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Investigating the Structural and Functional Effects of Mutating Asn Glycosylation Sites of Horseradish Peroxidase to Asp

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Abstract Horseradish peroxidase (HRP) has long attracted intense research interest and is used in many biotechnological fields, including diagnostics, biosensors, and biocatalysis. Enhancement of HRP catalytic activity and/or stability would further increase its applications. One of the problems with heterologus expression of HRP especially in prokaryotic host is lack of glycosylation that affects it's stability toward H_2O_2 and thermal inactivation. In this study, two asparagine residues which constitute two of the eight glycosylation sites in native HRP (Asn 13 and 268) with respectively 83% and 65% surface accessibility were substituted with aspartic acid in recombinant HRP. Both mutant proteins expressed in *Escherichia coli* showed increased stabilities against heat (increase in $t_{1/2}$ from 20 min in native rHRP to 32 and 67 min in N13D and N268D) and H_2O_2 (up to threefold). Unexpectedly, despite the distance of the mutated positions from the active site, notable alterations in steady-state k_{cat} and K_m values occurred with phenol/4-aminoantipyrine as reducing substrate which might be due to conformational changes. No significant alteration in flexibility was detected by acrylamide quenching analyses, but ANS binding experiments purposed lesser binding of ANS to hydrophobic patches in mutated HRPs. Double mutation was non-additive and non-synergistic.

Keywords Recombinant horseradish peroxidase · Site-directed mutagenesis · Glycosylation site · Protein stability · H_2O_2 inactivation

Introduction

Horseradish peroxidase (HRP, EC 1.11.1.7) catalyzes the oxidation of a wide range of substrates by hydrogen peroxides. HRP isozyme C (HRP C) is probably the most

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extensively studied member of the plant peroxidase superfamily [1]. This 308-amino acid enzyme is glycosylated at eight asparagine sites; it also contains a ferric heme prosthetic group and two calcium ions per molecule [2–5].

HRP is widely used as a reporter in medical diagnostics, histochemical staining, and in biosensors [6]. In addition, HRP is applicable in chemical synthesis [7, 8] and removal of phenols and aromatic amines from waste waters [9]. The stability of HRP is critical for its applicability and is affected by a number of variables; one of them is glycosylation. Nine potential *N*-glycosylation sites from the motif of Asn- X-Ser/Thr (where 'X' represents an amino acid residue other than Asp and Gly) can be recognized in the primary sequence; among them, eight sites are occupied in native HRP. The glycans of HRP appear to belong to the flexible protein surface shielding type [10, 11]. The presence of glycans was shown to double the stability of HRP C toward H_2O_2 [12] and significantly affect its kinetic stability [11]. Lack of post-translational modifications in prokaryotic hosts such as *Escherichia coli* is the main problem in heterologous expression of such proteins.

Initial HRP mutagenesis studies commenced to identify the critical residues involved in HRP catalysis [13]. Little information is available regarding the effects of rational, site-directed amino acid substitution on the HRP stability toward critical inactivating factors such as temperature and hydrogen peroxide. Till now, the sole reports concerning genetic manipulation of HRP in order to improve its thermal and peroxide stability are performed by Ryan and Fagain [12, 14]. In this research, Asn 13 and Asn 268 residues of recombinant HRP substituted with Asp, and the effect of these substitutions on the activity and stability of the enzyme was investigated.

Materials and Method

Chemicals

All chemicals were of analytical-grade purity. H_2O_2, 4-aminoantipyrine, isopropyl-β-D-thiogalactopyranoside (IPTG), oxidized glutathione, dithiothreitol were purchased from Sigma Aldrich (St. Louis, MO, USA). Thermostable DNA-polymerase from *Pyrococcus furiosus* and long-template Taq enzyme mix with proofreading activities were from Stratagene (USA). Restriction endonucleases and T4 DNA ligase were obtained from Fermentas (Germany). Molecular biology kits were from Bioneer (Korea). All other chemicals were from Merck (Darmstadt, Germany).

Bacterial Strains and Plasmids

E. coli XL1-blue cells and *E. coli* BL21 (DE3) cells, pET26b(+) vector were purchased from Novagen (USA). The recombinant HRP gene was a generous gift from Prof. Frances H. Arnold (Caltech, CA, USA).

Cloning

The DNA fragment encoding HRP was cloned in the pET26b vector which is characterized by a strong T7 promoter, the pelB leader and a C-terminal 6×histidine tag. Appropriate restriction sites were introduced at 5′- and 3′- termini of the synthetic gene, respectively, by polymerase chain reaction (PCR) using *P. furiosus* polymerase, upstream primer 5′-CGCGGATCCGATGCAGTTAAC-3′ and downstream primer 5′-

CCCAAGCTTAGAGTTGCTGTTGAC-3′. The respective BamHI and HindIII restriction sites for cloning into the expression vector are underlined. The DNA fragment was purified by PCR purification kit. The purified fragment was digested with BamHI and HindIII and subsequently cloned in the expression vector that had been previously digested with the same enzymes. Sequence analysis confirmed the correctness of cloning procedure (Macrogen, Korea).

Recombinant DNA Techniques

All DNA manipulations were carried out by standard techniques. Site-directed mutagenesis was carried out with some modifications in the Quick-Change-method described by Fisher [15]. Mega primers produced with a simple PCR were utilized to amplify the whole gene. Mutant primers were supplied by Alpha DNA (Montreal, Canada). One of these primers was complement to the hrp gene's 5′ or 3′ termini and the other harbored the mutation inside the gene. This PCR product that did not exceed 200–250 bp was used as both forward and reverse primers in the second round of PCR to produce complete pET26b (+) with desired mutation. PCR products were incubated in a digestion reaction with DpnI restriction endonuclease at 37 °C for 12 h to exclude original template DNA. Cloned fragments were checked for the desired mutations by sequencing (Macrogen, Korea).

Protein Expression

Bacteria transformed with pET26b (+) carrying the hrp gene were grown with vigorous shaking at 37 °C in 25 mL Luria-Bertani (LB) medium containing 50 μg/mL kanamycin. An overnight culture was inoculated into fresh 300 mL LB broth medium (1% inoculation) containing kanamycin (50 μg/mL) and incubation at 37 °C was continued until it reached an OD_{600} of 0.5. IPTG was added to a final concentration of 0.5 mM to induce protein expression. Bacteria were further grown at 30 °C for 3 h and harvested by centrifugation (8,000×*g*, 15 min, 4 °C).

Purification of Periplasmic Fraction

E. coli cytoplasm is a reducing environment, preventing correct formation of disulfide bond and leading to formation of inclusion bodies upon expression of cysteine-rich proteins like HRP [16]; one solution to this problem is fusion of the protein with a signal peptide for secretion to the bacterial periplasm. After translocation of the expressed protein, the signal peptide is cleaved off and properly folded protein with disulfide bonds is formed in the oxidative environment of periplasm [17]. This approach has been successfully applied for rHRP [18]. pET26 b(+) also carries pelB leader sequence for periplasmic secretion of the expressed protein. Purification of periplasmic fraction was performed as described by French and coworkers [18]. Recombinant HRP–His was bound to Ni–NTA agarose (Amersham Biosciences) and eluted with 20 mM Tris, pH 7.9. 0.5 M NaCI, 2 M urea, containing 200 mM imidazole. Prior to determination of the activity, the peroxidase samples were gel filtrated on Sephadex G25 column (Sigma Aldrich, St. Louis, MO, USA).

Purification of Cytosolic Fraction and Refolding

Extraction of the cytosolic fraction of expressed HRP and its refolding were also done as described by Grigorenko and coworkers with some modifications [19]; 0.1 mM of oxidized

L-glutathione was used in the refolding buffer. Refolded apo HRP was loaded on Ni–NTA agarose column (Amersham Biosciences) that had been equilibrated before with 20 mM Tris–HCl buffer at pH 8.5 containing 1.7 M urea and 4% glycerol. Bound apo HRP was eluted with 200 mM imidazole in tris buffered saline (TBS) buffer (10 mM Tris–HCl, pH 7.4, 0.15 M NaCl) supplemented with 2 M urea. Purity of the apo protein was confirmed on SDS-PAGE (12.5% acrylamide) according to Laemmli method [20]. The gel was stained by Coomassie brilliant blue R-250 [21]. Fractions containing the apo HRP were collected. The apo HRP concentration was determined by the Bradford assay using bovine serum albumin as standard [22]. In order to obtain holo enzyme, 1 mM stock solution of hemin in 0.1 M KOH was added drop by drop to the collected fragment. The final concentration of hemin did not exceed two times the concentration of apo HRP [19]. Activity was measured against phenol at different time intervals after addition of hemin. When activity reached a plateau, the sample was passed through a column of Sephadex G25 (Sigma Aldrich, St. Louis, MO, USA) equilibrated with 200 mM potassium phosphate buffer at pH 7.0 to remove unbound hemin, salts, and urea.

Enzyme Activity and Biochemical Characterization

HRP concentrations were determined by spectrophotometer considering an extinction coefficient of 102 mM^{-1} cm^{-1} at 403 nm [23–26]. Horseradish peroxidase activity was measured spectrophotometrically at room temperature using phenol/4-aminoantipyrine and H_2O_2 as substrates. In the assay mixture, oxidation of phenol/4-aminoantipyrine (42.5 and 0.625 mM, respectively) in 200 mM potassium phosphate buffer (pH 7.0) in the presence of the desired concentrations of H_2O_2 was measured by the increase in absorbance at 510 nm (ε= 6.58 mM^{-1} cm^{-1}) [25]. All assays were performed in triplicates. The change in absorbance per minute (ΔA/min) was calculated for each substrate concentration. The data were fitted to generate a Michaelis–Menten equation, and apparent kinetic values were calculated.

pH Profile

In order to find the optimal pH, horseradish peroxidase activity was studied in 50 mM mixed buffer of glycine, Tris and di-pottasium hydrogen phosphate, within the pH range of 3–9 using phenol/4-aminoantipyrine and H_2O_2 as substrates.

Thermal Stability

Thermal stability was determined at 50 °C in 200 mM potassium phosphate buffer (pH 7.0) for 1 h. Samples were removed periodically and incubated on ice; residual activities were determined upon re-warming to room temperature. A constant protein concentration of 1.5×10^{-6} M was used for all thermal inactivations to diminish possible effects of protein concentration on stability.

H_2O_2 Stability

H_2O_2 stability of recombinant HRP and its variants was determined by the method described by Hiner and coworkers [27]. A plot of the remaining activity versus Peroxide Concentration was generated. The incubation period was 4 h at 4 °C; the concentration of rHRP was fixed at 7.5×10^{-5} M, and the H_2O_2 was varied between 0 and 11.25 mM. Fifty-microliter aliquots were withdrawn, and the remaining catalytic activity was assayed.

ANS Binding Experiments

1-Anilino naphthalene-8-sulfonate (ANS) binding studies were performed in a Perkin Elmer luminescence spectrometer LS 50B. The spectra were measured in potassium phosphate buffer (200 mM) at pH 7.0 and 25 °C. The final concentration of ANS in the enzyme solutions was 10 μM, and the molar ratio of protein to ANS was 1:70. The ANS emission was scanned between 400 and 600 nm with an excitation wavelength of 380 nm after incubation for 20 min in the dark.

Acrylamide Quenching

Fluorescence quenching experiments were carried out by addition of aliquots of acrylamide stock solution (1 M) to the protein solution (1 μg/mL) at pH 7.0. The final concentration of acrylamide was varied between 0 and 200 mM, and the incubation time was 5 min. The enzyme was excited at 293 and 280 nm, and the emission spectra were scanned between 300 and 500 nm. The decrease in fluorescence intensity at λ_{max} of emission was analyzed according to the Stern–Volmer equation, $F_0/F = 1 + K_{SV}[Q]$ in which, F_o and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher, respectively, K_{sv} is the Stern–Volmer constant, and [Q] is the molar concentration of the quencher [28].

Results and Discussion

HRP has eight asparagine-linked glycans at Asn 13, Asn 57, Asn 158, Asn 186, Asn 198, Asn 214, Asn 255, and Asn 268 [29, 30]. It is believed that glycans restrict fluctuations within the protein domain [6] and may also protect proteins from uncontrolled proteolysis [31] or cross-linking in the presence of free radicals [27]. The sole report to date about mutation of these glycosylation sites is by Lin and coworkers who encountered an Asn 255 to Asp mutation relieved to produce more properly folded enzyme in an effort to develop functional expression of HRP in *E. coli* by directed evolution [32].

Online ASA view software was used to analyze surface accessibility of these nine residues. Asn 13 and Asn 268 with 83% and 65% surface accessibility were chosen for site-directed mutation analysis. The first candidate for these residues was aspartic acid. Asp may impel more rigidity in loop structures; it also prevents deamidation and therefore affecting protein stability. In addition, the negative charge of aspartic acid's side chain may somehow resemble the polarity of glycans and impede oxidation in the presence of reactive oxygen species generated during H_2O_2 catalysis. Asp substitution also maintain space-fill characteristic of the native residue [12]. These entire hypotheses were approved by I-Mutant and Eris softwares [33].

Both periplasmic and cytosolic fractions were extracted, and the enzyme was purified. Properly folded active enzyme was gained from periplasmic space, however, cytosolic fraction was selected for enzyme characterization, in the sake of attaining more active enzyme after refolding procedure. It was interesting that most of the expressed proteins in both mutants have been aggregated into inclusion bodies in comparison with native enzyme that mostly was secreted to the periplasmic region (data not shown). This observation controverts with what Lin and coworkers observed about N255D mutation that significantly affected refolding of recombinant enzyme [32]. Both mutants and native recombinant enzyme showed similar behavior in reconstitution with hemin, and their activities reached to a plateau in 2 h after hemin addition.

Kinetic parameters of plant HRP, rHRP, N13D, and N268D mutants were compared at pH 7.0 and 25 °C using phenol/4-aminoantipyrine as reducing substrates (Table 1). rHRP gave k_{cat} and K_m values of 38 s^{-1} and 0.42 mM, respectively. These parameters were 800 s^{-1} and 0.41 mM for plant HRP. Both mutants showed decreased values for K_m parameter up to 90% for N268D and 75% for N13D. Effects on k_{cat} are also remarkable, with an increase noted for both mutations (2.5-fold, N13D; and 2.6-fold, N268D). Substitution of uncharged polar side chain in Asn with charged polar side chain in Asp despite of their distance from the active site enhanced HRP's reactivity through increased k_{cat}'s and decreased K_m values (Table 1). Increase in catalytic efficiencies of mutant HRP with N268D substitution is comparable with plant HRPs. Asn 13 is located 24 Å far from the active site in the distal side of the enzyme immediately before helix A. Distance of Asn 268, the last residue of the helix I, from the active site is 14 Å and its position is directly opposite to the active site entrance channel in the proximal side of the enzyme (Fig. 1). There are few reports with improved kinetic properties of HRP by means of single mutations, for example, an increased k_{cat} of the enzyme ratio for 2,2′-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS) resulted from a single S35K substitution [34], a decreased K_m value for ABTS was obtained from a combination of five mutations [35] and an increased reactivity toward ABTS in E238Q and E239Q substitution was achieved [14]. An N70D substitution also decreased guaiacol oxidation rates [36]. In another research, a variant of HRP with enhanced enantioselectivity for L- or D-tyrosinol was introduced through yeast cell surface display [37].

An observation in this study concerning kinetic characterization of recombinant and mutated HRPs was the increase in k_{cat} value of enzymes after incubation in −20 °C and centrifugation. It seems that misfolded or unfolded polypeptide chains of newly refolded and purified HRP form aggregates that could be simply collected and removed by centrifugation. This phenomenon decreased apparent enzyme concentration (increased RZ value, i.e., A_{404}/A_{280}) and subsequently enhanced k_{cat} value till it reached a plateau; thereafter, these constant k_{cat} values were used for comparison of recombinant and mutant enzymes.

pH profile of mutants did not show any significant difference compared with native recombinant enzyme complying with wide pH range in native enzyme. Remaining activities of native recombinant and mutant HRPs in different H_2O_2 concentrations are shown in Fig. 2. Both mutants showed improvement in C_{50} value for H_2O_2 tolerance (see Table 1, twofold for N13D and 2.8-fold for N268D).

In the presence of excess H_2O_2 and without a reducing substrate, HRP undergoes H_2O_2-mediated suicide inactivation. A three-pathway model proposed by Hernández-Ruiz and coworkers has gained support to explain the inactivation mechanism. According to this

Table 1 Biochemical characteristics of recombinant HRP variants with substitutions in Asn-13 and Asn-268

Mutant	Phenol/4-aminoantipyrine steady-state kinetics			Thermal stability	H_2O_2 stability
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})	$T_{1/2}$ (min)	C_{50} (mM)
Recombinant HRP	38±2	0.420±0.02	90	20±1	1.87±0.10
N13D	94±5	0.100±0.005	940	32±2	3.75±0.19
N268D	100±5	0.050±0.003	2,000	67±3	5.25±0.25
Plant HRP	800±30	0.410±0.02	2,000	NC	NC

NC not calculated

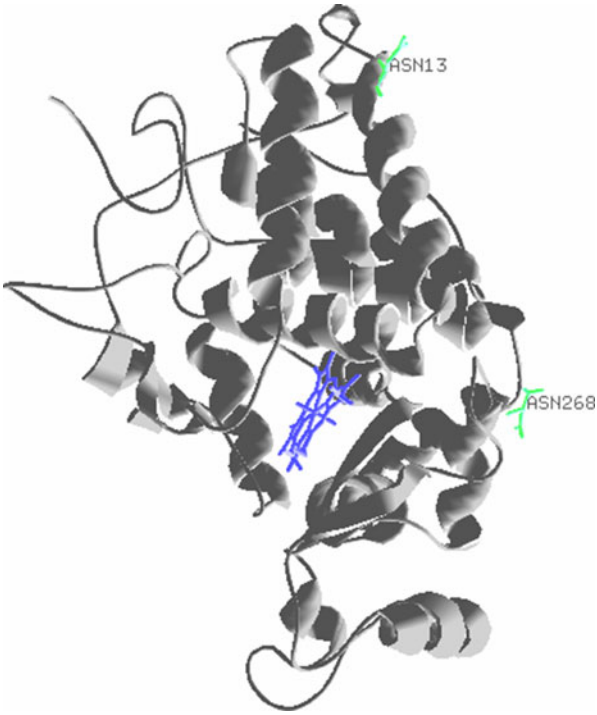

Fig. 1 Three-dimensional structure of HRP. HRP structure showing the location of the Asn-13 and Asn-268 to Asp mutations in the surface loops of HRP (PDB code: 1H55)

model, catalytic competition exists in the presence of excess H_2O_2, which may lead into one of the three possible outcomes as follows: (a) formation of the dead-end compound III, (b) a catalase-type reaction, and (c) complete inactivation. The first and second routes will result in enzyme survival [38]. However, in the long-term presence of excess H_2O_2, the enzyme gradually inactivates, and inactive verdohemochrome P670 will accumulate in the reaction mixture [39].

Thermal stabilities of plant HRP and recombinant, and mutant HRP variants at 50 °C are shown in Fig. 3; thermal stability of N13D showed a slight improvement over recombinant enzyme, but it was shown that N268D is more stable than the rHRP. Thermal activation was

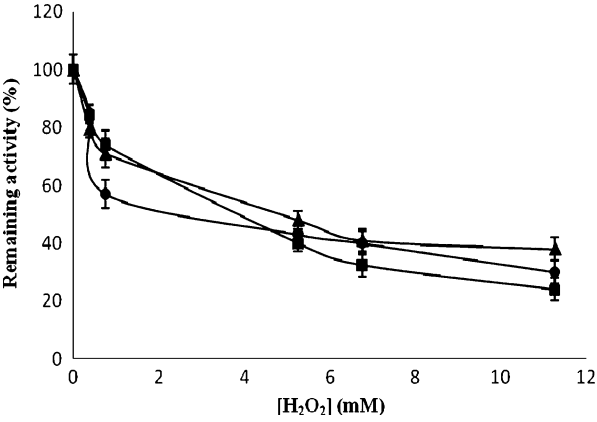

Fig. 2 H_2O_2 stability. Plot of percent remaining activity versus hydrogen peroxide concentration for rHRP and mutant variants over 4 h at 4 °C. (*filled circle*: rHRP; *filled square*: N13D; *filled triangle*: N268D)

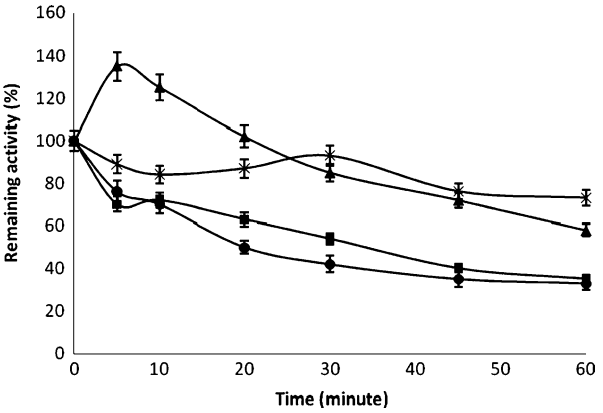

Fig. 3 Thermal stability. Thermostability of plant, recombinant, and mutant HRPs at 50 °C in potassium phosphate buffer (200 mM, pH 7.0). (*multiplication symbol*: plant HRP; *filled circle*: rHRP; *filled square*: N13D; *filled triangle*: N268D)

also observed in N268D mutant; it could be assumed that some conformational changes are induced in enzyme structure upon incubation at 50 °C that increases the enzyme activity. This phenomenon is also observed in some other enzymes such as laccases [40, 41]. In accordance with the model proposed by Klibanov for two-step thermoinactivation of proteins [42, 43], deamidation of asparagine is particularly important in the irreversible conversion of the unfolded enzyme to totally denatured inactive form. It is generally believed that the deamidation process which occurs through nucleophilic attack on the side chain carboxyl carbon of asparagines by the backbone nitrogen of the adjacent amino group will result in the formation of a five-membered succinimide intermediate [44–46]. Plots of the log of residual activity versus time were linear for native, rHRP, and N13D, indicating a first-order decay process under these conditions. N268D mutation did not assent with a first-order decay. Further experiments are needed to resolve thermal inactivation mechanism in this mutant enzyme.

Acrylamide quenching analyses were done; comparison of Stern–Vormer plots did not show any significant changes in the flexibilities of native recombinant and mutant enzymes (data not shown). ANS binding analysis showed decreased ANS emissions resulting from diminished binding of ANS to hydrophobic patches (Fig. 4). ANS is mainly non-fluorescent in aqueous solution, but its emission intensity increases in hydrophobic environments [47]. The negative charge of Asp may resist with the binding of ANS to neighboring hydrophobic sites.

In summary, both steady-state kinetic values and stability parameters improved in N13D and N268D mutations, but this improvement was particularly notable for N268D. This observation may be attributed to the Asn 268's extra surface accessibility and also its

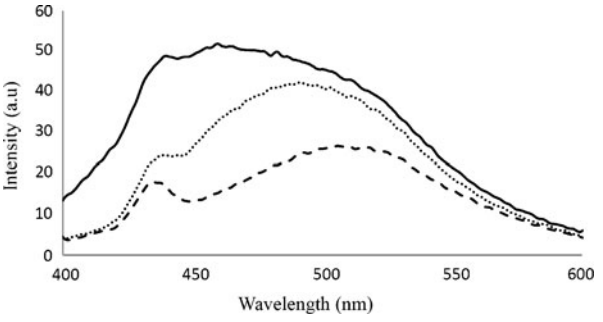

Fig. 4 ANS binding spectra. Fluorescence spectra of 10 μM ANS in the presence of recombinant and mutant HRPs in potassium phosphate buffer (200 mM, pH 7.0). The ANS emission was scanned between 400 and 600 nm with an excitation wavelength of 380 nm (*solid line*: rHRP; *broken line*: N268D; *dashed line*: N268D)

shorter distance from active site. N268D substitution may decrease the flexibility of the loop between helix I and J in the proximal side of the enzyme behind the substrate binding pocket that was not detectable by acrylamide fluorescence quenching analyses. This reduced flexibility could stabilize the binding between substrate and active site residues, thus increasing catalytic efficiency of the enzyme. Modest gains in the thermal and H_2O_2 stabilities presumed by improved hydrogen bonding prohibited deamidation and also lowered enzyme flexibility. The negative charge of Asp side chains may also hamper oxidation of oxygen reactive species during H_2O_2 inactivation process.

Double mutation enhanced enzyme stability toward H_2O_2, but no considerable improvement in thermal stability was gained. A severe decrease in the catalytic efficiency was also observed (data not shown).

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