



## Full length article

Role of hemoglobin from blood clam *Scapharca kagoshimensis* beyond oxygen transportBin Xu <sup>a,1</sup>, Jun Zhao <sup>a,1</sup>, Zhao Jing <sup>a,b,1</sup>, Yanan Zhang <sup>a,c</sup>, Ying Shi <sup>a</sup>, Tingjun Fan <sup>a,\*</sup><sup>a</sup> Department of Marine Biology, College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China<sup>b</sup> Department of Histology and Embryology, Medical College, Qingdao University, Qingdao 266021, China<sup>c</sup> Department of Biology, Medical College, Qingdao University, Qingdao 266021, China

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## ABSTRACT

The evolutionary race between hosts and pathogens has led to a variety of adaptations. Little is known about the immunological role of hemoglobin (Hb) in antimicrobial immune responses. Results showed that a 31.2 kDa monodimer Hb (skHbI) and a 57.8 kDa heterotetramer Hb (skHbII) from the blood clam, *Scapharca kagoshimensis*, had phenoloxidase (PO)-like activities and antimicrobial activities. Both were found capable of oxidizing *L*-DOPA, catechol and hydroquinone. Their PO-like activities were visibly greatly inhibited by oxidase inhibitors, EDTA, and divalent metal ions, and greatly enhanced by isopropanol and Fe<sup>2+</sup>, indicating that they have the properties of a metalloenzyme and a catecholase-type PO as well. They also showed obvious anti-bacterial activities against gram-positive bacteria but not against either gram-negative bacteria nor fungi. The anti-bacterial activities levels were a result of the generation of reactive oxygen species (ROS) of superoxide anions. These results indicate that skHbI and skHbII, not only function as iron-containing oxygen carriers, but also exert anti-bacterial activities and catecholase-type oxidizing activities. The fact that skHbII exerts high level of PO-like activity indicates different roles in the innate immunodefense system. These results may improve understanding of the multiple functions of invertebrate Hbs beyond serving as oxygen carriers and may provide insight into how the fundamental and universal mode of the innate immune system has persisted in respiratory proteins throughout the course of evolution.

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## 1. Introduction

Throughout evolution, pathogenic microbes have developed many ways to invade hosts by evading immune defenses, while hosts have evolved various individualized strategies to resist invasion by improving their autologous immune defenses [1]. The innate immune response is a multi-step process, which is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) and followed by successive cascade signal transduction to activate downstream antimicrobial effectors [2]. Though interesting results have been obtained for host receptors and signaling cascades, little is known about the ancestral forms of immunologic

surveillance, which might involve a direct link between pathogen sensors and antimicrobial effectors in hosts [3].

Antimicrobial activity mediated by prophenoloxidase (proPO) and reactive oxygen species (ROS) is the primary method of host defense in invertebrates [4] and vertebrates [5,6], respectively. Previous studies have shown that hemocyanin (Hc), a copper-containing invertebrate respiratory protein, can be converted into a PO-like oxidase [3,7–14], and has both antibacterial and antiviral properties [3,15,16]. Recently, hemoglobin (Hb), a widespread iron-containing respiratory protein found in vertebrates and some invertebrates [6], has been found capable of producing ROS [3,14,17,18] and of exerting pseudoperoxidase and deoxygenase activities [3,18–20], which are involved in host defense. However, whether Hb exerts or can be converted to exert PO-like oxidase activity is not fully understood.

The blood clam, *Scapharca kagoshimensis*, is a marine shellfish that uses Hb as an oxygen carrier. Hb from *S. kagoshimensis* (skHb) consists of a homodimer (skHbI<sub>2</sub>) and a heterotetramer (skHbIIA<sub>2</sub>skHbIIB<sub>2</sub>) (unpublished data), which are very similar to

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Hbs from *Scapharca inaequalis* [21]. They are less similar to Hbs from vertebrates in cDNA sequences, but there is high similarity with vertebrate Hb in secondary and tertiary structures (unpublished data). This makes the lower skHbs attractive as molecular models for studies of the immunoreactive activity of respiratory proteins. The present study was designed to investigate the PO-like and antibacterial activities of skHbs during the innate immune response. Its results provide new insight into the immunological functions and underlying mechanisms of Hb beyond its role as a respiratory protein.

## 2. Materials and methods

### 2.1. Blood clams and microbial strains

Fresh healthy blood clams of *S. kagoshimensis*, with an average size of  $3 \times 4$  cm, were obtained from Nanshan Fish Market (Qingdao, China). After being maintained for 24 h in aerated sterile seawater containing 1000 IU/ml of penicillin and 1000 µg/ml of streptomycin (Shandong Lukang Chenxin Pharmaceutical, China) at room temperature, haemolymph was collected. All bacteria, including gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Micrococcus tetragenus*) and gram-negative bacteria (*Escherichia coli*, *Clostridium perfringens*, *Proteus species*, *Vibrio anguillarum*, and *Vibrio harveyi*), and fungi, including molds (*Penicillium glaucum* and *Aspergillus niger*) and yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*), were obtained from the Microbiotechnology Laboratory in our college.

### 2.2. Purification of skHbs

Hemolymph was collected from the mantle sinusoids of 1.0 kg of clams using a 5.0 ml syringe containing anticoagulant solution, consisting of 2.05 g of glucose, 0.8 g of citrate and 0.42 g of NaCl brought to a volume of 100 ml with distilled water, then centrifuged at  $1000 \times g$  for 10 min. The precipitate was resuspended in 0.3 ml of 50 mM Hepes buffer (pH 7.2). After freezing and thawing 3 times, the blood cell suspension was centrifuged at  $8000 \times g$  for 45 min. The supernatant was considered to be a crude skHb preparation. The crude skHb was loaded onto a Sephacryl S-100 (1.6 cm  $\times$  40 cm) (GE, U.S.) gel-filtration column and eluted with 50 mM Hepes buffer (pH 7.2). The fractions were collected with 1.0 ml per tube every 2 min. Ultraviolet absorption was monitored at 280 nm and 415 nm. The enzymatic activities of the fractions were measured using 15 mM of *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) and fractions with high enzymatic activities were collected as purified skHbs. The molecular weight of purified skHbs were also determined by column chromatography at conditions as described above using bovine serum albumin (67.0 kDa), chicken ovalbumin (45.0 kDa) and cytochrome C (12.4 kDa) as standard molecular weight proteins. All of these above manipulations were performed at 4 °C.

### 2.3. SDS-PAGE and mass spectrometry analysis

Denaturing SDS-PAGE was performed using the method described by Laemmli on 12.5% gels, with low-molecular-weight protein markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; chicken egg ovalbumin, 42.7 kDa; carbonic anhydrase, 31 kDa; chicken egg lysozyme, 14.4 kDa) (Shanghai Biotech, China) [22]. After denaturation and electrophoresis, samples were stained with 0.05% of Coomassie brilliant blue R-250. For mass spectrometry analysis, bands of purified skHbs were carefully excised from the SDS-PAGE gel and destained in solution (30%

methanol and 10% acetic acid). After trypsinization, the bands were subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) analysis. The spectrums obtained were analyzed using the online Mascot software package ([www.matrixscience.com](http://www.matrixscience.com)).

### 2.4. PO-like activity assay

The PO-like activity of skHb was measured using a modified version of the method described by Ashida and Dohke [23]. Approximately 10 µl of skHb preparations and 10 µl of 20 mM *L*-DOPA were dissolved in 20 mM Tris–HCl buffer (pH 7.1). After 40 min of incubation at 28 °C, 280 µl of ice-cold distilled water was added to each sample to stop the reaction. Then the absorbance of the reaction mixture was measured at 490 nm with a UV-2450 spectrophotometer (Shimadzu, Tokyo, Japan). The PO-like activity was estimated as the increment in the rate of absorbance and an increase of 0.001 per min was defined as 1 unit (U): Activity =  $A_{490} \times 10^{-3}/\text{min}$ .

To assess the activation of PO-like activity of purified skHbs, the reaction mixture containing 10 µl of the different concentrations of isopropanol, urea, SDS and trypsin (all dissolved in 20 mM Tris–HCl buffer at pH 7.8) was incubated with 10 µl of the skHb solutions and subjected to spectrophotometric measurement. The optimal temperature and pH of the PO-like activity of the purified skHb were measured as described previously at different temperatures from 20 °C to 60 °C and pH from 5.0 to 9.0 [12]. The kinetic parameters of PO-like activity of purified skHbs on *L*-DOPA, catechol, and hydroquinone were determined using the Lineweaver–Burk plot method [24].

### 2.5. Effect of inhibitors and metal ions

The effects of inhibitors and metal ions on the PO-like activity of the purified skHbs were measured [12]. Briefly, 10 µl of different skHbs was incubated with 10 µl of different inhibitors, chelators or ions before spectrophotometric measurement, using sodium sulfite, ascorbic acid, citric acid, cysteine, benzoic acid, thiourea, 1-phenyl-2-thiourea, ethylenediaminetetraacetic acid (EDTA), diethyldithiocarbamate (DETC),  $\text{Fe}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Cu}^{2+}$ . The ability of some metal ions to restore PO-like activity was investigated using EDTA and DETC at different concentrations [25].

### 2.6. Preparation of bacterial and fungal suspension

Gram-positive bacteria (*S. aureus*, *B. subtilis*, and *M. tetragenus*) and gram-negative bacteria (*E. coli*, *C. perfringens*, *P. species*, *V. anguillarum*, and *V. harveyi*) were inoculated on nutrient agar medium (3 g of beef extract, 10 g of peptone, 5 g of NaCl, and 20 g of agar, brought to a volume of 1000 ml with distilled water, with a pH of 7.2–7.4) and cultured at 37 °C for 24 h. Molds (*P. glaucum* and *A. niger*) were inoculated on PDA medium (200 g of potato, 20 g of sucrose, 20 g of agar, increased to a volume of 100 ml with distilled water, with a pH of 7.0–7.2) and cultured at 28 °C for 48 h. Yeasts (*S. cerevisiae* and *S. pombe*) were inoculated on YPD medium (20 g of peptone, 10 g of yeast extract, 20 g of glucose, 20 g of agar, increased to 1000 ml with distilled water, with a pH of 5.0–5.5) and cultured at 28 °C for 48 h. The cultures of each type of bacteria and fungi were centrifuged at  $5000 \times g$  for 2 min. The precipitates from each strain were resuspended with 0.9% sterile saline to a final concentration of  $10^5$  cfu/ml, but mold cultures were filtrated with sterile gauze after resuspension.

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