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Expression of *Macrobrachium rosenbergii* lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP) in *Saccharomyces cerevisiae* and evaluation of its immune function

Jie Du[a,b], Huanxi Zhu[c], Chunlei Cao[d], Yan Ma[a,b,*]

[a] Institute of Agricultural Resources and Environment, Jiangsu Academy of Agricultural Sciences, Nanjing, China
[b] Key Laboratory of Agro-Environment in Downstream of Yangtze Plain, Ministry of Agriculture, Nanjing, China
[c] Institute of Animal Sciences, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China
[d] The National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China

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ABSTRACT

Pattern recognition proteins (PRPs) activate the innate immune system in invertebrates, and lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP) is an important PRP with various biological functions. Here, the open reading frame (ORF) of *Macrobrachium rosenbergii* LGBP (MrLGBP) was cloned into plasmid vector pHAC181, then integrated into downstream of the GAL1 promoter of *Saccharomyces cerevisiae* strain GAL1-ScRCH1 via homologous recombination, followed by its expression in the yeast eukaryotic system. The resulting recombinant LGBP contained a 3 × HA-tag at its C terminus and had a molecular weight of about 45 kDa, as evaluated by western blot analysis. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were ranged from 0.340 to 0.802 and 1.189–1.810 μM, respectively. The recombinant MrLGBP protein agglutinated almost all tested bacteria except *Bacillus thuringiensis* and *Staphylococcus aureus.* These results revealed that this recombinant protein exhibited antimicrobial activity against some Gram-positive and Gram-negative bacteria. *M. rosenbergii* prawns were fed with the recombinant yeast strain MrLGBP for 1 month and challenged with the most common crustacean pathogen, *Vibrio parahaemolyticus*. These prawns showed lower mortality and higher enzymatic activity and expression levels of immunity genes than did the control groups. All these results suggest that MrLGBP may play important roles in the innate immunity of crustaceans, and recombinant strain *S. cerevisiae* MrLGBP may be useful for the development of an effective immune feed additive in the future.

1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii*, has commercial value and has been commercially cultured in China and other Southeast Asian countries [1,2]. Nonetheless, prawn culture has been restricted, owing to epidemic infectious diseases such as white tail disease (WTD), extra small virus (XSV) infection [3,4], and spiroplasma disease caused by a novel pathogen, spiroplasma MR-1008 [5,6]. As a consequence, there is a huge business loss in the local economy. Therefore, research on the immunity of this prawn is desirable for control of diseases and to ensure long-term survival of the crustacean culture industry.

Crustaceans, like other invertebrates, lack the adaptive immune system and rely entirely on their innate immune responses for protection against invading pathogens [7]. Pattern recognition proteins (PRPs) perform important functions in the innate immune response by recognizing cell wall components of pathogens, such as β-1,3-glucans (BGs), lipopolysaccharides (LPSs), and peptidoglycans (PGs), as non-self molecules and trigger cellular and humoral responses [8]. In crustaceans, several PRPs, including β-1,3-glucan–binding protein (BGBP), LPS-binding protein (LBP), PG-binding protein (PGBP), and LPS- and BG-binding protein (LGBP), which recognize and respond to microbial intruders, are reported to be involved in the activation of the prophenoloxidase (proPO) system [9]. In recent years, many of these PRPs have been studied. For instance, complementary (c) DNA of BGBP from white shrimp *Litopenaeus vannamei* has been cloned and characterized [10]. In addition, LGBP cDNA from the freshwater crayfish *Pacifastacus leniusculus* has been cloned and characterized [8], as have cDNAs from tiger shrimp *Penaeus monodon* [11], kuruma shrimp *Marsupenaeus japonicus* [12], blue shrimp *Litopenaeus stylirostris* [13], white shrimp *L. vannamei* [14], fleshy prawn *Fenneropenaeus chinensis* [15], and *M. rosenbergii* [16]. Nevertheless, studies on the expression and

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* Corresponding author. Institute of Agricultural Resources and Environment, Jiangsu Academy of Agricultural Sciences, Nanjing, China.
E-mail address: myjaas@sina.com (Y. Ma).

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functions of PRPs in crustaceans are limited. Amparyup [17] has reported that *Pm*LGBP functions as a PRP for LPS and BG in the proPO activation system of *P. monodon*. Chen [18] has found that LGBP binds to seaweed polysaccharides and activates the proPO system in the white shrimp *L. vannamei*, whereas Chaosomboon recently reported the LGBP protein from *Fenneropenaeus merguiensis* and its function as a pattern recognition receptor along with its broad specificity for diverse pathogens [19]. All these proteins have been mainly expressed in an *Escherichia coli* prokaryotic system; the use of a yeast system for protein expression is relatively rare.

In comparison with *E. coli*, yeast has an advanced heterologous-protein–folding pathway. In addition, the yeast signal sequence allows yeast cells to secrete proteins that are properly folded and processed. With the broad application of the industrial fermentation technology, yeast species have shown great advantages in the expression of clinically and industrially important proteins [20,21]. As a generally regarded as safe (GRAS) organism, *Saccharomyces cerevisiae* is widely used in the food and beverage industry and has been successfully employed to express a variety of exogenous eukaryotic proteins such as a hepatitis B vaccine, human insulin, human granulocyte colony-stimulating factor, and a human blood vessel inhibitor [22]. On the other hand, few reports have described the application of an *S. cerevisiae* eukaryotic system to the expression of crustacean LGBP.

In this study, the important PRP-LGBP of *M. rosenbergii* (hereafter MrLGBP), was expressed in *S. cerevisiae* system. The open reading frame (ORF) of the MrLGBP gene was cloned into plasmid vector pHAC181, and the recombinant plasmid was integrated into downstream of the GAL1 promoter of *S. cerevisiae* strain GAL1-ScRCH1 using a homologous recombination technique. By means of D-galactose, the target protein was expressed in yeast cells, and tentative immune functions of the recombinant protein as well as its effect on the immunity of *M. rosenbergii* were determined. This study is intended to provide useful information and experimental basis for crustacean innate immunity to prevent and control aquatic diseases.

2. Materials and methods

2.1. Experimental animals, microbial strains, and media

Healthy *M. rosenbergii* prawns (average body weight of 20 g) were obtained from a commercial farm in Wuxi, Jiangsu province of China. The prawns were maintained in flat-bottomed glass tanks (200 L) with aerated and filtered freshwater at 28 ± 1 °C in the laboratory. They were daily fed with a commercial prawn diet at a rate of 4% body weight for 2 weeks before experimentation.

Plasmid vector pHAC181 (constructed by our laboratory) was cultured in the Luria Bertani (LB) liquid medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L sodium chloride (NaCl), pH 7.0) at 37 °C, whereas *S. cerevisiae* strain GAL1-ScRCH1 was grown at 30 °C in the YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose).

Four Gram-negative bacterial species (*E. coli*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus,* and *Pseudomonas aeruginosa*) and three Gram-positive bacterial species (*Bacillus subtilis*, *Bacillus thuringiensis*, and *Staphylococcus aureus*) were purchased from the Microbial Culture Collection Center (Beijing, China) and grown in LB broth at 37 °C.

2.2. RNA isolation from M. rosenbergii hemolymph and cDNA synthesis

Healthy *M. rosenbergii* were swabbed with 75% ethanol, and the hemolymph was withdrawn from the ventral part of the hemocoel of the second abdominal segment using a 1 mL sterile syringe containing 500 μL of modified phosphate-buffered saline (PBS) [6] (0.9 g/L disodium phosphate [Na_2HPO_4], 0.27 g/L monopotassium phosphate [KH_2PO_4], 0.6 g/L potassium chloride [KCl], 25.5 g/L NaCl, 1.0 g/L L-glucose, pH 7.2) as an anticoagulant. The diluted hemolymph was centrifuged at 3500 × *g* for 5 min at 20 °C, and the cells obtained were used for total RNA extraction with 500 μL of the TRIzol Reagent (TaKaRa, Japan). After thorough mixing, a 0.2 mL aliquot of chloroform was added to the mixture, the latter was vigorously shaken and allowed to stand for 15 min. The resultant mixture was centrifuged at 12,000 × *g* for 15 min at 4 °C. Isopropanol (0.5 mL) was added to the separated upper aqueous phase, and the reaction system was centrifuged at 12,000 × *g* for 10 min at 4 °C. The obtained pellet was washed with 75% ethanol, air died, and dissolved in 20 μL of diethyl pyrocarbonate (DEPC)-treated sterile water. The concentration and quality of RNA were assessed by measuring absorbance at 260/280 and 260/230 nm wavelengths on a UV–Visible spectrophotometer (Eppendorf, Germany). Only RNA with the $OD_{260}/_{280}$ ratio between 1.8 and 2.1 and $OD_{260}/_{230}$ ratio ≥2.0 was used for cDNA synthesis. The general integrity of total RNA was assessed by 1% agarose gel electrophoresis. A total of 1 μg of RNA was subjected to cDNA synthesis using the PrimeScript RT Reagent Kit (TaKaRa, Japan).

2.3. Construction of the expression plasmid and homologous recombination

The sequence of *M. rosenbergii* LGBP cDNA was retrieved from GenBank (accession No. GQ228481.1) [16], and LGBP ORF (1098 bp) was amplified by polymerase chain reaction (PCR) with LGBP-specific primers carrying *Pst*I and *Sph*I sites at their 5′ termini. We used plasmid pHAC181 [23] as the vector for the target gene cloning (pHAC181 is the multicopy plasmid constructed by our laboratory via insertion of three hemagglutinin (HA) tags into commercial plasmid YEplac181). After restriction enzyme digestion and a T_4 DNA ligase (TaKaRa, Japan) reaction, the mixture was transfected into competent cells *Trans*1-T1 (TransGen, China). Transformants were verified with restriction enzyme digestion, and recombinant plasmids were verified by DNA sequencing. The homologous recombination technique was employed to integrate the target gene (along with HA tags from the successful recombinant plasmids) downstream of the GAL1 promoter of *S. cerevisiae* strain GAL1-ScRCH1 (*S. cerevisiae* GAL1-ScRCH1, the *ScRCH1* gene promoter was replaced with the *GAL1* gene promoter in *S. cerevisiae* BY4741 strain [Sc04153268_s1] in our laboratory). The construction strategy for the *S. cerevisiae* expression system is described in Fig. 1A. High-fidelity PrimeSTAR GXL DNA Polymerase (TaKaRa, Japan) and the homologous primer pair (INT-F and INT-R) were used to amplify the large fragments, whereas detection primers served to verify whether the integration was successful (Table 1).

2.4. Expression of recombinant MrLGBP

The successfully integrated strains were cultured in the D-galactose induction medium YPG (20 g/L peptone, 20 g/L D-galactose, and 10 g/L yeast extract; D-galactose as an induction agent) with vigorous shaking at 220 rpm and 30 °C. After optical density of 1.0–1.5 at 600 nm wavelength (OD_{600}) was attained, total protein was extracted and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and western blot analysis was carried out to evaluate the expression of recombinant MrLGBP.

2.5. SDS-PAGE and western blot analysis

Standard SDS-PAGE in a 10% gel was conducted to analyze the expression of recombinant MrLGBP. The protein bands were visualized by staining with Coomassie Brilliant Blue.

Western blot analysis was performed to confirm the identity of the expressed recombinant MrLGBP. The proteins separated with SDS-PAGE were electrotransferred onto a nitrocellulose membrane (Bio-Rad) in a semidry electrophoretic transfer apparatus (*Trans*-blot SD, Bio-Rad) at 25 V for 30 min. The membrane was washed twice with Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 7.5) at 25 °C for 10 min and incubated in blocking buffer (3% bovine serum albumin [BSA] in TBS buffer) at room temperature for 2 h. The

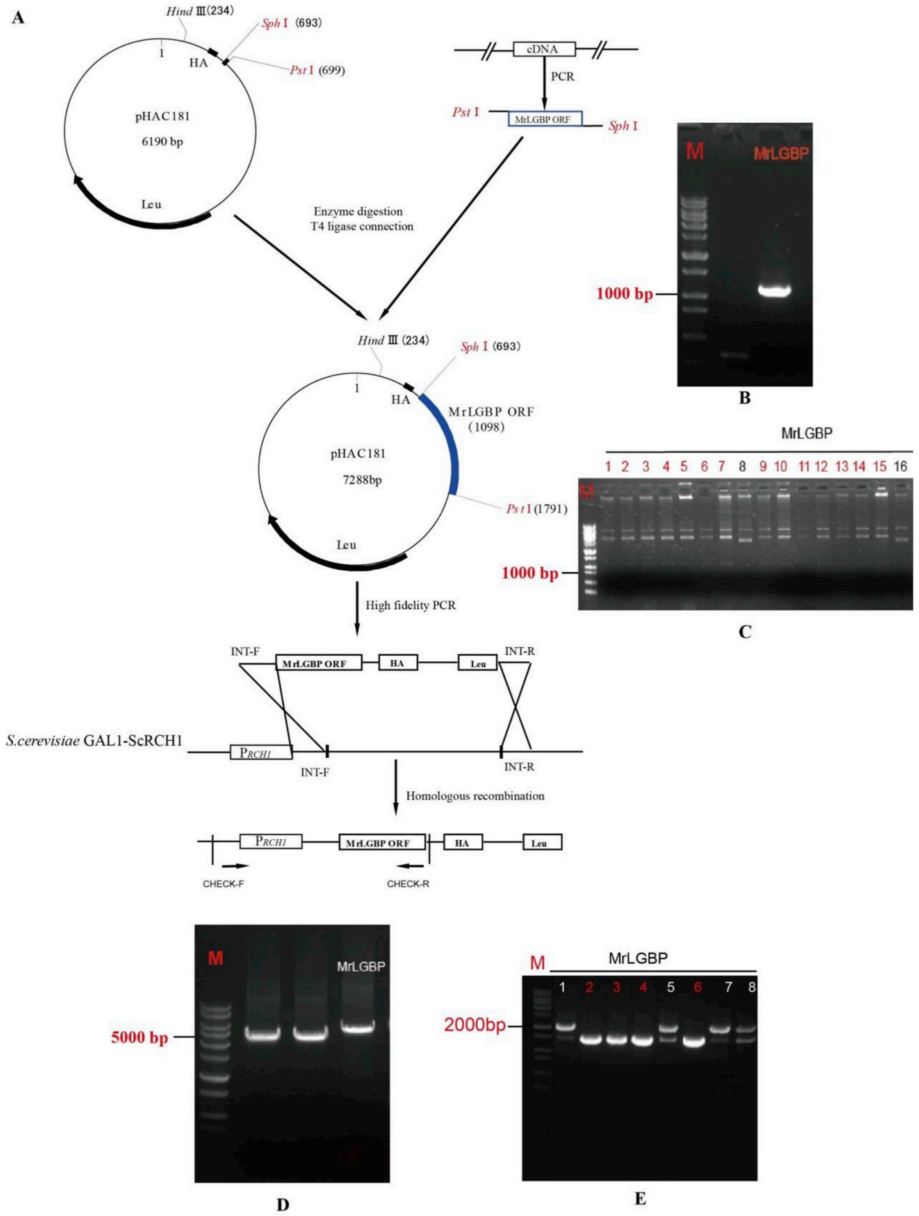

Fig. 1. The strategy for the construction of the expression system of *M. rosenbergii* LGBP in *S. cerevisiae.* (A) Construction process of yeast eukaryotic expression system. (B) Amplification of 1098 bp open reading frame (ORF) of LGBP from *M. rosenbergii* cDNA. (C) Verification of the positive transformants by restriction enzyme digestion. (D) Amplification of 5481 bp target fragment of the recombinant plasmid by homologous recombination using high fidelity PrimeSTAR GXL DNA Polymerase. (E) Verification of the 1381 bp target fragment in the successful transformants with detection primers via homologous recombination. Annotation: Red number indicates the correct positive transformation, whereas the unlabeled strips in B and D are incorrect amplified strips. M: DNA marker. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

membrane was next washed twice with TBS containing 0.05% (v/v) Tween 20 (TBST) at room temperature for 10 min and incubated with an anti-HA antibody (Abcam, UK) at room temperature for 2 h. After three washes with TBS, secondary antibodies conjugated with horseradish peroxidase (Abcam, UK) were added. Two hours later, the membrane was washed thrice, and the recombinant protein was detected by an exposure development method.

2.6. Purification of the recombinant MrLGBP protein

This protein was purified by means of rProtein A Sepharose™ Fast Flow (GE Healthcare, USA). PEB buffer (2 mM ethylenediaminetetraacetic acid [EDTA], 1 × phenylmethanesulfonyl fluoride [PMSF], and 50 mM Tris-HCl, pH 8.0) and buffer B (0.05 M NaH_2PO_4 and 0.05 M trisodium citrate [$C_6H_5Na_3O_7{\cdot}2H_2O$], pH 3.0) were used in this process. The purification steps were as follows: 500 µL of total protein extract was mixed with 25 µL of the anti-HA antibody at 4 °C overnight. A total of 200 µL of rProtein A beads (with 20% of ethanol) was centrifuged to remove ethanol and was washed twice with 1 mL of PEB buffer, followed by their resuspension in 500 µL of PEB buffer. The washed rProtein A beads were added to the antibody solution and incubated at 4 °C for 4 h, followed by centrifugation for 5 min at 4 °C and 500 × *g*. The supernatant was removed, and the anti-HA antibody and target protein associated with rProtein A in the form of a precipitate were resuspended in 100 µL of buffer B. The precipitate was mixed well and placed on ice for 10 min, followed by centrifugation at 500 × *g* for 5 min. The obtained supernatant served for the detection of antibacterial activity.

2.7. Antimicrobial activity assay

The bacterial strains chosen for this study included four Gram-negative species (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*) and three Gram-positive species (*B. subtilis*, *B. thuringiensis*, and *S. aureus*). For assessment of antibacterial activity, values of minimum inhibitory concentration (MIC) were determined by liquid growth inhibition assays, as previously described [24]. Briefly, 10 µL of each diluted MrLGBP protein sample (or protein from *S. cerevisiae* GAL1-ScRCH1, which without the homologous recombination gene MrLGBP served as a control) was incubated in sterile 96-well plates containing

Table 1
Specific primers used in these experiments.

Primer	Purpose	Sequence (5’ -3′)
MrLGBPF	cDNA amplification	CCG GAATTC TTAATTAA ATGGCGAAGTGCTTACAAGTC
MrLGBPR	cDNA amplification	ACAT GCATGC CTGTTTCGCGCTCTCCATTT
INT-F	Homologous recombination	CAAATGTAATAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACCCGGATCTCAAAATGAGGACCTTATACCTATTGCTAC
INT-R	Homologous recombination	TATGGACGAGGTAATAAGGAAACTCAGAACCAGAATAGTGGCATGAGCTCTCCAATTTAACATATTTGCCATTAGTGACCCGATGATAAGCTGTCAAACATG
Check-F	Check primer	CCTGGCCCCACAAACCTTC
Check-R	Check primer	CTGTTTCGCGCTCTCCATTT
QLGBP-F	qRT-PCR	AGGAACCGGCGGTTTCTT
QLGBP-R	qRT-PCR	GGTGTTGGACCAAGGCTTGT
QAKP-F	qRT-PCR	TGAGGAACTGGTGGCCTTCGAGG
QAKP-R	qRT-PCR	GTCAGGTTTGGTCTCTTTACGCC
QACP-F	qRT-PCR	GTTTACACTCGCCTTATCCTCCG
QACP-R	qRT-PCR	CTTTGTGCATGAACATGACCCTG
QCAT-F	qRT-PCR	AGCGAGATTGGCAAGAAGACACC
QCAT-R	qRT-PCR	AAGGATGGTGACCTGGTGCGTGG
QCZSOD-F	qRT-PCR	TCGCCTAACGAGGAGGTTCA
QCZSOD-R	qRT-PCR	CGGCTTCATCAGGATTTTGAG
β-actinF	qRT-PCR	GAGACCTTCAACACCCCAGC
β-actinR	qRT-PCR	TAGGTGGTCTCGTGAATGCC

100 μL of a suspension of a mid-logarithmic phase bacterial culture diluted in the culture medium to OD_{600} = 0.001. Poor-broth nutrient medium (10 g/L bactotryptone and 5 g/L NaCl, pH 7.5) was employed for the standard bacterial strain cultures. The bacteria were grown for 24 h with vigorous shaking at 30 °C. Bacterial growth was controlled by measuring OD_{600} after incubation for 24 h. MIC was recorded as the concentration of MrLGBP that inhibited the bacterial growth, whereas minimum bactericidal concentration (MBC) was the lowest concentration that caused 100% inhibition of bacterial growth [25]. Furthermore, to examine whether the antibacterial activity of the recombinant protein is bactericidal, bacteria were cocultured with different MrLGBP concentrations for 24 h, and 20 μL aliquots were then removed and plated on nutrient agar, then the number of colony-forming units was determined [26].

2.8. Bacterial agglutination assay

The bacterial recognition of MrLGBP was assessed by a bacterial agglutination test, as previously described [27]. Gram-negative bacteria (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*) and Gram-positive bacteria (*B. subtilis*, *B. thuringiensis*, and *S. aureus*) were resuspended in the LB medium at 1.0 × 10^7 cells/mL. A total of 90 μL of a bacterial suspension was added to 10 μL of recombinant MrLGBP (20 μg/μL), whereas the protein extract from yeast strain *S. cerevisiae* GAL1-ScRCH1, which without the homologous recombination gene MrLGBP served as a control. The mixtures were incubated overnight at 30 °C, and bacterial cells were observed under a light microscope.

2.9. Feeding experiment and pathogen challenge

The recombinant protein strain *S. cerevisiae* MrLGBP in the fermentation broth was concentrated to 1.0 × 10^9 cells/mL and sprayed directly onto the granulated feed. The yeast immune feed containing the immunoprotein was prepared after drying of the granular feed surface. A total of 200 M. *rosenbergii* prawns were held in a recirculation system containing filtered and UV-treated artificial freshwater with controlled light (12 h light/dark) and a regulated temperature (26–28 °C) regime. Prawns weighing 20–25 g were used for the study.

The healthy prawns were fed daily with different diets at a rate of 4% body weight for 4 weeks. The prawns were subdivided into the following groups: experimental group; the pellet feed mixed with yeast strain *S. cerevisiae* (GAL1-ScRCH1) that expressed the target protein MrLGBP; control group 1, the pellet feed mixed with the blank *S. cerevisiae* strain (yeast strain *S. cerevisiae* GAL1-ScRCH1 which without the homologous recombination gene MrLGBP); control group 2: common pellet feed without yeast strains. After feeding for 1 month, prawns were injected with 50 μL physiological saline (PBS) containing bacteria (*V. parahaemolyticus*, approximately 3.0 × 10^6 colony-forming units [CFU]), whereas the control prawns were injected with 50 μL of PBS. *V. parahaemolyticus* was selected as the pathogen is this study because it is one of the most common pathogens of crustaceans. After infection, the mortality of *M. rosenbergii* was evaluated at 0, 2, 6, 12, 24, and 48 h. Hepatopancreas samples were collected for subsequent experiments.

2.10. Determination of the activity of immune enzymes of M. rosenbergii

The collected hepatopancreas tissue was used to detect the change in the immune enzyme activity. For alkaline phosphatase (AKP) and acid phosphatase (ACP) activity assays, approximately 0.1 g of hepatopancreas was homogenized at 2000 × *g* for 1 min in 1 mL of ice-cold physiologic saline (0.6%) in an automatic homogenizer (Essen, Germany). Homogenized samples were centrifuged at 3500 × *g* for 10 min at 4 °C and the resulting supernatants were used for enzyme activity determination. AKP and ACP activities were determined by the method of King [28] using disodium phenyl phosphate (Sigma, America) as the substrate. The unit definition of AKP and ACP

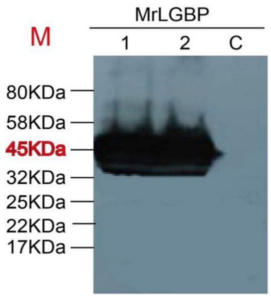

Fig. 2. Western blot analysis to detect the expression of recombinant MrLGBP protein using anti-HA antibody. A 45 kDa target band was detected in Lane 1 and 2 while the control protein showed no detectable bands. Lanes 1 and 2 represent positive homologous recombinant transformant of *S. cerevisiae* GAL1-ScRCH1 expressing the MrLGBP protein. C: Protein of control yeast strain *S. cerevisiae* GAL1-ScRCH1, which without the homologous recombination gene MrLGBP. M: protein marker.

enzymatic activities corresponded to the degradation of 1 g of phenol per gram protein at 37 °C within 15 and 30 min, respectively. Protein concentration in the supernatant was determined by Coomassie Brilliant Blue G250 staining [29], and the diagnostic reagent kit purchased from Nanjing Jiancheng Bioengineering Institute (China).

Superoxide dismutase (SOD) activity was assayed according to the method of Misra and Fridovich [30], on the basis of the oxidation of epinephrine to adreno-chrome by the enzyme. A total of 0.1 mL hepatopancreas homogenate was added to a tube containing 0.75 mL ethanol and 0.15 mL chloroform (chilled in ice) and centrifuged. The supernatant obtained (0.5 mL) was treated with 0.5 mL EDTA solution and 1 mL buffer. The reaction was initiated with the addition of 0.5 mL epinephrine and the increase in the absorbance at 550 nm wavelength was monitored at an interval of 30 s for 3 min. The enzyme activity was expressed as 50% inhibition of epinephrine auto-oxidation per minute for every milligram of a protein sample. Catalase (CAT) activity was detected by measuring the intensity of a yellow complex formed by molybdate and hydrogen peroxide (H_2O_2) at 405 nm after the addition of ammonium molybdate to terminate H_2O_2 degradation reaction catalyzed by CAT [31]. One enzymatic activity unit was defined as degradation of 1 mol of H_2O_2 per second for every milligram of protein.

Protein concentration in the supernatant was determined with Coomassie Brilliant Blue G250 [29] according to the provided protocol. The diagnostic reagent kit was purchased from Nanjing Jiancheng Bioengineering Institute (China).

2.11. Quantification of immune-related gene expression by real-time RT-PCR

Hepatopancreas of *M. rosenbergii* were collected, and RNA extracted at 0, 2, 6, 12, 24, and 48 h post challenge. First-strand cDNA was obtained by reverse transcription with PrimeScript® 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) and an Oligo-dT primer. The synthesized cDNA was used for RT-PCR and quantitative RT-PCR (qRT-PCR) analyses. QRT-PCR was performed to investigate the expression levels of five immunity-related genes (LGBP, AKP, ACP, CAT, and Cu,Zn-SOD) in the hepatopancreas from *M. rosenbergii* challenged with *V. parahaemolyticus*. The expression of β-actin mRNA served as a reference. All PCRs (volume 20 μL) comprised 10 mL of the SYBR Premix *Ex Taq* II (Takara, Japan), 1 μL of cDNA, 10 pmol of each forward and reverse primer (Table 1), and 7 μL of ultrapure water. The thermal cycling program was as follows: 95 °C for 30 s, 40 cycles of 94 °C for 5 s, and 60 °C for 30 s. Fluorescence yields obtained from three replicate reactions of each cDNA sample were analyzed using Mastercycler Ep Realplex (Eppendorf, Germany); three biological replicates were analyzed to ensure the validity and accuracy of experimental results. The relative expression levels of different genes in the hepatopancreas were calculated according to the $2^{-\Delta\Delta CT}$ method [32].

2.12. Statistical analysis

All experiments were performed thrice, and statistical analyses were carried out in the SPSS 17.0 software. The data analyzed using one-way ANOVA were represented as mean ± standard error, and statistical significance was defined as $P < 0.05$.

3. Results

3.1. Cloning of MrLGBP cDNA and homologous recombination

The construction strategy of the *S. cerevisiae* expression system is described in Fig. 1A. Total RNA was extracted from *M. rosenbergii* hemolymph and amplified with an LGBP-specific primer pair. The amplified ORF of the target cDNA was 1098 bp (Fig. 1B) and encoded a predicted protein of 366 amino acid residues. After restriction enzyme digestion and T_4 DNA ligase reaction, the cDNA fragment and vector pHAC181 were ligated and transfected into competent cells *Trans*1-T1. Positive transformants were verified by restriction enzymes (Fig. 1C). Homologous primers INT-F and INT-R amplified the large segment of 5481 bp (Fig. 1D), whereas the detection primers amplified 1381 bp in the correct homologous recombination (Fig. 1E).

3.2. Protein expression and western blot analysis

MrLGBP cDNA was expressed in the yeast eukaryotic expression system. The construct was designed to yield the recombinant MrLGBP with a 3 × HA-Tag at the amino terminus, showing a molecular weight of about 45 kDa. The recombinant plasmid was integrated into downstream of the GAL1 promoter of *S. cerevisiae* strain GAL1-ScRCH1, and the expression of MrLGBP was induced with D-galactose. Analysis of cell lysates by SDS-PAGE in a 10% gel showed that the induced cells, but not uninduced ones, contained the protein with an approximate size of 45 kDa. The identity of the recombinant MrLGBP protein was confirmed by western blotting with the anti-HA antibody such that the size of a target band was about 45 kDa (Fig. 2), the protein of control yeast strain *S. cerevisiae* GAL1-ScRCH1 which without the homologous recombination gene MrLGBP was used as control, and results showed that no specific band was detected.

3.3. Antibacterial activity of recombinant MrLGBP

MIC and MBC were determined to evaluate the bacteriostatic and bactericidal activities of MrLGBP. Recombinant MrLGBP inhibited the growth of four Gram-negative bacterial species (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*) and three Gram-positive bacterial species (*B. subtilis*, *B. thuringiensis*, and *S. aureus*) at different magnitudes. MIC and MBC ranged from 0.340 to 0.802 and 1.189–1.810 μM, respectively (Table 2).

3.4. Bacterial agglutination test

To test whether MrLGBP interacts with the microbial surface, we performed the agglutination assay using four Gram-negative bacterial species (*E. coli*, *A. hydrophila*, *V. parahaemolyticus* and *P. aeruginosa*) and three Gram-positive bacterial species (*B. subtilis*, *B. thuringiensis*, and *S. aureus*). All four Gram-negative strains were agglutinated by MrLGBP (Fig. 3A), whereas only one Gram-positive strain *B. subtilis* showed agglutination with MrLGBP (Fig. 3B). These results indicated that MrLGBP recognized the surface molecules on some Gram-negative bacteria and some Gram-positive bacteria.

Table 2
Antibacterial activity of recombinant MrLGBP protein.

Bacteria	MIC (μM)	MBC (μM)
Gram-negative		
Escherichia coli	0.802	1.547
Aeromonas hydrophila	0.447	1.810
Vibrio parahaemolyticus	0.587	1.217
Pseudomonas aeruginosa	0.406	1.310
Gram-positive		
Bacillus subtilis	0.340	1.189
Bacillus thuringiensis	ND	ND
Staphylococcus aureus	ND	ND

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; ND: not detectable.

3.5. Detection of M. rosenbergii mortality after bacterial challenge

The mortality of *M. rosenbergii* was calculated at 0, 2, 6, 12, 24, and 48 h after *V. parahaemolyticus* challenge. The results indicated that after 1-month feeding, *M. rosenbergii* fed with the pellet of *S. cerevisiae* MrLGBP had the lowest mortality rate (its highest mortality value was 50.2%), whereas those fed with the common pellet showed the highest mortality rate (87.5%) at 48 h after the challenge (Fig. 4). The mortality rate of *M. rosenbergii* fed with the pellet of blank *S. cerevisiae* was higher than the mortality rate of the group fed with the pellet of *S. cerevisiae* MrLGBP, but lower in comparison with the group consuming the common pellet diet. No significant difference was observed between the PBS control groups (Fig. 4).

3.6. Determination of the hepatopancreas enzymatic activity of M. rosenbergii

The immune enzymatic activity (AKP, ACP, SOD, and CAT) of *M. rosenbergii* was measured at 0, 2, 6, 12, 24, and 48 h after *V. parahaemolyticus* challenge. The results revealed that *M. rosenbergii* fed with the pellet of *S. cerevisiae* MrLGBP manifested a significant increase in AKP activity at 6 h (67.2 U/g, $P < 0.05$) as compared with those fed with blank *S. cerevisiae* and only pellet diet, the value increased and reached its maximum (80.3 U/g, $P < 0.01$) at 12 h (Fig. 5A). Then the value declined to 72.4 U/g ($P < 0.05$) at 24 h but was still higher than that reported for the group fed with the control pellet. During this period, the group fed with the pellet of blank *S. cerevisiae* and common pellet showed a similar enzymatic activity level, which was lower than that observed for the target protein group; little change was observed in the trend. No significant effect was observed in the PBS control group (data not shown).

The changes in ACP activity were similar to those observed for AKP. The group fed with the pellet of *S. cerevisiae* MrLGBP showed a visible increase in ACP activity at 6 h as compared with the other two groups, and the value reached its peak at 12 h (51.33 U/g, $P < 0.05$) after introduction of the pathogen. The groups fed with the blank *S. cerevisiae* pellet and common pellet showed similar results; their enzymatic activity levels underwent no obvious change during this period (Fig. 5B).

The evaluation of SOD activity revealed higher levels in the group fed with the target protein MrLGBP than in the other two groups from 2 to 12 h after *V. parahaemolyticus* infection (Fig. 5C). CAT activity significantly increased at 2 and 12 h in the group fed with the target protein MrLGBP as compared with those fed with blank *S. cerevisiae* pellet and common pellet (Fig. 5D). These results on immunoenzyme activity indicate that recombinant protein MrLGBP may enhance the immune activity of *M. rosenbergii* to a certain extent.

3.7. Expression of immunity-related genes in the hepatopancreas

Expression of immune-related genes (LGBP, AKP, ACP, CAT, and Cu,Zn-SOD) was measured by qRT-PCR in the hepatopancreas of *M. rosenbergii* at 0, 2, 6, 12, 24, and 48 h after a challenged with *V. parahaemolyticus*. As shown in Fig. 6, most genes (LGBP, ACP, CAT, and Cu,Zn-SOD) were significantly overexpressed during stimulation with pathogenic bacteria in the group fed with the pellet of *S. cerevisiae* MrLGBP as compared with those fed with blank *S. cerevisiae* and only pellet diet. Only the AKP gene showed slightly higher expression in the group fed with the pellet of *S. cerevisiae* MrLGBP at 12 h after stimulation with *V. parahaemolyticus*, whereas no obvious difference in overall expression was observed in the other two groups. We noted a remarkable increase in the expression of the LGBP gene in the hepatopancreas of prawns fed with the pellet of *S. cerevisiae* MrLGBP ($P < 0.05$ or $P < 0.01$) at 2, 6, and 12 h as compared with the other two groups. At 12 h, LGBP mRNA transcripts reached a peak value (440-fold increase). No obvious difference was reported between PBS-challenged control groups (data not shown).

4. Discussion

Invertebrates lack the ability to produce antibodies but have an efficient innate immune system to defend themselves against invading pathogens. PRPs perform an important function in invertebrates by activating the innate immune defense [8,9]. LGBP is one of the important PRPs that has various biological functions [33]. In this study, an *S. cerevisiae* eukaryotic system was used to express MrLGBP. *M. rosenbergii* LGBP cDNA ORF (1098 bp) was cloned into vector pHAC181, and the recombinant plasmid was integrated into downstream of the GAL1 promoter of *S. cerevisiae* strain GAL1-ScRCH1 via a homologous recombination technique. The resulting recombinant MrLGBP contained a 3 × HA-tag at its C terminus. The protein was expressed in the presence of D-galactose, and the yeast cells were collected, lysed, and analyzed by SDS-PAGE in a 10% gel. Western blot analysis revealed the molecular weight of recombinant MrLGBP to be about 45 kDa (Fig. 2).

In previous studies, crustacean LGBP transcripts were found to be significantly upregulated after challenges with bacteria such as *V. harveyi* [34], *V. alginolyticus* [15], *V. anguillarum* [35], or *A. hydrophila* [36]. Zhang et al. [37] have shown that LPS and BG significantly induce *Eriocheir sinensis* LGBP expression, suggestive of the role of EsLGBP in the innate immunity and defense against infections by Gram-negative bacteria and fungi. In our study, recombinant MrLGBP inhibited the growth of four Gram-negative and three Gram-positive bacterial species at various levels of effectiveness. MIC values ranged from 0.340 to 0.802 μM and MBC values varied between 1.189 and 1.810 μM, suggesting that MrLGBP exerts some antibacterial action on Gram-negative and Gram-positive bacteria, and that it exerts different antibacterial activities on different bacteria.

The agglutination assay was performed to test whether MrLGBP interacts with the microbial surfaces. The results revealed that the recombinant MrLGBP protein agglutinated all four Gram-negative strains (Fig. 3A); however, only one Gram-positive bacterial species (*B. subtilis*) appeared to be agglutinated by MrLGBP. *B. thuringiensis* and *S. aureus* showed no obvious aggregation. These results suggest that MrLGBP as a typical PRP is capable of recognizing and noncovalently binding to specific saccharide moieties and may efficiently immobilize the invading bacteria and therefore inhibit further tissue invasion. The bacterial agglutination activity of recombinant MrLGBP protein recognized the LPS of bacterial cell walls, and by binding to its substrate, MrLGBP efficiently distinguished the pathogens. Our results are in line with those reported by Du et al. [15], which highlight the selectivity of *F. chinensis* LGBP binding activity; the strongest binding activity was

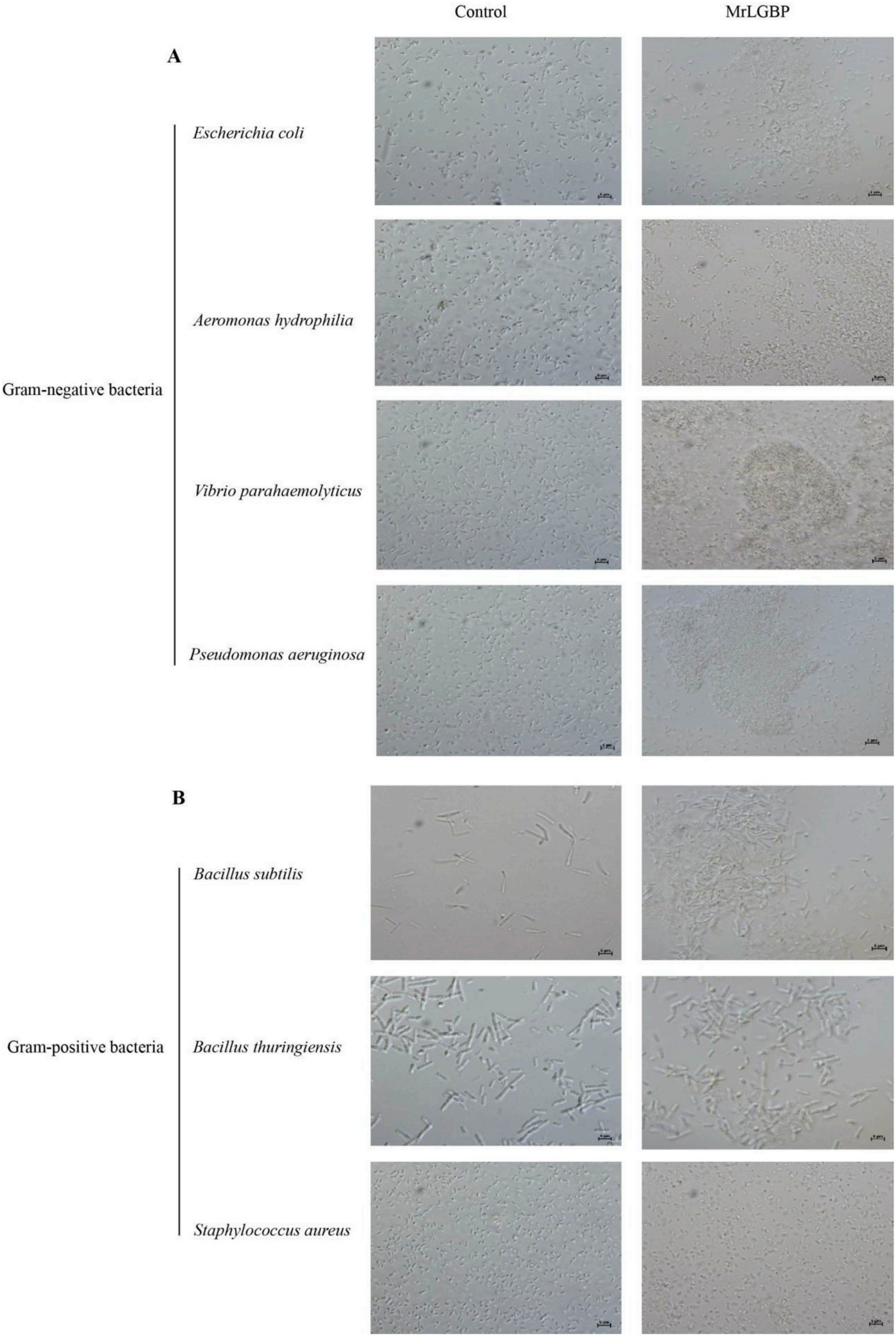

Fig. 3. Four Gram-negative bacterial species (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*) and three Gram-positive bacterial species (*B. subtilis*, *B. thuringiensis*, and *S. aureus*) were used in the bacterial agglutination test. The protein extracted from yeast strain *S. cerevisiae* GAL1-ScRCH1 which without the homologous recombination gene MrLGBP was served as control. All Gram-negative strains (Fig. 3A) and one Gram-positive bacterium *B. subtilis* were agglutinated by MrLGBP (Fig. 3B), whereas two Gram-positive strains (*B. thuringiensis* and *S. aureus*) showed no obvious agglutination phenomenon (Fig. 3B). Bar: 5 μm.

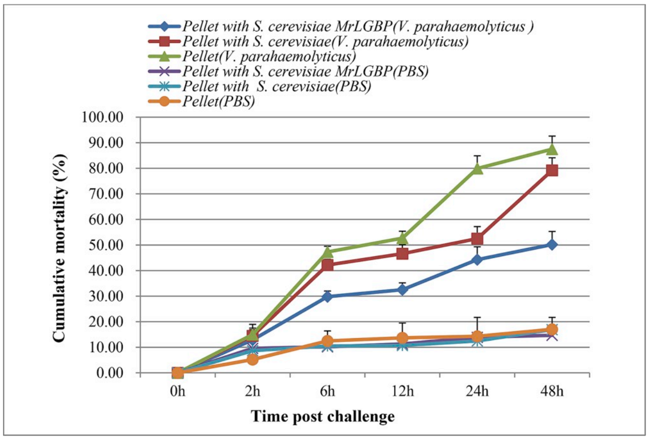

Fig. 4. Detection of *M. rosenbergii* mortality after *V. parahaemolyticus* challenge. *M. rosenbergii* fed with the pellet of *S. cerevisiae* MrLGBP showed the lowest mortality rate (highest mortality rate was 50.2%), whereas those fed with the common pellet showed the highest mortality rate of 87.5% at 48 h after bacterial challenge. *M. rosenbergii* group fed with the pellet of *S. cerevisiae* exhibited higher mortality than that fed with *S. cerevisiae* MrLGBP but lower than that fed with the common pellet. There was no significant difference between the PBS control groups. Annotation: Pellet with *S. cerevisiae* MrLGBP (the pellet feed mixed with yeast strain *S. cerevisiae* GAL1-ScRCH1 that expressed the target protein MrLGBP); Pellet with *S. cerevisiae* (means pellet with blank *S. cerevisiae*, the pellet feed mixed with yeast strain *S. cerevisiae* GAL1-ScRCH1, which without the homologous recombination gene MrLGBP); Pellet (common pellet feed without yeast strains). Similar notation is used hereinafter.

reported for Gram-negative bacteria, followed by little or no binding to Gram-positive bacteria and yeast. Our bacterial agglutination assay also indicated that MrLGBP plays important roles in the innate immunity of *M. rosenbergii.* Two species of pathogenic bacteria affecting crustaceans, *A. hydrophila* and *V. parahaemolyticus*, were effectively agglutinated, suggesting that MrLGBP may be a type of immunity-related protein in aquatic animals.

To verify the effect of the recombinant MrLGBP protein on the immune function of *M. rosenbergii*, recombinant yeast strain MrLGBP was concentrated in fermentation broth at 1×10^9 cell/mL and sprayed onto the pellet feed. Upon infection with *V. parahaemolyticus*, *M. rosenbergii* fed with the pellet of *S. cerevisiae* MrLGBP showed the lowest mortality rate, whereas the groups fed with the pellet of blank *S. cerevisiae* manifested lower mortality than did those fed with the common pellet diet. These results indicate that recombinant yeast strain *S. cerevisiae* MrLGBP enhanced the immune response of *M. rosenbergii*. The group fed with the blank yeast strain *S. cerevisiae* showed lower mortality than did the common pellet group, suggesting that *S. cerevisiae* itself may serve as a good immune feed additive. Because the target protein–expressing strain *S. cerevisiae* MrLGBP strengthened the immune function, the group fed with *S. cerevisiae* MrLGBP manifested the best immune effect against the pathogen.

The innate immune system of crustaceans is a complex proteolytic cascade system that may be triggered by components of pathogens. Antioxidant enzymes such as SOD and CAT constitute the major defense system against reactive oxygen species (ROS) [38,39]; hence, respiratory SOD and CAT activities have been widely used to evaluate the defense ability against pathogens in crustaceans. Moreover, AKP and ACP perform important functions in the defense and in cellular oxygen-carrying systems of crustaceans. In this study, enzymatic activity assays and qRT-PCR analysis were performed to assess the immune responses and related gene expression in the hepatopancreas of *M. rosenbergii* infected with *V. parahaemolyticus*. AKP activity significantly increased in the group fed with *S. cerevisiae* MrLGBP at 6 h after *V. parahaemolyticus* infection and reached its peak value at 12 h. The two other *M. rosenbergii* groups (fed with the pellet of blank *S. cerevisiae* and common pellet) showed lower enzymatic activity than did the group fed with the target protein and displayed no obvious variation. Similar to AKP, ACP activity increased in the group fed with the target protein at 6 h post challenge and reached its peak at 12 h after the challenge; no obvious change was seen in the other two groups. These results are in agreement with our previously reported [40], where AKP activity in the *M. rosenbergii* hepatopancreas increased and reached its peak 5 days after infection with spiroplasma MR-1008, and ACP activity increased from 5 days and reached its peak at 10 days. Zhang et al. [41] have observed an increase in the expression of AKP and ACP in the hepatopancreas of *F. chinensis* in response to white spot syndrome virus (WSSV) and zymosan A; AKP and ACP activities were higher in the hepatopancreas than in lymphoid organs and gills. In contrast, AKP and ACP enzymatic activities induced by *V. parahaemolyticus* in our study were significantly higher than those caused by virus WSSV [41] or spiroplasma MR-1008 [40]. This observation may be attributed to the differences in tolerance to different pathogens. The evaluation of RNA transcript levels showed an obvious increase in ACP gene expression in the group fed with the target protein versus the other two groups after the *V. parahaemolyticus* challenge, and the value reached its peak at 24 h (81-fold, $P < 0.01$); however, AKP mRNA transcripts showed only a slight increase in the expression at 12 h. These results indicate that the hepatopancreas generated higher AKP and ACP activities to resist the invasion by *V. parahaemolyticus*, and the mRNA transcript levels of AKP and ACP in the hepatopancreas could be induced effectively. Thus, the recombinant protein *S. cerevisiae* MrLGBP enhanced the immune ability of *M. rosenbergii*.

In the present study, we report a significant increase in SOD and CAT enzymatic activities in the group fed with the target protein at 2 h until 12 h after the *V. parahaemolyticus* challenge. CAT enzymatic activity markedly increased at 2 h and reached its peak at 12 h; these results were consistent with those reported in a previous study (with *M. rosenbergii* infected by spiroplasma MR-1008 [40]) but differed from findings in another study, which revealed a significant reduction in SOD activity in WSSV-infected tissues of *P. monodon* [42]. Moreover, CAT activity in crabs exposed to the highest dose of a *Microcystis aeruginosa* aqueous extract significantly decreased at the end of the experiment [43]. In this work, SOD and CAT gene expression levels showed similar trends, with obvious upregulation observed at 6 h after the *V. parahaemolyticus* challenge; these values reached their peaks at 12 h (a 210-fold increase for SOD and a 30-fold increase for CAT), followed by a decline in the expression of the two genes until 48 h. These results are similar to those previously reported for shrimps infected with bacteria or WSSV [44,45], wherein CAT mRNA expression increased. Taken together, the increases in SOD and CAT mRNA expression levels may increase SOD and CAT activities for the self-protection of crustaceans against the invading pathogen. Thus, activation of the antioxidant defense system may result in the direct or indirect ROS generation after pathogenic infection.

Protein LGBP serves as a PRP and participates in the activation of the proPO system in crustacean infections during the early stages. The infection of *M. rosenbergii* with *V. parahaemolyticus* caused an increase in the mRNA expression of LGBP at 2 h, and the level reached its peak value at 12 h (440-fold increase, $P < 0.01$). The expression of hepatopancreas LGBP mRNA in *P. stylirostris* was significantly upregulated with the progression of WSSV infection [46], but LGBP expression was lower than that seen in our study. The present study indicated that when *V. parahaemolyticus* infected with *M. rosenbergii*, LGBP as one of the PRPs recognizing the non-self material that has gained entrance into the prawn body immediately, so it increased significantly at the early stage. With infection of pathogen, enzymes in oxygen antibacterial system (SOD and ACT) were synthesized rapidly in hepatopancreas in

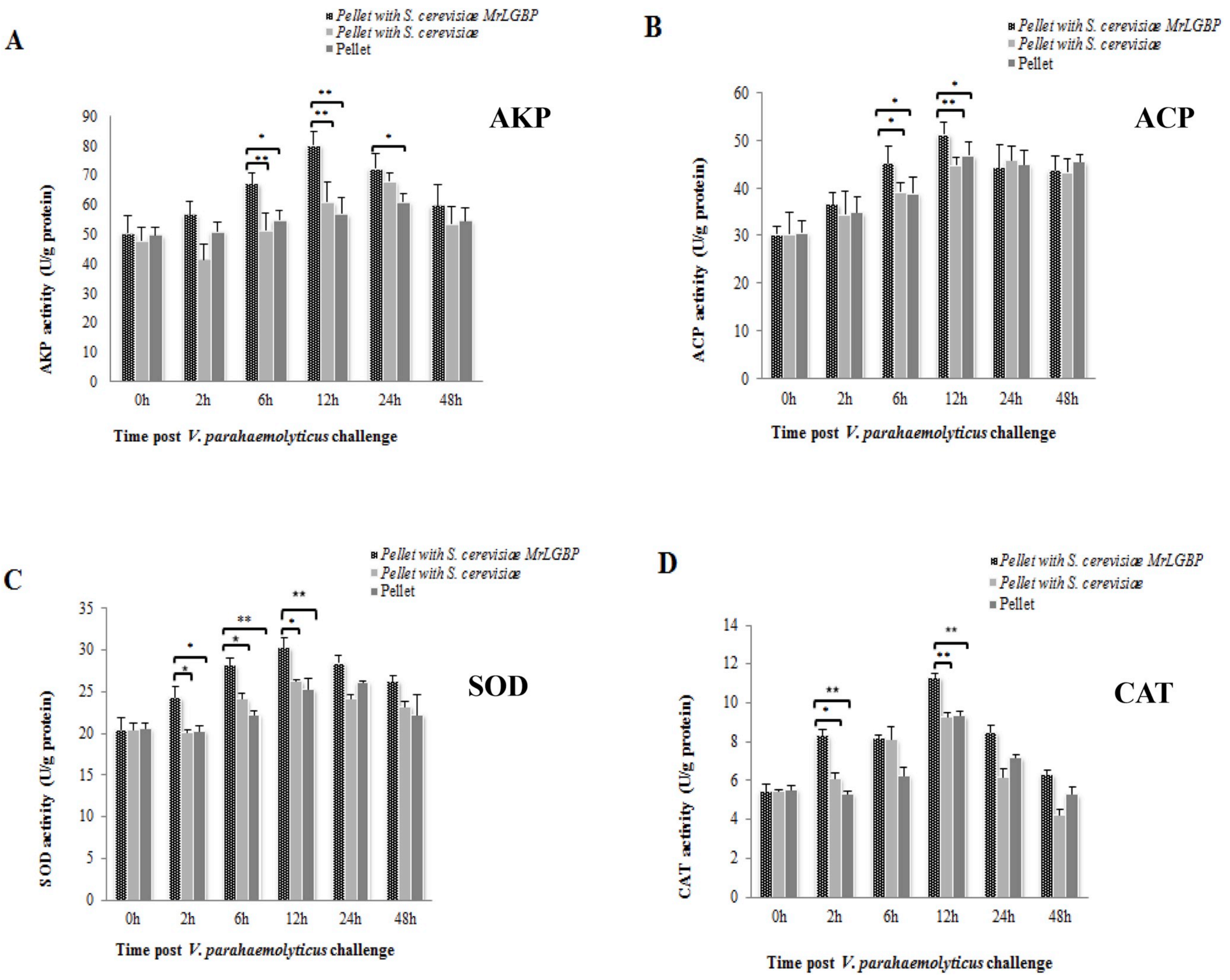

Fig. 5. Changes in the relative immune enzymatic activity in *M. rosenbergii* hepatopancreas at 0, 2, 6, 12, 24, and 48 h after *V. parahaemolyticus* infection. *M. rosenbergii* were fed with three different diets for a month. Prawns challenged with PBS were maintained as controls; no obvious difference was observed between the three treatment groups following the PBS challenge (data not shown). A: AKP activity. B: ACP activity. C: SOD activity. D: CAT activity. Each bar represents a mean value from six determinations with standard error. The asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$).

response to stimulation of *V. parahaemolyticus*, meanwhile, the AKP and ACP phosphatases enzymes also participate in this immune response actively, these immune factors participated in prawn innate immunity and played a positive and effective role in resisting the *V. parahaemolyticus*. Furthermore, the group fed with *S. cerevisiae* MrLGBP showed higher enzymatic activity and mRNA expression levels than did the control groups, indicating that the recombinant *S. cerevisiae* MrLGBP strain expressing the target protein is important for the enhancement of the immune function of *M. rosenbergii* against the pathogen.

It is noteworthy that some gene mRNA levels in the hepatopancreas were not consistent with their enzyme activities, similar phenomena have been found in our previous research work [40], this situation was widespread and can be explained by the dynamics of cellular transcription and translation, translational and post-translational regulation of antioxidant enzymes is important in determining changes in the levels of activity in response to oxidative stress, thus making mRNA levels less reliable in estimating enzyme functional states. The delay in the decline of mRNA transcripts relative to enzyme activities could be the result of post-transcriptional regulatory mechanisms or simply to the stability of the enzyme proteins themselves. Maier [47] reported many parameters can influence mRNA-protein correlation, transcription and translation are far from having a linear and simple relationship. Some studies have revealed increases in enzyme activity that follow increases at mRNA levels [48,49], or increases in activities without changes of mRNA levels [50]. In recent years, Liu [51] came to a conclusion that transcript levels by themselves are not sufficient to predict protein levels in many scenarios. Thus explained the up regulation of some genes expression at some time points were not consistent with their enzyme activity, even increasing in enzyme activity that occurred before the up regulation of gene expression in this study.

In conclusion, MrLGBP was expressed in *S. cerevisiae* eukaryotic system and had a predicted molecular weight of 45 kDa. Evaluation of the antibacterial activity of the recombinant protein suggested that MrLGBP might serve as an important immune-related protein in the crustacean innate immune system. The recombinant protein strain can improve the immune activity level of *M. rosenbergii* and induce and upregulation the immune genes expression level simultaneously. These genes cooperation and participate in immune response effectively and enhance the immunity of *M. rosenbergii* to against the invading

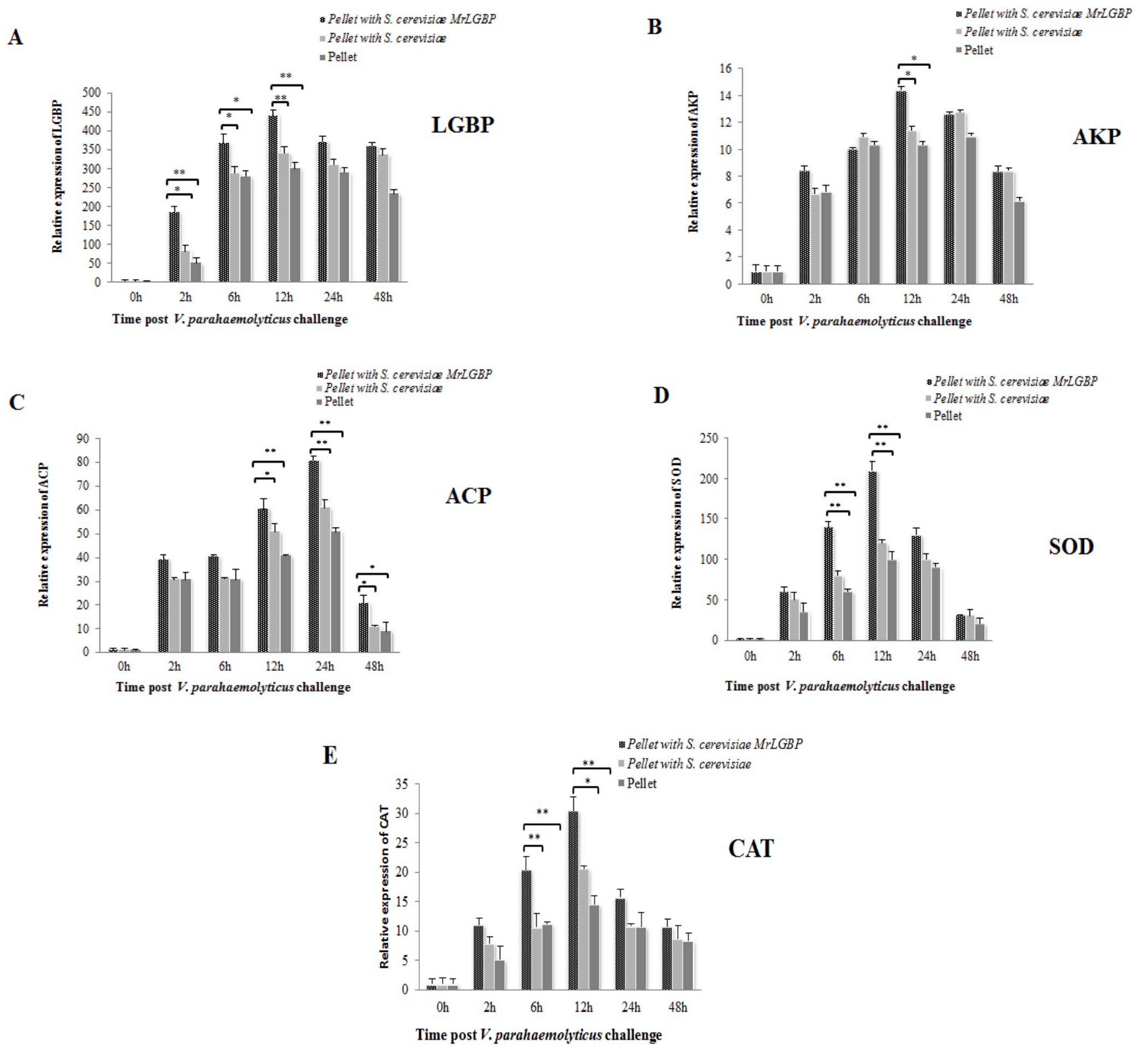

Fig. 6. Analysis of expression of immunity-related genes in *M. rosenbergii* hepatopancreas by qRT-PCR at 0, 2, 6, 12, 24, and 48 h after *V. parahaemolyticus* infection. *M. rosenbergii* were fed with three different diets for a month. Prawns challenged with PBS were maintained as controls; no obvious difference was observed between the three treatment groups after the PBS challenge (data not shown). The mRNA levels of immunity related genes were analyzed and standardized according to β-actin mRNA level. A: Relative expression of LGBP. B: Relative expression of AKP. C: Relative expression of ACP. D: Relative expression of SOD. E: Relative expression of CAT. Each bar represents a mean value from six measurements with standard error. The asterisks indicate significant differences ($*P < 0.05$, $**P < 0.01$).

pathogen *V. parahaemolyticus*. Thus, this potential antimicrobial peptide may be used in the aquaculture sector to control bacterial infections and may be useful for the development of an effective feed additive for strengthening the crustacean immunity.

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