Fish & Shellfish Immunology 32 (2012) 914–921

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Identification and expression analysis of goose-type lysozyme in half-smooth tongue sole (*Cynoglossus semilaevis*)

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ARTICLE INFO

Article history:
Received 30 July 2011
Received in revised form
18 December 2011
Accepted 11 January 2012
Available online 2 February 2012

Keywords:
G-type lysozyme
Cynoglossus semilaevis
Gene expression
Bacterial infection
Innate immunity

ABSTRACT

Lysozymes are considered to be potent innate immune molecules against the invasion of bacterial pathogens. The goose-type lysozyme is one of the three major distinct lysozyme types identified in the animal kingdom including teleosts. In this report, we identified, sequenced, and characterized the goose-type lysozyme gene (*CsGLys*) from half-smooth tongue sole (*Cynoglossus semilaevis*). The full-length cDNA of *CsGLys* is 1191 bp in length from the transcription start site to polyadenylation site, including a 91 bp 5′-terminal untranslated region (UTR), a 452 bp 3′-terminal UTR and a 648 bp open reading frame (ORF) of encoding a polypeptide with 215 amino acids. The deduced amino acid sequence of *CsGLys* possesses a Goose Egg White Lysozyme (GEWL) domain with three conserved residues (E91, D104 and D121) essential for catalytic activity. The *CsGLys* gene consisting of 2535 bp, was similar to those of other teleost species such as Japanese flounder and large yellow croaker with five exons interrupted by four introns. The 5′-flanking region of *CsGLys* gene shows several transcriptional factor binding sites related to immune response. Tissue expression profile analysis by quantitative real-time reverse transcription PCR showed that *CsGLys* mRNA was constitutively expressed in all examined tissues with the predominant expression in skin and the weakest expression in heart. The expression of *CsGLys* after challenged with bacteria *Vibrio anguillarum* was up-regulated in blood, head kidney, liver and spleen at 12 h post-infection and it reached the peak level at the same time point with a 19.89-, 4.21-, 14.45- and 10.37-fold increase, respectively, while the *CsGLys* expression was down-regulated to lower level than the normal level in each tested tissues except in liver from the 48 h until 96 h. These results suggest that *CsGLys* might play an important role in half-smooth tongue sole host defense against the bacteria infection.

1. Introduction

Lysozyme is the ubiquitous enzyme in the innate immune system of all major taxa of living organisms, which catalyzes the hydrolysis of 1, 4-beta-linkages between N-acetyl-D-glucosamine and N-acetylmuramic acid in peptidoglycan heteropolymers of bacterial cell walls, leading to breakdown of bacteria [1,2]. Thus, the enzyme could act as an innate defense molecule against bacterial infection. Based on their differences in structure, catalytic character and original source, six types of lysozymes had been identified including chicken-type lysozyme (c-type lysozyme), goose-type lysozyme (g-type lysozyme), invertebrate-type lysozyme (i-type lysozyme), phage lysozyme, bacterial lysozyme and plant lysozyme [3–5]. While, in the animal kingdom, three major distinct lysozyme types have been identified: the c-type lysozyme, the g-type lysozyme and the i-type lysozyme [6]. So far, g-type lysozymes (or at least their corresponding genes) are found in members of the Chordata, as well as in some bivalve mollusks belonging to the invertebrates [6]. The g-type lysozyme owes its name to its initial identification in egg whites of the Embden goose [7] and subsequently had been characterized in several avian species such as chicken, black swan and ostrich [6]. The first teleost g-type lysozyme gene, as well as the first occurrence of g-type lysozyme outside the class of birds, was discovered in Japanese flounder (*Paralichthys olivaceus*) by Hikima et al. [8] and the g-type lysozyme was considered as one of the important anti-bacterial molecules in fish [9]. Since then, genes of g-type lysozymes have been identified in a number of fish species, including common carp (*Cyprinus carpio* L.) [10], orange-spotted grouper (*Epinephelus coioides*) [11], mandarin fish (*Siniperca chuatsi*) [12], Atlantic salmon (*Salmo salar*) [13], large yellow croaker (*Larimichthys crocea*) [14], brill [15],

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doi:10.1016/j.fsi.2012.01.010

Atlantic cod (*Gadus morhua* L.) [16], grass carp (*Ctenopharyngodon idellus*) [17] and turbot (*Scophthalmus maximus*) [18]. The research on fish g-type lysozyme has been attracting increasing attention recently.

Half-smooth tongue sole, *Cynoglossus semilaevis*, is mainly distributed in the East Asia. Wild resources of half-smooth tongue sole have declined sharply in the recent decades probably due to over-exploitation. Owing to high commercial value, fast growth rate and delicious flavor, this species has become one of the most important cultured species in China since 2003. However, culture of half-smooth tongue sole has been severely threatened by bacterial and viral pathogens. Innate immunity represents the first line of defense against invading pathogens and is believed to respond efficiently in fish [19]. The information is still limited about the innate immune system and the defense mechanisms that this species displays against bacterial infections. In the present study, we described the characterization and expression pattern of the g-type lysozyme gene of half-smooth tongue sole (*CsGLys*), previously identified by expressed sequence tag (EST) analysis of a normalized mix immune tissues cDNA library [20]. Main lysozyme features were identified, and phylogenetic analysis was carried out. Gene structure and putative transcription binding site in the 5′ flank region of *CsGLys* genomic DNA were analyzed. Gene expression analysis in various tissues and challenge experiments with bacteria *Vibrio anguillarum* were performed in order to elucidate the role of g-type lysozyme in the response against bacteria in half-smooth tongue sole.

2. Materials and methods

2.1. Identification and sequencing of CsGLys cDNA

BLAST searches were used to identify cDNA encoding for CsGLys from half-smooth tongue sole expressed sequence tags (ESTs) from previous sequencing efforts [20]. The clone csgmma0_0011_A02 (GenBank ID: GH229796) containing the putative complete cDNA with 14 bp 5′-UTR as revealed by BLAST searches was completely sequenced to generate the full-length cDNA sequence. T7 and SP6 primers were used for sequencing the clone csgmma0_0011_A02 (Table 1). Sequencing reactions were performed using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols in Beijing Genome Institute in China and samples were sequenced on an ABI 3730XL automated DNA sequencer (Applied Biosystems). To obtain more upstream region including the transcription start site, rapid amplification of cDNA ends (RACE) was performed using a Smart RACE cDNA Amplification Kit (Clontech/BD sciences, Mountain View, CA) per the manufacturer's instructions.

Table 1
Sequence and experimental conditions for primers used in the present study.

Primers	Sequence (5′ → 3′)	Utilization
SP6	ATTTAGGTGACACTATAG	Sequencing cDNA clone
T7	TAATACGACTCACTATAGGG	Sequencing cDNA clone
CsGL-F1	CGGGGGAACTACGGTGACA	CsGLys genomic DNA
CsGL-R1	CCCTGATTGTAGGCTGCGA	
CsGL-5′RACE	GGAGATGATGGCAGCAATAAGACACGGT	CsGLys 5′RACE PCR
CsGL-RT-F	TCTTATTGCTGCCATCATCTCC	qPCR
CsGL-RT-R	GTCTTTTTGGGCTCCAGTCTCC	
18S rRNA-RT-F	GGTCTGTGATGCCCTTAGATGTC	qPCR internal
18S rRNA-RT-R	AGTGGGGTTCAGCGGGTTAC	control
CsGL-SP1	ATGATGGCAGCAATAAGACACGGT	Genome
CsGL-SP2	CCAGTTTGTCCTGTTGAGCAGTCTT	walking PCR
CsGL-SP3	TTCCAGGAAGCTCCACTAGTAGGTATC	

2.2. Genomic CsGLys DNA sequencing

To determine the CsGLys genomic DNA sequence, initially one pair of primer (Table 1) was designed against the 5′- and 3′-flanking sequences of the obtained full-length cDNA. Genomic DNA was extracted from blood using the method as described previously [21]. A total of 100 ng genomic DNA was used for PCR. The program of PCR was performed with an initial denaturation step of 4 min at 94 °C, and then 34 cycles were run as follows: denaturation of 30 s at 94 °C, annealing of 40 s at 55 °C and extension of 50 s at 72 °C, followed by final extension of 10 min at 72 °C and storage at 4 °C. The PCR products were purified and cloned into pBS-T vector (TIANGEN Beijing, China). Two positive clones of different fragments were sequenced. The 5′-flanking region was obtained using a genome walking approach, by constructing genomic libraries with a Universal Genome Walker™ kit (Clontech/BD sciences, Mountain View, CA). A total of three 5′ gene specific primers (Table 1) were designed for gene walking. The putative transcription binding sites in 5′-flanking region of CsGLys genomic DNA were predicted by TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html) [22].

2.3. Phylogenetic analysis

G-type lysozyme coding sequences from various species were retrieved from GenBank for multiple sequence alignment using ClustalW. A phylogenetic tree was constructed using the neighbor-joining method within the Molecular Evolutionary Genetics Analysis (MEGA 4) package [23]. Data were analyzed using Poisson correction, and gaps were removed by complete deletion. The topological stability of the trees was evaluated by 10 000 bootstrap replications.

2.4. Fish, samples, challenge experiments and RNA extraction

Half-smooth tongue sole, about 1.5 years with an average weight 247 g (average 34.5 cm of body length), was obtained from Mingbo Mariculture Farm in Laizhou, Shandong, China. A total of 27 fish were acclimated at 25 °C in aerated seawater for 15 days before artificial bacterial challenges. To determine *CsGLys* gene expression in various tissues, 12 tissues including blood, brain, gill, heart, head kidney, intestine, liver, muscle, ovary, skin spleen and stomach were collected from healthy group.

Challenge experiments were conducted on the fish aquariums following established protocols for *V. anguillarum* challenges with modification [24]. In brief, 2×10^7 CFU (colony forming units) *V. anguillarum* per fish were injected intraperitoneally whereas control fish were injected the same volume of PBS (200 μl). The blood, head kidney, intestine, liver, skin and spleen were collected from challenge group at 6 time points after the challenge: 0 h, 12 h, 24 h, 36 h, 48 h, 72 h and 96 h post-injection. Each tissue was collected from each group at each time point from three fish and pooled for RNA isolation. The tissue samples were flash frozen in liquid nitrogen immediately after collection, and stored at −80 °C until RNA extraction.

TRIzol reagent (Invitrogen, Carlsbad CA, USA) was used for RNA extraction and the concentration of total RNA was quantified by Ultrospec 1100pro (Amersham Biosciences, Piscataway, NJ, USA). Total RNAs were subsequently used for the analysis of gene expression.

2.5. Gene expression analysis

The quantitative real-time PCR (qRT-PCR) was conducted on Applied Biosystems 7500 Real-Time PCR System (Applied

Biosystems, Foster City, CA, USA) with SYBR® Premix Ex Taq™ (Takara, Dalian, China) for gene expression pattern analysis. The cDNA samples of tissues from normal fish, *V. anguillarum* infected fish and control fish were used for CsGLys expression patterns with primers CsGL-RT-F and CsGL-RT-R (Table 1). The expression of 18S rRNA was used as an internal control [25] with primers 18S rRNA-RT-F and 18S rRNA-RT-R (Table 1).

The 20-μl qRT-PCR mixture consisted of 1 μl cDNA sample, 10 μl SYBR® Premix Ex Taq™, 0.4 μl ROX Reference Dye II, 0.4 μl PCR forward/reverse primers (10 μM) and 7.8 μl nuclease-free water. The PCR was conducted at 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 58 °C for 34 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The results were analyzed with the $2-^{\Delta\Delta}$CT method using the SDS 4.1 software. Finally, the data were submitted to one-way ANOVA (analysis of variance) test followed by an unpaired, two-tailed *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of CsGLys cDNA

The full-length cDNA of *CsGLys* was submitted to GenBank with an accession number of JF927973. *CsGLys* cDNA contains a 648 bp open reading frame (ORF) encoding a putative protein of 215 amino acids with a 91 bp 5′-untranslated region (UTR) from start transcription site and a 452 bp 3′-UTR. The typical polyadenylation signal AATAAA was found 18 bp before the poly A signal (Fig. 1). Analysis of the deduced amino acid sequences by multiple sequence alignments indicated that the g-type lysozyme gene is moderately conserved through evolution (Fig. 2). The CsGLys showed 51–69% identity with those lysozyme protein sequences of *Scophthalmus rhombus*, *Solea senegalensis*, *P. olivaceus*, *Danio rerio*, *Takifugu rubripes*, *S. salar*, *Ctenopharyngodon idella*, *Xenopus laevis* and *Anser anser anser*, and 37–40% identity with mouse (*Mus musculus*) and human (*Homo sapiens*) lysozyme protein. Typical signal peptide was not found at the N-terminus of CsGLys, neither the conserved cysteine residues. In silico analysis for conserved domain (NCBI website: http://blast.ncbi.nlm.nih.gov/Blast.cgi, find sequences with similar conserved domain architecture) revealed that CsGLys possesses a Goose Egg White Lysozyme (GEWL) domain (residues 35–211), which contains the conserved catalytic residues (E91, D104 and D121) and seven N-acetyl-D-glucosamine binding sites. Sequence alignment showed that both lower and higher vertebrate g-type lysozymes are conserved at a number of residues and regions located in the GEWL domain, including those involved in catalysis (Fig. 2).

3.2. Structural analysis of CsGLys gene

Half-smooth tongue sole g-type lysozyme genomic DNA sequence was submitted to GenBank with an accession number of JF914942. It is 1810 bp in length from the first transcriptional start site to the polyadenylation site and composed of 5 exons and 4 introns. The transcription start site is indicated by +1, located at 90 bp upstream of the start codon ATG. Each of exon/intron junctions showed the canonical intron splicing motifs (GT/intron/AG). The *CsGLys* gene consists of 5 exons and 4 introns, its gene structure was quite similar to Japanese flounder and large yellow croaker, as well as exon 3 and 4 each containing the same number of nucleotides in three species, while exon 1, 2 and 5 of *CsGLys* gene are longer than that of Japanese flounder and large yellow croaker (Fig. 3).

A total of 725 bp from putative transcription start site in 5′ flank region of *CsGLys* gene was analyzed for prediction of the transcription factor binding sites by TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html). As shown in Fig. 1, a number of putative transcription factor binding sites were identified including 8 SRY binding sites (-622, -550, -307, -213, -26, -27, -11, -15), 3 CdxA binding sites (-551, -138, -63), 2 upstream stimulatory factor (USF, -673, -40), 2 AML-1a (-252, -192), 2 GATA (-199, -106), an NF-κB (p65) binding site (-605) as well as c-Rel binding site, an NF-κB 2 binding site (-606), a CP2 binding site (-500), a Nkx-2 binding site (-415), a octamer factor 1 (Oct-1) binding site (-422), a c-myc binding site (-352), a hepatic nuclear factor 3β (HNF-3β, a liver-enriched transcription factor) binding site (-349), a CREB (-327), a Sox5 binding site (-212), an AP-1 binding site (-141) and a ROPalp binding site (-121). Some of them such as NF-κB, AP-1 and Oct-1 were involved in innate immune response.

3.3. Phylogenetic analysis

In order to analyze CsGLys gene in the larger context of vertebrate g-type lysozyme genes, phylogenetic analysis was conducted based on the deduced amino acid sequences from 25 taxa including 19 kinds of fish, 2 kinds of amphibian, 2 kinds of birds and 2 kinds of mammals, as shown in Fig. 4. The vertebrate g-type lysozyme homologs are supported by very strong bootstrap values. The phylogenetic tree clearly showed that the g-type lysozyme genes formed 4 clades as teleosts clade, amphibian clade, avian clade and mammal clade. The teleost g-type lysozyme genes formed two distinct sub-clades, CsGLys clustered with all of marine fish gene except mandarin fish, while all of the fresh water fish and anadromous fish such as salmon and cod were grouped together in the same sub-clade.

3.4. Tissue expression of CsGLys gene

qRT-PCR was used to determine tissue distribution of the *CsGLys* gene expression under normal physiological conditions. As shown in Fig. 5, *CsGLys* was expressed ubiquitously in the examined tissues, it was expressed most abundantly in intestine and skin with the relative expression value as 8.82 and 8.77 ($p < 0.05$), respectively, and also at a relatively high level in brain, gill, stomach and muscle. The lowest transcript level in heart and spleen with the relative expression value was 0.48 and 0.45, respectively.

3.5. Expression profiles of the CsGLys after V. anguillarum infection

To investigate the role of *CsGLys* in immune response, qRT-PCR was performed to detect the transcriptional levels of the *CsGLys* in several immune-related tissues at different time points after infection with *V. anguillarum*. As shown in Fig. 6. *CsGLys* relative expression level was significantly changed in blood, head kidney, intestine, liver, skin and spleen after infection. The gene expression showed similar profiles in blood, head kidney and spleen, while expression level of *CsGLys* was up-regulated drastically and reached the peak value with 19.89-, 4.21-, 10.37-fold increase at 12 h after infection respectively (all $p < 0.05$), then gradually decreased and recovered near to the pre-injection level from 24 h to 36 h and was down-regulated from 36 till 96 h post-injection (Fig. 6B, C and D). *CsGLys* gene expression pattern in skin and intestine against *V. anguillarum* infection was similar to that above tissues and showed up-regulation excepted that the peak value was appeared at 24 h post-infection and then decreased to the normal or near level from 36 h to 96 h after challenge (Fig. 6E and F). While, the *CsGLys* gene expression in liver after infection was up-regulated fluctuating and reached two peaks at 12 h (14.45-fold) and 72 h (10.16-fold), respectively (Fig. 6A). *CsGLys* gene expression was down-regulated at 12 h post-injection in intestine, and then up-

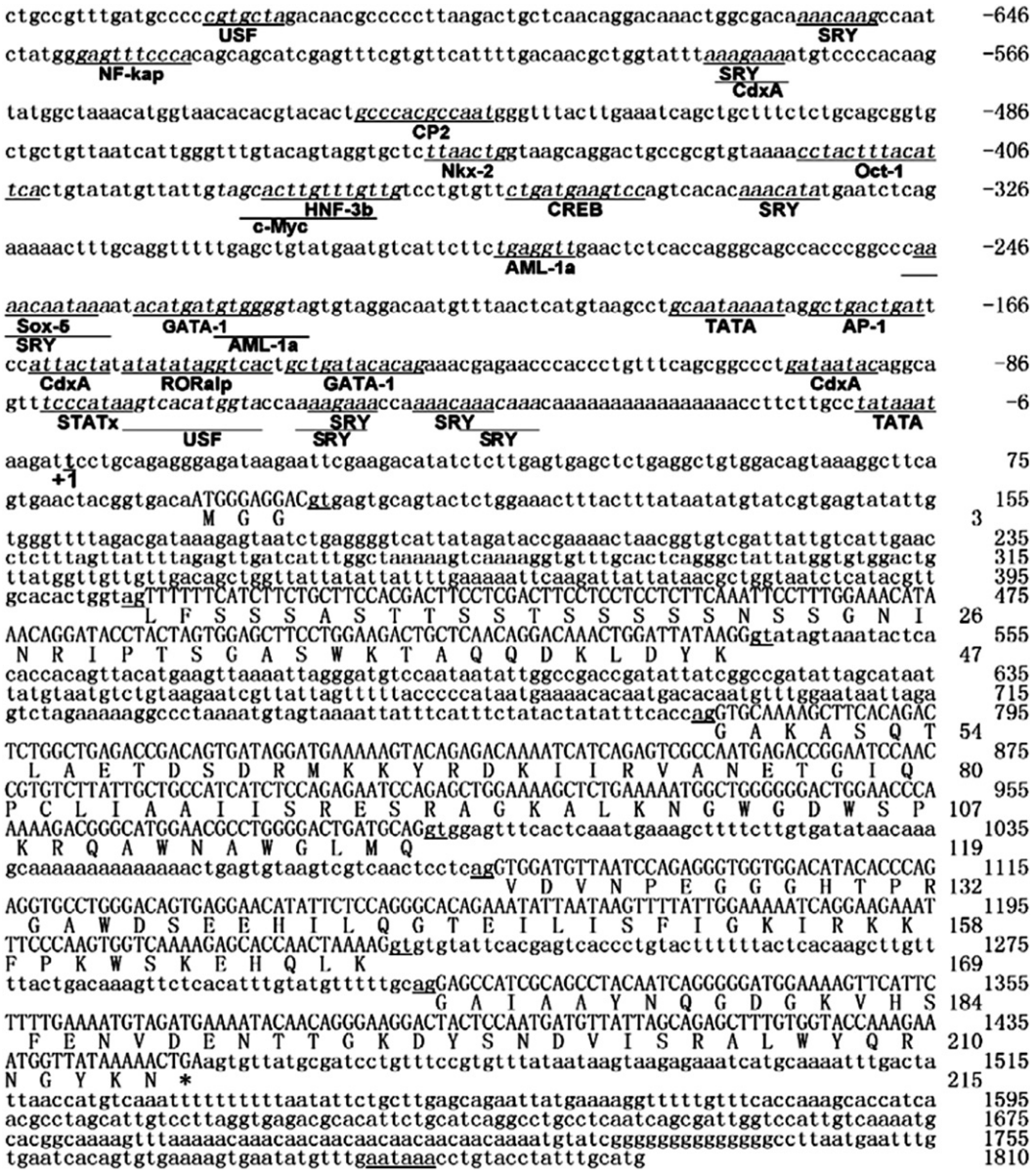

Fig. 1. Nucleotide sequence of the half-smooth tongue sole g-type lysozyme gene. Coding regions are indicated by uppercase letters. Deduced amino acid sequences are shown below the coding regions. The asterisk indicates the stop codon. Untranslated regions are indicated by lowercase letters. The exon/intron junction site (gt-ag) and polyadenylation signal (aataaa) is underlined. The transcription start site is indicated by '+1'. The consensus elements which could interact with transcription factor are underlined in the region of promoter.

regulated from 24 h to 96 h after infection, which the peak value appeared at 24 h with 4.54-fold increase (Fig. 6E). These data indicate that *CsGLys* gene may contribute to host innate immune defense upon exposure to gram-negative bacteria or their products.

4. Discussion

In the present paper, we identified and characterized the novel g-type lysozyme gene from half-smooth tongue sole, and analyzed its expression and regulation by bacterial infections. *CsGLys* contains longer deduced amino acids of 215 residues than in other fish species. For instance, the number of deduced amino acids of g-type lysozyme cDNA from the most reported fish species such as Japanese flounder, orange-spotted grouper, larger yellow croaker, mandarin fish, brill and turbot was 193–195 [8,10,12,15,17]. While, some fish species possess the shorter lysozyme protein, such as common carp and grass carp with 185 amino acids [10,17], some fish with longer deduced lysozyme protein such as Atlantic cod lysozyme 1 that contains 217 amino acids [16]. Although different fish species contain different ORF length of g-type lysozyme cDNA, the conserved domain of deduced g-type lysozyme protein was much similar. The deduced *CsGLys* protein contains typical GEWL domain with 185 amino acid residues and three conserved catalytic residue E, D and D, which exist in most fish species with known g-type lysozyme sequence as well as in birds and mammals, and are thought to have strong catalytic activity for bird egg g-type lysozyme [6]. Further more, neither the signal peptide nor conserved cysteine residues was found in CsGLys, which is similar to g-type lysozyme gene from some fish such as Japanese flounder, mandarin fish, large yellow croaker and grass carp, and totally different with avians and mammals. In birds and mammals, signal peptide sequence was found at the N-terminus of g-type lysozymes [17],

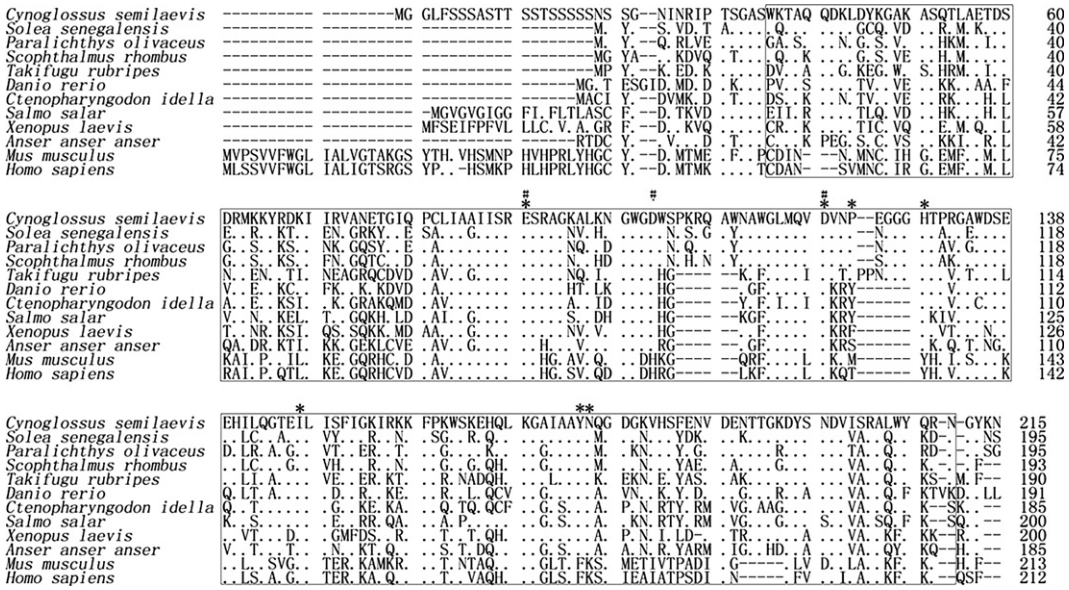

Fig. 2. Amino acid sequence alignment of g-type lysozymes homologs. Dashes represent amino acid deletion, dots denote identical amino acid residues. The Goose Egg White Lysozyme (GEWL) domain is boxed (CDD:29563). "#" indicates the catalytic residues. "*" indicates the N-acetyl-D-glucosamine binding site. The GenBank accession numbers of the aligned sequences are as follows: half-smooth tongue sole *Cynoglossus semilaevis*, JF927973; senegal sole *Solea senegalensis*, BAG14278; Japanese flounder *Paralichthys olivaceus*, Q90VZ3; brill *Scophthalmus rhombus*, BAF75845; *Takifugu rubripes*, NP_001027764; zebrafish *Danio rerio*, XP_002664417; grass carp *Ctenopharyngodon idella*, ACF41165; Atlantic salmon *Salmo salar*, ACI69359; platanna *Xenopus laevis*, NP_001088153; goose *Anser anser*, P00718; mouse *Mus musculus*, AAI47568; human *Homo sapiens*, AAI00883.

and four conserved Cys residues form two intramolecular disulphide bonds in the mature proteins in avian g-type lysozyme [26,27], Whereas, the disulphide bonds for g-type lysozyme activity was not essential for the correct folding of the catalytically active conformation [28]. Even one cysteine, as in *C. carpio* [10] and *S. salar* [13], or two, as in *D. rerio* and *S. maximus* [17], but with no potential to form an intramolecular disulphide bond [27], suggesting that CsGLys as well as fish g-type lysozyme was an intracellular protein.

The 5′ RACE result demonstrated the full-length CsGLys cDNA generated from the clone cagmma0_0011_A02 containing CsGLys was exactly correct and the transcription start site was confirmed. According to mechanism of BD SMART™ cDNA synthesis described in the manual of BD SMART™ RACE cDNA Amplification Kit (Clontech), the transcription start site should be following several G residues, In our result, we found the BD SMART II Oligonucleotide in front of 5′ end of RACE sequence, the first nucleotide behind G residues should be the transcription start site as T residues (Fig. 7). TATA box is at the 6 bp upstream from the transcription start site. The gene structure of CsGLys is similar to its homologs in fish species such as Japanese flounder, mandarin fish and yellow croaker [8,12,14], consisting of five exons and four introns, especially exon 3 and exon 4 were exactly conserved that each exon encoded the same number of amino acid in different fish (Fig. 3). The high conservation of gene structure among fish g-type lysozymes suggests that they might have same functions.

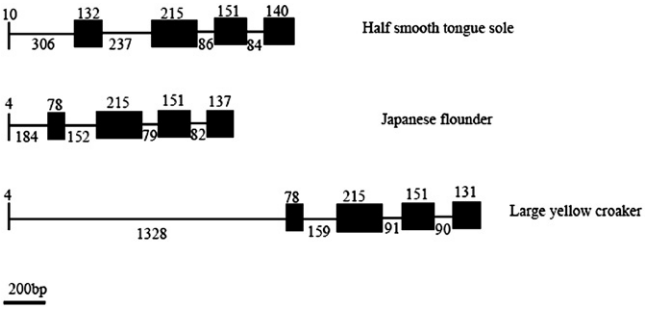

Fig. 3. Schematic representation of g-type lysozyme gene structure and organization from half-smooth tongue sole, Japanese flounder and large yellow croaker. Exons are represented by solid boxes and exon sizes in base pairs are shown on the top of the boxes. Introns are represented by a line and the size in base pairs is shown under the line.

So far, the information of transcriptional regulation of g-type lysozyme gene is still limited. In the present study, some transcript binding sites such as NF-κB, USF, AML-1a, AP-1 and OCT-1 binding sites were found in the 5′ flank region of *CsGLys* gene. NF-κB is a major transcription factor that regulates genes responsible for both the innate and adaptive immune response. NF-κB transcription binding sites were also found in the 5′-flanking region of g-type lysozyme genes in Japanese flounder, mandarin fish and yellow large croaker [8,12,14]. AP-1 is another important transcription factor to regulate inflammation reaction [29,30]. It dominantly participates in the regulation of inducible expression of granulysin, an antimicrobial protein, after stimulation with *Acholeplasma laidlawii* [30]. OCT-1, as a stress sensor [31], is widely expressed in adult tissues and has been proposed to regulate a large group of target genes. Since a functionally similar protein in different species may have similar regulatory mechanisms, NF-κB, AP-1 and OCT-1 are proved as immune-related transcriptional factors in previous study, and the putative transcriptional factor binding sites found in the half-smooth tongue sole may be involved in the immune response. The results reported here would benefit for a further understanding of the transcriptional regulation of *CsGLys* gene, and the function of *CsGLys* in innate immunity.

Phylogenetic tree based on the deduced amino acid sequences of lysozymes revealed the relationship of collected g-type lysozymes. The CsGLys had higher homology with other fish species than non-fish species and was closer with marine fish (Fig. 2). Placement of g-type lysozyme sequences into tight clades closely mirroring the evolutionary relationships of the fish species indicated rapid divergence of g-type lysozyme sequences following speciation. According to the traditional taxonomy, half-smooth tongue sole and *S. senegalensis* belong to the same family, and

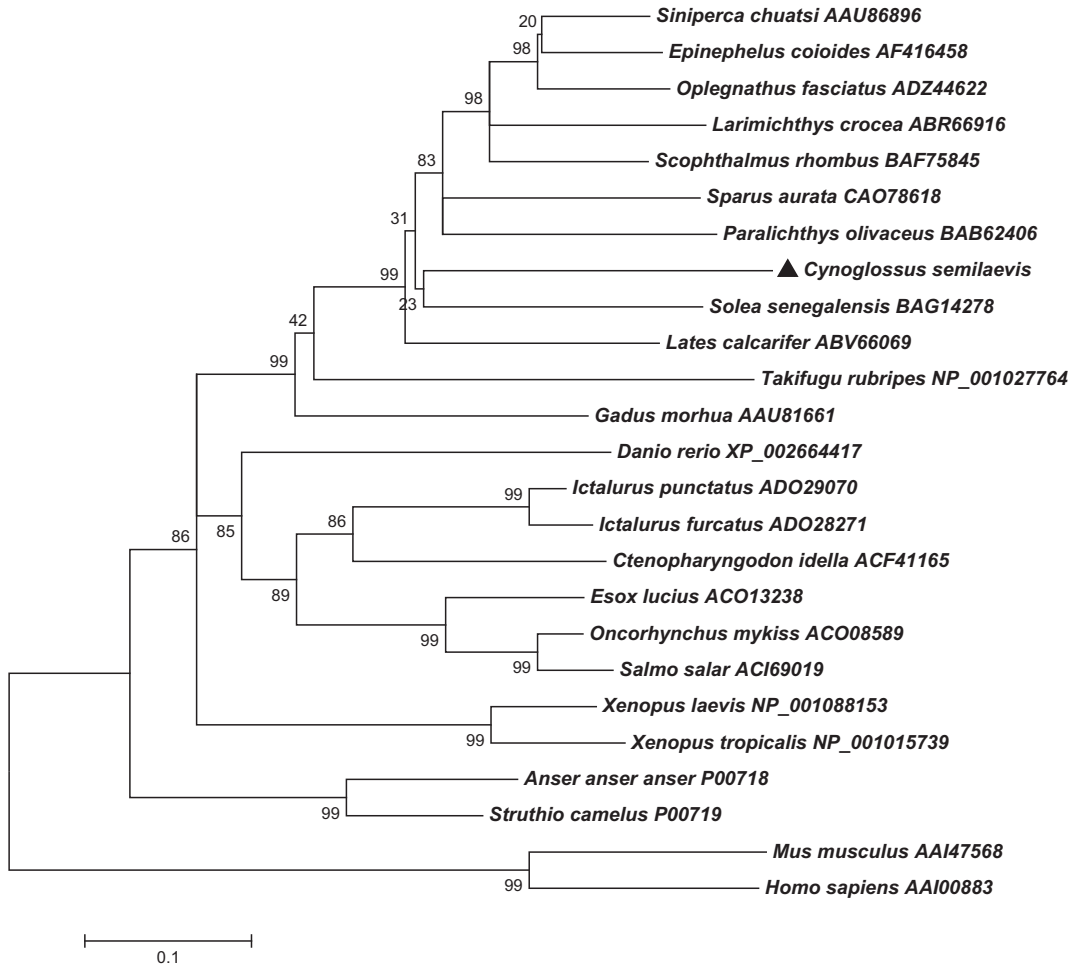

Fig. 4. Phylogenetic analysis of g-type lysozyme genes with related vertebrate lysozyme sequences. The phylogenetic tree was constructed based on the amino acid sequences of g-type lysozymes using the neighbor-joining method within MEGA 4 package. The accession number of the protein sequence is behind the name of species. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor-joining tree was evaluated by 10 000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. The scale bar indicates evolutionary distance in base substitutions per site. *CsGLys* gene is marked with black triangle.

g-type lysozyme gene of both fish cluster together means that they shared the higher similarity than other fish on the molecular level. It provided molecular evidence for the comparative anatomy result and was consistent with the traditional taxonomy.

The expression of g-type lysozyme gene showed different patterns in various species. The gene expression patterns were more restricted in birds and mammals than in fish. For instance, the g-type lysozyme gene was found to be expressed in lung and non-

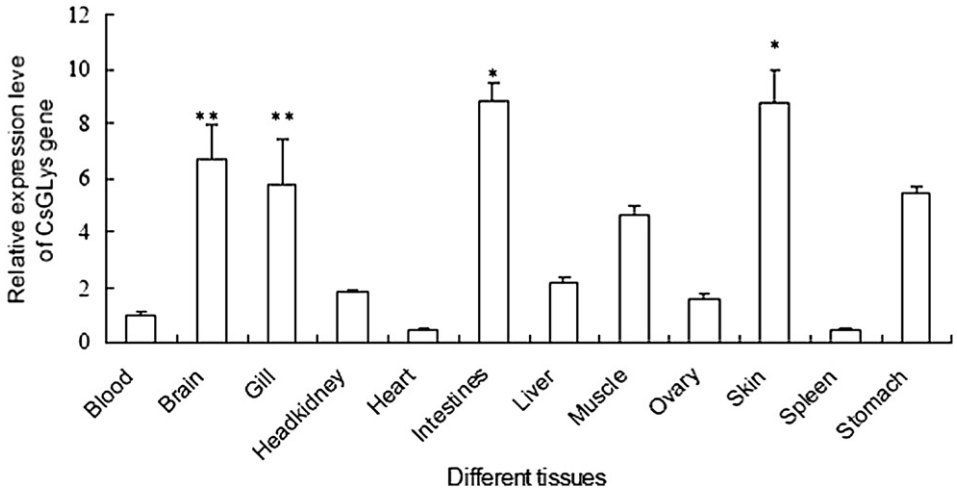

Fig. 5. *CsGLys* expression in various healthy tissues. *CsGLys* expression level in the brain, gill, heart, blood, liver, spleen, head kidney, intestine, muscle, skin and ovary was determined by real-time quantitative RT-PCR. The expression level of CsGLys in blood was set as 1. Vertical bars represent mean ± SE ($N = 3$). Significances between *CsGLys* expressions in blood and other tissues are indicated with asterisks. $^{**}p < 0.01$, $^{*}p < 0.05$.

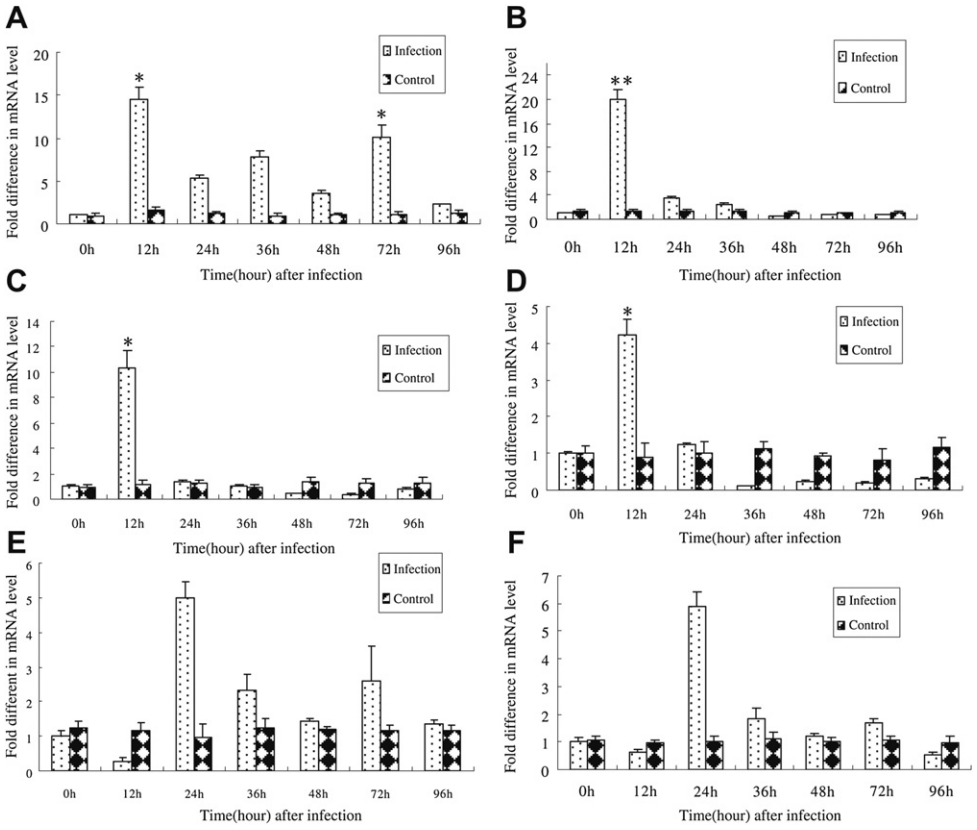

Fig. 6. Analysis of expression of *CsGLys* after bacteria *Vibrio anguillarum* infection. *CsGLys* expression was determined in liver (A), blood (B) spleen (C), head kidney (D), intestine (E) and skin (F) by real-time quantitative PCR method. The samples were analyzed at 0 h, 12 h, 24 h, 36 h, 48 h, 72 h and 96 h post-treatment. Expression of 18S rRNA was as an internal control for qRT-PCR. Each experiment was performed at least in triplicate. Data are shown as mean ±SE ($N = 3$). At each of the examined time points, the lysozyme mRNA level of PBS-challenged fish was set as 1. Significances between PBS- and bacterium challenged fish are indicated with asterisks. $^{**}p < 0.01$, $^{*}p < 0.05$.

adherent bone marrow cells in chicken [32] and was also expressed at low levels in the adult kidney of mammals [27]. In contrast, g-type lysozyme gene was found in most tissues and organs with a broad expression spectrum at different expression levels in fish. In the present study, CsGLys transcript was observed in all 12 kinds of tested tissues such as blood, brain, head kidney, heart, gill, intestine, liver, muscle, ovary, skin, stomach and spleen in half-smooth tongue sole (Fig. 5), indicating that the g-type lysozyme gene in fish may have a similar expression pattern. The wide distribution in fish suggested that g-type lysozyme was an intracellular protein, which was consistent with the absence of signal peptide in the protein molecules [17]. However, the most predominant expression was found in skin, and the weakest expression of them was found in the heart and head kidney, which expression level in different tissues showed a minor difference with the g-type lysozyme from Japanese flounder [8], mandarin fish [12] and yellow croaker [17], which may be due to the different fish species or the different cultured environment. The changing expression of g-type lysozyme was found in fish infected with bacteria or treated with lipopolysaccharide (LPS) in former studies. The g-type lysozyme expression was increased in intestine, heart and blood of Japanese flounder after injection with *Edwardsiella tarda* [8]. In orange-spotted grouper injected with *V. alginolyticus*, g-type lysozyme transcripts increased in the stomach, spleen, anterior kidney, posterior kidney, heart, brain, and leucocytes at 72 h after injection [10]. Large yellow croaker g-type lysozyme mRNA levels in intestine, spleen and head kidney were up-regulated 10.32-, 10.2- and 8.26-fold, respectively [13]. Brill g-lysozyme mRNA level was significantly increased in head kidney after LPS inoculation or infection with *Photobacterium damselae* [15]. Expression level of g-type lysozymes was up-regulated in grass carp challenged with *Aeromonas hydrophila* and the strongest up-regulation was detected in both spleen and liver [17]. In the present study, the variation of *CsGLys* gene in various tissues was detected at different time points after *V. anguillarum* induction by qRT-PCR. The results revealed that *CsGLys* mRNA level in blood, liver, spleen and head kidney was significantly up-regulated 19.89-, 14.45-, 10.37- and 4.21-fold and reached the peak value at 12 h post-infection, and 4.97- and 5.41-fold increased in intestine and skin at 24 h post challenge, respectively. *CsGLys* gene then decreased gradually to the normal or lower level in blood, head kidney, intestine, skin and spleen, while in liver, *CsGLys* gene expression level reached another peak value by 10.16-fold at 72 post-infection. These data indicate that *CsGLys* mRNA levels increase after the bacterial challenge, although there are species-specific expression profiles. Our results may suggest that the *CsGLys* gene plays an important role in immunity response, and probably cooperates as an acute protein due to the quick variation

AAGCAGTGGTATCAACGCAGAGTACGCGGGGTCCTGCAGAGGGAGATAAGAATTCGA
AGACATATCTCTTGAGTGAGCTCTGAGGCTGTGGACAGTAAAGGCTTCACTGAACTAC
GGTGACAATGGGAGGACTTTTTTCATCTTCTGCTTCCACGACTTCCTCGACTTCCTCCT
CCTCTTCAAATTCCTCTGGAAACATAAACAGGATACCTACTAGTGGAGCTTCCTGGAAG
ACTGCTCAACAGGACAAACTGGATTATAAGGGTGCAAAAGCTTCACAGACTCTGGCTG
AGACCGACAGTGATAGGATGAAGAAGTACAGAGACAAAATCATCAGAGTTGCCAATG
AGACCGGAATCCAACCGTGTCTTATTGCTGCCATCATCTCCAATCGAATTCCTGCAGCC
CGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGTGAGCACAATTCC

Fig. 7. 5′-RACE result for confirming the transcription start site. Box indicates the sequence of BD SMART II™ A Oligonucleotide, the 5′-RACE gene specific primer is marked with underline, the first nucleotide is shown with the red bold letter. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of *CsGLys* gene expression level in blood, head kidney, intestine, liver, skin and spleen during the short time after pathogen challenge. Without bacterial induction, the expression of *CsGLys* gene was lower in liver, spleen and head kidney than in intestine, gill and skin, which may illuminate that *CsGLys* gene had contribution to make the first barrier line against external pathogen and intestine, gill and skin mostly plays important roles in innate immunity.

In conclusion, our results support the hypothesis that *CsGLys* gene is an effector of the innate immune system and is activated during bacterial encounter. The results reported here would be helpful for a further understanding of the transcriptional profiles and regulation of g-lysozyme gene during different development stage with different sex, in response to different bacteria, and the function of g-lysozyme in innate immunity of fish.

Acknowledgments

The work was supported by grants from the National Major Basic Research Program (Grant No. 2010CB126303), the Qingdao Scientific Foundation (10-3-4-11-3-jch), and the Taishan Scholar Project of Shandong Province.

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