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Macrobrachium rosenbergii Cu/Zn superoxide dismutase (Cu/Zn SOD) expressed in *Saccharomyces cerevisiae* and evaluation of the immune function to *Vibrio parahaemolyticus*

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ABSTRACT

Superoxide dismutases (SODs) are important antioxidant enzymes that occur in virtually all oxygen-respiring organisms, and copper/zinc SOD (Cu/ZnSOD) is one of the most important SODs. In the present study, *Macrobrachium rosenbergii* Cu/Zn-SOD was expressed in a yeast eukaryotic system. The open reading frame (ORF) of MrCu/ZnSOD was cloned into the plasmid vector pHAC181, and the recombinant plasmid was integrated into the downstream region of the GAL1 promoter in *Saccharomyces cerevisiae* strain GAL1-ScRCH1 via homologous recombination. The resulting recombinant MrCu/ZnSOD consisted of a 3 × HA-tag at its C-terminal. Via western blot, the molecular weight of the recombinant MrCu/ZnSOD was estimated at about 30 kDa. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of this recombinant MrCu/ZnSOD ranged from 0.556 to 0.840 μM, and from 0.967 to 2.015 μM, respectively. The recombinant MrCu/ZnSOD protein was able to agglutinate four Gram-negative bacterial strains, as well as two of three Gram-positive strains (except *Staphylococcus aureus*). This demonstrated that the recombinant protein possessed some antimicrobial activity against certain Gram-positive and Gram-negative bacteria. *M. rosenbergii* were fed with the recombinant yeast strain MrCu/ZnSOD for 4 weeks and then challenged with the most common crustacean pathogen, *Vibrio parahaemolyticus*. This group of prawns presented lower mortality, higher enzymatic activity, and higher expression of the mRNA of immune-related genes than that in the control groups. Taken together, these results suggest that MrCu/ZnSOD is an antioxidant enzyme and antimicrobial peptide involved in the crustacean innate immune system and offers protection to the host against pathogenic bacteria.

1. Introduction

Macrobrachium rosenbergii is a large freshwater prawn that has been commercialized in China and Southeast Asian countries [1,2]. However, the cultivation of this prawn has been restricted due to epidemic infectious diseases in recent years, including parvovirus extra small virus (XSV), white tail disease (WTD) [3,4], and diseases caused by spiroplasma [5,6], which can cause considerable business losses to the local economy. Therefore, studying the prawn innate immunity is the first step towards controlling such diseases and ensuring the long-term survival of the crustacean aquaculture industry.

Crustaceans do not possess an acquired immune system. Instead, they have an innate immune system that confers protection against pathogens [7]. The innate immune response includes melanization by the prophenoloxidase-activating system (proPO system), clotting, encapsulation of foreign material, phagocytosis, cell agglutination, and production of a diverse array of general and specific antimicrobial peptides (AMPs) [8]. Circulating hemocytes play a crucial role in the innate immune response of crustaceans, via clot formation, phagocytosis, and packaging, to regulate physiological functions such as blood coagulation, hardening of lymph exoskeleton, and confinement of invasive particles or microorganisms [9–11]. Once the microbes are engulfed by hemocytes, they activate the host NADPH-oxidase, which, in turn, produces a series of reactive oxygen intermediates (ROIs) such as superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$), all of which possess high anti-microbial activity [12]. However, high levels of superoxide anion and other ROIs may be cytotoxic to the host [13,14]. ROIs are effectively and

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rapidly eliminated by antioxidant defense mechanisms, including superoxide dismutases (SODs), which scavenge the superoxide anion [15]. SODs are important antioxidant enzymes that occur in virtually all oxygen-respiring organisms. They are classified into three distinct groups depending on their metal content: iron SOD (Fe-SOD), manganese SOD (Mn-SOD), and copper/zinc SOD (Cu/Zn-SOD) [16]. Cu/Zn-SOD has been cloned in several teleost species, including black porgy *Acanthopagrus schlegelii* [17], red seabream *Pagrus major* [18], grouper *Epinephelus malbaricus* [19], *E. coioides* (AAW29025), Atlantic salmon *Salmo salar* (BG936553), zebra fish *Danio rerio* [20], and Pacific oyster *Crassostrea gigas* [21]. However, only a small number of studies have cloned and characterized Cu/Zn-SOD in decapod crustaceans [16,22,23]. Furthermore, most studies have focused on cloning and analyzing the full-length cDNA sequence of the genes and their expression following stimulation with pathogenic bacteria. In contrast, studies on their protein expression and function are relatively scarce. Furthermore, to date, SOD in crustaceans has been typically expressed in the *Escherichia coli* prokaryotic system [20], and very few reports have described the application of the *S. cerevisiae* system for expression of crustacean SOD.

Compared with *E. coli*, yeast has an advanced heterologous protein-folding pathway. In addition, use of the yeast signal sequence allows yeast cells to secrete proteins that are properly folded and processed. With the wide application of industrial fermentation technology, yeast has presented great advantages for the expression of clinically and industrially important proteins [24,25]. *S. cerevisiae* is a yeast species that is generally regarded as a safe (GRAS) organism and is used widely in the food and beverage industry [26,27]. In addition, active dry yeast, yeast culture, and yeast extract are widely used as feed additives in aquaculture industry [28–30]. *S. cerevisiae* has been used to successfully express a variety of exogenous eukaryotic proteins, including the hepatitis B vaccine, human insulin, human granulocyte colony stimulating factor, and human blood vessel inhibitor [31]. However, the use of a *S. cerevisiae* system to express *M. rosenbergii* Cu/ZnSOD has not been reported to date.

In this study, we used *S. cerevisiae* to express the innate immunity and antioxidant factor Cu/Zn SOD of *M. rosenbergii* (hereafter referred to as MrCu/ZnSOD). The open reading frame (ORF) of the MrCu/ZnSOD gene was cloned into the plasmid vector pHAC181, and the recombinant plasmid was integrated downstream of the GAL1 promoter in *S. cerevisiae* GAL1-ScRCH1 strain via homologous recombination. Under D-galactose induction, the target protein was expressed in yeast cells, and a preliminary study on the antibacterial function of the recombinant protein was performed. Moreover, tentative immune functions of the recombinant protein as well as its effect on the immunity of *M. rosenbergii* were determined. This study aimed to provide useful information and empirical data for crustacean innate immunity, as well as prevention and control diseases of aquatic animal.

2. Materials and methods

2.1. Experimental animals, microbial strains, and culture media

Healthy *M. rosenbergii* prawns (average body weight 20 g) were obtained from a commercial farm in Nanjing, Jiangsu province, China. The prawns were kept in a recirculation system containing filtered and UV-treated artificial freshwater, under a controlled light (12-h light: 12-h dark) and regulated temperature (26–28 °C) regime. The prawns were fed daily with a commercial prawn diet (Nantong Bada Feed Co., Ltd, China) at a rate of 4% body weight, for 2 weeks prior to experimentation.

Plasmid vector pHAC181 (constructed in our laboratory) was cultured in Luria Bertani (LB) liquid medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) at 37 °C, while *S. cerevisiae* strain GAL1-ScRCH1 (constructed in our laboratory) 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 cultured in LB broth at 37 °C.

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

Healthy *M. rosenbergii* individuals 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 modified phosphate-buffered saline (PBS) (0.9 g/L Na_2HPO_4, 0.27 g/L KH_2PO_4, 0.6 g/L KCl, 25.5 g/L NaCl, 1.0 g/L L-glucose, pH 7.2) as an anticoagulant [6]. 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 TRIzol Reagent (TaKaRa, Japan), performed in accordance with the standard manual [6]. The concentration and quality of RNA were assessed by measurement of absorbance at 260/280 and 260/230 nm wavelengths on a UV–Visible spectrophotometer (Eppendorf, Germany). Only RNA with an $OD_{260}/_{280}$ ratio of 1.8–2.1, and an $OD_{260}/_{230}$ ratio ≥ 2.0, was used for cDNA synthesis. The general integrity of the total RNA was assessed using 1% agarose gel electrophoresis. A 1-μg sample of the 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 *M. rosenbergii* Cu/ZnSOD cDNA sequence (GenBank accession No. DQ121374.1) [23] was amplified by PCR using Cu/ZnSOD-specific primer pairs, with *EcoR* I and *sph* I site at their 5′ terminal ends, respectively. Plasmid pHAC181 [32] was used as a vector for target gene cloning (pHAC181 is a multicopy plasmid that was constructed in our laboratory by inserting three hemagglutinin [HA] tags into the commercial plasmid YEplac181). After enzyme digestion, and ligation using T_4 DNA ligase (Takara, Japan), the mixture was transformed into competent *Trans*1-T1 cells (TransGen, China). The transformants were confirmed using restriction enzymes, and the recombinant plasmids were verified via DNA sequencing. Homologous recombination was used to integrate the target gene along with the HA tags from the recombinant plasmids, into the downstream region of GAL1 promoter in *S. cerevisiae* strain GAL1-ScRCH1 (wherein the *ScRCH1* gene promoter was replaced by the *GAL1* gene promoter in *S. cerevisiae* BY4741 strains [Sc04153268_s1] in the laboratory). High-fidelity PrimeSTARGXL DNA Polymerase (Takara, Japan) and the homologous primer (Table 1) were used to amplify the large fragments, and detection primers were used to confirm successful integration (Table 1).

2.4. Expression of recombinant MrCu/ZnSOD

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 was used as an induction agent) under 30 °C, with vigorous shaking at 220 rpm. Once an optical density of 1.0–1.5 was attained at 600 nm wavelength (OD_{600}), the total protein was extracted and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and western blot analysis was performed to evaluate the expression of recombinant MrCu/ZnSOD.

2.5. SDS-PAGE and western blot analysis

Standard 10% SDS-PAGE was used to analyze the expression of recombinant MrCu/ZnSOD. The protein bands were visualized by staining with Coomassie Brilliant Blue.

Western blot analysis was used to confirm the identity of the expressed recombinant MrCu/ZnSOD. Proteins separated by SDS-PAGE

Table 1
Specific primers used in these experiments.

Primer	Purpose	Sequence (5′ -3′)
MrCu/ZnSODF	cDNA amplification	CCG GAATTC TTAATTAA ATGGCGAAGTGCTTACAAGTC
MrCu/ZnSODR	cDNA amplification	ACAT GCATGC ATTTTCTAAGTCGTGGGGCG
INT-F	Homologous recombination	CAAATGTAATAAAAGTATCAACAAAAAATTGTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACCCGGATCTCAAAATGGCGAAGTGCTTACAAGTC
INT-R	Homologous recombination	TATGGACGAGGTAATAAGGAAACTCAGAACCAGAATAGTGGCATGAGCTCTCCAATTTAACATATTTGCCATTAGTGACCGATGATAAGCTGTCAAACATG
Check-F	Check primer	CCTGGCCCCACAAACCTTC
Check-R	Check primer	ATTTTCTAAGTCGTGGGGCG
QLGBP-F	qRT-PCR	AGGAACCGGCGGTTTCTT
QLGBP-R	qRT-PCR	GGTGTTGGACCAAGGCTTGT
QLectin-F	qRT-PCR	CTAACAACGACACTGAGGCAA
QLectin-R	qRT-PCR	ACCGACATCCACAAAGAAAAC
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
QCu/ZnSODF	qRT-PCR	TCGCCTAACGAGGAGGTTCA
QCu/ZnSODR	qRT-PCR	CGGCTTCATCAGGATTTTGAG
β-actinF	qRT-PCR	GAGACCTTCAACACCCCAGC
β-actinR	qRT-PCR	TAGGTGGTCTCGTGAATGCC

were electro-transferred onto a nitrocellulose membrane (Bio-Rad), using a semi-dry electrophoretic transfer apparatus (*Trans*-blot SD, Bio-Rad) at 25 V for 30 min. The membrane was then washed twice with Tris-buffered saline (TBS, 10 mM Tris-HCl, 150 mM NaCl, pH 7.5) at room temperature (20–25 °C) for 10 min and incubated in a blocking buffer (3% BSA in a TBS buffer) at room temperature for 2 h. Subsequently, the membrane was washed twice with TBS containing 0.05% (v/v) Tween 20 (TBST) at room temperature for 10 min, and incubated with anti-HA antibodies (Abcam, UK) at room temperature for 2 h. After the membrane was washed thrice with TBS, secondary antibodies conjugated to horseradish peroxidase (Abcam, UK) were added. Two hours later, the membrane was washed thrice, and the recombinant protein was detected following exposure.

2.6. Purification of the recombinant MrCu/ZnSOD protein

The recombinant MrCu/ZnSOD protein was purified using rProtein A Sepharose™ Fast Flow (GE Healthcare, USA), with PEB buffer (2 mM EDTA, 1 × 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). The purification steps were as follows: 500 μL of the total protein extract was mixed with 25 μL of the anti-HA antibody and incubated overnight at 4 °C. The rProtein A beads (200 μL; with 20% ethanol) were centrifuged to remove the ethanol, washed twice with 1 mL PEB buffer, and resuspended in 500 μL PEB buffer. The washed rProtein A beads were added to the antibody solution and incubated at 4 °C for 4 h, followed by centrifugation at 4 °C and 500 × *g* for 5 min. 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 a 100 μL volume 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 antibacterial activity of the obtained supernatant was then determined.

2.7. Antimicrobial activity assay

The bacterial strains used in this study included four Gram-negative bacteria (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*) and three Gram-positive bacteria (*B. subtilis*, *B. thuringiensis,* and *S. aureus*). For antibacterial activity, the minimum inhibitory concentration (MIC) was determined by liquid growth inhibition assays, as previously described [33]. In brief, 10 μL of each diluted MrCu/ZnSOD sample (*S. cerevisiae* GAL1-ScRCH1 was used as a control) was incubated in sterile 96-well plates containing a 100 μL 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, 5 g/L NaCl, pH 7.5) was used to culture the standard bacterial strains. The bacteria were grown for 24 h under vigorous shaking at 30 °C. Bacterial growth was controlled based on the OD_{600} values after incubation. The MIC value was recorded as the concentration of MrCu/ZnSOD that inhibited the bacterial growth, whereas minimum bactericidal concentration (MBC) was the lowest concentration that caused 100% inhibition of bacterial growth [34]. To examine whether the antibacterial activity of the recombinant protein is bactericidal, the bacteria were co-cultured with different MrCu/ZnSOD concentrations for 24 h, following which, 20 μL aliquots were removed and plated on nutrient agar, and the number of colony-forming units was determined [35].

2.8. Bacterial agglutination assay

The bacterial recognition of MrCu/ZnSOD was assessed by a bacterial agglutination test following a previously described method [36] with minor modifications. Gram-negative bacteria *E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*, and Gram-positive bacteria *B. subtilis*, *B. thuringiensis,* and *S. aureus*, were suspended in LB medium at

1.0×10^7 cells/mL. A 90 μL bacterial suspension was added to 10 μL of recombinant MrCu/ZnSOD at a concentration of 20 μg/μL, while the total protein extract from *S. cerevisiae* GAL1-ScRCH1, which did not express the target protein, was used as a control. The mixtures were incubated overnight at 30 °C, following which, the bacteria were observed under a light microscope.

2.9. Feeding experiment and V. parahaemolyticus challenge

The recombinant protein strain *S. cerevisiae* MrCu/ZnSOD and strain *S. cerevisiae* (without the homologous recombination gene MrCu/ZnSOD) were concentrated to $1.0 \times l0^9$ cells/mL in fermentation broth, and sprayed directly onto the commercial common pellets feed (Nantong Bada Feed Co., Ltd, China) respectively. Yeast immune feed (containing the immunoprotein) and the yeast control feed were prepared after the granular feed surface had dried. Three hundred *M. rosenbergii* prawns were held in a recirculation system containing filtered and UV-treated artificial freshwater, under a controlled light (12 h light/dark) and regulated temperature (26–28 °C) regime. Prawns weighing 20–25 g were used for the experiment.

Healthy prawns were fed daily with different diets, at a rate of 4% body weight for 4 weeks. Based on the diet they were on, the prawns were subdivided into three groups. The experimental group had pellets feed mixed with yeast strain *S. cerevisiae* MrCu/ZnSOD, which expressed the target protein MrCu/ZnSOD. Control group 1 had pellets feed mixed with the *S. cerevisiae* strain (*S. cerevisiae* GAL1-ScRCH1, without the homologous recombination gene MrCu/ZnSOD). Control group 2 had common pellet feed (without any *S. cerevisiae* strain). After feeding for 4 weeks, the prawns were injected with 50 μL physiological saline (PBS) containing approximately 3.0×10^6 colony-forming units [CFU] of the bacterium *V. parahaemolyticus*, whereas the control prawns were injected with 50 μL of PBS. *V. parahaemolyticus* was selected as the pathogen in 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, while hepatopancreas samples were collected for subsequent experiments.

2.10. HE stain of the M. rosenbergii hepatopancreas

Three prawns in different feeding groups were randomly selected at the mid-infection stage (24 h), and following fixation in Davidson's AFA fixative, processed for routine histology according to standard methods [37,38]. The prawns were dissected, the hepatopancreas was prepared, and the tissues were sliced. Continuous 4-mm-thick sections were prepared from synthetic paraffin blocks, and for each pair of consecutive slices, one of the slices was dyed with HE dye. The stained sections were examined and photographed under an inverted phase-contrast microscope (Nikon, Japan).

2.11. Determination of the immune enzyme activity in M. rosenbergii

Hepatopancreatic tissue was used to detect the changes in the activity of immune enzymes. Six *M. rosenbergii* were randomly selected per treatment group at each time point, and the hepatopancreatic tissues were used for enzyme activity determination. SOD activity was assayed according to the method described by Misra and Fridovich [39], based on the oxidation of epinephrine to adreno-chrome by the enzyme. A 0.1 mL volume of hepatopancreatic homogenate was added to a tube containing 0.75 mL ethanol and 0.15 mL chloroform (ice-chilled) 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 0.5 mL epinephrine, and the increase in absorbance at 550 nm was monitored at 30 s intervals for 3 min. The enzyme activity was expressed as 50% inhibition of epinephrine auto-oxidation per minute per milligram of protein. 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 the H_2O_2 degradation reaction catalyzed by CAT [40]. One unit of enzymatic activity was defined as the degradation of one mole of H_2O_2 per second per milligram of protein.

For alkaline phosphatase (AKP) and acid phosphatase (ACP) activity assays, approximately 0.1 g of hepatopancreas was homogenized at $2000 \times g$ for 1 min in 1 mL of ice-cold physiologic saline (0.6%) in an automatic homogenizer (Essen, Germany). Homogenized samples were centrifuged at $3500 \times g$ for 10 min at 4 °C, and the resulting supernatants were used to determine the enzyme activity. AKP and ACP activity were determined as described by King [41], using disodium phenyl phosphate (Sigma, America) as the substrate. One unit of AKP and ACP enzymatic activity 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 with Coomassie Brilliant Blue G250 [42], according to the standard protocol. The diagnostic reagent kit was purchased from Nanjing Jiancheng Bioengineering Institute (China).

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

Six *M. rosenbergii* were randomly sampled from each group, and their hepatopancreatic tissues were collected and mixed for RNA extraction at 0, 2, 6, 12, 24, and 48 h post *V. parahaemolyticus* 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 of six immune-related genes (Cu/Zn-SOD, CAT, AKP, ACP, LGBP, and Lectin) in the hepatopancreas of *M. rosenbergii* challenged with *V. parahaemolyticus*. The expression of β-actin mRNA served as a reference. All PCR (volume 20 μL) mixtures comprised 10 μL of the SYBR Premix *Ex Taq* II (Takara, Japan), 1 μL of cDNA, 10 pmol each of the 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 the experimental results. Relative expression of different genes in the hepatopancreas was calculated according to the $2^{-\Delta\Delta\ CT}$ method [43].

2.13. Statistical analysis

All experiments were performed in triplicate, and statistical analyses were carried out with SPSS 20.0 software. Data were analyzed using one-way ANOVA and represented as mean ± standard error, with statistical significance defined at $P < 0.05$.

3. Results

3.1. Cloning of MrCu/ZnSOD cDNA and homologous recombination

Fig. 1A describes the construction of the *S. cerevisiae* expression system in our study. Total RNA was extracted from the *M. rosenbergii* hemolymph and amplified using Cu/ZnSOD-specific primer pairs. The amplified open reading frame (ORF) of the target cDNA was 603 bp (Fig. 1B), which encoded a predicted protein of 201 amino acids (without the stop codon). After enzyme digestion, the cDNA fragment and vector pHAC181 were ligated using T_4 DNA ligase and transformed into *Trans*1-T1 competent cells. Positive transformants were verified using restriction enzymes (Fig. 1C). Homologous primers INT-F and INT-R amplified the large segment of 4985 bp (Fig. 1D), while the detection primers amplified 886 bp segments (Fig. 1E).

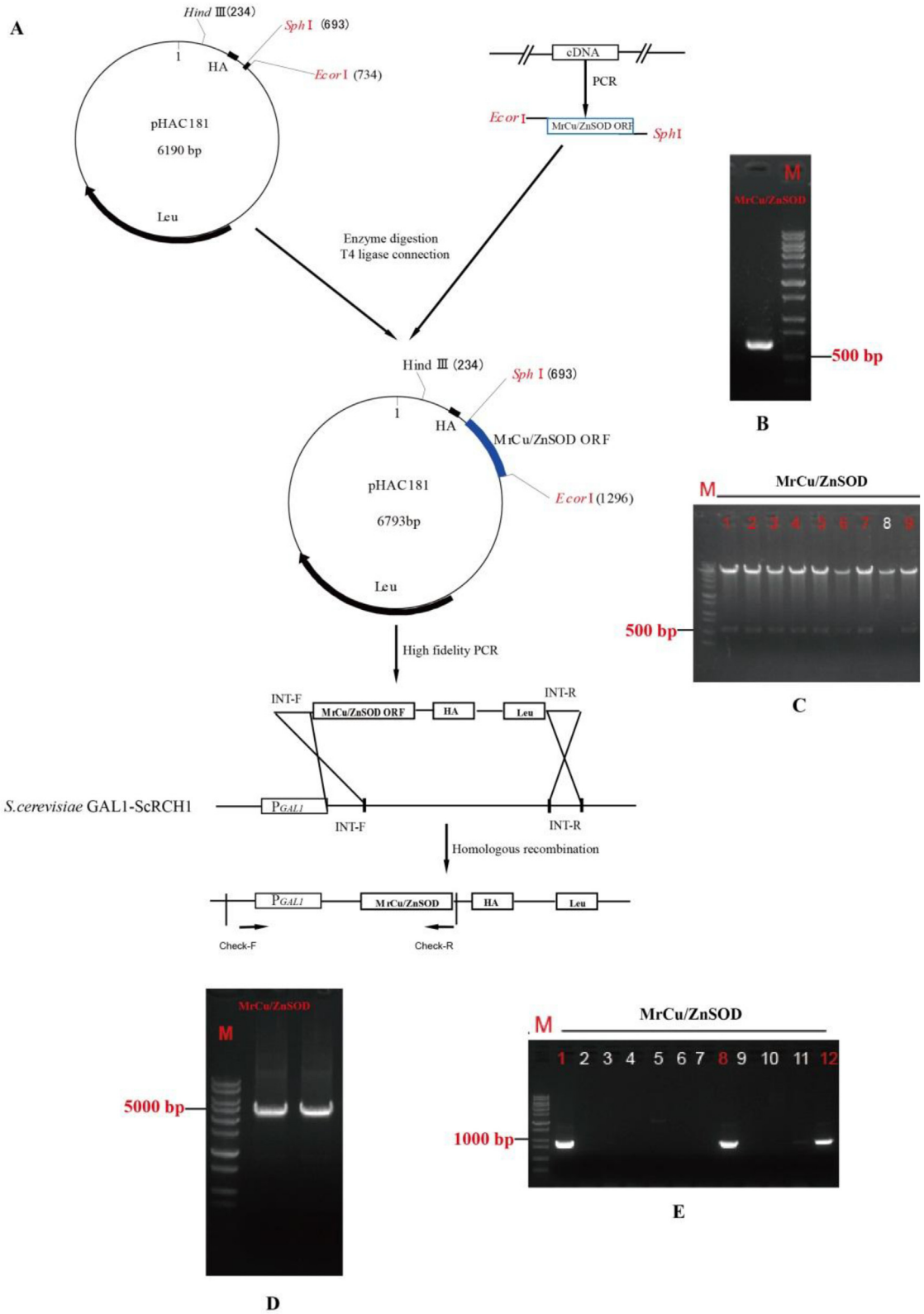

Fig. 1. Construction of the *M. rosenbergii* Cu/ZnSOD expression system in *S. cerevisiae.* Fig. A showed the construction of the yeast eukaryotic expression system; Fig. B showed that the size of the amplified open reading frame (ORF) of Cu/ZnSOD from *M. rosenbergii* cDNA (603 bp); Fig. C indicated the verification of positive transformation by enzyme digestion. The recombinant plasmid was amplified by homologous recombination primer using high-fidelity PrimeSTARGXL DNA Polymerase, where the length of the target fragment was 4985 bp (Fig. D). Detection primers were used to verify successful transformation via homologous recombination where the target fragment was 886 bp (Fig. E). Annotation: red numbers indicate correct positive transformation; 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.)

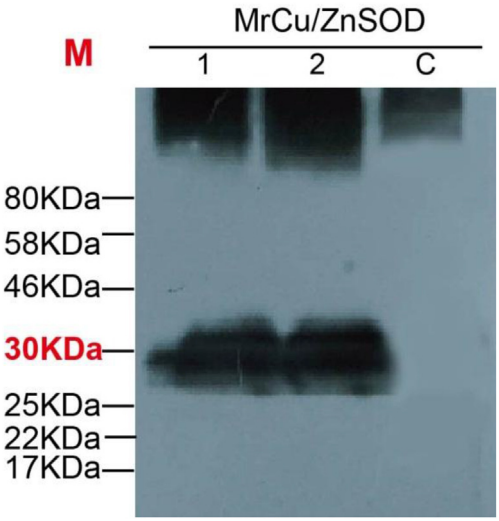

Fig. 2. Western blot analysis showing the expression of recombinant MrCu/ZnSOD protein using an anti-HA antibody. A 30 kDa target band was detected in Lanes 1 and 2, while the control protein presented no detectable bands. Lanes 1 and 2 represented the positive homologous recombinant transformant of *S. cerevisiae* GAL1-ScRCH1 expressing the MrCu/ZnSOD protein. C: Protein of the control yeast strain *S. cerevisiae* GAL1-ScRCH1, which without the homologous recombination gene MrCu/ZnSOD. M: protein marker.

3.2. Protein expression and western blot analysis

MrCu/ZnSOD cDNA was expressed in the yeast eukaryotic expression system. The construct was designed to yield recombinant MrCu/ZnSOD with a 3 × HA-Tag at the amino terminus, and therefore, the molecular weight of MrCu/ZnSOD was estimated to be about 30 kDa. The recombinant plasmid was integrated downstream of the GAL1 promoter in *S. cerevisiae* strain GAL1-ScRCH1, and the expression of MrCu/ZnSOD was induced by D-galactose. YPG (20 g/L Peptone, 20 g/L D-galactose, 10 g/L Yeast extract) was used as the induction medium. Electrophoretic analysis of cell lysates on 10% SDS-PAGE revealed that only induced cells contained the protein with an approximate size of 30 kDa (data not shown). The identity of the recombinant MrCu/ZnSOD protein was confirmed by western blot using an anti-HA antibody. A band of approximately 30 kDa was identified (Fig. 2).

3.3. Antibacterial activity of recombinant MrCu/ZnSOD

To determine the antibacterial activity of MrCu/ZnSOD, both the MIC and the MBC were determined to evaluate the bacteriostatic and bactericidal activities, respectively. Recombinant MrCu/ZnSOD inhibited the growth of four Gram-negative bacterial species (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, *P. aeruginosa*) and three Gram-positive bacterial species (*B. subtilis*, *B. thuringiensis* and *S. aureus*) by different degrees. The MIC and MBC values ranged from 0.556 to 0.840 μM and from 0.967 to 2.015 μM, respectively (Table 2).

3.4. Bacterial agglutination test

To test whether MrCu/ZnSOD can interact with the surface of microorganisms, we performed an agglutinating assay using four Gram-negative bacteria species (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*) and three Gram-positive species (*B. subtilis*, *B. thuringiensis,* and *S. aureus*). The four Gram-negative strains were agglutinated by MrCu/ZnSOD with different intensity and states (Fig. 3A), while two of the three Gram-positive bacterial strains, except for *S. aureus*, appeared to be agglutinated (Fig. 3B). These results indicated that MrCu/ZnSOD recognized the surface molecules on both Gram-negative and Gram-positive bacteria. It is worth noting that two bacterial strains that are pathogenic to crustaceans, *A. hydrophila* and *V. parahaemolyticus,* were agglutinated, suggesting that MrCu/ZnSOD serves an important immune function in *M. rosenbergii* against pathogenic bacteria.

Table 2
Antibacterial activity of recombinant MrCu/ZnSOD protein.

Bacteria	MIC (μM)	MBC (μM)
Gram-negative bacteria		
Escherichia coli	0.598	1.869
Aeromonas hydrophila	0.692	1.325
Vibrio parahaemolyticus	0.556	0.967
Pseudomonas aeruginosa	0.840	ND
Gram-positive bacteria		
Bacillus subtilis	0.677	1.533
Bacillus thuringiensis	0.761	2.015
Staphylococcus aureus	ND	ND

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

3.5. Detection of M. rosenbergii mortality after V. parahaemolyticus 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 4 weeks of feeding, *M. rosenbergii* fed with the pellets of *S. cerevisiae* MrCu/ZnSOD had the lowest mortality rate (the highest mortality value was 68.2%), whereas those fed with the common pellets showed the highest mortality rate (89.3%) at 48 h after the *V. parahaemolyticus* challenge (Fig. 4). The mortality rate of *M. rosenbergii* fed with *S. cerevisiae* pellets was higher than that of the group fed with *S. cerevisiae* MrCu/ZnSOD pellets, but lower than that of the group consuming common pellets. No significant difference was observed between the PBS control groups (Fig. 4).

3.6. HE stain

When *M. rosenbergii* was inoculated with *V. parahaemolyticus*, prawns in the three feeding groups began to show obvious infection symptoms: weakness without eating, lying down and unable to stand, swimming weakness, and death in the midinfection stage (6–24 h). We randomly selected three prawns from each group for HE stain at 24 h post *V. parahaemolyticus* infection. Fig. 5A showed that the hepatopancreas in the PBS control group was intact and smooth, which was typical of non-damaged epithelium and connective tissue. In the *S. cerevisiae* MrCu/ZnSOD feeding group, the epithelium and connective tissue of hepatopancreas were slightly damaged, although most of them were relatively complete and smooth. Interestingly, many fat bodies were found in the hepatopancreas of the *S. cerevisiae* MrCu/ZnSOD feeding group (Fig. 5B). In comparison, in the *S. cerevisiae* feeding group, the breakage and vacuolation of hepatopancreas was more obvious at 24 h post *V. parahaemolyticus* infection (Fig. 5C), whereas the epithelium and connective tissue of the infected hepatopancreas in the common pellets feeding group showed a several instances of tissue fragmentation and diffused structure (Fig. 5D).

3.7. Determination of hepatopancreas enzymatic activity of M. rosenbergii

The immune enzymatic activity (SOD, CAT, AKP, and ACP) of *M. rosenbergii* was measured at 0, 2, 6, 12, 24, and 48 h after the *V. parahaemolyticus* challenge. The results revealed that SOD activity increased markedly at 2, 12, and 48 h post infection in MrCu/ZnSOD pellets group, with a peak value of 82.11 U/g at 48 h. The other two control groups (groups fed with *S. cerevisiae* pellets and common pellets) also presented a continuous increase, although these were less marked than

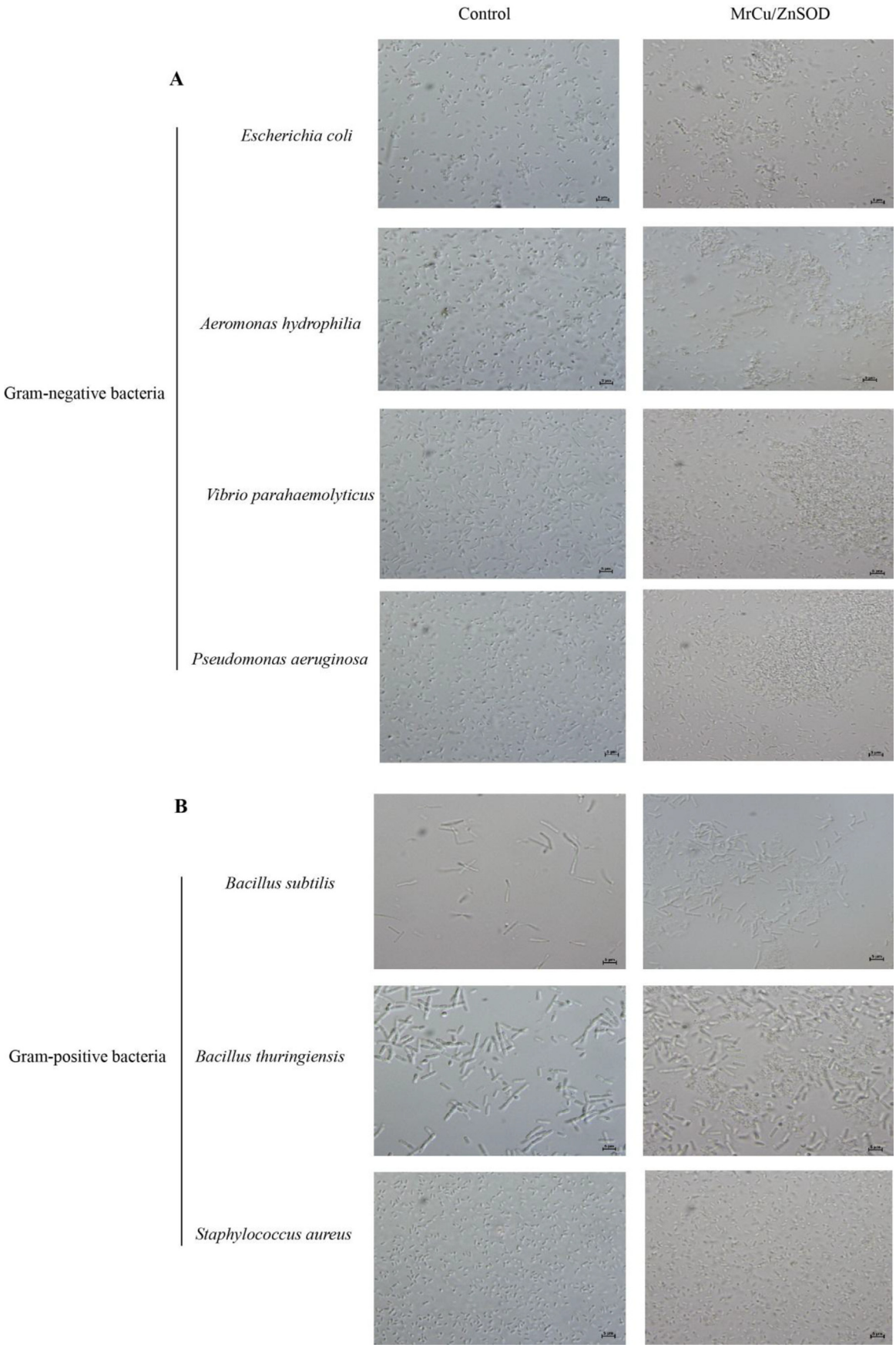

Fig. 3. Four Gram-negative bacteria (*E. coli*, *A. hydrophila*, *V. parahaemolyticus,* and *P. aeruginosa*) and three Gram-positive bacteria (*B. subtilis*, *B. thuringiensis*, and *S. aureus*) were used in a bacterial agglutination test. Protein extracted from yeast strain *S. cerevisiae* GAL1-ScRCH1, which without the homologous recombination gene MrCu/ZnSOD, served as the control. All four Gram-negative strains were agglutinated by MrCu/ZnSOD (Fig. 3A), while two of the three Gram-positive strains (*B. subtilis* and *B. thuringiensis*) appeared to be agglutinated compared with the control protein, except for *S. aureus* (Fig. 3B). Bar: 5 μm.

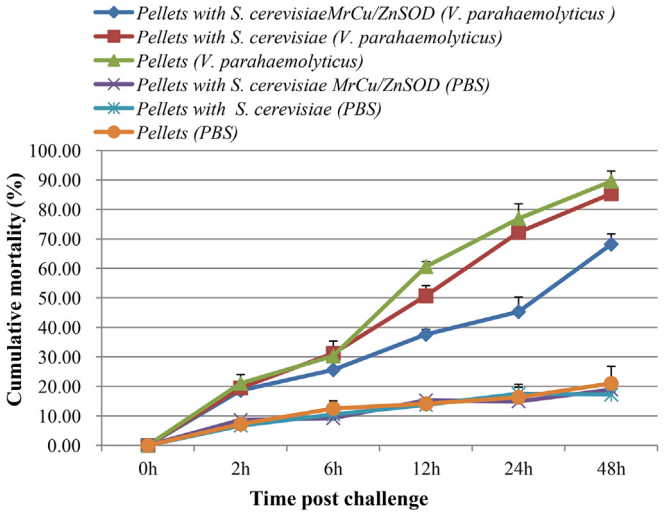

Fig. 4. *M. rosenbergii* mortality after *V. parahaemolyticus* challenge. *M. rosenbergii* fed with the pellets of *S. cerevisiae* MrCu/ZnSOD showed the lowest mortality rate (the highest mortality rate was 68.2%), whereas those fed with the common pellets showed the highest mortality rate of 89.3% after bacterial challenge (48 h). The *M. rosenbergii* group fed with the pellets of *S. cerevisiae* exhibited a higher mortality compared with the group fed with *S. cerevisiae* MrCu/ZnSOD, however, it was lower than the group fed with the common pellets. There was no significant difference between the PBS control groups. Annotation: Pellets with *S. cerevisiae* MrCu/ZnSOD (the pellets feed mixed with yeast strain *S. cerevisiae* GAL1-ScRCH1, which expressed the target protein MrCu/ZnSOD); Pellets with *S. cerevisiae* (pellets feed mixed with yeast strain *S. cerevisiae* GAL1-ScRCH1, which without the homologous recombination gene MrCu/ZnSOD); Pellets (common pellets feed without any *S. cerevisiae* strains).

that observed in the experimental group (Fig. 6A). No significant effect was observed in the PBS control group (data not shown). CAT activity increased markedly at 6 and 24 h in the group fed with MrCu/ZnSOD, and peaked at 35.488 U/g. The groups fed with *S. cerevisiae* pellets and common pellets presented similar levels of enzymatic activity, which were lower than that observed for the target protein group (Fig. 6B).

AKP activity in *M. rosenbergii* fed with *S. cerevisiae* MrCu/ZnSOD pellets started increasing from 2 h post infection, and peaked (54.27 U/g, $P < 0.01$) at 24 h (Fig. 6C). Subsequently, this value declined to 46.6 U/g ($P < 0.05$) at 48 h. During this period, the groups fed with *S. cerevisiae* pellets and common pellets presented a similar level of enzymatic activity, which was lower than that observed for the target protein group, and little great change was observed in the trend.

ACP activity started to increase at 2 h post *V. parahaemolyticus* infection, following which it increased and peaked at 24 h (53.41 U/g, $P < 0.01$) in the group fed with *S. cerevisiae* MrCu/ZnSOD pellets. Although the enzyme activities increased in the two control groups as well, they performed lower than the experimental group (Fig. 6D). These results indicate that the recombinant *S. cerevisiae* MrCu/ZnSOD strain enhanced the immune activity of *M. rosenbergii*.

3.8. Immunity-related genes mRNA expression in the hepatopancreas of *M. rosenbergii*

QRT-PCR was used to determine the expression of the mRNA of immune-related genes (Cu/Zn-SOD, CAT, AKP, ACP, LGBP, and Lectin) in the hepatopancreas of *M. rosenbergii* at 0, 2, 6, 12, 24, and 48 h after challenge with *V. parahaemolyticus*. As shown in Fig. 7, these genes were up-regulated in different levels following stimulation with pathogenic bacteria in the group fed with *S. cerevisiae* MrCu/ZnSOD pellets compared with the control groups (fed with *S. cerevisiae* pellets and

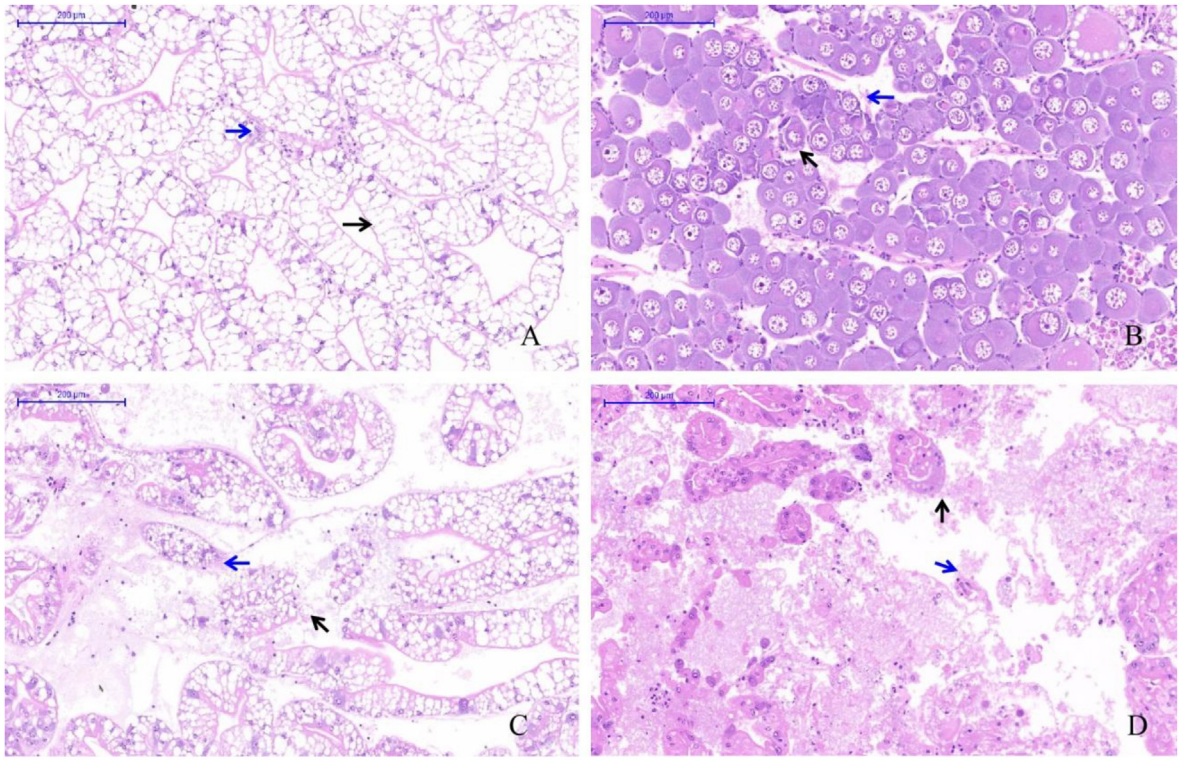

Fig. 5. HE stains showing different groups of hepatopancreas in *M. rosenbergii*. (A) Hepatopancreas in *M. rosenbergii* which inoculated with PBS post 24 h (control). (B, C, D) Hepatopancreas in *M. rosenbergii* which infected with *V. parahaemolyticus* post 24 h (in mid-infection stage) after feeding with different diets for 4 weeks. (B) Hepatopancreas in *M. rosenbergii* which fed with the pellets of *S. cerevisiae* MrCu/ZnSOD. (C) Hepatopancreas in *M. rosenbergii* which fed with the pellets of *S. cerevisiae* (which without the homologous recombination gene MrCu/ZnSOD). (D) Hepatopancreas in *M. rosenbergii* which fed with the common pellets (which without any *S. cerevisiae* stains). Bar length: 200 μm. Annotation: The black arrow represents epidermal tissue and the blue arrow represents connective tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

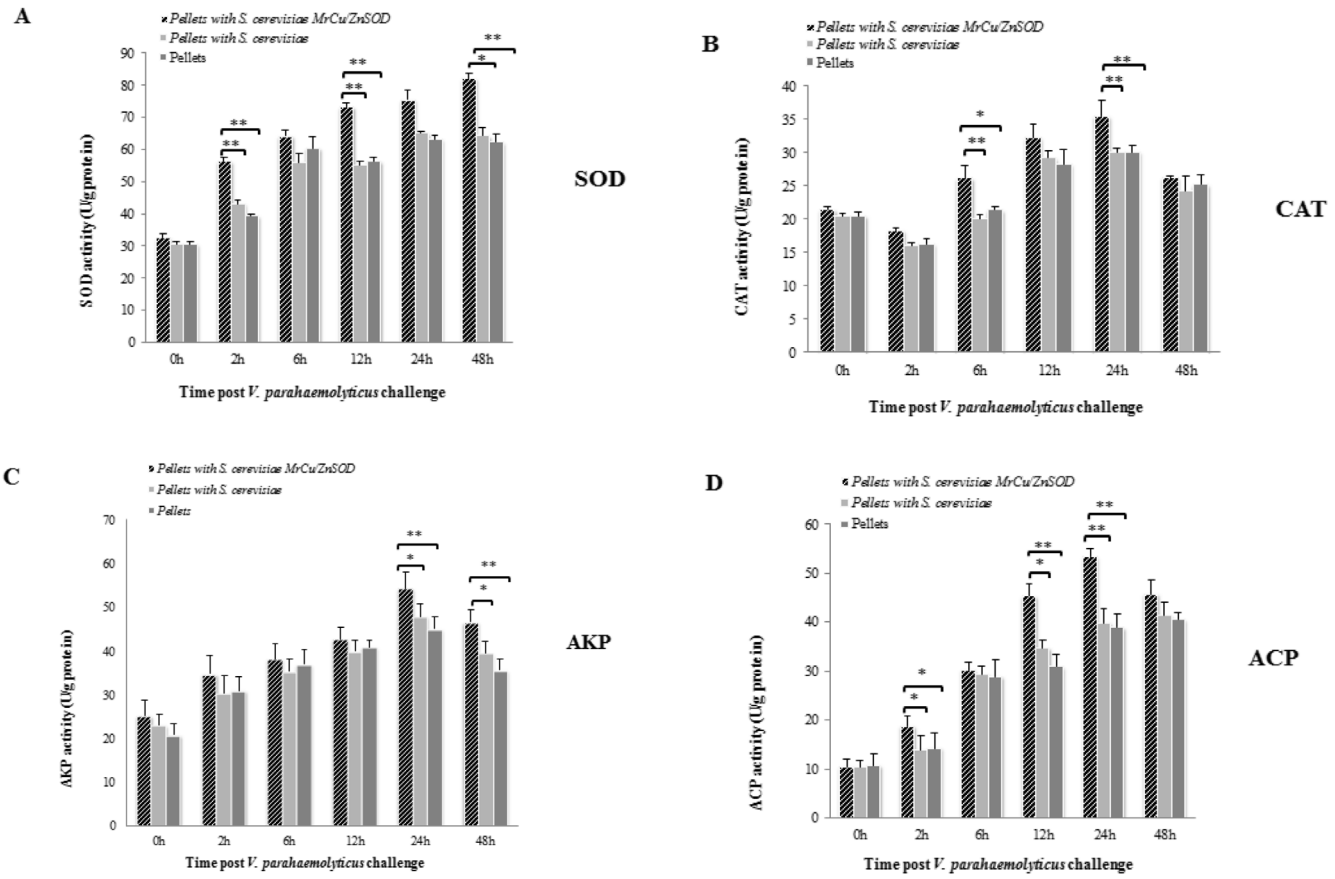

Fig. 6. Changes in the relative immune enzymes activity in *M. rosenbergii* hepatopancreas at 0, 2, 6, 12, 24, and 48 h after *V. parahaemolyticus* infection. A: SOD activity, B: CAT activity, C: AKP activity, D: ACP activity. Each bar represents the mean of six determinations with standard error. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$). Prawns challenged with PBS were maintained as controls, no significant difference was observed across the three treatment groups following the PBS challenge (data not shown).

the common pellets). The mRNA expressions of some genes (LGBP, AKP, and CAT) reached their peak when infection with *V. parahaemolyticus* after 12 h (Fig. 7E, C, 7B), whereas the mRNA expression of ACP (Fig. 7D) and lectin peaked at 24 h post infection. Cu/ZnSOD mRNA transcription (Fig. 7A) started to increase at 6 h and continued to increase up to 48 h when it reached a maximum with 210-fold increase ($P < 0.01$). In addition, the expression of Cu/ZnSOD and Lectin (Fig. 7F) was higher than that of other genes, with their maximum of 210- and 166-fold increases, respectively. No obvious difference was reported between the PBS control groups (data not shown).

4. Discussion

SODs are enzymes that scavenge molecular oxygen radicals and thus prevent the harmful effects of reactive oxygen in aerobic organisms. SODs are metalloproteins that can be divided into three types (Mn−, Fe−, and Cu/Zn-SOD) according to the metal present in the active site. In this study, Cu/Zn-SOD of *M. rosenbergii* was expressed in the *S. cerevisiae* system. We cloned the *M. rosenbergii* Cu/Zn-SOD cDNA ORF (603 bp) into the vector pHAC181, and the recombinant plasmids were integrated into the downstream of the GAL1 promoter in *S. cerevisiae* strain GAL1-ScRCH1 via homologous recombination. The resulting recombinant MrCu/ZnSOD contained a 3 × HA-tag at its C-terminal. MrCu/ZnSOD was overexpressed by addition of D-galactose into the culture medium, following which, the yeast cells were collected, lysed, and analyzed via 10% SDS-PAGE. Western blot analyses revealed that the molecular weight of the recombinant MrCu/ZnSOD was about 30 kDa (Fig. 2).

MIC values were determined by liquid growth inhibition assays to assess the antibacterial activity of MrCu/ZnSOD, in this study, the antimicrobial activity of the recombinant MrCu/ZnSOD was assessed using four Gram-negative bacteria (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*) and three Gram-positive bacteria (*B. subtilis*, *B. thuringiensis,* and *S. aureus*). Recombinant MrCu/ZnSOD inhibited the growth of all four Gram-negative strains (*A. hydrophila*, *E. coli*, *V. parahaemolyticus,* and *P. aeruginosa*) and two of the Gram-positive strains (*B. subtilis* and *B. thuringiensis*). The MIC and MBC values ranged from 0.556 to 0.840 μM, and from 0.967 to 2.015 μM, respectively (Table 2). While no previous study has reported the antibacterial activity of recombinant Cu/ZnSOD, many studies have investigated other genes associated with innate immunity, especially antimicrobial peptides. For example, Chia-Chen [35] reported that the MIC and MBC values of recombinant MrALF ranged from 0.273 to 0.592 μM, and from 0.684 to 0.969 μM, respectively. In a recent study, we demonstrated that recombinant MrLGBP also exhibits antibacterial activity, with MIC ranging from 0.340 to 0.802 μM, and MBC ranging from 1.189 to 1.810 μM [44].

An agglutination assay was performed to test whether MrCu/ZnSOD can interact with bacteria. The results revealed that all four Gram-negative bacterial strains (*E. coli*, *A. hydrophila*, *V. parahaemolyticus*, and *P. aeruginosa*) were agglutinated by MrCu/ZnSOD (Fig. 3A), while two of the three Gram-positive strains (*B. subtilis* and *B. thuringiensis*) were agglutinated, except *S. aureus* (Fig. 3B). In these agglutination experiments, the agglutination effect of recombinant MrCu/ZnSOD differed across bacteria. A likely explanation for this is that different pathogens have their own specific characteristics, and the recombinant protein has a different tolerance for these pathogens. Moreover, the agglutinated bacteria presented partial bacterial lysis and death. Peroxide free

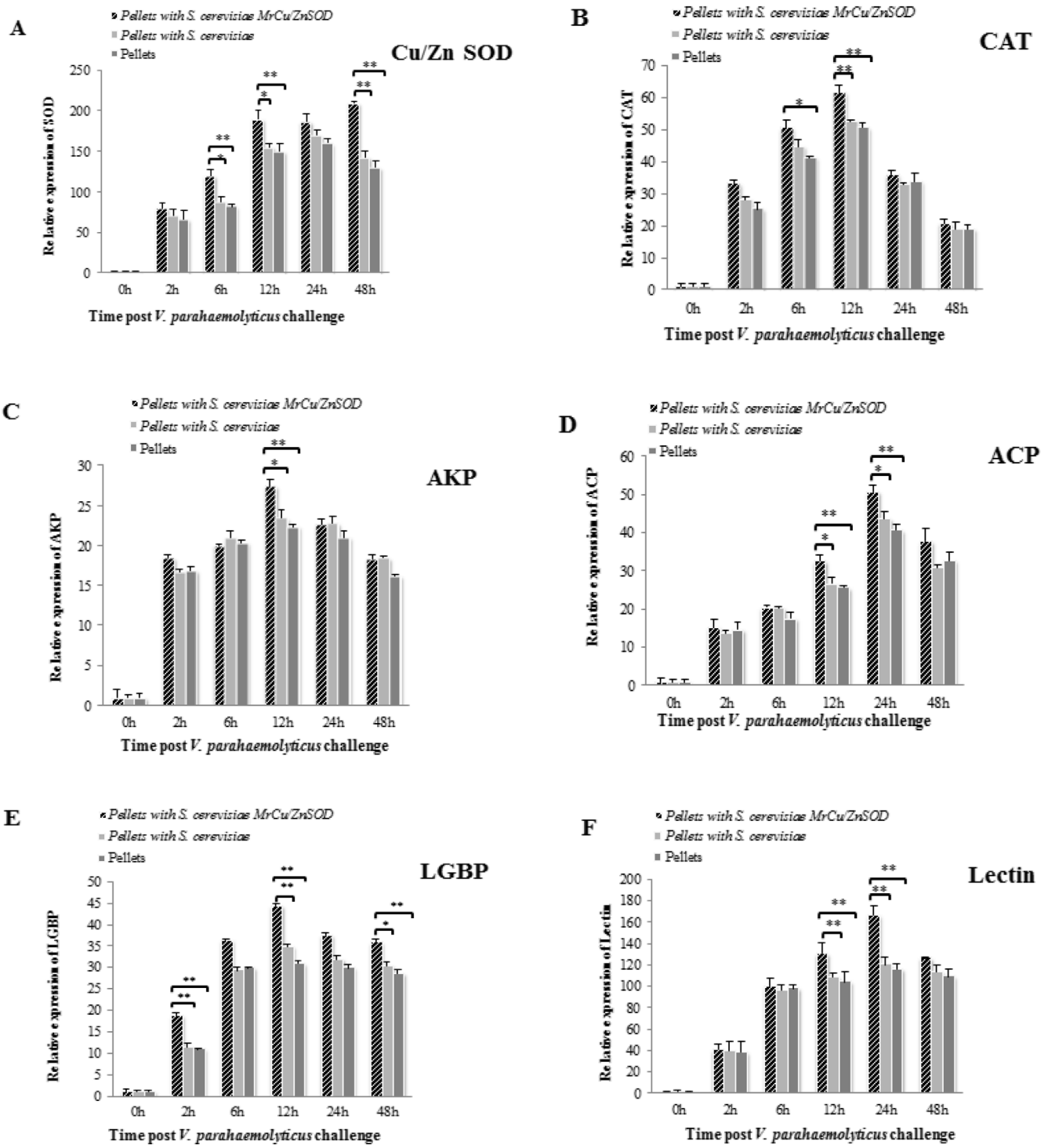

Fig. 7. Expression of immune-related genes in the *M. rosenbergii* hepatopancreas, assessed by qRT-PCR at 0, 2, 6, 12, 24, and 48 h after *V. parahaemolyticus* infection. Prawns challenged with PBS were maintained as controls; no significant difference was observed across the three treatment groups after the PBS challenge (data not shown). The mRNA levels of immune-related genes were analyzed and standardized to that of β-actin mRNA. A: Relative expression of SOD. B: Relative expression of CAT. C: Relative expression of AKP. D: Relative expression of ACP. E: Relative expression of LGBP. F: Relative expression of Lectin. Each bar represents the mean of six measurements with standard error. Asterisks indicate significant differences ($*P < 0.05$, $**P < 0.01$).

radicals can damage organisms, and SOD can promote the conversion of peroxide free radicals into H_2O_2 and oxygen, thereby eliminating the peroxide free radicals during inflammation and producing a strong anti-inflammatory effect. In the present study, the MrCu/ZnSOD protein adversely affected the growth and proliferation of bacteria, thus, inhibiting bacterial growth and even inducing their death. The results of our bacterial agglutination assay indicated that MrCu/ZnSOD plays an important role in the innate immunity of *M. rosenbergii.* Simultaneously, *A. hydrophila* and *V. parahaemolyticus*, two species of pathogenic bacteria that affect crustaceans, were effectively agglutinated, suggesting that MrCu/ZnSOD is an important immune protein against aquatic pathogens.

To validate the effect of recombinant MrCu/ZnSOD on *M. rosenbergii* immunity, the recombinant yeast strain MrCu/ZnSOD was concentrated

in fermentation broth at 1×10^9 cell/mL and sprayed onto the pellet feed for dry. After 4 weeks of feeding, *M. rosenbergii* was infected with *V. parahaemolyticus*, and the results for different feeding groups showed different mortality rates: *M. rosenbergii* fed with *S. cerevisiae* MrCu/ZnSOD pellets presented the lowest mortality rate, whereas the group fed with *S. cerevisiae* pellets manifested slightly lower mortality than that of the group fed with common pellets. These results indicate that the recombinant yeast strain *S. cerevisiae* MrCu/ZnSOD enhanced the immune response of *M. rosenbergii*. In this study, the mortality of the group fed with the *S. cerevisiae* yeast strain was slightly lower than that of the group fed with common pellets, that's because *S. cerevisiae* by itself can serve as a good immune feed additive. Moreover, *S. cerevisiae* MrCu/ZnSOD strengthened the immune function, and thus, the group fed with *S. cerevisiae* MrCu/ZnSOD presented the best immune response against the pathogen *V. parahaemolyticus*.

Besides hemolymph, hepatopancreas is also an important organ for innate immunity, playing a key role in the crustacean immune response [45]. The hepatopancreas of crustaceans is susceptible to various kinds of pathogens, and therefore, many diseases can be diagnosed according to their pathological changes and physiological abnormalities. In this study, the hepatopancreas of the *V. parahaemolyticus*-infected prawn showed a significant pathological response, with the connective and epithelial tissue presenting signs of morbidity, including tissue fragmentation, tubular vacuoles, and the emergence of dispersed structures. Similar pathological structures were observed in the hepatopancreas of *M. rosenbergii* infected with spiroplasma MR1008 in our previous study [46]. However, in this study, the pathological status of hepatopancreas was different across groups. The group of *M. rosenbergii* fed with *S. cerevisiae* MrCu/ZnSOD pellets showed a slight damage in the hepatopancreatic tissue, whereas prawns fed with the common pellets manifested severe fragmentation of the epithelial and connective tissue, accompanied with the appearance of a large number of vacuoles and diffused structures. The pathological state of hepatopancreas in *M. rosenbergii* fed with *S. cerevisiae* pellets was in between the states of the two groups described above. Moreover, we found a significantly high number of fat bodies in the hepatopancreas of *M. rosenbergii* fed with *S. cerevisiae* MrCu/ZnSOD pellets, which might be due to the higher dietary protein nutrition enhancing the growth and physical fitness of *M. rosenbergii*.

The innate immune system is very complicated in crustaceans, involving several important immune factors. SODs are an important group of antioxidant enzymes in living organisms. They have a special physiological activity and serve as the primary agents for scavenging of free radicals. They can resist and prevent the damage caused by oxygen free radicals to cells, repair damaged cells in time, and repair the specific damage caused by free radicals. In addition, CAT is also part of the defense system against ROS [15,47]; therefore, respiratory SOD and CAT activity has been widely used to evaluate the defense against pathogens in crustaceans. AKP and ACP, which are found in abundance in the hepatopancreatic tissues, are associated with immunity and metabolism. The determination of AKP and ACP activity has become an important means for the diagnosis and monitoring of various diseases. In this study, the enzymatic activities of these immune factors were determined to assess the immune response in the hepatopancreas of *M. rosenbergii* that infected with *V. parahaemolyticus*.

Marked increases in SOD activity were observed at 2, 12, and 48 h post infection in pellets with the target protein group, with a peak value of 82.11 U/g at 48 h. CAT activity was increased at 6 and 24 h in the MrCu/ZnSOD group compared with the groups fed with pellets of *S. cerevisiae* and common pellets. These results are consistent with those reported in *M. rosenbergii* infected by spiroplasma MR-1008 [46], but contrary to those from another study, which reported a significant reduction in SOD activity in WSSV-infected tissues of *P. monodon* [48]. In our recent report on the recombinant protein MrLGBP, the SOD activity in the MrLGBP feeding group increased during the early stage of bacterial infection with *V. parahaemolyticus* (2–12 h), whereas in this study, the SOD activity remained relatively high during the 48 h infection progress. In addition, the trend for CAT enzymatic activity during *V. parahaemolyticus* infection was consistent with that observed in the recombinant MrLGBP feeding group [44].

AKP activity in the group fed with *S. cerevisiae* MrCu/ZnSOD increased slowly and peaked at 24 h, and then decreased at 48 h after *V. parahaemolyticus* infection. Lower enzymatic activity was observed in the other two *M. rosenbergii* groups (fed with *S. cerevisiae* pellets and common pellets) compared with the group fed with the target protein. ACP activity increased significantly from 2 to 12 h, and peaked at 24 h post infection. Similar to the results of the previous study [46], AKP and ACP activity in the *M. rosenbergii* hepatopancreas increased after infection with *V. parahaemolyticus* or spiroplasma. However, the increase in enzymatic activity and the time taken to reach the highest value are different for different pathogens.

QRT-PCR analysis was performed to assess the expression of immune-related genes (Cu/ZnSOD, CAT, AKP, ACP, LGBP, and Lectin) in the hepatopancreas of *M. rosenbergii* infected with *V. parahaemolyticus*. As observed in previous studies [6,44], the mRNA levels of some genes were not consistent with their enzymatic activities. This is a common observation that can be explained by the dynamics of cellular transcription and translation. Maier [49] noted that many parameters can influence the correlation between mRNA and protein levels, and the relationship between transcription and translation is neither linear nor simple. Liu [50] concluded that transcript levels are not sufficient to predict the protein levels in many scenarios.

SODs serve important roles within the antioxidant defense pathways in response to oxidative stress [51,52]. SOD constitutes a first line of defense against oxidative damage by catalyzing the dismutation of the superoxide radical to molecular oxygen and hydrogen peroxide [12]. Cu/ZnSOD is an important SOD because of its physiological function and therapeutic potential [53]. SODs have been used as biomarkers, and their activities and mRNA levels are closely associated with immune stimulation, disease, and health status in aquatic organisms [54–57]. In the present study, Cu/ZnSOD mRNA expression in *M. rosenbergii* hepatopancreas was upregulated at 6 h after infection with *V. parahaemolyticus* and continued to increase up to 48 h when a peak increase of 210-fold was observed ($P < 0.01$). This value is much higher than that reported by Xiu for *Macrobrachium nipponense* Cu/ZnSOD [58], wherein injection with *A. hydrophila* resulted in the upregulation of mRNA expression, followed by a marked decrease and then an increase to a peak levels of 4-fold increase ($P < 0.05$). However, in contrast to our results, Cheng [23] showed that Cu/Zn-SOD mRNA expression in *M. rosenbergii* hepatopancreas injected with *L. garvieae* decreased at 3 h post infection, but subsequently it recovered.

CAT is an important component of the antioxidant enzyme defense system, which can partially balance ROS levels. In the present study, CAT mRNA expression was upregulated at 6 and 12 h post injection with *V. parahaemolyticus*, following which the mRNA transcripts were down regulated from 24 to 48 h. This is similar to the other reports in shrimp, whereby the CAT mRNA production also increased after bacteria and white spot syndrome virus (WSSV) infection [59,60]. Compared with protein levels, CAT mRNA transcripts peaked at 12 h, while CAT enzyme activity was not prominent at 12 h and maximum levels appeared at 24 h post infection. This may have happened because the translational and post-translational regulation of antioxidant enzymes are important for determining changes in activity levels in response to oxidative stress. The delayed decline in mRNA expression relative to enzymatic activity may be due to post-transcriptional regulatory mechanisms or the stability of the enzyme proteins.

AKP mRNA transcripts in the hepatopancreas increased and peaked at 12 h post *V. parahaemolyticus* infection. ACP mRNA transcripts increased significantly at 12 h and peaked at 24 h, with a 51-fold increase ($P < 0.01$). This is consistent with our previous findings, wherein infected with the spiroplasma in *M. rosenbergii,* AKP mRNA expression in the hepatopancreas of infected *M. rosenbergii* was enhanced and peaked

at 15 days, while expression of ACP mRNA transcripts peaked at 5 days [46]. These results suggest that, as the main digestive gland in prawns, the hepatopancreas exhibited high AKP and ACP activity against pathogens, and the mRNA expression of AKP and ACP in the hepatopancreas could be induced by *V. parahaemolyticus*.

The LGBP, which acts as a pattern-recognition protein, is important for the activation of the proPO system in crustaceans during the early stages of infection. In this study, when *M. rosenbergii* was challenged with *V. parahaemolyticus*, the mRNA expression of LGBP was upregulated after 2 h and reached a peak at 12 h post inoculation. The result is consistent with those from a study on *Penaeus stylirostris*, which LGBP mRNA expression in the hepatopancreas was significantly upregulated during WSV infection [61]. Similarly, as one of the PRRs present in crustaceans, lectins also perform an important function in the initial response against foreign pathogens. Lectins are ubiquitous proteins found in the invertebrate hemolymph and hepatopancreas and are active participants in cellular and humoral immune recognition [62,63]. In this study, upon infection with *V. parahaemolyticus,* the level of lectin mRNA transcription clearly increased at 12 h then peaked at 24 h. All these results indicated that within the early stages of *V. parahaemolyticus* infection in *M. rosenbergii*, PRPs such as LGBP and Lectin showed a significant increase for enhanced pathogen recognition and host protection.

Generally, when *M. rosenbergii* was infected with *V. parahaemolyticus*, the PRPs (LGBP and Lectin) immediately recognized the non-self-matter that had gained entry into the prawn body. Following with the pathogenic bacteria's infection, not only the enzymes involved in the antioxidant antibacterial system (SOD, CAT) were synthesized rapidly in the hepatopancreas, the phosphatase enzymes (AKP, ACP) also actively participated in this immune response. It should be noted that, although these immune-related genes were up- or down-regulated in the two control groups (fed with pellets of *S. cerevisiae* and common pellets) as well, their changes were not significant compared with those in the experimental group (fed with pellets of *S. cerevisiae* MrCu/ZnSOD).

In conclusion, MrCu/ZnSOD was expressed in the *S. cerevisiae* eukaryotic system with a predicted recombinant molecular weight of 30 kDa. Antibacterial activity of the recombinant protein indicated that MrCu/ZnSOD is an important immune-related protein in the crustacean innate immune system. Moreover, the recombinant *S. cerevisiae* MrCu/ZnSOD strain could improve the *M. rosenbergii* immune activity level, induce and simultaneously up-regulate the expression levels of immune-related genes. These genes participated in the immune response and enhanced the immunity of *M. rosenbergii* against the invading pathogen *V. parahaemolyticus* in a cooperative manner. This *S. cerevisiae* MrCu/ZnSOD strain may be useful for developing immune feed additives, as well as for prevention and control of the aquatic animal diseases in the future.

Acknowledgements

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