrophobic core by the leucine residue had a greater influence than restrictions of flexibility by the proline residue. We previously reported that the leucine substitution of the serine residue in *P. aeruginosa* lipase improved the organic solvent-stability.[12] Taken together, these data indicate that stabilization of the hydrophobic core of an enzyme may be a promising strategy for improving the enzyme stability using rational design approaches.

Table 2. Specific Activity, T_{50}, and 1-Propanol Stability of Wild-Type and Mutant BPO-A1 Haloperoxidases

BPO-A1*	Specific activity (kU/g)	T_{50} (°C)	Residual Activity in 40% (v/v) 1-Propanol (%)†
Wild-type	56.4	80.6	25.9 ± 3.0
Mutant OST48	49.0	81.3	46.5 ± 4.3
Mutant P123L	46.1	81.1	81.7 ± 3.5
Mutant P241A	56.3	81.6	6.92 ± 2.0
Mutant OST959	83.3	84.3	18.6 ± 2.1
Mutant H53Y	70.3	80.1	33.4 ± 1.8
Mutant G162R	62.9	85.3	17.4 ± 0.9

*Mutants OST48 and OST959 were generated using directed evolution. The single amino acid-substitution mutants P123L, P241A, H53Y, and G162R were constructed using site-directed mutagenesis.

†Data are presented as the mean of two independent experiments.

Although the organic solvent-stability of the OST959 mutant BPO-A1 haloperoxidase was not higher than that of wild-type, thermostability and specific activity were higher than those of wild-type (Figure 3 and Table 2). In addition, native PAGE analyses showed that this mutant had higher dimerization stability at elevated temperatures. In agreement, improved enzyme thermostability has been shown following the enhancement of subunit interactions.[17,18] Thus, higher thermostability of the OST959 mutant may reflect increased stability of the dimer, suggesting that identification of quaternary protein enzyme structures may provide a promising strategy for improving enzyme stability using rational design approaches.

The present 3-D structure analyses indicated that the G162R substitution in the OST959 mutant BPO-A1 haloperoxidase formed two additional hydrogen bonds with R162 and Q225

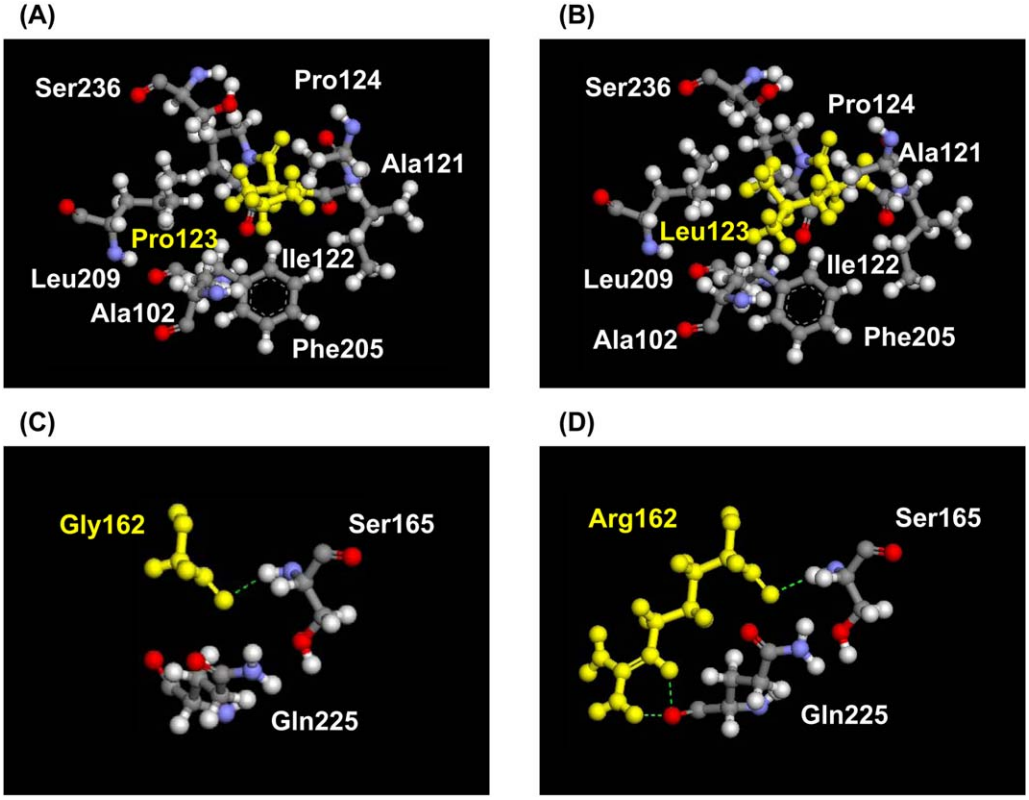

Figure 7. Partial structures of wild type and mutant BPO-A1 haloperoxidases.

Structures of residue 123 and neighboring residues of wild type (A) and mutant P123L (B) BPO-A1 haloperoxidases. Structures of residue 162 and neighboring residues of wild type (C) and G162R mutant (D) BPO-A1 haloperoxidases. Hydrogen bonds are represented as dashed lines.

(Figures 7C,D), which may improve thermostability. Moreover, the G162R substitution was central to the high thermostability of the OST959 mutant BPO-A1 haloperoxidase (Table 2), potentially reflecting the presence of additional hydrogen bonds.[31,32] Thus, increasing the number of hydrogen bonds may be a promising strategy to improve enzyme stability using rational design approaches. Furthermore, the OST959 mutant BPO-A1 haloperoxidase had higher specific activity than that of the wild type, potentially reflecting the synergistic effect of H53Y and G162R substitutions. However, the associated mechanisms are poorly understood.

In this study, the organic solvent-stability and thermostability of *S. aureofaciens* BPO-A1 haloperoxidase were successfully improved using random mutagenesis and subsequent selection of mutants with higher residual activity in the presence of organic solvents. In addition, the related amino acid residues were identified using single amino acid-substituted mutants. The resulting improvements in the organic solvent-stability and thermostability of BPO-A1 haloperoxidases reflected stabilization of the hydrophobic core and presence of additional hydrogen bonds, respectively. Although stabilization of the hydrophobic core and addition of hydrogen bonds generally improves the conformational stability, organic solvent-stability and thermostability of the present enzymes were improved independently.

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