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RESEARCH REPORT

Hemoglobin from the blood clam *Tegillarca granosa* (Tg-HbIIA, Tg-HbIIB): expression and antibacterial activity of recombinant proteins

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Abstract

The hemoglobins produced by *Tegillarca granosa* have antibacterial activity toward some Gram-positive and Gram-negative bacteria. In this study, the genes encoding the recombinant proteins Tg-HbIIA and Tg-HbIIB were cloned from *T. granosa* hemocytes by RT-PCR, and the proteins were expressed in *Escherichia coli* Transetta (DE3). The proteins were purified using a HisTrap FF affinity chromatography column under denaturing conditions and refolded at 4 °C by urea gradient dialysis, and the antibacterial activity of the recombinant proteins was determined. The Tg-HbIIA protein had antibacterial activity toward *Vibrio harveyi* and *Pseudomonas putida*, with the minimum inhibition concentration (MIC) values of 65.8 ug/ml and 4.11 ug/ml, respectively. The Tg-HbIIB protein had antibacterial activity toward *V. harveyi*, *P. putida* and *Acinetobacter baumanii*, with MIC values of 158 ug/ml, 39.5 ug/ml and 79 ug/ml, respectively. They had no antibacterial activity against *Staphylococcus aureus*, *E. coli*, *B. firmus*, *B. subtilis*, *S. epidermidis*, or *V. parahaemolyticus*. This study provides a basis for further research on the antibacterial function and mechanism of hemoglobin.

Key Words: *Tegillarca granosa*; hemoglobin; recombinant protein; antibacterial activity

Introduction

Hemoglobin (Hb) is a respiratory protein containing iron that has multiple biological functions and is one of the most researched proteins in the protein family (Riggs, 1991; Terwilliger, 1998; Jiang *et al*., 2007; Katsu *et al*., 2010). Hb is rare in molluscs, with most harboring copper-containing hemocyanin instead (Lieb *et al*., 2006). However, *Tegillarca granosa*, which belongs to the family Arcidae, contains abundant Hb in circulating erythrocytes (Suzuki *et al*., 1992). In our previous studies, full-length cDNAs and genomic DNA of Tg-HbIIA and Tg-HbIIB were cloned from *T. granosa.* Sequence and structural analyses revealed that Tg-HbIIA and Tg-HbIIB form A_2B_2 heterotetramers (Wang, 2012). The expression of Tg-Hbs mRNA in *T. granosa* hemocytes was markedly upregulated after *Vibrio parahaemolyticus*, LPS or PGN challenge (Bao *et al*., 2011). The purified heterotetramers of Tg-HbII exhibited antibacterial activity against *Pseudomonas putida* but no antibacterial activity against *Staphylococcus aureus*, based on an agarose diffusion test and the minimal inhibitory concentration (MIC) (Bao *et al*., 2013). And the polypeptides from Tg-HbII also exhibited antibacterial activity against *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* (Wang *et al*., 2015). These results indicated that Tg-Hbs might be involved in the immune responses against bacterial infection.

Many animals are known to that their Hb can limit the growth of or kill bacteria as a defense mechanism. Parish *et al.* (2001) found that intact Hb tetramers, alpha and beta subunits, from human, exhibited considerable activity against Gram-positive and Gram-negative bacteria, and fungi (Parish *et al.*, 2001). Many previous studies demonstrated that bovine Hb is a rich source of antimicrobial peptides (Daoud *et al*., 2005; Nedjararroume *et al*., 2008; Przybylski, 2015). Antibacterial activities of Hb from non-mammalian vertebrates such as *Crocodylus siamensis* and Japanese eel (*Anguilla japonica*) also have been confirmed. Hb, α-chain, β-chain and fragmented Hb of *C. siamensis* can inhibit growth of Gram-positive bacteria *B. subtilis*, *B. amyloliquefaciens*, and *B. pumilus* (Srihongthong *et al*., 2012). Zhang *et al.* (2013) isolated an antibacterial

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Table 1 Primers used in the present study

Gene	Genbank number	Primer sequence	Product size
Tg-HbIIA	HQ729976	F: GGATCCGTTGATGCAGCAGTTGCAAAT R: AAGCTTCCATTGATGGTTGGTCCAGAT	492bp
Tg-HbIIB	HQ149306.1	F: GGATCCGGTGTCAACGAAGCAATCAAAG R: AAGCTTAATAGTCGTTTTTTCTCATGC	483bp

peptide from Hb alpha in the liver of Japanese eel. And they found that its synthetic peptide also had strong antibacterial activities against Gram-positive and Gram-negative bacterias. There are several Hb of invertebrate have been reported. It reported that *Scapharca Kagoshimensis* Hb had antibacterial activity against *S. aureus, B. subtilis, Micrococcus tetragenus* (Xu *et al*., 2015). In a word, nature Hb has broad-spectrum antibacterial activity and different sources of Hb with different antimicrobial activity. However, few study reported that recombinant Hb have antibacterial activity.

In the present study, we constructed Tg-HbIIA and Tg-HbIIB expression vectors, expressed these vectors by IPTG induction, and purified the proteins by affinity chromatography. In addition, through the analysis of the recombinant Hb antibacterial activity by *in vitro* bacteriostatic experiments, this work provides the basis for the further study of the antibacterial immunity function of Hb and its mechanism.

Materials and Methods

Experimental clams, collection of hemocytes and experimental bacteria

T. granosa (average shell length approximately 30 mm) were purchased from an aquaculture farm (Ningbo, China). After the *T. granosa* were vivisected and the blood was quickly drawn, the hemolymphs were centrifuged at 1000 rpm for 10 min to harvest the hemocytes. The cells were flash-frozen in liquid nitrogen and then stored at -80 °C for RNA extraction. *E. coli* (ATCC 35218), *P. putida* (CGMCC 1.0593), *S. aureus* (ATCC 29213), *V. harveyi*, *V. parahaemolyticus,* and *V. alginolyticus, B. subtilis, S. epidermidis, B. firmus*, and *A. baumanii* are provided by Zhejiang Wanli College Microbiology and Environmental Engineering Key Laboratory.

Cloning and expression vector construction

Total RNA was isolated from the *T. granosa* hemocytes using Trizol (Invitrogen, USA) and was reverse transcribed using a Reverse Transcription System Kit (Promega, USA). The primers were designed to amplify the ORF of the full-length Tg-HbIIA and Tg-HbIIB cDNAs. All primers were synthesized by Sangon Biotechnology (China) and are shown in Table 1. The amplified products with a size of approximately 500 bp were cloned into the pMD-18T vector (TaKaRa, Japan), and the recombinant plasmids were named pMD-18T/Tg-HbIIA and pMD-18T/Tg-HbIIB. The recombinant plasmids were digested with *BamHI* and *HindIII* (New England biolabs, UK), which were then inserted into the corresponding sites of the expression vector pET30a (TaKaRa) to generate pET30a/Tg-HbIIA and pET30a/Tg-HbIIB. Three positive clones for each product were sequenced at Sangon Biotechnology (China).

Expression and purification of Tg-HbIIA and Tg-HbIIB

The pET30a/Tg-HbIIA and pET30a/Tg-HbIIB plasmids were separately transformed into *E. coli* Transetta (DE3) (TransGen, China). For the expression of Tg-HbIIA and Tg-HbIIB recombinant proteins, the positive Transetta (DE3) cells were grown at 37 °C in 1 L LB liquid medium with 50 mg/ml of kanamycin. When the OD_{600} reached between 0.5 and 0.8, the culture was induced with 1 mM IPTG (Aladdin USA). To determine the optimal conditions for the expression, the expression of the target proteins at 37 °C, 30 °C, 28 °C, 25 °C, and 20 °C after 2 h, 3 h, 4 h, 5 h and 6 h of induction was examined. Then, a large number of recombinant proteins were expressed in the best condition. Finally, the cells were harvested by centrifugation at 6,000 rpm for 20 min at 4 °C and stored for next step of the experiment. All of the wet cells were resuspended in lysis buffer (50 mM $NaH_2PO_4{\cdot}2H_2O$, 2 mM EDTA, 100 mM NaCl, 0.5 % Triton X-100) and disrupted by sonication on ice. The cell lysate was centrifuged at 12,000 rpm for 30 min at 4 °C. Because the Tg-HbIIA and Tg-HbIIB were found in inclusion body, the insoluble fraction (*i.e*., inclusion body) from the lysate was resuspended in solubilization buffer (50 mM $NaH_2PO_4{\cdot}2H_2O$, 10 mM Tris-HCl, 8 M urea, pH 7.2) at 5 ml per gram of wet inclusion body and centrifuged at 12,000 rpm for 30 min to pelletize the cellular debris. To purify the proteins, a 5 ml HisTrap FF affinity chromatography column (GE Healthcare) was used in the AKTA pure protein chromatography system (GE Healthcare). As a first step, the HisTrap FF column was equilibrated with 2~3 column volumes of distilled water and equilibrating buffer (50 mM $NaH_2PO_4{\cdot}2H_2O$, 300 mM NaCl, 8 M urea, 10 mM imidazole, pH 7.2) at a flow rate of 0.5 ml/min. Then, protein samples were loaded into the column by using equilibrating buffer. Subsequently, the desired protein was eluted using elution buffer (50 mM $NaH_2PO_4{\cdot}2H_2O$, 300 mM NaCl, 8 M urea, 200 mM imidazole, pH 7.2), automatically

collected in a fraction collector and stored at 4 °C. The collected proteins were analyzed by SDS-PAGE to verify the purity of the proteins. The components of the proteins were identified by mass spectrometry, and the concentrations were estimated using a Bradford protein assay. The purified protein was denatured, so we needed to make the proteins form complexes. We added 1 mg of the purified recombinant Tg-HbIIA or Tg-HbIIB into a dialysis bag and added a slight excess of heme, ~1 mg. The samples were then dialyzed for 12 h at 4 °C using renaturation buffer (50 mM $NaH_2PO_4 \cdot 2H_2O$, 50 mM NaCl, 1 mM EDTA, 2 M reduced glutathione, 0.02 M oxidized glutathione, 10 % glycerol, 10 % glycine, pH 7.2), which contained 6 M, 4 M, 2 M or 0 M urea.

Antibacterial activity of the recombinant protein

An agar diffusion method was used for the preliminary detection of the antibacterial activity of the recombinant proteins (Tg-HbIIA, Tg-HbIIB) against the Gram-negative bacteria *E. coli, A. baumanii*, *P. putida, V. harveyi, V. parahaemolyticus,* and *V. alginolyticus* and the Gram-positive bacteria *S. aureus, B. subtilis, S. epidermidis,* and *B. firmus*. *S. aureus*, *E. coli*, *P. putida*, *A. baumanii*, *B. firmus*, *B. subtilis* and *S. epidermidis* were spread on LB solid medium and grown at 37 °C for 12 h. Then, colonies were picked into 50 ml of LB in a liquid culture flask and cultured at 37 °C for 5 h with shaking. *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* were spread on seawater solid medium in a liquid culture flask and grown at 28 °C for 12 h. Then, colonies were picked into 50 ml of seawater medium and cultured at 28 °C for 5 h with shaking. All of the bacteria were counted using a hemocytometer, diluted into 10^5 CFU/ml bacterial suspensions, and 200 µl of each test microorganism suspension was spread onto LB or seawater solid medium. Bacterial plates were cultured for 10min, and three oxford cups were then put into the medium. The recombinant proteins (Tg-HbIIA, Tg-HbIIB) and the phosphate buffer negative control (50 mM $NaH_2PO_4 \cdot 2H_2O$, 50 mM NaCl, 1 mM EDTA, 2 M reduced glutathione, 0.02 M oxidized glutathione, 10 % glycerol, 10 % glycine, pH 7.2) were added to the oxford cup. *S. aureus, E. coli*, *P. putida*, *A. baumanii*, *B. firmus*, *B. subtilis*, and *S. epidermidis* were cultured at 37 °C for 12 h, and *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* were cultured at 28 °C for 12 h. Then, the oxford cups were unplugged, and the zone of inhibition was observed. To ensure the accuracy of the bacteriostatic experiment, we performed three replicates for each kind of bacterium. The MIC of recombinant proteins (Tg-HbIIA, Tg-HbIIB) was determined using the broth dilution method. The bacterial suspensions and serially diluted recombinant proteins were added to 96-well plates at a ratio of 4:1 in a final volume of 100 µl for 10 wells. The 11th well without added recombinant protein served as a growth control. The 12th well contained 0.1 mg/ml trypsin as a positive control. The microplates of *S. aureus*, *E. coli*, *P. putida*, *A. baumanii*, *B. firmus*, *B. subtilis*, and *S. epidermidis* were incubated at 37 °C with continuous shaking. The microplates of *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* were incubated at 28 °C with continuous shaking. After 16 ~ 20 h, the OD value of each well was measured with an automatic microplate reader (SpectraMax 190, Molecular Devices, USA) set at 600 nm. The experiment was repeated 3 times. Finally the clear bacterial growth was observed both in the growth control and in the 96-well plates, and the concentrations of the wells with no bacterial growth were designated as the value of the MIC.

Results and Discussion

Cloning and expression vector construction

We cloned the Tg-HbIIA and Tg-HbIIB genes from *T. granosa* hemocytes by RT-PCR and the PCR products were inserted into pMD-18T vectors. The coding region of the mature Tg-HbIIA was amplified from the plasmid pMD-18T/Tg-HbIIA and cloned into the expression vector pET30a (+) to generate the pET30a/Tg-HbIIA. The plasmid was confirmed by a double restriction enzyme digestion of pET30a/Tg-HbIIA with *BamHI* and *HindIII*, and using PCR, we showed that the coding region of the mature Tg-HbIIA gene was 492 bp, as expected. In the same way, the coding region of the mature Tg-HbIIB gene was confirmed and was shown to be 483 bp, as expected. The result was also confirmed by sequencing (data not shown). Then, the pET30a/Tg-HbIIA and pET30a/Tg-HbIIB were introduced into *E. coli* Transetta (DE3). To confirm the positive Transetta (DE3), Tg-HbIIA and Tg-HbIIB were amplified using PCR with T7 primers and were then detected by 1% agarose gel electrophoresis. We observed a single band of approximately 750 bp (Fig. 1). It was confirmed that the recombinant expression vector was successfully constructed.

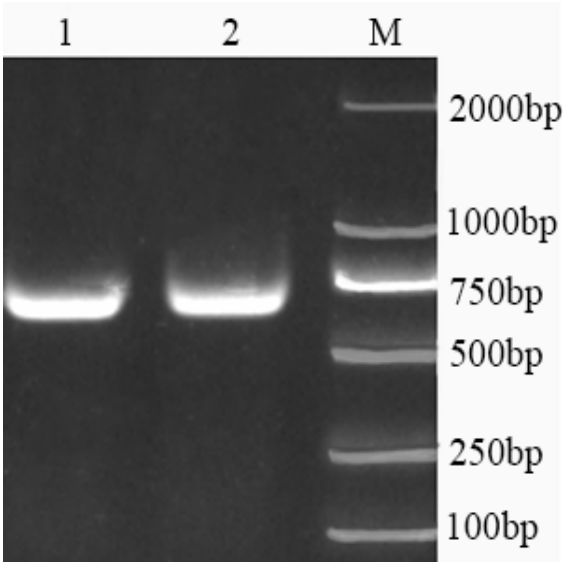

Fig. 1 Agarose gel electrophoresis analysis of recombinant expression vector. Lane 1, pET-30a/Tg-HbIIA; Lane 2, pET-30a/Tg-HbIIB; Lane M, DNA Marker.

Expression and purification of Tg-HbIIA and Tg-HbIIB

The approach used to produce the recombinant Tg-HbIIA and Tg-HbIIB proteins at high levels was to determine the optimal expression conditions by evaluating the induction time and temperature. After induction with IPTG, the Tg-HbIIA gene was successfully expressed, and the highest expression was induced by 1 mmol/L IPTG at 37 °C for 5 h (Suppl. Fig. 1). In the same way, the highest expression of Tg-HbIIB was induced by 1 mmol/L IPTG at 28 °C for 4 h (Suppl. Fig. 2). The largest amounts of Tg-HbIIA and Tg-HbIIB were found in the precipitate when analyzed by SDS-PAGE analysis, after the samples were ultrasonicated. This result showed that they were mostly in insoluble inclusion bodies. To isolate the Tg-HbIIA and Tg-HbIIB, bacterial cells were collected and disrupted using ultrasonication. The inclusion bodies were obtained by centrifugation and dissolved in the solubilization buffer. The cell debris was removed by centrifugation, and the solubilized protein was purified by the HisTrap FF column in the denaturing condition. After purification and SDS-PAGE, single bands of the Tg-HbIIA and Tg-HbIIB at approximately 24 kDa and 25 kDa, respectively, were observed (Fig. 2). To further determine the components of the proteins, mass spectrometry was performed. The results see Figure 3 and in Supplementary Figure 3. The concentrations of Tg-HbIIA and Tg-HbIIB were 3.26 mg/ml and 3.71 mg/ml, respectively, as determined by the Bradford protein assay. Exogenous gene expression in *E. coli* is affected by many factors, such as induction temperature, IPTG concentration, inducing time and genetic structure (Lilie *et al.,* 1998). Experiments found that the expression of the vectors pET30a/Tg-HbIIA and pET30a/Tg-HbIIB was mainly affected by inducing time. Although the expression of exogenous genes in *E. coli* is one of the most economic and convenient expression systems, the greatest obstacle is the inability to correctly fold recombinant proteins and form active proteins. Gradient dialysis, via the ultrafiltration centrifugal method, can induce recombinant protein folding *in vitro* but this method is unable to restore the original

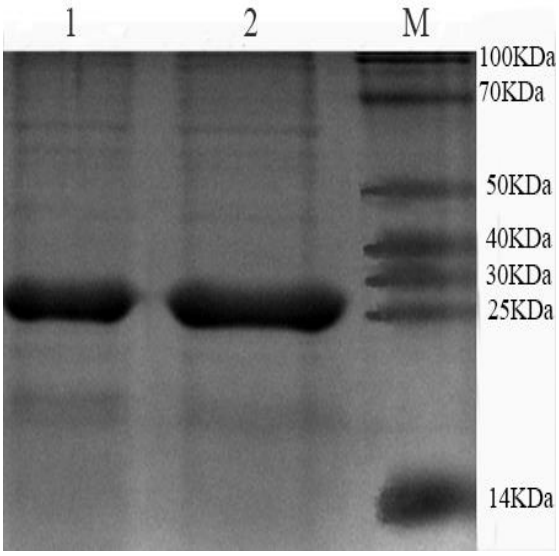

Fig. 2 SDS-PAGE analysis of purified recombinationt hemoglobins. Lane 1, Purified recombinationt Tg-HbIIB; Lane 2, Purified recombinationt Tg-HbIIA; lane M, Broad range protein molecular weight markers

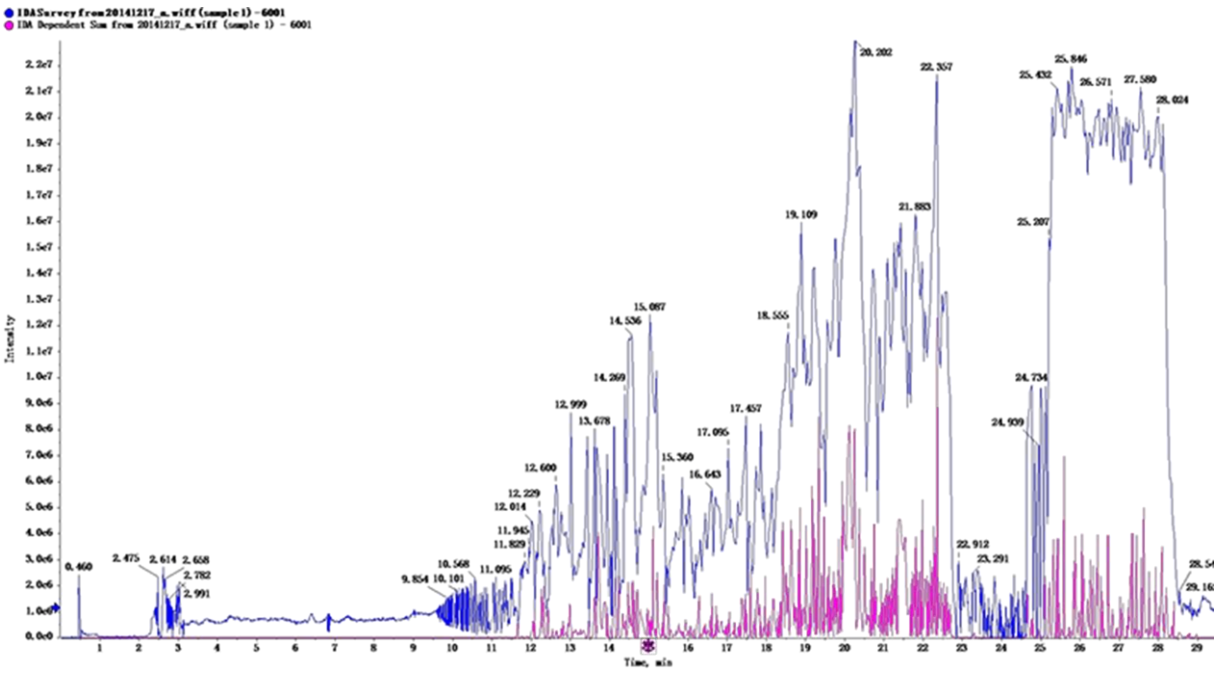

Fig. 3 The mass spectrogram of recombinationt HbIIA from *Tegillarca granosa.*

Table 2 Antibacterial activity of recombinant hemoglobin from *Tegillarca granosa*

Bacteria	Gram	The minimum inhibition concentration (MIC) (μg/ml)	
		Tg-HbIIA	Tg-HbIIB
V. harveyi	-	65.8	158
P. putida	-	4.11	39.5
A. baumanii	-	---	79
E. coli	-	---	---
V. parahaemolyticus	-	---	---
V. alginolyticus	-	---	---
S. aureus	-	---	---
B. subtilis	+	---	---
S. epidermidis	+	---	---
B. firmus	+	---	---

level (Mayer and Buchner, 2004). If the recombinant proteins were solubilized as inclusion bodies and refolded in the right process, then they would have biological activity (Sings and Panda, 2005). The recombinant proteins (Tg-HbIIA, Tg-HbIIB) in dialysis renaturation required the addition of a slight excess of heme in proportion. Heme is an important component of Hb, but it is also the active center of Hb. Without adding the heme, the protein could not be folded into its normal active protein form (Perutz, 1979). Each Hb subunit needs one auxiliary heme. The Fe^{2+} of the heme is oxidized easily in the renaturation process, but it may reduce the activity of the recombinant protein. Additionally, heme was dissolved in ethanol. Therefore, the excess heme and the residual urea also likely affected the protein activity. It was found that when 1 mg of recombinant protein was added to 1 mg heme, the result of renaturation was good. Protein renaturation is a complex process that is affected by many factors.The protein spatial structure needs to be formed slowly, so the urea should be diluted gradually (Vallejo and Rinas, 2004; Yu and Tao, 2007). To prevent protein denaturation, the entire process should be performed at 4 °C.

Antibacterial activity of the recombinant protein

The results of the bacteriostatic experiment are shown in Table 2. We found that recombinant Tg-Hb exhibits great differences in its effects on various bacteria. The recombinant proteins have no antibacterial activity against *S. aureus*, *E. coli*, *B. firmus*, *B. subtilis*, *S. epidermidis*, *V. parahaemolyticus* or *V. alginolyticus*. The antibacterial activity of the recombinant proteins to *V. harveyi*, *P. putida* and *A. baumanii* is shown in Figure 4. In the previous study, the Tg-HbllA and Tg-HbllB had different amino acids, but they had similar tertiary structure, like 8 alpha helix. We also found that the recombinant proteins without heme have no antimicrobial activities in the pre experiment. And the potential heme binding sites of Tg-HbllA, Tg-HbllB and *S. inaequivalvis* HbII were highly conserved, indicating that the function of Hb play mainly related to its tertiary structure and function domain(Jr *et al*., 1994; Jr *et al*., 1995; Wang, 2012). Compared with the previous study, we found that the effects of the recombinant proteins on antibacterial activity on *P. putida*, *V. parahaemolyticus* and *S. aureus* are similar to those of natural Tg-HbII (Wang *et al*., 2014). This result indicated that recombinant

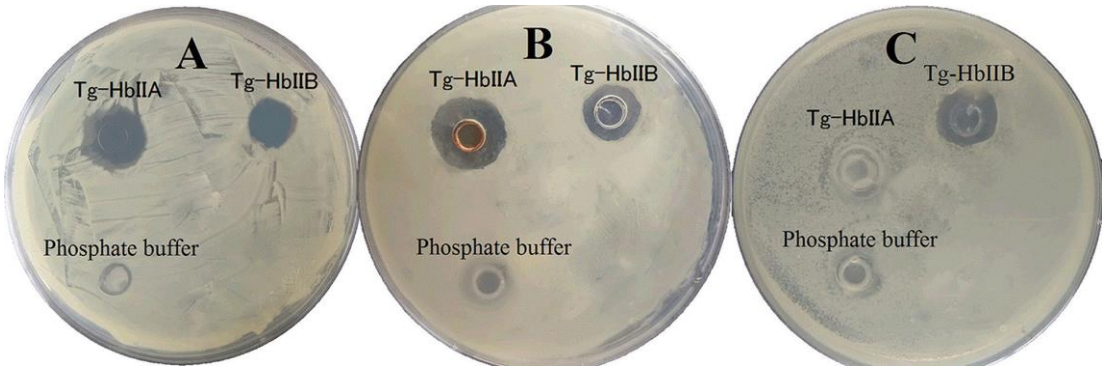

Fig. 4 The inhibition zone of purified recombinationt hemoglobin from *Tegillarca granosa*. A, *V. harveyi*; B, *P. putida*; C, *A. Baumanii.*

proteins are inducible and have high antibacterial activity against Gram-negative bacteria, similar to natural protein. There are also some differences. The nature Tg-HbII heterotetramers obtained by gel exclusion chromatography was exhibited antibacterial activity against *E. coli*, *B. subtilis*, *B. firmus*, and had no antibacterial activity against *V. harveyi*, *A. baumanii* (Bao *et al*., 2013; Bao *et al*., 2016). The Tg-HbII was an A_2B_2 heterotetramers formed by Tg-HbIIA and Tg-HbIIB. The interaction between Tg-HbIIA subunit and Tg-HbIIB subunit may lead to different antibacterial results. In the previous study, the expression of Tg-HbIIA and Tg-HbIIB mRNA was markedly upregulated after *V. parahaemolyticus*, but the recombination Hb had no antibacterial activity against *V. parahaemolyticus*. We speculate that polypeptides from Tg-HbIIA and Tg-HbIIB by enzymatic hydrolysis can resist *V. parahaemolyticus* (Wang *et al*., 2015). In addition, that the hemolytic effects of the hemolysins produced during *V. Parahaemolyticus* infection are responsible for enhanced hematopoeisis (Honda and Iida, 1993).

Our results also confirmed that protein with antibacterial activity can be expressed and assembled in *E. coli* (De-La-Re-Vega *et al*., 2006; Zhao *et al*., 2007; Mai *et al*., 2009). So far, most have focused on the natural Hb and its peptides antibacterial research, while the recombinant Hb and its peptides is less. Niu and Chen (2016) reported that recombinant Hb from *Urechis unicinctus* has antibacterial activity against *S. aureus, M. luteus, E. coli,* and *V. parahaemolyticus*. And their results are different from ours, which indicated that the antibacterial mechanism of different sources of Hb may be different. At present, the antibacterial mechanism of Hb is unclear. Though Hb has been found capable of producing ROS and of exerting pseudoperoxidase and deoxygenase activities, which are involved in host defense (Adachi *et al*., 2003; Cheng *et al*., 2011; Goodarzi *et al*., 2014). Jiang *et al*. (2007) showed that human Hb is oxidized to ferric iron when stimulated by virulence factors, resulting in a superoxide anion that can produce toxic derivatives such as hydroxyl radicals and hypochlorous acid and has a bactericidal effect. Wang *et al*. found that purified Tg-Hbs catalyzed the oxidation of several phenol compounds in the presence of H_2O_2, with high affinity to guaiacol. And the predicted structure at their heme pocket was highly similar to that of horseradish peroxidase (HRP) and myeloperoxidase (MPO). It indicated that Tg-Hbs may function as peroxidase in the clam's hemocytes. The authors considered that the antibacterial effect may be generated by the peroxidase activity (Wang *et al.,* 2014; Wang *et al*., 2017). The heme moiety of Hb can either act as an iron chelator or an oxidant, leading to bacterial and fungal cell wall damage (Katsu *et al*., 2010). The diversity of antimicrobial activity of Tg-Hbs might be due to their antibacterial mechanism. The research of antibacterial mechanism of nature and recombinant Tg-Hb is under way.

Acknowledgments

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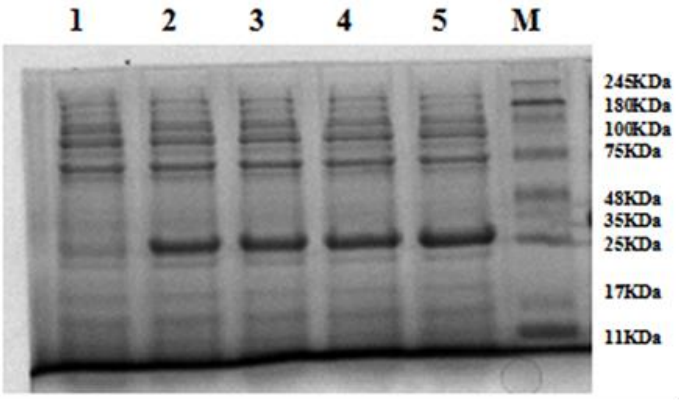
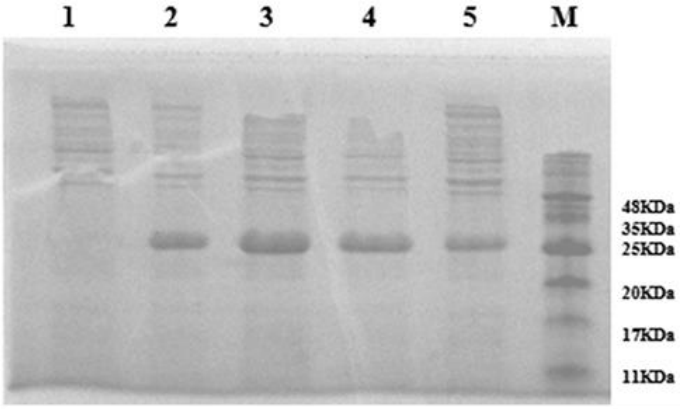
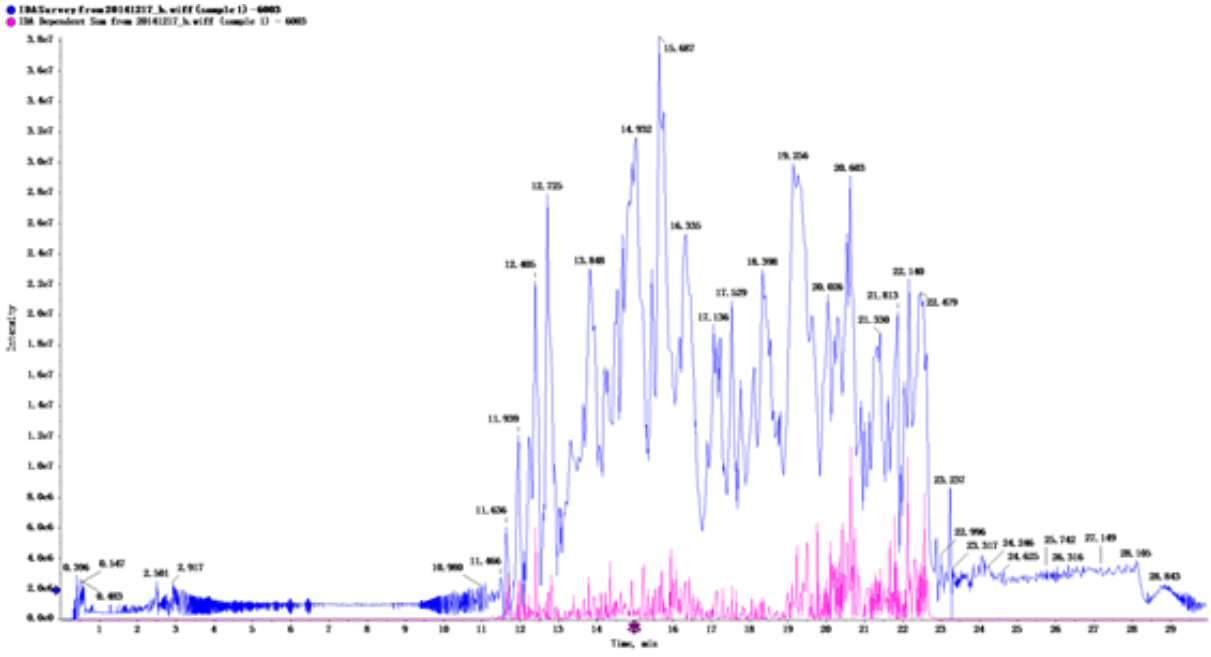


Suppl. Fig. 1 SDS-PAGE analysis of recombinant Tg-HbIIA. Lane 1, non-induced; Lane 2, after induction by 37 °C, 2h; Lane 3, after induction by 37 °C, 3 h; Lane 4, after induction by 37 °C, 4 h; Lane 5, after induction by 37 °C, 5 h; lane M, Broad range protein molecular weight markers.



Suppl. Fig. 2 SDS-PAGE analysis of recombinant Tg-HbIIB. Lane 1, non-induced; Lane 2, after induction by 28 °C, 3 h; Lane 3, after induction by 28 °C, 4 h; Lane 4, after induction by 28 °C, 5 h; Lane 5, after induction by 28 °C, 6 h; lane M, Broad range protein molecular weight markers.



Suppl. Fig. 3 The mass spectrogram of recombinationt HbIIB from *Tegillarca granosa*.