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The first chordate big defensin: Identification, expression and bioactivity

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ABSTRACT

Defensins are broadly present in plants, invertebrates and vertebrates, but little information is available about it in amphioxus, a protochordate holding a key phylogenetic position. In this study, a big defensin cDNA was identified from the amphioxus *Branchiostoma japonicum* (termed *Bjbd*). The cDNA contained an open reading frame (ORF) of 354 bp encoding a 117 amino acid protein, which had an N-terminal signal sequence followed by a propeptide and the mature big defensin. The mature peptide had the hydrophobic region GAAAVT(A)AA at N-terminus and the consensus pattern C-X6-C-X3-C-X13(14)-C-X4-C-C at C-terminus as well as four α-helices, four β-sheets, and three disulfide bridges (C1–C5, C2–C4 and C3–C6) in the predicted tertiary structure. This is the first big defensin gene ever identified in the phylum Chordata. Quantitative real-time PCR analysis revealed that *Bjbd* was constitutively expressed in most of the tissues examined, and its expression was remarkably up-regulated following the challenge with LPS, LTA, *Aeromonas hydrophila* and *Staphylococcus aureus*. Moreover, the recombinant BjBD was shown to be able to inhibit the growth of *S. aureus*, *Escherichia coli* and *A. hydrophila*. Taken together, these suggest that BjBD is a molecule involved in the removal of invading pathogens.

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1. Introduction

Innate immunity is the first defense line of organisms against the invasion of microorganisms. Antimicrobial peptides (AMPs) are ancient innate immune components and have been identified in organisms throughout the plant and animal kingdoms. Defensins constitute a family of AMPs with antimicrobial activity and immunomodulatory property [1,2]. Based on their disulfide patterns, defensins are classified into three subfamilies, α-, β- and θ-defensins. Both α- and β-defensins have been documented in all vertebrate species examined, but θ-defensins are only isolated in *Rhesus macaque* [3–6]. Defensin and defensin-like peptides have also been found in invertebrates [7] and plants [8–10].

Most defensins are small peptides (12–50 amino acids), display cationic property and possess an amphipathic (containing both hydrophobic and hydrophilic domains) structure, enabling them to interact with microbial membranes [2,11]. Interestingly, a defensin consisting of 79 amino acids, termed big defensin, was also purified from the horseshoe crab *Tachypleus tridentatus* by Saito et al. [12]. Since then, big defensins have been identified in protostome invertebrates including *Ruditapes philippinesis* [13], *Argopecten irradians* [14], *Venerupis philippinarum* [15] and *Mercenaria mercenaria* [16]. To our knowledge, no big defensins have ever been reported in deuterostomes including chordate.

As ancient molecules showing perspective as substitutes for traditional antibiotcs, defensins have attracted much attention of biologists and biochemists and have been extensively studied biochemically, genetically and evolutionarily [1,2]. However, no information is available to date regarding defensins in amphioxus, a primitive chordate holding a key phylogenetic position bridging from invertebrates to vertebrates [17], thereby leaving a clear gap for gaining insights into the evolution of structure and function of defensins. The aims of this study are thus to clone the big defensin gene (termed *Bjbd*) from the primitive chordate amphioxus, *Branchiostoma japonicum*, and to examine its expression and bioactivity.

2. Materials and methods

2.1. Cloning and sequence analysis

The gut cDNA library of adult *B. japonicum* was constructed with SMART cDNA Library Construction Kit (CLONTECH, CA, USA), and

Abbreviations: BjBD, *Branchiostoma japonicum* big defensin; AMPs, antimicrobial peptides; qRT-PCR, quantitative real-time PCR; LPS, lipopolysaccharide; LTA, lipoteichoic acid; Trx, thioredoxin; IPTG, isopropyl-β-D-thiogalactoside; PB, phosphate buffer; rBjBD, recombinant BjBD; ORF, open reading frame; UTR, untranslated region.

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more than 5000 clones were sequenced and analyzed for coding probability with the DNATools program. Sequence comparison against the GenBank protein database performed using the BLAST network server at the NCBI revealed that one cDNA clone of 594 bp was homologous to previously identified big defensin from *T. tridentatus* [18], which was selected for further study. The deduced protein, designated as BjBD, was analyzed by the SMART program (http://smart.embl-heidelberg.de/). The signal peptide was predicted with the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The mature peptide was analyzed using the Antimicrobial Peptide Predictor program (http://aps.unmc.edu/AP/prediction/prediction_main.php). Multiple protein sequences were aligned using the MegAlign program by the CLUSTAL W method in DNAStar software package.

The tertiary structure of BjBD was predicted with a homology-modeling method via SWISS-MODEL using ANOLEA mean force potential and Gromos96 force field energy [19,20]. The visualization and characterization of the three-dimensional structure of the BjBD was performed with software PyMOL [21].

2.2. Assays for expression of Bjbd in different tissues

Semi-quantitative real-time PCR (qRT-PCR) was performed to determine the expression of *Bjbd* gene in the different tissues. The total RNAs were prepared with Trizol (Gibco) from the whole animals and the different tissues gill, hepatic caecum, hind-gut, notochord, muscle, ovary and testis. The total RNAs were digested with RQ1 RNase-free DNase (Promega) to eliminate the genomic contamination, and the cDNAs were synthesized with reverse transcription system (Promega) using oligo d(T) primer and used as templates. The SYBR Green RT-PCR assay was carried out in an ABI PRISM 7300 Sequence Detection System. The amplifications were conducted in triplicates in a total volume of 20 μl. Two primers, P1: 5′-GTTAGCCGTGCCCCTGGT-3′ and P2: 5′-GAGTGGCTGTCATTGTCGGAG-3′, specific for *Bjbd* cDNA, were used to amplify a product of 129 bp. The β-actin gene was selected as reference gene for internal standardization [22]. All the data were presented in terms of relative mRNA expressed as mean ± S.E. ($n = 3$).

2.3. Assays for effects of LPS, LTA and bacterial challenge on Bjbd expression

Adult *B. japonicum* were acclimatized in sterilized filtered seawater for two days, divided into four groups and immersed in 200 ml of filtered seawater containing 10 μg/ml LPS (a Gram-negative bacterium-specific component) (Sigma, USA), 10 μg/ml LTA (a Gram-positive bacterium-specific component) (Sigma, USA), 10^9 cells/ml *Aeromonas hydrophila* (a Gram-negative bacterium) or 10^9 cells/ml *Staphylococcus aureus* (a Gram-positive bacterium) at 25 °C [23,24]. A total of 8 animals were sampled at intervals of 0, 1, 2, 4, 8, 16 and 32 h, individually, following the challenge, and the total RNAs were prepared with Trizol (Gibco). The untreated animals were processed similarly as control. The temporal expression of *Bjbd* was determined by qRT-PCR as above. All data were given in terms of relative mRNA expressed as means ± SD. The data obtained from qRT-PCR analysis were subjected to one-way analysis of variance (ANOVA) followed by an unpaired, two-tailed *t*-test to determine differences in the mean values among the treatments. Significance was concluded at $P < 0.05$. Statistical analysis was performed using SPSS 13.0 for Windows.

2.4. Expression and purification of BjBD

The cDNA fragment (246 bp) encoding the mature peptide (82 amino acids) of BjBD was amplified by PCR with the specific primers, P3: 5′-CCGGAATTCGCGGTGCCGTTAGCCGTGC-3′ and P4: 5′-CCGCTCGAGTTAGTAGCGTGGGCGGCAGC-3′, with *EcoR* I and *Xho* I sites (underlined) at their 5′ end, respectively. The PCR product was digested with *EcoR* I and *Xho* I, and subcloned into the plasmid expression vector pET32a (Novagen) previously cut with the same restriction enzymes. The identity of the insert was verified by sequencing, and the plasmid was designated pET32a-*Bjbd*. The recombinant plasmid pET32a-*Bjbd* was transformed into *Escherichia coli* BL21(DE3)pLysS (Novagen). Positive clones were screened by PCR reaction with the primers P3 and P4. The pET-32a vector without insert fragment was selected as a negative control, which could express a thioredoxin (Trx) with 6 × His-tag. The positive transformants and negative control were both incubated in 200 ml LB medium at 37 °C with shaking at 140 rpm until the culture reached OD_{600} of 0.5–0.7. To induce expression, isopropyl-β-D-Thiogalactoside (IPTG) was added to the medium to a final concentration of 0.1 mM. The mediums were then cultured at 25 °C with shaking at 100 rpm for 16 h. The recombinant BjBD (rBjBD) was purified by Ni-NTA resin column (Amersham). The column was washed with the washing buffer (20 mM PBS containing 500 mM NaCl and 20 mM imidazole, pH 8.0) and eluted with the elution buffer (20 mM PBS containing 500 mM NaCl and 250 mM imidazole, pH 8.0). The purity of the eluted samples was analyzed by 12% SDS-PAGE, and stained with Coomassie brilliant blue R-250. To remove imidazole and NaCl, the eluted samples were ultrafiltrated by Amicon® Ultra-4 Centrifugal Filter Units (Millipore) in 10 mM PB (phosphate buffer; pH 8.0). The concentrations of rBjBD and Trx-His-tag protein were determined by BCA Protein Assay Kit (Beyotime).

2.5. Antimicrobial activity assay

The antimicrobial activity of rBjBD was assayed according to the method described by Zhang et al. [25] with slight modification. The Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli* were grown in LB medium, and the Gram-negative bacterium *A. hydrophila* was grown in tryptic soy broth (TSB medium), to mid-logarithmic phase, and then harvested by centrifugation at 5000 g for 10 min. The bacterial pellets were washed in 10 mM PB three times, and re-suspended and diluted to a density of 10^3 cells/ml. The rBjBD was filtered through 0.22 μm Millipore paper, and diluted two-folds serially (starting at 500 μg/ml) with 10 mM PB (pH 8.0), giving concentrations of 500, 250, 125, 62.5 and 31.25 μg/ml. An aliquot of 30 μl of each rBjBD solution was mixed with 5 μl of the bacterial suspension, and the mixtures were pre-incubated at 25 °C for 2 h. Each mixture was added into 3 wells (10 μl/well) in a sterile 96-well plate, and 200 μl LB or TSP medium was then added into every well. After incubation at 37 °C for 16 h, the absorbance at 595 nm of each well was measured using a Microplate Reader (TECAN GENIOS PLUS, Switzerland). Serial diluted Trx-His-tag solution and 10 mM PB solution were used as negative control and blank control, respectively, and processed similarly. All data (OD values) were given as means ± SD and then subjected to ANOVA followed by an unpaired, two-tailed *t*-test. Difference was considered to be significant at $p < 0.05$.

3. Results

3.1. cDNA cloning and sequence of Bjbd

The full-length cDNA (GenBank ID: AY175378) of *Bjbd* was 594 bp long, which contained an open reading frame (ORF) of 354 bp, a 5′-untranslated region (UTR) of 75 bp and a 3′-UTR of 165 bp with a polyadenylate tail (Fig. 1A). The ORF encoded a protein of 117 amino acids with a predicted molecular mass of approximately 12.9 kDa. SMART program analysis revealed that the

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