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Molecular characterization and expression analysis of the IκB gene from pearl oyster *Pinctada fucata*

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ABSTRACT

Inhibitor of NF-κB (IκB) is one important member of NF-κB signal pathway and plays a pivotal role in regulating the innate immune response of invertebrate. Herein, we described the isolation and characterization of pearl oyster *Pinctada fucata* IκB gene (designated as poIκB). The poIκB cDNA was 1975 bp long and consisted of a 5′ untranslated region (UTR) of 73 bp, a 3′ UTR of 807 bp with three RNA instability motifs (ATTTA) and a polyadenylation signal (AATAAA) at 13 nucleotides upstream of the poly (A) tail, and an open reading frame (ORF) of 1095 bp encoding a polypeptide of 364 amino acids with an estimated molecular mass of 40.11 kDa and theoretical isoelectric point of 4.61. A conserved degradation motif ($DS_{35}GFSS_{39}$) and six ankyrin repeats were identified in the poIκB by SMART analysis. Homology analysis of the deduced amino acid sequence of the poIκB with other known IκB sequences by MatGAT software revealed that the poIκB shared 23.5–63.3% similarities with other known IκB isoforms. The poIκB mRNA was constitutively expressed in all studied tissues with the most abundant mRNA in the haemocyte. The poIκB mRNA was up-regulated and increased 4.13- and 5.28-fold after LPS and *Vibrio alginolyticus* stimulation, respectively. These results suggested that the poIκB was a constitutive and inducible acute-phase protein that perhaps involved in the immune defense of pearl oyster.

1. Introduction

Nuclear factor-κB (NF-κB) pathway is highly conserved from insects to mammal and has been studied extensively for its role in innate immune system [1–3]. Inhibitor of NF-κB (I-κB) is one important member of NF-κB pathway and interacts with the Rel-homology domain (RHD) of NF-κB and masks the nuclear localization signal (NLS) of NF-κB, so the I-κB prevents NF-κB nuclear translocation under normal condition, which makes NF-κB protein retain in the cytoplasm in an inactive form [4]. In response to a large variety of extracellular stimuli, the I-κB protein is rapidly phosphorylated by I-κB kinases (IKKs), leading to its ubiquitination and ultimately proteolytic degradation by the proteosome. NF-κB is subsequently free to translocate to the nucleus where it stimulates the transcription of various immune-related genes [5].

In mammal, the I-κB family is composed of I-κBα, I-κBβ, I-κBγ, I-κBε, I-κBζ, bcl-3, p100 and p105 [6–9]. The members of I-κB family all contain five to seven copies of about 30–33 amino acid sequence called ankyrin repeats (AR) which are responsible for interaction with NF-κB proteins [4,5,9]. Despite their structural similarities, the members of I-κB family appear to play different roles in vivo. Individual I-κB member has different affinities for individual NF–κB complexes and is expressed in a tissue specific manner [5,6]. In fish, the I-κB mRNA is up-regulated after LPS or bacteria stimulation [10,11], which suggests that activation of NF-κB induces I-κB expression, and an auto-regulatory loop exists in the NF-κB pathway, which elevates I-κB mRNA expression after initial degradation of I-κB, thereby restoring the inhibition of NF-κB [5,11].

The previous studies have indicated the existence of I-κB in many invertebrates, such as fruit fly [12], mosquito (EAT48251), sea sleeve [4], honey bee (XP_394485), horseshoe crab [3] and sea urchin (XP_001192604), some of which have been demonstrated to participate in innate immune signal transduction. Recently, pacific oyster I-κB has been characterized and its expression in different tissues also has been investigated [13]. However, there are very few I-κB genes characterized in bivalve mollusk so far, and whether the bivalve I-κB functions as the insect and mammal I-κB in innate immune response remains unknown. Therefore, to further understand the functions of bivalve I-κB in innate immune signal transduction, it is necessary to isolate and characterize more genes of I-κB family from bivalve mollusk.

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Pearl oyster *Pinctada fucata* is distributed over southern coast of China and is the most popular farming shellfish for pearl production. However, since the mid-1990s, pearl oyster begins to die largely due to disease outbreaks in China and leads to a dramatic decline in South China Sea pearl production [14,15]. In order to control disease and enhance the pearl yields, it is necessary to research the innate immune defense mechanisms of pearl oyster, which lacks the adaptive immune system. NF-κB pathway is very important in innate immune signal transduction. To isolate and characterize the related genes of NF-κB pathway may provide new insights into disease control in pearl oyster farm. In the present study, the main objectives are (1) to clone the full-length cDNA of the I-κB from pearl oyster *P. fucata* (named as poI-κB), and compare it with other known I-κB genes, (2) to investigate the distribution of poI-κB mRNA in different tissues and its expression profile in haemocytes challenged by lipopolysaccharide (LPS) or *Vibrio alginolyticus*, (3) to provide clues on the presence of NF-κB pathway in pearl oyster, which participate in immune regulation.

2. Materials and methods

2.1. Pearl oyster and immune challenge

Pearl oyster *P. fucata* (shell length 4.5–5.2 cm, body weight 18.2–22.5 g) were obtained from pearl oyster culture base of South China Sea Fisheries Research Institute in Xincun village, Hainan province, China and maintained at 25–27°C in tanks with the recirculating seawater for one week before experimental. The pearl oyster were fed twice daily on *Tetraselmis suecica* and *Isochrysis galbana*. The experiment contained four groups: the blank group, the control group, the LPS stimulation group and bacterial challenge group, each group was divided into three replicates. Five individuals of each replicate were randomly sampled at the same time point, which were pooled together as one sample.

The LPS stimulation group was performed by injecting with 50 μl of LPS (*Escherichia coli* 055:B5, #62326, Sigma–Aldrich, Munich, Germany) dissolved in PBS (LPS 10 μg ml^{-1}) into the adductor muscles of each pearl oyster. The bacterial challenge group was performed by injecting with 50 μl of *V. alginolyticus* resuspended in PBS to $OD_{600} = 0.4$ (1 OD = 5×10^8 bacteria ml^{-1}) into the adductor muscles of each pearl oyster. The control group was performed by injecting with 50 μl of PBS. The untreated pearl oysters were used as the blank. At the each time point (0, 2, 4, 8, 12, 24 and 36 h), the haemolymph from the blank, the LPS stimulation group and the bacterial challenge group were collected from the adductor muscles using a syringe and immediately centrifuged at 5000×g, 4°C for 10 min to harvest the haemocytes. The haemocyte pellets were immediately used for RNA extraction.

2.2. cDNA library construction and EST analysis

A cDNA library was constructed from the whole body of a pearl oyster challenged by *V. alginolyticus*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 6741 successful sequencing reactions. BLAST analysis of all the EST sequences revealed that an EST of 537 bp (EST no. pmpca 0_002154) was homologous to the I-κB of *Crassostrea gigas* (ABB52821). This EST was selected for further cloning of the I-κB gene of pearl oyster.

2.3. Cloning the full-length cDNA of poI-κB

Based on the identified EST sequence, two gene-specific primers IKB-F1 and IKB-R1 (Table 1) were designed to amplify the full-length cDNA of poI-κB by rapid amplification of cDNA ends (RACE) technique. To obtain 5′-end of the poI-κB cDNA, PCR reaction was performed in a T-1 Thermocycler (Biometra) by using the T3 and IKB-R1 primers (Table 1) in a 25 μl of reaction volume, containing 2.5 μl of 10 × PCR buffer, 1.5 μl of $MgCl_2$ (25 mmol L^{-1}), 2.0 μl of dNTP, (2.5 mmol L^{-1}), 1 μl of each primer (10 μmol L^{-1}), 15.8 μl of double-distilled water, 0.2 μl (1.0 U) of *Ex* Taq (TaKaRa) and 1 μl of 100-fold diluted cDNA library as template . The cycle condition was one initial denaturation cycle of 94 °C for 2 min, then 35 PCR cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 2 min, and a final extension step at 72 °C for 10 min. PCR amplification of 3′-end of the poI-κB was carried out using the T7 and IKB-F1 primers (Table 1), the PCR temperature profile was 94 °C for 2 min, following by 35 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 2 min, and then an additional extension of 72 °C for 10 min. The PCR products were separated by agarose gel (1.2%) electrophoresis, and then the bands of the desired size were excised and purified using a DNA Gel Extraction Kit (KeLi, China). Finally, the purified DNA fragments were cloned into the pMD18-T vector (TaKaRa) and sequenced.

2.4. Sequence analysis of poI-κB

The poI-κB amino acid sequence was deduced using DNAstar software. The percentage of similarity and identity of the known I-κB sequences was calculated by the MatGat [16] program with default parameters. The protein domain was predicted with the simple modular architecture research tool (SMART) version 4.0 program [17,18] (http://www.smart.emblheidelberg.de/). The protein sequence of the poI-κB was compared to its counterpart sequences currently available in GenBank, retrieved using the BLAST program [19] (http://www.ncbi.nlm.nih.gov). Multiple alignment of poI-κB was carried out with the ClustalW program (http://www.ebi.ac.uk/clustalw/). The phylogenetic tree was constructed with MEGA program version 3.1 [20] based on amino acid sequences alignment. The phylogenetic tree was tested for reliability using 1000 bootstrap replications.

2.5. Semi-quantitative RT-PCR analysis of poI-κB gene expression

For the tissue distribution analysis, total RNA was extracted from haemocytes, gill, digestive gland, mantle, gonad and adductor muscle of three healthy pearl oysters. For the temporal expression analysis, total RNA was extracted from the above mentioned haemocyte pellets using Trizol reagent (Invitrogen). The single-strand cDNA was synthesized based on manufacture's instruction of PrimerScript™ 1st Strand cDNA Synthesis Kit (TaKaRa) using the DNase I (Promega)-treated total RNA as template and adaptor-dT primer (Table 1). cDNA mix was diluted to 1:5 and stored at −80 °C for subsequent semi-quantitative RT-PCR.

Two poI-κB gene-specific primers, IKB-F and IKB-R (Table 1), were designed to amplify a product of 239 bp. A housekeeping gene, the beta-actin gene, was used as an internal control to verify the RT-PCR reaction and adjust the cDNA templates. The beta-actin gene was identified as an EST sequence (EST no. pmpca 0_006148)

Table 1
Primers for gene amplification and characterization.

Primer name	Sequence (5′–3′)	Application
IKB-F1	ACGACGAAATCGTGGAAATAC	For RACE PCR
IKB-R1	GTGGGGTTTGTAGAAGGTTG	
IKB-F	GGCAGATCCAGCTTCTATGC	For semi-quantitative PCR
IKB-R	CGACCATCAACATACGAACG	
Beta-Actin-F	CGGTACCACCATGTTCTCAG	
Beta-Actin-R	GACCGGATTCATCGTATTCC	
Adaptor-dT	$GGCCACGCGTCGACTAGTACT_{17}$	For reverse transcription
T3	AATTAACCCTCACTAAAGGG	For sequencing and RACE PCR
T7	GTAATACGACTCACTATAGGGC	