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Improvement of the stability and activity of the BPO-A1 haloperoxidase from *Streptomyces aureofaciens* by directed evolution

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ABSTRACT

Haloperoxidases are oxygenases that catalyze the halogenation of a range of organic compounds without the need for additional high-cost cofactors. Thus, haloperoxidases with high activity and stability are desired for industrial application. In this study, a directed evolution approach was adopted to improve the thermostability of the homodimeric BPO-A1 haloperoxidase from *Streptomyces aureofaciens*. Among 1000 mutant BPO-A1 haloperoxidases, 2 mutants HT177 and HT507, having higher thermostabilities than the wild-type BPO-A1 haloperoxidase, were obtained by directed evolution. The residual activities of mutants HT177 and HT507 were 2.3- and 5.1-fold higher than that of wild-type BPO-A1, respectively, after incubation at 80 °C for 1 h. In addition, mutant HT177 showed higher stability in organic solvents, such as methanol, ethanol, dimethyl sulfoxide, and *N*,*N*-dimethylformamide, than the wild-type BPO-A1 haloperoxidase. Furthermore, mutant HT507 showed higher specific activity. Based on the evaluation of single-amino-acid-substituted mutants, stabilization of the α-helix conformation, substitution of amino acid residues located at the surface of the protein molecule, and enhancement of the interaction between subunits may account for the improvement in thermostability, organic solvent stability, and specific activity. Consequently, the thermostability, organic solvent stability, and specific activity of *S. aureofaciens* BPO-A1 haloperoxidase were successfully improved by a directed evolution approach.

1. Introduction

Haloperoxidases are oxygenases that catalyze the halogenation of organic compounds in the presence of halide ions and hydrogen peroxide (Burton, 2003). Recently, this enzyme has attracted considerable attention because of its ability to halogenate a range of organic compounds and the reaction is important in the industrial and pharmaceutical fields. Furthermore, most oxygenases require high-cost cofactors for oxidation reactions, posing a challenge for industrial application of oxygenases. However, haloperoxidases require only the inexpensive hydrogen peroxide as a cofactor for oxidation reactions. Thus, haloperoxidases with high activity and stability are desired for industrial application.

Haloperoxidases are classified into 3 distinct classes: heme-containing, vanadium-containing, and metal-free haloperoxidases (Littlechild, 1999). These classes have different protein structures and reaction mechanisms, and those haloperoxidases catalyze various types of reactions including halogenation, sulfoxidation, epoxidation, and aromatic hydroxylation. Heme-containing and vanadium-containing haloperoxidases have been well studied. And the effect of calcium and vanadium ions on thermal and organic solvent stabilities had been investigated using of a vanadium-containing haloperoxidases from *Corallina pilulifera* (Garcia-Rodriguez et al., 2005). However, research on metal-free haloperoxidases is limited. Among metal-free haloperoxidases, BPO-A1 from *Streptomyces aureofaciens* has been relatively well studied (Pelletier et al., 1994; Hofmann et al., 1998). However, because it has a homodimeric structure (Weng et al., 1991), enzyme deactivation could be caused by subunit dissociation (Fernandez-Lafuente, 2009). Thus, the development of a stable metal-free haloperoxidase is desired for industrial application.

For industrial application of enzymes, thermostability and organic solvent stability are desirable, because enzymatic reaction at elevated temperature and/or with organic solvents may often be beneficial in many ways (Doukyu and Ogino, 2010; Haki and Rakshit, 2003). For example, substrate solubility could be improved at elevated temperature and/or with organic solvents, and as a result, the productivity could be improved. Some mechanisms for improvement of enzyme thermostability, such as stabilization of the α-helix conformation (Facchiano et al., 1998; Petukhov et al., 1997), oligomerization of the enzyme (Maes et al., 1999; Villeret et al., 1998), improvement of the interaction between subunits

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(Kirino et al., 1994; Akanuma et al., 1999), and enhancement of electrostatic interaction (Cambillau and Claverie, 2000; Arnott et al., 2000), have been reported. In contrast, studies on organic solvent stability have been limited. We previously reported that disulfide bonding was an important factor in organic solvent stability (Ogino et al., 2001). Amino acid residues located at the surface of the protein molecule are also important for enzyme stability in the presence of organic solvents (Ogino et al., 2007; Kawata and Ogino, 2009). However, the mechanisms underlying thermostability and organic solvent stability and their correlations are not completely resolved.

Directed evolution is one of the approaches for improving the function of enzymes. It involves the generation of large mutant libraries and recombination followed by high-throughput screening or selection methods to obtain desired mutants. To obtain biocatalysts that have high thermostability, random mutagenesis via directed evolution has been attempted by several investigators. Zhang et al. (2003) generated lipase from *Candida antarctica* with >20-fold increase in half-life at 70 °C compared with wild-type lipase by directed evolution. Miyazaki et al. (2000) enhanced the half-life of psychrophilic protease subtilisin up to 500-fold at 60 °C compared with wild-type subtilisin. In contrast to the rational protein design approach, detailed information such as the functional domain and three-dimensional (3-D) structure about the target enzyme is not necessary for the directed evolution approach (Lehmann and Wyss, 2001). Thus, directed evolution could be a promising approach for improving the thermostability of various types of enzymes.

The goal of this study was to improve the thermostability of *S. aureofaciens* BPO-A1 haloperoxidases by directed evolution. First, mutant BPO-A1 haloperoxidases were constructed using error-prone PCR, and high-throughput screening of thermostable haloperoxidases was performed. Further, thermostable mutant BPO-A1 haloperoxidases were purified and their thermostabilities and organic solvent stabilities were evaluated. Finally, single-amino-acid-substituted mutants were constructed by site-directed mutagenesis and their properties were evaluated.

2. Materials and methods

2.1. Organisms

Escherichia coli JM109 was used as the cloning host for recombinant DNA manipulations. *E. coli* Rosetta 2 (DE3) (Novagen, Madison, WI, USA) was 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.

2.2. Plasmids

The BPO-A1 haloperoxidase gene conjugated with a hexahistidine tag expressing plasmid pET42-b_BPO-A1-His was constructed as follows. The BPO-A1 haloperoxidase gene was amplified using *S. aureofaciens* NBRC 12843 genomic DNA as the template, a set of 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 the LA Taq DNA polymerase (Takara Bio Inc., Shiga, Japan). The amplified DNA fragment was digested with XhoI and StuI and ligated into the XhoI/PshAI site of pET42-b (Novagen). The resulting plasmid was designated pET42-b_BPO-A1-His.

2.3. Construction of a plasmid library containing randomly mutated genes

Mutant BPO-A1 haloperoxidase genes were constructed using error-prone PCR. The BPO-A1 haloperoxidase gene was amplified using the pET42-b_BPO-A1-His plasmid as the template, a set of 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, USA). The plasmid library containing randomly mutated genes was obtained by ligation of the amplified DNA fragment digested with NdeI and XhoI into the NdeI/XhoI site of pET42-b_BPO-A1-His.

2.4. Substitution of residues by site-directed mutagenesis

Site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene) following the supplier's protocol. The primers were designed so that the mutation position was located in the center of the primer, with 10–15 bases functioning as the template complementary sequence on both sides. The DNA fragment was digested with the restriction enzyme DpnI to digest the parental methylated DNA. DpnI-digested DNA was transformed into competent *E. coli* JM109 cells, and transformants were obtained from LB agar media containing 30 mg/L kanamycin sulfate. Each mutation was confirmed by nucleotide sequencing.

2.5. High-throughput screening of thermostable BPO-A1 haloperoxidase

E. coli Rosetta 2 (DE3) cells transformed with the plasmid library containing mutant BPO-A1 haloperoxidase genes were obtained from LB agar media containing 30 mg/L kanamycin sulfate and 17 mg/L chloramphenicol. High-throughput screening of thermostable haloperoxidases was performed using a 96-well plate-based cultivation and enzyme assay. The transformants were cultivated in 100 μL of LB media containing 30 mg/L kanamycin sulfate and 17 mg/L chloramphenicol. After 18 h of cultivation 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. After 135 min of cultivation at 37 °C with rotary shaking at 150 rpm, 11 μL of 10 mM isopropyl-β-D(−)-thiogalactopyranoside (IPTG) was added and cultivated for 7 h at 37 °C with rotary shaking at 150 rpm to induce the expression of mutant BPO-A1 haloperoxidases. After induction, the cells were collected by centrifugation at 825 × *g* for 10 min and resuspended in 100 μL of 0.2 M Tris–H_2SO_4 (pH 8.3). Subsequently, the cell suspensions were incubated at 80 °C for 25 min to lyse the cells and inactivate low-thermostable mutant BPO-A1 haloperoxidases. The resulting cell suspensions were used for assaying haloperoxidase activity.

Haloperoxidase activity was assayed by the halogenation of monochlorodimedone (MCD) as described previously (Pfeifer et al., 1992) with some modifications. The enzyme solution (10 μL) was mixed with the substrate mixture [150 μL of 2 M sodium acetate buffer (pH 5.5), 6 μL of 5 M NaBr, 3 μL of 1 M NaN_3, 1 μL of 2.64 M H_2O_2, 1 μL of 14.4 mM MCD, and 129 μL of deionized water]. The enzyme and substrate mixture was incubated at 30 °C for 3 min and absorbance at 290 nm was measured 18 times using the microplate reader Multiskan GO (Thermo Scientific, Rochester, NY, USA). Enzyme activity was calculated by the reduction rate of MCD.

2.6. Preparation of purified BPO-A1 haloperoxidases

To prepare purified wild-type and mutant BPO-A1 haloperoxidases, *E. coli* Rosetta 2 (DE3) transformants were cultivated in a

500-mL baffled Erlenmeyer flask containing 200 mL of LB medium supplemented with 30 mg/L kanamycin sulfate and 17 mg/L chloramphenicol. The transformants were initially grown at 37 °C with shaking at 150 rpm. When the optical density at 600 nm reached approximately 0.75, IPTG was added to a final concentration of 1 mM. Cultivation was continued for a further 6 h at 37 °C with shaking, and the cells were harvested by centrifugation at 3000 × *g* for 10 min at 4 °C.

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

Soluble fractions prepared from *E. coli* transformants were incubated at 4 °C for 30 min in 50% saturated ammonium sulfate solution with agitation. The precipitate was collected by centrifugation at 20,000 × *g* for 10 min at 4 °C. The collected precipitates were dissolved in 50 mL of 50 mM sodium phosphate buffer (pH 7.4) containing 20 mM imidazole and purified by immobilized nickel ion affinity chromatography using a HisTrap HP column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The dissolved precipitates were applied to the HisTrap HP column. The adsorbed proteins were washed with 20 mL of 50 mM sodium phosphate buffer (pH 7.4) containing 100 mM imidazole and eluted with 10 mL of 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), and protein concentration was evaluated using the Bradford method (Bradford, 1976).

2.7. Evaluation of the thermostability of BPO-A1 haloperoxidases

The purified enzyme solutions (3 μg/L) were incubated for 1 h at temperatures ranging from 30 °C to 80 °C and cooled on ice. Thereafter, residual activity was measured as described in Section 2.9.

In addition, the purified enzyme solutions (3 μg/L) were incubated for 10 min at temperatures ranging from 80 °C to 87 °C and cooled on ice. Thereafter, residual activity was measured as described in Section 2.9 and the temperature at which the residual activity reached 50% (T_{50}) was calculated.

2.8. Evaluation of organic solvent stability of BPO-A1 haloperoxidases

The 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, residual activity was measured as described in Section 2.9.

In addition, the 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). Thereafter, residual activity was measured as described in Section 2.9.

2.9. Assay of purified BPO-A1 haloperoxidase activity

Purified haloperoxidase activity was assayed as described in the section "High-throughput screening of thermostable BPO-A1 haloperoxidase" with some modifications. The enzyme solution (100 μL, 3 μg/L) was mixed with the substrate mixture [1.5 mL of 2 M sodium acetate buffer (pH 5.5), 60 μL of 5 M NaBr, 30 μL of 1 M NaN_3, 10 μL of 2.64 M H_2O_2, 10 μL of 14.4 mM MCD, and 1.29 mL of deionized water]. The enzyme and substrate mixture was incubated at 25 °C and absorbance at 290 nm was measured continuously using a spectrophotometer (UV-2500PC; Shimadzu, Kyoto, Japan). Enzyme activity was calculated from the reduction rate of MCD. One unit of haloperoxidase activity was defined as the amount of enzyme that reduces 1 μmol of MCD per min at 25 °C.

2.10. Structural analysis of BPO-A1 haloperoxidases

Substituted amino acid residues in mutant BPO-A1 haloperoxidases were mapped on the 3-D structure of the wild-type BPO-A1 haloperoxidase (PDB 1A8Q) (Hofmann et al., 1998). Calculation of solvent accessibilities were performed using Discovery Studio (Accelrys, Inc., San Diego, CA, USA).

2.11. Native polyacrylamide gel electrophoresis

Each purified enzyme solution (10 μL, 60 μg/mL) was mixed with 10 μL native polyacrylamide gel electrophoresis (PAGE) treatment solution consisting of 10% (w/v) glycerol and 31.25 mM Tris–HCl buffer (pH 6.8). The sample was run in a stacking gel consisting of 4.0% (w/v) polyacrylamide and 0.2% (w/v) *N*,*N*′-methylenebisacrylamide and a separating gel containing 14.8% (w/v) polyacrylamide and 0.2% (w/v) *N*,*N*′-methylene bisacrylamide under the conditions developed by Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue R-250 and analyzed using Gel-Pro Analyzer (Media Cybernetics, Inc., Rockville, MD, USA).

3. Results

3.1. Screening of thermostable mutants of BPO-A1 haloperoxidases by directed evolution

Approximately 1000 *E. coli* transformants with mutant BPO-A1 haloperoxidases were cultured on LB media in 96-well plates and their residual haloperoxidase activities were evaluated. Approximately 40 *E. coli* transformants showed higher residual activity than the transformant expressing the wild-type enzyme (data not shown). Among them, 2 candidate transformants with thermostable mutant BPO-A1 haloperoxidases were selected. These mutant BPO-A1 haloperoxidases were named HT177 and HT507.

3.2. Thermostability of mutant BPO-A1 haloperoxidases

To evaluate the thermostability of mutant BPO-A1 haloperoxidases, wild-type and mutant BPO-A1 haloperoxidases were incubated at elevated temperature for 1 h and their residual activities were measured (Fig. 1). Both mutant and wild-type BPO-A1 haloperoxidases were stable below 70 °C. At 80 °C, the wild-type BPO-A1 haloperoxidase showed 8.7% residual activity. In contrast, the mutant BPO-A1 haloperoxidases HT177 and HT507 showed 20.4% and 44.2% residual activities, respectively, and they had 2.3- and 5.1-fold higher thermostabilities than the wild-type BPO-A1, respectively. Thus, mutations contributing to improved thermostability could be inserted into the mutant BPO-A1 haloperoxidases HT177 and HT507.

3.3. 1-Propanol stability of mutant BPO-A1 haloperoxidases

1-Propanol is one of very useful water soluble organic solvents to improve solubility of various substrates. And the solubility depends on the concentration of 1-propanol. At first, wild-type and mutant BPO-A1 haloperoxidases were incubated at 30 °C in various concentrations of 1-propanol and their residual activities after 1 h-incubation were shown in Fig. 2. The activities of both mutant and wild-type BPO-A1 haloperoxidases decreased with increasing 1-propanol concentrations. In the presence of 40% 1-propanol, the wild-type BPO-A1 haloperoxidase showed 25.9% residual activity.

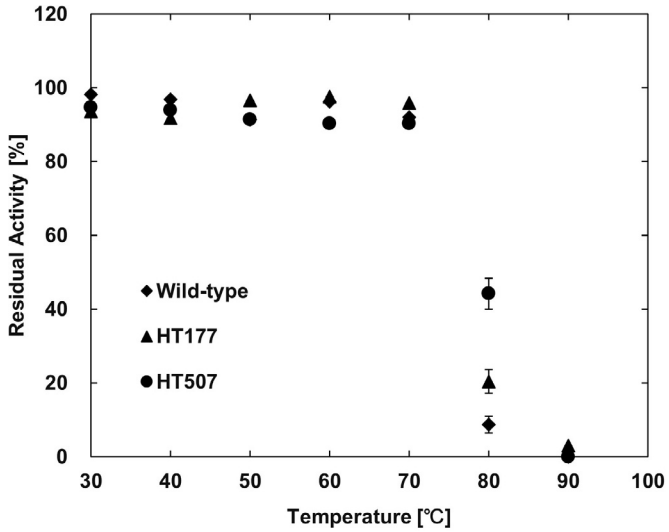

Fig. 1. Thermostability of wild-type and mutant BPO-A1 haloperoxidases. Wild-type and mutant BPO-A1 haloperoxidases were incubated at various temperatures for 1 h. Thereafter, residual activities were measured at 25 °C. The data at 80 °C represent the averages of 2 independent experiments.

Under the same condition, the residual activity of the mutant BPO-A1 haloperoxidase HT177 was 55.4% and 2.1-fold higher than that of the wild-type BPO-A1 haloperoxidase. In contrast, the activity of the mutant BPO-A1 haloperoxidase HT507 was 15.2% and 1.7-fold lower than that of the wild-type BPO-A1 haloperoxidase. This result indicates that a mutation contributing to improvement of 1-propanol stability could be inserted into the mutant BPO-A1 haloperoxidase HT177.

3.4. Stability of mutant BPO-A1 haloperoxidases in various organic solvents

Next, the organic solvent stabilities of mutant BPO-A1 haloperoxidases were investigated using various water-soluble organic solvents. The residual activities of wild-type and mutant BPO-A1 haloperoxidases after incubation in 40% (v/v) of various organic

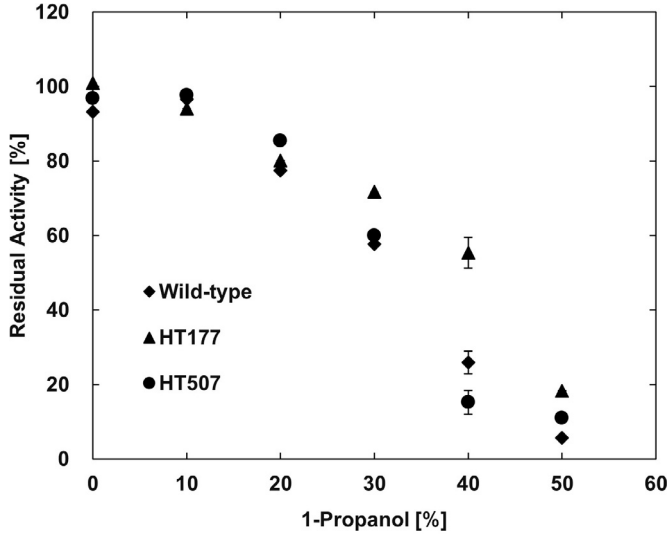

Fig. 2. 1-Propanol stability of wild-type and mutant BPO-A1 haloperoxidases. Wild-type and mutant BPO-A1 haloperoxidases were incubated in various concentrations of 1-propanol for 1 h at 30 °C. Thereafter, residual activities were measured at 25 °C. The data for 40% (v/v) 1-propanol represent the averages of 2 independent experiments.

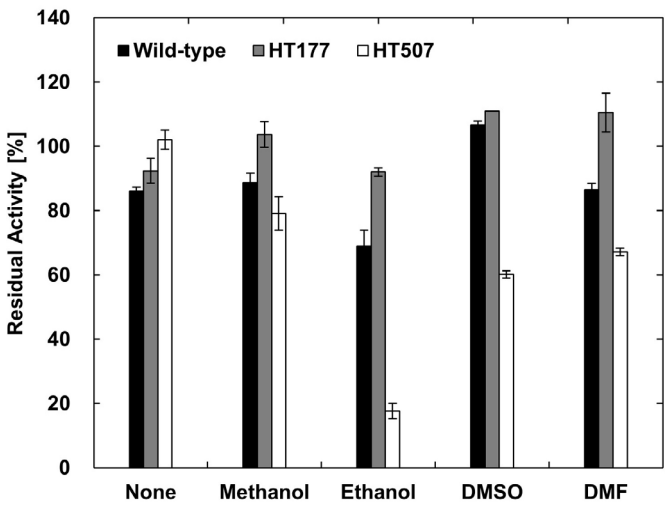

Fig. 3. Organic solvent stability of wild-type and mutant BPO-A1 haloperoxidases. Wild-type and mutant and BPO-A1 haloperoxidases were incubated in 40% (v/v) of various organic solvents for 48 h at 30 °C. Thereafter, residual activities were measured at 25 °C. The data represent the averages of 2 independent experiments.

solvents for 48 h at 30 °C were shown in Fig. 3. After incubation in the presence of methanol, ethanol, DMSO, or DMF, the mutant BPO-A1 haloperoxidase HT177 showed higher residual activity than wild-type BPO-A1; in contrast, the mutant BPO-A1 haloperoxidase HT507 showed lower residual activity than wild-type BPO-A1.

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

To evaluate the relationship between thermostability and dimerization, wild-type and mutant BPO-A1 haloperoxidases were incubated at 80 °C for 1 h and dimerization was analyzed by native PAGE (Fig. 4). Single protein bands were obtained from purified protein solutions of wild-type and mutant BPO-A1 haloperoxidases before incubation. In contrast, double protein bands were obtained after incubation at 80 °C for 1 h. Because high-molecular-weight compounds could be speculated to have low mobility in native PAGE, protein bands A and B were inferred to be dimeric and monomeric forms, respectively. The relative intensities of upper bands A of wild-type, and mutants HT177 and HT507 after incubation compared with those before incubation were 12.3%, 18.9%, and 45.6%, respectively, and they were well correlated to the residual activities after incubation at 80 °C for 1 h (Fig. 1). Thus the higher thermostabilities of the mutant BPO-A1 haloperoxidases HT177 and HT507 were believed to result from the dimerization stability of the BPO-A1 haloperoxidase.

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

To evaluate the effect of incubation in the presence of 1-propanol on dimerization, wild-type and mutant BPO-A1 haloperoxidases were incubated in 40% (v/v) 1-propanol for 1 h at 30 °C and then analyzed by native PAGE (Fig. 5). All haloperoxidases showed single protein bands before incubation in the presence of 1-propanol; in contrast, double protein bands were obtained after incubation. In the case of incubation in the presence of 1-propanol, the relative intensities of upper bands of wild-type, mutants HT177, and HT507 in native-PAGE were 11.8%, 24.9%, and 1.3%, respectively, and their residual activities were 25.9%, 55.4%, and 15.2%, respectively. However the residual activities of wild-type, mutants HT177, and HT507 were higher than their relative intensities of

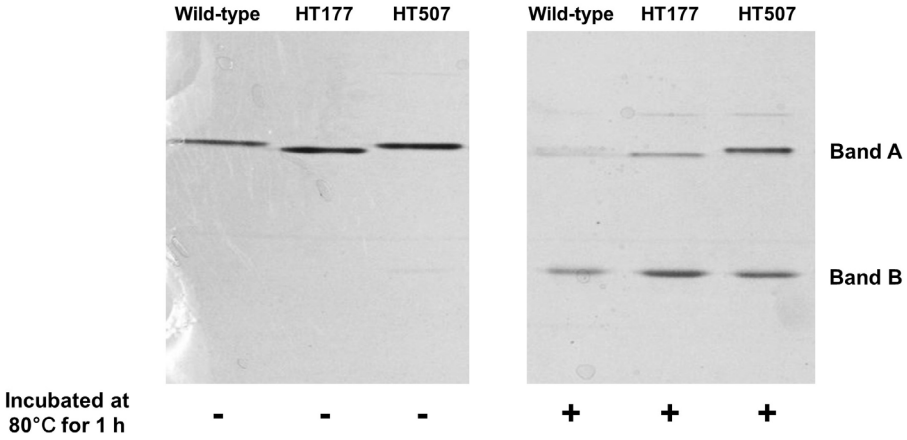

Fig. 4. Evaluation of the effect of heat treatment on dimerization of wild-type and mutant BPO-A1 haloperoxidases by native PAGE. Wild-type and mutant BPO-A1 haloperoxidases were incubated at 80 °C for 1 h. Thereafter, the original and incubated enzyme solutions were subjected to native PAGE.

Table 1
Relevant features of substituted amino acid residues in mutant BPO-A1 haloperoxidases.

Mutants	Substituted residues	Secondary structure	Solvent accessibility [%]
HT177	R114H	Sheet	37.4
	N146H	Helix	63.5
HT507	G106S	Helix	43.5
	V148I	Helix	1.72

upper bands. When BPO-A1 haloperoxidases were incubated in the presence of 1-propanol, monomerized BPO-A1 haloperoxidases might have catalytic activity. This result indicates that the mutant BPO-A1 haloperoxidases HT177 and HT507 had higher and lower dimerization stability, respectively, than wild-type BPO-A1 in the presence of 1-propanol.

3.7. Analysis of the 3-D structures of mutant BPO-A1 haloperoxidases

Plasmids containing the mutant BPO-A1 haloperoxidases HT177 and HT507 genes were extracted from their transformants and the nucleotide sequences were determined. The amino acid sequences of both mutant BPO-A1 haloperoxidases HT177 and HT507 were discriminated from that of the wild-type BPO-A1 haloperoxidase by 2 different amino acid residues (Table 1). The substituted residues of mutant BPO-A1 haloperoxidases were mapped onto the 3-D structure of *S. aureofaciens* BPO-A1 haloperoxidase (PDB 1A8Q) (Fig. 6). The substituted amino acid residues G106S, N146H, and V148I were located in helix regions and the substituted amino acid residue R114H was located in a sheet region. The high solvent accessibility of the substituted amino acid residues 106, 114, and 146 indicated that these residues were located on the surface of the protein.

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

To evaluate the effect of each amino acid residue substitution in mutant BPO-A1 haloperoxidases on specific activity and stability, 4 types of mutated BPO-A1 haloperoxidases with single substitutions at amino acid residues 106, 114, 146, and 148 were constructed by site-directed mutagenesis and named mutants G106S, R114H, N146H, and V148I, respectively. The specific activities and stabilities of the wild-type and single-amino-acid-substituted mutants were evaluated and are shown in Table 2. Mutants G106S, N146H, and V148I showed higher specific activities than wild-type BPO-A1, and mutants G106S, R114H, and V148I showed higher T_{50} than wild-type BPO-A1. Furthermore, mutants R114H and G106S showed higher 1-propanol stability, whereas mutants N146H and V148I showed lower 1-propanol stability than the wild-type BPO-A1 haloperoxidase.

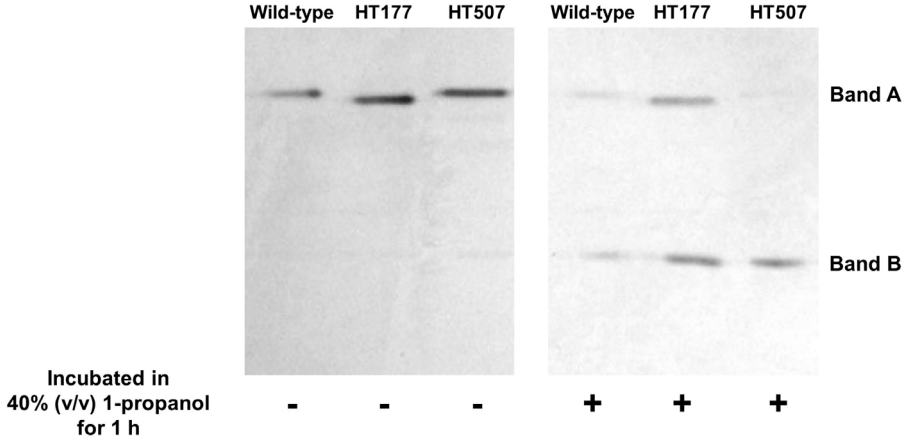

Fig. 5. Evaluation of the effect of 1-propanol on the dimerization of wild-type and mutant BPO-A1 haloperoxidases by native PAGE. Wild-type and mutant BPO-A1 haloperoxidases were incubated in 40% (v/v) 1-propanol for 1 h. Thereafter, the original and incubated enzyme solutions were subjected to native PAGE.

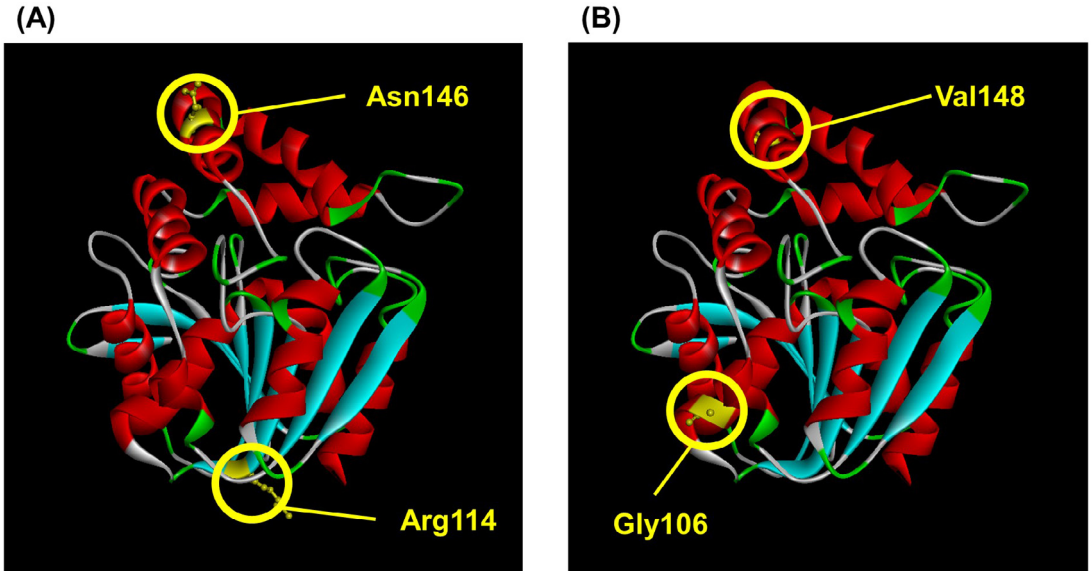

Fig. 6. Positions of substituted amino acid residues in mutant BPO-A1 haloperoxidases. Substituted residues in (A) mutant HT177 and (B) mutant HT507.

4. Discussion

In this study, thermostable *S. aureofaciens* haloperoxidases were constructed by directed evolution, and their thermostabilities and organic solvent stabilities were evaluated. In addition, single-amino-acid-substituted mutants were constructed, and the amino acid residues responsible for thermostability and organic solvent stability were identified.

Both mutant BPO-A1 haloperoxidases HT177 and HT507 had higher thermostabilities than wild-type BPO-A1 (Fig. 1, Table 2). In contrast, only mutant HT177 had higher organic solvent stability for 1-propanol, methanol, ethanol, DMSO, and DMF than wild-type BPO-A1 (Figs. 2 and 3, Table 2). Mutant HT507 had lower stability than wild-type BPO-A1 in all organic solvents used in this study. Although both mutant BPO-A1 haloperoxidases were selected as thermostable mutants, only mutant HT177 showed organic solvent stability. Petukhov et al. (1997) reported that stabilization of the α-helix conformation is important for the thermostability of enzymes. On the other hand, in our previous studies (Ogino et al., 2007; Kawata and Ogino, 2009), we reported that amino acid residues located at the surface of the protein play an important role in the organic solvent stability of enzymes. Based on the evaluation of single-amino-acid-substituted mutants, the V148I substitution, which was located in the helix formation area, could have contributed to thermostability (Tables 1 and 2). On the other hand, the R114H and G106S substitutions which were located at the surface of the protein, could have contributed to organic solvent stability. Thus, some effects of amino acid substitutions on thermostability and organic solvent stability could be explained rationally from previous studies. However, other effects, such as synergistic effects of multiple amino acid substitutions or week aggregation, are still under investigation.

Table 2
Specific activity, T_{50}, and 1-propanol stability of wild-type and mutant BPO-A1 haloperoxidases.

BPO-A1[b]	Specific activity [kU/g]	T_{50} [°C]	Residual activity in 40% (v/v) 1-propanol [%][a]
Wild-type	56.4	80.6	25.9 ± 3.0
Mutant HT177	59.6	82.0	55.4 ± 4.1
Mutant R114H	50.3	81.9	63.2 ± 4.3
Mutant N146H	78.7	80.4	20.3 ± 5.3
Mutant HT507	101.7	84.5	15.2 ± 3.2
Mutant G106S	99.4	81.1	33.5 ± 2.4
Mutant V148I	117.5	83.7	7.60 ± 1.2

[a] The data represent the averages of 2 independent experiments.
[b] Mutants HT177 and HT507 were obtained by directed evolution. Mutants R114H, N146H, G106S, and V148I having single-amino-acid-substitutions were constructed by site-directed mutagenesis.

The single-amino-acid-substituted mutant BPO-A1 haloperoxidases G106S and V148I showed higher specific activities and T_{50} values than wild-type BPO-A1 (Table 2). In general, because thermostable enzymes have rigid structures, they show low activities at mild temperatures (Vieille and Zeikus, 2001). However, the thermostability and specific activity of BPO-A1 were simultaneously improved by directed evolution in this study. Some reports have indicated that there is no correlation between the rigid structure and thermostability of enzymes (Fitter and Haber-Pohlmeier, 2004; LeMaster et al., 2005). Although the detailed mechanisms related to the improvement of thermostability and specific activity remain unknown, directed evolution could be useful for simultaneously improving multiple functions of enzymes.

The *S. aureofaciens* BPO-A1 haloperoxidase used in this study has a homodimeric structure, and dimerization stability was important for thermostability and organic solvent stability (Figs. 4 and 5). Some reports have indicated that enhancement of the interaction between subunits is important for improving enzyme thermostability (Kirino et al., 1994; Akanuma et al., 1999). The crosslink of subunits, immobilization, or chemical modification seems to an effectible approach to prevent subunit dissociation. In this study, because the quaternary protein structure of the BPO-A1 haloperoxidase has not been resolved, a rational design to enhance the interaction between subunits could not be applied. Thus, resolution of the quaternary protein structures could also be important for improving the stability of multi-subunit enzymes.

In this study, the thermostability, organic solvent stability, and specific activity of *S. aureofaciens* BPO-A1 haloperoxidase were successfully improved by a directed evolution approach. In addition, the amino acid residues responsible for thermostability and organic solvent stability were identified by evaluating single-amino-acid-substituted mutants. Possible explanations of the mechanisms related to the improvement of thermostability and organic solvent stability were stabilization of the α-helix conformation, substitutions of amino acid residues located at the surface of the protein, and enhancement of the interaction between subunits.

However, other stabilization mechanisms, such as synergistic effects between 2 amino-acid substitutions, are still under investigation. Besides, because hydrogen peroxide is able to deactivate enzyme (Hernandez et al., 2012), to improve hydrogen peroxide stability in haloperoxidase could be also important. Accumulation of additional knowledge is essential for improving enzyme stability by the rational design approach.

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