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Research Article

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

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. fasciatus* and conducted phylogeny 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.

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. The innate immune system is of more importance to the lower vertebrates like fish than to the higher vertebrate. As one of the most representative parts of innate immune system, lysozyme facilitates the hydrolysis of N-acetyl glucosamine and N-acetyl muramic acid in the peptidoglycan layer of bacterial cell walls and destroys the cell walls, thus arresting the spread of bacteria. 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.

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. Since then, a c-type lysozyme gene was found in 13 kinds of fish: *Danio rerio*, *Oryzias latipes*, *Cyprinus Carpio*, *Oreochromis aureus*, *Perca flavescens*, *Takifugu rubripes*, *Scophthalmus rhombus*, *Scophthalmus maximus*, *Solea senegalensis*, *Paralichthys olivaceus*, *Salmo salar*, and *Oncorhynchus mykiss* and *Esox lucius*. It was registered in Genbank database [1]. 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 [2-5].

A c-type lysozyme gene has easily been expressed in abalones and the invertebrates such as mammals, birds, and reptiles, as well as fish. The gene has been found in other biota, some of which have shown a distinct form or tissue-specific gene expression patterns [6]. 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. In mice, it has been reported that a c-type lysozyme is predominantly expressed in small intestines and less predominantly in several tissues [7].

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 *Cyprinus carpio*, *Danio rerio*, *Paralichthys olivaceus*, and *Scophthalmus rhombus* [8,9]. A c-type lysozyme has recently been identified in an invertebrate, *Haliotis (Nordotis) discus hannai* [10]. 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 [1].

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 fasciatus* and conducted phylogeny analysis. Furthermore, tissue-specific expression analysis of genes verified the roles of c-type lysozyme in general circumstances.

Materials and Methods

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 Signal P 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.

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. 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°C for 4 min and 95°C for 10 min, followed by 45 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with a final dissociation at 95°C for 15 sec, 60°C for 30 sec and 95°C 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 \pm standard deviation (SD).

Table 1: Primers used in this study.

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

Results

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 (Figure 1).

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 domestica* P12068), 64% with House mouse (*Mus musculus* P08905), 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) (Figure 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 (Figure 3).

Detection of RbLysC in various tissues of healthy fish

The tissue distribution pattern of RbLysC mRNA transcripts was determined by Quantitative real-time PCR of PBLs, head kidney, trunk kidney, spleen, liver, intestine, gill and muscle of healthy individuals (Figure 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

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1 CAACGCTTCGCTGCAGTTTCGGGTGCACTTCTCACTGTAAAAGAACATCCAGCAGCAG 60
61 CTGTGTACACATTTTGTCTCTGCCAGCAGAGAGTTTGTCTATGAGGAGTCTGGTCTTCTG 120
M R S L V F L 7
121 CTCTTGGTGGCTTTGCCAGCGCTAAAGTCTACCAGCGCTGTGAATGGGCCAGAGTGGCTG 180
L L V A L A S A K V Y Q R C E W A R V L 27
181 AAAATCATGGGATGGAGGGCTACCAGGCTACAGCCTGGCTAACTGGTCTTGTCTGTCA 240
K N H G M D G Y Q G Y S L A N W V C L S 47
241 CAGTGGGAGTCTGCTACAAACCAGAGCCATCAACCACAACATGATGGCTCCACTGAC 300
Q W E S S Y N T R A I N H N T D G S T D 67
301 TACGGCATCCTCCAGATCAACAGCCGATGGTGGTGTGACGACGGCCGACCCAGTCTTCG 360
Y G I L Q I N S R W W C D D G R T Q S S 87
361 AATGCATGCGCATCAGGTGCGAGGCTTCTGACCGATGATGCCAGCGTGGCAATCAAC 420
N A C S I R C S E L L T D D A S V A I N 107
421 TGTGCCAAACGTGTAGCTAGGGATCCCAACGGCATCAAAGCCTGGGTGGCTGGCGCAAT 480
C A K R V A R D P N G I K A W V A W R N 127
481 CACTGCCAGGCGCTGACCTGAGCCCTATCTGGCAGGATGTGGTGTTAATCAACACC 540
H C Q G R D L S P Y L A G C G V * 143
541 GAAGATGGACGTTGTATCAACATAATGAAATTTCTCCATCTTCAGCTGCAGTGGATTGA 600
601 AACGTTCTATTTGTCGTATGCTGATCTAACAGAACTAAAACCTACAGTTACTGAAGCT 660
661 ACAGATGATCTTTTCAGATGAAATTAATCAAATCAGTATATTTGGTTAACTTTACT 720
721 TGATTTAACAGATTGAATAGTGAAGAATGTTAAATGTTGATTTCCACTAACATTAA 780
781 CAGCTGTTGAATTAACATGAATGAACCCGGTTTCCATGAAAATAAACACACAGCATTCCT 840
841 TTAAGTAAACATGATGTCCTCATAATCCCGGAGAGGAAGTGGAGAGGGTGGACTCAT 900
901 ACAAGTACCTTGGAGTCCAAATCAATAAAAACCTGGACTGGTCTCATAACGCTGAAAAA 960
961 AAAAAAAAAAAAAAAAAAAAAA 985

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Figure 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.

Rockbream	1:----MRSLVFLLVVALA-SAKVYQRC	EWARVLK	NHGM	DGYQ	GYSLAN	VVLSQ	WESSYNT	55										
Yellow perch	1:----MRCLVLLFVALA-NAKVYER	CEWARVL	KNYG	MDGYR	GISLAD	WVCLSH	RHSYNT	55										
Turbot	1:----MRCLLLLLVAVA-GAKVFER	CEWARLL	KRNGM	SYRGI	SLADW	VCLSQ	WESSYNT	55										
Sole	1:----MKLVFVLLVALS-SAKVFER	CEWARKL	RSHGM	DGVGY	NLANW	VCLTK	GESDYNT	55										
Atlantic salmon	1:----MRAVVVLLVAVA-SAKVYDR	CELARAL	KAYGM	DYAGN	SLPNW	VCLSK	WESSYNT	55										
Abalone	1:MMGPTTILSLLAVAMVAVDGRF	YSKDL	ASEL	--VSQ	HGINR	DDAPD	WVCM	FAESSINT	58									
Frog	1:MNSAVLFWGGIFIFTVT-DGK	VFER	CEL	LAGIM	KKMLD	GYRGS	LPNV	CTAFFESSYNT	59									
Chicken	1:--MRSLILVLCFLPLAA-LGK	VFER	CEL	LAAM	KRHGL	DNYRGS	SLGNW	VCAAFESNNT	58									
House mouse	1:--MKTLLTLGLLLLSVTA-QAK	VYER	CE	FART	LKRNG	MAGYY	GVSL	ADWVCLAQHSYNT	58									
Pig P12068	1:--MKTLLVLAALLLSVSV-QAK	VYDR	CE	FARIL	KKS	GMDGYR	GVSLAN	VCLAKWESDYNT	58									
Human	1:--MKALIVLGLVLLSVIV-QGK	VFER	CEL	ARTL	KRLG	MDGYR	GISLAN	WMCLAKWESGYNT	58									
*********									
Rockbream	56:RAINH-NTD	GSTDY	GILQ	INSRW	DDGR	TOSS-NA	C	SIR	SELLT	DDASVAIN	CAKRVA	113						
Yellow perch	56:AAINPYNTD	GSDY	GIFQ	INSRW	CNNG	VIP	TS-NG	C	NIR	SELLT	DNVRVAIN	CAKRVA	114					
Turbot	56:RATNR-NTD	GSDY	GIFQ	INSRW	DDNG	QIPT	S-NA	C	GIS	C	ALLTDDV	GAAI	CAKHVV	113				
Sole	56:RATNR-NTD	GSDY	GIFQ	INSRW	CNNG	QOPT	S-NA	C	GIS	C	SELLK	DDVTA	AARV	CKRVV	113			
Atlantic salmon	56:QATNR-NTD	GSDY	GIFQ	INSRY	WDD	GRTP	GAKN	VCGIR	C	S	QLLTD	DDITV	AVS	CAKRVA	114			
Abalone	59:AATNFNSRGN	STDY	GILQ	INSRY	WDD	PQD	GRKTK	NGCG	HPC	SDY	QNSN	IGDD	VTC	VKQLL	117			
Frog	60:DRTNFNRGN	STDY	GILQ	INSRW	CND	NKTP	GS	HNA	C	N	IC	ALLS	DDIT	QSVI	CAKRVA	119		
Chicken	59:QATN-RNTD	GSDY	GILQ	INSRW	CND	GRTP	GS	RNL	C	NIP	C	ALLS	DDIT	AVS	CAKRVV	117		
House mouse	59:RATNYNRGD	STDY	GIFQ	INSRY	WDD	NGK	TPRA	VN	C	G	INC	S	ALLQ	DDIT	AAIQ	CAKRVA	118	
Pig P12068	59:KAINHNVG--	STDY	GIFQ	INSRY	WDD	NGK	TPRA	VNA	C	HIS	C	KVLL	DD	DD	SQDIE	CAKRVA	116	
Human	59:RATNYNAGD	RSTDY	GIFQ	INSRY	WDD	NGK	TPRA	VNA	C	HLS	C	S	ALLQ	DD	DD	ADAVA	CAKRVA	118
	..* ..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*	..*.*
Rockbream	114:RDPNGIKAWVAWRNH	C	QGR	DLSP	YLAC	GV----	143											
Yellow perch	115:RDPQIGAWVAWRNH	C	KNR	NLS	SYV	AGCGL----	144	75%										
Turbot	114:RDPNGIGAWVAWRNH	C	QGD	LSS	YV	AGCGV----	143	74%										
Sole	114:QDPNGIRAWVAWRH	C	EGR	DLSS	YV	RGCGV----	143	69%										
Atlantic salmon	115:LDPNGIGAWVAWRH	C	QND	LR	YP	VDGCGV----	144	73%										
Abalone	118:REHRGWGFSYGHGAQ	C	SSVT	--SS	Y	SGCTY----	146	33%										
Frog	120:RDPQMEAWVGRNH	C	KGR	DL	SQ	WKDKLDLIP	153	54%										
Chicken	118:SDNGMNAWVAWRNH	C	KG	T	DVQ	AWIRGRL----	147	57%										
House mouse	119:RDPQIRAWVAWRH	C	QNR	DL	SQ	YIRNCGV----	148	64%										
Pig P12068	117:RDPNGIKAWVAWRH	C	QNK	D	V	SQYIRGCKL----	146	65%										
Human	119:RDPQIRAWVAWRNH	C	QNR	D	V	RQYVQCGV----	148	59%										
	...*.*	...*.*	...*.*	...*.*	...*.*	...*.*	...*.*	...*.*										

Figure 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 residue are shown as arrow. The positions of cysteine residues identical in all sequences are shaded blue.

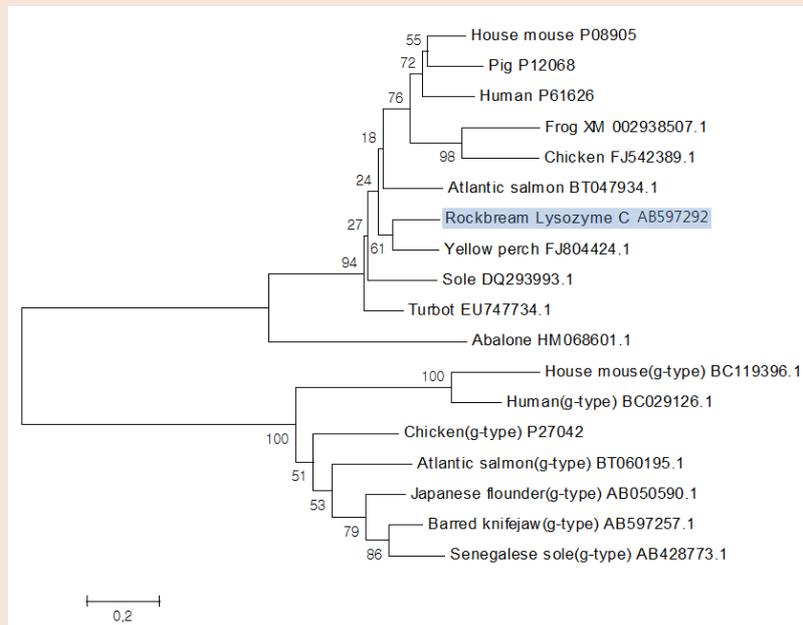


Figure 3: Neighbor-joining phylogenetic tree of Lysozyme C amino acid sequences reported in representative taxa. The bootstrap confidence values shown at the nodes of the tree are based on 2000 bootstrap replications.

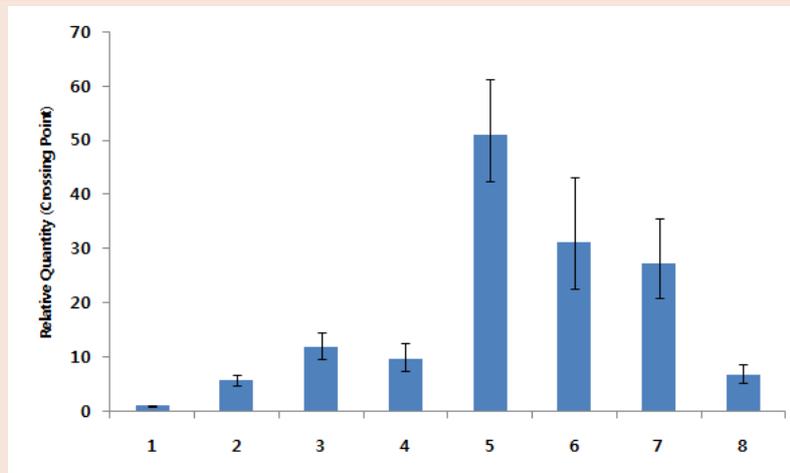


Figure 4: Expression of RbLysC cDNAs in various tissues of healthy Rock breem as determined by Real-time PCR. PBLs, head kidney, trunk kidney, spleen, liver, intestine, gill, and muscle were examined. The asterisk indicates a statistically significant difference ($P < 0.05$).

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.

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 fasciatus*, 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 Glu⁶⁴. The sequence also preserves 8 cysteine and two catalytic residues of Glu⁵⁰ and Asp⁷¹ 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. 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. 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 [10].

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. 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 (Figure 2).

Our phylogeny 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 [11].

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 c-type lysozymes were predominantly expressed in the liver and stomach [6]. If lysozymes can potentially degrade chitin by attacking the internal links between N-acetylglucosamine, it is interpreted that the g-type functions as a digestive mechanism and the c-type as a defensive one [6]. Therefore, further studies should investigate expression of and the roles of the c-type lysozymes in *Oplegnathus fasciatus* 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 [1], whereas the g-type lysozymes in head kidney, posterior kidney, spleen, skin, muscles, heart, and brain [8]. 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 [13].

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 anguillarum*. On the contrary, the g-type lysozymes were the most predominantly identified in spleen and liver [14]. 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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