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Identification and characterization of multiple β-glucan binding proteins in the Pacific oyster, *Crassostrea gigas*

Naoki Itoh *, Ryo Kamitaka, Keisuke G. Takahashi, Makoto Osada

Aquacultural Biology Laboratory, Graduate School of Agricultural Science, Tohoku University, Tsutsumidori Amamiya-machi, Aoba-ku, Sendai, Miyagi 981-8555, Japan

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ABSTRACT

The present study reports on the characterization of two cDNAs coding β-glucan binding proteins (βGBPs), designated as Cg-βGBP-1 and Cg-βGBP-2, from the Pacific oyster, *Crassostrea gigas*. Cg-βGBP-1 consists of 555 amino acid residues and possesses two possible integrin recognition sites. The other protein, Cg-βGBP-2, is composed of 447 amino acid residues without integrin recognition sites. Domain structures of both Cg-βGBPs are similar to other invertebrate βGBPs, but phylogenetic positions and major expression tissues for these proteins are different. Cg-βGBP-1 is expressed in circulatory hemocytes and Cg-βGBP-2 in digestive glands. Functional assays using recombinant proteins revealed that Cg-βGBP-2 enhanced the phenoloxidase (PO) activity of hemocyte suspensions under the presence of laminarin, but Cg-βGBP-1 did not show this enhancement. It is suggested that Cg-βGBPs in the Pacific oyster have evolved to obtain different immunological functions. Cg-βGBP-1 possibly evolved for hemocyte-related functions through integrin, and Cg-βGBP-2 for the PO activation system.

1. Introduction

Recognition of invasive pathogens is an essential step for the activation of immune responses. In invertebrates, this important step is accomplished by binding of pattern recognition receptors (PRRs) to pathogen-associated molecule patterns (PAMPs). The PRRs and PAMPs complexes subsequently activate appropriate immune responses. To date, various types of invertebrate PRRs have been reported, such as peptideglycan recognition proteins (PGRPs) [1,2], C-type lectins [3,4], lipopolysaccharide (LPS)-binding proteins [5], and scavenger receptor proteins [6].

β-Glucan binding proteins (βGBP), also called gram-negative binding proteins or β-glucan recognition proteins, are known as one of the most important PRRs in invertebrates. This protein molecule recognizes β-glucan, a major cell wall component of yeast cells and some bacterial species. Presence of βGBPs has been reported for a variety of invertebrate phyla, including Annelida [7,8], Mollusca [9–12] and Arthropoda [13–18]. The presence of these proteins in such a wide range of invertebrates may indicate that the defense systems against β-glucan containing microorganisms are crucial for these organisms.

In Crustacea, it has been known that βGBP recognition is related to immune responses in different systems and processes, such as the prophenoloxidase system [14], opsonization for phagocytosis [15], and hemocyte adhesion and degranulation [16]. Thus, the importance of βGBPs against viral infection in this taxonomic group has attracted the attention of various researchers [17,18]. In contrast, little direct knowledge has been generated for bivalves and their βGBP defense mechanisms, although related experimental evidence suggests the presence of βGBP-related defense mechanisms in marine bivalves [10–12,19].

In the present study, we identified mRNA of β-glucan binding proteins (*Cg*-βGBPs) from the Pacific oyster, *Crassostrea gigas*. Tissue expression patterns of these proteins were analyzed by reverse-transcription PCR, and characterization of *Cg*-βGBPs molecules were conducted using recombinant proteins. Based on these results, *Cg*-βGBP-related immune responses are discussed for Pacific oysters.

2. Materials and methods

2.1. cDNA cloning of βGBPs in Pacific oysters

Pacific oysters (*C. gigas*) were sampled in Matsushima Bay, Miyagi, Japan in November 2006. Total RNA was extracted from digestive gland and mantle tissues, and cDNAs for 5′- and 3′ rapid amplification cDNA ends (RACE) were prepared from 1 μg of the extracted total RNA using a SMART RACE cDNA Amplification Kit (Takara Bio Inc., Shiga, Japan).

* Corresponding author. Tel.: +81 22 717 8725; fax: +81 22 717 8727.
E-mail address: nitoh@bios.tohoku.ac.jp (N. Itoh).

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Table 1
List of primers used in the present study.

Primer	Usage	Direction	Sequence	Position
GNRP1-3GSP-1	3′-RACE for *Cg*-PGBP-1	Sense	5′-GGA CCA GTA TAT GGC TAT GAC CCT TAC G-3′	1218–1245[a]
GNRP2-3GSP-1	3′-RACE for *Cg*-pGBP-2	Sense	5′-CCC CTA CCA TAA CAT AGA GGA CCC CTG-3′	1096–1122[b]
GNRP1-5GSP-1	5′-RACE for *Cg*-PGBP-1	Antisense	5′-GGA GGA GAC ACG TTC ATG ACT TCC TGT-3′	1352–1378[a]
GNRP2-5GSP-1	5′-RACE for *Cg*-pGBP-2	Antisense	5′-AAA GAA ACC GCT GGT TCC TCC CAC TAC-3′	1181–1207[b]
18S-RT-F	RT-PCR for18SrRNA	Sense	5′-TCC CAG TAA GCG CGA GTC AT-3′	1596–1615*
18S-RT-R		Antisense	5′-ACG GGC GGT GTG TAC AAA G-3′	1642–1660*
bGRP1-f-sp	RT-PCR for *Cg*-PGBP-1	Sense	5′-TGC CTC ATA GAA ATG CGT ATG G-3′	1081–1102[a]
bGRP1-r-sp		Antisense	5′-GTT GCC TCT AGA TTC CAC AAC GT-3′	1129–1151[a]
bGBP2-RT-Fm	RT-PCR for *Cg*-pGBP-2	Sense	5′-GAC CAC CAT TCC TTC AAC GAA-3′	539–559[b]
bGBP2-RT-Rm		Antisense	5′-CAT ATT CCG CGG ACA TCC A-3′	582–600[b]
bGBP1-NcoI-F	Construction for recombinant *Cg*-pGBP-1	Sense	5′-TGG CCA TGG CCA TAC AAC CCG C-3′	TGG CCA TGG CC+ 90–100[a]
bGBP1-XhoI-R		Antisense	5′-GGT GCT CGA GTG GCT TCA TCT T-3′	GGT GCT CGA G+ 1686–1697[a]
bGBP2-NcoI-F	Construction for recombinant *Cg*-pGBP-2	Sense	5′-TTC CAT GGC CTC GCC CAC GAT T-3′	TTC CAT GGC C+ 68–79[b]
bGBP2-XhoI-R		Antisense	5′-TGG TGC TCG AGA TAC TGG ATC AT-3′	TGG TGC TCG AG+ 1346–1357[b]

Location for primers are indicated as positions of nucleotide sequences in *Cg*-βGBP-1[a] and *Cg*-βGBP-2[b]. Locations of 18S-RT-F and 18S-RT-R (*) are indicated as positions of the Pacific oyster, *Crassostrea gigas*, 18S rRNA gene sequence (**AB064942**).

A tBLASTn search in the NCBI revealed that two different expression sequence tags from *C. gigas* – **CB617438** described as similar to LGBP [20], and **CF369243** described as unknown protein mRNA [Tanguy et al., published in database only] – had high homology with a gram-negative bacterial binding protein from the gastropod, *Biomphalaria glabrata* (**ABO40828**) [11]. In order to obtain complete sequences from these ESTs, gene-specific primers for 3′- and 5′-RACE (Table 1) were designed in the EST sequence regions, and RACE PCR analyses were performed according to the manufacturer's manual in the SMART RACE cDNA Amplification Kit (Takara). Since the first RACE primers failed to give the distinct band of interest, nested-RACE PCR analyses were performed using the nested-RACE primers. The RACE-PCR products were separated and visualized by electrophoresis on 1.5% agarose gel containing SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR, USA). The DNA was extracted and purified from the band corresponding to the expected molecular size using a QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA, USA). The purified PCR products were cloned by pCR II-TOPO vector and One Shot TOP 10 *Escherichia coli* competent cell (Invitrogen Corp., Carlsbad, CA, USA), and three clones were randomly selected and sequenced.

2.2. Computational sequence analyses

Amino acid sequences were deduced from obtained cDNAs, and molecular masses and isoelectric points were calculated using the software, Genetyx Mac Ver. 10.1.6 (Software Development, Tokyo, Japan). Sequence similarities with other known sequences were analyzed using the Protein-protein BLAST program on the NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Presence of signal peptides was predicted by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), and domain searches were conducted in the CD-search in NCBI [21] and Pfam sequence search (http://pfam.sanger.ac.uk/search?tab=searchSequenceBlock).

In order to compare their structures, *Cg*-βGBPs amino acid sequences were aligned with lipopolysaccharide and β-1,3-glucan binding protein (LGBP) of kuruma shrimp (**ABY89089**) using ClustalX [22] and modified visually and manually. βGBPs sequences from mollusks and arthropods were aligned as described above. The alignment sequences were used to create a Neighbor-Joining tree with the gap-free model of the software PAUP*4.0b [23]. Bootstrap analysis was performed using 1000 replicates.

2.3. Semi-quantitative reverse-transcription PCR for *Cg*-β*GBPs*

To determine tissue expression patterns of *Cg*-βGBPs, three Pacific oysters were sampled from Matsushima Bay, Myagi, Japan, in August 2007. About 500 μl of hemolymph was drawn from the adductor muscle sinus of each oyster with a 1 ml syringe and a 25 gauge needle, and immediately centrifuged at 800 × *g* for 2 min at 4 °C. Collected hemocyte pellets were immersed in RNA*later* (Qiagen) and stored at −20 °C. Concurrently, the oysters were shucked and small pieces of the mantle, gills and digestive gland were excised and stored in RNA*later* (Qiagen). The total RNA was extracted from each tissue fragment using the RNeasy Mini Kit (Qiagen). RNA quantities were measured using a Gene Quant RNA/DNA calculator (Amersham Biosciences, Piscataway, NJ, USA). A 100 ng sub-sample of the total RNA was used to synthesize cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

PCR primers for each oyster's βGBPs were designed (Table 1). For PCR, 1 μl of the synthesized cDNA in 15.1 μl of water was mixed with 0.6 μl of 20 μM of each primer, 0.1 μl of Takara Ex Taq DNA polymerase, 2 μl of 10 × PCR buffer and 1.6 μl of dNTP (Takara). The PCR conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s. In order to confirm specificity of each primer pair, 5 ng of plasmids containing each βGBP cDNA were applied. PCR products were separated and visualized as described above.

Gene expression of 18S rRNA was used as the internal control. Preparation of PCR mixtures was the same as described above with the exception of primers (Table 1). The PCR conditions for primers were as follows: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing and extension at 60 °C for 60 s.

2.4. Expression of recombinant *Cg*-β*GBP-1 and -2 (rCg*-β*GBP-1 and -2)*

In order to express *Cg*-βGBP-1 and *Cg*-βGBP-2, the cDNA fragments encoding mature proteins, 90-1697 of *Cg*-βGBP-1 (Fig. 1) and 68-1357 of *Cg*-βGBP-2 (Fig. 2), were generated by PCR using the primers listed in Table 1. The amplified products were cloned into pET22b (+) (Takara), and the cloning reactions were used to transform One Shot TOP 10 *Escherichia coli* (Invitrogen). Cloned expression vectors were then transferred into BL21 Star (DE3) One Shot Chemically Competent cell *E. coli* for expression. Induction of recombinant protein expression was conducted by addition of IPTG at a final concentration of 1 mM.

Expressed recombinant proteins were purified using TALON Metal Resin (Takara) under denaturing conditions in 8 M urea, following the manufacture's instructions. Purified recombinant *Cg*-βGBPs (r*Cg*-βGBPs) were renatured by dialysis in PBS, and their purity was analyzed by SDS-PAGE, and then they were stained with Coomassie Brilliant Blue.