Fish & Shellfish Immunology 51 (2016) 9–16

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

A preliminary study on the antibacterial mechanism of *Tegillarca granosa* hemoglobin by derived peptides and peroxidase activity

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ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form
30 January 2016
Accepted 5 February 2016
Available online 12 February 2016

Keywords:
Tegillarca granosa
Hemoglobin
Peptides
MIC
Antibacterial activity
Peroxidase activity

ABSTRACT

The blood clam, *Tegillarca granosa*, is one of the few bivalve molluscs containing hemoglobin (Hb). In the present study, we purified two types of *T. granosa* hemoglobin, Tg-HbI and Tg-HbII, using size exclusion chromatography and measured their antibacterial and peroxidase activities. We also tested antibacterial activities of peptides prepared by trypsin digestion of purified Tg-Hb and reversed-phase high-performance liquid chromatography purification. Purified Tg-HbI and Tg-HbII showed antibacterial activity against *Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis*, and *Bacillus firmus*, with differences in minimal inhibitory concentrations (MICs), but lacked antibacterial activity against *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio harveyi* and *Staphylococcus aureus*. In contrast, 7 Tg-Hb derived peptides exhibited varying degrees of antibacterial activity against *V. alginolyticus* (MICs: 12–200 μg/ml), *V. parahaemolyticus* (11–100 μg/ml) and *V. harveyi* (1–200 μg/ml). The antibacterial activity of Hb derived peptides was confirmed by fluorescence microscopy. In addition, peroxidase activity was detected in Tg-HbI and Tg-HbII. The results indicated that in addition to functioning as a respiratory protein *T. granosa* hemoglobins likely play a role in host antibacterial defense probably via a peroxidase activity of native molecules and some internal peptides released from the proteins.

1. Introduction

The blood clam *Tegillarca granosa* is a bivalve mollusc of the Arcidae family. It inhabits intertidal mudflats along the coasts of most Indo-Pacific region, and is particularly common and commercially important in East and Southeast Asia. Unlike most molluscs and other invertebrates that use hemocyanin for oxygen transportation, *T. granosa* and other Arcidae species have hemoglobin enclosed in red blood cell [1].

Hemoglobin represents a major respiratory protein, transporting oxygen (O_2) and carbon dioxide (CO_2) in vertebrates and a few invertebrate species that have erythrocytes [1–4]. Hemoglobin usually exists as polymers, but the polymerization forms differ between host species. Human hemoglobin, for example, is present as a tetramer consisting of 2 α subunits and 2 β subunits in adults, and 2 α subunits and 2 γ chains in infants [5]. On the other hand, the hemoglobin of *Scapharca inaequivalvis*, a blood clam species belonging to the same family (i.e., Arcidae) as *T. granosa*, has been characterized by X-ray crystallography to be present in 2 forms, a homodimer and heterotetramer [6,7]. We have identified 3 genes (i.e., *Tg-HbI*, *Tg-HbIIA* and *Tg-HbIIB*) that appear to encode hemoglobin subunits in *T. granosa* [8,9]. The native status of *T. granosa* hemoglobin however remains to be characterized.

There have also been studies indicating the involvement of hemoglobin in other physiological reactions [10–18], and of particular interest is the finding that vertebrate hemoglobin has antibacterial activity [19–21]. In addition to the entire hemoglobin molecules, peptides derived from hemoglobin have also been found to antibacterial activities [22]. Human hemoglobin and its derivative peptides for example exhibited inhibitory activities observed against a variety of Gram-positive and -negative bacteria, fungi and parasites [12]. Similarly intact and fragmented Hb of crocodiles (*Crocodylus siamensis*) has demonstrated antibacterial activities [23]. In addition, peptides obtained from hemoglobin of several other vertebrate species such as bovine, porcine and Japanese eel

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http://dx.doi.org/10.1016/j.fsi.2016.02.004
1050-4648/

have been reported to have antibacterial activities against a variety of bacteria [21,24–27]. We have found that Tg-Hb gene expression was significantly up-regulated in clams challenged by *Vibrio parahaemolyticus*, lipopolysaccharide (LPS) and peptidoglycan, and Tg-Hb genetic polymorphisms were associated with hosts' resistance to *V. parahaemolyticus* infections [8]. However there is still no evidence for the presence of antibacterial activities in hemoglobin and related derivative peptides from invertebrates.

The objectives of the present study were thus to (1) purify and characterize *T. granosa* hemoglobin, (2) prepare internal peptides from purified *T. granosa* hemoglobin, (3) determine the antibacterial activities of the native *T. granosa* hemoglobin and its derivative peptides, and (4) analyze the peroxidase activity of the purified *T. granosa* hemoglobin. Results of the study generated direct evidence for *T. granosa* hemoglobin to function as an antibacterial protein.

2. Materials and methods

2.1. Purification of hemoglobins

Blood clams, about 30 mm in shell length, were collected from a clam farm in Ningbo, Zhejiang Province, China, and acclimatized in seawater (25 °C, 30‰) for one week before processing. Hemolymph collected from the mantle sinusoids of the clams using a 2.0 ml syringe was mixed with citrate anticoagulant buffer (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 Mm EDTA, pH 4.6) and centrifuged for 10 min at 1000*g* and 4 °C. After supernatant was removed, hemocyte pellets were washed 3 times in 0.5% NaCl and then suspended in 0.05 mol/ml phosphate-buffered saline (PBS, pH 7.4). The hemocyte suspension was homogenized with PBS and centrifuged for 30 min at 8000*g* under 4 °C. The supernatant was then collected and an aliquot of 5 ml at 19.55 mg/ml total proteins was loaded into a Sephacryl S-100 high HR column (2.6 × 60 cm) pre-equilibrated with 0.05 mol/ml PBS and 0.15 mol/ml NaCl for a size exclusion chromatography. The column was eluted with the same buffer at a flow rate of 0.2 ml/min. The elution was monitored for absorbance at 280 nm and 415 nm and fraction-collected according to absorbance peaks. Collected fractions were analyzed using Tis-Tricine SDS-PAGE in 10% separation gel and Coomassie blue staining for 2 h [28] and antibacterial activities as described below. The gel pieces containing protein of interest were cut and the proteins were identified by LC-MS/MS.

Protein concentration was measured using Bradford assay with bovine serum albumin (BSA) as the standard [29]. Protein molecular weight was estimated by size exclusion chromatography in a Sephacryl S-100 column with 3 protein standards according to the method reported by Ref. [30]. The three standard proteins included bovine haemoglobin (67 kDa), chicken ovalbumin (45 kDa) and cytochrome c (12.5 kDa).

2.2. Preparation of Tg-Hb derived peptides

Trypsin hydrolysis was used to release internal peptides from Tg-Hb. An aliquot of purified Tg-Hb was mixed with trypsin (Sigma, USA) to a final protease: sample ratio of 1:50 (w/w). After incubation at 37 °C for 2 h, ice-cold trichloroacetic acid (TCA) was added into the mixture to a final concentration of 10% to precipitate proteins and released peptides.

Peptides were purified from the precipitant by reversed-phase high-performance liquid chromatography (RP-HPLC) in a C-18 column (1.0 × 25 cm). After sample loading, the column was eluted with a gradients of 0%–60% acetonitrile over 20 min at a flow rate of 3 ml/min. The elution was monitored for absorbance at 220 nm and fraction-collected according to absorbance peaks. Collected fractions were vacuum-dried and dissolved in 50 mM PBS for antibacterial activity measurement and amino acid sequencing.

Molecular weight and amino acid sequence of the purified peptides were determined using matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometer (MALDI-TOF-MS) operated in linear mode. The theoretical pI and molecular mass were estimated with ExPASy (http://www.expasy.ch/tools/peptide-mass.html). Secondary structure was predicted using the Garnier-Osguthorpe-Robson (GOR) method (http://www.expasy.org/tools/). Antibacterial activities of each purified peptide were measured as described below.

2.3. Antibacterial assays

Antibacterial activities of Tg-Hbs and their derived peptides were first detected using inhibition zone assays on Gram-negative bacteria *Escherichia coli*, *Vibrio alginolyticus*, *V. parahaemolyticus*, and *Vibrio harveyi*, and Gram-positive *Bacillus subtilis*, *Bacillus firmus*, and *Staphylococcus aureus* by the inhibition zone radius method. The antibacterial activity was measured as the diameter of clear zone of growth inhibition by comparison to a positive control which was chloramphenicol, and to a negative control: sodium phosphate buffer in Petri plates. Samples that showed antimicrobial activity in the inhibition zone assay were then measured using minimum inhibitory concentration assays (MICs). MICs were conducted by mixing 100 μl of bacterial suspensions with 100 μl of samples in wells of a 96-well microtiter plates. After determination of bacterial cell density of each well using nutrient agar counting method, the plate was incubated for 24 h at 28 °C for *Vibrio* species or at 37 °C for other bacteria. Bacterial density of each well was measured again on nutrient agar. The lowest protein or peptide concentration that caused 100% growth inhibition was recorded. All assays were done in triplicate.

To confirm that detected antibacterial activity was exerted by Tg-Hb or its peptides, imaging of bacterial clearance was conducted using the Live/Dead BacLight Bacterial Viability Kit (Invitrogen, USA) according to the manufacturer's instruction, with bovine serum albumin (BSA) dissolved in eluent as control. Briefly, 5 μl of bacterial suspension at 2×10^6 CFU were mixed with 5 μl of protein or peptide samples at 0.12 mg/ml in PBS and 5 μl of SYTO 9 dye dissolved in dimethyl sulfoxide at 1.67 mM. After incubation for 10 min in dark at room temperature, 1 μl of the mixture was examined by fluorescence microscopy. PBS in place of protein or peptide samples was used as control. Examinations were carried out in three replicates.

2.4. Peroxidase activity assay

Peroxidase activity was measured as described by Jiang et al. [31] with modifications. Briefly the assay was performed by mixing 1 ml of 50 mM sodium phosphate (pH 7.0) with 4 mM guaiacol, 2 mM H_2O_2, 10 μg of Tg-HbI or 20 μg of Tg-HbII in a semimicro cuvette and the absorbance at 470 nm was then monitored continuously using a spectrophotometer at 25 °C for 2 min. To further verify that the antibacterial activity was indeed attributable to ROS producing from peroxidase activity, superoxide ions were quenched with 40 mmol GSH. Controls comprised incubation of bacteria *Pseudomonas putida* with TgHb, H_2O_2 and GSH separately or in combination.

3. Results and discussion

3.1. Purification of Tg-Hbs

Proteins released from *T. granosa* hemocytes were separated in 3 absorbance peaks in size exclusion chromatography (Fig. 1A), and