Lysozymes in molluscs

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ABSTRACT

Invertebrates lack antibody-mediated humoral immune systems; however, they are believed to possess efficient host defense mechanisms involving humoral defense molecules that are similar in function to antibodies. Lysozymes are a group of enzymes that cleave the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine (two amino sugars) in the peptidoglycans that form bacterial cell walls. In bivalves, lysozymes are especially important antibacterial molecules because of their bactericidal ability. Recently, the presence of multiple lysozymes has been found in several species of bivalve molluscs such as the blue mussel, *Mytilus edulis*, and the eastern oyster, *Crassostrea virginica*. Therefore, to determine the molecular and biochemical properties of bivalve lysozymes, we have identified the cDNA sequences of three different lysozymes (CGL-1, -2, and -3) from the Pacific oyster, *C. gigas*, and have produced recombinant lysozymes (rCGL) using the methylotrophic yeast *Pichia pastoris*. The lysozyme CGL-1 mRNA was expressed in all tissues except for those of the adductor muscle. In contrast, CGL-2 gene was only expressed in digestive diverticula. Interestingly, in digestive diverticula, CGL-1 gene expression was detected in the same digestive cells as that of CGL-2. It is therefore possible that CGL-1 and CGL-2 play complementary roles in digestive organs. *In situ* hybridization revealed that CGL-3 mRNA was highly expressed in the mantle and haemocytes. These results suggest that CGL-1 and -2 serve as digestive enzymes for enteric and engulfed bacteria in digestive organs and that CGL-3 is involved in biodefense against invading microbes. Based on the results from experiments using recombinant lysozymes, we found significant differences among the characteristics of the three lysozymes, suggesting that these lysozymes have different functions in *C. gigas*.

Key words: biodefense mechanism, lysozyme, oyster, *Crassostrea gigas*, bivalve molluscs

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INTRODUCTION

Bivalve molluscs and microorganisms coexist in the biosphere in numerous ways. Thus, bivalves have evolved sensitive mechanisms for recognizing pathogens and an array of strategies to defend themselves against attacks by microorganisms such as bacteria, fungi, and parasites. However, bivalve molluscs, as well as other invertebrates, lack an antibody-mediated adaptive immune system. They have an innate immune system that comprises haemocytes and non-specific humoral defense molecules. Therefore, to combat infection, bivalves rely on multiple defense reactions. The point of biodefense mechanisms is to recognize and eliminate various types of pathogens. Some defense molecules are able to respond to typical microbial pathogen-associated molecular patterns (PAMPs), and these molecules are used to distinguish pathogens from host cells. In insects, for example, peptidoglycan recognition proteins (PGRPs) play a crucial role in the recognition of bacterial pathogens and in the induction of various immune reactions. However, in bivalve molluscs, although the genes of PGRP have been cloned and sequenced from the scallops *Argopecten irradians* and *Chlamys farreri* (Ni *et al.*, 2007; Su *et al.*, 2007) and from the Pacific oyster, *Crassostrea gigas* (Itoh and Takahashi, 2008; 2009), their functions are still unclear. Although humoral responses to bacterial pathogens remain unknown in bivalves, it is considered that lectins might be the main recognition molecules as they function as a binding receptor for pathogens and as an opsonin for phagocytosis by haemocytes (summarized by Muroga and Takahashi, 2007). Lysozymes are a cleaving enzyme that break the glycosidic bonds in bacterial peptidoglycans, and these enzymes have been found in many species of bivalves and are considered to be one of the most important types of defense molecule.

BRIEF REVIEW ON BIVALVE MOLLUSC LYSOZYME STUDIES

Lysozymes (EC 3.2.1.17) occur in a wide variety of cells, tissues, and secretions from bacteriophages to mammals. They are a family of glucoside hydrolases that cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycans forming bacterial cell walls. Thus, lysozymes are bacteriolytic enzymes and play a major biological role in biodefense, as these enzymes can act as antibacterial and immune-modulating agents. In addition, lysozymes function as important digestive enzymes in some animals. Since Mcdade and Tripp (1967a, b) reported the detection of lysozyme activity in the hemolymph and skin mucus from the American oyster, *C. virginica*, lysozyme and lysozyme-like activity have been found in various bivalve molluscs (summarized by Muroga and Takahashi, 2007).

Three families of lysozymes have been identified in animals, they are the chicken type (c-type), goose type (g-type), and a new type of lysozyme; i.e., the invertebrate type (i-type). The existence of the i-type lysozyme was first proposed as a new type of lysozyme based on analysis of the quantitative amino acid composition and the N-terminal sequence of a lysozyme from the common starfish, *Asterias rubens* (Jollès and Jollès, 1975). In the late 1990s, a lysozyme from the clam *Tapes japonica* (*Ruditapes philippinarum*) was isolated, and its complete amino acid sequence at the protein level was determined

(Ito *et al*., 1999). This was the first bivalve mollusc lysozyme to be identified as an i-type lysozyme. Subsequently, cDNA coding lysozymes have been cloned and sequenced from the Icelandic scallop, *Chlamys islandica* (Nilsen *et al*., 1999; Nilsen and Myrnes, 2001) *T. japonica* (Takeshita *et al*., 2004), *C. gigas* (Matsumoto, Nakamura and Takahashi, 2006; Itoh and Takahashi, 2007), *C. virginica* (Itoh *et al*., 2007; Xue *et al*., 2007), and the Mediterranean mussel, *Mytilus galloprovincialis* (Li *et al*., 2008). Alignment and phylogenic analyses using six bivalve lysozymes have demonstrated that i-type lysozymes form a monophyletic family (Bachali *et al*., 2002). The gene of a lysozyme from *C. islandica* (designated chlamysin) was shown to have four exons of 38 to 252 bp separated by large introns (Nilsen and Myrnes, 2001). The overall gene organization of chlamysin resembled that of the c-type lysozyme genes found in invertebrates, but it differed from the invertebrate c-type lysozyme genes by having a three-exon structure. Furthermore, based on crystal structure and mutation analyses of *T. japonica* lysozyme (TJL), Goto *et al*. (2007) demonstrated that TJL formed a dimer by electrostatic interactions between catalytic residues (Glu-18 and Asp-30), and that the TJL dimer (inactive form) remained intact under low salt concentrations but that it dissociated into TJL monomers (active form) under high salt conditions. Moreover, with increasing salt concentrations, the chitinase activity of TJL markedly increased. In general, the lytic activity of bivalve lysozymes has been shown to be sensitive to the ionic strength and salt concentration of solutions (Muroga and Takahashi, 2007).

By using enzymatic analyses, the functions of bivalve lysozymes were estimated as digestion (McHenery and Birkbeck, 1979; McHenery, Birkbeck and Allen 1979; Jollès, Fiala-Médioni and Jollès, 1996) and host defense (Jollès and Jollès, 1984). Bacteria are the chief source (nitrogen and phosphorous) of food in bivalve molluscs as well as in other invertebrates. Bivalve molluscs are also classically known to feed through heterotrophic processes on marine bacteria and/or other planktonic organic materials filtered from seawater. In addition, Jollès, Fiala-Médioni and Jollès (1996) suggested a digestive role for the lysozymes in deep-seawater bivalve molluscs, which rely on symbiotic bacteria in their gills for nutrition. In fact, digestive lysozymes from many bivalve species were isolated or purified from the digestive glands and crystalline styles, e.g., *M. edulis* (McHenery and Birkbeck, 1979; McHenery, Birkbeck and Allen, 1979; Olsen *et al*., 2003); *T. japonica* (Ito *et al*., 1999); *C. virginica* (Xue *et al*., 2007); *C. islandica* (Nilsen *et al*., 1999); and the brackish water clam *Corbicula japonica* (Miyauchi, Matsumiya and Mochizuki, 2000). The crystalline style is a rod-shaped organ in the bivalve stomach in which various digestive enzymes are stored in high concentrations. In *T. japonica*, TJL may be rendered inactive by dimer formation in the crystalline style under highly concentrated conditions (Goto *et al*., 2007). When bivalves feed, they ingest bacteria present in the seawater, which has a high salt concentration. With increasing salt concentration in the stomach, the inactive TJL dimer is converted to the active TJL monomer (Goto *et al*., 2007). Recently, the presence of multiple lysozymes with different biochemical properties has been demonstrated in *M. edulis* (Olsen *et al*., 2003) and *C. virginica* (Xue *et al*., 2004; Xue *et al*., 2007). A *C. virginica* lysozyme purified from plasma (CVL-1) was found to be unique in its N-terminal amino acid sequence and showed optimal activity at high ionic strength (Xue *et al*., 2004). CVL-1

possesses strong antimicrobial activity, which suggested that its main role is in host defense (Itoh *et al*., 2007). Furthermore, a different lysozyme, designated CVL-2, was purified from the crystalline styles and digestive gland in *C. virginica* (Xue *et al*., 2007). CVL-2 showed high amino acid sequence similarity to other bivalve lysozymes, but its biochemical and molecular properties, distribution in the oyster body and site of gene expression suggested that its role was in digestion (Xue *et al*., 2007). These facts indicate that each species of bivalve mollusc possesses multiple lysozymes in various tissues such as the hemocytes and plasma or the digestive gland and crystalline style and may use different lysozymes for different functions (host defense or digestion, for example). These findings also indicate that the application of bacteria lysis tests using *Micrococcus luteus* (*lysodeikticus*) in classical studies is of limited use for characterizing unpurified lysozyme because a mixture of multiple lysozymes might be measured and characterized as lysozyme activity. However, it is never made particularly clear what that distinction between digestive and immune lysozymes. Thus, to better understand the physiological roles of lysozymes, it is necessary to identify each lysozyme molecule and determine its molecular and biochemical properties.

RECENT ADVANCES IN THE FIELD OF OYSTER LYSOZYMES AND THEIR ROLE IN BIODEFENSE MECHANISMS

The Pacific oyster, *C. gigas*, is the most important commercial species in the world and is a physiologically well-studied bivalve species. The lysozyme activity of *C. gigas* was first recognized by Mori *et al.* (1980) as bactericidin. Takahashi, Mori and Nomura (1986) concluded that lysozyme is a chief component of bactericidin, and Matsumoto, Nakamura and Takahashi (2006) identified a cDNA sequence of lysozyme (CGL-1) from *C. gigas*. However, it remained unknown whether *C. gigas* possesses multiple lysozymes, and it was still unclear whether the lysozymes in *C. gigas* were able to function as a digestive enzyme and/or as a host-defense molecule. Thus, to clarify the function of lysozymes in host defense mechanisms in *C. gigas*, we have been investigating molecular and biochemical characteristics of lysozymes since 2007. We have attempted to clone and identify each lysozyme cDNA; to demonstrate sites of lysozyme gene expression; to produce recombinant lysozymes; to characterize the properties of recombinant lysozymes; to purify each lysozyme molecule; and to determine the tissue distributions of each lysozyme molecule.

We obtained and identified a second lysozyme cDNA from the digestive gland of *C. gigas* (Itoh and Takahashi, 2007). A 536-bp of cDNA encoding a novel lysozyme was identified and designated as CGL-2. The encoded lysozyme is comprised of 142 amino acid residues including 20 residues of a signal peptide. The amino acid sequence of CGL-2 was different from that of CGL-1. CGL-1 was expressed in multiple tissues of *C. gigas*, especially the mantle, hemocytes, and digestive gland (Matsumoto, Nakamura and Takahashi, 2006). The CGL-2 has a lower isoelectric point and fewer peptide recognition sites; *in situ* hybridization revealed CGL-2 mRNA transcription in the digestive cells of the digestive tubule, suggesting that CGL-2 has evolved to adapt to digestive environments. Moreover, CGL-1 gene expression was also detected in the same digestive cells as CGL-2 (Itoh and Takahashi, 2007). It is predicted that CGL-1 and CGL-2 play complementary roles

in the digestive organs in *C. gigas*. To investigate the biochemical characteristics of each lysozyme, we produced recombinant CGL-1 and CGL-2 (rCGL-1 and rCGL-2) using the methylotrophic yeast *Pichia pastoris*. rCGL-1 showed relatively high activity within a broad range of pH and ionic strengths (Fig. 1). The maximum activity of rCGL-1 was detected in salt concentrations in excess of 150 mM. In contrast, rCGL-2 expressed more than 90% of its maximum activity in the pH range of 5.4 to 6.4 when the salt concentration was very low (Fig. 1). rCGL-2 activity was almost completely inhibited at higher ionic strengths.

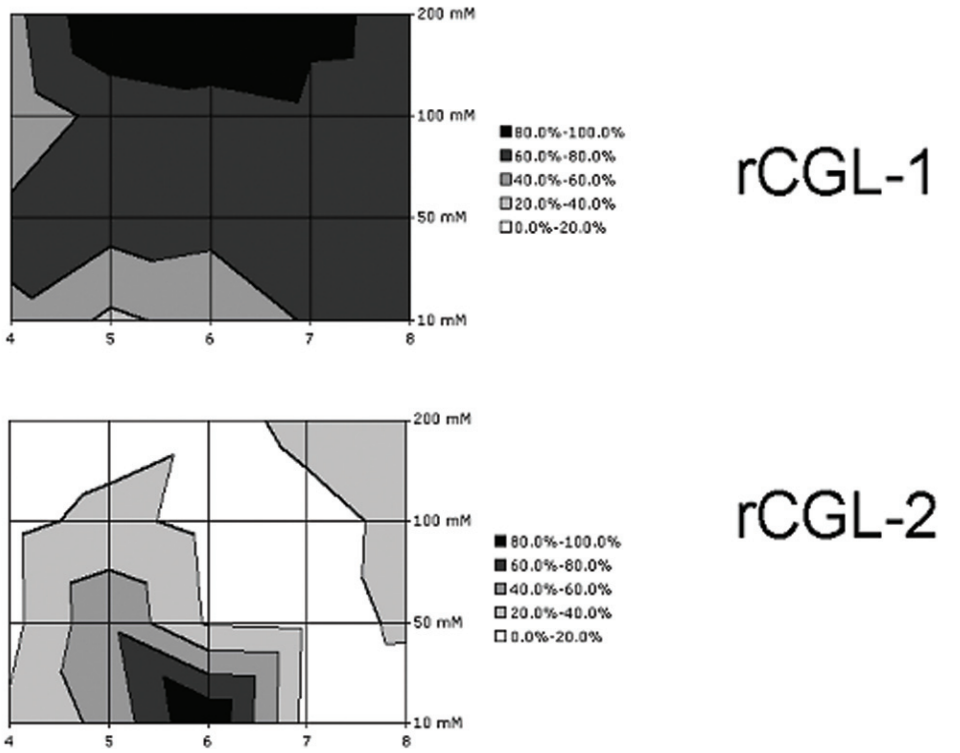

Figure 1. Optimal pH and ionic strength for activities of recombinant lysozymes (rCGL-1 and rCGL-2) produced by yeast *Pichia pastoris*.

We also cloned and identified a third lysozyme cDNA from the mantle tissues in *C. gigas* (Itoh and Takahashi, unpublished). This lysozyme was designated CGL-3. A protein-protein BLAST search in GenBank revealed that the amino acid sequence deduced from the CGL-3 cDNA is homologous to bivalve i-type lysozymes (Fig. 2). CGL-3 has a signal peptide sequence comprising 20 residues, and mature CGL-3 consists of 122 amino acid residues. Furthermore, multiple alignments with bivalve i-type lysozymes showed that the important residues responsible for lysozyme activity, Glu34, Asp45 and Trp61, were present in CGL-3. Thus, it is considered that all three lysozymes, CGL-1, CGL-2, and CGL-3, have true lysozyme activity in the body of *C. gigas*. We also measured the antibacterial activities of the three recombinant lysozymes. Results are expressed as the minimum concentration (MIC) of lysozymes that significantly inhibited bacterial growth.

As shown in Table 2, antibacterial activity differed greatly among the three lysozymes. rCGL-3 revealed the strongest activity, as it significantly inhibited the growth of *Escherichia coli* at a concentration of 1.6 µg/ml. rCGL-2 was significantly less effective than both rCGL-1 and rCGL-3. A comparison of the molecular and biochemical characteristics of CGL-1, CGL-2, and CGL-3 is shown in Table 1.

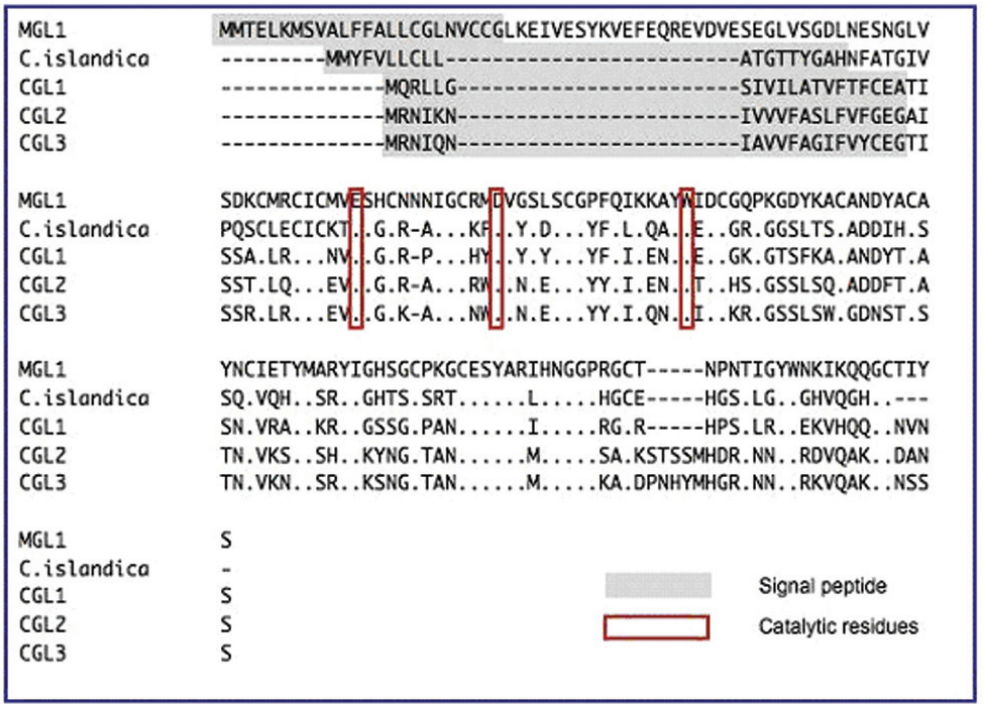

Figure 2. Multiple amino acid sequence alignments of the CGL in *C. gigas* and representative bivalve i-type lysozymes. The residues of possible signal peptides are shaded in bold letters. Catalytic residues for lysozyme activity are enclosed with red squares. The sequences used for alignment were from *Mytilus galloprovincialis* MGL1 (AAN16210); *Chlamys islandica* (CAC34834); and *C. gigas* CGL-1 (BAD19059), CGL-2 (BAF48044), and CGL-3 (BAF94156).

Significant differences were found in the characteristics of the three lysozymes, suggesting that they have different functions in *C. gigas*. Firstly, CGL-2 was mainly expressed in the digestive gland. Secondly, the lower isoelectric point of CGL-2, compared to those of CGL-1 and CGL-3, suggests that CGL-2 functions in an acidic environment. Thirdly, the number of arginine residues and protease cutting sites in CGL-2 was lower than in the other two lysozymes, suggesting that CGL-2 has an increased resistance to proteolytic digestive enzymes and has adapted to digestive conditions. Thus, we concluded that the main role of CGL-2 is related to digestion. In contrast, CGL-1 showed high activity in relatively high salt concentrations, suggesting that CGL-1 is better suited to function in the mantle tissues and the hemolymph, where pH and salt concentrations are usually high. Therefore, it is suggested that CGL-1 functions as both a host defense molecule and a digestive enzyme. CGL-3 was

Table 1
Comparison of CGL-1 molecule characteristics with those of CGL-2 and CGL-3

	CGL-1	CGL-2	CGL-3
Molecular weight	13079.5	13494.7	13698.2
Predicted pl	8.03	6.03	8.27
Total AA residues	117	122	122
No. argenine residues	8	5	7
Protease cutting sites			
Trypsin	1	10	14
Thermolysin	22	18	18
Tissue expression	Multiple	Digestive gland	Mantle
Optimal salinity	>150mM	<20 mM	ND

Table 2
Antibacterial activity of recombinant CGL

Antibacterial activity (MIC, µg/ml)	rCGL-1	rCGL-2	rCGL-3
Escherichia coli	12.5	50	1.6
Vibrio tubiashii	50	100	100
Marinococcus halophilus	0.8	2.5	0.8

found to be mainly synthesized in the mantle and hemocytes. In addition, CGL-3 showed the highest antibacterial activity among the three lysozymes. Thus, we propose that the main role of CGL-3 is related to host defense. However, we were unable to elucidate the function of each lysozyme in *C. gigas* because it is possible that the lysozymes can perform more than one function simultaneously.

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