

## Research Paper

# Chimeric Antibody-Binding *Vitreoscilla* Hemoglobin (VHb) Mediates Redox-Catalysis Reaction: New Insight into the Functional Role of VHb

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Experimentation was initiated to explore insight into the redox-catalysis reaction derived from the heme prosthetic group of chimeric *Vitreoscilla* hemoglobin (VHb). Two chimeric genes encoding chimeric VHbs harboring one and two consecutive sequences of Fc-binding motif (Z-domain) were successfully constructed and expressed in *E. coli* strain TG1. The chimeric ZVHb and ZZVHb were purified to a high purity of more than 95% using IgG-Sepharose affinity chromatography. From surface plasmon resonance, binding affinity constants of the chimeric ZVHb and ZZVHb to human IgG were  $9.7 \times 10^7$  and  $49.1 \times 10^7$  per molar, respectively. More importantly, the chimeric VHbs exhibited a peroxidase-like activity determined by activity staining on native PAGE and dot blotting. Effects of pH, salt, buffer system, level of peroxidase substrate and chromogen substrate were determined in order to maximize the catalytic reaction. From our findings, the chimeric VHbs displayed their maximum peroxidase-like activity at the neutral pH (~7.0) in the presence of high concentration (20-40 mM) of hydrogen peroxide. Under such conditions, the detection limit derived from the calibration curve was at 250 ng for the chimeric VHbs, which was approximately 5-fold higher than that of the horseradish peroxidase. These findings reveal the novel functional role of *Vitreoscilla* hemoglobin indicating a high trend of feasibility for further biotechnological and medical applications.

Key words: *Vitreoscilla* hemoglobin, peroxidase-like activity, Fc-binding motif, Z-domain, redox-catalysis reaction

## 1. Introduction

Hemoglobins are oxygen-carrying metalloprotein, which have been found in all vertebrates and some invertebrates including bacteria, fungi, and higher plants [1]. These heme proteins involve various functional vitality of cells e.g. electron transfer, storage and transfer of molecular oxygen, and major metabolism of cell. Hemoglobins are considered to be superlative molecules for studying reactions of electron transfer of heme protein owing to the well-defined structure and commercial availability [2]. Enzyme mechanisms among electron transporting system have been explored to provide a platform for fabricating biosensors. Construction and application of amperometric biosensors basing on molecular hemoglobins for determination of H<sub>2</sub>O<sub>2</sub> and nitrite have extensively been reported [3-5].

*Vitreoscilla* hemoglobin (VHb) is an oxygen binding protein produced by the obligate aerobic bacterium *Vitreoscilla* sp. [6]. It represents the most versatile tool for metabolic engineering of cell biotechnological processes. For circumstances, expression of VHb within various heterologous hosts

(e.g. bacteria, yeasts, fungi, and plant cells), particularly under hypoxic conditions, results in enhancement of cell propagation, oxidative metabolism, antibiotic and enzyme production, and the detoxification of nitric oxide (for recent review see [7]). It may also give rise to an increase yield of total proteins or intermediate metabolites and induction of bioremediation activity [8-10]. The proposed function of VHb is to serve as oxygen storage trap or to facilitate oxygen diffusion to the membrane terminal oxidases [11, 12]. Discovery on the consequences of VHb expression will provide a greater understanding on the functional role of VHb under oxygen-limiting conditions. Moreover, this will expand valuable information and pave the way for future applications of VHb.

Many attempts have been geared towards the evolutionary and functional significance of VHb in cellular metabolism. Expression of VHb improves the efficiency of microaerobic respiration and growth [12]. Highly susceptible to killing by H<sub>2</sub>O<sub>2</sub> has been revealed on cells-expressing VHb [13]. However, little is known on the biochemical reactivity towards the heme ligands of VHb as well as other related functions.

The VHb consists of two identical subunits of  $M_r$  15.8 kDa along with two protoheme IX per molecule. Protoheme IX is common to P<sub>450</sub>-type cytochromes, peroxidases, catalases, and other related enzymes. Therefore, VHb is anticipated to exhibit some of the activities assigned to these enzymes [14]. Supportive evidences have been drawn on the discovery of peroxidase-like activity of hemoglobins [15-17]. This motivates the use of hemoglobins in place of peroxidase in many purposes e.g. water purification [18], H<sub>2</sub>O<sub>2</sub> determination [19] and oxygen sensors [20].

To prove whether the heme prosthetic group of VHb can provide such a catalytic reaction as those obtained from the mammalian hemoglobins, the peroxidase-like activities of the purified VHbs have been tested *in vitro*. Herein, two chimeric VHbs carrying one and two consecutive regions of Fc-binding motif (Z-domain) have been constructed. The "Z" domain has been derived from a synthetic DNA fragment based on the sequence of the B-domain, a well-known motif of native staphylococcal protein A (SpA) that can bind specifically to the Fc portion of IgG [21]. This domain has been fused to the VHb in order to i) assist one-step protein purification via IgG-Sepharose affinity chromatography, ii) create a chimeric protein possessing dual characteristics of both IgG-binding and catalytic reaction, and iii) apply as an immobilization tool to allow the correct orientation of chimeric protein on the sensor surface. The purified forms of chimeric proteins designated as ZVHb and ZZVHb have then been characterized for their IgG-binding affinities via the surface plasmon resonance as well as their peroxidase-like activities. Maximization of the peroxidase-like activities of the chimeric VHbs has been performed in comparison with those obtained from the horseradish peroxidase and bovine hemoglobin.

## 2. Materials and methods

### Bacterial strains and culture

*E. coli* strain TG1 (*supE*, *hsdΔ5*, *thiΔ*(lac-proAB), F'[*traD36 proAB+ lacI<sup>q</sup> lacZΔM15*]) was used as host for gene cloning and protein expression. Luria-Bertani (LB) broth supplemented with ampicillin (100 mg/L) was used as a growth medium and protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG).

### Enzymes and chemicals

High Fidelity *Taq* DNA polymerase, restriction endonucleases and T4 DNA ligase were purchased from Roche (Mannheim, Germany). All other chemicals and reagents were of analytical grade.

### Construction of chimeric genes encoding chimeric VHbs harbouring one and two-consecutive Fc-binding motifs

DNA fragments coding for the Z and ZZ binding motifs were obtained by PCR amplification using plasmid pEZZ18 (Amersham Biosciences, Stockholm,

Sweden) as template and the two primers (sense: 5'-AAAAGAATTCATGGTAGACAACAAATT  
CAACAAAGAAC-3', antisense: 5'-  
AAAATCTAGATTCGGCGCCTGAGCATC-3'),  
(MWG-Biotech AG). Since the primers contain 5' overhang of *Eco*RI site in the sense and *Xba*I site in the antisense, therefore, the PCR products were digested with these enzymes and subsequently inserted into the VHb expression plasmid, pQVD [22]. These resulted in the in-frame fusion of the Z and ZZ encoding genes at the 5'-end of the *vhb* gene. All cloning procedures were performed according to the standard protocol as described by Sambrook *et al.* [23]. The newly constructed plasmids, designated pZV and pZZV, were verified for correct insertion by digestion of restriction endonucleases and further confirmed by DNA sequencing.

### Purification of chimeric ZVHb and ZZVHb using IgG-Sepharose affinity chromatography

*E. coli* cells carrying plasmids pZV and pZZV were grown for 8 hrs at 37°C with shaking at 200 rpm in 500 mL baffled flasks containing 200 mL of LB medium supplemented with 100 mg/L ampicillin. IPTG was then added to a final concentration of 1 mM and the culture was further cultivated at 25°C for 4 hrs. After harvesting by centrifugation, cells were washed and resuspended in IgG-Sepharose running buffer composed of 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6 (Tris-saline Tween; TST). Cell lysis was performed by ultrasonication (W380 Heat System Ultrasonic). Cell debris was removed by centrifugation at 15,000 rpm for 15 min and the crude supernatant containing solubilized fusion protein was collected. The crude extract was filtered through the millipore filter (0.45 μm) then subsequently applied to an IgG-Sepharose (Amersham Biosciences) column (∅ 1.0 cm, 3 mL drained gel) by gravity flow. Removal of unbound proteins was performed by washing with 10 column volumes of TST buffer followed by 2 column volumes of 5 mM NH<sub>4</sub>Ac, pH 5.0. The chimeric proteins were eluted from the column using 0.3 M acetic acid, pH 3.4. The eluted protein fraction was immediately neutralized with 0.5 M Tris, pH 10.0 and then dialysed against 0.1 M Tris-HCl, pH 8.0. Proteins were quantified using the Bio-Rad protein assay, based on the Bradford Coomassie blue dye-binding procedure [24].

### Characterization of chimeric VHbs harbouring one and two-consecutive Fc-binding motifs

#### Molecular size estimation by SDS-PAGE

Protein electrophoresis was carried out in a discontinuous buffer system as described by Laemmli [25]. A stacking gel (4%) and a separating gel (14%) were cast using the Mini Protean III apparatus (Bio-Rad Laboratories). Samples mixed with SDS-loading buffer were boiled at 100°C for 10 min before applying to the gel. After complete electrophoresis was reached the gel was stained with Coomassie brilliant blue.

### Native PAGE and activity staining

Native PAGE and activity staining were performed under non-reducing conditions. The stacking (4%) and separating gel (10%) used for electrophoresis and activity staining were prepared without addition of sodium dodecyl sulfate. Samples were mixed with loading buffer containing no reducing agent and loaded directly to the gel without heat denaturation. Immediately after electrophoresis, the gel was rinsed with deionized water and incubated for approximately 30 min in 0.1 mM Tris-HCl, pH 8.0 containing 1% dimethyl sulfoxide (DMSO) and 2 mM 1, 8-diaminonaphthalene (DAN). Hydrogen peroxide was added to a final concentration of 10 mM prior to immersion of the gel. In order to darken the DAN oxidation products, the gel was immersed in 20% trichloroacetic acid. Additionally, the color of activity stained proteins was further enhanced by counterstaining the gel with Coomassie brilliant blue dye [26].

### Absorbance spectra of chimeric VHbs

UV-Vis absorption spectra of chimeric VHbs were collected using a Shimadzu spectrophotometer model 1650 (Shimadzu, Japan). Scanning of the spectra was started from 300-700 nm.

### Analysis of IgG-binding affinity of chimeric VHbs harbouring one and two-consecutive Fc-binding motifs

To characterize the IgG binding capability of chimeric ZVHb and ZZVHb, the interaction between the fusion proteins and human IgG was investigated in real time using the BIACORE<sup>®</sup> 3000 instrument (Biacore AB, Uppsala, Sweden). Human purified IgG (DAKO) was utilized as an immobilized ligand and the amine-coupling procedure was performed [27]. In brief, the carboxylated dextran layer of a Biacore CM5 sensor chip was activated by N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-diethylaminopropyl)-carbodiimide (EDC). The IgG solution used for immobilization was prepared in 0.1 M sodium acetate, pH 5.6 to a concentration of 100 nM. The immobilized response of approximately 1,800 resonance units (RU) was reached before surface deactivation using ethanolamine hydrochloride solution. Activated and deactivated of blank flow cells (without IgG immobilization) were used as reference. The chimeric proteins were diluted in HBS-P buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% Surfactant P20, pH 7.4) to final concentrations of 100, 50, 25, 12.5 and 6.25 nM. One hundred fifty microlitres of each diluted sample was injected at the flow rate of 30  $\mu$ L/min over the immobilized IgG surface. The association and dissociation rates of ZVHb and ZZVHb to the IgG ligand were monitored and the results were displayed as sensograms. The data obtained were subsequently evaluated using the BIAevaluation 3.2 software (Biacore AB). The association rate constant ( $k_a$ ), dissociation rate constant ( $k_d$ ) and affinity constant ( $K_A$ ) were calculated.

### Analysis of peroxidase-like activity of chimeric VHbs

Peroxidase-like activity of chimeric VHbs was determined by measuring the rate of ABTS oxidation in the presence of H<sub>2</sub>O<sub>2</sub> at ambient temperature. Formation of the oxidised product, ABTS<sup>•+</sup>, was followed spectrophotometrically at 415 nm ( $\epsilon_{415} = 3.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ). The assay mixture contained 100  $\mu$ L of 50 mM ABTS, 200  $\mu$ L of 0.5 M Tris-HCl, pH 8.0 in a total volume of 0.9 mL. The reaction was initiated by addition of 100  $\mu$ L of 50 mM H<sub>2</sub>O<sub>2</sub>. The peroxidase activity of bovine hemoglobin (BHb; Sigma) was also measured for comparison.

### Maximization of peroxidase-like activity of chimeric VHbs

Maximization of peroxidase-like activity of the chimeric VHbs was performed as follows:

#### Optimum pH for peroxidase-like activity

The peroxidase-like activity of the chimeric VHbs was measured at various pHs ranging from 3.0-8.0 using phosphate citrate buffer.

#### Effect of salts and buffer systems

The peroxidase-like activity of the chimeric VHbs was measured in various kinds of buffer system including Na-phosphate buffer, K-phosphate buffer, phosphate-buffered saline, HEPES buffer and phosphate citrate buffer. The pH of all the buffer systems was maintained at 7.0.

#### Optimum concentration of peroxide substrate (H<sub>2</sub>O<sub>2</sub>)

Various concentrations of freshly prepared H<sub>2</sub>O<sub>2</sub> (1.25 – 80 mM) were tested in the assays of peroxidase-like activity of chimeric VHbs. Phosphate citrate buffer at pH 7.0 was used in the assay.

#### Optimum concentration of ABTS substrate

Various concentrations of ABTS ranging from 0.5 – 8 mM were tested. The reaction composed of 20 mM of H<sub>2</sub>O<sub>2</sub> in phosphate citrate buffer at pH 7.0.

For further experiments, the peroxidase-like activity of the chimeric VHbs at various amounts was analyzed in phosphate citrate buffer, pH 7.0 containing 2 mM ABTS and 20 mM H<sub>2</sub>O<sub>2</sub>. The catalytic reaction was recorded at OD 405 nm at 1 minute time interval for 3 min.

## 3. Results and discussion

### Chimeric gene construction and protein expression

In this study, two chimeric genes encoding chimeric *Vitreoscilla* hemoglobin harbouring one and two-consecutive Fc-binding motifs (Z-domain) were successfully constructed and expressed in *E. coli*. As shown in Figure 1, a markedly increase of chimeric protein expression up to approximately 20% of total protein was found upon induction by IPTG. The chimeric proteins could be conveniently purified by IgG-Sepharose affinity chromatography yielding protein with more than 95% purity as judged by SDS-PAGE (Figure 2) using AlphaImager<sup>TM</sup> 2200 Documentation and Analysis system (Alpha Innotech

Corp, USA). The molecular weights of the purified ZVHb and ZZVHb were at 22.6 and 29.3 kDa, respectively.

Although both of the chimeric ZVHb and ZZVHb were expressed in high levels in *E. coli*, however, less than 10% was successfully purified. Only 1.0 mg of the chimeric protein could be obtained from a 200 mL culture. This is correspondingly due to the tendency of the VHb to form inclusion bodies as those reported previously. Results from the immunogold labeling of VHb clearly indicated a cytoplasmic localization and VHb was concentrated near the periphery of the cytosolic face of the *E. coli* inner membrane [28]. From the absorption spectra measurements supportively demonstrated an additional peak at 620 nm beside the major peak ranged at 408-410 nm (Figure 3). The presence of major peak at 410 nm is denoted as a maximum absorption of hemoglobin. The  $A_{410}/A_{280-nm}$  has widely been applied for estimation of VHb purity [28, 29]. Meanwhile, it is conceivable that the minor peak ranged around 620 - 625 nm might be attributed to the spectra of phospholipids containing cyclopropanated fatty acids co-aggregated with VHb during the stationary phase of *E. coli* [30]. In order to recover the VHb from insoluble fraction, attempts have been geared towards the use of denaturation agents e.g. urea and guanidine hydrochloride or detergent solubilization [31]. However, it should be noted herein that adjusting of growth condition or addition of Triton X-100 increased the amount of protein in the soluble form, but after purification the protein showed no peroxidase-like activity (data not shown). Hart and coworkers [32] have identified that the VHb expressed in *E. coli* in insoluble form lacks the heme prosthetic group (apoVHb) whereas the soluble form (holoVHb) contains heme in the molecule. Since the peroxidase-like activity of VHb depends on the heme group, this may explain the inactive form of ZVHb and ZZVHb in some protein portion.

#### Evaluation of the kinetic parameters of ZVHb and ZZVHb binding activities to human IgG

Binding affinity to human IgG of the two chimeric proteins were investigated using the SPR biosensor. Our findings clearly showed that after being fused to VHb, the Z-domain possessed a strong binding affinity to human IgG. The kinetic parameters including the association rate constant ( $k_a$ ), dissociation rate constant ( $k_d$ ) and affinity constant ( $K_A$ ) were calculated as summarized in Table 1. The affinity constants to IgG of the chimeric ZVHb and ZZVHb were at  $9.7 \times 10^7$  and  $49.1 \times 10^7/M$ , respectively. These values were in the same range as those of the native Z or ZZ reported. This indicates that the Z-domain retains its native structure in the chimeric proteins.

#### Analysis of peroxidase-like activity of chimeric VHbs

Verification of the peroxidase-like activity of the chimeric ZVHb and ZZVHb were performed using  $H_2O_2$  and the reducing substrate ABTS as indicator.

Specific activities for bovine hemoglobin (BHb), VHb6H (native VHb carrying an additional hexahistidine), ZVHb and ZZVHb were 0.3, 1.7, 3.9 and 1.5 unit/mg, respectively. Moreover, the peroxidase activities of the chimeric proteins could be visualized by activity staining (Figure 4) as well as by dot blotting (Figure 5), where the proteins were applied on the polyacrylamide gel and nitrocellulose membrane and directly detected with DAN substrate.

To maximize the peroxidase-like activity of the chimeric VHbs, various parameters including the effect of pH, effect of salts and buffer systems, concentrations of  $H_2O_2$  and ABTS were applied into the assay reaction. Figure 6 illustrates that the pH optimum for the peroxidase-like activity of all the chimeric VHbs was approximately 7.0 while the HRP was shown to exhibit the highest activity at pH around 4.0. The phosphate citrate buffer at pH 7.0 was proven to provide a greater extent for the peroxidase-like activity of the chimeric VHbs. A gradual increase of activity correspondingly to the concentrations of hydrogen peroxide (up to 20 and 40 mM) was found in the case of chimeric VHbs (ZVHb and ZZVHb, Figure 7). Contrarily, for HRP, our results advocate the former finding that increase concentration of  $H_2O_2$  substrate markedly suppressed the peroxidase activity [33]. Among these parameters, the optimum concentration of ABTS was found to be at 2 mM to generate the highest activity. Therefore, varied amounts of chimeric VHbs were conducted for peroxidase activity under the optimum condition to generate the enzyme response curve as represented in Figure 8. The peroxidase-like activities for the chimeric VHbs were in the order of ZVHb > ZZVHb > VHb6H. The detection limits were down to 250 and 50 ng for the chimeric ZVHb or ZZVHb and HRP, respectively. Notification has to be made that the HRP provides 5-fold higher sensitivity than those of the chimeric VHbs.

To investigate the physical stability of the chimeric VHbs, effects of heat treatment on the peroxidase-like activity were tested. The ZVHb, ZZVHb, VHb6H and bovine Hb were diluted to a concentration of 10  $\mu M$  in Tris-HCl, pH 8.0, and the protein solutions were then subjected to heat treatment at various temperatures ranging from 30 to 90°C for 10 min and immediately cooled down on ice. The percentage of remaining peroxidase activities of these proteins were then determined (Figure 9). Our results revealed that the activity of VHb6H could be enhanced up to 135% when the protein was treated at 50°C for 10 min and the protein retained more than 70% activity after heating to 80°C. In the case of bovine hemoglobin, the activity was mostly enhanced at 40°C and it retained more than 60% activity after heated up to 80°C. For the chimeric proteins, the peroxidase-like activities were slightly increased in the case of ZVHb but not in ZZVHb, after heating the proteins, and totally disappeared when the proteins were heated up to 70°C. However, the ZVHb and

ZZVHb retained nearly 100% activity after heating at 50°C.

According to the previous studies, the structure of heme proteins has been found to be affected by heat treatment or denaturing agents. Diederex *et al* [34] have recently reported on the increase of peroxidase activity of cytochrome *c* upon addition of guanidine hydrochloride. Chattopadhyay [35] and his colleagues have demonstrated the conformational changes of horseradish peroxidase (HRP) by the effects of temperature and pH. By circular dichroism and fluorescence studies, they have concluded that at neutral pH, changes of the tertiary structure around the heme pocket occurred at the temperature range of 40-50°C while the overall secondary structure of the enzyme remained non-significant change. However, at higher temperature ( $T_m = 74^\circ\text{C}$ ), the heme was completely removed from the enzyme active site. Moreover, acidity caused the splitting of the hemes from the apoproteins as indicated by the drop of their Soret bands in methemoglobin [15]. Interestingly, the VHB6H and BHB give rise to more pronounced activity to heat enhancement than those of the ZVHb and ZZVHb. Since peroxidase activity of hemoglobin is also based on the prosthetic heme group, the enhancement of specific activity upon heat treatment is probably due to the rearrangement of tertiary structure nearby the heme pocket resulting in unmasking of the heme pocket to the environment.

### Conclusion and future perspectives

New insight into the catalytic reaction based on heme-prosthetic group of the *Vitreoscilla* hemoglobin (VHb) has been firstly revealed in this study. The peroxidase-like activity of the chimeric VHbs carrying the Fc-binding motif has been attained. This novel function opens up a high feasibility to further apply the chimeric VHb in biotechnological and medical approaches. The chimeric ZVHb and ZZVHb can further be accessed as a secondary antibody-peroxidase conjugate in various types of immunoassay. Since the peroxidase is widely used to tag the secondary antibody in enzyme immunoassays. Detection signal can be generated from a wide variety of chromogenic substrates. The development of HRP-labeled SpA based on chemical conjugation has earlier been reported by Zhou *et al* [36]. However, the chemical reaction is complicated and degree of conjugation is hardly controlled. Titration or validation is required due to batch to batch variation. In addition, expression of native HRP in bacteria is intrinsically difficult on the account of the high tendency to form inclusion bodies [37]. Meanwhile, conjugation via gene fusion generates exact chimeric protein of the fusion partners. The chimeric VHbs possess both the peroxidase-like activity and the IgG-binding affinity precisely as gene-engineered design. Although the activity of the chimeric VHbs after maximization is 5-fold lower than that of the HRP. This in turn provides more advantages on the lowering background interference as compared to the HRP while augmenting an appropriate sensitivity for

the detection. Interestingly, an optimum activity of the VHb is catalyzed on the high level of peroxide substrate which either of endogenous peroxidase or the HRP can be negligible [33]. Our findings lend support the high feasibility of using the chimeric VHbs in immunohistochemistry purposes. To further expand their applicability in other types of immunoassay, site-directed mutagenesis can then be applied to create mutation on the heme-neighbouring residues in order to achieve a higher catalytic mutant [38, 39]. Furthermore, the chimeric VHbs can be incorporated on to the sensor surface via the IgG-binding interaction and applied as an electrochemical-redox sensor for biotechnological applications in the future [20].

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### Conflict of interest

The authors have declared that no conflict of interest exists.

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## Tables and Figures

Table 1. Kinetic parameters of binding between fusion protein and human IgG\*

Protein	$k_a(\text{s}^{-1}\text{M}^{-1}\times 10^5)$	$k_d(\text{s}^{-1}\times 10^{-3})$	$K_A(\text{M}^{-1}\times 10^7)$	References
ZVHb	2.62 (±0.01)	2.69 (±0.03)	9.7	this study
ZZVHb	4.33 (±0.01)	0.88 (±0.01)	49.1	this study
Z	0.54	2.7	2.0	(40)
Z	1.9	3.2	5.9	(41)
Z	0.78	6.9	1.1	(42)
ZZ	1.1	0.33	33.0	(40)
ZZ	3.5	0.51	68.0	(41)

\* $k_a$  is an association rate constant and  $k_d$  is a dissociation rate constant.

$K_A$  is an affinity constant ( $K_A = k_a/k_d$ ).

Figure 1. SDS-PAGE represents protein profiles of cell lysate of *E. coli* carrying chimeric genes of ZVHb and ZZVHb before (lane 1 and 3) and after induction by IPTG (lane 2 and 4). Two major bands at molecular masses of approx. 22 and 29 kDa are shown after induction of cells expressing chimeric ZVHb and ZZVHb, respectively. *M* indicates molecular weight marker.

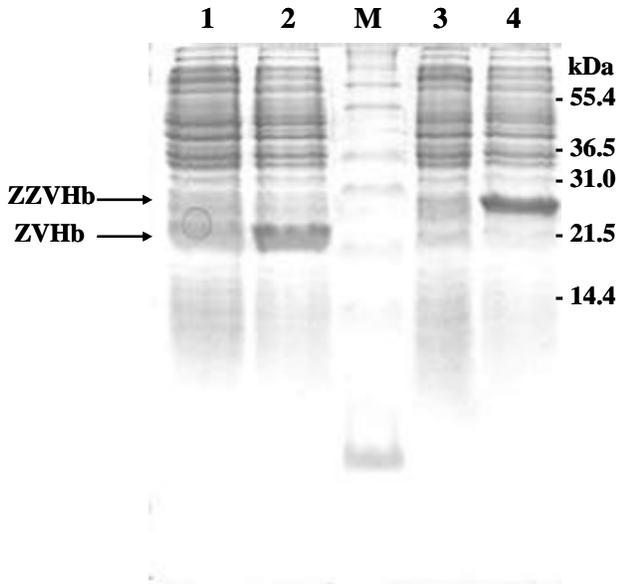


Figure 2. SDS-PAGE of the purified fraction of chimeric ZVHb (lane 1) and ZZVHb (lane 2) from IgG sepharose column. *M* indicates molecular weight marker.

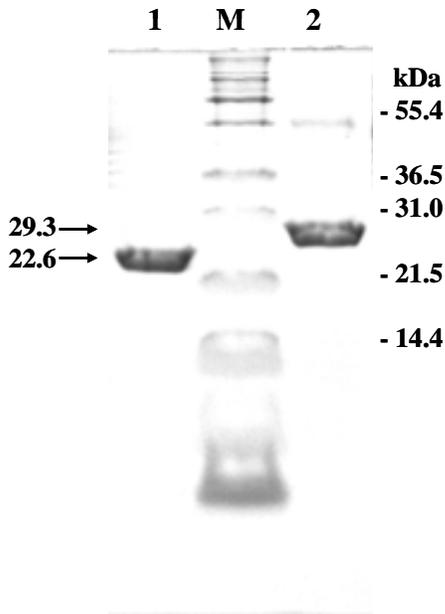


Figure 3. UV-Vis absorption spectra (300-700 nm) of the chimeric ZVHb (solid line), ZZVHb (dash-dot line) and Vhb6H (VHb with hexahistidine; dot line). Inset represents the spectra with high magnification.

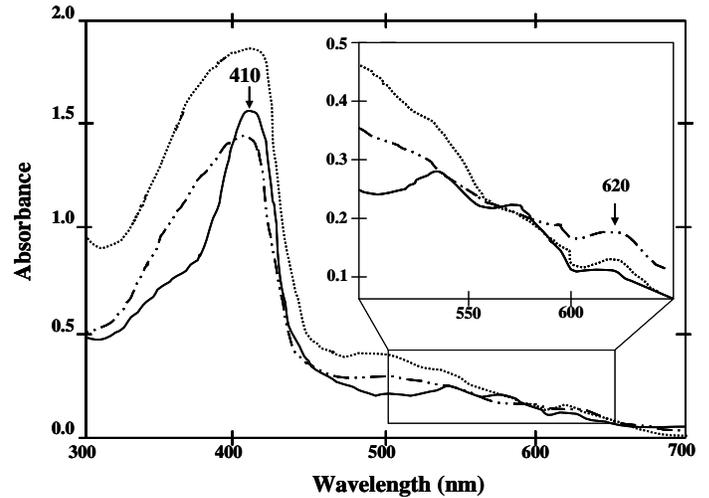


Figure 4. Activity stain of electrophoresis gel. The gel was loaded with 1.0 nmol of HRP, BHb, Vhb6H, ZVHb and ZZVHb (lane 1, 2, 3, 4 and 5, respectively). Peroxidase-like activity was detected by trichloroacetic acid enhanced DAN staining (A) without and (B) with CBB counterstained.

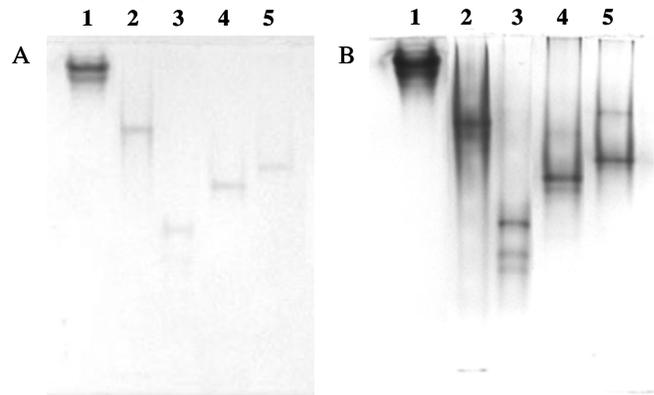


Figure 5. Dot blot peroxidase-like activity. Five microgram of each protein (HRP, bovine Hb, Vhb6H, ZVHb and ZZVHb) was dotted on nitrocellulose membrane and allowed to dry at 4°C. The membrane was then soaked in 0.1 mM Tris, pH 8.0 containing 1% dimethyl sulfoxide and 2 mM 1,8-diaminonaphthalene (DAN). A chimeric H6GFPZ was applied as a negative control (data not shown). H<sub>2</sub>O<sub>2</sub> was added to a final concentration of 10 mM.



Figure 6. Peroxidase-like activity of chimeric ZVHb (closed circle), ZZVHb (opened circle), VHb6H (closed triangle) and HRP (opened triangle) at different pHs

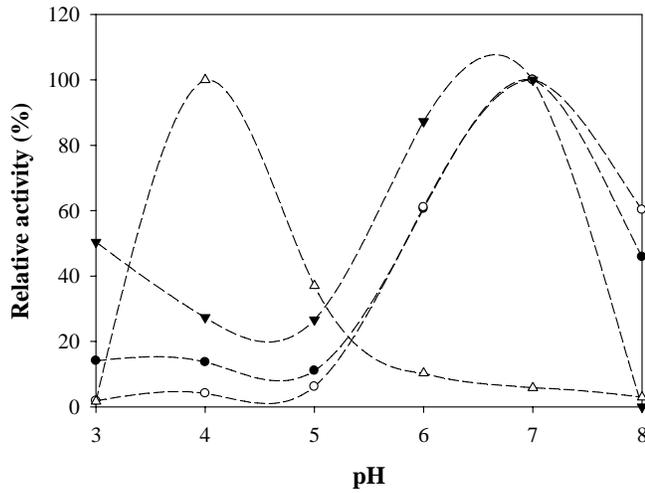


Figure 7. Analysis of peroxidase-like activity of the chimeric ZVHb, ZZVHb, VHb6H and HRP in the presence of various concentrations of hydrogen peroxide. The derivative of Absorption (A) as a function of time (t) is represented by dA/dt.

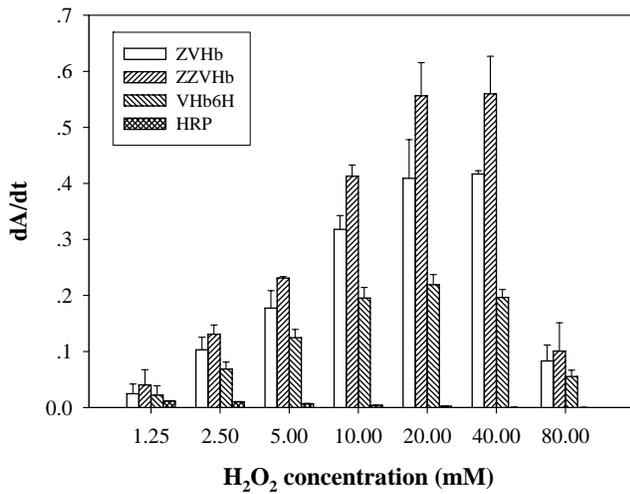


Figure 8. Enzyme-response curve of the peroxidase-like activity of ZVHb (closed circle), ZZVHb (opened circle), VHb6H (closed triangle), HRP (opened triangle) and H6GFPZ (square; applied as a negative control). The derivative of Absorption (A) as a function of time (t) is represented by dA/dt.

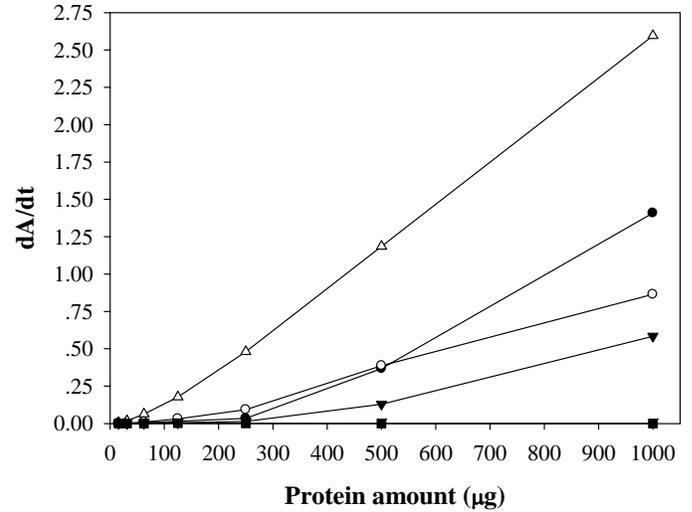


Figure 9. Percentage of residual peroxidase-like activity of ZVHb (closed circle), ZZVHb (opened circle), VHb6H (closed triangle), and BHb (opened triangle) after heat treatment at various temperatures for 10 min. The values are mean values of triplicate measurements and the error bars indicate standard deviations.

