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Molecular cloning, expression of a big defensin gene from bay scallop *Argopecten irradians* and the antimicrobial activity of its recombinant protein

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Abstract

Antimicrobial peptides are important components of the host innate immune responses by exerting broad-spectrum microbicidal activity against pathogenic microbes. The first mollusk big defensin (designated AiBD) cDNA was cloned from bay scallop *Argopecten irradians* by expressed sequence tag (EST) and rapid amplification of cDNA ends (RACE) techniques. The scallop AiBD consisted of 531 nucleotides with a canonical polyadenylation signal sequence AATAAA and a poly(A) tail, encoding a polypeptide of 122 amino acids. The high similarity of AiBD deduced amino acid sequence with big defensin from *Tachypleus tridentatus* and *Branchiostoma belcheri tsingtaunese* indicated that AiBD should be a member of big defensin family. The expression of AiBD in various tissues was measured by using Northern blotting analysis. mRNA transcripts of AiBD could be detected in haemocytes of unchallenged scallops. The temporal expression of AiBD in haemolymph after *Vibrio anguilarum* challenge was recorded by quantitative real time PCR. The relative expression level of AiBD in haemolymph was up-regulated evenly in the first 8 h, followed by a drastic increase, and increased 131.1-fold at 32 h post-injection. These results indicated that AiBD could be induced by bacterial challenge, and it should participate in the immune responses of *A. irradians*. Biological activity assay revealed that recombinant AiBD could inhibit the growth of both Gram-positive and Gram-negative bacteria, and also showed strong fungicidal activity towards the expression host. Recombinant expression of AiBD made it possible to further characterize its functions involved in immune responses, and also provided a potential therapeutic agent for disease control in aquaculture.

Keywords: *Argopecten irradians*; Big defensin; mRNA expression; Quantitative real time PCR; Recombinant protein; Antimicrobial activity

1. Introduction

Scallop aquaculture is a big industry and contributes enormously to the economic development of coastal provinces in China (Guo et al., 1999). Since the summer of 1997, large-scale mortality of cultured scallop has caused catastrophic losses to scallop aquaculture, which resulted in the production decreasing drastically.

Mollusk lack an acquired immune system and their defense mechanisms mainly rely on innate immune responses, which include cellular responses mediated by haemocytes and humoral immune responses that employ constitutive and inducible antimicrobial peptides to lyse invading microorganisms (Hoffmann et al., 1999; Roch, 1999). Increased resistance of bacteria towards antibiotic drugs has stimulated intensive efforts for discovery and characterization of antimicrobial effectors as sources or templates for the design of new therapeutic antibiotics (Patrzykat and Douglas, 2003). Moreover, advances in characterization of immune effectors may lead to a better understanding of the immune defense mechanisms of mollusk and give new insights into health management and diseases control in mollusk aquaculture.

Antimicrobial peptides (AMPs) are often small cationic molecules widely distributed in the whole living kingdom, and they are thought to be essential for organisms lacking adaptive immunity (Hancock and Diamond, 2000; Zasloff, 2002). Since the original discovery of cecropin in *Hyalophora cecropia*

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(Steiner et al., 1981), a rapid expansion with over 800 AMPs has been reported from various species according to the Antimicrobial Sequences Database (http://www.bbcm.univ.trieste.it/~tossi/search.htm). It is now well established that the antimicrobial peptide response is a common feature of innate immunity in animals.

Most AMPs display hydrophobic and cationic properties, have a molecular mass below 10 kDa, and adopt an amphipathic structure (α-helix, hairpin-like β-sheet, β-sheet, or α-helix/β-sheet mixed structures) that is believed to be essential for their antimicrobial action (Bulet et al., 2004). Based on amino acid sequences, secondary structures, and functional similarities, cationic AMPs are tentatively classified into three distinct groups (Boman, 1998): (i) linear cysteine-free peptides with α-helix conformation; (ii) cyclic and open-ended cyclic peptides with one to four intramolecular disulfide bonds forming hairpin-like β-sheet or α-helix/β-sheet mixed structures; (iii) peptides with an over-representation of some amino acids. More recently, a series of novel AMPs have been discovered as processed forms of large inactive proteins involved in various functions, such as histones and hemocyanin (Destoumieux-Garzón et al., 2001; Cho et al., 2002; Lee et al., 2003; Fernandes et al., 2003, 2004).

In mollusk, AMPs were mainly characterized in bivalves, the mussels *Mytilus edulis* and *Mytilus galloprovincialis*. Based on biochemical approach and molecular cloning, four families of cysteine-rich AMPs, including *M. galloprovincialis* defensins (MGDs), myticins, mytilins and mytimycin, have been identified in *Mytilus* spp. (Charlet et al., 1996; Mitta et al., 2000a). MGDs and myticins appear to be more active against Gram-positive bacteria compared to Gram-negative bacteria and fungi (Mitta et al., 1999a,b). The mytilin isoforms B, C and D are active against both Gram-negative and Gram-positive bacteria, whereas the G1 isoform displays potent only against Gram-positive bacteria (Hubert et al., 1996; Mitta et al., 2000b). Additionally, mytimycin identified from *M. edulis* haemolymph was strictly antifungal (Hubert et al., 1996). Among the characterized mollusk AMPs, properties and three-dimensional structure of MGD1 have been well studied and the corresponding mechanisms have also been proposed (Mitta et al., 2000c; Yang et al., 2000; Romestand et al., 2003).

Big defensin is one of the AMPs which possess remarkable microbicidal activity against Gram-positive, Gram-negative bacteria and fungi. The only well characterized big defensin was purified from *Tachypleus tridentatus*, which consists of 79 amino acid residues, including a C-terminal defensin domain and a highly hydrophobic N-terminus. It is noteworthy that the N-terminal region of horseshoe crab big defensin displays a more potent activity against Gram-positive bacteria, while the C-terminal defensin domain is more potent against Gram-negative bacteria (Saito et al., 1995).

To our knowledge, molecular features and functional studies of immune effectors in mollusk remained deficient. The main objectives of this study were: (1) to clone the full-length cDNA of AiBD from bay scallop *Argopecten irradians*, (2) to investigate the expression profile of AiBD after being infected by bacterial pathogen, and (3) to characterize the antimicrobial activity of the recombinant protein.

2. Materials and methods

2.1. Animals, immune challenge and haemolymph collection

Bay scallops *A. irradians*, averaging 55 mm in shell length, were collected from a scallop farm and incubated at 15 °C for 2 weeks before processing. For the bacterial challenge experiment, 400 scallops were employed and kept in eight aerated tanks (50 individuals in each tank). Fifty microliters of live *Vibrio anguillarum* resuspended in PBS ($O.D._{600} = 0.4$) was injected into the adductor muscles of 300 scallops. The untreated scallops and scallops injected with 50 μl PBS were used as the blank group and the control group, respectively. The injected scallops were returned to seawater tanks and 20 individuals were randomly collected at 2, 4, 6, 8, 16, and 32 h post-injection. Subsequently, within each $n = 20$ group, haemolymphs were pooled per subgroup of $n = 5$ to minimize individual variability, and four replicates were employed for each time point during the challenge experiment. The haemolymphs from the blank, control and stimulated groups were collected using a syringe from the adductor muscles and centrifuged at $1000 \times g$, 4 °C for 10 min to harvest the haemocytes. Total RNA was immediately extracted using Unizol reagent according to the manufacture's protocol (Biostar).

2.2. cDNA library construction and EST analysis

A cDNA library was constructed from the whole body of a bay scallop challenged with *V. anguillarum*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 5828 successful sequencing reactions (Song et al., in press). BLAST analysis of all the EST sequences revealed that one EST (EST No. rscae_0349; length: 471 bp) was homologous to the big defensin in *T. tridentatus* (GenBank accession no. P80957). The EST sequence was then selected for further cloning of the AiBD gene from bay scallop.

2.3. Cloning the full-length cDNA of AiBD

Two specific primers, sense primer P1 and reverse primer P2 (Table 1), were designed based on the sequence of EST (No. rscae_0349) to clone the full sequence of AiBD cDNA. PCR reaction to get 5′ end of AiBD cDNA was performed in a PTC-100 Programmable Thermal Controller Cycler (MJ Research) by using sense primer T3 and reverse primer P2 in a 20 μl reaction volume containing 2 μl of 10× PCR buffer, 1.2 μl of $MgCl_2$ (25 mmol l^{-1}), 1.6 μl of dNTP (2.5 mmol l^{-1}), 1 μl of each primer (10 μmol l^{-1}), 12 μl of PCR-grade water, 0.2 μl (1 U)of Taq polymerase (Promega) and 1 μl of cDNA mix. The PCR temperature profile was 94 °C for 5 min followed by 34 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min and the final extension step at 72 °C for 10 min. PCR amplification of the 3′ end of AiBD was carried out using sense primer P1 and reverse primer T7, and the PCR program was 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C