1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

![Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showing purified proteins of all Vitreoscilla hemoglobin (VHb) variants.](image1)

The spacer gel was 5%, the separation gel was 12%. M, protein marker; lane 1, wild-type VHb; lane 2, Q53H; lane 3, P54C; lane 4, Q53H/P54C.

2. The effect of pH on removal efficiency

The effect of pH on the removal efficiency was evaluated. The reactions were carried out in different buffers (pH 2.5~10.5) containing five different enzymes (10 µM), methylene blue (30 mg/ml) and an appropriate amount of H₂O₂ at 25°C for one hour. For HRP, 1.5 mM of H₂O₂ was used. For VHb and VHb variants, 7.5 mM of H₂O₂ was employed. The buffers used were Na₂HPO₄-citric acid (pH 2.5~7.5) and Gly-NaOH (pH 8.5~10.5), respectively.

As shown in Fig. S2, when VHb variants and wild-type VHb act as catalysts, the highest removal efficiencies appear at pH 7.5, and the removal efficiencies decrease under both acidic and alkaline conditions. In contrast, the removal efficiency of the dye mediated by HRP significantly decreases with the increase of pH (2.5~10.5), which is agreement with Wangikar’s viewpoint that the dye is stronger competitive inhibitor of HRP at higher pH values [1]. The experimental results demonstrate that VHb variants and wild-type VHb are more stable than HRP between pH 2.5~10.5.

3. The effect of H₂O₂ concentration on removal efficiency

The effects of H₂O₂ concentration on removal efficiencies were studied. The reactions were carried out in buffer containing HRP (10 µM), methylene blue (30 mg/ml) and H₂O₂ (1.5~15.5 µM) at 25°C for one hour. For HRP, Na₂HPO₄-citric acid buffer (0.2 M, pH 2.5) was used. For VHb and VHb variants, Na₂HPO₄-citric acid buffer (0.2 M, pH 7.5) was employed.

The experimental results show that, the removal efficiency of methylene blue remains constant with the increasing H₂O₂ concentrations ranging from 1.5 mM to 7.5 mM when VHb and VHb variants are employed as catalyst, and then sharply decreasing with the further increase of H₂O₂.
H₂O₂ concentrations (9.5~15.5 mM) (Fig. S3). The removal efficiency of the dye mediated by HRP continuously decreases with the increasing H₂O₂ concentrations ranging from 1.5 µM to 15.5 µM. The experiment results indicate that the H₂O₂-resistance of VHb and VHb variants is superior to that of HRP when H₂O₂ concentration changes from 1.5 mM to 7.5 mM.

4. Structure analysis of VHb mutants

Circular dichroism measurements were as follows: samples used for circular dichroism spectrum (CD) experiments were prepared at a concentration of VHb Q53H, P54C, and Q53H/P54C of 5 µM in phosphate buffer (0.02 M) and pH 7.5. All CD experiments were performed at 25 °C on a J-810 spectropolarimeter (JASCO, Japan) with a 1 mm cuvette. Spectra in the far-UV region (190–240 nm) were recorded after averaging 3 consecutive scans.

Circular dichroism (CD) spectra revealed the different secondary structure of VHb and its variants and the structural differences resulted in changes in the enzyme activity. The minima at 208 and 222 nm and a maximum at 193 nm represent helical structures; the absolute values of these points are, therefore, associated with the percentage of helical residues in the protein. In the natural state, VHb has high helical polypeptide chains to form a 6 helical bundle (D folded state is unknown) and the rest of the residues are in coil states. Fig. S4 shows that the P54C variant has the same helical structured compared with wild-type VHb and only slightly decreased absolute values at 193, 208, and 222 nm in the spectra. The absolute value of these 3 points increased in the variants Q53H and Q53H/ P54C compared with wild-type VHb and the protein turns into a higher helical state. Replacing glutamine at position 53 with histidine rendered the amino acid facing towards the heme surface [4, 6]. This change increased the helical structure of variants Q53H and Q53H/P54C.

5. Fluorescence spectrum analysis of the tertiary structure

Fluorescence spectroscopy measurements were as follows: the fluorescence emission spectra of 5 µM VHb Q53H, P54C, and Q53H/P54C in 0.02 M phosphate buffer of different pH (6.0–8.0) were collected by using a RF-5301PC fluorescence spectrometer (Shimadzu, Japan) with a 1cm quartz cell. The excitation wavelength was set at 295 nm. The widths of the excitation slit and the emission slit were set at 5 nm and 5 nm, respectively.

The amino acid residue of tryptophan (Trp) included in the protein structures could act as fluorescence probe [3]. The Fluorescence spectrum (FS) should reflect the ratio of the contributions of buried and exposed Trp. Trp buried in nonpolar regions of the protein has an absorption peak at λmax = 330–332 nm [2]. VHb contains a Trp residue in each subunit (Trp122). The experimental results show that the absorption peak is at 331 nm for VHb and at 329 nm for Q53H, P54C, and Q53H/P54C (Fig. S5). The emission wavelength and fluorescence intensity is less in the variants Q53H and P54C. Variant Q53H/P54C has a compact structure and the results are consistent with CD data (higher helical content). The glutamine at position 53 was substituted by histidine,
an amino acid that faces towards the heme surface [4, 6]. The distances between the distal histidine and the ferric heme iron are normally 5.5–6.0 Å for peroxidases [2, 5].