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Molecular cloning, expression of orange-spotted grouper goose-type lysozyme cDNA, and lytic activity of its recombinant protein

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ABSTRACT: Lysozyme acts as a non-specific innate-immunity molecule against the invasion of bacteria pathogens. A leukocyte cDNA library of orange-spotted grouper *Epinephelus coioides* was constructed and the goose-type (g-type) lysozyme cDNA was isolated. The complete cDNA consists of an open reading frame of 585 bp encoding a protein of 194 amino acids. This protein shows a 72.2% amino acid sequence identity with the flounder g-type lysozyme. Similar to most other species, the glu catalytic residue in g-type lysozymes of the grouper is conserved. Furthermore, like the flounder and carp, the 4 conserved cysteine residues identified in avian and mammalian g-type lysozymes were also absent from the grouper. Northern blot analysis indicated that the g-type lysozyme was expressed in intestine, liver, spleen, anterior kidney, posterior kidney, heart, gill, muscle and leukocytes. In addition, RT-PCR analysis detected the g-type lysozyme transcripts in the stomach, brain and ovary. When an orange-spotted grouper was injected with *Vibrio alginolyticus*, the number of lysozyme mRNA transcripts detected in the stomach, spleen, anterior kidney, posterior kidney, heart, brain and leucocytes increased 72 h after injection. Recombinant grouper g-type lysozyme produced in the *Escherichia coli* expression system showed lytic activity against *Micrococcus lysodeikticus*, *V. alginolyticus* from *Epinephelus fario*, *V. vulnificus* from culture water, *Aeromonas hydrophila* from soft-shell turtle, *A. hydrophila* from goldfish and *V. parahaemolyticus*, *Pseudomonas fluorescens* and *V. fluvialis* from culture water.

KEY WORDS: Goose-type lysozyme · Orange-spotted grouper · Lytic activity · cDNA library

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INTRODUCTION

Lysozyme (muramidase, EC.3.2.1.17) catalyzes the hydrolysis of the cell walls of most bacteria and results in a non-specific innate-immunity response against the invasion of bacterial pathogens (Jollès & Jollès 1984). In vertebrates, there are 2 types of lysozyme: chicken-type (c-type) and goose-type (g-type) (Jollès & Jollès 1984). c-type lysozymes have been isolated from many vertebrates and insects (Prager & Jollès 1996, Qasba & Kumar 1997). In fishes, complete coding sequences of the c-type lysozymes of Japanese flounder (Hikima et al. 1997) (GenBank #AB050469), turbot (GenBank #AJ250732), rainbow trout (Dautigny et al. 1991) (GenBank #X59491), zebra fish (GenBank #AF402599) and common carp (GenBank #AB027305) have been reported. G-type lysozymes have also been characterized in geese, chickens, black swans and ostriches (Canfield & McMurry 1967, Simpson et al. 1980, Schoentgen et al. 1982, Nakano & Graf 1991), and g-type lysozyme full-length cDNA sequences have been reported from chickens (Nakano & Graf 1991), flounder (Hikima et al. 2001), carp (GenBank #AB084624), mice (GenBank #AK009014) and humans (GenBank #BC029126). Despite this wealth of sequence information for g-type lysozymes, expression studies have only been reported in chickens and flounder (Nakano & Graf 1991, Hikima et al. 2001).

In fishes, lysozymes have a lytic effect on pathogenic bacteria. A g-type lysozyme from Japanese flounder showed lytic activity against *Vibrio anguillarum* (Hikima et al. 2001), and Lysozyme II from rainbow

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trout can kill *V. anguillarum* and *V. salmonicida* in Atlantic salmon, *Aeromonas* spp., *A. salmonicida* ssp. *achromogenes*, *Yersinia ruckeri* and *Flavobacterium* sp. in many fish species (Grinde 1989). These and other pathogenic bacteria could cause diseases in cultured fishes (Kusuda & Salati 1993, Liao et al. 1996). The orange-spotted grouper *Epinephelus coioides* is an important cultured marine fish in southern China. Recently, bacteria that caused ulceration and high mortalities (50 to 70%) in *E. coioides* cultures were reported in China (Liu et al. 1994, Zhu et al. 2000).

Expressed sequence tags (ESTs) are derived by partial, single-pass sequencing of the inserts from randomly selected cDNA clones. This approach has been used to identify many novel genes (Adams et al. 1991, 1993). In this study, we constructed a cDNA library from leukocytes of orange-spotted grouper for EST study and isolated a g-type lysozyme cDNA. Using Northern blot and RT-PCR analyses, we examined the expression of g-type lysozyme in different tissues. To study the function of the g-type lysozyme, we produced recombinant protein in bacteria and examined its lytic activity against *Micrococcus lysodeikiticus* and 7 strains of fish disease pathogens.

MATERIALS AND METHODS

Construction of leukocyte cDNA library. An orange-spotted grouper (602 g) was injected intramuscularly with Poly I:C (Amersham Pharmacia) at 1 mg kg^{-1} body weight and peripheral blood was collected from the caudal artery 72 h after injection. Leukocytes were isolated from the blood sample using Ficoll-paque plus (density 1.077 g ml^{-1}) (Amersham Pharmacia). Blood cell suspensions were layered over Ficoll-paque plus, and centrifuged at 400 $\times g$ for 30 min at room temperature. Cells recovered from the medium-Ficoll interface were transferred to clean tubes, centrifuged at 200 $\times g$ for 15 min at room temperature and washed twice with 0.9% sodium chloride.

Total RNA was isolated from leukocytes with Tripure reagents (Roche Diagnosis). The cDNA library was constructed using a SMART cDNA library construction kit (Clontech). Recombinant phages were packaged with Max Plax lambda packaging extract (Epicentre) and amplified in *Escherichia coli* XL1-Blue cells.

Molecular cloning of orange-spotted grouper g-type lysozyme cDNA. Individual phage plaques were isolated from plates and diluted in 0.5 ml dilution buffer (0.1 M NaCl, 0.01 M $MgSO_4$, 0.035 M Tris-HCl pH 7.5, 0.01% gelatin). The phage solution was used as a template for PCR amplification. TriplEx 5′ and 3′ long distance-insert screening amplimers (5′: CTCGGGAAGCGCGCCATTGTGTTGGT, 3′: ATACGACTCATATAGGGCGAATTGGCC) were used as primers. After 10 min at 94°C for phage lysis, PCR was performed for 35 cycles at a denaturing temperature of 94°C for 30 s, and at an annealing and extending temperature of 68°C for 3 min. PCR products were analyzed on 1% agarose gel. DNA > 500 bp were purified with a DNA gel extraction kit (Sangon) and sequenced using an ABI PRISM Big Dye terminator kit on an ABI PRISM 377 sequencer. The sequences of inserts were compared with GenBank Database using the BLASTX program at www.ncbi.nlm.nih.gov/BLAST/. The clone with the sequence most similar to the Japanese flounder g-type lysozyme was isolated.

Expression analysis by Northern blot and RT-PCR. For Northern blot analysis, 2 orange-spotted groupers were used: 1 fish (600 g) was injected intramuscularly with 2×10^5 cells of *Vibrio alginolyticus* and sacrificed for total RNA extraction (Tripure Reagents) after 72 h. The other healthy fish (612 g) was also sacrificed for RNA extraction. For each fish, duplicate RNA samples (25 µg) from different tissues were separated on 2 agarose gels (1%) and transferred on 2 nitrocellulose membranes; 1 membrane was used for hybridization with a grouper g-type lysozyme probe ([α-^{32}P] deoxycytidine triphosphate) using a random primer DNA labeling kit (Takara). The other membrane was used for hybridization with a grouper β-actin probe. Hybridization was carried out in a hybridization buffer (1% BSA, 7% sodium dodecyl sulfate [SDS]; 0.5 M phosphate-buffered saline, pH 6.8; 1 mM EDTA, pH 8.0) at 68°C overnight. After hybridization, the membrane was washed in Buffer I (0.5% BSA; 5% SDS; 40 mM phosphate buffer, pH 6.8; 1 mM EDTA) at 68°C for 15 min and then in Buffer II (1% BSA, 40 mM phosphate buffer, pH 6.8; 1 mM EDTA) at 68°C for 15 min. The membranes were wrapped in plastic bags and exposed to X-ray film for 72 h with an intensifying screen at –80°C.

For RT-PCR analysis reverse transcription of RNA from different tissues of a healthy fish (625 g) was performed in a final concentration of 1 × transcription buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiothreitol), 2.5 µM random primers; and 1 Unit of Moloney murine leukemia virus reverse transcriptase (Promega). The reaction mixture (20 µl) was incubated at 37°C for 1 h. For PCR, a pair of primers containing the *Bam*HI and *Eco*RI restriction sites (Primer I: 5′-GCGGATCCATGGGTTATGGAAACAT-3′; Primer II: 5′-CGGAATTCTTAGAAG CCTT-TCTGGG-3′) were designed from the open reading frame of grouper g-type lysozyme cDNA. The final PCR mixture (50 µl per reaction) consisted of 10 mM Tris-HCl pH 8.5, 1.5 mM $MgCl_2$, 50 mM KCl, 10 pmol of each primer, and an aliquot (1 µl) of the reverse transcription product. The touch-down PCR included 30 cycles of 95°C for 30 s, annealing at gradient temperatures of 60 to 45.5°C (each cycle 0.5°C

lower than the former) for 30 s and extension at 72°C for 1 min. An additional 20 cycles were performed: each at 94°C for 30 s, at 45°C for 30 s and an extension at 72°C for 1 min. At the end of the last cycle, the PCR mixture was incubated at 72°C for 10 min for the completion of DNA synthesis.

Construction of recombinant protein. The PCR-amplified g-type lysozyme gene fragment was digested with *Bam*HI and *Eco*RI, ligated into the pRSET A expression vector (Invitrogen) linearized with the same enzymes and transformed to XL1-Blue cells. After sequencing to ensure inframe insertion, the DNA construct was transformed to the host BL21 (DE3) pLysS strain for protein expression. The fusion protein was expressed by isopropyl-beta-D-thiogalactopyranoside (IPTG) induction, and SDS polyacrylamide gel (SDS-PAGE) electrophoresis was performed. To detect expression of recombinant fusion protein, Western blot was carried out using Ni-NTA AP conjugate (alkaline-phosphatase conjugated nickel-nitrilotriacetic acid) (Qiagen). Recombinant bacteria (1.5 ml) was collected, the pellet was resuspended in 500 µl of phosphate buffer at neutral pH, lysed in 3 consecutive freeze (–80°C) and thaw (room temperature) cycles, and lysed by sonication on ice. The lysate was centrifuged at 10 000 × *g* for 10 min at 4°C. The supernatant was freeze-dried and resuspended in 50 µl of water, and used for lytic activity assays.

Lytic activity assays. The lytic activity assay used *Micrococcus lysodeikticus* as a substrate according to the method of Hikima et al. (2001). The *M. lysodeikticus* in 1% warm (50°C) melting agarose (50 mM phosphate buffer, pH 6.2) was poured onto 80 mm plates. The concentration of *M. lysodeikticus* was adjusted to 0.2 absorbance units at 600 nm (about 10 mg *M. lysodeikticus* to 100 ml 1% agarose). The recombinant samples (10 µl), the negative control samples and 250 µg of hen egg-white lysozyme (Sigma) were put in individual wells in the agarose plates and incubated at 30°C for 24 h. The diameters of the lysed circular zone were measured. Sonicated protein extracts from bacteria containing empty vectors were used for the controls.

To determine the lytic activity of the recombinant lysozyme against known fish disease pathogens, different bacteria (*Vibrio alginolyticus* from *Epinephelus fario*, *V. vulnificus* from the water in which the fish were cultured [culture water], *Aeromonas hydrophila* from soft-shell turtle, *A. hydrophila* from goldfish, *V. parahaemolyticus*, *Pseudomonas fluorescens* and *V. fluvialis* from culture water) were cultured overnight in Luria-Bertani medium and used as substrates in the lysoplate assay. For comparison, *E. coli* DH5α and *Micrococcus lysodeikticus* were used as substrates in the assay.

RESULTS

Nucleotide sequence of orange-spotted grouper g-type lysozyme cDNA

The g-type lysozyme cDNA isolated from orange-spotted grouper was 788 bp in length. The longest open reading frame consisted of 585 bp and encoded a protein of 194 amino acid residues (Fig. 1). The cDNA consisted of a start codon (ATG), a stop codon (TAA) and a putative polyadenylation signal site (AATAAA) at 10 bp upstream from the poly (A) tail. The calculated molecular mass of the g-type lysozyme was 21.18 kDa, with a PI (isoelectric point) of 5.93 using the Compute pI/MW program at www.us.expasy.org/tools/pi_tool.html. However, no signal peptide was found using the SignalP program at www.cbs.dtu.dk/services/SignalP-2.0 (Nielsen et al. 1997).

Comparison of g-type lysozyme of orange-spotted grouper with those of other species

Comparison of the deduced amino acid sequence of grouper g-type lysozyme with other known g-type lysozymes showed that the highest sequence identity was with that of Japanese flounder (72.2% overall: Fig. 2). The next highest degree of sequence identity was with carp (50.3%: Table 1). The Glu71 catalytic residue in the grouper g-type lysozyme sequence was conserved as in other species listed in Table 1 except for the human sequence.

Expression of g-type lysozyme in different tissues

Northern blot analysis indicated that the g-type lysozyme was expressed in the intestine, liver, spleen, anterior kidney, posterior kidney, heart, gill, muscle and leukocytes, but not in the stomach, brain and ovary (Fig. 3). Using RT-PCR, the g-type lysozyme mRNA transcripts were detected in all tissues examined (Fig. 3). The additional detection of g-type lysozyme expression in stomach, brain and ovary using the more sensitive RT-PCR indicated low expression levels in these tissues. Furthermore, When an orange-spotted grouper was injected with *Vibrio alginolyticus*, the number of lysozyme mRNA transcripts detected in the stomach, spleen, anterior kidney, posterior kidney, heart, brain and leukocytes increased 72 h after injection (Fig. 3).

SDS-PAGE and Western blot

A 24.4 kDa protein was detected in the recombinant bacteria but not in the negative control (Fig. 4). West-

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  1  GGC ACC AGA TGA GAC AGA GGG ACA CTG CGT GAC TGT TCT CGT ATT CCG   48
 49  CTT TCA AGG AGA CCT GTA GAA GAG GAA ACG ATT AAA ATG GGT TAT GGA   96
  1                                                   M   G   Y   G     4
 97  AAC ATC ATG AAT GTT GAA ACT ACT GGT GCA TCA TGG CAA ACG GCT CAG  144
  5   N   I   M   N   V   E   T   T   G   A   S   W   Q   T   A   Q    20
145  CAG GAC AAG CTG GGA TAC TCA GGT GTG AGG GCA TCA CAC ACC ATG GCA  192
 21   Q   D   K   L   G   Y   S   G   V   R   A   S   H   T   M   A    36
193  AAC ACT GAC TCA GGC AGA ATG GAG AGG TAC AGG TCT AAA ATC AAC TCC  240
 37   N   T   D   S   G   R   M   E   R   Y   R   S   K   I   N   S    52
241  GTG GGA GCA AAA TAC GGA ATC GAT CCA GCT CTG ATT GCC GCC ATC ATC  288
 53   V   G   A   K   Y   G   I   D   P   A   L   I   A   A   I   I    68
289  TCC GAA GAG TCC AGG GCT GGA AAT GTA TTA CAT GAT GGC TGG GGA GAC  336
 69   S   E   E   S   R   A   G   N   V   L   H   D   G   W   G   D    84
337  TAT GAC TCA AAC AGA GGA GCG TAC AAC GCC TGG GGA CTG ATG CAG GTT  384
 85   Y   D   S   N   R   G   A   Y   N   A   W   G   L   M   Q   V   100
385  GAT GTT AAT CCA AAT GGA GGT GGA CAC ACT GCG CGG GGT GCA TGG GAC  432
101   D   V   N   P   N   G   G   G   H   T   A   R   G   A   W   D   116
433  AGT GAG GAA CAC CTC TCT CAA GGC GCA GAG ATC TTG GTT TAT TTT ATT  480
117   S   E   E   H   L   S   Q   G   A   E   I   L   V   Y   F   I   132
481  GGA CGC ATC CGC AAC AAG TTT CCT GGC TGG AAC ACG GAG CAG CAG CTG  528
133   G   R   I   R   N   K   F   P   G   W   N   T   E   Q   Q   L   148
529  AAA GGA GGA ATA GCA GCC TAC AAT ATG GGG GAT GGG AAC GTC CAC TCT  576
149   K   G   G   I   A   A   Y   N   M   G   D   G   N   V   H   S   164
577  TAT GAT AAT GTG GAT GGC AGA ACA ACA GGT GGA GAC TAC TCC AAT GAT  624
165   Y   D   N   V   D   G   R   T   T   G   G   D   Y   S   N   D   180
625  GTT GTT GCC AGA GCT CAG TGG TAC AAA ACC CAG AAA GGC TTC TAA AAC  672
181   V   V   A   R   A   Q   W   Y   K   T   Q   K   G   F   *       195
673  CTG AAG CTG TGT TCA CAT CAG AAA TCA CTG TAA CTG TGC AAT GTG TGC  720
721  TCT GTG TCA AAA CCT TTC CTA AAT AAA CAT AAT GCC CAA AAA AAA AAA  768
769  AAA AAA AAA AAA AAA AAA AA                                       788
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Fig. 1. *Epinephelus coioides.* Nucleotide sequence of orange-spotted grouper goose-type (g-type) lysozyme. Amino acid sequences represented by single capital letter below the respective nucleotide sequence; polyadenylation signal underlined

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grouper.PRO     MG---YGNIMNVETTGASWQTAQQDKLGYSGVRASHTMANTDSGRMERYRSKINSVGAKYGIDPALI   64
flounder.PRO    MS---YGQIRLVETSGASGATSQQDNLGYSGVKASHKMAEIDSGRMSKYKSKINKVGQSYGIEPALI   64
carp.PRO        MA-YIYGDTMKIDTTGASEATAKQDKLTIKGVEAPKKLAEHDLARGEKYKNMITKVGKAKKMDPAVI   66
blackswan.PRO   RT-DCYGNVNRIDTTGASCKTAKPEGLSYCGVPASKTIAERDLKAMDRYKTIIKKVGEKLCVEPAVI   66
ostrich.PRO     RT-GCYGDVNRVDTTGASCKSAKPEKLNYCGVAASRKIAERDLQSMDRYKALIKKVGQKLCVDPAVI   66
goose.PRO       RT-DCYGNVNRIDTTGASCKTAKPEGLSYCGVSASKKIAERDLQAMDRYKTIIKKVGEKLCVEPAVI   66
chicken.pro     GT-GCYGSVSRIDTTGASCRTAKPEGLSYCGVRASRTIAERDLGSMNKYKVLIKRVGEALCIEPAVI   66
mouse.PRO       SSWGCYGNIRTLDTPGASCRIGRRYGLTYCGVRASERLAEVDRPYLLRHQPTMRLVGQKYCMDPAVI   67
human.PRO       SNWGCYGNIQSLDTPGASCGIGRRHGLNYCGVRASERLAEIDMPYLLKYQPMMQTIGQKYCMDPAVI   67

                    *           *          *                        *
grouper.PRO     AAIISEESRAGNVLHDGWGDYDSNRGAYNAWGLMQVDVNPNGGGHTARGAWDSEEHLSQGAEILVYF  131
flounder.PRO    AAIISRESRAGNQLKDGWGDWNPQRQAYNAWGLMQVDVNPNGGGHTAVGGWDSEDHLRQATGILVTF  131
carp.PRO        AAMISRESRAGAVLKNGWEPAG------NGFGLMQVDKRS----HTPVGAWDSEQHVTQATEILIGF  123
blackswan.PRO   AGIISRESHAGKVLKNGWGDRG------NGFGLMQVDKRS----HKPQGTWNGEVHITQGTTILTDF  123
ostrich.PRO     AGIISRESHAGKALRNGWGDNG------NGFGLMQVDRRS----HKPVGEWNGERHLMQGTEILISM  123
goose.PRO       AGIISRESHAGKVLKNGWGDRG------NGFGLMQVDKRS----HKPQGTWNGEVHITQGTTILINF  123
chicken.pro     AGIISRESHAGKILKNGWGDRG------NGFGLMQVDKRY----HKIEGTWNGEAHIRQGTRILIDM  123
mouse.PRO       AGVLSRESPGGNYVVDL-GNIGS------GLGMVK------ETKFYPPTAWKSETWVSQKTQTLTSS  121
human.PRO       AGVLSRKSPGDKILVNM-GDRTS----------MVQ------DPGSQAPTSWISESQVSQTTEVLTTR  118
                     #           $
grouper.PRO     IGRIRNKFPGWNTEQQLKGGIAAYNMGDGNVHSYDNVDGRTTGGDYSNDVVARAQWYKTQKGF    194
flounder.PRO    IERIRTKFPGWSKEKQLKGGIAAYNMGDKNVHSYEGVDENTTGRDYSNDVTARAQWYR-DNGYSG  195
carp.PRO        IKEIKVNFPKWTQEQCFKGGIAAYNKGVSRVTSYENIDVKTTGLDYSSDVVARAQWFR-SKGY    185
blackswan.PRO   IKRIQKKFPSWTKDQQLKGGISAYNAGAGNVRSYARMDIGTTHDDYANDVVARAQYYK-QHGY    185
ostrich.PRO     IKAIQKKFPRWTKEQQLKGGISAYNAGPGNVRSYERMDIGTTHDDYANDVVARAQYYK-QHGY    185
goose.PRO       IKTIQKKFPSWTKDQQLKGGISAYNAGAGNVRSYARMDIGTTHDDYANDVVARAQYYK-QHGY    185
chicken.pro     VKKIQRKFPRWTRDQQLKGGISAYNAGVGNVRSYERMDIGTLHDDYSNDVVARAQYFK-QHGY    185
mouse.PRO       IKEIKTRFPTWTADQHLRGGLCAYSKGPNFVRSNQDLNC-----DFCNDVLARAKYFK-DHGF    178
human.PRO       IKEIQRRFPTWTPDQYLRGGLCAYSGGAGYVRSSQDLSC-----DFCNDVLARAKYLK-RHGF    175
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Fig. 2. *Epinephelus coioides.* Comparison of amino acid sequence of grouper g-type lysozyme with those of Japanese flounder, common carp, black swan, ostrich, goose, chicken, mouse and human. Under alignment of sequences, : cysteine residues of mouse, human, black swan, ostrich, goose and chicken sequences; #: catalytic glu residue; and $: conserved asp residue in grouper, flounder, human, black swan, ostrich, goose and chicken sequences

Table 1. Identities of mature g-type lysozyme amino acid sequences in grouper *Epinephelus coioides* and other species

Species	Grouper	Flounder	Carp	Black swan	Ostrich	Goose	Chicken	Mouse	Source/GenBank Ref. No.
Flounder	72.2								Hikima et al. (2001)
Carp	50.3	51.9							#AB084624
Black swan	49.7	48.6	55.7						Simpson et al. (1980)
Ostrich	49.7	49.7	56.2	82.2					Schoentgen et al. (1982)
Goose	48.6	48.6	56.8	96.8	83.8				Canfield & McMurry (1967)
Chicken	47.0	48.1	54.6	81.1	77.8	80.5			Nakano & Graf (1991)
Mouse	31.5	31.5	35.4	40.4	41.0	39.9	39.9		#AK009014
Human	31.4	30.9	33.1	40.6	40.6	40.0	41.1	68.6	#BC029126

ern blot analysis indicated that the 24.4 kDa protein contained the 6× His tag sequence.

Lysozyme activity

The recombinant protein clearly caused lysis of *Micrococcus lysodeikticus* at pH 6.2 and 30°C (Fig. 5). In order to determine the lytic activity of the grouper g-type lysozyme against fish disease pathogens, 7 bacteria strains (*Vibrio alginolyticus* from *Ephinephelus fario*, *V. vulnificus* from culture water, *Aeromonas hydrophila* from soft-shell turtle, *A. hydrophila* from goldfish and *V. parahaemolyticus*, *Pseudomonas fluorescens* and *V. fluvialis* from culture water) and *Escherichia coli* DH5α were used as substrate. All the examined fish disease pathogens were lysed by the recombinant grouper g-type lysozyme (Fig. 6). However,

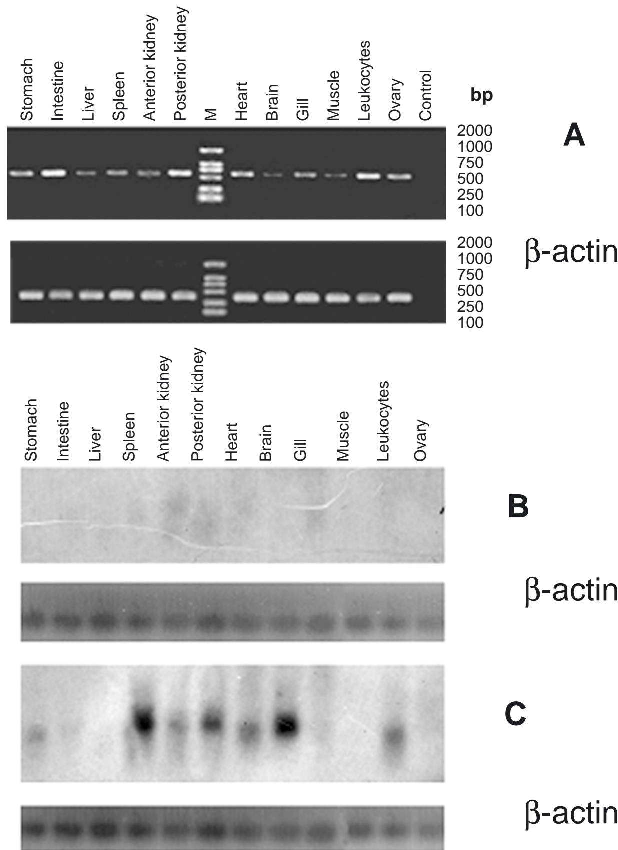

Fig. 3. Expression of grouper g-type lysozyme in different tissues. (A) RT-PCR detection of lysozme in different tissues; (B) Northern blot analysis of lysozyme in healthy fish; (C) Northern blot analysis of lysozyme in fish infected with 2×10^5 cells of *Vibrio alginolyticus.* M: DL-2000 DNA ladder from Takara

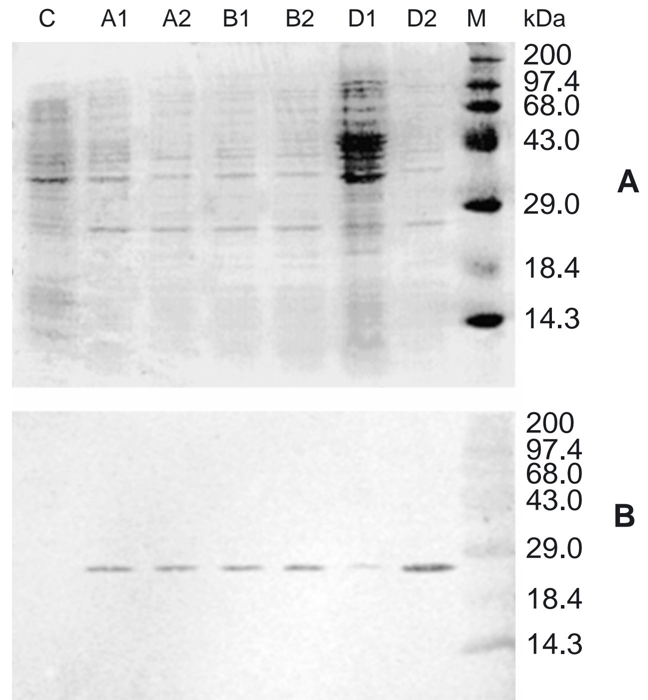

Fig. 4. *Epinephelus coioides.* Analysis of recombinant g-type lysozymes. (A) SDS-PAGE; (B) Western Blot. A1, B1, D1: total proteins of recombinant bacteria without isopropyl-beta-D-thiogalactopyranoside (IPTG) induction; A2, B2, D2: total proteins of recombinant bacteria with IPTG induction; C: total proteins of bacteria with empty plasmid pRSET A (Invitrogen) without IPTG induction. M: pre-stained protein molecular weight standards from Invitrogen

Fig. 5. *Epinephelus coioides.* Lytic activity of recombinant grouper g-type and hen egg-white c-type lysozyme against *Micrococcus lysodeikticus.* Small central circles are to same scale and represent the wells containing the samples; larger circles represent lysed halos formed by the lysozyme on *M. lysodeikticus* substrate. control: supernatant after sonication of the bacteria with empty pRSET A vector; G-lzm: supernatant after sonication of recombinant bacteria; HEW: hen egg-white lysozyme

the recombinant protein did not show a lytic effect on *E. coli* DH5α.

DISCUSSION

All the known avian g-type lysozymes contain 4 cysteine residues located in the N-terminal half of the chain (Nakano & Graf 1991). The recently reported putative g-type lysozymes of humans and mice contain the cysteine residues at the same location (GenBank #BC029126, AK009014). Despite the fact that deduced g-type lysozymes of grouper and flounder lack these cysteine residues, recombinant g-type lysozyme from these fishes caused lysis of *Micrococcus lysodeikticus* (Hikima et al. 2001). This supports the hypothesis that disulfide bonding resulting from the cysteine residues is not necessary for lysozyme activity (Hikima et al. 2001). Additionally, the newly published g-type lysozyme from common carp also lacks cysteine residues (GenBank #AB027305).

The chicken g-type lysozyme contains a signal peptide of 26 amino acid residues (Nakano & Graf 1991). Using the SignalP program, a signal peptide of 19 amino acid residues can be identified in the human (GenBank #BC029126) and mouse (GenBank #AK0-09014) g-type lysozyme. However, the signal peptide sequence could not be detected from grouper g-type lysozyme. Using the carp g-type lysozyme sequence (GenBank #AB027305), SignalP program was also unable to detect any signal peptide sequence. Hikima et al. (2001) obtained a similar result for flounder. This is a very interesting find, and further research is necessary to investigate this similarity between g-type lysozymes in grouper, flounder and carp.

It has been suggested that Glu35 and Asp52 are important residues for lytic activity in c-type lysozymes, whereas Glu73 and Asp86 are the corresponding catalytic residues of avian g-type lysozymes (Prager & Jollès 1996). The results from a higher resolution crystallographic study (Weaver et al. 1995) supported the equivalence of the Glu residues. However, Weaver et al. (1995) found no Asp52 counterpart in g-type lysozyme and suggested that only 1 acidic residue is essential for the catalytic activity of g-type lysozyme and that Asp52 might not be required for catalysis by the c-type lysozyme. The latter suggestion was supported by work in which an engineered chicken c-type lysozyme with Asn52 had 5% of wild-type activity (Malcolm et al. 1989). The Glu71 and Asp84 of grouper and flounder g-type lysozymes were conserved with Glu73 and Asp86 of avian g-type lysozymes (Fig. 2). However, the mouse and carp g-type lysozymes contain no Asp84. If further studies are able to demonstrate that mouse and carp g-type lysozymes do cause lytic effects, this would support Weaver et al.'s suggestion.

The predicted molecular weight of the grouper g-type lysozyme (21.18 kDa) is similar to that of Japanese flounder (22 kDa) (Hikima et al. 2001). Expression of the g-type lysozyme together with the N-terminal tag appears to have resulted in an increase of approximately 3 kDa. As shown from SDS-PAGE and Western blot analyses, the recombinant protein has a molecular weight of 24.4 kDa, making its size close to the expected value.

In chickens, the g-type lysozyme is only expressed in the bone marrow and lung (Nakano & Graf 1991). However, the Japanese flounder g-type lysozyme is expressed in the anterior kidney, posterior kidney, spleen, liver, skin, muscle, heart, brain, intestine, ovary and

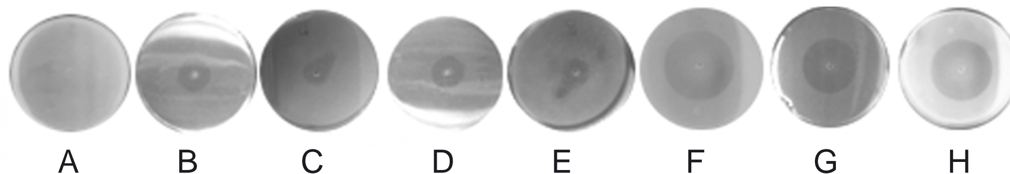

Fig. 6. *Epinephelus coioides.* Lytic activity of recombinant grouper g-type lysozyme against 7 strains of fish disease pathogen and *Escherichia coli* DH5α. Small central circles are to same scale and represent the wells containing the samples; larger circles represent the lysed halos formed by lysozyme on bacteria substrate. (A) *E. coli* DH5α; (B) *Vibrio alginolyticus* from *E. fario*; (C) *V. vulnificus* from culture water; (D) *Aeromonas hydrophila* from soft-shell turtle; (E) *A. hydrophila* from gold fish; (F) *V. parahaemolyticus* from culture water; (G) *Pseudomonas fluorescens* from culture water; (H) *V. fluvialis* from culture water

whole blood (Hikima et al. 2001). In orange-spotted grouper, RT-PCR analysis indicated that g-type lysozyme is also transcribed in these tissues, as well as in the stomach and gills. Thus, the pattern of expression of the lysozyme in orange-spotted grouper is similar to that in flounder but different from that in chickens.

Based on differences in cysteine content and expression profiles in different tissues, we suggest that fish g-type lysozymes may belong to a different subtype than that of the avian and mammalian g-type lysozymes.

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