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REVIEW

Characterization and roles of lysozyme in molluscs

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Abstract

Lysozyme can hydrolyse the β-1.4-glycosidic linkage between the N-acetylmuramic (NAM) and N-acetylglucosamine (NAG). The three major categories of lysozymes in molluscs are Goose-type, Chicken-type and Invertebrate-type lysozymes. The function of lysozymes is served as an innate immune protection against exogenous microbial invasion. The tyoical c-, g- and i-type mollusc lysozymes are secreting type, have signal peptide and eight, six and fourteen cysteine residues, respectively. The c- and g-type lysozymes are highly expressed in hepatopancreas, hemocytes and gills, and weakly expressed in foot and goand tissues of muscle. The i-type lysozyme gene is high expression in different tissues. The three type lysozymes exhibit antibacterial and digestive activity, and i-type lyaozyme also has antifungi activity. Furthermore, this review includes current knowledge regarding to the genomic structure, tissue distribution of mollusc lysozyme, the antimicrobial function and mechanism. The evolution of three type lysozymes in molluscs is also discussed. These lysozymes research may help to understand the basic knowledge and to use it in the production of molluscs.

Key Words: c-type; g-type; i-type; mollusc; antimicrobial

Introduction

Molluscs possess as much as approximately 200,000 species, which widely distribute in various ecosystem, including terrestrial, freshwater and marine environments (Ponder and Lindberg, 2008), and rely on innate immune systems to mediate cellular and humoral components for defense against pathogens (Loker *et al*., 2004). In recent years, mollusc aquaculture has been facing a set back due to challenges emanating from pathogenic infections. *Haliotis discus hannai* suffers from abnormal deaths, and results in the considerable reduction of abalone output throughout the world (Zhang *et al.,* 2004; Sawabe *et al.,* 2007). The effector of mollusc immune is crucial to better understand the immune defense mechanisms and provides the potentially feasible solutions for disease control.

The innate immune system is of great importance to protect invertebrate against a wide range of microbial pathogens and encompasses a complex array of defense reactions, in which mainly focusing on immune recognition, signal transduction and effector synthesis involved in cellular and humoral immunity in the field of mollusc immunity. Lysozyme is identified a classic mollusc immune effector in innate immune (Wang *et al.,* 2013), which is originally found to dissolves bacterial cell walls in human saliva and tears (Haug *et al.,* 2004), and was subsequently described in other vertebrates and invertebrate (Zhao *et al*., 2007; Whang *et al*., 2011; He *et al*., 2012; Wang *et al*., 2012; Umaasuthan *et al*., 2013). The enzyme is a ubiquitous bacteriolytic enzyme, which is produced by diverse groups of organisms, ranging from bacteria and bacteriophages to fungi, plants and animals (Bathige *et al*., 2013), is characterized by their ability to bacterial peptidoglycan between two amino sugars, *N*-acetylmuramic acid and *N*-acetylglucosamine and cause bacterial cell lysis (Chipman and Sharon, 1969; Prager and Jollès, 1996), and has bactericidal and digestive ability (Dobson *et al*., 1984; Itoh and Takahashi, 2007). Besides antimicrobial activity, lysozymes have also proved to perform many other functions, such as growth

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stimulation, digestion, antiviral, anti-inflammatory, and even association with tumors (Irwin, 2004; Wang and Zhang, 2010; Lee *et al.* 2015; Xin *et al.*, 2015), which are regarded to play important roles in the innate immunity and physiological activities, is a first line defensive protein that acts as a barrier to resist bacterial pathogen invasion in innate immune systems of invertebrates, and is widespread in many tissues and secretions (Bachali *et al.*, 2002; Liu *et al.*, 2006). Extensive studies have been devoted to their structure, catalytic mechanism, relationship between structure and activity, phylogeny, immunology, and genetics (Jollès, 1996). The types of lysozymes are different in amino acid sequences, biochemical and tissue distribution. The present review attempts to mainly focus on classification, distribution and function of mollusc lysozyme. It will help to improve the current knowledge about lysozyme of molluscs.

Classification and charecteristics of mollusc lysozyme

Lysozyme (EC 3.2.1.17) catalyzes the hydrolysis of 1, 4-beta-linkages between N-acetyl-dglucosamine (NAG) and N-acetylmuramic acid (NAM) in peptidoglycan heteropolymers of prokaryotic cell walls, and leads to the breakdown of bacterial cells (Fleming, 1922; Jollès and Jollès, 1984a). The enzyme are generally classified into six types based on differences in structural, catalytic and immunological characteristics, including chicken-type (c-type), goose-type (g-type), plant, bacteria, T4 phage, and invertebrate-type (i-type) lysozymes (Inouye *et al.*, 1970; Matthews *et al.*, 1981; Joskova *et al.*, 2009). These types of lysozymes have been described in organisms (Jollès and Jollès, 1984). Three types lysozymes, c, g and i-type, have been recorded in molluscs (Wang *et al.* 2013; Guo *et al.*, 2014; Zhu *et al.*, 2016). The distribution and properties of lysozyme in molluscs are shown in Table 1.

Table 1 the tissue distribution and characteristics of three types mollusca lysozymes

species	type	distribution	Lysozyme gene	Accession number(s)	Number of amino acids
Haliotis discus discus	c	pallium, muscle, gill, digestive gland	HdLysC	ADR70995	146
Haliotis discus discus	c	pallium, muscle, gill, digestive gland	HdLysC	ADR70996	146
Ruditapes philippinarum	c	pallium, gill, hepatopancreas	VpCLYZ-1	AGO06638	156
Ruditapes philippinarum	c	pallium, gill, hepatopancreas	VpCLYZ-2	AGO06639	153
Mytilus galloprovincialis	g	crystalline, digestive gland	MgLYZ1	AFF18185	206
Mytilus galloprovincialis	g	crystalline, digestive gland	MgLYZ2	AFF18186	*206*
Argopecten irradians	g	pallium, gill, hepatopancreas	-	AY788903	200
Physella acuta	g	hepatopancreas	PALYsG	ADV36303	198
Chlamys farreri	g	hepatopancreas, gill	CFLysG	ABB53641	200
Mizuhopecten yessoensis	g	gill, pallium, hemocytes	MyLysoG	AEY77130	201
Meretrix meretrix	i	hepatopancreas, gill	Mmelys	ADL27913	146
Cristaria plicata	i	pallium, gill, hemocytes	CpLYZ2	AFN66526	161
Cristaria plicata	i	pallium, gill, hemocytes	CpLYZ1	AFN66527	160
Crassostrea gigas	i	digestive gland, basophil	CGL	BAF48044	142
Crassostrea gigas	i	gill, hemocytes	CgLys	BAD19059	137
Haliotis discus discus	i	pallium, muscle, gill, digestive gland	AbLysG	AGQ50336	131
Crassostrea virginica	i	digestive gland, hemocytes,	cv-lysozyme 3	BAE93114	135
Ruditapes philippinarum	i	pallium, gill, hepatopancreas	Tj-lysozyme	BAC15553	136
Ruditapes philippinarum	i	mantles, gill, hepatopancreas	RpiLYZ-2	AMS37097	156

The c-type lysozyme is originally isolated from chicken-egg (Itoh *et al.*, 2007b), and subsequently is reported in other vertebrates and invertebrates, including amphibians, reptiles, mammalia, insect, crustacean and mollusc (Jollès *et al.*, 1996; Ito *et al.*, 1999; Miyauchi *et al.*, 2000; Olsen *et al.*, 2003; Liu *et al.*, 2006). The c-type lysozymes have two 2 catalytic residues (Glu^{53} and Asp^{70}) and 8 cysteine residues that can form 4 disulfide bonds to stabilize the protein structure (Hikima *et al.*, 2001; Jimènez-Cantizano *et al.*, 2008; Ye *et al.*, 2008). Several c-type lysozymes have recently been determined in *Mytilus galloprovincialis* (Wang *et al.*, 2013), abalone *Haliotis discus hannai* (Umasuthan *et al.*, 2013), and manila clam *Venerupis philippinarum* (Yang *et al.*, 2017). Comparison with c-type lysozyme of vertebrates, that of mollusc counterparts have not been well characterized. The c-type lysozyme of *H. discus hannai* is firstly described, the full-length cDNA of HdLysC is 586 bp, and contains an open reading frame of 441 bp encoding a 147-amino acid protein with a calculated molecular mass of 15.64 kDa, an isoelectric point being 4.87, and a polyadenylation signal (AATAA). The genomic length of HdLysC is 2865 bp, and has four exons interrupted by three introns, 2 catalytic residues (Glu^{53} and Asp^{70}), as well as the 8 cysteine residues involved in disulfide bond formation (Ding *et al.*, 2011). The homologous structure of c-type lysozymes also exists in the genome of *V. philippinarum* and *M. galloprovincialis* (Wang *et al.*, 2013; Yang *et al.*, 2017). The genome of c-type lysozyme possess 4 exons interspaced by relatively large introns in vertebrate (Hikima *et al.*, 2000), which do 3 exons separated by relatively smaller introns in invertebrate (Liu *et al.*, 2006), and even is lost in *Drosophila* (Kylsten *et al.*, 1992). The typical genomic structures of c-type lysozymes, number and size of both exons and introns, which exist in chicken, amphioxus, mosquito and silk moth, is also found in *abalone H.discus hannai*, and seem difference due to the changes of the introns length (Ding *et al.*, 2011). The results indicate that the c-type lysozyme gene must have undergone unknown evolutionary events, *e.g.,* a recombination, insertion or deletion in different lineages during evolution (Larsen *et al.*, 2009). The lysozymes could usually be divided into the calcium binding and the noncalcium binding lysozymes according to the presence/absence of conversed calcium binding residue Asp (Nitta *et al.*, 1987), which of birds and mammals belong to calcium binding lysozyme (Lemos *et al.*, 1993), which of fish has not yet found calcium bindin lysozyme (Saurabh *et al.*, 2008). Due to lack of calcium binding Asp residue, manila clam *V. philippinarum* is also categorized into the non-calcium binding lysozymes family (Yang *et al.*, 2017).

The g-type lysozyme is initially identified from egg of the whites Embden goose (Canfield, 1967), many of which is recently described from birds and fishes (Nakano and Graf; 1991; Thammasirirak, 2001; Larsen *et al.*, 2009). The enzyme of molluscs is originally detected in *Argopecten irradians* (Zou *et al.* 2005; Zhao *et al.*, 2007), and is subsequently reported in other molluscs (He *et al.* 2012; Wang *et al.*, 2012; Zhang *et al.*, 2012; Guo *et al.*, 2014). The g-type lysozymes in birds and mammals are secreting type, and have four conserved cysteine in signal peptide that can make secreted proteins to form a more stable three-dimensional structure (Jollès and Jollès, 1975)**.** All of known g-type lysozymes from *Argopecten irradians*, *Chlamy farreri, Mytilus edulis, Physa acuta* and *H. discus hannai* contain signal peptides, have similar three active center with (Glu^{82}, Asp^{97}, Asp^{108}), and share one conserved cysteine that also exists in birds and mammals. The six conserved cysteines are observed in mollusc g-type lysozymes, except the lysozyme of *Oncomelania hupensis* that contains eight conserved cysteine. Comparison with other g-type lysozymes of scallops, that of *O. hupensis* has two additional cysteines (Zhang *et al.*, 2012), and shares some features with other g-type lysozymes, such as the substrate binding sites, a signal peptide, the catalytic residues critical for the fundamental structure and function of g-type lysozymes (Nakai *et al.*, 2005, 2007). *P. acuta* can survive better in polluted water environment than other snails. A better understanding the immune mechanisms of *P.acuta* may be lead to important advances in the innate immune system of invertebrate. Comparison with g-type lysozyme of other molluscs, the lysozyme of *P. acuta* shares the same substrate binding sites, the catalytic residues and the same six cysteines (Hikima *et al.*, 2001; Zhao *et al.*, 2007; Itoh *et al.*, 2009). The same six cysteines also appear in *P. acuta*，which possibly constitute disulphide bridge to result in a compact structure, and are specific in molluscs (Zhao *et al.* 2005, 2007).

The i-type lysozyme is originally described from starfish *Asterias rubens* (Jollès and Jollès,1975), which is identified in phylogenetically diverse organisms of invertebrates, including porifers, molluscs, annelids, nematodes, echinodermates, hemichordates, and arthropoda (Ito *et al.*, 1999; Van Herreweghe and Michiels, 2012). The first i-type enzyme is described in marine shellfish (Hikima *et al.*, 2001), and recently identified in other molluscs, including *Chlamys islandica*, *Mytilus edulis*, *M. galloprovincialis*, *Crassostrea gigas*, *Crassostrea virginica*, *Ruditapes philippinarum* (Nilsen *et al.* 1999; Olsen *et al.*, 2003; Yue *et al.*, 2011; Zhu *et al.*, 2016). The complete amino acid sequence of the enzyme is cloned from *Tapes japonica* (Nilsen and Myrnes, 2001). The enzymes of *T. japonica* and *C. islandica* contain the same as fourteen cysteine residues (Ito *et al.*, 1999; Nilsen *et al.*, 1999), that of *Meretrix meretrix*, *R. philippinarum* and *C. gigas* have a signal peptide and fourteen conservative cysteine residue, and all structure domains are destabilase (Naoki *et al.*, 2007; Xin *et al.*, 2011; Yang *et al.*, 2017).

The typical g-type lysozyme of molluscs has six cysteine residues (Zou *et al.* 2005), which of numbers vary ranging from zero to ten in different species (Irwin and Gong 2003; Nilsen, 2003). The typical c-type lysozyme of molluscs has eight cysteine residues, which also exist in digestive organ (Xin *et al.*, 2011; Yang *et al.*, 2017). The high content of cysteine residues of g-type and i-type lysozymes

in molluscs are proposed to maintain more stable proteins that can possess a compacter structure in high osmolarity seawater and in the digestive (Ito *et al.*, 1999; Zhao *et al.*, 2007). Meanwhile, the three type lysozymes of molluscs are secreting type, and have signal peptide (Ito *et al.*, 1999). The genetic structure of i-type lysozyme has is similar to that of c-type lysozyme, and both have 4 exons and 3 introns (Nilsen *et al.*, 1999, 2001; Paskewitz *et al.*, 2008). However, the lysozyme with 5 exons and 2 exons was also found in Mytilidae. The i-type lysozyme genome of *M. edulis* comprises 5 exons instead of the classical 4 exons of the c-type lysozyme gene (Bachali *et al.*, 2002; Paskewitz *et al.*, 2008). The maximum number of exons in g-type lysozyme genome possess 7 exons from human (Irwin and Gong, 2003), and chicken and mice have 6 exons (Nakano and Graf, 1991). The g-type lysozyme of abalone *H. discus discus* has 7 exons and 6 introns (Ding *et al.*, 2011), and that of *M. galloprovincialis* do 6 exons and 5 introns (Hui *et al.*, 2008). Therefore, it is suggested that i and c-type lysozyme may originate from the same ancestor. The genetype of lysozyme is more than 2 in molluscs (Li *et al.*, 2008; Wang *et al.*, 2012; Wen *et al.*, 2015; Yang *et al.*, 2017). Three hypotype of the g-type lysozymes are firstly found in one species of molluscs (Zhu *et al.*, 2016), and c-, i- and phage-type lysozymes are described in *R. philippinarum* (Zhao *et al.*, 2010; Ding *et al.*, 2014). However, relatively little is known about the hypotype of lysozymes in vertebrates.

Phylogenetic analysis showed that the major lysozyme genes were clustered into two main clades (Fig. 1) that include g-, c- and i-type lysozyme sequences. It is indicated that c- and i-type lysozyme belong to the near-edge parallel macromolecules, and the c- and g-type lysozyme is a parallel evolution. Except for abalone *H. discus discus*, the c- and i-type lysozymes were clustered into two main clades in molluscs. Phylogenetic analysis of lysozyme gene also showed that the i-type lysozymes were clustered into main clades. *Chlamys islandica* and *Calyptogena sp* were clustered to the corresponding subgroup in the phylogenetic tree (Fig. 2).

Antimicrobial protection and mechanism

Lysozyme is antibacterial and digestion of bacteria in the major functions, and widely distribute in the tissues or secretions of vertebrates and invertebrates (Hultmark *et al.*, 1996; Irwin *et al.*, 1996). The transcript expression of c-type lysozyme is obvious in kidney, spleen, brain and ovary tissues from *Paralichthys olivaceus* (Hikima *et al.*, 2001). The g-type lysozyme gene replication is common in vertebrates, except for cartilaginous fish (Irwin, 2014). The expression of the g-type lysozyme is a high level in the kidney of *Oncorhynchus* (Miyauchi *et al.*, 2000), by liquidchromatography tandem mass spectrometry (LC-MS/MS), that of c-type lysozyme increase significantly in the blood cells and blood lymphocytes of *Biomphalaria glabrata* (Mollusca) after stimulated by the live *Bacillus megaterium* (Cheng *et al.*, 1978). The activity of mollusc lysozyme is detected in the hepatopancreas, hemolymph, gills, mantles, and digestive organs, by transcripts were detected inall tissues tested (He *et al.*, 2012; Wang *et al.*, 2012; Wen *et al.*, 2015). The distribution of the lysozymes in molluscs is shown in Table 1. The c-type lysozyme transcripts are highly expressed in hepatopancreas, hemocytes and gills from *V. philippinarum* and *H. discus hannai* (Yu *et al.*, 1999; Yang *et al.*, 2017). The g-type lysozyme from the

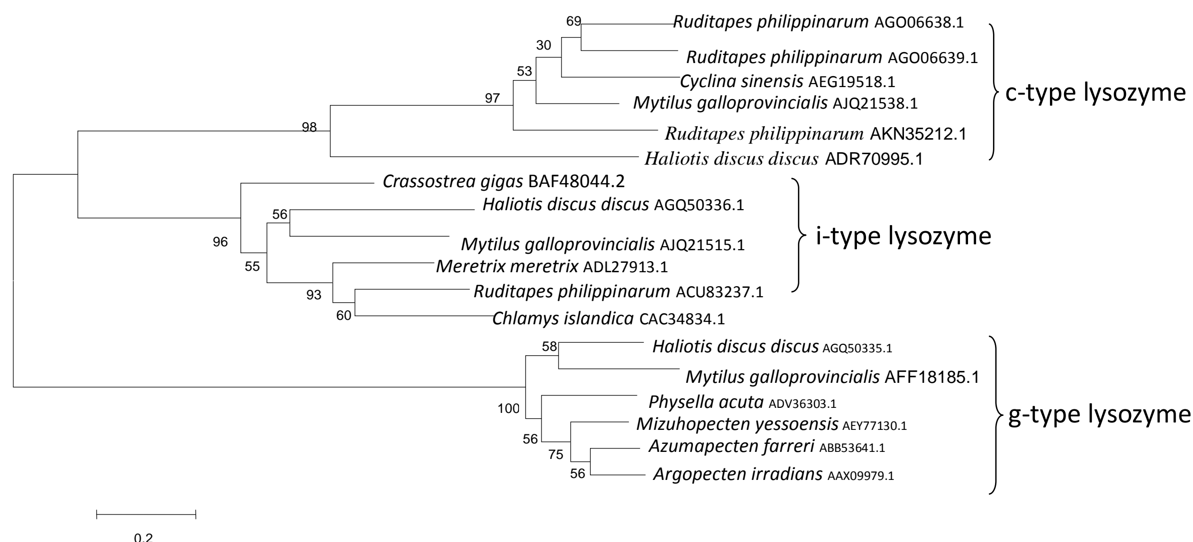

Fig. 1 Phylogenetic tree constructed by the neighbor-joining method in MEGA software based on the c, g, i-type lysozyme sequences. Bootstrap support values for the NJ tree are shown at the nodes (out of 1000 replicates).

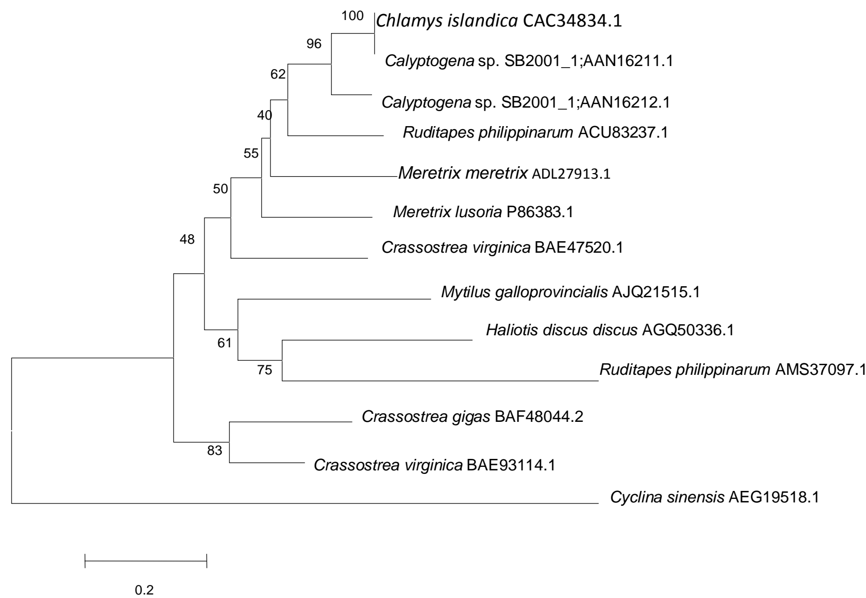

Fig. 2 Phylogenetic tree constructed by the neighbor-joining method in MEGA software based on the i-type lysozyme sequences. Bootstrap support values for the NJ tree are shown at the nodes (out of 1,000 replicates).

Mizuhopecten yessoensis is the highest expression in the hepatopancreas, gills and mantle (He *et al.* 2012). The i-type lysozymes of *Meretrix meretrix* and *Octopus ocellatus* mainly present in hepatopancreas, blood cells and gills (Hultmark *et al.*, 1996; Zhao *et al.*, 2010), that of *C. virginica* mainly exists in digestive gland and hemolymph (Xue *et al.*, 2007), that of *R. philippinarum* is the highest expression in mantle (Zhu *et al.*, 2016). The expression of i-type lysozyme in mantle is higher than that in gills, digestive glands and hemocytes from *Crassostrea virginica*, and is abundant in the tissues of gills, hepatopancreas and haemocytes from *V. philippinarum* (Itoh *et al.*, 2007). The c- and g-type lysozymes are highly expressed in hepatopancreas, hemocytes and gills, and are weakly expressed in the tissues of muscle, foot and goand (Zou *et al.*, 2005; Zhao *et al.*, 2007; Ding *et al.*, 2011; Wang *et al.*, 2013; Umasuthan *et al.*, 2013; Guo *et al.*, 2014; Yang *et al.*, 2017). The expression pattern of i-type lysozyme gene in different tissues probably indicate that the different biological functions of the enzyme occur during their evolution, that of g- and c-type lysozymes in different organs/tissues also suggested that they may serve as some extent reflect their functional role.

The major biological role of lysozymes can act as antibacterial and immune-modulating agents (Hikima *et al.*, 2001). The mRNA of lysozymes from *Mizuhopecten yessoensis*, *H. discus hannai* and *M. galloprovincialis* predominately express and execute its antibacterial activity in hepatopancreas, gills and mantle (Nilsen *et al.*, 1999; Li *et al.*, 2008; Wang *et al.*, 2011; He *et al.*, 2012). The expression of c-type lysozyme from *C. farreri* is in the hepatopancreas, gill and gonad, and the higher expression level in gills may contribute to the clearance of bacteria (Zhao *et al.*, 2007). The g-type lysozyme possess combined features of the immune and digestion, and also gain the lytic activities to inhibit gram-positive and gram-negative bacteria in vitro, the g-type lysozyme s of *C. farreri*, *M. galloprovincialis* and *M. yessoensis* can inhibite *Micrococcus lysodikicus*, that of *Physa acuta* is beyond restraint to *S. aureus* (Zhao *et al.*, 2007; Wang *et al.*, 2013). The g-type lysozyme gene of *O. hupensis* is mainly expressed in hepatopancreas, and antibacterial activity was stronger than the c-t ype lysozyme (Zhang *et al.*, 2012).

The i-type lysozymes are detected in hemocytes from Ruditapes decussatus and *R. philippinarum* (Yue *et al.*, 2011). The activity of i-type lysozyme in hemocytes from Mytilus edulis is higher than that from *R. decussatus* and *R. philippinarum* (Pipe, 1990; Carballal et al., 1997; Lopez et al., 1997). The gills often face to the invasion of all kinds of pathogens, which construct of only a single layer of fragile cells and covered with a thin layer of protective mucus, were constantly flushed with water that contained pathogens (Callewaert and Michiel, 2010). The antimicrobial activities of two lysozymes from *V. philippinarum* (rVpCLYZ-1 and rVpCLYZ-2) are investigated against *Staphyloccocus aureus*, *Micrococcus luteus*, *Vibrio anguillarum*, *Enterobacter cloacae*. rVpCLYZ-1 displays broad spectrum antibiotic activities, and they possess strong microbicidal activities against *M. luteus* and *V. anguillarum*. rVpCLYZ-2 has strong inhibitory activity against all detected bacteria, but is less effective against *P. pastoris* KM71. The turbidimetric assay is also performed to measure thelysozyme activity of rVpCLYZs against *M. luteus* and *V. anguillarum* (Yang *et al.*, 2017). The recombinant CpLYZ1 has bacteriolytic activity against *E. coli* DH5a, *A.*

hydrophila, *Staphyloccocus aureus*, *Streptococcus* sp. and *Staphylococcus epidermidis*, and the bacteriolytic activity of CpLYZ1 against *B. subtilis* is the strongest, while the relative activity is 50 %. Its relative activity against *E. coli* DH5 a, *A. hydrophila*, *S. aureus* and *Streptococcus* sp. is 19 % - 28 %, and against *S. epidermidis* is only 16 %. The bacteriolytic activity of standard lysozyme against *A. hydrophila*, *S. aureus*, *B. subtilis*, *Streptococcus* sp. and *S. epidermidis* are higher than the recombinant CpLYZ1, but its bacteriolytic activity against *E. coli* DH5 a is lower than the recombinant CpLYZ1(Wu *et al.*, 2013). Therefore, the lysozyme in gills of *V. philippinarum* shows strong antibacterial activity against Gram positive and Gram negative bacteria. The high expression level of mollusc lysozyme in gills implies that it has a significant contribution in prevention of microbial exploitation (Matsumoto *et al.*, 2006). However, some i-type lysozymes from *Venerupis philippinarum* and *Ruditapes decussates* also express in haemocytes, and exhibit antibacterial activity against gram-positive bacteria and gram-negative bacteria (Lopes C, 1997; Itoh *et al.*, 2007). Besides killing bacteria, the c-type lysozyme of *R. philippinarum* shows high antimicrobial activities, and the i-type lysozyme of *V. philippinarum* also has antifungi activity (Goto *et al.*, 2007). Most lysozymes exhibit muramidase activity, and also do chitinase activity- enzymatic hydrolysis of chitin to produce N- acetyl glucosamine (Yang *et al.*, 2017; Bathige *et al.*, 2013). The result is probably the similarity between peptidoglycan (heteropolymer of β-1,4 linked Nacetylmuramic acid and N-acetylglucosamine), the natural substrate of lysozymes, chitin (homopolymer of β-1,4 linked N-acetylglucosamine), and the natural substrate of chitinases. Besides warding off pathogenic bacteria infections, the lysozymes have also other clear function of the chitinase activity, which of *V. philippinarum*, *Tapes Japonica* and *Crassostrea virginica* are reported to possess chitinase activity, (McHenery and Birkbeck, 1982; Ito *et al.* 1999; Nilsen *et al.* 1999; Miyauchi *et al.* 2000; Xue *et al.* 2004). The quaternary structure in Vp-ilys crystal is revealed dimer formation by *Venerupis philippinarum* lysozyme (Vp-ilys) molecules, which is assumed to result from the dissociation of the Vp-ilys dimer at high ionic strength with a high salt concentration (≥ 133 mM NaCl), thereby increasing chitinase and muramidase activity (Goto *et al.*, 2007). The activity of lysozyme originated from *glycosidic hydrolases* is powerful to hydrolyze PGN and chitin (Takeshita *et al.*, 2003; Goto *et al.*, 2007; Callewaert and Michiels, 2010). The degradation of PGN and chitin in bacterial cell wall may lead to rapid killing of bacteria and fungi (Elmogy *et al.*, 2015).

The lysozymes serve as the function of important digestive enzymes in some animals (Dobson *et al.*, 1984; Stewart *et al.*, 1987; Lemos *et al.*, 1993; Kornegay *et al.*, 1994; Hultmark *et al.*, 1996; Prager, 1996). While the enzymes are present in a high concentration, they are a major digestive enzyme in the true stomach of ruminants (Dobson *et al.*, 1984; Jollès and Jollès, 1984; Irwin, 1996). Three-type lysozymes of molluscs are detected in digestive systems, and are regarded as digestive lysozymes (Nilsen *et al.*, 1999; Olsena *et al.*, 2003; Zhao *et al.*, 2007). Digestive gland has an important lymphoid site in molluscs, and the hepatopancreas may act as a major site for the production of lysozymes (Mchenery *et al.*, 1979; Jollés *et al.*, 1996; Tan *et al.*, 2007). The i-type lysozyme of *C. gigas* plays complementary role in digestive organs, it has been reported that the basophil cells have an intense enzyme activity, demonstrating that lysozyme is synthesized in the digestive tubule basophil cells. The i-type lysozyme genes in the hepatopancreas of *Hyriopsis cumingii* are down-regulated, which can inhibite bacteria to attack the host immune organs, and also promote the acid digestion of bacteria in molluscs (Zhang *et al.*, 2010). Therefore, bacteria may protect themselves from lysozyme-induced digestion by down-regulating i-type lysozyme genes.

The nutriments of molluscs are harvested to produce by autotrophic bacteria, the c- and i-type lysozyme of *C. farreri* are detected to serve as digestive lysozymes in digestive tract (Nilsen *et al.*, 1999; Olsena *et al.*, 2003; Zhao *et al.*, 2007). It is postulated that lysozymes of deep-sea bivalves are similar to that of ruminants in digestive function (Jollès *et al.*, 1996). The lysozyme of *M. edulis* is also involved in digestion, since lysozymes from the digestive gland-associated crystalline style are believed to be purified from the digestive gland (Olsen *et al.*, 2003). In two i-type lysozymes (Cv-iLys1, 2) of eastern oyster *C. virginica*, Cv-iLys2 is mainly found in the digestive gland, which is lower amounts in the crystalline style, and is expressed in basophil cells of digestive tubules. In contrast, Cv-iLys1 is mainly found in lips and mantle, and is lower amounts in gills, style sac, midgut, digestive gland and gonads (Zobel *et al.*, 1938; McHenery *et al.*, 1985; Langdon *et al.*, 1990). The molluscs are also ability to utilize bacteria as food. The deepwater molluscs rely on symbiotic bacteria in gills for nutrition (Jollès *et al.*, 1996). The biochemical and molecular information about mollusc lysozymes is obtained from digestive systems (McHenery *et al.*, 1979; Jollès *et al.*, 1996; Ito *et al.*, 1999; Miyauchi *et al.*, 2000; Olsen *et al.* 2003; Liu *et al.*, 2006). The lysozymes of molluscs not only possess combined features of immunity and digestion, but also can inhibit gram-positive and gram-negative bacteria. Therefore, it is suggested that the digestive lysozymes apparently evolve from parallel in different species, and acquire the ability to function in highly acidic and protease-rich environments (Jollès *et al.*, 1984; Stewart *et al.*, 1987; Kornegay *et al.*, 1994; Prager, 1996; Regel *et al.*, 1998). The lysozyme can also induce regulation of the synthesis and secretion of other immune factors in vivo of animal software (Zobel *et al.*, 1983), and involve in digestion, promoting reproduction, stimulating growth, and cancer related functions, besides the common function of lysis of bacterial and fungal cell wall (Irwin, 2004; Zhang *et al.*, 2005; Kanda *et al.*, 2007). The lysozyme of *O. hupensis* not only has the function of resisting the removal of foreign pathogenic microorganisms, but also does the function of hydrolyzing fibrin. Other potential activities include isopeptidase activity and perhaps chitinase activity that is detected in both c-type (Chipman and Sharon, 1969; Callewaert and

Michiels, 2010) and i-type lysozymes (Jollès and Jollès, 1984; Takeshita *et al*., 2003; Goto *et al*., 2007; Xue er al., 2007). However, molluscs constantly encounter various potential pathogenic microorganisms in their living environment, and the content of lysozyme is affected by a variety of environmental factors and pathogens (Irwin *et al*., 1996). The lysozyme of *M. meretrix* shows strongly antibacterial activity against gram-positive and gram-negative bacteria, and the gene expression of lysozyme increases following *Vibrio parahaemolyticus* challenge, the recombinant g-type lysozyme shows strong antibacterial activity against *Micrococcus luteus* (Xin *et al*., 2011). The expression levels of c-type lysozymes increase after bacterial (*Vibrio anguillarum*) stimulation from *V. philippinarum*, *H. discus hannai* and *Cyclina sinensis*, and the recombinant lysozyme also shows bacteriolytic activity against both gram-positive and gram-negative bacteria (Goto *et al*., 2007; Yang *et al*., 2017). The two lysozymes are identied from *V. philippinarum*, the recombinant proteins of lysozymes (rVpCLYZ-1 and rVpCLYZ-2) possess strong microbicidal activities against *M. luteus* and fungi. Comparison with rVpCLYZ-1 and rVpCLYZ-2, the lysozyme from chicken egg-white shows lower activity against *M. luteus* (Yang *et al*., 2017). The mRNA expression of i-type lysozymes from *M. galloprovincialis* can be induced by *Vibrio anguillarum* (Hui *et al*., 2008). The lysozymes of *R. philippinarum* are designed as RpiLYZ-1, RpiLYZ-2, the expression of RpiLYZ-1, 2 are induced after *Vibrio anguillarum* stimulation, VpLYZ mRNAs are down-regulated sharply from 6 to 12 h post-infection. Then, the expression level increase to the peak at 72 h, and recover to the original level at 96 h (Yang *et al*., 2017). Therefore, mollusc lysozymes have obvious antibacterial activity against *V. anguillarum* (Bassem *et al*., 2006; Pan *et al*., 2010; Yue *et al*., 2011). While *O. hupensis* is infected by schistosome, the g-lysozyme gene expression significantly increase (Zhu *et al*., 2016), *P. acuta* (PALysG) possess to inhibite capacity against *M. lysodikicus*, and *C. farreri* (CFLysG) can not inhibite *S. aureus* (Zhao *et al*., 2007). These results reveal that the c-type lysozyme is involved in the non-specific immune of molluscs. The external environment parameters, such as pH, temperature, and ion strength, can influence on the lytic activity of lysozymes (Ye *et al*., 2010). Generally, the optimal pH of the lytic activity is below 7 from mollusc lysozymes, c-type of *M. galloprovincialis* and *R. philippinarum*, g-type of *O. hupensis,* i-type of *Crassostrea virginica* (Umasuthan *et al*., 2013; Wang *et al*., 2013). While pH is less than 7, the lytic activity of g-type mollusc lysozymes changes to follow pH (Huang, 2014). However, the optimal pH of the lytic activity is generally ranging from 7 to 10 from c-type lysozymes of mammal and chicken (Hui *et al*., 2017; Yang *et al*., 2017). Moreover, high lytic activities are detected at pH 9.5 - 10. Similar phenomenon is also observed in lysozyme from chicken egg white with high activity at both pH 6.2 and 9.2 (Davies *et al*., 1969). The existence of a wide range of optimal conditions for the activity of c-type lysozyme is suggested that these conditions are perhaps species-specific (Bathige *et al*., 2013)**.**

The antibacterial activity of lysozyme in *O. hupensis* is examined. While the temperature is less than 50 ℃, the activity of lysozyme changes to follow temperature. Therefore, the optimum temperature of lysozyme activity was 50 ℃, and the optimum pH was 7.0 (Saurabh *et al*., 2008; Ye *et al*., 2008). At temperature ranging from 15 ℃ to 50 ℃, while the temperature increased, the bacteriolytic activity of i-type lysozyme from *Cristaria plicata* gradually increased. The relative activity declined when the temperature was above 50 ℃. The effect of pH on the enzyme of *Cristaria plicata* between pH 4.5 - 8.5 shows that pH of the highest activity was 5.5. The optimal pH and temperature for the enzyme activity of *C. plicata* were 5.5 and 50 °C (Wu *et al*., 2013; Dai *et al*., 2015). Meanwhile, the activity of i-type lysozyme from *V. philippinarum* is high in low temperature, and the optimal temperature is 20 ℃. The lysozyme of *V. philippinarum* has activity at low temperature, which is in agreement with the characteristic of coldblooded aquatic animals (Yang *et al*., 2017). The expression profiles of mollusc lysozymes further indicate the coexistence of multiple types of lysozymes in molluscs.

The most known function of lysozyme is antibacterial activity by catalyzing the hydrolysis of bacterial cell walls, and can kill bacteria using non-enzymatic bactericidal domains (Dobson *et al*., 1984; Stewart *et al*., 1987; Lemos *et al*., 1993). Meanwhile, the mechanisms of action are different for gram-positive bacteria and gram-negative bacteria, the cell walls of gram-positive bacteria are exposed so that lysozyme can act directly on the cell walls and cause lysis of cell walls, and the cell wall components of gram negative bacteria, such as lipopolysaccharide (LPS), PliI and MliC/PliC, affect the cell wall of bacteria (Callewaert *et al*., 2008; Vanderkelen *et al*., 2011). Therefore, lysozyme should be combined with other components of the immune system in order to lysis the cell wall structure of gram negative bacteria, resulting in bacterial lysis death (Cheetham *et al*., 1992). The lytic activity of lysozyme against bacteria and fungi is suggested to be associated with the muramidase and chitinase activities. The c-type lysozymes typically possess muramidase activity that cleaves the β-1, 4-glycosidic bond of peptidoglycan (PGN) in microbial cell walls, and cause the lysis of bacteria (Vocadlo *et al*., 2001; Supungul *et al*., 2010). The lysozyme is also served as a model for studies on enzyme structure and function (Peters *et al*., 1989; Prager and Jollès, 1996). Typical i-type lysozymes exhibit muramidase activity and generate bactericidal activity by hydrolyzing the cell wall, which show bacteriolytic activity against both Gram-positive and Gram-negative bacteria **(**Zhao *et al*., 2010; Zhou *et al*., 2017).

Conclusion and perspective

Lysozymes are present in variety of organisms, ranging from viruses to plants and animals. Although all lysozymes perform the same enzymatic function, and exhibit overall similarity in three dimensional (3D) structures, the primary amino acid sequences of these lysozymes is rarely the same. It is speculated that the 3D structure and function of the enzymes

are analogous, and the genes of the enzymes are not homologous. The various types of lysozymes are generated by convergence during evolution, and can coexist in the same taxon. For example, the c- and g-type lysozymes are in vertebrates. The c- and i-type lysozymes are present in arthropod, the c-, i- and g-type lysozymes exist in molluscs. The question of evolutionary relationship is raised among different types of lysozymes.

The phylogenetic tree analysis shows that i-type lysozyme is more closely related to c-type one than g-type one in molluscs. The partial sequence of i- and c-type lysozyme gene is homology. The central exon of lysozyme genome from *M. galloprovincialis* is homologous to the second exon of that from chicken, and both belong to the c-type lysozyme (Wang *et al.*, 2013). It is suggested that c- and i-type lysozyme belong to the near - edge parallel macromolecules, and is believed that c- and i-type lysozyme gene evolved from a single complete gene. The i-type of *C. gigas*, g-type of *H. discus discus* and *M. galloprovincialis*, was also clustered to the corresponding subgroup in the phylogenetic tree. However, the other evolutionary relationship of three type lysozymes also is supposed (Jollès and Jollès, 1984; Bachali *et al.*, 2002). Some studies assumed that i-type lysozymes were more closely related to g-type lysozymes, and suggested that c-type was basal (implication ancestral) to g- and i-type lysozymes (Hikima *et al.*, 2003). The i- and g-type lysozymes diverge from an ancestor of c-type. Others believe the g-type lysozyme is considered as the common ancestor to c- and i-type ones (Thunnissen *et al.*, 1995). The i-, g- and c-type lysozymes are detected in molluscs, and this may provide some clues to clarify the relationship of the three types of lysozyme (Xin *et al.*, 2011). These results consist with the notion that the three type lysozymes diverge from a common precursor, and c-type lysozyme is closed to the ancestor. Further, the molluscs encounter a greater range of bacterial strains or species in the marine environment, and the varied composition and structure of the bacterial cell wall may promote a type of 'substrate-induced evolution' of lysozymes (Jollès and Jollès, 1984).

Two conserved amino acid Glu^{54} and Asp^{70} are critical for the c-type lysozyme lytic activity to bacterial cell wall, and the motif that flanking Asp^{70} is also conserved in the c-type lysozyme (Vocadlo *et al.*, 2001). These results indicate that the mature c-type lysozyme of molluscs may possess the antimicrobial activity as well as that of other species. Other potential activities, such as isopeptidase activity and perhaps chitinase activity, are detected in c-type lysozymes (Chipman and Sharon, 1969; Callewaert and Michiels, 2010). In conclusion, the c-type lysozymes are characterized from some molluscs, and their expression profiles and antimicrobial activities are also investigated. These results provide helpful evidence for further understanding the innate immunity of molluscs. More investigation should be directed to understand the interaction mechanisms of c-type lysozymes with membranes or cell walls of bacteria. *O. ocellatus* has three conservative enzyme activity center (Glu^{40}, Glu^{49}, Ser^{52}) and 12 conserved cysteines that form 4 pairs of protein disulfide bonds and the stable conformation. The characteristics of i-type mollusc lysozyme structure possess two catalytic domains exhibiting muramidase and isopeptidase activities (Jollès and Jollès, 1984b; Ito *et al.*, 1999; Takeshita *et al.*, 2003; Xue er al., 2007; Goto *et al.*, 2007).

Although lysozyme research is described in 1960s, the data about lysozyme is increasingly abundant. So far, the lysozymes are studied to remain one of the hot spots in life science, that of some animals has been studied more thoroughly, and that of molluscs still needs further to do improvement. The origin and evolution of mollusc type lysozyme will especially require more experimental data and bioinformatic analyses.

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