Fish & Shellfish Immunology 51 (2016) 401–404

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Identification, expression, and innate immune responses of two insulin-like peptide genes in the razor clam *Sinonovacula constricta*

Donghong Niu [a, 1], Fei Wang [a, 1], Honggang Zhao [a, b], Ze Wang [a], Shumei Xie [a], Jiale Li [a, *]

[a] Shanghai Engineering Research Center of Aquaculture and College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China
[b] Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL 36849, USA

ARTICLE INFO

Article history:
Received 19 June 2015
Received in revised form
8 March 2016
Accepted 10 March 2016
Available online 12 March 2016

Keywords:
Sinonovacula constricta
Insulin-like peptide
ILP
Innate immunity

ABSTRACT

Insulin-like peptide (ILP) has emerged as a cell regulatory factor with multiple functions in vertebrates and invertebrates. In the present study, we identified and characterized two ILP genes, ILP1 and ILP2, in the razor clam *Sinonovacula constricta*. Both ILPs have a signal peptide and a mature domain consisting of six strictly conserved cysteines. The tertiary structure is divided into three main α-helices with a C-domain loop that separates helix 1 from helix 2. Both of ILPs were found to be regulated according to tissue type and developmental stage. After challenge with *Vibrio anguillarum*, *Vibrio parahaemolyticus* and *Micrococcus lysodeikticus*, the expression of two ILP genes was significantly up-regulated in the liver, hemocytes and mantle tissues, suggesting that the ILPs may play roles in the innate immunity in the razor clam *Sinonovacula constricta*.

1. Introduction

In vertebrates, the insulin/insulin-like growth factors (IIS) pathway includes the ligands insulin, insulin-like growth factors (IGFs) and relaxins [1], which play important roles in growth, metabolism, hormone secretion, cell osmotic pressure, and immunologic function [2]. Insulin and IGF are among the most important insulin-like peptides (ILPs) [3]. In invertebrates, multiple insulin-like peptides (ILPs) were found and bombyxin was the first reported ILP [4]. Subsequently, 211 types of ILPs were found in 38 species, including insects, molluscs and deuterostomes [5,6]. These ILPs combine with the insulin-like receptor and enter the signal protein network [7,8].

The insulin-like family can regulate diverse physiological processes. IGF-1 typically functions as a promoter of increased appetite and growth in fishes, as evidenced by the positive correlation between circulating IGF-1 and growth rate that has been observed in several fish species [9–12]. Furthermore, ILPs were proven to play a role in the cellular transformation of glucose into glycogen, and thus participate in catabolism, lipid synthesis, and proteometabolism [13]. Besides, IGF-1 may play a role in antimicrobial activity during critical periods in postpartum dairy animals by delaying and reversing the effects of lipopolysaccharides [14]. However, there are very few studies on the function of ILP in bivalves [15–17].

The razor clam, *Sinonovacula constricta*, is a commercially important bivalve that is widely distributed in the lower-to-mid intertidal zones along the coasts of the Western Pacific Ocean. In recent years, this razor clam are exceedingly susceptible to the degradation of germplasm resources, pollution as a result of high-density aquaculture, as well as pathogenic infection [18]. Aquatic invertebrate animals with an open circulatory or semi-open circulatory system are always exposed to the environment, as a result of which they may come into contact with various potential pathogens and bacteria [19]. For example, many *Vibrio* species constitute the primary route for disease transmission, and infection is often grossly evident in all life stages of bivalve molluscs [20]. In the present study, we aimed to examine the structure and characteristics of the *ILP* genes, its expression patterns and its involvement in immune responses against *Vibrio anguillarum*, *Vibrio parahaemolyticus* and *Micrococcus lysodeikticus* in the razor clam.

* Corresponding author. Shanghai Engineering Research Center of Aquaculture and College of Fisheries and Life Science, Shanghai Ocean University, 999 Hucheng Huan Road, Shanghai 201306, China.
E-mail address: jlli2009@126.com (J. Li).
[1] Both authors contributed equally to the manuscript.

http://dx.doi.org/10.1016/j.fsi.2016.03.012
1050-4648/

2. Materials and methods

2.1. Database search and phylogenetic analysis

The related sequences obtained from the transcriptome library were analyzed using *BlastX* to confirm that the expressed sequence tags (ESTs) were aligned to the ILP genes. These sequences were further confirmed in both directions with forward and reverse primers (Supplementary Table 1) using the Sanger method on the ABI3730 platform (Applied Biosystems, Forster City, CA, USA). The ILP gene open reading frame (ORF) was predicted using the Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the signal peptides and mature domains were identified using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and the Simple Molecular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Based on the transcriptome data and using the ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/), the arginine and lysine propeptide cleavage sites were mapped. The predicted tertiary structure of ILPs was determined using Protein Homology/analogY Recognition Engine V 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The phylogenetic tree was constructed using the neighbor-joining (NJ) method of the Molecular Evolutionary Genetics Analysis (MEGA5.0) package based on the deduced full-length amino acid sequences with 10000 bootstrapping replications. The data were analyzed using Poisson correction, and gaps were removed by complete deletion.

2.2. Biological material

All experimental razor clams were obtained from Yuejingyang Farm, Ninghai City, Zhejiang Province, China. Embryos and larvae from different developmental stages, including post-fertilization embryos, trochophores (6 h after fertilization), veliger larvae (1 day after fertilization), umbo larvae (2–5 days after fertilization), creeping larvae (6 days after fertilization), and juvenile clams (7–39 days after fertilization), were collected using silk screens with different mesh sizes and stored in 1.5-ml micro tubes with RNA storage liquid.

2.3. Bacterial challenge and collection of tissue samples

Bacterial challenge with *Vibrio anguillarum*, *V. parahaemolyticus* and *Micrococcus lysodeikticus* was conducted on clams with an average body weight of 9.2 g and an average body length of 5.5 cm. Before the challenge, the clams were acclimatized in the laboratory with aeration at 25–27 °C for 1 week. There were three challenge groups (OD600 = 0.4): the *V. anguillarum*-challenged group, the *V. parahaemolyticus*-challenged group, and the *M. lysodeikticus*-challenged group. Three corresponding control groups (phosphate-buffered saline [PBS], pH = 7.2–7.4) were also used. These treatments were conducted by injecting 50 μL of solution into the clam foot.

To determine the level of expression of the ILP genes in healthy tissues, including mantle, foot, siphon, gill, liver, and gonads, and hemocytes (four pools for each tissue and the equal amounts of tissue from six clams in each pool) were isolated and flash-frozen in liquid nitrogen and stored at −80 °C. Similarly, the liver, hemocytes and mantle tissues were isolated for RNA extraction at 0, 4, 8, 12, 24, 48, 72 h after bacterial injection. Concurrently, a control sample was taken at each time point as a negative control.

2.4. RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted using the RNAsimple total RNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The purity of RNA was checked by electrophoresis on a 1% agarose gel. First-strand cDNA synthesis was performed in a volume of 20 μL with 1 μg total RNA and 4 μL 5 × PrimeScript RT Master Mix (Perfect Real Time) using the PrimeScript RT reagent kit (Takara, Otsu, Shiga, Japan) according to the manufacturer's protocol, and all the cDNA samples were stored at −20 °C.

Quantitative real-time PCR (qRT-PCR) was performed using the CFX Real Time Detection System (BioRad, Hercules, CA, USA). Primers were designed and listed in Supplementary Table 1. The reaction volumes containing the following components: 10 μL of 2 × SYBR Premix Ex taq TM (Takara, Otsu, Shiga, Japan), 0.8 μL of each gene-specific primer (10 μM), 1.6 μL of cDNA, and 6.8 μL of nuclease-free water. The qRT-PCR protocol was as follows: one cycle at 95 °C for 30 s; 39 cycles at 95 °C for 5 s and 55 °C for 30 s; and dissociation curve analysis at 95 °C/10 s, 65 °C/5 s and 95 °C/15 s to verify the amplification of a single product.

For qRT-PCR, the 18S rRNA gene was used as the reference gene. The triplicate fluorescence intensities of the control and treatment products for each gene, as determined from the crossing-point values, were compared and converted into fold differences by the relative quantification method using Relative expression Software Tool 384 v. 1 (REST) [21] and assuming 100% efficiency. The differences in expression between the control and challenge groups were assessed for statistical significance ($p < 0.05$) using a randomization test with the REST software.

3. Results and discussion

3.1. Identification of ILP genes

Two ILP genes were identified from the transcriptome of *Sinonovacula constricta* [22]. The ORFs of two ILPs were 525 bp and 537 bp in length, and encoded 174 and 178 amino acids, respectively (Supplementary Fig. 1), referred to as ILP1 (KR534869) and ILP2 (KR534870). Both ILP genes comprised of a signal peptide followed by a B-chain, a connecting peptide (C-peptide), and an A-chain. The two ILP genes are composed of six cysteines in the A and B peptides, with the signature amino acid sequence CCX(3)CX(8)C. The two terminals of the ILP1 and ILP2 C-chain contain the classical arginine and lysine propeptide cleavage sites (Supplementary Fig. 2). The propeptide is converted into a mature form by proteolytic cleavage of the C-peptide [23]. The putative disulfide bonds have been assumed to form two inter-chain bridges across the two chains and one intra-chain bond on the A-chain (Supplementary Fig. 3), which was deemed to be pivotal for the functional structure of the mature dimeric peptide [24]. Compared with the primary structure, the two ILP genes lack D and E domains, which suggest that they are insulins rather than IGF orthologues [25]. The tertiary structure of both the genes is divided into three main α-helices with a C-domain loop that separates helix 1 from helix 2 (Supplementary Fig. 4). This structure allows optional occupancy of space and creates a strong hydrophobic core that contributes to biosynthesis functions and shares similarities with human proinsulin and IGF-1 [26].

3.2. Phylogenetic analysis of ILPs

The phylogenetic tree was constructed to clarify the phylogenetic relationship among insulin, IGFs, relaxin, and ILPs in this study. The results showed that the ILPs of molluscs and the ILPs of *Drosophila* spp. are clustered. Moreover, phylogenetic analysis indicated that insulin, IGF1, IGF2, and relaxin genes in vertebrates can be classified into their respective clades (Supplementary Fig. 5). Multiple sequence alignment of members of the insulin family