Production of *Vitreoscilla* Hemoglobin - Sarcosine Oxidase Fusion Protein for Future Use in Quantification of Sarcosine in Biological Samples

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ABSTRACT

Sarcosine or N-methylglycine has recently been proposed as a potential biomarker for detection of prostate cancer. The level of sarcosine in urine was found to elevate during prostate cancer progression and metastasis. In this study, a fusion protein between sarcosine oxidase (SOX) from *Bacillus* sp. BSD-8 and *Vitreoscilla* hemoglobin (VHb) was constructed for further development of a simple colorimetric method for quantification of sarcosine. The fusion protein was expressed in *E. coli* and purified to >95% homogeneity by IMAC. One liter of culture yielded 11 milligrams protein. Study of spectral properties showed that the protein had a soret peak at 412-414 nm indicating the existence of heme with iron in ferric (Fe^{3+}) state. Enzymatic characterization demonstrated that the fusion protein retained 46% and 36% of relative peroxidase and sarcosine oxidase activities, respectively. This bi-functional protein will be further applied for quantification of sarcosine in biological samples especially urine.

Keywords: Sarcosine, Fusion protein, Prostate cancer

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Introduction

Prostate cancer (PCa) is the cancer of prostate gland. It has been estimated as a second leading cause of cancer death in Americans and its incidence is significantly increasing worldwide (Siegel *et al*., 2016). Normally, the cancer does not present any symptoms until it progresses to locally advanced or metastatic stage. Therefore, the development of its early diagnostic test is much necessary. Nowadays, serum prostate-specific antigen (PSA) testing and digital rectum examination (DRE) have been used for the screening (Leewansangtong *et al*., 1999). However, their limitations, such as low sensitivity and specificity are still major problems. In many cases, DRE did not reveal any abnormalities, even the cancer progressed. Moreover, beside prostate cancer, the elevated level of serum PSA can be caused by benign prostate hypertrophy (BPH), prostate inflammation, prostate infection and trauma. Consequently, these limitations often lead to misdiagnosis, unnecessary biopsy and the side effects of overtreatment (Polascik *et al*., 1992; Stamey *et al*., 2004). To overcome these limitations, free PSA/total PSA ratio (fPSA/PSA ratio) has been considered to evaluate the status of the disease (Kuriyama *et al*., 1998). However, fPSA/PSA ratio only increases the sensitivity up to 56%. Therefore, the finding of new and efficient biomarkers has become an essential challenge for improving the efficiency of early diagnosis. In 2009, sarcosine or N-methylglycine, a metabolite of glycine, has been proposed to be a potential biomarker for the estimation of prostate cancer progression (Sreekumar *et al*., 2009). In biological samples, sarcosine concentration in serum and urine has been determined to be 1.59 ± 1.08 mM and 1-20 mM, respectively (Cernei *et al*., 2012). In PCa patients, although sarcosine concentration in serum did not significantly increase, the urinary sarcosine of PCa patients increased more than 2 times when compared with healthy urine samples (Lan *et al*., 2014). Recently, analytical methods for direct detection of sarcosine in urine, especially chromatography with mass spectroscopy were reported (Jiang *et al*., 2010; Shamsipur *et al*., 2013). These techniques have very high sensitivity and specificity. However, they also have several disadvantages e.g., high instrumentation cost, requirement of complicate sample preparation and skilled operator, making these methods unsuitable for routine application. Beside direct assays, indirect assays have also been developed (Burton C *et al*., 2012; Rebelo *et al*., 2014). The enzymatic method using sarcosine oxidase (SOX) and peroxidase is one of the most common methods used for sarcosine detection. Nevertheless, in the present, none of the reported methods can be applied with urine samples.

To solve this problem and to save cost in enzyme production, in this study, a fusion protein between sarcosine oxidase (SOX) from *Bacillus* sp. BSD-8 and hemoglobin with peroxidase activity from *Vitreoscilla* spp. (VHb) was constructed. . The fusion protein was expressed in *Escherichia coli* and purified to homogeneity by IMAC. Spectral properties and enzymatic activity were also studied.

Objective of the study

To produce a fusion protein between SOX and VHb in *E. coli* using genetic and protein engineering techniques.

Materials and Methods

Bacterial strains, gene and plasmids

E. coli Novablue and *E. coli* BL21 (DE3) were purchased from Novagen (EMD Bioscience, Germany). Plasmid containing *vhb* gene (pET46VHb) and *Bacillus* sp. BSD-8 sarcosine oxidase gene (pET20SOX) are maintained in our laboratory.

Chemicals

Sarcosine, δ-Aminovulinic acid and Amplex red were purchased from Sigma–Aldrich (MO, USA). Imidazole, ampicillin, isopropyl β-D-1-thiogalactopyranoside and glycerol were obtained from Bio Basic Inc. (ON, Canada). Hydrogen peroxide (H_2O_2) 30% was purchased from Merck (Schuchandt, Germany).

DNA manipulation for expression of VHb-SOX fusion protein

Construction of plasmid expressing recombinant VHb (pET46VHb) was previously described (Isarankura-Na-Ayudhya *et al*., 2010). The pET20SOX plasmid was previously prepared in our laboratory. To construct the VHb-SOX fusion protein, *sox* gene was PCR amplified. The primers used for PCR amplification were 5'-CAGGATCCATGAGCACGCATTTTG-3' (forward primer, the BamHI restriction site is underline.) and 5'-CAACTCGAGTTATTTTGCTGCTTCC-3' (reverse primer, the XhoI restriction site is underlined). After pET46VHb was treated with BamHI and XhoI, the vectors and PCR products (~1,200 bp) were ligated and transformed into *E. coli* Novablue. The resulting plasmid was designated as pET46VHb-SOX (Fig. 1). To verify the correctness of cloning procedure, the constructed plasmid was checked by colony PCR and DNA sequencing.

Protein expression and purification by immobilized-metal affinity chromatography (IMAC)

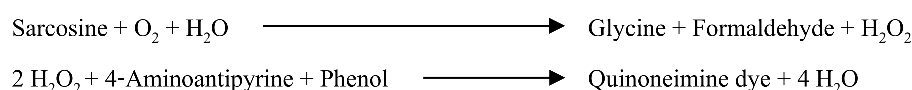
The pET46VHb, pET20SOX and pET46VHb-SOX plasmid were transformed into *E. coli* BL21 (DE3) using heat-shock technique. Cells were cultured in 6 L of modified terrific (TB) containing 100 μg/ml ampicillin at 37°C, with shaking at 180 rpm for 10 h. To induce protein expression, the cultures were added with 1 mM isopropyl beta-D-1 thiogalactopyranoside (IPTG) and 0.3 mM delta-aminolevulinic acid (δ-ala). The culture was continued for 16 h at 30°C with shaking at 120 rpm. The cells were harvested and resuspended in sonication buffer, 50 mM Tris-HCl containing 500 mM NaCl, pH 7.8. To disrupt the cells, the cells were freezed-thawed at least 3 times and sonicated on ice for 15 cycles of 2 min with 1 min interval. The supernatant and pellet fractions were separated by centrifugation at 14,000 rpm, 4°C for 10 minutes. Prior to protein purification, crude extracts were filtered through a 0.45 μm membrane filter. Then, sterile-crude extract was loaded onto IMAC column pre-equilibrated with sonication buffer. The target protein was eluted with gradient imidazole in the same buffer solution. Protein fractions were subjected to SDS-PAGE analysis. Highly purified fractions were pooled and desalted by Amicon® centrifugal filter devices 10 kDa (VHb), 30 kDa (SOX) and 50 kDa (VHb-SOX). Each protein was measured for its concentration by Bradford assay and kept at -80°C with 15-20% glycerol until use.

Spectral property assay

Spectral properties of VHb, SOX and VHb-SOX fusion protein were studied. Protein samples were adjusted to 1 mg/ml in sonication buffer. The absorbance ranging from 250-700 nm was measured in quartz cuvette.

SOX activity assay

Sarcosine oxidase catalyzed the oxidation of sarcosine to yield glycine, formaldehyde, and H_2O_2. The amount of H_2O_2 was measured from the amount of quinoneimine dye caused by the oxidation of 4-aminopyrine and phenol in the presence of horseradish peroxidase (HRP). To measure SOX activity, protein samples (SOX and VHb-SOX fusion protein) were prepared in 50 mM Tris-HCl buffer pH 7.8, 500 mM NaCl to the concentration of 115.6 and 125.2 µg/ml, respectively. The reaction mixture (1 ml) contained 100 µl of diluted protein, 1 mM sarcosine, 6 mM phenol, 1 mM 4-aminoantipyrine and 7 units of HRP (Inouye Y *et al*., 1987). The catalyzed reactions are as follows:



Under such conditions, the absorbance of quinoneimine dye ($\varepsilon = 13.3\ mM^{-1}\ cm^{-1}$) was measured at 500 nm in every 5 seconds for 5 minutes. All assays were performed in triplicate. The specific SOX activity was determined as the unit per milligram protein. One unit of SOX activity (U) was defined as the amount of enzyme that catalyzed the formation of 1 µmol H_2O_2 per min at 37°C. The relative activity of rVHb-SOX was calculated in comparison with the specific activity of rSOX, which was regarded as 100%.

Peroxidase activity assay

Peroxidase assay was slightly modified from that described by Suwanwong (Suwanwong *et al*., 2006). VHb and VHb-SOX fusion protein were prepared in 50 mM Tris-HCl buffer containing 500 mM NaCl, pH 7.8 to the concentration of 70.25 µg/ml. The mixture contained 100 µl of diluted proteins, 100 µl of 2 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 700 µl of the same buffer. Then, the reaction was initiated by addition of 100 µl of 6.25 mM H_2O_2. The absorbance of ABTS radical ($\varepsilon = 36\ mM^{-1}\ cm^{-1}$) was measured at 415 nm in every 5 second for 5 minutes. All assays were performed in triplicate. The specific peroxidase activity was determined as the unit per milligram protein. One unit of peroxidase activity (U) was defined as the amount of enzyme that catalyzed the formation of 1 µmol H_2O_2 per min at 37°C. The relative activity of rVHb-SOX was calculated in comparison with the specific activity of rVHb, which was regarded as 100%.

Results

DNA manipulation

Schematic representation of DNA manipulation is shown in Fig. 1. After gene cloning process, the results showed that all clones possess a correct DNA sequence (data not shown). This indicated that the pET46VHb-SOX plasmid was successfully constructed.

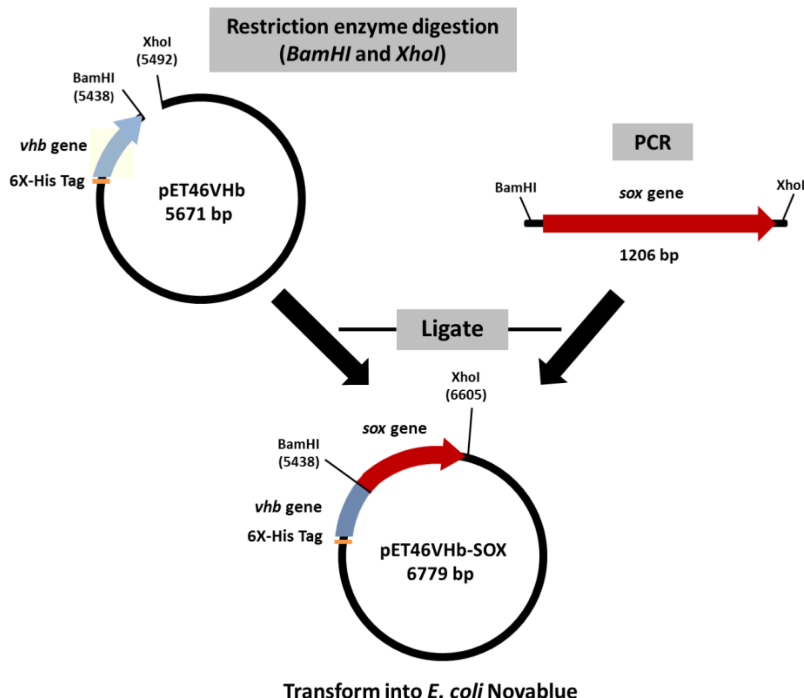


Figure 1: Construction of plasmid expressing VHb-SOX fusion protein.

Protein expression and purification

Protein expression and purification were done as indicated in the "materials and methods" section. For each protein, after IMAC purification, all fractions containing the target proteins were pooled and removed imidazole (Fig. 2). All purified proteins were determined their purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Fig. 3, the purity of each protein was achieved at up to 95% homogeneity. The yield of rVHb, rSOX and rVHb-SOX production were 46, 10, 11 mg/L of culture, respectively. All purified proteins contained the intense protein bands which were closely related to the theoretical molecular weight of the target proteins.

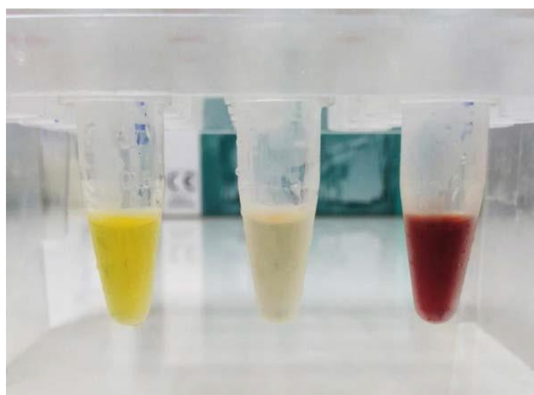


Figure 2: The produced recombinant proteins. Left: rSOX, middle: rVHb-SOX, right: rVHb.

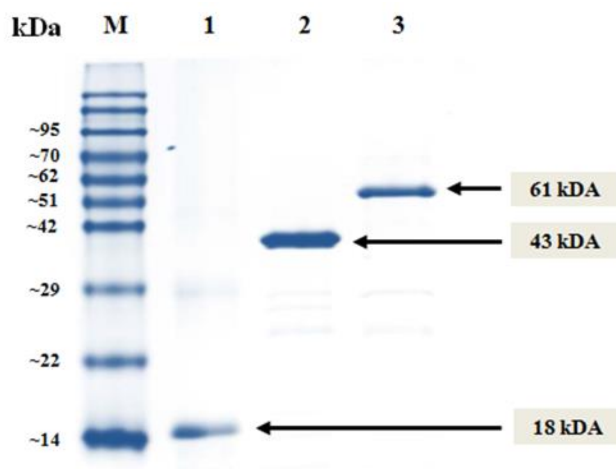


Figure 3: SDS-PAGE analysis of recombinant protein. Lane M: protein marker, lane 1: rVHb (18 kDa), lane 2: rSOX (43 kDa) and lane 3: rVHb-SOX (61 kDa).

Spectral properties

As shown in Fig. 4, the soret peak at 412-414 nm was observed from rVHb. This peak indicates the presence of heme prosthetic group with iron in ferric state (Fe^{3+}), which is essential for peroxidase activity of VHb. For rSOX, the soret peak at 455 nm was elucidated. This peak indicates the presence of flavin-adenine dinucleotide (FAD) in the oxidized form, which contributes to SOX activity. For rVHb-SOX, the heme peak (412-414 nm) was obviously presented. However, the FAD peak (455 nm) was hardly observed.

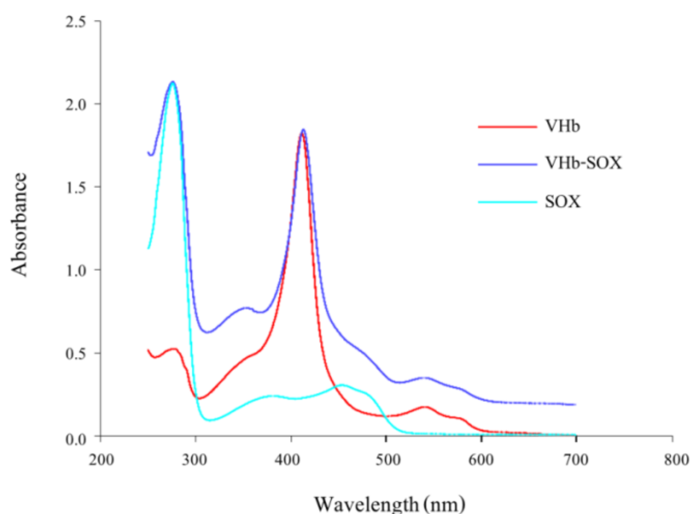


Figure 4: Spectral properties of recombinant proteins.

Peroxidase and SOX activities

The correlation between the absorbance intensity and the formation of oxidized co-substrate for peroxidase and SOX activities were shown in Fig. 5 and 6, respectively. The specific activity of peroxidase and SOX were calculated as indicated in the “materials and methods” and shown in Table 1. The fusion protein retained 46% of

relative peroxidase activity and 36% of relative SOX activity in term of unit/mg protein. However, when compared in term of unit/micromole, the relative peroxidase activity increased from 46% to 164% and the relative SOX activity increased from 36% to 51%.

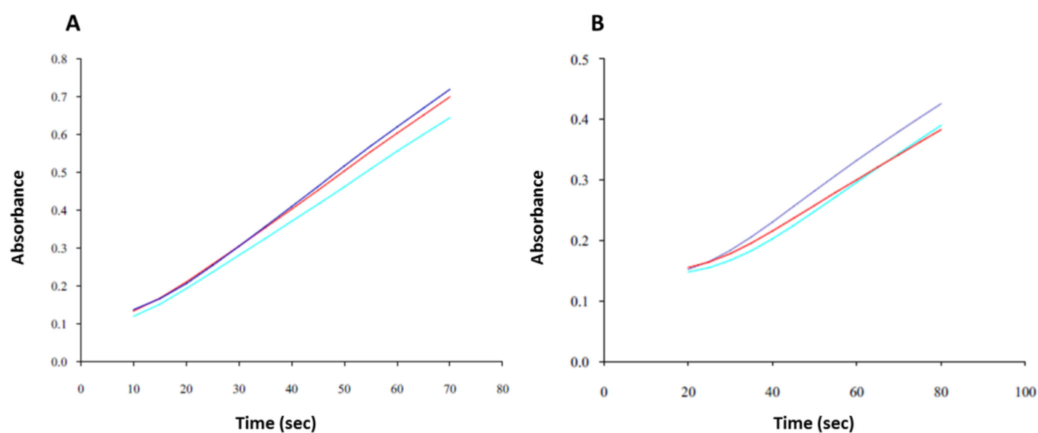


Figure 5: The correlation between the absorbance intensity and the formation of oxidized co-substrate ($ABTS^{+}$) for peroxidase activity. A: the reaction catalyzed by rVHb, B: the reaction catalyzed by rVHb-SOX

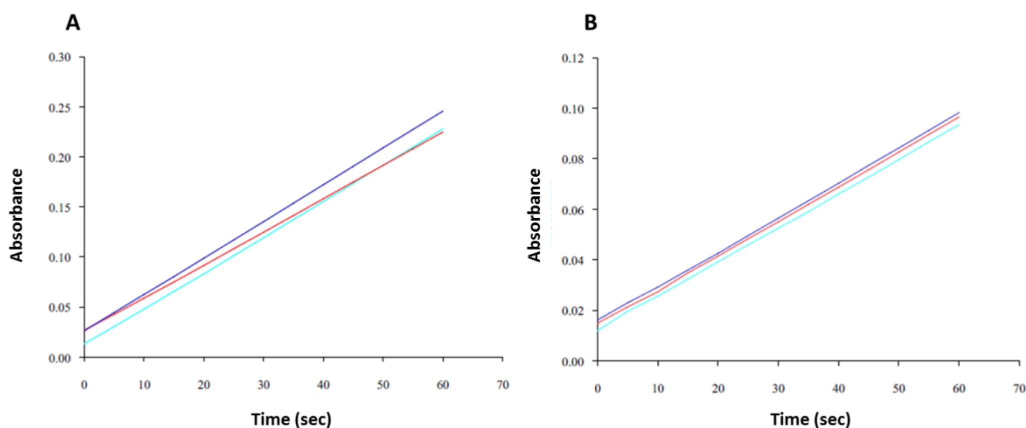


Figure 6: The correlation between the absorbance intensity and the formation of quinoneimine dye for SOX activity. A: the reaction catalyzed by rSOX, B: the reaction catalyzed by rVHb-SOX

Table 1: Relative peroxidase and SOX activities for rVHb, rSOX and rVHb-SOX

Recombinant proteins	Peroxidase activity				SOX activity			
	Specific activity		Relative activity		Specific activity		Relative activity	
	U/mg	U/μmol	U/mg	U/μmol	U/mg	U/μmol	U/mg	U/μmol
rVHb	2.2 ± 0.117	36.37 ± 1.932	100	100				
rSOX					2.74 ± 0.146	117.87 ± 6.283	100	100
rVHb-SOX	1.00 ± 0.092	58.26 ± 5.501	46	164	1.00 ± 0.003	58.46 ± 0.208	36	51

Discussion

Sarcosine oxidase from *Bacillus* sp. BSD-8 has been recognized as a thermostable monomeric enzyme (Guo K *et al*., 2006). These features render it is proper for making fusion protein; it is expected to have less hindrance after fusing with the other protein, comparing to multimeric protein, and it should be more stable comparing to non-thermostable protein. To incorporate peroxidase activity, *Vitreoscilla* hemoglobin, a dimeric bacterial hemoglobin from *Vitreoscilla* spp., which was reported to exhibit peroxidase activity by our group was chosen. VHb was reported to dissociate into monomer at pH 7-8. However, the protein exhibits maximal peroxidase activity at this condition (Kvist M *et al*., 2007). Therefore, these properties make SOX and VHb a perfect pair for generating a fusion protein with SOX and peroxidase activities. In this study, genes encoding both proteins were tandemly fused head-to-tail. Two versions of fusion gene were generated, SOX-VHb and VHb-SOX. However, mature protein with expected enzymatic activities can be obtained only from VHb-SOX fusion. The direct head-to-tail fusion of SOX-VHb may, somehow, prevent the protein from proper folding and maturation, which resulted in protein aggregation and loss of enzymatic activity.

After purification, SDS-PAGE analysis revealed that all recombinant proteins were produced with >95% homogeneity (Fig. 3). The recombinant VHb, SOX and VHb-SOX fusion protein yielded 46, 10 and 11 mg protein/Liter of culture, respectively. This indicated that construction of the fusion protein does not have any effects on the yield of the protein. In spectral property assay, the fusion protein showed heme absorbance peak (412-414 nm) similar to that of rVHb. This peak indicated the presence of heme with iron in ferric (Fe^{3+}) state, which plays an important role in peroxidase activity. In addition, the absorbance peak at 455 nm was observed from rSOX indicating the presence of oxidized FAD, which functions as a cofactor for enzyme catalysis to complete the reaction of the protein activity. However, the FAD peak (455 nm) was hardly observed. The FAD peak might be hindered by the heme peak since these two peaks were in proximity but the absorptivity of the heme group was much more than that of the FAD group. The produced recombinant proteins were further measured for enzymatic activity. The relative

peroxidase and SOX activities of the fusion protein were reduced to <50%. Nevertheless, these results indicated that the engineering strategy affects structure and, hence, activity. The fusion partner may hinder the active site of each other. To solve this problem, a short linker such as $(Gly_4Ser)_2$ may be placed between the two protein to create a proper distance. In addition, a bigger protein such as maltose binding protein (MBP) may also be used. The MBP will not only function as a spacer but also help to increase the solubility of the protein.

In conclusion, the VHb-SOX fusion protein was successfully produced through genetic and protein engineering. The protein retained peroxidase and SOX activity up to 46% and 36%, respectively. Although the enzymatic activities of the fusion protein were partially reduced, the protein contained both peroxidase and SOX activities, expecting to be able to use for sarcosine determination in blood and urine samples in the future.

Acknowledgements

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