Appl Microbiol Biotechnol (2017) 101:5689–5697
DOI 10.1007/s00253-017-8299-0

Enhancing H_2O_2 resistance of an esterase from *Pyrobaculum calidifontis* by structure-guided engineering of the substrate binding site

Pengfei Zhou[1] · **Dongming Lan**[2] · **Grzegorz Maria Popowicz**[3] · **Xuping Wang**[2] · **Bo Yang**[1] · **Yonghua Wang**[2]

Received: 19 January 2017 / Revised: 6 April 2017 / Accepted: 12 April 2017 / Published online: 17 May 2017

Abstract Green technologies are attracting increasing attention in industrial chemistry where enzymatic reactions can replace dangerous and environmentally unfriendly chemical processes. In situ enzymatic synthesis of peroxycarboxylic acid is an attractive alternative for several industrial applications although concentrated H_2O_2 can denature the biocatalyst, limiting its usefulness. Herein, we report the structure-guided engineering of the *Pyrobaculum calidifontis* esterase (PestE) substrate binding site to increase its stability and perhydrolysis activity. The L89R/L40A PestE mutant showed better tolerance toward concentrated H_2O_2 compared with wild-type PestE, and retained over 72% of its initial activity after 24-h incubation with 2 M H_2O_2. Surprisingly, the half-life ($t_{1/2}$, 80 °C) of PestE increased from 28 to 54 h. The k_{cat}/K_m values of the mutant increased 21- and 3.4-fold toward pentanoic acid and H_2O_2, respectively. This work shows how protein engineering can be used to enhance the H_2O_2 resistance and catalytic efficiency of an enzyme.

Keywords *Pyrobaculum calidifontis* · Esterase · Stability · Structure-guided engineering · Perhydrolysis activity

✉ Yonghua Wang
yonghw@scut.edu.cn

[1] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, People's Republic of China

[2] School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, People's Republic of China

[3] Institute of Structural Biology, Helmholtz ZentrumMünchen Deutsches Forschungszentrum für Gesundheitund Umwelt (GmbH), Ingolstädter Landstrasse l, 85764 Oberschleißheim, Germany

Introduction

Oxidation reactions, such as epoxidation, hydroxylation, N-oxidation, S-oxidation, and Baeyer-Villiger oxidation, are attractive for organic synthesis of value compounds for science and industry purposes (Hernandez et al. 2012). Although the high catalytic efficiency of oxidation reactions can be achieved by using chemical methods, the toxicity and environmental pollution arising from side products and reaction residues deserve our attention. Alternatively, oxidases including peroxidase (Ullrich et al. 2008), peroxygenase (Kluge et al. 2009), P450 (Bernhardt 2006), chloroperoxidase (Colonna et al. 1992), haloperoxidase (Dembitsky 2003), and Baeyer-Villiger monooxygenase (Fraaije et al. 2005) are promising biocatalysts for these reactions because of their high catalytic efficiency under mild reaction conditions and low toxicity to the environment. Additionally, esterases and lipases can catalyze the reaction of carboxylic acids and H_2O_2 to generate peroxycarboxylic acid, which can transfer its oxygen to olefins (Björkling et al. 1990; Björkling et al. 1992; Moreira et al. 2005; Ankudey et al. 2006; Wang et al. 2015), cyclic ketones (Kotlewska et al. 2011; Drozdz et al. 2015), amines (Irimescu and Kato 2004), and organic sulfur compounds (Nieuwenhuizen 1949; Lou et al. 2008). Thus, in situ enzymatic synthesis of peroxycarboxylic acid by esterases and lipases is an attractive alternative for a number of industrial applications compared with the use of a chemical in situ generation method (Björkling et al. 1990; Rusch et al. 2002).

Unfortunately, except for its role as a substrate, H_2O_2 has a strong detrimental effect on the chemical structure of proteins because of its ability to oxidize amino acids. Amino acids, such as Arg, Pro, Lys, Met, Cys, Tyr, and His, are sensitive to a high concentration of H_2O_2, which destabilizes enzymes and limits their applications in oxidation reactions (Villegas et al. 2000; Kroutil et al. 2004;

Kitajima et al. 2008; Hernandez et al. 2012). To solve this problem, several strategies have been developed to increase the tolerance of enzymes toward H_2O_2. Immobilization of enzymes on suitable supporting materials can improve their resistance to extreme reaction conditions, while chemical modification of enzymes is an alternative method to achieve similar effects. However, in most cases, these strategies may sacrifice enzyme activity for enhanced protein stability (Hernandez et al. 2012). Unlike physical and chemical methods, genetic engineering is a simple way to increase the protein stability by substituting the H_2O_2-sensitive amino acid with an insensitive one, and its feasibility has been confirmed by many researchers (Villegas et al. 2000; Miyazaki and Takahashi 2001; Kuo et al. 2004; Ryan and Ó'Fágáin 2007; Kitajima et al. 2008; Ogola et al. 2010). The manganese peroxidase from *Phanerochaete chrysosporium* showed higher resistance to H_2O_2 inactivation when the H_2O_2-sensitive Met in the H_2O_2-binding pocket was replaced with Leu (Ryan and Ó'Fágáin 2007). Three mutants of horseradish peroxidase, K232N, K241F and T110V, showed higher H_2O_2 tolerances of 25-, 18-, and 12-fold compared with that of the parent enzyme (Miyazaki and Takahashi 2001).

PestE, an esterase from the hyperthermophilic archeon *Pyrobaculum calidifontis,* has potential industrial applications because of its extreme stability in the presence of heat and organic solvents (Palm et al. 2011). However, it is not stable in the presence of a high concentration of H_2O_2, limiting its application in peroxycarboxylic acid production. In this study, we selected PestE as a model enzyme to increase its stability toward H_2O_2 by engineering its substrate binding pocket by structure-guided mutagenesis. The H_2O_2-tolerance, thermostability, and biochemical characterization of the PestE mutants were investigated. We present a new approach for enhancing the H_2O_2 stability of PestE esterase by introducing mutations that allow stabilization of H_2O_2 in the enzyme active site. Surprisingly, these modifications dramatically enhanced both enzymatic activity and H_2O_2 stability.

Materials and methods

Chemicals and reagents

The artificial substrates *p*-nitrophenyl (*p*NP) acetate (C2), *p*NP butyrate (C4), *p*NP caproate (C6), *p*NP caprylate (C8), *p*NP caprate (C10), *p*NP laurate (C12), *p*NP myristate (C14), *p*NP palmitate (C16), and oleic acid were purchased from Sigma-Aldrich (Shanghai, China). Formic acid, acetic acid, propanoic acid, butyric acid, pentanoic acid, n-hexylic acid, hydrogen peroxide (30% wt), and sodium bromide were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China), and 2-chloro-5,5-dimethyl-1,3-cyclohexanedione (monochlorodimedone) was from Alfa Aesar (Shanghai, China). All other regents were of analytical grade.

Expression and purification of PestE and its mutants

The gene-encoding PestE (GenBank accession number: AB078331.1) was chemically synthesized and cloned into the pET23a vector as a cellulose-binding domain (CBD) fusion at the N terminus and His tag at the C terminus. The protein was expressed in *Escherichia coli* BL21(DE3). *E. coli* BL21(DE3) cells harboring the plasmid pET23a encoding the target genes were grown at 37 °C for 8 h in Luria-Bertani (LB) medium containing 100 μg ampicillin mL^{-1}, and then the microorganisms were inoculated into auto-induction medium until the optical density (OD_{600}) reached 0.8, and then the gene expression was induced by setting the temperature at 20 °C for 12 h at 200 rpm as previously described (Studier 2005).

Cells were collected, disrupted by sonication, and the protein of interest was recovered by adsorption to cellulose. The CBD tag was removed by proteolysis with 3C protease and centrifugation and the protein was recovered in the supernatant. The recombinant enzyme was further purified by metal ion affinity chromatography. The protein concentration in the final preparation was determined using the Lowry Bradford method (Sedmak and Grossberg 1977) and bovine serum albumin was used as a standard. Mutants were obtained by site-directed mutagenesis and expressed exactly as the wild type. All mutants were verified by sequencing of the relevant constructs.

General assays

The stability of PestE and its mutants in the presence of H_2O_2 was determined by incubating the test enzyme (0.05 μM) in phosphate buffer containing different concentrations of H_2O_2 (5 mM to 2 M) at room temperature. Samples were withdrawn at different time points and the residual perhydrolysis activity was determined. The residual activity was expressed as a fraction of the residual activity of the reference sample treated in the same way except for the addition of H_2O_2. The thermal stability of PestE and its mutants was measured by using *p*NP butyrate as the substrate. Enzymes were incubated in phosphate buffer at pH 7.0 at 80 °C for 24 h. Samples were taken from the media at different time intervals. The optimal pH for the perhydrolysis reaction was determined by varying the pH of the pentanoic acid buffer.

The temperature dependence of the perhydrolysis reaction was tested by varying the temperature from 40 to 55 °C. The dependence of the hydrolysis rate on the length of the aliphatic chain of the carboxylic acid substrate was determined by exchanging the pentanoic acid buffer into a buffer containing the tested acid.

Hydrolysis assay

The hydrolytic activity of the recombinant PestE and its variants was determined using the chromogenic substrate *p*NP butyrate. The reaction was carried out at 80 °C in 50 mM NaOH/glycine buffer at pH 9.0. The specific hydrolytic activity was expressed in units, where 1 U represents the amount of enzyme required to release 1 μmol of *p*NP per minute under the reaction conditions. The kinetic parameters were determined under the same conditions at a constant enzyme concentration (0.05 μM) by varying the *p*NP butyrate concentration and determining the initial velocity of *p*NP production.

Perhydrolysis assay

The perhydrolysis activity was determined following a general protocol by Bernhardt and colleagues (Bernhardt et al. 2005) where aliphatic acid perhydrolysis is monitored by a coupled reaction of the oxidation of 2-chloro-5,5-dimethyl-1,3-cyclohexanedione (monochlorodimedone, MCD). The test was carried out at 45 °C with pentanoic acid as the substrate (in 0.1 M pentanoic acid buffer at pH 5.5 containing 90 mM NaBr and 180 μM MCD) in the presence of 100 mM H_2O_2. The activity was determined spectrophotometrically and expressed in specific activity units where 1 U represents the amount of enzyme required to produce 1 μmol of MCD per minute under the reaction conditions.

The kinetic parameters for pentanoic acid were determined under the same conditions at constant enzyme (0.05 μM) and H_2O_2 (100 mM) concentrations by varying the pentanoic acid concentration and determining the initial velocity of the peracid (oxidized MCD) production. The kinetic parameters for H_2O_2 were determined under the same conditions at constant enzyme (0.05 μM) and pentanoic acid (100 mM) concentrations by varying the H_2O_2 concentration and determining the initial velocity of peracid (oxidized MCD) production.

Molecular modeling

The model of the L89R mutant was constructed by homology modeling based on the crystal structure of PestE (PDB code: 3ZWQ) using the Modeller package (Discovery Studio3.5, Accelrys Inc.). All structure models were visualized using PyMOL software.

The published PestE was hydrogenated using Yasara and tested mutations were introduced. Steepest descent minimization was performed in vacuo using the YASARA-NOVA force field to relax the structure and remove steric clashes (Krieger et al. 2002). The cell was parametrized using the AMBER14 force field and neutralized (Giambaşu et al. 2014). The resulting structure was time simulated using implicit solvent.

Epoxidation reaction

The epoxidation of oleic acid was carried out in a 10-mL Erlenmeyer flask; the reaction mixture contained 0.5 mmol oleic acid, 5.0 mg purified lyophilized esterase (PestE-WT or L89R/L40A), 1.0 mmol 30% hydrogen peroxide, and 1.0 mL phosphate buffer (100 mM, pH 5.0). The 30% hydrogen peroxide was added in three portions in 1-h intervals. The reaction mixtures were placed at different temperature from 40 to 70 °C, stirred at a speed of 300 rpm. Samples (20 μL) were withdrawn from the mixtures at specific time. All experiments were carried out in triplicate.

Analytical methods

Oleic acid and 9,10-epoxystearic acid were determined using a high-performance liquid chromatography (Waters 1525) equipped with refractive index detector (HPLC-RID) (Waters 2487, USA). RP-C18 column (4.6 mm × 250 mm, 5 mm, Wasters, USA) was used to separate these components. The column was operated at 35 °C with methanol:water:formic acid = 95:5:0.5 (*v*/*v*/*v*) as the mobile phase at the flow rate of 1 mL/min. Peak of oleic acid and 9,10-epoxystearic acid in HPLC were identified and calculated by the calibration curves which were made with the reference standard compounds.

Results

Structure-guided design of mutations

The crystal structure of PestE (PDB: ID 3ZWQ) has been solved previously (Palm et al. 2011). Structural analysis demonstrated that the active site and presumed H_2O_2 binding site is completely hydrophobic (Fig. 1a, b). We reasoned that introduction of hydrogen bond donors in the catalytic pocket could help to stabilize H_2O_2 in an optimal conformation to increase both structure stability and catalytic efficiency. Therefore, the hydrophobic amino acids G85, L89, I208, L209, and F217 were selected to substitute with serine, arginine, threonine, threonine, and tyrosine, respectively, to introduce hydrogen bond donors in the presumed H_2O_2 interaction site. Furthermore, to facilitate better substrate penetration into the catalytic site, amino acids with bulky side chains located around the substrate binding pocket, such as L40, F33, F20, L213, and F289, were replaced with alanine that contains small side chain to "open" the active site entrance and potentially increase the catalytic activity.

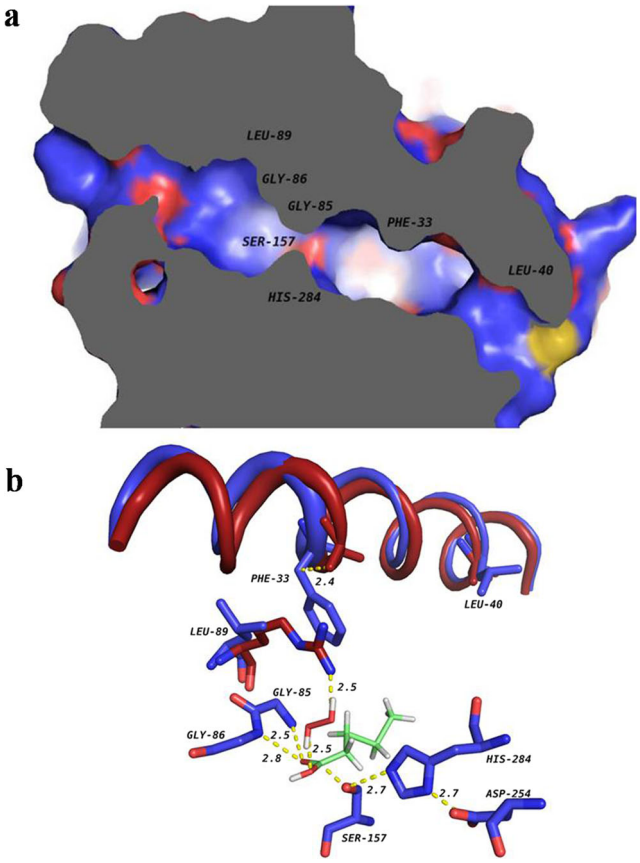

Fig. 1 **a** The catalytic channel of PestE. Ser157 and His284 of the catalytic triad together with candidate sites for mutation are shown. **b** Model of the active site architecture for peroxidation. The L89R mutation introduces a hydrogen bond with H_2O_2. The carbonyl oxygen of the pentanoic acid is located in the oxyanion hole. The H_2O_2 molecule is located optimally for attack on the tetrahedral intermediate. Distances are given in angstrom.

Production and H_2O_2 tolerance analysis of PestE mutants

All the PestE mutants (listed in Fig. 2) were produced as fusion proteins with a CBD tag at the N terminus and a His tag at the C terminus (Fig. 2a). The CBD tag facilitated isolation of the recombinant protein from the cellular proteins by adsorption onto cellulose. Then, the tag-free recombinant PestE mutants (after 3C protease digestion of the CBD fusion proteins) were further purified by a nickel affinity column. The final yields of each purified protein (with purity >90%, Fig. 2b) were about 20 to 50 mg from 1 L of culture media.

The H_2O_2 tolerance of purified PestE wild type (WT) and its mutants were investigated by incubating the enzymes with 1 M H_2O_2 for 24 h followed by measurement of their residual activity (Fig. 2c). The mutant L89R showed higher perhydrolysis activity (1.7 U/mg) and better H_2O_2 tolerance (retained 67.5% of its initial activity) than that of PestE–WT, indicating that introduction of a hydrogen bond donor at this site can increase the H_2O_2 tolerance and activity of PestE. However, the G85S mutant did not show a similar positive effect. It should also be noted that G85 together with G86 is involved in the formation of an oxyanion hole in the PestE structure. Mutation at amino acid 85 might negatively affect the configuration of the oxyanion hole resulting in a decrease of enzyme activity and stability. With the exception of mutant L89R/L209T, introduction of an additional hydrogen bond donor at I208 and F217 had a beneficial effect on the stability of mutant L89R toward H_2O_2. "Open" the entrance of the catalytic pocket may allow better penetration of the substrate to the catalytic site. Thus, substitution of L40 with alanine was found to increase the stability (retained 85.8% of its initial activity) and activity (2.77 U/mg) of the L89R mutant. The triple mutants L89R/L40A/L213A, L89R/L40A/F289A, and L89R/F20A/F33A did not show any obvious increase in stability and activity compared with that of the mutant L89R/L40A. Among the enzymes tested, L89R/L40A had the best H_2O_2 tolerance and activity, and thus was selected for the subsequent evaluation study.

Biochemical characterization of the mutant L89R/L40A

Comformational changes of the substrate binding pocket will occur after site-directed mutagenesis and will affect not only the structure stability but also the substrate specificity, catalytic efficiency, and other physical properties. Therefore, the biochemical properties of L89R/L40A were characterized after the initially H_2O_2-tolerance screening.

To investigate the H_2O_2 tolerance profile of L89R/L40A in more detail, its activity was monitored after incubation with different H_2O_2 concentrations at various time intervals. As shown in Fig. 3, the L89R/L40A mutant did not lose its activity under conditions of low H_2O_2 concentrations (5 and 50 mM) regardless of the incubation time, while PestE-WT retained 65 and 50% of its initial activity after a 24-h incubation with 5 and 50 mM of H_2O_2, respectively. At the higher H_2O_2 concentrations (0.5 and 2 M), the PestE mutant still retained over 80% of its initial activity after a 24-h incubation.

An analysis of the heat inactivation of mutant L89R/L40A was also conducted. Its residual activity after incubation at 80 °C was measured as shown in Fig. 4 and the mutant L89R and PestE-WT were used as controls. After a 24-h incubation, L89R/L40A and L89R retained over 71% of their initial activities. The half-lives ($t_{1/2}$, 80 °C) of L89R/L40A (54 h) and L89R (52 h) were higher than that of PestE-W (28 h), indicating that substitution of L89 with arginine can stabilize the PestE structure. Replacing L40 with alanine made only a marginal contribution to the thermostability of L89R based on the observation that L89R/L40A and L89R had similar half-lives.

The substrate selectivity of mutant L89R/L40A toward *p*-nitrophenyl (*p*NP) esters with various acyl-chain lengths (C2–C16) was evaluated by measuring its hydrolysis activity. As shown in Fig. 6a, mutant L89R/L40A showed a change in

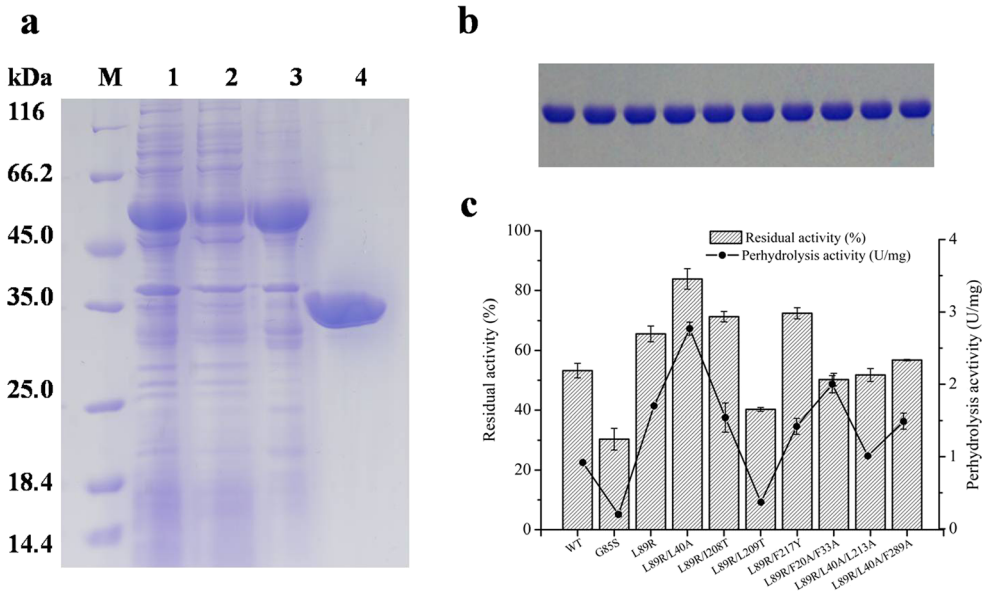

Fig. 2 Production of PestE mutants and H_2O_2 resistance. **a** Expression of the PestE mutant in *E.coli*. *Lane 1*: total cellular protein; *lane 2*: supernatant of lysate; *lane 3*: insoluble cellular pellet; *lane 4*: purified protein. **b** Purified PestE and its mutants. **c** Residual activity of enzymes tested after incubation with 1 M H_2O_2 for 24 h at room temperature. The data are expressed as the percentage of residual activity compared with the original activity of each enzyme tested (%)

its substrate preference, from shorter (C4) to short- and medium-chain length *p*NP esters (C4–C8), compared with PestE-WT.

To measure perhydrolysis activity, various carboxylic acid substrates including formic acid, acetic acid, propanoic acid, butyric acid, pentanoic, and n-hexylic acid were tested (Fig. 6b). L89R/L40A preferred similar substrates as PestE-WT, and the highest activity was obtained using pentanoic acid as a carboxyl donor. The kinetic constants for L89R/L40A and PestE-WT toward pentanoic acid were determined. The K_m value significantly decreased from 16.1 to 2.8 mM for the mutant indicating that it had higher affinity for pentanoic acid than PestE-WT, while its catalytic efficiency significantly increased from 6.22 to 23.08 s^{-1} (Table 1).

The perhydrolysis activities of PestE-WT and mutant L89R/L40A under various temperatures and pH were measured (Fig. 6c and d). Both enzymes maintained high activity from 45 to 50 °C with the optimal activity at 45 °C. Mutant

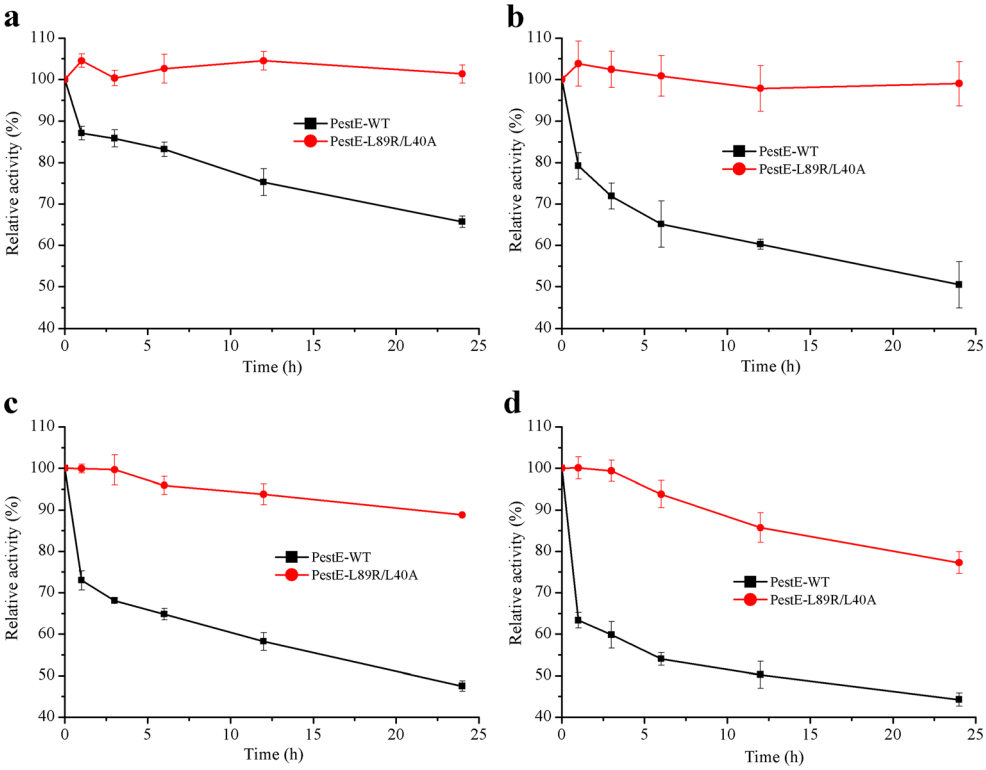

Fig. 3 Effects of various concentrations of H_2O_2 on the perhydrolysis activity of PestE-WT and mutant L89R/L40A. **a** 5 mM, **b** 50 mM, **c** 500 mM, **d** 2 M

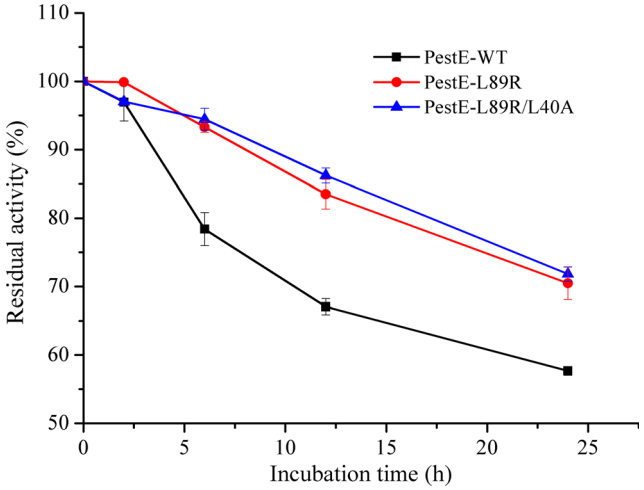

Fig. 4 Thermostability of PestE wild-type, mutant L89, and L89R/L40A. The enzymes were incubated at 80 °C for 24 h. Residual activity was measured by using 4-nitrophenyl butyrate as a substrate at various time intervals. The initial activity of each enzyme is expressed as 100%

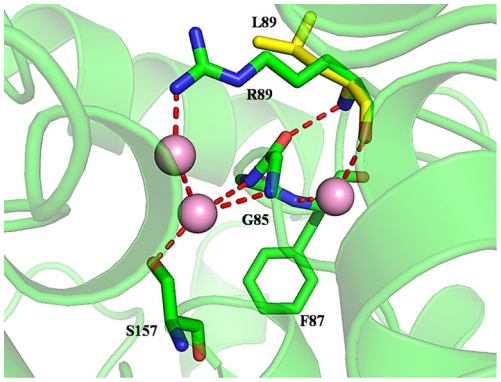

Fig. 5 The hydrogen bond network near the catalytic site in mutant L89R. G85, F87, R89, and S157 are shown as *green sticks*, while the L89 residue is shown as a *yellow stick*. The water molecules are displayed as *pink balls*

L89R/L40A exhibited maximum activity at pH 5.5, while the optimal pH of PestE-WT was pH 5.0.

Enzyme-catalyzed epoxidation of oleic acid

The initial rates of epoxidation of oleic acid catalyzed both by PestE-WT and mutant L89R/L40A depended linearly on temperature. However, after 48 h, the maximum conversion of oleic acid by PestE-WT and mutant L89R/L40A was 40.2 and 75.6%, respectively, both obtained at 60 °C. This means nearly doubled conversion efficiency of the mutant after this period. The conversion of epoxidation products decreased at 70 °C as the result of the higher temperature possibly leading to hydrogen peroxide decomposition and enzyme inactivation.

Discussion

H_2O_2 has a Janus role to oxidases. It is an indispensable substrate for those enzymes, while shows detrimental effects to the proteins. Unfortunately, the most biocatalysts are unstable in the presence of concerted H_2O_2 due to their inherent nature. Biocatalyst with better H_2O_2 tolerance ability is highly desired for industrial applications. Protein engineering is an acknowledged method of modifying enzymatic properties, such as catalytic efficiency, thermostability, and substrate specificity (Kim et al. 2010; Guo et al. 2015; Lan et al. 2015a). And most of the efforts focus on substitution of the H_2O_2 sensitive amino acids with non-sensitive one to enhance the enzyme stability against H_2O_2 (Miyazaki and Takahashi 2001; Lin et al. 2003; Kitajima et al. 2008). In this work, we try to figure out whether introduction of hydrogen bond donors in the catalytic pocket of enzyme to stabilize H_2O_2 can modify its structure stability and catalytic behaviors. Using PestE as model enzyme, polar amino acids with a hydroxyl group (Ser, Thr, and Tyr) or guanidinium group (Arg) were introduced into the substrate binding site by site-directed mutagenesis.

Substitute of Leu89 with Arg resulted in a better ability of H_2O_2 resistance. We reasoned that L89R introducing hydrogen bond donors in the vicinity of the presumed H_2O_2 interaction site could help stabilize H_2O_2 in an optimal conformation. This might alleviate the harmful effect to H_2O_2-sensitive amino acids around the catalytic pocket when the H_2O_2 accesses. The L89R has higher perhydrolysis activity, indicating that stabilization of H_2O_2 in the substrate binding pocket also

Table 1 Kinetic constants of PestE wild-type and the mutant L89R/L40A

Enzymes	Substrates	k_{cat} (s^{-1})	K_m(mM)	k_{cat}/K_m(s^{-1} M^{-1})
Perhydrolysis[a]				
PestE-WT	Pentanoic acid	6.22 ± 0.5	16.01 ± 1	388.24
	Hydrogen peroxide	10.06 ± 0.5	101.4 ± 2	98.6
PestE-L89R/L40A	Pentanoic acid	23.08 ± 0.68	2.8 ± 0.1	8242
	Hydrogen peroxide	29.01 ± 1.5	86.76 ± 12.8	334

[a] Monochlorodimedone (MCD, 0.18 mM), different concentrations of H_2O_2, and NaBr (90 mM) in different concentrations of pentanoic acid buffer (optimal pH 5.0 or 5.5) at 45 °C (optimal temperature). The control experiments were carried out under the same conditions

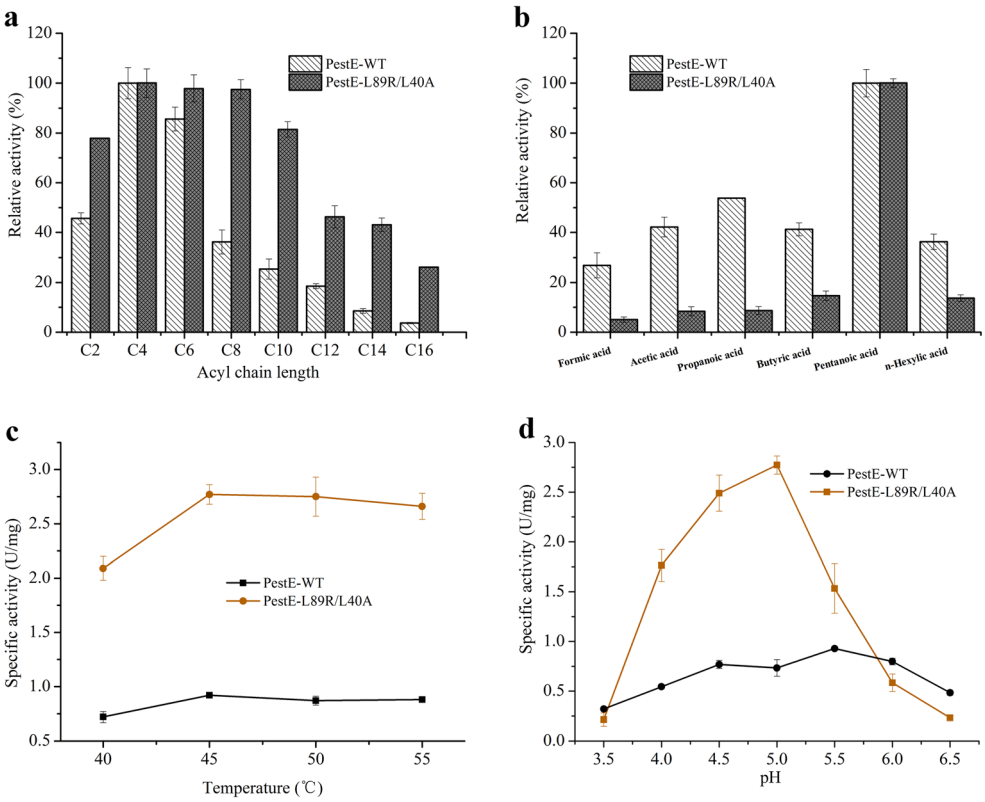

Fig. 6 Biochemical characteristics of PestE wild-type and mutant L89R/L40A. **a** Substrate selectivity for the *p*NP ester with various chain lengths. **b** Substrate preference for the carboxylic acid substrate. **c** Effect of temperature on the activity of the enzymes. **d** Effect of pH on the activity of enzymes

benefits the catalytic activity. Meanwhile, arginine is well suited in this mutant since it partially retains the hydrophobic interactions of leucine within the enzyme structure, which does not affect structural conformation. However, significant synergistic effect to the stability has not been seen by introduction additional hydrogen bond donors (Fig. 2c).

Many literatures have described that amino acids with bulky side chain will affect the substrate selectivity and catalytic efficiency (Gao et al. 2014; Lan et al. 2015a; Lan et al. 2015b). To investigate the effect of bulky amino acid in the substrate binding pocket on the catalytic behavior of PestE, alanine were introduced. Among the alanine-substituted mutants, L89R/L40A mutant showed highest perhydrolysis activity. Kinetic study found that the k_{cat}/K_m values of the mutant increased 21- and 3.4-fold toward pentanoic acid and H_2O_2 as compared to that of PestE-WT, respectively. The substitute with non-bulky alanine may facilitate better substrate access into the catalytic site resulting in increasing the catalytic efficiency. Due to the more "open" of the catalytic pocket after mutagenesis, L89R/L40A mutant showed more preference to *p*NP ester of C6 and C8 compared to that of PestE-WT in the hydrolysis reaction. But it was unexpected that L89R/L40A had a better ability of H_2O_2 tolerance as compared to other mutants.

Apart from the desired activity, stability at reaction conditions is of primary importance in industrial applications. The

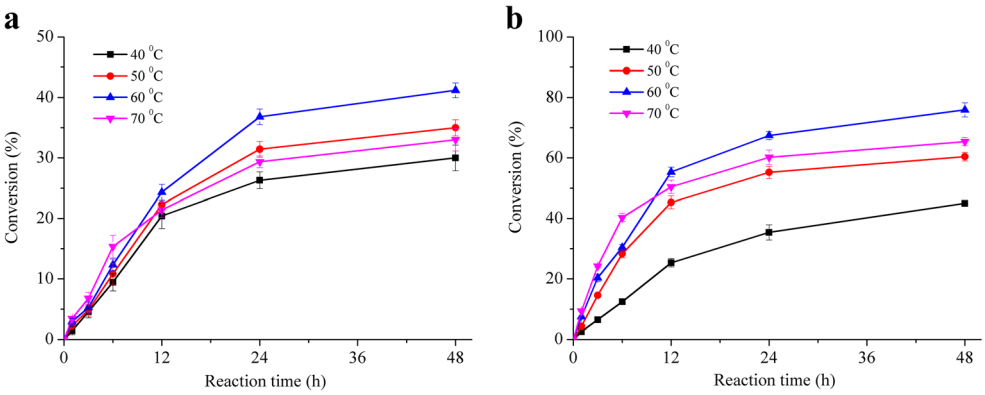

Fig. 7 Epoxidation oleic acid catalyzed by **a** PestE-WT and **b** PestE-L89R/40A

half-lives ($t_{1/2}$, 80 °C) of mutant L89R and L89R/L40A were 52 and 54 h, which are roughly twofold increase to that of PestE-WT. By analysis of the structural model of L89R, substitution of Leu89 with Arg in the hydrophobic catalytic cleft can generate an extra hydrogen bond with a water molecule (Fig. 5) and form a hydrogen bond network with G85, F87, R89, S157, and three surrounding water molecules. This hydrogen bond network can stabilize the loop (H83–S91) located in the catalytic pocket and nucleophilic serine (S157) to maintain the conformation of PestE. Hydrogen bonds occur not only within and between polypeptide chains but with the surrounding aqueous medium, which can contribute to stabilizing the conformation and enhancing the thermostability of proteins (Eijsink et al. 1992; Li et al. 2005). This proposed hydrogen bonding network explains why mutant L89R/L40A showed high tolerance toward concentrated H_2O_2 and elevated temperature.

Epoxides are important intermediates in organic synthesis due to the high reactivity of oxirane rings (Milchert et al. 2015; Zhou et al. 2017). Traditional synthetic routes for the epoxidation of products entail stoichiometric use of peracids such as peracetic acid under acidic reaction conditions. The challenges of these methods reside with the hazard of peracid. Therefore, in situ peracid generation approach was developed using H_2O_2 and carboxylic acid catalyzed by enzyme. However, oxidative inactivation by H_2O_2 represents a major challenge to enzyme stability and therewith to the robustness of the chemical transformation. In this study, a double mutant L89R/L40A significantly enhanced the tolerance of H_2O_2 and high temperature was reported. The epoxidation reaction test was found that the conversion of epoxidation of oleic acid of the mutant L89R/L40A increased approximately twofold as compared to that of PestE-WT (Fig. 7). The creating mutant L89R/L40A is promising as a novel biocatalyst for epoxidation reaction.

In summary, engineering of the substrate binding site by structure-guided mutagenesis is a potent way to modify enzymes. By introducing an extra hydrogen bond donor near the catalytic site and "opening" the catalytic entrance of PestE, mutant L89R/L40A efficiently catalyzes peroxyacid formation and is stable in concentrated H_2O_2. Because of its thermophilic origin and ease of recombinant production, our PestE mutant is an excellent candidate for industrial applications in green chemistry. Furthermore, these results open new opportunities for designing industrial biocatalysts with tolerance for high concentrations of H_2O_2 and catalytic efficiency.

Acknowledgements This work was supported by the National Natural Science Foundation of China (21406076, 31501412, and 31471690), Science and Technology Planning Project of Guangdong Province (2014B020204003), and Youth Science and Technology Innovation Leader of Guangdong Province (2015TX01N207).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Ankudey EG, Olivo HF, Peeples TL (2006) Lipase-mediated epoxidation utilizing urea-hydrogen peroxide in ethyl acetate. Green Chem 8: 923–926. doi:10.1039/b604984b

Bernhardt P, Hult K, Kazlauskas RJ (2005) Molecular basis of perhydrolase activity in serine hydrolases. Angew Chem Int Ed 44:2742–2746. doi:10.1002/anie.200463006

Bernhardt R (2006) Cytochromes P450 as versatile biocatalysts. J Biotechnol 124:128–145. doi:10.1016/j.jbiotec.2006.01.026

Björkling F, Godtfredsen SE, Kirk O (1990) Lipase-mediated formation of peroxycarboxylic acids used in catalytic epoxidation of alkenes. J Chem Soc Chem Commun 1:1301–1303. doi:10.1039/c39900001301

Björkling F, Frykman H, Godtfredsen SE, Kirk O (1992) Lipase catalyzed synthesis of peroxycarboxylic acids and lipase mediated oxidations. Tetrahedron 48:4587–4592. doi:10.1016/S0040-4020(01)81232-1

Colonna S, Gaggero N, Casella L, Carrea G, Pasta P (1992) Chloroperoxidase and hydrogen peroxide: an efficient system for enzymatic enantioselective sulfoxidations. Tetrahedron Asymmetry 3:95–106. doi:10.1016/S0957-4166(00)82316-2

Dembitsky VM (2003) Oxidation, epoxidation and sulfoxidation reactions catalysed by haloperoxidases. Tetrahedron 59:4701–4720. doi:10.1016/S0040-4020(03)00701-4

Drozdz A, Erfurt K, Bielas R, Chrobok A (2015) Chemo-enzymatic Baeyer-Villiger oxidation in the presence of *Candida antarctica* lipase B and ionic liquids. New J Chem 39:1315–1321. doi:10.1039/C4NJ01976H

Eijsink VG, Vriend G, Van der Zee JR, Van den Burg B, Venema G (1992) Increasing the thermostability of the neutral proteinase of *Bacillus stearothermophilus* by improvement of internal hydrogen-bonding. Biochem J 285:625–628

Fraaije MW, Wu J, Heuts DPHM, Van Hellemond EW, Spelberg JHL, Janssen DB (2005) Discovery of a thermostable Baeyer-Villiger monooxygenase by genome mining. Appl Microbiol Biotechnol 66:393–400. doi:10.1007/s00253-004-1749-5

Gao C, Lan D, Liu L, Zhang H, Yang B, Wang Y (2014) Site-directed mutagenesis studies of the aromatic residues at the active site of a lipase from *Malassezia globosa*. Biochimie 102:29–36. doi:10.1016/j.biochi.2014.02.004

Giambaşu GM, Luchko T, Herschlag D, York DM, Case DA (2014) Ion counting from explicit-solvent simulations and 3D-RISM. Biophys J 106:883–894. doi:10.1016/j.bpj.2014.01.021

Guo S, Xu J, Pavlidis IV, Lan D, Bornscheuer UT, Liu J, Wang Y (2015) Structure of product-bound SMG1 lipase: active site gating implications. FEBS J 282:4538–4547. doi:10.1111/febs.13513

Hernandez K, Berenguer-Murcia A, Rodrigues C, R, Fernandez-Lafuente R (2012) Hydrogen peroxide in biocatalysis. A dangerous liaison. Curr Org Chem 16:2652–2672. doi:10.2174/138527212804004526

Irimescu R, Kato K (2004) Lipase-catalyzed enantioselective reaction of amines with carboxylic acids under reduced pressure in non-solvent

system and in ionic liquids. Tetrahedron Lett 45:523–525. doi:10.1016/j.tetlet.2003.10.210

Kim HS, Le QAT, Kim YH (2010) Development of thermostable lipase B from *Candida antarctica* (CalB) through in silico design employing B-factor and Rosetta Design. Enzym Microb Technol 47:1–5. doi:10.1016/j.enzmictec.2010.04.003

Kitajima S, Kitamura M, Koja N (2008) Triple mutation of Cys26, Trp35, and Cys126 in stromal ascorbate peroxidase confers H_2O_2 tolerance comparable to that of the cytosolic isoform. Biochem Biophys Res Commun 372:918–923. doi:10.1016/j.bbrc.2008.05.160

Kluge M, Ullrich R, Dolge C, Scheibner K, Hofrichter M (2009) Hydroxylation of naphthalene by aromatic peroxygenase from *Agrocybe aegerita* proceeds via oxygen transfer from H_2O_2 and intermediary epoxidation. Appl Microbiol Biotechnol 81:1071–1076. doi:10.1007/s00253-008-1704-y

Kotlewska AJ, van Rantwijk F, Sheldon R a, Arends IWCE (2011) Epoxidation and Baeyer–Villiger oxidation using hydrogen peroxide and a lipase dissolved in ionic liquids. Green Chem 13:2154–2160. doi:10.1039/c1gc15255f

Krieger E, Koraimann G, Vriend G (2002) Increasing the precision of comparative models with YASARA NOVA—a self-parameterizing force field. Proteins Struct Funct Genet 47:393–402. doi:10.1002/prot.10104

Kroutil W, Mang H, Edegger K, Faber K (2004) Biocatalytic oxidation of primary and secondary alcohols. Adv Synth Catal 346:125–142. doi:10.1002/adsc.200303177

Kuo LY, Hwang GY, Yang SL, Hua YW, Chen W, Lin LL (2004) Inactivation of *Bacillus stearothermophilus* leucine aminopeptidase II by hydrogen peroxide and site-directed mutagenesis of methionine residues on the enzyme. Protein J 23:295–302. doi:10.1023/B:JOPC.0000027854.56051.e4

Lan D, Popowicz GM, Pavlidis IV, Zhou P, Bornscheuer UT, Wang Y (2015a) Conversion of a mono- and diacylglycerol lipase into a triacylglycerol lipase by protein engineering. Chem BioChem 16:1431–1434. doi:10.1002/cbic.201500163

Lan D, Wang Q, Xu J, Zhou P, Yang B, Wang Y (2015b) Residue Asn277 affects the stability and substrate specificity of the SMG1 lipase from *Malassezia globosa*. Int J Mol Sci 16:7273–7288. doi:10.3390/ijms16047273

Li WF, Zhou XX, Lu P (2005) Structural features of thermozymes. Biotechnol Adv 23:271–281. doi:10.1016/j.biotechadv.2005.01.002

Lin LL, Lo HF, Chiang WY, Hu HY, Hsu WH, Chang CT (2003) Replacement of methionine 208 in a truncated *Bacillus* sp. TS-23 α-amylase with oxidation-resistant leucine enhances its resistance to hydrogen peroxide. Curr Microbiol 46:211–216. doi:10.1007/s00284-002-3846-y

Lou FW, Liu BK, Wu Q, Lv DS, Lin XF (2008) *Candida antarctica* lipase B (CAL-B)-catalyzed carbon-sulfur bond addition and controllable selectivity in organic media. Adv Synth Catal 350:1959–1962. doi:10.1002/adsc.200800207

Milchert E, Malarczyk K, Kłos M (2015) Technological aspects of chemoenzymatic epoxidation of fatty acids, fatty acid esters and vegetable oils: a review. Molecules 20:21481–21493. doi:10.3390/molecules201219778

Miyazaki C, Takahashi H (2001) Engineering of the H_2O_2-binding pocket region of a recombinant manganese peroxidase to be resistant to H_2O_2. FEBS Lett 509:111–114

Moreira MA, Bitencourt TB, Graça M, Moreira MA, Bitencourt TB (2005) Optimization of chemo- enzymatic epoxidation of cyclohexene mediated by lipases. Synth Commun 35:2107–2114. doi:10.1081/SCC-200066705

Nieuwenhuizen TM (1949) Organic peracids. Chem Rev 45:1–68

Ogola HJO, Hashimoto N, Miyabe S, Ashida H, Ishikawa T, Shibata H, Sawa Y (2010) Enhancement of hydrogen peroxide stability of a novel *Anabaena* sp. DyP-type peroxidase by site-directed mutagenesis of methionine residues. Appl Microbiol Biotechnol 87:1727–1736. doi:10.1007/s00253-010-2603-6

Palm GJ, Fernández-Álvaro E, Bogdanović X, Bartsch S, Sczodrok J, Singh RK, Böttcher D, Atomi H, Bornscheuer UT, Hinrichs W (2011) The crystal structure of an esterase from the hyperthermophilic microorganism *Pyrobaculum calidifontis* VA1 explains its enantioselectivity. Appl Microbiol Biotechnol 91:1061–1072. doi:10.1007/s00253-011-3337-9

Rusch GKM, Steffens K, Patett N (2002) Biocatalytic peroxy acid formation for disinfection. J Mol Catal B Enzym 19–20:499–505. doi:10.1016/S1381-1177(02)00204-7

Ryan BJ, Ó'Fágáin C (2007) Effects of single mutations on the stability of horseradish peroxidase to hydrogen peroxide. Biochimie 89:1029–1032. doi:10.1016/j.biochi.2007.03.013

Sedmak JJ, Grossberg SE (1977) A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal Biochem 79:544–552. doi:10.1016/0003-2697(77)90428-6

Studier FW (2005) Protein production by auto-induction in high-density shaking cultures. Protein Expr Purif 41:207–234. doi:10.1016/j.pep.2005.01.016

Ullrich R, Dolge C, Kluge M, Hofrichter M (2008) Pyridine as novel substrate for regioselective oxygenation with aromatic peroxygenase from *Agrocybe aegerita*. FEBS Lett 582:4100–4106. doi:10.1016/j.febslet.2008.11.006

Villegas JA, Mauk AG, Vazquez-Duhalt R (2000) A cytochrome c variant resistant to heme degradation by hydrogen peroxide. Chem Biol 7:237–244. doi:10.1016/S1074-5521(00)00098-3

Wang X, Tang Q, Popowicz GM, Yang B, Wang Y (2015) A mechanistic study into the epoxidation of carboxylic acid and alkene in a mono, di-acylglycerol lipase. Biochem Biophys Res Commun 460:392–396. doi:10.1016/j.bbrc.2015.03.044

Zhou P, Wang X, Yang B, Wang Y (2017) Chemoenzymatic epoxidation of alkenes with *Candida antarctica* lipase B and hydrogen peroxide in deep eutectic solvents. RSC Adv 7:12518–12523. doi:10.1039/C7RA00805H