[2] Fish and Shellfish Bio-Defense

inhibiting bacterial or viral activities, protection against stress, elimination of leftover, damaged or infected harmful cells, microbe recognition, activation of signaling pathways involve in immune responses and in maintaining normal aerobic metabolism.

Glossary

AMPs: Antimicrobial Proteins or Peptides

4. SHELLFISH BIO-DEFENSE

Keisuke G. Takahashi, Naoki Itoh and Makoto Osada

4.1. Synopsis

Human has exploited shellfish as important bio-resources for multiple purposes; for example, seafood and pearl production. Aquaculture of shellfish is one of the most important fishery industries worldwide. Therefore, interest in shellfish immunity has developed due to the importance of aquaculture and their role in the aquatic environment. Shellfish, as well as other invertebrates, do not possess adaptive immunity. Therefore, to combat infection, shellfish rely on an innate immune system, which is comprised of multiple bio-defense reactions employing circulating hemocytes and multiple defense molecules. Circulating hemocytes, which possess strong migration ability in response to invading microorganisms and subsequently actively phagocytose these invaders, are the most responsible in bio-defense in shellfish. Humoral defense factors comprise molecules of two types, those which act in bio-defense with the recognition of pathogenic microorganisms and those that mediate microbial killing and macromolecular degradation.

4.2. Introduction

Shellfish belongs to the phylum Mollusca and is mainly comprised of bivalves and gastropods. The Phylum Mollusca is one of the largest and numerous groups in the animal kingdom. Shellfish 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. An oft-asked question is how invertebrates including shellfish survive against pathogenic microorganisms without an adaptive immune system. Indeed, invertebrates do not have lymphocytes and do not produce antibodies (Loker et al, 2004; Rowley and Powell, 2007). They have only an innate immune system that comprises hemocytes and non-specific humoral defense molecules (Bachère et al, 2004; Song et al, 2010). Therefore, to combat infection, bivalves rely on multiple bio-defense reactions. The point of bio-defense mechanisms is to recognize and eliminate various types of pathogens (Loker et al, 2004; Rowley and Powell, 2007; Bachère et al, 2004; Song et al, 2010). Circulating hemocytes, which possess strong migratory ability in response to invading microorganisms and subsequently actively phagocytose these invaders, are the most responsible factor in bio-defense in shellfish (Cheng, 1996; Hine, 1999). Humoral defense factors comprise molecules of two types, those which act in bio-defense with recognition and binding to typical microbial pathogen-associated molecular patterns (PAMPs), and those which mediate microbial killing and macromolecular

degradation (Gestala et al, 2008; Lemaitre and Hoffmann, 2007). It is considered, in invertebrates including shellfish, that the former might be lectins and peptidoglycan recognition proteins (PGRPs) and that the latter might be antimicrobial peptides (AMPs) and various defense-related enzymes such as lysozymes. Here, we review current knowledge of the innate immunity of shellfish, especially bivalve mollusks, focusing on phagocytosis by hemocytes, microbicidal reaction of lysozymes, and immune recognition.

4.3. Cellular Bio-Defense in Shellfish

4.3.1. Hemocytes

Shellfish hemocytes morphologically resemble mammalian phagocytic leukocytes and, like these leukocytes, have ability to recognize, engulf, and degrade pathogenic microorganisms (Cheng, 1996; Hine, 1999; Takahashi and Muroga, 2008; Canesi et al, 2002). Different forms and functions of bivalve molluscan hemocytes have been reviewed in depth (Cheng, 1996; Hine, 1999). A classification of the hemocytes has resulted in the recognition of two categories of cells, which have been designated as granulocytes and hyalinocytes (agranulocytes) (Figure. 4.1). Granulocytes are distinguished from other hemocytes by the presence of many cytoplasmic granules (Cheng, 1996; Canesi et al, 2002). Hyalinocytes meanwhile, are further classified into the following two subtypes: common hyalinocytes and small agranulocytes (Takahashi and Muroga, 2008; Canesi et al, 2002).

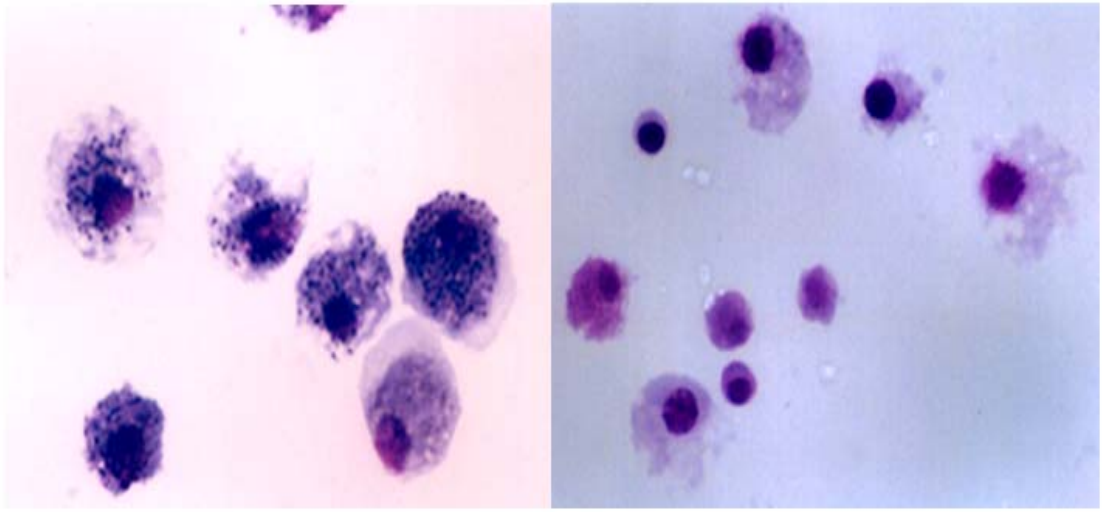

Figure 4.1. Photomicrographs of *C. gigas* hemocytes (×400). Left panel: Granulocytes. Right panel: Hyalinocytes.

Cheng (Cheng, 1996) described that the differences in ages, physiological states, and environmental factors influence the number of circulating hemocytes in each individual mollusks and cause large fluctuations in both the total number of hemocytes and the ratios between the hyalinocytes and granulocytes. Therefore, the establishment of baseline counts of hemocytes in oysters or other molluscan species is difficult. For instance, the hemocytic density in *C. gigas* hemolymph exhibited a remarkable seasonal change. The total hemocyte count in each *C. gigas* individual collected from the same

hanging-place in Onagawa Bay varied from 617 ± 149 (February, 2007) to 3,121 ± 267/mm^3 (June, 2007).

The proportion of hyalinocytes to granulocytes also varied during the year; however, the number of hyalinocytes was always greater than that of granulocytes. The hyalinocyte ratio varied from about 68.2% to 88.3% of the total number of hemocytes in *C. gigas* that were examined. In contrast, in the American oyster *C. virginica* hemocytes, the number of granulocytes is much greater than that of the hyalinocytes (agranulocytes). For instance, granulocytes comprised about 87.5% of the total number of hemocytes in *C. virginica* (Cheng, 1996).

4.3.2. Phagocytosis

The phagocytic process of hemocytes is characterized by the following four phases: (1) recognition of non-self materials, (2) binding of non-self materials to hemocytes (surface attachment), (3) engulfment of non-self materials into phagosomes, and (4) intracellular killing and degradation of non-self materials in most instances (Figure 4.2). In many species of bivalve mollusks, it is well documented that the hemocytes are capable of phagocytizing bacteria and subsequently degrade them intracellularly, suggesting that the presence of bio-defense mechanisms is mainly mediated by phagocytosis against invading bacteria (Takahashi and Muroga, 2008). Hine (1999), summarized the phagocytic characterization by both hyalinocytes and granulocytes: granulocytes exhibit a high phagocytic ability against various foreign particles; on the other hand, agranulocytes may have a non-phagocytic ability or a lower phagocytic ability than granulocytes.

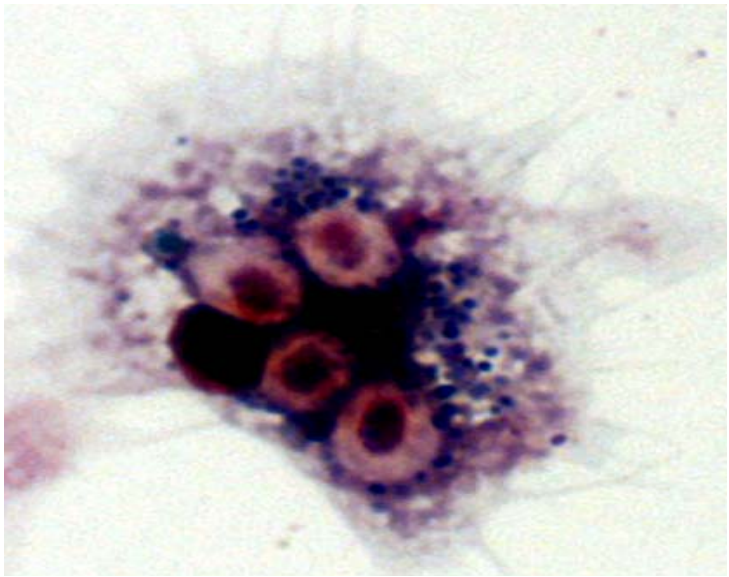

Figure 4.2. Photomicrograph of a *C. gigas* granulocyte phagocytosing yeast cells (×1000).

We examined the phagocytic ability of both hyalinocytes and granulocytes against three different particles. Both the hyalinocytes and granulocytes exerted phagocytic ability against all foreign particles tested (Figure 4.3). Granulocytes were more active phagocytes against *Escherichia coli* cells. Yeast cells were also extensively phagocytized

by granulocytes, but hyalinocytes showed little phagocytic activity for yeast cells. These results suggest that most foreign particles, if not all, are more actively phagocytized by granulocytes than by hyalinocytes.

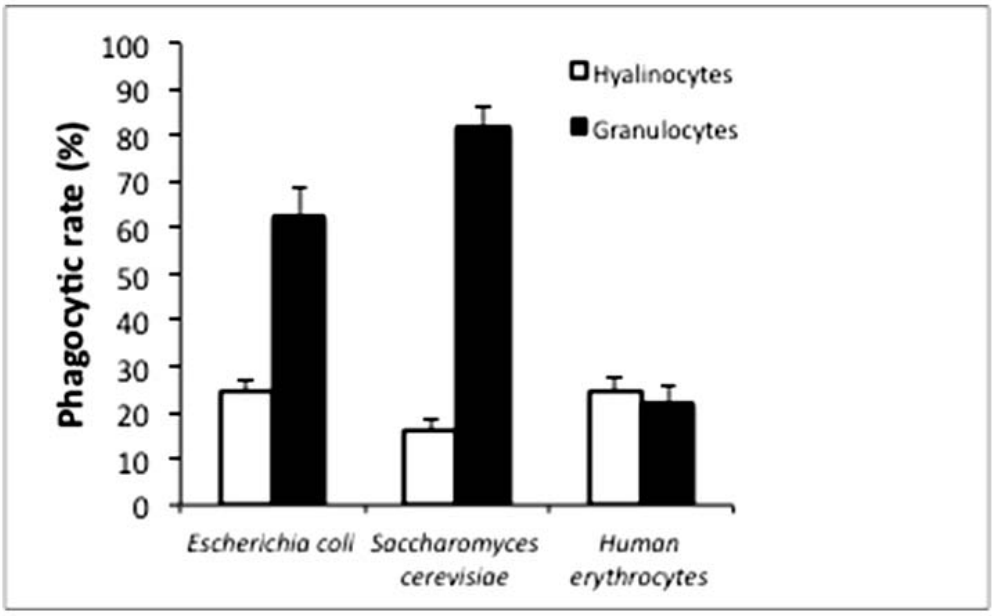

Figure 4.3. Phagocytosis of three different particles by hyalinocytes and granulocytes of *C. gigas*. The percent exhibiting phagocytosis (phagocytic rate) was calculated as number of hemocytes engulfing at least one particle/total number of hemocytes counted.

4.4. Humoral Bio-Defense in Shellfish

4.4.1. Microbicidal Factors

Lysozymes

Lysozymes (EC 3.2.1.17) occur in a wide variety of cells, tissues, and secretions from bacteriophages to mammals (Song et al, 2010). They are a family of glucoside hydrolases that cleave the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycans forming bacterial cell walls. Thus, lysozymes are bacteriolytic enzymes and play a major biological role in bio-defense, as these enzymes can act as antibacterial and immune-modulating agents (Takahashi and Itoh, 2011). In addition, lysozymes function as important digestive enzymes in some animals. Lysozyme activity was firstly detected in the hemolymph and skin mucus from *C. virginica*, and since then, lysozyme and lysozyme-like activity have been found in various bivalve mollusks (Song et al, 2010). Three families of lysozymes have been identified in animals: chicken type (c-type), goose type (g-type), and a new type of lysozyme; i.e., the invertebrate type (i-type) (Gestala et al, 2008).

By using enzymatic analyses, the functions of bivalve lysozymes were revealed to be involved in digestion and bio-defense (Takahashi and Itoh, 2011). Bacteria are the chief source (nitrogen and phosphorous) of food in bivalve mollusks as well as in other invertebrates. Recently, the presence of multiple lysozymes with different biochemical properties has been demonstrated (Gestala et al, 2008; Xue et al, 2010). For instance, 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. CVL-1 possesses strong antimicrobial activity, which suggested that its main role is in bio-defense (Gestala et al, 2008). Furthermore, a different lysozyme, designated 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, 2010). Moreover, a third lysozyme (CVL-3) was identified from shell liquor of *C. virginica* (Xue et al, 2010). The biochemical properties of CVL-3 suggest it represents a transitional form between CVL-1 and CVL-2 used for bio-defense and digestion (Xue et al, 2010).

4.4.2. Self/Non-Self Recognition Molecules

Peptidoglycan Recognition Proteins (Pgrps)

In bivalve mollusks, recognition of bacteria is achieved through the recognition and binding of specific forms of peptidoglycan (PGN) by peptideglycan recognition proteins (PGRPs). PGN, composed of *N*-acetylglucosamine and *N*-acetylmuramic acids, is an essential component of bacterial cell walls of both Gram-negative and Gram-positive bacteria. Since eukaryotic organisms do not contain PGN in their cellular structures, PGN is an ideal target molecule for detecting bacterial invasion in eukaryotic organisms. PGN is a highly complex and fast-evolving molecule with marked differences from one bacterium to another.

While vertebrate PGRPs are antimicrobial peptides, invertebrate PGRPs are involved in immune functions through more complicated ways (Lemaitre and Hoffmann, 2007). In *C. gigas*, we reported that four types of PGRPs have different tissue expression patterns, and suggested that these PGRPs are utilized to survey bacterial invasion in various tissues (Itoh and Takahashi, 2009). Additionally, some of them seemed to function as antimicrobial peptides to kill bacteria, like vertebrate PGRPs. Moreover, we have identified of a fifth PGRP cDNA from *C. gigas* (Itoh and Takahashi, 2009). This novel PGRP contained two domains, amidase/PGRP and goose-type (g-type) lysozyme. These findings suggest that the PGRP molecule may be a bi-functional protein, PGRP and lysozyme.

Lectins

Lectins are protein complexes with carbohydrate-specific binding properties that have been widely expressed in plants, invertebrates, and vertebrates and may serve a wide variety of physiological functions. Six lectin families have so far been identified; legume lectins, cereal lectins, P-type lectins, C-type lectins, galectins, and pentraxins. Of the latter four occurring in animals, galectins, pentraxins and C-type lectins are implicated in bio-defense (Arason, 1996). Lectins are good candidates for the recognition role because they can bind and opsonize foreign material with recognition specificity to PAMPs (Arason, 1996; Vasta et al, 1999). Therefore, lectins may act as an agglutinating molecule and opsonin for phagocytosis by hemocytes in bivalve mollusks (Arason, 1996; Vasta et al, 1999; Tasumi and Vasta, 2007). Additionally, it is believed that

bivalve C-type lectins have different carbohydrate-binding specificities and function to be a kind of antibody in non-self recognition (Song et al, 2010).

Invertebrate lectins have been demonstrated in the plasma of the hemolymph and bound to hemocyte membrane (Vasta et al, 1999; Tasumi and Vasta, 2007). Lectins have been isolated and characterized from the hemolymph of many species of bivalve mollusks (Vasta et al, 1999).

In marine bivalves, using potent invasive microorganisms such as marine bacteria requires investigation into the functional roles of lectins. For instance, in clam *Ruditapes philippinarum*, a C-type lectin MCL-4 enhanced the phagocytic ability of hemocytes to eliminate bacteria via recognition of terminal carbohydrate residues on the microbe surface (Song et al, 2010). In *C. gigas*, the hemolymph contains two erythrocyte lectins with the ability to agglutinate horse RBC (Gigalin E) and human RBC (Gigalin H), respectively. Gigalin E is a C-type lectin. Gigalin H has a high affinity for sialic acid residues in glycoprotein and has strong agglutinating activity against bacteria (Yamaura et al, 2008).

Glossary

PGN: Peptidoglycan,

PGRPs: Peptideglycan recognition proteins,

PAMPs: Pathogen-associated molecular patterns

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