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Analysis of EST and lectin expressions in hemocytes of Manila clams (*Ruditapes philippinarum*) (Bivalvia: Mollusca) infected with *Perkinsus olseni*

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Abstract

The hemocytes of invertebrates play key roles in both cellular and humoral immune reactions by phagocytosis or delivering immune factors such as lectin and anti-microbial peptides. Bacterial infection causes changes in components such as lectins, anti-bacterial peptides, and lysosomal enzymes of plasma or hemolymph in molluscs. Previously, we found that infection with the protozoan parasite, *Perkinsus,* increases lectin synthesis in hemocytes. In order to investigate the patterns of genes expressed in Manila clams (*Ruditapes philippinarum*) infected with the protozoan parasite *Perkinsus olseni*, we constructed a cDNA library and sequenced 1850 clones (expressed sequence tags). A total of 79 ESTs, were related to 29 functional immune genes such as C-type lectin, lysozyme, and cystatin B, in Manila clams. Lectins were the largest group of immune-function ESTs found in our Manila clams library. Among 7 lectin clones, two full length cDNAs of lectins were cloned. MCL-3, which is a simple C-type lectin composed of 151 amino acids, has a relatively short signal sequence of 17aa and single carbohydrate-recognition domain (CRD) of ~130 residues. It is highly homologous to eel C-type lectin. The sequence of mc-sialic acid-binding lectin consists of 168 amino acid residues with molecular weight of 19.2 and shows high homology to sialic acid-binding lectin from the snail, *Cepaea hortensis*.

The expression of 7 different lectins in hemocytes was analyzed by RT-PCR using gene-specific primers. Hemocytes from *Perkinsus*-infected clam expressed different sets of lectins than with *Vibrio* infection. These results demonstrate that several lectins are involved in Manila clam innate immunity and different challenges induce expression of different lectins.
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Keywords: Manila clam; *Ruditapes philippinarum*; Perkinsus olseni; Expressed sequence tag; Innate immunity; C-type lectin; Sialic acid-binding lectin

1. Introduction

The ability of the host to discriminate self from potentially harmful non-self, and act appropriately is a crucial feature of immune defense. Invertebrate

Abbreviations: ESTs, expressed sequence tags cDNA; complementary DNA; NCBI, National Center for Biotechnology Information; MCL, Manila clam lectin

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animals lack an adaptive immune system but have developed various innate immune systems that respond to pathogen-associated molecular patterns (PAMPs) [1]. The PAMPs are evolutionarily conserved and this led to the emergence of pattern recognition receptors (PRRs) which specifically recognize PAMPs and deliver appropriate signals to cells of the innate immune system [1]. Lectins [2,3] are believed to be important PRRs.

The animal lectins are a group of carbohydrate-binding proteins that are involved in a variety of processes, including the innate immune response critical for the detection of infectious micro-organisms [4]. Most of them can be classified into two categories: calcium-dependent animal lectins (C-type lectins) and metal-independent galectins. Recently, C-type lectins were implicated as the main players in carbohydrate recognition within the immune system as PRRs [5].

Lectins have been reported as PRR in few marine invertebrates [6]. In the chelicerate arthropods, a group of lectins termed tachylectins were found in the hemocytes of horseshoe crab, and shown to recognize PAMPs. These lectins have different carbohydrate-binding specificities and they were also induced in response to LPS [6]. In the tunicate, *Styela plicata,* in vivo challenge with the inflammatory agent zymosan causes generation of a collectin-like protein that was secreted into hemolymph after 96 h [7].

Perkinsus is a deadly protozoan parasite of commercially important marine molluscs, including the American oyster, *Crassostrea virginica,* the manila clam, *R. philippinarum* and the Australian black rib abalone, *Haliotis rubra* [8]. In clams, cellular defensive activities to *Perkinsus* induce pathologic symptoms such as hemocytic infiltration, digestive tubule atrophy, and nodule formation on mantle and foot due to inflammation [9–13]. Since *Perkinsus* is believed to be responsible for mass mortalities in clam populations, recently [10,13], more detailed studies on immune responses of bivalves against *Perkinsus* infection have started.

Our previous studies [14] showed that *Perkinsus* infection in the Manila clam increases the hemagglutination titer of body lysate 1000-fold. Manila clam lectin (MCL) is one of the lectins responsible for this increase, although, other factors seem to be involved in this change. It has been reported that bacterial infection causes changes in the components of plasma or hemolymph in molluscs, such as agglutinins, anti-bacterial peptides, and lysosomal enzymes [15–18]. However, most of these assays were done on protein levels and little is known about the nature of these changes and factors responsible for their synthesis. In this study, we collected and analyzed EST sequences from Manila clams infected with *Perkinsus olseni* to analyze the change of immune-related genes upon *Perkinsus* infection. While attempting to identify expression of the immune related gene, we isolated two C-type lectin clones and five partial lectin-like clones. We further studied the expression patterns of each of the lectins against protozoan or bacterial challenges.

2. Materials and methods

2.1. Preparation of hemocytes

To assess the level of *P. olseni* infection, Manila clam tissues were stained with Lugol's iodine and examined under a light microscope after incubation of the tissues in Ray's fluid thioglycollate medium (FTM). Approximately 1 ml of hemolymph was collected from the posterior adductor muscle sinus using 1 ml syringes with 26G × 1/2″ needles through the shell hinge [19]. Shell length of the clams used in the present study ranged from 25 to 32 mm with a mean value of 27 mm. The supernatant was taken for hemocyte free-hemolymph and the pellet contained the hemocytes. Hemocytes were resuspended in PBS (6.7 mM $Na_2H\ PO_4$/ KH_2PO_4, pH 7.4) containing 0.15 M NaCl.

2.2. RNA isolation and cDNA library construction

Total RNA was isolated using the TRIzol procedure (Invitrogen, Frederick, MO, USA) and precipitated in ethanol. Messenger RNA was isolated from the total RNA using oligotex mRNA spin columns (Qiagen). The cDNA library was constructed using a ZAP-cDNA synthesis kit and a ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene, La Jolla, CA, USA). The procedures were carried out as suggested by the manufacturer. First-strand cDNA synthesis used an oligo-dT primer with a *Xho* I restriction site: Double-strand cDNA was synthesized using RNase H and DNA polymerase I. The double-strand cDNA was ligated to an EcoR I adapter. Double- strand cDNA was cloned into Uni-ZAP XR vector, which was packaged into a λ-phage using a Gigapack III Gold Cloning kit. The cDNA library was then titered.

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