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Analysis on the Molecular Biologic Characteristics and Expression of Lysozyme C Separated from *Oplegnathus fasciatus*

Dong-Hee Jo [a], Dae-Won Park[b], Cheul Min An [c], Bo-Hye Nam [c], Ji-Min Jeong [a], Ju-Won Kim [a], Chan-il Park [a, *]

[a]Department of Marine Biology & Aquaculture, College of Marine Science, Gyeongsang National University, 455, Tongyeong, 650-160, Republic of Korea
[b] Gyeongsangnam-do Fisheries Resources Research Institute, Tongyeong, Gyeongnam 650-947, Republic of Korea
[c]Biotechnology Research Division, National Fisheries Research and Development Institute, 216 Gijanghaean-ro, Gijang-eup, Gijang-gun, Busan, 619-705, Republic of Korea
Corresponding author. Tel.: +82-55-772-9153; Fax: +82-55-772-9159.
E-mail address: vinus96@hanmail.net (C.I. Park).

Abstract

Background and Aim: lysozyme has been known as a significant component of the innate immune system of fish. It is reported that the g-type and c-type lysozymes have been identified in fish. *Oplegnathus fasciatus* is one of the economically important cultured species, generating higher market value and demands in Korea. Contrary to the heavy consumption of *O. fasciatus,* however, there has been very limited information on their immune system and immunity genes responding against diseases.

Methods: This study clarified the molecular biological characteristics of cDNA of the C-type lysozyme from *O. fasicatus* and conducted phylogency analysis. Furthermore, tissue-specific expression analysis of genes verified the roles of c-type lysozyme in general circumstances.

Results: The full-length RbLysC cDNA was 985 bp long and contained an ORF of 432 bp that encoded 143 aa residues, the 5'-UTR of 120 bp and the 3'-UTR of 444 bp. Signal peptide was not found in the terminal and flanking active aspartate region are conserved in RbLysC, and a polyadenylation signal and poly A-tail are present in the 3'-UTR of RbLysC. RbLysC presented the closest distant relationship with sequences from Yellow perch lysozyme C.

The highest RbLysC gene expression was observed in the liver, which was about 50-fold relative to that of the PBLs.

Keywords: c-type lysozymes; *Oplegnathus fasciatus*; Korea; molecular biological characteristics

1. Introduction

The immune system protects organisms from external hazards in tandem with physical barriers like skin and is promptly activated upon the invasion by pathogens (Magnadottir et al., 2006). The innate immune system is of more importance to the lower vertebrates like fish than to the higher vertebrate (Globler et al., 1994). As one of the most representative parts of innate immune system, lysozyme facilitates the hydrolysis of N-acetyl glucosamine and N-actyl muramic acid in the peptidoglycan layer of bacterial cell walls and destroys the cell walls, thus arresting the spread of bacteria (Jollet et al., 1984). In gram-positive bacteria, lysozyme is immediately activated, destroying the top layer of peptidoglycan. In gram-negative bacteria, it creates a synergy with positive antibacterial peptide and decomposes external cell walls, thus exposing the peptidoglycan layer (Hancock et al., 2000).

In addition, lysozyme has been known as a significant component of the innate immune system of fish. It is reported that the g-type and c-type lysozymes have been identified in fish. A c-type lysozyme gene of fish was identified in Rainbow Trout (Oncorhynchus mykiss) first (Dautigny et al., 1991). Since then, a c-type lysozyme gene was found in 13 kinds of fish: *Danio rerio, Oryzias latipes, Cyprinus Carpio, Oreochromis aureus, Perca flavescens, Takigfugu rubripes, Scophthalmus rhombus, Scophthalmus maximus, Solea senegalensis, Paralichthys olivaceus, Salmo salar, and Oncorhyncus mykiss and Esox lucius*. It was registered in Genbank database (Dautigny et al., 1991, Hikima et al., 1997, Pernandez et al., 2008). A c-type lysozyme gene serves a variety of functions. It not only stimulates digestion, anti-bacterial effects, reproduction, and growth, but also influences tumors (Irwin et al., 2004, Irwin et al., 1992, Kanda et al., 2007, Yuen et al., 1998, Zhang et al., 2005).

A c-type lysozyme gene has easily been expressed in abalones and the invertebrates such as mammals, birds, and reptiles, as well as fish (Hikima et el., 2003, Kylsten et al., 1992). The gene has been found in other biota, some of which have shown a distinct form or tissue-specific gene expression patterns (Rosa et al., 2008). In cows, it has been reported that a c-type lysozyme gene is most abundant in intestines and also expressed in a stomach, kidney, respiratory tract, and mammary gland (Irwin et al., 2004). In mice, it has been reported that a c-type lysozyme is predominantly expressed in small intestines and less predominantly in several tissues (Cross et al., 1988, Cortopassi et al., 1990).

A g-type lysozyme gene as well as a c-type lysozyme gene has been found in diverse fish species. It is noteworthy that the both types of lysozyme are simultaneously found in *Cyprio carpio, Danio rerio, Paralichthys olivaceus, and Scophthalmus rhombus* (Jimenez et al., 2008, Hikima et al., 2001). A c-type lysozyme has recently been identified in an invertebrate, Haliotis (Nordotis) discus hannai (Hikima et al., 2001).

There have been consistent research efforts on the expression patterns and genetic regulation of the two lysozyme genes of some fish. In particular, it was reported that the number of the genes significantly increases after experiment testing bacterial attacks. For *Paralichthys olivaceus*, the gene was identified most abundantly in head kidney, spleen, and ovary (Hikima et al., 1996, Hikima et al., 1997).
Oplegnathus fasciatus belongs to class of Chordata, Actinopterygii, and perciformes, and is temperate zone fish usually inhabiting in rock zones of coasts. They are distributed in all coastal areas of Korea, the Japanese coastal areas, and the Chinese coastal areas. A male *Oplegnathus fasciatus* matures faster than a female *Oplegnathus fasciatus* . *Oplegnathus fasciatus* is one of the economically important cultured species, generating higher market value and demands in Korea. Contrary to the heavy consumption of *Oplegnathus fasciatus*, however, there has been very limited information on their immune system and immunity genes responding against diseases. This study clarified the molecular biological characteristics of cDNA of the C-type lysozyme from Oplegnathus fasicatus and conducted phylogency analysis. Furthermore, tissue-specific expression analysis of genes verified the roles of c-type lysozyme in general circumstances.

2. Materials and Methods

2.1. Molecular characterization of RbLysC cDNA

Been transferred to the laboratory in the purchase rock bream fish farms located in Tongyeong, Gyeongsangnam-do and acclimation were used in the study was 24 hours, feed was not available. Full-length RbLysC (Rock bream Lysozyme C) cDNA was obtained from expressed sequence tags (ESTs) analysis of liver from rock bream stimulated with lipopolysaccharide (LPS) (GenBank accession number AB597292).
Nucleotide sequences of RbLysC were compared lysozyme sequences from other species registered in peptide sequence databases of National Center for Biotechnology Information (NCBI), amino acid homology was found with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of NCBI. Multiple sequence alignments were analysed using GENETYX ver. 7.0 (SDC Software Development, Japan), positions of the signal peptide were identified using the SignalP program (http://www.cbs.dtu.dk/services/SignalP), phylogeny was inferred using the Mega 4 software and distance analysis using the neighbour-joining method, bootstrap sampling was performed with 2,000 replicates.

2.2. Analysis of RbLysC gene expression in various tissues of healthy fish

To evaluate RbLysC gene expression, various tissues including the head kidney, trunk kidney, spleen, liver, intestine, gill and muscle were isolated from three healthy rock breams(Weight 68.5 ± 10 g; body length 14.3 ± 1 cm). Peripheral blood leukocytes (PBLs) were isolated using Percoll density gradients (Sigma-Aldrich, St. Louis, MO, USA), as described previously (Nam et al., 2000). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and first strand cDNA synthesis was carried out using a first-strand cDNA synthesis kit (Takara, Shiga, Japan)

according to the manufacturer's instructions. Quantitative real-time PCR was performed with SYBR Green Master Mix (Takara, Shiga, Japan) according to the manufacturer's protocol. Quantitative real-time PCR was carried out using cDNA templates for each tissue and specific primer sets for the RbLysC gene (Table 1). Amplification was performed by initial denaturation at 50℃ for 4 min and 95℃ for 10 min, followed by 45 cycles at 95℃ for 30 sec, 60℃ for 30 sec and 72℃ for 30 sec, with a final dissociation at 95℃ for 15 sec, 60℃ for 30 sec and 95℃ for 15 sec. The relative expression of the RbLysC gene was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using β-actin expression as a reference. Data were reported as the RbLysC mRNA levels relative to that of β-actin mRNA, expressed as mean ± standard deviation (SD).

Table 1. Primers used in this study.

Primer name	**Sequence (5'-3')**
RbLysC-F	TTTCTGCTCTTGGTGGCTTT
RbLysC-R	TTGTACGACGACTCCCACTG
β-actin-F	TTTCCCTCCATTGTTGGTCG
β-actin-R	GCGACTCTCAGCTCGTTGTA

3. Results

3.1. Molecular characterization of RbLysC

The full-length RbLysC cDNA was 985 bp long and contained an open reading frame (ORF) of 432 bp that encoded 143 amino acid (aa) residues, the 5'-untranslated region (UTR) of 120 bp and the 3'-UTR of 444 bp. Signal peptide was not found in the terminal and flanking active aspartate region (64-73aa) are conserved in RbLysC, and a polyadenylation signal (AATAAA) and poly A-tail are present in the 3'-UTR of RbLysC (Fig. 1).

Fig. 1. The full-length cDNA and deduced amino acid sequences of Lysozyme C and amino acid sequences from rock bream, *Oplegnathus fasciatus*. The primers that were used in the study are indicated with arrow. The conserved flanking active aspartate is shaded. Cysteine residues are boxed. The polyadenylation signal (AATAAA) is indicated single underline.

Eight cysteine residues involved in the tertiary structure formation of protein and two amino acid residues that are considered as potentially important for the lysozyme catalytic activity, Glu50 and Asp71, were found to be completely conserved in RbLysC. There was also a flanking active aspartate region is well preserved in most of the fish lysozyme C.

Indicated that the deduced amino acid sequences of RbLysC shared significant identity with other reported lysozyme C. RbLysC presented 75% identity with Yellow perch (Perca flavescens FJ804424.1), 74% with Turbot (Psetta maxima EU747734.1), 73% with Atlantic salmon (Salmo salar BT047934.1), 69% with Sole (Solea solea DQ293993.1), 65% with Pig (Sus scrofa domesticus P12068), 64% with House mouse (mus musculusP08905), 59% with Human (Homo sapiens P61626.gnu), 57%

with Chicken (Gallus gallus FJ542389.1), 54% with Frog (Leiopelma archeyi XM_002938507.1) and 33% with Abalone (Haliotis discus HM068601.1) (Fig. 2).

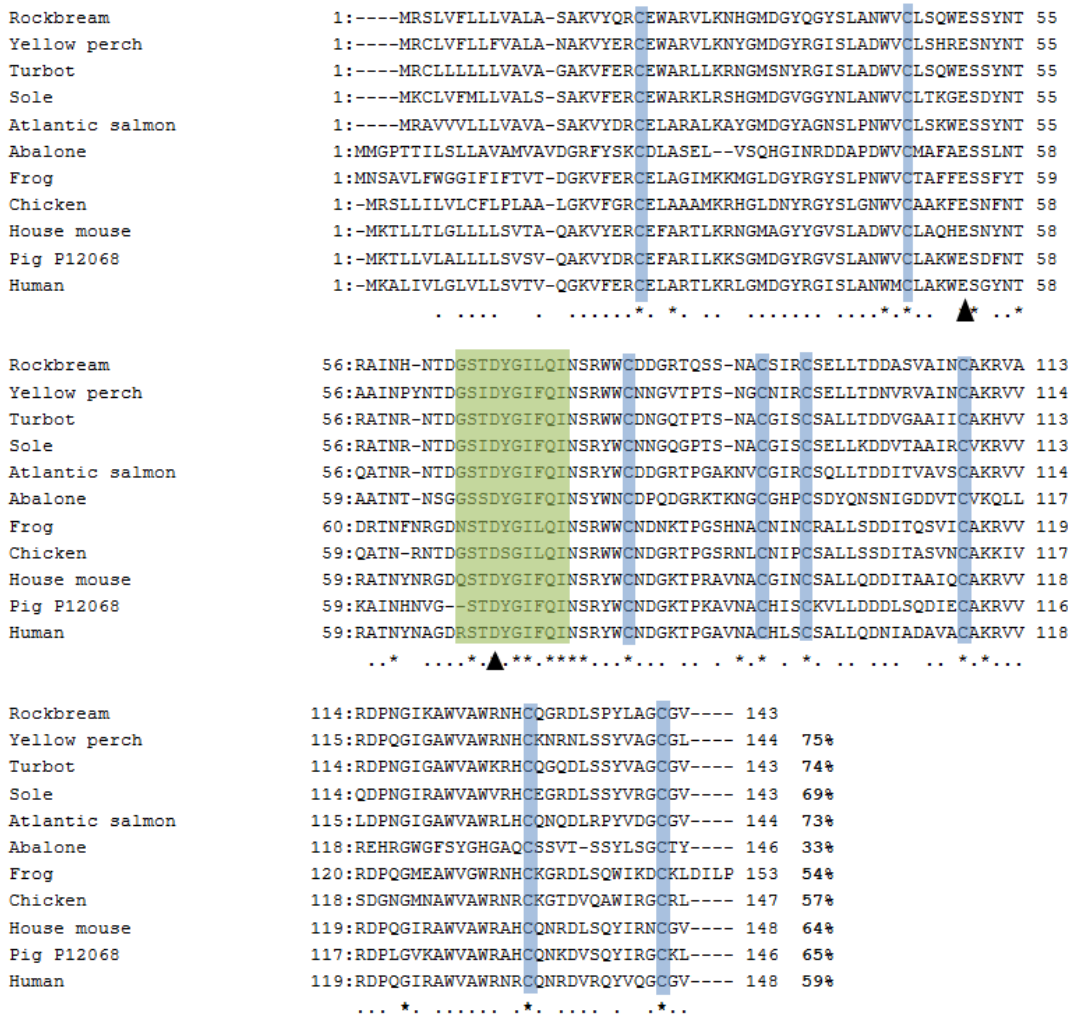

Fig. 2. Multiple alignment of amino acid sequences of the *O. fasciatus* Lysozyme C and other Lysozyme C. Identical (*) and similar (.) amino acid residues are indicated. Gaps (−) were introduced to maximize the alignment. The conserved flanking active aspartate region is shaded green. The two essential catalytic residuce are shown as arrow. The positions of cysteine residues identical in all sequences are shaded blue.

3.2. Phylogenetic analysis of RbLysC

Phylogenetic analysis based on the amino acid sequences of other known lysozymes was constructed. The overall topology of the phylogenetic tree revealed two separate groups. As expected, RbLysC was clearly grouped in the same clade as other lysozymes C. The g-type lysozymes also formed individual cluster. The relationship revealed in this phylogenetic tree agreed with homology comparisons using other

lysozymes C of teleosts; RbLysC presented the closest distant relationship with sequences from Yellow perch lysozyme C (Fig. 3).

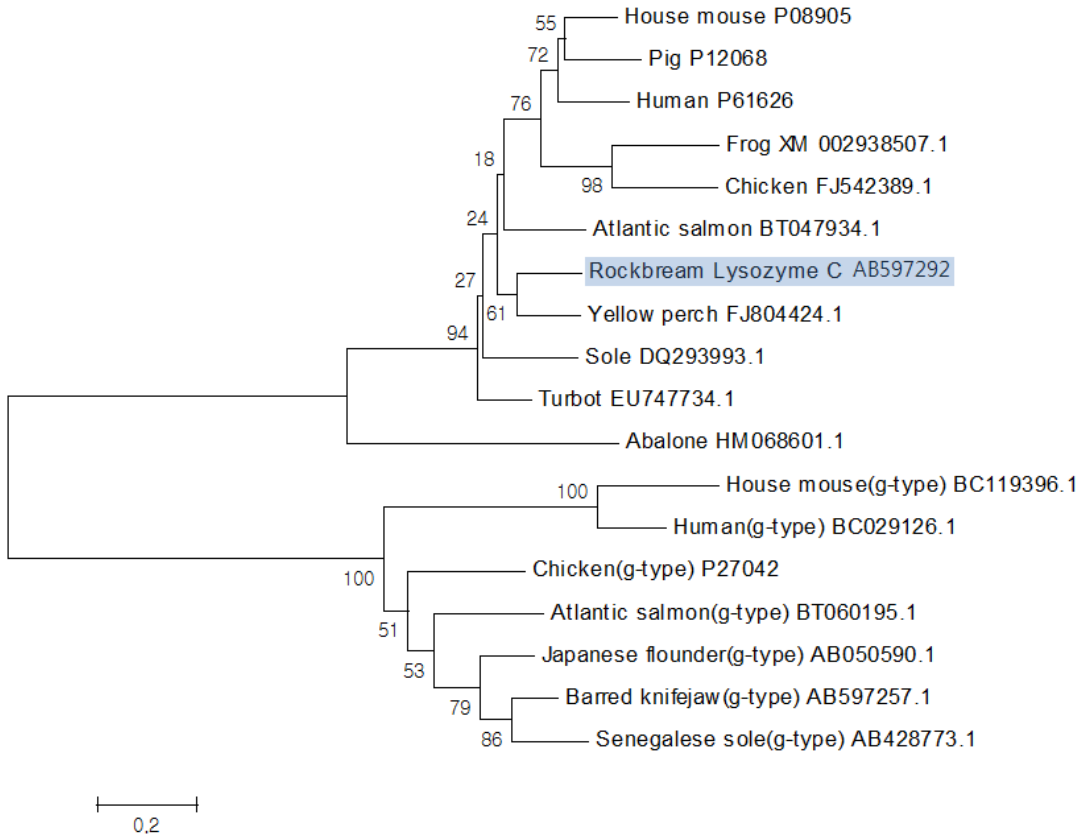

Fig. 3. Neighbor-joining phylogenetic tree of Lysozyme C amino acid sequences reported in representative taxa. The bootstrap confidence values shown at the nodes of the tree are based on 2000 bootstrap replications.

3.3. Detection of RbLysC in various tissues of healthy fish

The tissue distribution pattern of RBgLyz mRNA transcripts was determined by Quantitative real-time PCR of PBLs, head kidney, trunk kidney, spleen, liver, intestine, gill and muscle of healthy individuals (Fig. 4). The expression level for each of the tissues examined was normalized to that of β-actin. Relative expression fold differences were calculated based on the expression in PBLs to determine the tissue expression profile. RbLysC gene expression was ubiquitous in all tissues tested. The highest RbLysC gene expression was observed in the liver, which was about 50-fold relative to that of the PBLs. RbLysC was highly expressed in the intestine (about 30-fold), gill, trunk kidney, spleen, muscle and head kidney relative to that of the PBLs.

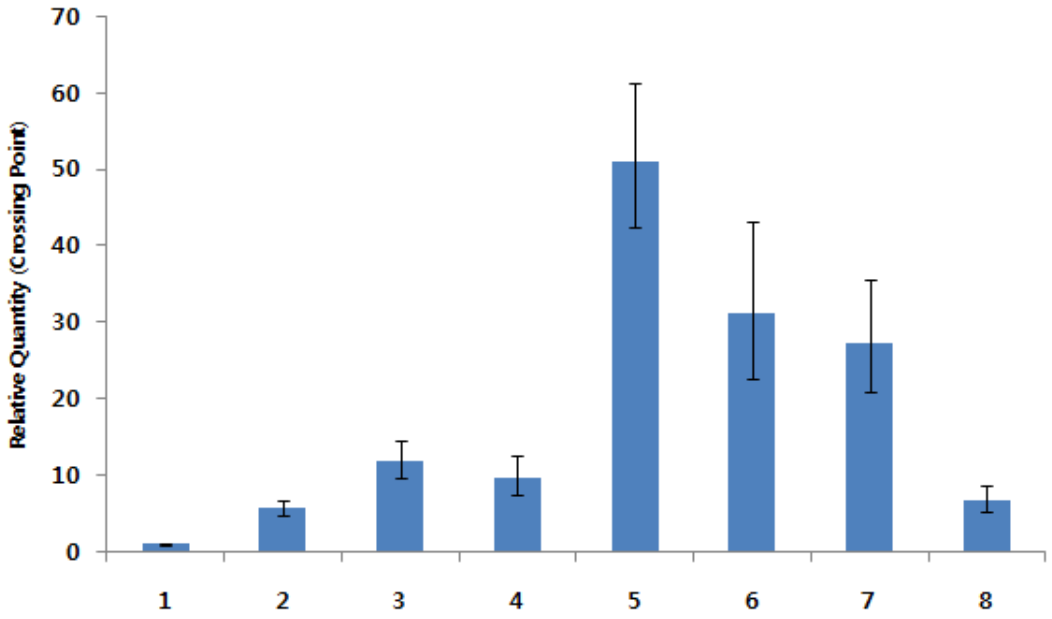

Fig. 4. Expression of RbLysC cDNAs in various tissues of healthy Rock bream as determined by Real-time PCR. PBLs, head kidney, trunk kidney, spleen, liver, intestine, gill, and muscle were examined. The asterisk indicates a statistically significant difference ($P < 0.05$).

4. Discussion

In this research, we analyzed the molecular biologic characteristics, multiple alignments, and identified the tissue-specific expression of the c-type lysozymes of *Oplegnathus faciatus*, one of the major cultured species in Korea.

According to our analysis, the amino acid sequence has ORF of 432bp, which encodes amino acid sequence, and the flanking active aspartate (64-73aa) in Glu^{64}. The sequence also preserves 8 cysteine and two catalytic residues of Glu^{50} and Asp^{71} engaged in four disulfide bonds. Related with three-dimensional structure of a protein, the disulfide bond is a very powerful covalent bond, which indicates that the lysozyme C is strongly bonded (Jimenez et al., 2003). The three-dimensional protein structures of c-type and g-type lysozyme have very similar glutamine residues, which suggests that the two types of lysozyme basically serve as a dissolver (Xie et al., 2010). Considering that there have been no significant differences in amino acid sequence alignment of the two types of the lysozyme, it can be said that lysozyme G is engaged mainly in digestion and antibacterial function (Grutter et al., 1983, Weaver et al., 1984, Gavilanes et al., 1984).

Lysozyme C is largely divided into calcium binding lysozyme and non-calcium binding ones. In general, a catalytic residue of asparaginic acid is conserved in 101, 106, and 107 of calcium binding group, to which birds and mammals belong (Parager et al., 1996). Like the lysozyme C of the other fish, that of the *Oplegnathus fasciatus*

belongs to non-calcium binding group, where a residue of asparaginic acid is not conserved (Fig.2).

Our phylogency analysis reveals that lysozyme C and lysozyme G form different groups. The lysozyme C of *Oplegnathus fasciatus* was the first to be separated from abalones. This leads us to expect that the abalones and vertebrates have experienced different evolutionary processes. The cDNA of the Yellow perch was the most phylogenetically similar to the lysozyme C of *Oplegnathus fasciatus*. Both belonging to the *Perciformes* (order), the two species are thought to undergo a similar generation process.

Considering that the lysozyme C has more complicated evolutionary process than the lysozyme G. and that the two species are grouped with different fish communities, the two species seem to have different genetic ancestors. However, it is too early to conclude anything about the origin of the two species (Bachali et al., 2002, Nilsen et al., 2003).

Our experiments on the tissue-specific gene expression of several species suggest that the lysozyme C has acquired two roles. In the case of the brill, the g-type lysozyme was broadly expressed in all tissues, whereas the g-type lysozymes were predominantly expressed in the liver and stomach (Rosa et al., 2008). If lysozymes can potentially degrade chitin by attacking the internal links between N-acetylglucosamine (Krogdahl et al., 2005), it is interpreted that the g-type functions as a digestive mechanism and the c-type as a defensive one (Rosa et al., 2008). Therefore, further studies should investigate expression of and the roles of the c-type lysozymes in *Oplegnathus fasiatus* through additional experiments.

In some species, however, the c-type was expressed predominantly in tissues of the immune system. This suggests that the c-type lysozymes still basically function as a defensive mechanism. In *Paralichthys olivaceus*, the c-type lysozymes were predominantly identified in head kidney, posterior kidney, spleen, brain, and ovary (Hikima et al., 1997),whereas the g-type lysozymes in head kidney, posterior kidney, spleen, skin, muscles, heart, and brain (Hikima et al.,2001). In *Ctenopharyngodon idella*, the cDNA of the c-type lysozymes were broadly expressed in all parts, of which they are the most highly expressed in the head kidney (Wen et al., 1999).

In an attack experiment, the expression patterns of c-type and of g-type were reported to be very different from each other. In the Brill, the c-type were not detected in all tissues nine hours after the infection by the *Vibrio angularium*. On the contrary, the g-type lysozymes were the most predominantly identified in spleen and liver (Xing et al,. 2010). This result implies that the g-type and c-type evolved to acquire differentiated functions of bacteriolytic action and digestion. Therefore, attack experiments are to be conducted to clearly determine the functions of lysozymes in *Oplegnathus fasciatus*.

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