Construction and Characterization of *Vitreoscilla* Hemoglobin (VHb) with Enhanced Peroxidase Activity for Efficient Degradation of Textile Dye

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Pollution resulting from the discharge of textile dyes into water systems has become a major global concern. Because peroxidases are known for their ability to decolorize and detoxify textile dyes, the peroxidase activity of *Vitreoscilla* hemoglobin (VHb) has recently been studied. It is found that VHb and variants of this enzyme show great promise for enzymatic decolorization of dyes and may play a role in achieving their successful removal from industrial wastewater. The level of VHb peroxidase activity correlates with two amino acid residues present within the conserved distal pocket, at positions 53 and 54. In this work, site-directed mutagenesis of these residues was performed and resulted in improved VHb peroxidase activity. The double mutant, Q53H/P54C, shows the highest dye decolorization and removal efficiency, with 70% removal efficiency within 5 min. UV spectral studies of Q53H/P54C reveals a more compact structure and an altered porphyrin environment (\(\lambda_{\text{Soret}} = 413\) nm) relative to that of wild-type VHb (\(\lambda_{\text{Soret}} = 406\)), and differential scanning calorimetry data indicate that the VHb variant protein structure is more stable. In addition, circular dichroism spectroscopic studies indicate that this variant’s increased protein structural stability is due to an increase in helical structure, as deduced from the melting temperature, which is higher than 90°C. Therefore, the VHb variant Q53H/P54C shows promise as an excellent peroxidase, with excellent dye decolorization activity and a more stable structure than wild-type VHb under high-temperature conditions.

**Keywords:** *Vitreoscilla* hemoglobin, peroxidase activity, dye, decolorization

**Introduction**

Wastewater from the textile industry is considered to be one of the largest sources of pollution among all industrial sectors, requiring appropriate treatment technologies [8]. There is a great need to develop an effective way of dealing with textile dye effluents [35]. Biodegradation appears to be a promising technology; however, analyses of contaminated soil and water have shown that toxic dye pollutants persist, even in the presence of microorganisms that are completely capable of mineralizing the pollutants [16, 35]. Therefore, enzymatic treatment of synthetic dyes should be used in conjunction with conventional treatments [23, 28, 30, 31, 38, 39, 44, 50], and peroxidases have already shown potential for efficient decolorization of industrial dye solutions and textile effluents [14, 19, 27, 45].

Peroxidases are heme-containing proteins that generally contain a metal, such as copper or iron, and use H\(_2\)O\(_2\) or related compounds to catalyze the conversion of a variety of organics and inorganics [7]. In recent years, peroxidases...
have been used to purify industrial wastewater containing toxic organic compounds such as phenols and aromatic amines. In a previous study, peroxidase degraded industrial wastewater dyes with high efficiency [2]. Peroxidase has also been shown to remove phenols from coal mine wastewater, casting wastewater, and bleaching wastewater [6]. Meanwhile, another peroxidase has been applied to effect polyaniline polymerization [24].

Vitreoscilla hemoglobin (VHb) is a type of oxygen-binding protein that contains two homologous oxygen-binding subunits (16 kDa per subunit), each containing a single porphyrin b cofactor. VHb has been thought to maintain an essential level of intracellular, dissolved oxygen [47]. As both an oxygen carrier and storage protein, VHb enables Vitreoscilla and other obligate aerobic bacteria to live under hypoxic conditions by increasing cell density, accelerating oxidative metabolism, and facilitating engineered product formation [33]. Thus, VHb engineering technology has been applied to the industrial-scale production of polyhydroxyalkanoates and antibiotics [49, 51]. VHb has also become a versatile tool in current biotechnological applications, especially for large-scale fermentation, since heterologous expression of VHb during cultivation can enhance cell growth and productivity under oxygen-depleted conditions [9, 17, 18]. In addition to its role in O$_2$ delivery, VHb has been involved in various cellular activities, such as protein translation efficiency [1, 36], cellular metabolism [12], and alleviation of nitrosative stress [10, 15].

Recently, the peroxidase activity of VHb has been elucidated [13, 20, 21, 42] and this activity endows VHb with additional industrial applicability. However, before VHb can be efficiently employed as a peroxidase, alterations of its active site are needed to improve its activity and stability. The protein sequence of horseradish peroxidase (HRP), a commonly used peroxidase exhibiting very high activity, contains a catalytically important distal histidine (E7) that is missing in VHb, possibly accounting for the lower observed VHb peroxidase activity. Therefore, in this work, site-directed mutagenesis of VHb residues at Q53 (E7) and/or P54 (E8) were performed. VHb variants possessing distal histidine and cysteine residues were constructed and the peroxidase kinetics were determined in an assay reaction using H$_2$O$_2$ and an aromatic substrate.

Materials and Methods

Materials

Materials used for creation and cloning of site-directed mutants included pUC19 plasmid (TaKaRa, Japan), pfu polymerase (Fermentas, ThermoFisher Scientific, Inc., Grand Island, NY, USA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) markers (Promega, Madison, WI, USA), and P-60 Bio-Gel polyacrylamide beads and DEAE Support (Bio-Rad Hercules, CA, USA). Methylene blue and 30% H$_2$O$_2$ (v/v) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All aqueous solutions were prepared using water purified using a Milli-Q system (Millipore, Bedford, MA, USA). Other reagents were of analytical grade and commercially available. HRP was obtained from Beijing Solarbio Science & Technology Co., Ltd., China (P8020, RZ 3.0).

VHb Mutation Selection

In order to investigate the ability of VHb to function as a peroxidase, VHb variants were generated via site-directed mutagenesis (Q53H, P54C, and Q53H/P54C) in which two amino acids within the distal pocket of VHb, 53 and/or 54, were substituted with histidine and/or cysteine, respectively. The VHb gene was amplified using primers containing a single mutation. After PCR, template DNA was completely digested by addition of the DpnI restriction enzyme to the PCR product (37°C for 2 h).

Construction of Expression Vectors and Protein Expression

DNA cloned from wild-type VHb and its variants were transformed into Escherichia coli (E. coli) BL21 (DE3) competent cells and cultured overnight at 37°C on Luria-Bertani (LB) medium plates supplemented with 100 µg/ml ampicillin (Amp). Verification of the correct mutation in each plasmid was carried out using DNA sequencing.

E. coli BL21 (DE3) clones containing DNA sequences of wild-type VHb or its variants (P54C, Q53H, P54C/Q53H) were grown in LB medium with 100 µg/ml ampicillin (Amp) and shaken in an aerobic environment at 37°C, 170 ×g, in order to cultivate the bacteria. Once the cell density (OD$_{600}$) reached 0.6–0.8, recombinant VHb proteins were actively produced because of gene induction due to the anaerobic environment. The bacteria expressing recombinant VHb proteins were cultivated at 25°C and 120 ×g for ~36 h.

Protein Purification

Protein purification was carried out according to the methods proposed by Rinas et al. [34] and Webster and Liu [48]. Cells were collected, centrifuged, washed with phosphate-buffered saline (PBS) (0.02 M, pH = 7.5), and sonicated for 40 min (3 sec on, 2 sec off). After removal of debris by centrifugation at 12,000 ×g for 30 min, the crude extract was fractionated using ammonium sulfate. The soluble fraction at a saturation of 45% was separated from the insoluble material by centrifugation and was precipitated at a saturation of 65%. The pellet was resuspended in PBS (0.02 M, pH 7.5) and dialyzed for 2 days against the same buffer. The solution was then centrifuged for 60 min at 12,000 ×g and the precipitate was discarded. The supernatant was collected, loaded...
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on a DEAE column (Bio-Rad), and eluted using a linear gradient of 0–0.5 M NaCl. A UV-2550 detection system (Shimadzu Corp., Japan) was employed to determine the “Reinheitszahl” (Rz) of the sample, in order to determine whether the purity met necessary requirements.

The samples were collected and loaded onto a gel filtration column (Bio-Gel P-60). The UV-2550 detection system was used to determine the Rz of the sample, and the fraction with Rz > 3 was collected. Protein concentrations were determined using the Bradford dye-binding assay with bovine serum albumin as a standard. The molecular mass of the protein was determined using SDS-PAGE.

Methylene Blue Enzymatic Degradation

The ability of VHb to decolorize aqueous solutions of methylene blue was initially investigated using the following conditions: dye concentration of 30 mg/l, hydrogen peroxide concentration of 7.5 mM, pH 7.5, and variable temperatures ranging from 25°C to 75°C. Solutions were prepared in phosphate buffer (0.02 M, pH 7.5), and the decolorization isotherms were recorded at 664 nm for VHb, Q53H, P54C, and Q53H/P54C, each at a concentration of 10 µM. The concentration of methylene blue was determined using UV-visible (UV-Vis) spectroscopy at 664 nm (ε664 nm = 9.5 × 10^4 M^-1 cm^-1) [4]. The initial rate of decolorization was also investigated as a function of the temperature (25°C, 35°C, 45°C, 55°C, 65°C, and 75°C). All UV-Vis measurements were obtained using a UV-2550 detector.

UV-Vis Absorption Spectrum

UV-Vis absorption spectra of VHb, and the Q53H, P54C, and Q53H/P54C variants were recorded at 25°C on a UV-2550 detector (350–700 nm). PBS (pH 7.5, 0.02 M) was used as the background control.

Differential Scanning Calorimetry Analysis

High-sensitivity differential scanning calorimetry (DSC) was carried out using a differential scanning microcalorimeter (VP-DSC; Microcal, LLC, Northampton, MA, USA) with 10 µM VHb, Q53H, P54C, Q53H/P54C, or HRP in PBS buffer. The calorimetry cells were pressurized and heated from 20°C to 100°C at a rate of 90°C/h. DSC data were analyzed to determine the transition temperature (Tm) using the software package Origin 7.0 (OriginLab, Northampton, MA USA).

Results and Discussion

Protein Molecular Mass and Purity

Three VHb mutants (Q53H, P54C, and Q53H/P54C) were constructed using the wild-type VHb DNA as a template. After cloning, expression in E. coli, and protein purification using a DEAE column and gel filtration column (P-60), the wild-type VHb and its variants showed a single band at ~16 kDa (see Fig. S1).

Treatment of Textile Dye by Wild-Type VHb

Methylene blue is a potent cationic dye that is commonly used in the textile industry. The UV-Vis spectrum showed the absorption peak of methylene blue to be 664 nm (Fig. 1). Dye decolorization after peroxidase treatment results in reduced UV absorption [45]. The absorption peak was reduced after decolorization by VHb after a contact time of 60 min, while the signal at 413 nm, representing the heme absorption peak of VHb-H₂O₂, increased. Therefore, VHb is able to completely decolorize methylene blue in the presence of H₂O₂.

The removal efficiency of methylene blue was determined using VHb and H₂O₂ at 25°C. The efficiency of decolorization as a function of contact time with the enzyme is shown in Fig. 2. Different reaction times were observed for the methylene blue dye; the degree of decolorization was 66% after 60 min of contact with VHb. However, within 10 min of contact with VHb, the degree of decolorization almost reached 40%. We can conclude that the enzymatic reaction of VHb is rapid, with most of the color being

Fig. 1. UV-VIS spectra of methylene blue before and after decolorization with 10 µM VHb in 0.02 M PBS buffer.

Fig. 2. Effect of the reaction time on decolorization of the dye with 10 µM VHb.
removed within the first 10 min of contact with the enzyme. In similar studies using wild-type VHb peroxidase, Bhunia et al. [2] showed that most of the degradation of the dye occurs within 3 h [2].

**Dye Degradation of VHb Variants**

The objective of this study was to achieve the maximum degree of dye degradation for a given amount of enzyme. Therefore, an active site containing histidine and cysteine residues, characteristic of commonly used peroxidases, was created within the distal pocket of VHb at positions 53 and 54, respectively, to create three variants of VHb (Q53H, P54C, and Q53H/P54C). Neither single mutant of VHb exhibited any appreciable improvement in dye degradation activity. The decolorization efficiencies of Q53H and P54C single mutants were 65% and 68%, respectively (Fig. 3). However, the Q53H/P54C double mutant showed the strongest dye degradation ability and the highest catalytic efficiency; within 5 min, the degree of methylene blue decolorization by mutant Q53H/P54C reached 70%.

In wild-type VHb, position 53 is occupied by a glutamine residue, which points to the heme pocket [43]. The hydrophilic nature of residues lining the distal site has been verified as being crucial for the interaction with polar substrates such as H$_2$O$_2$ for all classes of peroxidase enzymes [46]. The distal histidine is believed to be a catalyst by first acting as a base to withdraw an electron from the substrate, H$_2$O$_2$, and then functioning as an acid to donate a hydrogen atom to heme-bound peroxide [26, 46], facilitating the cleavage of the peroxide bond to allow the release of a water molecule. The cysteine side chain has been shown to participate in the hydrophobic bonding system of micelles as a result of recent studies describing the hydrophobic nature of the cysteine side chain in proteins [11, 29]. It is now known that the cysteine thiol side chain often participates in enzymatic reactions by serving as a nucleophile. The thiol group is susceptible to oxidation to create the disulfide derivative of cysteine, which has as an important structural role in many proteins. Thus, the VHb variant of Q53/P54, in which glutamine and proline have been substituted by histidine and cysteine, respectively, showed a significant increase in catalytic ability.

**Effect of reaction parameters on removal efficiency**

The pH, temperature, and H$_2$O$_2$ concentration are three critical factors that may affect the performance of peroxidase. Thus, these factors were surveyed for obtaining the optimal reaction conditions in which the dye would be removed to the maximum extent.

Temperature often influences an enzyme’s catalytic activity. The effect of temperature on the decolorization of the dye mediated by HRP, VHb, and VHb variants was studied at different temperatures ranging from 25°C to 75°C (Fig. 4). The results show that the decolorization of methylene blue was not affected by variation in temperature when using VHb or VHb variants as catalysts. The Q53H/P54C variant still exhibited a very high decolorization activity at high temperatures (75°C, 89%). In contrast, the removal efficiency of the dye mediated by HRP continuously decreased with the increase of temperature ranging from 25°C to 75°C. A previous report showed a 50% reduction in the decolorization efficiency of the dyes bromophenol and methyl orange by HRP at 40°C [23]. This behavior can be related to the loss of HRP enzymatic activity at high temperatures, which was also observed in other studies.

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**Fig. 3.** Decolorization of the dye with 10 µM VHb, Q53H, P54C, and Q53H/P54C in 0.02 M PBS buffer.

**Fig. 4.** Influence of temperature on the decolorization of textile dyes.
While VHb and its variants demonstrate good thermal stability, as shown by their abilities to decolorize dye at high temperatures. Therefore, VHb and its variants should be especially useful for dye decolorization in the textile industry, where high temperatures may be present. Moreover, the effects of pH and H$_2$O$_2$ concentration on removal efficiency were measured. The results affirm that VHb and its variants possess better pH stability and H$_2$O$_2$-resistance compared with HRP (see Figs. S2 and S3). On the basis of these results, the optimal reaction conditions for the degradation of the dye were determined.

**Comparison of Activities of HRP, VHb, and VHb Variants**

The activities of HRP, VHb, and VHb variants were compared under optimal reaction conditions to evaluate their performance for the degradation of the dye.

As can be seen in Table 1, the VHb variant Q53H/P54C showed a 7.9-fold increase in activity as compared with HRP. Although dyes often act as a strong competitive inhibitor of HRP at neutral pH [2, 5], our experimental data suggest that VHb (Q53H/P54C) can overcome this competitive inhibition to some extent, and thus exhibits enhanced activity as compared with HRP. Moreover, the activity of the VHb Q53H/P54C variant was 1.4 times higher than that of VHb, and the Q53H/P54C variant exhibited an increase in activity of 147% as compared with VHb (Q53H). The activity of VHb (Q53H/ P54C) was enhanced 1.3-fold as compared with VHb (P54C).

**UV-Vis Absorption Spectrum Properties**

To investigate the spectral properties of VHb proteins in

![Fig. 5. UV-Vis absorption spectra of VHb and VHb variants.](image)

The ferrous or reduced form was obtained following addition of excess dithionite to ferric-VHb. The absorption spectra in the visible region starting at 450–650 nm were magnified. (A) VHb; (B) Q53H; (C) P54C; (D) Q53H/P54C.
ferric and ferrous states, sodium dithionite and ammonium persulfate were added, respectively, in excess to the hemoglobin solutions prior to acquiring their spectra. The Soret peak of untreated wild-type VHb appears at 406 nm (Fig. 5A) and additional characteristic peaks were detected at 540 and 577 nm. These peak positions are similar to the oxy-form peak, but the VHb variants lack these typical peaks at 540 and 577 nm, known as α-bands [22]. UV–Vis spectroscopy of the VHb variants revealed some unusual properties for these mutant proteins. Variant Q53H exhibited a Soret maximum at 413 nm (Fig. 5B), the usual position for the oxy-form; the spectrum lacked characteristic bands typical for oxy-forms at 540 and 577 nm, but instead exhibited broad bands around 536 and 560 nm. P54C exhibited its Soret peak at 394 (Fig. 5C), with additional characteristic bands at 500 and 530 nm. The Soret peak of Q53H/P54C coincided with that of Q53H (413 nm) and exhibited the same characteristic bands at 536 and 563 nm (Fig. 5D). VHb and its variants each showed broad minor peaks at 536–540 nm and 577 nm (Figs. 5A–5D), which are characteristic bands of a typical six-coordinate porphyrin absorption spectrum [40]. Moreover, these findings indicate that all protein samples were present in the ferric form. VHb proteins exposed to excess dithionite produced a peak at 430 nm for wild-type VHb and the Q53H/P54C variant, a peak at 428 nm for the Q53H variant, and a peak at 433 nm for the P54C variant. Soret peaks of all proteins were slightly shifted in the ferrous states (432 nm) and slightly different peaks in α-bands for each protein were observed (Figs. 5A–5D).

**Fig. 6.** Differential scanning calorimetry analysis with 10 µM VHb and VHb variants in 0.02 M PBS buffer. (A) VHb; (B) Q53H; (C) P54C; (D) Q53H/P54C, (E) HRP.
where a peak could be seen at 555–560 nm. Replacement of glutamine with a histidine residue caused a substantial spectral shift in both ferric and ferrous states, to 413 nm and 428 nm, respectively, for the Q53H and Q53H/P54C variants (Figs. 5B and 5D).

Substitution of the distal histidine with an aliphatic residue such as leucine in myoglobin has been demonstrated to shift the absorption spectra resulting from the loss of the stabilizing residue to the heme-bound water molecule [26]. In order to assess the oxidation state of the heme metal in the VHb proteins, the CN-bound form was generated by addition of potassium cyanide to the VHb and VHb variant solutions. The Soret peaks of Q53H and Q53H/P54C appeared at 415 nm, while the α-band absorption peaks remained at 540 nm. Studies by Bolognesi et al. [3] have shown that binding of cyanide to ferric VHb is independent of the distal heme pocket. In general, the reactivity of cyanide towards hemoproteins is influenced mainly by the presence of proton acceptor groups in the heme pocket, which are believed to assist in the deprotonation of the incoming ligand. In fact, cyanide dissociation of ligated ferric VHb is affected by proton donor group(s) catalyzing the protonation of the outgoing ligand [43].

**Thermal Denaturation Analysis by DSC**

To study their structural stabilities, the thermal denaturation of VHb, Q53H, P54C, and Q53H/P54C were investigated using differential scanning calorimetry. It is well known that DSC is a sensitive and useful tool for studying the unfolding of proteins and gaining information about protein folding and stability [32, 41], as well as investigating the conformational changes of mutated proteins. Fig. 6 shows that the melting temperature was greater than 90°C (90.3°C and 92.3°C, Figs. 6B and 6D, respectively) for the VHb variants Q53H and Q53H/P54C. The melting temperatures of wild-type VHb and variant P54C were almost as high, 88.04°C and 89.5°C (Figs. 6A and 6C, respectively). HRP exhibited the lowest melting temperature (80.5°C, Fig. 6E), when compared with VHb and its variants. Moreover, these results conform to the results for HRP, VHb, and VHb variants in the thermal stability assay. Using circular dichroism (CD) and fluorescence spectra, we also verified that VHb variant Q53H/P54C has a compact structure, which can help to improve its thermal stability (see Supplementary Figs. S4 and S5). Replacement of Q53 with a histidine residue caused an increase in the helical content (CD data), moving histidine into an orientation where it faces the heme surface. Accordingly, this structural change likely also makes porphyrin-surrounding structures more proximal to the reaction site.

In conclusion, our experimental data showed that the activity of VHb is 5.7-fold higher than that of HRP. To improve the decolorization ability of VHb, two catalytically conserved residues were introduced into the distal pocket of VHb. As a result, the resulting VHb variant Q53H/P54C exhibited a 1.4-fold increase in activity as compared with wild-type VHb. Moreover, VHb variant Q53H/P54C possessed better thermal stability, pH stability, and H₂O₂-resistance compared with wild-type HRP. DSC analysis demonstrated that the melting temperature of VHb (Q53H/P54C) is higher than wild-type HRP, wild-type VHb, and the other VHb variants. UV-Vis, circular dichroism, and fluorescence spectra also verified that VHb variant Q53H/P54C has a compact structure. Thus, the VHb variant Q53H/P54C shows great promise for its applicability as a dye decolorization enzyme. This VHb variant can soon play a role as part of a larger pollution removal strategy for use by the textile industry.

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**References**


