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Molecular cloning of an invertebrate goose-type lysozyme gene from *Chlamys farreri*, and lytic activity of the recombinant protein

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Abstract

Lysozyme is a widely distributed hydrolase possessing lytic activity against bacterial peptidoglycan, which enables it to protect the host against pathogenic infection. In the present study, the cDNA of an invertebrate goose-type lysozyme (designated CFLysG) was cloned from Zhikong scallop *Chlamys farreri* by expressed sequence tag (EST) and rapid amplification of cDNA ends (RACE) techniques. The full-length cDNA of CFLysG consisted of 829 nucleotides with a canonical polyadenylation signal sequence AATAAA and a poly(A) tail, and an open reading frame (ORF) of 603 bp encoding a polypeptide of 200 amino acid residues with a predicted molecular weight of 21.92 kDa and theoretical isoelectric point of 7.76. The high similarity of CFLysG with goose-type (g-type) lysozymes in vertebrate indicated that CFLysG should be an invertebrate counterpart of g-type lysozyme family, which suggested that the origin of g-type lysozyme preceded the emergence of urochordates and even preceded the emergence of deuterostomes. Similar to most g-type lysozymes, CFLysG possessed all conserved features critical for the fundamental structure and function of g-type lysozymes, such as three catalytic residues (*Glu* 82, *Asp* 97, *Asp* 108). By Northern blot analysis, mRNA transcript of CFLysG was found to be most abundantly expressed in the tissues of gills, hepatopancreas and gonad, weakly expressed in the tissues of haemocytes and mantle, while undetectable in the adductor muscle. These results suggested that CFLysG could possess combined features of both the immune and digestive adaptive lysozymes. To gain insight into the in vitro lytic activities of CFLysG, the mature peptide coding region was cloned into *Pichia pastoris* for heterogeneous expression. Recombinant CFLysG showed inhibitive effect on the growth of both Gram-positive and Gram-negative bacteria with more potent activities against Gram-positive bacteria, which indicated the involvement of CFLysG in the innate immunity of *C. farreri*.
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Keywords: *Chlamys farreri*; Goose-type lysozyme; Gene cloning; Innate immunity; Northern blot; Recombinant protein; Lytic activity

1. Introduction

Chlamys farreri is one of the economic scallop species cultivated widely in the coastal provinces of north China. Since the summer of 1997, large-scale mortality of *C. farreri* has been reported in China and has caused catastrophic economic losses to scallop aquaculture. Mollusk lack the adaptive immune system that is characteristic of vertebrate organisms and they therefore rely exclusively on the innate immune responses, which include cellular responses mediated by haemocytes and humoral immune responses that employ constitutive and inducible antimicrobial molecules to lyse invading microorganisms (Hoffmann et al., 1999; Roch, 1999; Rolff and Siva-Jothy, 2003).

The abuse of antibiotics in aquaculture has accelerated the production of drug-resistant bacteria, and such problem has stimulated intensive effort for discovery and characterization of antimicrobial molecules with original mode of action as sources or templates for the design of novel therapeutic agents (Patrzykat and Douglas, 2003). Moreover, advances in the characterization of immune effectors will provide a better understanding of the immune defense mechanisms of mollusk and can give new insights into health management and diseases control in mollusk aquaculture (Seo et al., 2005). However, molecular features and functional studies of immune effectors in mollusk are still deficient compared with those of insects and vertebrates.

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Lysozyme (muramidase, EC 3.2.1.17) is a ubiquitous enzyme existing in numerous phylogenetically diverse organisms such as bacteria, bacteriophages, fungi, plants and animals, which catalyzes the hydrolysis of the β-1,4-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine alternating sugar residues in the bacterial peptidoglycan, and causes bacterial cell lysis (Jollès and Jollès, 1984; Prager and Jollès, 1996; Qasba and Kumar, 1997). It has been widely accepted that lysozyme functions as a crucial biodefense effector in innate immunity (Grunclová et al., 2003; Hultmark, 1996; Kollien et al., 2003; Regel et al., 1998; Simser et al., 2004; Sun et al., 1991), especially for aquatic animals against the invasion of bacterial pathogens (Foley and Cheng, 1977; Hikima et al., 2000, 2003; Lindsay, 1986; Minagawa et al., 2001; Nilsen et al., 1999; Pipe, 1990; Xue et al., 2004; Yano, 1996; Yin et al., 2003). Recent studies have demonstrated that lysozymes could augment the activity of antibacterial peptides (AMPs) through a synergistic mechanism (Chalk et al., 1994; Hancock and Scott, 2000; Patrzykat et al., 2001; Yan and Hancock, 2001). In addition to the enzymatic bacteriolytic activity, some lysozymes have been demonstrated to contain independent non-enzymatic bactericidal domains (Düring et al., 1999; Ibrahim et al., 2001; Masschalck and Michiels, 2003; Mine et al., 2004). For examples, the peptides Ile98-Trp108 and Ala107-Arg112 in chicken egg-white lysozyme exhibited inhibitory activity against *Escherichia coli* K12 (Hunter et al., 2005; Mine et al., 2004).

Based on their differences in structural, catalytic and immunological characters, lysozymes have been traditionally categorized into six types: chicken-type (c-type) lysozyme, goose-type (g-type) lysozyme, invertebrate-type (i-type) lysozyme, phage lysozyme, bacterial lysozyme and plant lysozyme (Bachali et al., 2002, 2004; Fastrez, 1996; Beintema and Terwisscha van Scheltinga, 1996; Hikima et al., 2003; Hultmark, 1996; Ito et al., 1999; Jollès and Jollès, 1984; Jollès, 1996; Liu et al., 2006; Nilsen and Myrnes, 2001; Prager, 1996; Qasba and Kumar, 1997). Most of the animals investigated appear to possess a single type of lysozyme only, but multiple types of lysozymes have been demonstrated to coexist in certain animals, for instance the coexistence of c- plus g-type lysozymes in birds and c- plus i-type lysozymes in *Penaeus vannamei* (Chong et al., 2002; Gross et al., 2001; Nakano and Graf, 1991; Sotelo-Mundo et al., 2003). Until now, there are no reports describing the coexistence of multiple types of lysozymes in mollusk.

As filter-feeding organisms, mollusks expose to various potential pathogens in the aquatic environment. For defensive and nutritional purpose, mollusk lysozymes were therefore expected to possess digestive capability in addition to bactericidal effect towards prokaryotic cells (Nilsen et al., 1999, 2003). Lysozyme activities had been detected in the haemolymph, gills, mantle and digestive organs from a variety of mollusks (Haug et al., 2004; Ito et al., 1999; McDade and Tripp, 1967; McHenery and Birckbeck, 1982; Myrnes and Johansen, 1994; Olsen et al., 2003). However, it was not until very recently, with the original discovery of i-type lysozyme from *Asterias rubens* (Jollès and Jollès, 1975; Bachali et al., 2004), about 10 lysozymes had been isolated from mollusks, and they were all classified into the novel invertebrate class of the lysozyme family.

g-Type lysozyme was initially identified as an antibacterial enzyme from the egg whites of various bird species (Canfield and McMurry, 1967). To the best of our knowledge, all the g-type lysozyme sequences published were from vertebrate, such as goose, chicken, black swan, Japanese flounder and common carp (Canfield et al., 1971; Hikima et al., 2001; Savan et al., 2003; Simpson et al., 1980; Simpson and Morgan, 1983), with exceptions from *Oikopleura dioica* (Nilsen et al., 2003). There was no experimental evidence for the existence of g-type lysozyme in invertebrate so far.

More recently, two g-type lysozyme genes were cloned from bay scallop (Zou et al., 2005) and Zhikong scallop, respectively. The main objectives of this study were: (1) to clone the full-length cDNA of an invertebrate g-type lysozyme gene from Zhikong scallop *C. farreri*, (2) to investigate the tissue distribution of CFLysG transcript, and (3) to characterize the lytic activity of recombinant CFLysG against various bacteria with preference to pathogenic *Vibrio* species.

2. Materials and methods

2.1. Construction of cDNA library and EST analysis

A cDNA library was constructed from the whole body of an adult *C. farreri* challenged by *Vibrio anguillarum*, using the ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 6935 successful sequencing reactions. BLAST analysis of all the 6935 EST sequences revealed that one EST of 678 bp (no. *Scag_8720*) was highly similar to previously identified vertebrate g-type lysozyme, and this EST sequence was selected for further cloning of the full-length cDNA of CFLysG.

2.2. Cloning and analysis of the full-length cDNA of CFLysG

Based on the EST sequence of clone *Scag_8720*, the 3′ and 5′ ends of CFLysG cDNA were obtained by RACE approaches, using gene-specific primers and vector primers (Table 1). The PCR reaction to get 5′ end of CFLysG cDNA was performed with T3 and LysR1 in a 20 μl reaction volume containing 2 μl of 10× PCR buffer, 1.2 μl of $MgCl_2$ (25 mmol l^{-1}), 1.6 μl of dNTP mix (2.5 mmol l^{-1}), 1 μl of each primer (10 μmol l^{-1}), 12 μl of PCR-grade water, 0.2 μl (1 U) of Taq polymerase (Promega) and 1 μl of cDNA mix. The PCR temperature profile was 94 °C for 5 min followed by 34 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min and the final extension step at 72 °C for 10 min. PCR amplification of the 3′ end of CFLysG cDNA was carried out using LysF1 and T7. The cycling protocol was 1 cycle of 94 °C for 5 min and 32 cycles of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min, followed by an additional extension at 72 °C for 10 min. The PCR products were gel-purified and cloned into the pMD18-T simple vector (Takara). After transformed into the

Table 1
Sequences of primers used in this study

Primer	Sequence (5′–3′)	Sequence information
LysF1 (forward)	CATCCGCTACCACTCCTGTC	3′ RACE primer
LysR1 (reverse)	CAACTACGTCATTGCTGTAGTC	5′ RACE primer
LysF2 (forward)	AGTCGAGAATCACGTGGAGGG	Northern blot primer
LysR2 (reverse)	TCATTGCTGTAGTCATTTCC	Northern blot primer
AF (forward)	TATGCCCTCCCTCACGCTAT	β-Actin primer
AR (reverse)	GCCAGACTCGTCGTATTCCT	β-Actin primer
rLysF (forward)	GAATTCGCGTCCTACACCTGCCATGGTGAC	Recombinant primer
rLysR (reverse)	GCGGCCGCTTAATGCCAGCCGTGGCTTGAAATA	Recombinant primer
T3 (forward)	AATTAACCCTCACTAAAGGG	Vector primer
T7 (reverse)	GTAATACGACTCACTATAGGGC	Vector primer

competent cells of *E. coli* JM109, the recombinants were identified through blue-white color selection in ampicillin-containing LB plates. The positive clones were sequenced in both directions, and the resulting sequences were verified and subjected to cluster analysis.

The searches for nucleotide and protein sequence similarities were conducted with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/), and protein domain features of CFLysG were determined by using Simple Modular Architecture Research Tool (Schultz et al., 1998). Multiple alignment of CFLysG with g-type lysozyme proteins from other organisms was performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment show program (http://www.bio-soft.net/sms/index.html).

2.3. Phylogenetic analysis

The deduced amino acid sequence of CFLysG was aligned with the corresponding sequences from various animals using the ClustalX software (Thompson et al., 1997). Based on this alignment, a phylogenetic tree was constructed with MEGA software (Kumar et al., 2001) by using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis (Felsenstein, 1985) was used with 1000 replicates to test the relative support for the branches produced by the NJ analysis. All the analyzed sequences of g-type lysozymes were retrieved from GenBank and SWISS-PROT database and their accession numbers were listed in Table 2.

2.4. Northern blot analysis

CFLysG gene-specific primers, LysF2 and LysR2, were used to obtain the transcription template for probe synthesis. PCR products were subcloned into pGEM-T easy vector (Promega) with negative orientation, and the plasmid was linearized by restriction enzyme *Nde*I. The cRNA probe was transcribed in vitro by T7 RNA polymerase as recommended in the manufacture's protocol (MEGAshortscript high yield transcription kit, Ambion). Once synthesized, the cRNA was labeled by using the BrightStar Psoralen-Biotin nonisotopic labeling kit (Ambion). Meanwhile, the primers AF and AR were used to amplify the transcription template for scallop β-actin, and the cRNA probe of β-actin was prepared as described above.

Table 2
Sequences used for multiple alignment and phylogenetic analysis

Species	Common name	Taxonomy	Accession numbers
Homo sapiens	Human	Vertebrate: Mammalia	AAI00886
Mus musculus	House mouse	Vertebrate: Mammalia	BAE21171
Gallus gallus	Chicken	Vertebrate: Aves	NP_001001470
Anser anser anser	Western graylag goose	Vertebrate: Aves	P00718
Cygnus atratus	Black swan	Vertebrate: Aves	P00717
Rhea americana	Greater rhea	Vertebrate: Aves	JC7955
Casuarius casuarius	Southern cassowary	Vertebrate: Aves	Q7LZR3
Struthio camelus	Ostrich	Vertebrate: Aves	P00719
Epinephelus coioides	Orange-spotted grouper	Vertebrate: Teleostei	AAL08021
Siniperca chuatsi	Chinese perch	Vertebrate: Teleostei	AAU86896
Takifugu rubripes	Fugu rubripes	Vertebrate: Teleostei	NP_001027764
Cyprinus carpio	Common carp	Vertebrate: Teleostei	Q8JFR1
Paralichthys olivaceus	Bastard halibut	Vertebrate: Teleostei	Q90VZ3
Danio rerio	Zebrafish	Vertebrate: Teleostei	AAH76099
Oikopleura dioica	NO	Urochordates	CAD92342
O. dioica	NO	Urochordates	CAD92344
Argopecten irradians	Bay scallop	Invertebrate: Mollusca	AY788903
Chlamys farreri	Zhikong scallop	Invertebrate: Mollusca	DQ227696

Total RNA was extracted from various tissues, including mantle, gills, haemocytes, hepatopancreas, gonad and adductor muscle, according to the protocol of TRIzol reagent (Invitrogen). Northern blot analysis of the CFLysG transcript expression was carried out by using formaldehyde-based NorthernMax kit (Ambion). Ten micrograms of the RNA was denatured by incubation with formaldehyde load dye at 65 °C for 15 min, resolved on a 1.2% agarose gel, and blotted onto a nylon membrane. The blot was hybridized with biotin-labeled cRNA probe as suggested by the NorthernMax protocol. After hybridization, the membrane was washed twice, each of 5 min, in 2× SSC containing 0.1% SDS at room temperature, and twice in 0.1× SSC, 0.1% SDS at 68 °C for 15 min, and then was detected with the BrightStar BioDetect nonisotopic detection kit (Ambion).

2.5. Construction of expression cassette for CFLysG expression

PCR fragment encoding the mature peptide of CFLysG was amplified with specific primers rLysF and rLysR. For the convenience of cloning, an *Eco*RI site was added to the 5′ end of the rLysF and a *Not*I site was added to the 5′ end of rLysR after the stop codon. These primers were designed according to codon usage preference of *Saccharomyces cerevisiae* (Bennetzen and Hall, 1982). The purified PCR product was first cloned into pMD18-T simple vector (Takara), and digested with the restriction enzymes *Eco*RI and *Not*I, and then cloned into *Eco*RI/*Not*I site of the *Pichia* expression vector pPIC9K (Invitrogen). The resulting plasmid was transformed into *E. coli* JM109 for nucleotide sequencing to ensure in-frame insertion.

2.6. Pichia pastoris transformation and selection of recombinant clones

The constructed recombinant plasmid was linearized by *Sac*I and transformed into competent cells of *P. pastoris* GS115 by PEG method as recommended by manufacturer's instructions (Invitrogen). The cells were then spread on MD plates (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2% dextrose and 2% agar) for selection of positive clones. Positive transformants were pooled in sterile water and spread on YPD plates (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) supplemented with G418 at a final concentration of 1.0, 1.5 and 2 mg ml^{-1} for multi-copy selection.

2.7. Expression of recombinant CFLysG in P. pastoris

One positive clone with high resistance toward G418 was inoculated into BMGY medium (1% yeast extract, 2% peptone, 100 mmol l^{-1} potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin and 1% glycerol) and grew at 30 °C until the culture reached OD_{600} = 2–6. The cells were harvested by centrifuging the medium at 1500 × *g* for 5 min at room temperature, and cell pellet was resuspended in BMMY medium (1% yeast extract, 2% peptone, 100 mmol l^{-1} potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin and 0.5% methanol) to 1.0 absorbance unit at 600 nm. To induce expression, methanol was added every 24 h to a final volume concentration of 0.5% for successive 4 days. The fermentation supernatant was collected and dialyzed against distilled water using a Spectra/Por6 membrane (MWCO 10,000, Spectrum). The resulting solution was further purified by ultrafiltration on an Amicon Ultra-15 centrifugal filter device with 30 kDa cut off (Millipore). The filtrate was lyophilized, reconstituted in distilled water and subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). After electrophoresis, the protein bands were visualized by Coomassie brilliant blue R250 staining. The concentration of recombinant CFLysG was determined using the Bradford Protein Assay kit (Beyotime).

2.8. Lytic activity assay

Antimicrobial activity of the recombinant CFLysG was assessed with the radial diffusion assay as described by Minagawa et al. (2001). Seven bacterial strains were used as substrates, including Gram-negative bacteria *E. coli* JM109, *V. anguillarum*, *Vibrio splendidus*, *Vibrio parahaemolyticus*, and Gram-positive bacteria *Micrococcus lysodikicus*, *Micrococcus luteus* and *Staphylococcus aureus*. The bacterial concentration in medium was adjusted to 0.1 absorbance unit at 600 nm and poured onto 90 mm plates. Wells (diameter: 5 mm) were cut into the freshly poured plates after the solidification of the agar. For radial diffusion assays, 50 μg of recombinant protein were pipetted into individual wells and the agar plates were incubated at 25 °C. After 48-h incubation, the radius of the clearing zone was measured.

3. Results

3.1. cDNA cloning and sequencing of the CFLysG gene

Randomly sequencing of the scallop cDNA library with T3 primer yielded 6935 EST sequences, and these sequences were clustered into 686 contigs and 2191 singletons. Blast analysis revealed that a 678-bp EST (no. *Scag_8720*) was highly similar to the previously identified g-type lysozymes in vertebrate species. Based on this EST sequence, two gene-specific primers were designed to clone the full-length cDNA of CFLysG. By 5′ RACE technique with primers T3 and LysR1, a 674-bp fragment was amplified. PCR reaction with primers T7 and LysF1 resulted in a 534-bp product that contained the 3′ end of the gene. An 829-bp nucleotide sequence representing the complete cDNA sequence of CFLysG was obtained by overlapping the two fragments mentioned above with EST *Scag_8720*.

The full-length cDNA sequence of CFLysG was deposited in GenBank under accession no. DQ227696 and the deduced amino acid sequence was shown in Fig. 1. The complete sequence of CFLysG cDNA contained a 5′-terminal untranslated region (UTR) of 21 bp, a 3′-terminal UTR of 205 bp with a canonical polyadenylation signal sequence AATAAA and a poly(A) tail, and an open reading frame (ORF) of 603 bp encoding a polypeptide of 200 amino acids with a predicted molecular weight of 21.92 kDa and theoretical isoelectric point of 7.76, respectively.

Fig. 1. Nucleotide (GenBank accession no. DQ227696) and deduced amino acid sequence of CFLysG in *Chlamys farreri*. The nucleotides and amino acid residues are numbered on the left. The putative signal peptide is underlined and the SLT domain in the C-terminus is shadowed. The start and stop codons, and the classical polyadenylation signal in the 3′-UTR, are marked in bold. The *cysteine* residues are doubly underlined. The dot line indicates the catalytic residues (*Glu* 82, *Asp* 97, *Asp* 108).

The N-terminus had the features consistent with a signal peptide as defined by the PSORT II program analysis (National Institute for Basic Biology, Japan) with a putative cleavage site located after position 18 (AWA-AS), conforming to the ‘(−3, −1) rule’ (Von, 1986). The deduced mature peptide was of 182 amino acid residues with a theoretical mass of 20.01 kDa and a *p*I of 7.88.

3.2. Homology analysis of CFLysG

The deduced amino acid sequence of CFLysG shared high similarity with the g-type lysozyme proteins of *Gallus gallus* (39% identity; $E = 7e{-}33$), *Anser cygnoides* (39% identity; $E = 2e{-}32$), *Anser anser anser* (39% identity; $E = 3e{-}32$), *Cygnus atratus* (39% identity; $E = 2e{-}31$) and *O. dioica* (36% identity; $E = 4e{-}25$). SMART program analysis revealed that CFLysG contained an SLT domain in the C-terminus (Bacterial lytic transglycosylases domain), which was involved in the cleavage of the β-1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan of bacterial cell walls. Multiple alignment of CFLysG with g-type lysozymes from other organisms presented the conserved amino acids critical for the fundamental structure and function of g-type lysozyme, such as substrate binding sites (*Ile* 104, *Ile* 125, *Gly* 156, *Gly* 176), the catalytic residues (*Glu* 82, *Asp* 97, *Asp* 108) and their neighboring amino acids (Fig. 2). Furthermore, amino acid residues bearing charge were relatively conserved in the mature peptide of CFLysG, which resulted in a net charge of +17. The sequence similarity and the common structure features strongly suggested that CFLysG was a marine invertebrate homologue of mammalia, bird and fish g-type lysozymes.

Another remarkable characteristic in CFLysG was that the mature peptide harbored six *cysteine* residues other than four *cysteines* (C_1–C_4, C_2–C_3) conserved in avian and mammalian g-type lysozymes, which rendered the disulfide pattern of CFLysG quite different from other animal lineages. Pairwise alignment of CFLysG with the deduced amino acid sequence of bay scallop g-type lysozyme (accession no. AY788903) revealed that six *cysteine* residues in these two mollusk proteins shared the same location (Zou et al., 2005).

3.3. Phylogenetic analysis

To evaluate the molecular evolutionary relationships of CFLysG against other g-type lysozymes, a phylogenetic tree was constructed based on the protein sequences of 18 g-type lysozymes by the neighbor-joining method (Fig. 3). According to the phylogenetic tree, CFLysG was first clustered with bay scallop g-type lysozyme and formed an independent sister group to the urochordate g-type lysozymes, which suggested that scallop and urochordate g-type lysozymes underwent fewer divergences than those of scallop and vertebrate lysozymes. The relationships revealed in the phylogenetic tree were in agreement with the concept of traditional taxonomy. Evidently, CFLysG provided direct evidence that g-type lysozyme was not unique to vertebrate, and the origin of g-type lysozyme was traced back to invertebrate.

3.4. Tissue distribution of the CFLysG transcripts by Northern blot

Northern blot analysis was employed to determine the tissue specificity of CFLysG mRNA with biotin-labeled cRNA probe. CFLysG mRNA was found to be constitutively expressed in a wide range of tissues. However, the expression of CFLysG transcript was predominantly detectable in the tissues of gills, hepatopancreas and gonad, and to a lesser degree in the tissues of haemocytes and mantle, while no hybridization signal were detected in adductor muscle (Fig. 4).

3.5. Lytic activity of recombinant CFLysG protein

One clone with high resistance toward G418 was selected for secreted expression. After 96 h of induction, the fermentation supernatant was subject to dialysis and ultrafiltration for protein purification. The resulting protein was applied to SDS-PAGE analysis under reducing conditions, and the 20 kDa target protein

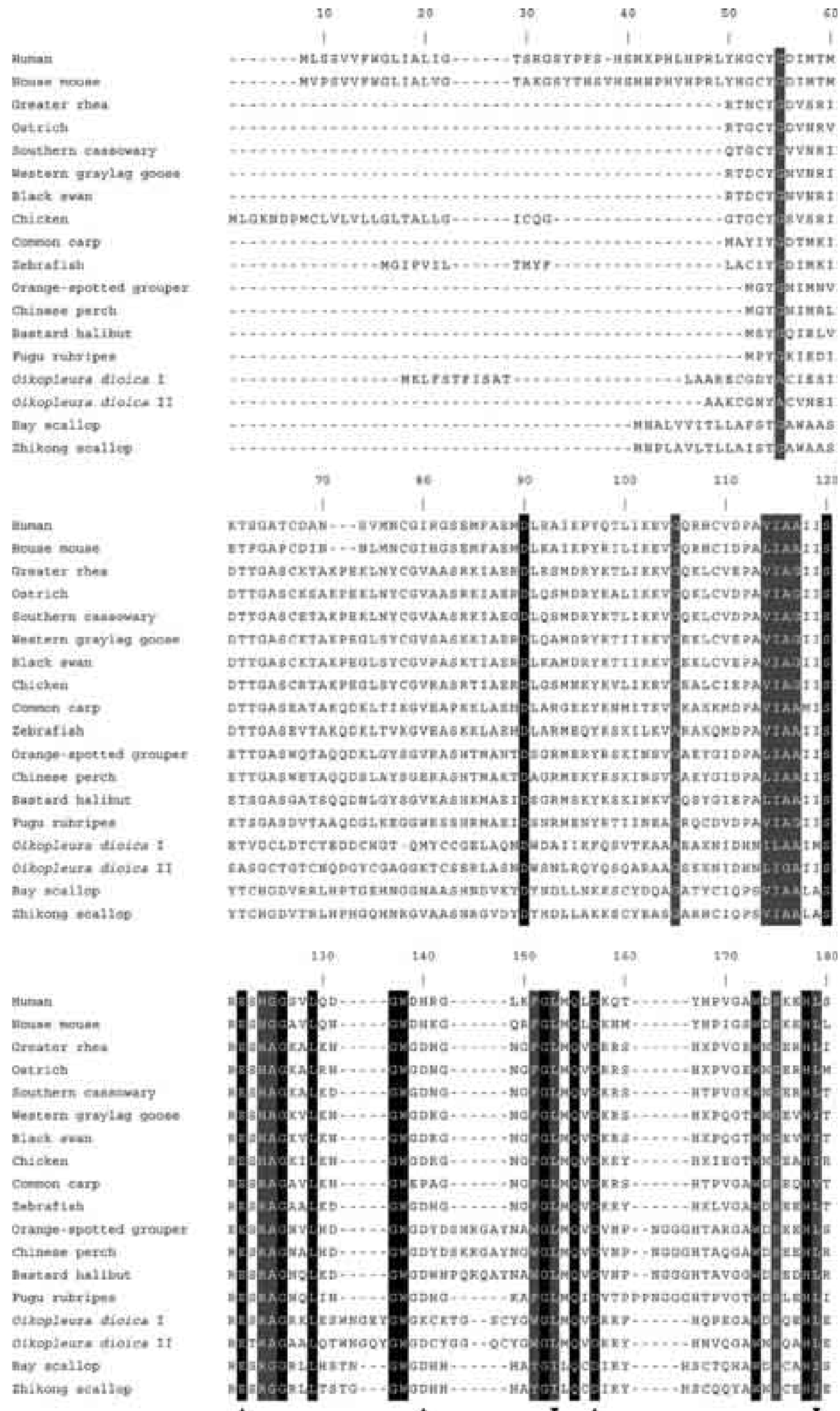

Fig. 2. Multiple alignment of CFLysG with g-type lysozymes from various animals. Identical amino acids are in white letters with black background, and gray background indicates high levels of amino acid similarity. The triangle (▲) and inverted triangle (▼) indicate the catalytic residues and the substrate binding sites of g-type lysozymes, respectively. Gaps are indicated by dashes to improve the alignment.

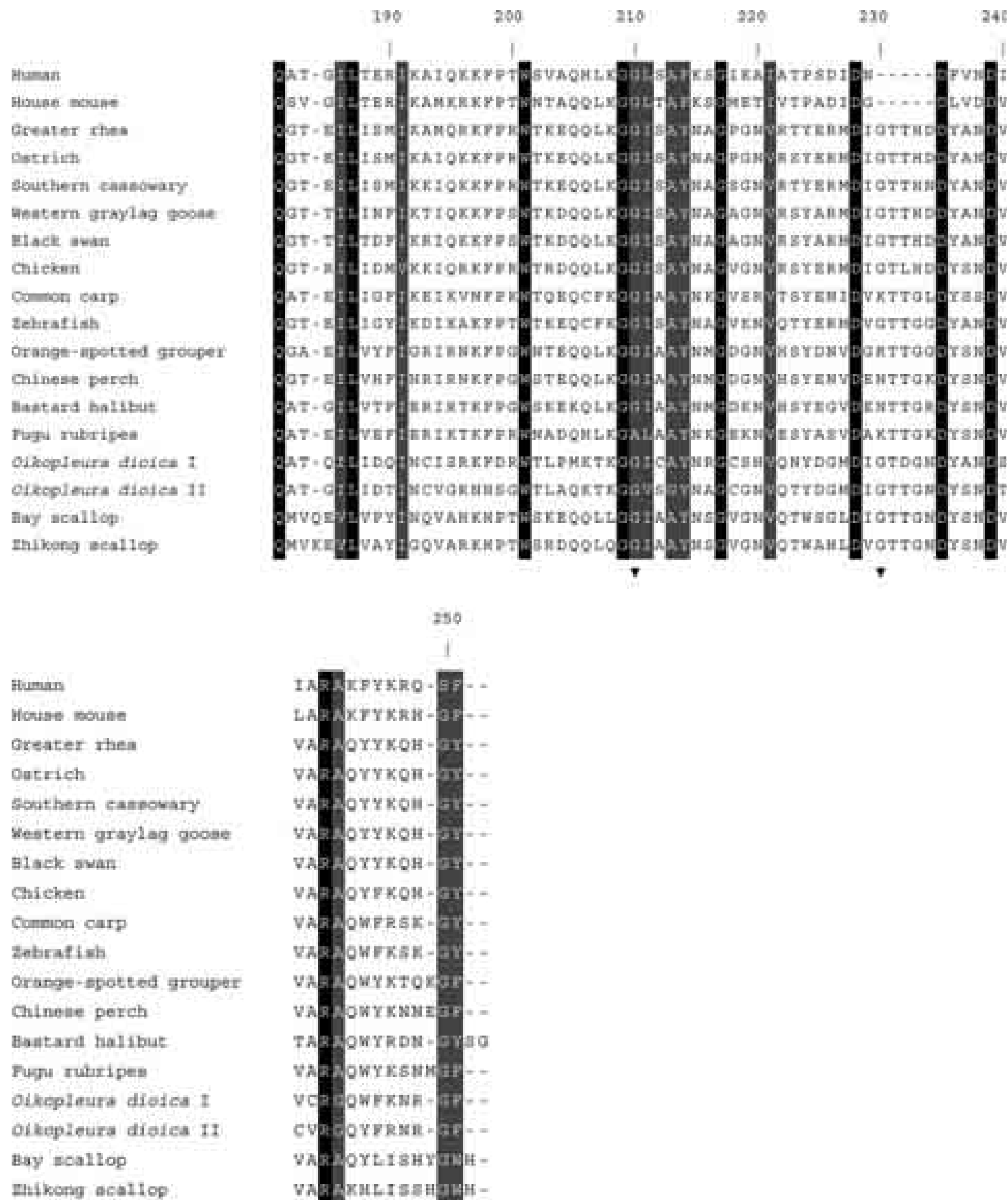

Fig. 2. (*Continued*).

was visualized by Coomassie Bright Blue R250 staining, which agreed with the expected molecular mass of the mature protein (Fig. 5).

Radial diffusion assay was employed to evaluate the antimicrobial spectrum of recombinant CFLysG against several Gram-positive and Gram-negative bacteria, including three mollusk pathogenic vibrios. Based on the radius of the antimicrobial zone, recombinant CFLysG was found to harbor remarkable in vitro inhibitive effect on Gram-positive bacteria with the exception of *S. aureus*, while lytic activity against Gram-negative bacteria was relatively weak (Fig. 6). Among the tested Gram-negative bacteria, recombinant CFLysG exerted marginally bactericidal activity against *V. parahaemolyticus*, *V. splendidus* and *V. anguillarum*, and no lytic effect was detected on *E. coli* JM109.

4. Discussion

Mollusk aquaculture is a big industry and contributes enormously to the economic development of coastal provinces in China (Guo et al., 1999). However, the intensification of mollusk farming has also been accompanied by the outbreak of infectious diseases. Understanding the immune defense mechanisms of mollusk may be conductive to the development of better disease management strategies in mollusk farming. The identification and characterization of antimicrobial effector are now considered to be essential for the elucidation of defense mechanisms and for disease control because of their potential uses as therapeutic agents and genetic improvement biomarkers on disease-resistant strain selection. However, the understanding of mollusk immunity was still in its very infancy, and the

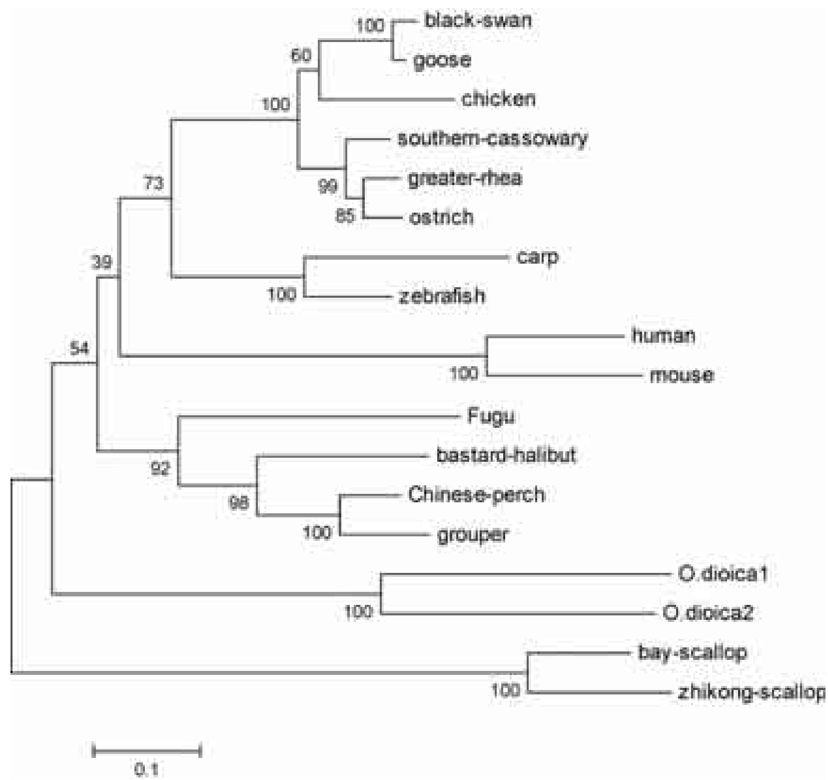

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method in MEGA software based on the g-type lysozyme sequences. Bootstrap support values for the NJ tree are shown at the nodes (out of 1000 replicates). The accession numbers retrieved in this study are listed in Table 2.

molecular characterization and functional studies of defensive molecules remains poorly investigated in mollusk.

In the present study, a cDNA encoding an invertebrate g-type lysozyme (designated CFLysG) was identified from *C. farreri* by EST approach and RACE techniques. Like g-type lysozymes found in birds and mammalian, CFLysG had the characteristic hydrophobic signal peptide at the N-terminus and an SLT domain in the C-terminus. The structurally important amino acid residues characteristic for g-type lysozymes, such as the substrate binding sites and active sites involved in catalysis (Nakai et al., 2005), were demonstrated to be highly conserved

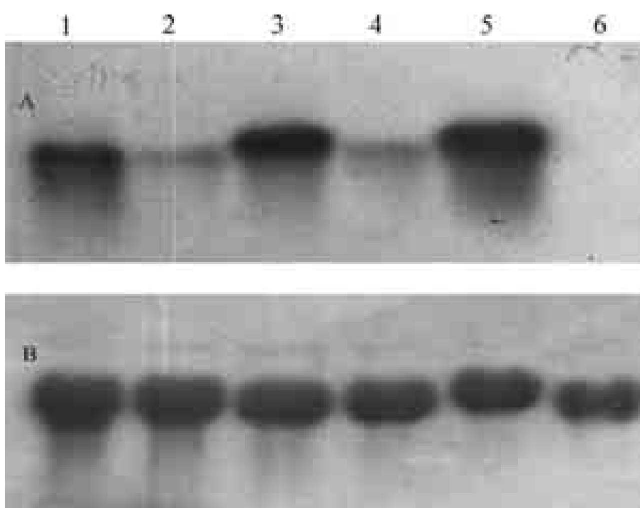

Fig. 4. Tissue distribution of CFLysG transcript revealed by Northern blot. Ten micrograms of RNA from different tissues was subjected to electrophoresis and blotted onto a nylon membrane. The membrane was hybridized with (A) the biotin-labeled CFLysG gene probe and (B) the scallop β-actin probe, and visualized with chemiluminescent detection. Lane 1: gills; lane 2: mantle; lane 3: gonad; lane 4: haemocytes; lane 5: hepatopancreas; lane 6: adductor muscle.

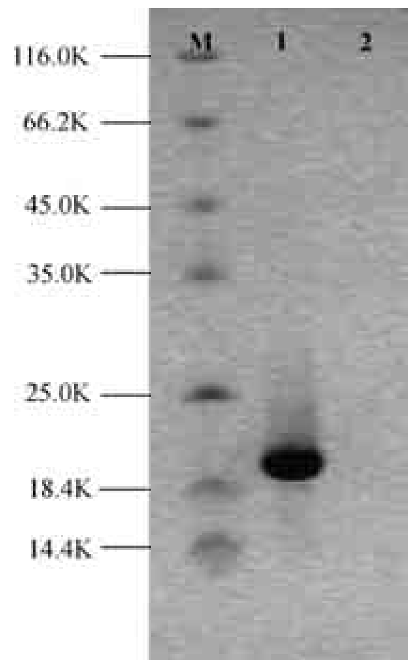

Fig. 5. SDS-PAGE analysis of recombinant CFLysG. After electrophoresis, the gel was visualized by Coomassie brilliant blue R250 staining. Lane M: protein molecular standard; lane 1: purified recombinant CFLysG; lane 2: negative control.

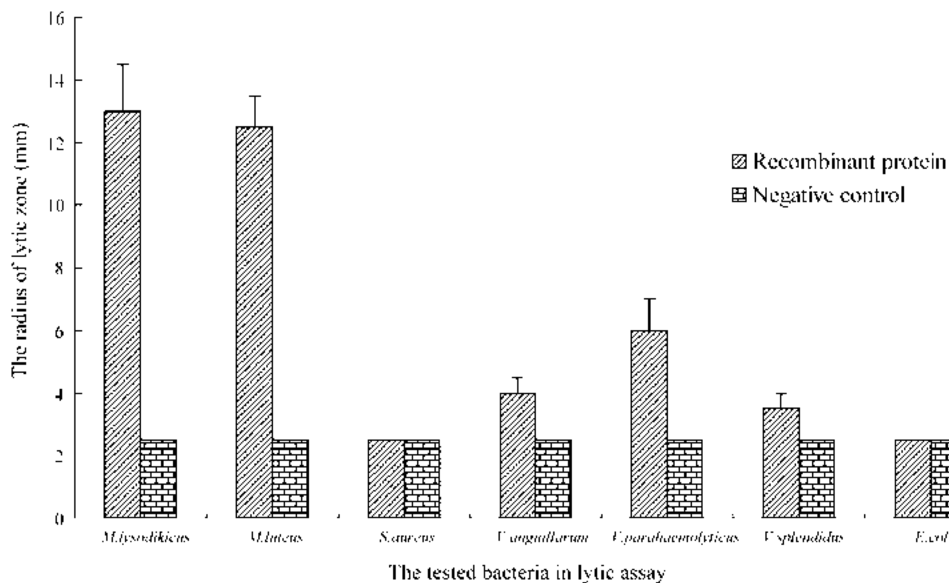

Fig. 6. Lytic activities of the recombinant CFLysG against *Micrococcus lysodikicus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Vibrio splendidus*, *Escherichia coli* JM109 by a lysoplate assay. Fifty micrograms of recombinant sample was applied to each treatment and values were shown as means ± S.E., $n = 3$.

within the amino acid sequence of CFLysG. However, the well-conserved four *cysteine* residues in the N-terminus of avian g-type lysozymes were absent in the mature peptide of CFLysG. Most notable was the presence of another six *cysteines* in mollusk g-type lysozymes that were not reported in other species. The connectivity of the disulfide bridges had still to be worked out, but given the *cysteine* array in the primary sequence, it was clearly distinctive from those of the hitherto characterized g-type lysozymes. The high content of *cysteine* residues had also been identified in i-type lysozyme from *Tapes japonica* and *Chlamys icelandic*, which contained as many as 14 *cysteine* residues (Ito et al., 1999; Nilsen et al., 1999). At this point, it was postulated that mollusk lysozymes (both g-type and i-type) harbored more *cysteine* residues, possibly rendering them more stable in high-osmolarity seawater with a compacter structure. Furthermore, compact structure could protect themselves from the proteases coexisting in the digestive organs (Ito et al., 1999).

g-Type lysozymes were first identified from avian species (Canfield and McMurry, 1967), and recently it was found to be present in the urochordates (Nilsen et al., 2003). However, no g-type lysozyme had been reported in invertebrate so far. Taken together with our previous results on g-type lysozyme from bay scallop (Zou et al., 2005), it was demonstrated that g-type lysozyme was not vertebrate-specific and its origin should precede the divergence of invertebrate and vertebrate. Results from our molecular phylogenetic analysis indicated that CFLysG was more closely related to the urochordate g-type lysozymes than the vertebrate counterparts. However, more information on the genomic structure and organization of CFLysG gene were required to better understand the evolutionary relationships of different g-type lysozymes. Recently, different lysozyme types were reported to be encoded within the genome of certain individual species (Qasba and Kumar, 1997). Considering the discovery of i-type lysozymes in mollusk, especially the chlamysin isolated from *C. icelandic* (Nilsen et al., 1999), it was reasonable to assume that i-type and g-type of lysozymes were coexistent in marine mollusk. The significantly divergent amino acid sequences between mollusk i-type and g-type lysozymes perhaps conferred functional differences, indicating that mollusk possessed different types of adapted lysozymes that were spatially and temporally differentially expressed.

Expression pattern of g-type lysozyme genes has been investigated in a variety of organisms. The g-type lysozymes in avian were found to be expressed in the lung and non-adherent bone marrow cells (Nakano and Graf, 1991). In contrast to the restricted expression patterns in birds, g-type lysozyme in Japanese flounder was found to be expressed in all tissues examined, although predominantly expressed in hematopoietic and the tissues exposed to external environment (Hikima et al., 2001). The variation in g-type lysozyme gene expression pattern among the species probably indicated that the biological functions might have altered during their evolution. In the present study, the CFLysG transcript was predominantly expressed in the tissues of hepatopancreas, gills and gonad. The enrichment of CFLysG mRNA in hepatopancreas indicated that CFLysG served as a digestive enzyme against the bacteria in the digestive organs, which had been verified by lysozyme activities in hepatopancreas of several marine bivalves (McHenery et al., 1979; Jollès et al., 1996). The gills, constructed of only a single layer of fragile cells and covered with a thin layer of protective mucus, are constantly being flushed with water that contains pathogens. The high expression level of CFLysG mRNA in gills implied that CFLysG had a significant contribution in prevention of microbial exploitation. Moreover, CFLysG mRNA was also expressed in non-digestive tissues such as haemocytes, which supported the possible immune functions for this enzyme, since mollusk haemocytes were thought to play extremely important roles in defense not only by direct sequestration and killing of

foreign invaders, but also by synthesis and exocytosis of bioactive molecules (Hoffmann et al., 1999; Roch, 1999; Tincu and Taylor, 2004). These results suggested that CFLysG was a multifunctional molecule both for the digestive function and also for the host immune responses.

It has been proposed that lysozymes of marine invertebrates have a wider range of activities than those of terrestrial invertebrates in order to cope with a greater range of bacterial strains and species in the marine environment (Hikima et al., 2003). In order to characterize CFLysG in terms of in vitro lytic activity, CFLysG was recombined in *P. pastoris* to facilitate the correct linkage of disulfide bonds. The recombinant CFLysG displayed a wide range of lytic activities with more potent activity against the Gram-positive bacteria than the Gram-negative bacteria, which was in agreement with the g-type lysozymes from Japanese flounder and orange-spotted grouper. However, the recombinant protein exhibited no lytic activity toward *S. aureus*, primarily due to the presence of 6-*O*-acetylated peptidoglycan in the cell wall of this bacterium (Rau et al., 2001). The marginal activity of recombinant CFLysG towards several vibrios indicated that this protein might be less effective in controlling vibrios infection in vivo. This was consistent with the fact that the *Vibrio* genus was one of the most devastating pathogens for Zhikong scallop. To better understand the immune functions of CFLysG, it would be of prime importance to further analyze the lysozyme activity, expression kinetics and localization of CFLysG in response to infection.

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