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Comparison of recombinant α-hemoglobin from *Crocodylus siamensis* expressed in different cloning vectors and their biological properties

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ABSTRACT

Hemoglobin (Hb) is an important component in red blood cells of the vertebrate. It is a major respiratory protein with oxygen or carbon dioxide transport function. Hb has been reported to contain bioactive peptides which have antibacterial and antioxidant activities. In this study, the alpha-chain hemoglobin(αHb) gene of *Crocodylus siamensis* was cloned into the three different expression vectors and expressed in *Escherichia coli* BL21 (DE3). The recombinant αHb proteins from all constructs could be expressed and purified. The result from UV–visible absorption spectra showed a similar pattern of all recombinant proteins to the oxy-hemoglobin form of intact Hb. The different recombinant αHb could exhibit antioxidant activities. All recombinant proteins could inhibit the growth of *Bacillus* spp. Especially, most of the recombinant proteins could inhibit the growth of *Bacillus amyloliquefaciens* TISTR 1045 better than intact one. The result obtained from this study can provide us further information about the possibility using of αHb as a supplementary food.

1. Introduction

The Siamese crocodile (*Crocodylus siamensis*) is a critically endangered species of freshwater crocodiles. Crocodiles can stay in water for an hour without coming up to breathe [1]. This is possibly due to their high hemoglobin (Hb) transfer capability to deliver oxygen from lungs to tissues efficiently. Interestingly, crocodilians live with opportunistic bacterial infection but normally suffer no adverse effects. They are not totally immune to microbial infection, but their resistance is remarkably effective [2]. The explanation for this may be due to the presence of some molecules in crocodile blood that could accelerate the promote process [3]. Crocodile bloods in various tissues, such as lung and adrenal have been reported as being antibacterial [4]. Plasma [5], serum [6–8] and white blood cell of crocodile (*Alligator mississippiensis*) have been shown to inhibit bacterial growth with broad-acting properties [8,9]. Likewise, plasma and serum of *C. siamensis* were reported to exhibit antibacterial properties [10,11]. They were observed to improve wound healing in a mouse model (unpublished data). More recently, Leucrocin I-IV from white blood cell extract of *C. siamensis* had been shown strong antibacterial activity toward *Staphylococcus epidermidis*, *Salmonella typhi* and *Vibrio cholerae* [12]. In addition, Hb also contains many bioactive peptides [13].

Hb is the metalloprotein, contains in mainly component of the red blood cells in all vertebrates. It's an oxygen transporting protein which is a heterotetramer molecule, consisting of two identical alpha subunits and two identical beta subunits. Each subunit associates with a heme group. Numerous reports have been disclosing Hb as an antimicrobial protein which inhibits the growth of some Gram-negative, Gram-positive bacteria and fungi [14–16]. In addition, crocodile Hb was also reported to be an antioxidant substance [17,18]. In previous studies, Parish and coworkers elucidated that the antimicrobial activity is derived from the protein in the Hb itself and not from the heme since removal of

Abbreviations: Hb, hemoglobin; αHb, alpha-chain hemoglobin; βHb, beta-chain hemoglobin; IPTG, isopropyl-β-D-thiogalactopyranoside; IMAC, immobilized metal affinity chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTT, dithiothreitol; GST, glutathione S-transferase; Trx, thioredoxin; PMSF, phenylmethylsulfonyl fluoride.

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the heme prosthetic group did not lead to decreases in its potency [15]. However, heme iron played an important role for the antioxidant property of bovine Hb [19]. Furthermore, other studies found that the structure of Hb from crocodile blood exhibits high oxygen-transfer capability and showed strongly an antioxidant property [17]. Moreover, alpha-chain Hb (αHb), beta-chain Hb (βHb) and fragmented Hb of *C. siamensis* demonstrated both antibacterial and antioxidant activities [18]. It was shown that the αHb exhibited higher antioxidant activity than the βHb [18]. In addition, the hydrophobicity of the αHb significantly rises more than the βHb [18]. The antioxidant property might be associated with some amino acid residues, such as Tyr, Met, His, Lys and Trp [18]. The increasing degree of their hydrophobicities is related to their antioxidant properties [9,20–24]. In previous work, the gene encoding the alpha- and beta-chain Hb from *C. siamensis*, a species of freshwater crocodile in Thailand, was successfully cloned and analyzed. Both constructs were made in pET vector (pET-17b) and induced by IPTG within *Escherichia coli* Rosetta pLysS [3]. However, one major drawback to this system is that the vast majority of protein expressed was found within insoluble inclusion bodies and thus recovery of soluble protein was limited. In addition, the first time that heme-bound crocodile αHb have been expressed in bacteria without *in vitro* heme reconstitution in pET-17b and induced by an auto-induction system with in *E. coli* BL21 (DE3) [25]. However, most of the overexpressed αHb protein was found to be aggregated proteins. Both the monomeric and the dimeric protein configuration formed by intermolecular disulfide bond could be purified as soluble protein [25]. Refolding of the expressed protein is generally considered as a difficult task due to the insolubility of the recombinant protein which is based on its property inherently derived from amino acid sequence. The amino acid sequence in turn determines the physical properties of the protein, including its stability, pI, hydrophobicity, and molecular weight. All of which can directly affect the expression levels and solubility of the protein [13]. To provide information about biological function and structural characterization of this important protein, various systems were developed to produce recombinant αHb in *E. coli*. However, sometime it is difficult to express eukaryotic proteins in *E. coli* since the bacterium lacks the machinery necessary for eukaryotic post-translational modifications, and inclusion bodies are often observed [14]. Adding fusion protein tags, such as glutathione S-transferase (GST), thioredoxin (Trx), and maltose binding protein (MBP) often improves the solubility of recombinant expression products. Moreover, Trx and GST are the two most frequently used carrier proteins for fusion expression of antimicrobial peptides [26].

In this work, the gene encoding the αHb was cloned in different expression vectors in order to compare their properties. Afterwards, UV-spectra profile, antimicrobial and antioxidant activities of different recombinant αHb proteins were studied.

2. Materials and methods

2.1. Cloning of the alpha-chain hemoglobin

The reaction was performed in a 25 μL reaction mixture containing *Taq* polymerase buffer, 10 mM dNTP, 25 mM $MgCl_2$, 10 μM of primers (Table 1), 2.5 U of *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 2 μL of αHb gene construct in pET-17b was used as template. The PCR condition following previously described. The PCR products were cloned into *Nco*I/*Xho*I site of pET-32a vector (Novagen, Medison, WI, USA), *Bam*HI-HF/*Xho*I site of pET-28a vector (Novagen, Medison, WI, USA) and transformed into *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) by electroporation. The transformed cells were plated and cultivated in Luria–Bertani (LB) medium and auto-induction medium. The plasmid was purified and the cloned product was confirmed by sequencing.

Table 1
The nucleotide sequence of primers used in this experiment.

Primer name	Sequence (5′→3′) (restriction sites underlined)
αHb_p28a, F1	CGCGGATCCATGGTGCTGTCTTCGGATGAT
αHb_p28a, R1	CCCCGCTCGAGACGGTACTTGGAGGTCAGC
αHb_p32a, F2	GTGCTGTCTTCGGATGATAAGTG
αHb_p32a, R2	CCCCGCTCGAGACGGTACTTGGAGGTCAGC

2.2. Expression of the recombinant proteins

A single colony of *E. coli* strain BL21 (DE3) containing the recombinant plasmid αHb in pET-28a and pET-32a vector was expression by using auto-induction system of the method of Studier [27]. In auto-induction method, the *E. coli* cell containing recombinant plasmid was inoculated in a 20 mL of ZYM-5052 starter medium (10 g/L tryptone, 5 g/L yeast extract, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.05% w/v glucose, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, and 50 mM NH_4Cl) supplemented with antibiotic (ampicillin/kanamycin (50 μg/mL)) incubated at 37 °C with agitation at 180 rpm, overnight. Ten milliliters of inoculum was placed in an Erlenmeyer flask containing 1,000 mL of ZYP-5052 medium (10 g/L tryptone, 5 g/L yeast extract, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.01% v/v glycerol, 0.001% w/v glucose, 0.004% w/v α-lactose, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, and 50 mM NH_4Cl) with antibiotic (ampicillin/kanamycin (50 μg/mL)). The culture was incubated at 37 °C with vigorous shaking at 180 rpm until the optical density measured at 600 nm (OD_{600}) reached a value of ~1.0, at which point the culture was continue incubated for 6 h. After induction, the culture was centrifuged at 6,000 rpm for 20 min at 4 °C and pellet was kept at −20 °C for next step experiment.

2.3. Purification of the recombinant proteins

The *E. coli* cells containing recombinant plasmid of αHb gene were overexpressed in 1,000 mL of auto-induction medium with supplemented suitable of antibiotic. After centrifugation at 6,000 rpm for 20 min at 4 °C, the cell pellets were resuspended in 10 mL of lysis buffer (20 mM sodium phosphate, 500 mM sodium chloride, 20 mM imidazole, pH 7.4). Then, the bacteria cells suspension were added 0.1 mg/mL of lysozyme and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Calbiochem, CA) and incubated on ice for 1 h. Glycerol (20% v/v) (Carlo Erba, France) was added to bacteria cells suspension in order to prevent hydrophobic interaction between proteins and stabilizes proteins [28], before the cells were disrupted by sonication and centrifuged at 12,000 rpm for 10 min at 4 °C. The clarified supernatants were filtered through a 0.2 μm before purification using an immobilized Ni^{2+} affinity chromatography column HisTrap FF (1 mL, GE Healthcare). The soluble proteins were loaded onto a column that was equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4 and 5 mM DTT). Then the column was washed with the same buffer, containing 150 mM imidazole. After washing the column, then the protein was eluted from the column with 500 mM imidazole elution buffer. The eluted protein was dialyzed with dialysis buffer (20 mM sodium phosphate buffer, 5 mM DTT, and 20% v/v glycerol). The clarified protein was concentrated by vacuum concentrators (Savant Instrument, Inc. USA) following manufacturer's instructions. The purified protein was analyzed by SDS-PAGE and confirmed by western blot analysis.