Fish and Shellfish Immunology 95 (2019) 688–696

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Lectin-like and bacterial-agglutinating activities of heat shock proteins Hsp5 and Hsp90α from amphioxus *Branchiostoma japonicum*

Lan Yao[a], Baozhen Qu[a], Zengyu Ma[a], Ying Chen[a], Yunxia Tan[a], Zhan Gao[a,**], Shicui Zhang[a,b,*]

[a] Institute of Evolution & Marine Biodiversity and Department of Marine Biology, Ocean University of China, Qingdao, 266003, China
[b] Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao, 266003, China

ARTICLE INFO

Keywords:
Heat shock protein
Amphioxus
Branchiostoma
Agglutination
Lectin-like

ABSTRACT

Previous studies have shown that heat shock proteins (Hsps) are broadly associated in immune responses in a variety of animals. However, it remains largely unknown about the direct roles of Hsps during a bacterial infection. In this study, we have cloned and characterized the cDNAs of two Hsp genes in the amphioxus *Branchiostoma japonicum*, termed *Bjhsp5* and *Bjhsp90α*, the first ones in this evolutionarily important animal. Both *Bjhsp5* and *Bjhsp90α* showed distinct tissue expression patterns, and were inducible by challenge with lipopolysaccharide (LPS) and lipoteichoic acid (LTA), suggesting they may be involved in anti-infectious responses. We also showed that both BjHsp5 and BjHsp90α displayed lectin-like property with affinity to both the Gram-negative and -positive bacteria as well as their signature molecules LPS and LTA, hinting they may both act as a pattern recognition receptor, capable of identifying pathogens. In addition, we found that BjHsp5 and BjHsp90α were both able to agglutinate the Gram-negative and -positive bacteria in the presence of Ca^{2+}, suggesting they may be able to trap the invading pathogens together *in vivo*, avoiding them moving around and thereby protecting the host from pathogenic attack. These data provide a new angle to the roles of Hsps in immune defense.

1. Introduction

Heat shock proteins (Hsps) are molecular chaperones that are produced in virtually all organisms, ranging from bacteria to mammals, in response to high temperature and other environmental stresses. Based on their molecular mass and sequence similarities, Hsps have been classified into six families: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small molecular weight Hsps [1,2]. In general, Hsps are stress-inducible by high temperature [3], ultraviolet radiation [4], heavy metals [5], parasitic infection [6], and chemicals [7]. Most Hsps are expressed intracellularly. However, some Hsps are secreted to the cell exterior particularly in response to stress [8,9]. Hsps play a central role in proteostasis via promoting correct protein refolding, protein trafficking and protein complex assembly/disassembly [10]. In addition, Hsps exhibit a variety of functions in immune response [8,11]. For example, both Hsp90 and Hsp70 have been shown related with bacterial challenge in fish [12–16], scallop [17,18] and shrimp [19–21]. Similarly, Hsp70 has been shown involved in antiviral responses in grass carp [22] and shrimp [23], and Hsp27 involved in the response against nervous necrosis virus infection in sea perch [24]. In addition, Hsp40 and Hsp70 have been found associated with regulation of immune NF-κB signaling in humans and fruit-fly [25]. Despite these enormous studies, however, our understanding of the role of Hsps during a bacterial infection remains rather limited.

Amphioxus or lancelet, the basal extant chordate lineage, has a vertebrate-like body plan including dorsal neural tube, notochord, segmented somites and pharyngeal gill slits, but it is less complex than vertebrates, having a genome uncomplicated by extensive genomic duplication [26]. It is hence an important reference to the origin and evolution of vertebrate species. In particular, the findings of the presence of the complement system [27–30], the acute phase response [31–33], and the constituent elements of key molecules involved in adaptive immunity [34–37], together with the relative structural and genomic simplicity have made amphioxus a rising star for gaining insights into the origin and evolution of the vertebrate immune system [33]. However, little information is available to date regarding Hsps in this evolutionarily important animal, though Li et al. [38] has isolated and analyzed the promoter of amphioxus Hsp70. In this study, we have cloned Hsp5 and Hsp90α cDNAs, designated *Bjhsp5* and *Bjhsp90α*, from Qingdao amphioxus *Branchiostoma japonicum*, and shown that their

* Corresponding author. Room 320, Darwin Building, 5 Yushan Road, Ocean University of China, Qingdao, 266003, China.
** Corresponding author. Room 213, Darwin Building, 5 Yushan Road, Ocean University of China, Qingdao, 266003, China.
E-mail addresses: gaozhan@ouc.edu.cn (Z. Gao), sczhang@ouc.edu.cn (S. Zhang).

https://doi.org/10.1016/j.fsi.2019.10.074
Received 22 August 2019; Received in revised form 19 October 2019; Accepted 30 October 2019
Available online 02 November 2019
1050-4648/

expression are both inducible by lipopolysaccharide (LPS). We also demonstrated that both recombinant BjHsp5 and BjHsp90α exhibited lectin-like and bacterial-agglutinating activities. Our results provide a new insight into the role of Hsps in immune response.

2. Materials and methods

2.1. RNA extraction and cDNAs synthesis

Adult amphioxus (*Branchiostoma japonicum*) were collected during the breeding season in the vicinity of Qingdao, China and cultured in aerated seawater at room temperature for one week. Total RNAs were extracted with Trizol (TaKaRa, Dalian, China) from *B. japonicum* according to the manufacturer's instructions. After digestion with recombinant DNase I (RNase free) (TaKaRa) to eliminate the genomic contamination, the cDNAs were synthesized with reverse transcription system (Promega) using oligo d(T) primer. The reaction was carried out at 42 °C for 50 min and inactivated at 75 °C for 15 min. The cDNAs synthesized were stored at −20 °C until use.

2.2. Gene cloning and sequencing

Based on the sequences of heat shock protein 5 (Hsp5) gene (No. 105210R) and heat shock protein 90 α (Hsp90α) gene (No. 312830F) in the database of *B. belcheri* genome (http://mosas.sysu.edu.cn/genome/index.php), two pairs of primers P1 and P2, and P3 and P4 (Supplementary Table 1) were designed using Primer Premier 5.0 program. The PCR amplification reaction was carried out at 94 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 3 min, and a final extension at 72 °C for 7 min. After purification with DNA gel extraction kit (Omega), the PCR products were cloned into the pGEM-T vector (Invitrogen) at 4 °C overnight, and transformed into Trans5α *Escherichia coli* (TransGen). The positive clones were selected and sequenced to verify for authenticity.

2.3. Sequence analyses

The domain of deduced protein was analyzed using the SMART program (http://smart.embl-heidelberg.de/). Signal peptide was predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight (MW) and isoelectronic points (pI) of the mature protein were determined using ProtParam (http://www.expasy.ch/tools/protparam.html). Homology searches in the GenBank database were carried out by BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple protein sequences were aligned using the MegAlign program of the LASERGENE software suite (DNASTAR).

The information of exon-intron organization of Hsp5 and Hsp90α genes were obtained from NCBI database (http://www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed by MEGA6.0 using p-distance based on the neighbor-joining method [39]. The reliability of each node was estimated by bootstrapping with 1000 replications.

2.4. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was used to examine the expression profiles of amphioxus Hsp5 gene, *Bjhsp5,* and Hsp90α gene, *Bjhsp90α,* in the different tissues of *B. japonicum*. Total RNAs were extracted with Trizol from the different tissues collected from 5 individuals, including the hepatic caecum, hind-gut, gill, muscle, notochord, testis and ovary dissected out of *B. japonicum*. After digestion with RNase-free DNase to eliminate the genomic contamination, the cDNAs were synthesized with reverse transcription system using oligo d (T) primer, and used for qRT-PCR. The PCR primer pairs P5 and P6, P7 and P8 as well as P9 and P10 (Supplementary Table 1) specific of *Bjhsp5*, *Bjhsp90α* and *EF1α* were designed using primer 5.0 program. The *EF1α* gene was selected as the reference for internal standardization. The amplification efficiency of each primer set was assessed using the hind-gut cDNA serially diluted 2-fold (Supplementary Table 2). qRT-PCR was performed on ABI 7500 Realtime PCR system (Applied Biosystems, USA) as described by Wang et al. [40]. The expression levels of *Bjhsp5* and *Bjhsp90α* relative to that of *EF1α* were calculated by the comparative C_T method ($2^{-\triangle\triangle CT}$) of Livak et al. [41].

qRT-PCR was also performed to assay the expression profiles of *Bjhsp5* and *Bjhsp90α* in response to challenge with the bacterial signature molecules lipopolysaccharide, LPS and lipoteichoic acid, LTA [42,43]. A total of 90 adult amphioxus were acclimatized in sterilized filtered seawater for two days, divided into three groups (30 animals/group) and then exposed to sterilized seawater with either 10 μg/ml of LPS (Sigma, USA) or 10 μg/ml LTA (Sigma, USA) or sterilized seawater alone (control). Three animals were sampled from each group at 0, 2, 4, 8, 12, 24, 48 and 72 h post exposure. RNA extraction, cDNA synthesis and qRT-PCR were performed as above.

2.5. Construction of expression vector

The sequences encoding mature BjHsp5 and BjHsp90α were amplified by PCR using the primer pairs P11 and P12 as well as P13 and P14 (Supplementary Table 1) with *EcoR* I and *Hind* III sites in the forward and reverse primers, respectively. The PCR products were subcloned into the plasmid expression vector pET-28a (Novagen) previously cut with the restriction enzymes *EcoR* I and *Hind* III. The identity of inserts was verified by sequencing, and the constructed plasmids were designated *pET-28a/BjHsp5* and *pET-28a/BjHsp90α*, individually.

2.6. Expression and purification of recombinant proteins

The plasmids *pET28a/BjHsp5* and *pET28a/BjHsp90α* were transformed into *E. coli* transetta (DE3), respectively, and the transformed *E. coli* cells were cultured overnight in Luria-Bertani (LB) broth containing kanamycin (50 μg/ml). The cultures were diluted 1:50 with LB broth and further incubated at 37 °C for about 5 h. The expression of recombinant proteins, rBjHsp5 and rBjHsp90α, was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to the cultures at a final concentration of 0.1 mM. After further incubation at 20 °C for 8 h, the bacterial cells were harvested by centrifugation at 5000*g* at 4 °C for 20 min, re-suspended in 30 ml PBS (pH7.4) consisting of 8 mM Na_2HPO_4, 136 mM NaCl, 2 mM KH_2PO4 and 2.6 mM KCl, and sonicated on ice. After centrifugation at 12,000 g at 4 °C for 20 min, the supernatants were collected, filtered through 0.22 μm filter membrane, and loaded onto a Ni-NTA resin column (GE Healthcare). The column was successively washed with PBS containing 10 mM, 20 mM, 30 mM and 40 mM imidazole, respectively, and then eluted with PBS containing 250 mM imidazole. The purified rBjHsp5 and rBjHsp90α were dialyzed at 4 °C against PBS, which were changed every 6 h until imidazole was removed completely. For control, recombinant thioredoxin with His Tag (rTRX) was also expressed, purified and processed as above.

The purity of the eluted samples and purified proteins were analyzed by a 12% SDS-PAGE gel and stained with Coomassie brilliant blue R-250. The concentrations of the recombinant proteins were determined by BCA method.

2.7. Western blotting

The extracts of *E. coli* Transetta (DE3) containing *pET-28a/BjHsp5* and *pET-28a/BjHsp90α* before and after IPTG induction as well as the purified recombinant proteins were run on a 12% SDS-PAGE gel. The dissolved proteins on the gels were electroblotted onto PVDF membrane (Merck Millipore) by a semi-dry technique (Bio-RAD). After blocking with 4% bovine serum albumin (BSA) in PBS (pH7.4) at room temperature for 2 h, the PVDF membranes were incubated with anti-His tag

mouse monoclonal antibody (CWBIO, China) diluted 1:4000 with 4% BSA in PBS at 4 °C overnight. The membranes were washed four times with PBS containing 0.1% Tween-20 (PBST), and then incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (CWBIO, China) diluted 1:8000 with 4% BSA in PBS at room temperature for 1 h. The bands were visualized using diaminobenzidine (DAB) kit (CWBIO, China) according to the manufacturer's instructions.

2.8. Bacterial binding assay

To test the bacterial binding activity of rBjHsp5 and rBjHsp90α, the Gram-negative bacteria *E. coli* and *Aeromonas hydrophila* (ATCC 35654) as well as the Gram-positive bacteria *S. aureus* and *Bacillus subtilis* (ATCC 6633) were cultured to mid-logarithmic phase, and collected by centrifugation at 6000*g* for 5 min. After washing twice with PBS, the bacteria were re-suspended in PBS, giving a density of 1×10^8 cells/ml. Aliquots of 300 μl of the bacterial suspensions were mixed with 150 μl of 100 μg/ml rBjHsp5, rBjHsp90α or rTRX (control), incubated at 25 °C for 1 h, and centrifuged at 6000 g at 4 °C for 5 min. The bacterial pellets were washed three times with PBS and re-suspended in 300 μl PBS. The bacterial suspensions were subjected to 12% SDS-PAGE gel and the binding activity was determined by Western blotting as described above.

2.9. Ligand binding assay

An enzyme-linked immunosorbent assay (ELISA) was performed to test if rBjHsp5 and rBjHsp90α could bind to LPS, LTA and peptidoglycan (PGN). LPS, LTA and PGN were individually labelled with biotin hydrazide (Sigma-Aldrich) as described by Wang et al. [44]. Aliquots of 50 μl of 40 μg/ml rBjHsp5, rBjHsp90α and rTRX (control) were applied to each well of a 96-well microplate and air-dried at 16 °C overnight. The plate was incubated at 60 °C for 30 min to further fix the proteins, and then each well was blocked with 200 μl of 20 mg/ml BSA in PBS (pH7.4) at 37 °C for 2 h. After washing five times with 200 μl of PBS (pH7.4) containing 0.5% Tween-20 (PBST), a total of 50 μl PBS containing 1 mg/ml BSA plus different concentrations of biotinylated LPS, LTA or PGN (0, 0.25, 0.5, 1, 2, 4, 8 and 16 μg/ml) was added into each well and incubated at 25 °C for 3 h. The wells were each washed five times with 200 μl PBST, and 100 μl of streptavidin-HRP (CWBIO) diluted to 1:3000 with 10 mM PBS (pH7.4) containing 1 mg/ml BSA was added to each well. After incubation at room temperature for 1 h, the wells were washed five times with 200 μl PBST, added with 100 μl of 0.4 mg/ml O-phenylenediamine (Amresco) in the buffer consisting of 51.4 mM Na_2HPO_4, 24.3 mM citric acid and 0.045% H_2O_2 (pH5.0) and reacted at 37 °C for 10 min. Subsequently, 50 μl of 2 M H_2SO_4 was added to each well to terminate the reaction, and absorbances at 492 nm were monitored by a microplate reader (Multikan GO; Thermo Scientific).

2.10. Assay for effects of sugars on binding of rBjHsp5 and rBjHsp90α to ligands

To test if sugars could inhibit the binding of rBjHsp5 and rBjHsp90α to the ligands, a competitive ELISA using various monosaccharides was performed as described by Gao et al. [45]. In brief, the wells coated with rBjHsp5 or rBjHsp90α (2 μg/well) were blocked with BSA. Then, 50 μl of 16 μg/ml biotinylated LPS, LTA or PGN containing 100 mM of $_D$-glucose, $_D$-galactose, $_D$-mannose, N-acetyl-$_D$-glucosamine (GlcNAc), N-acetyl-$_D$-galactosamine (GalNAc) and N-acetyl-$_D$-mannosamine (ManNAc), were added into each well. After incubation at 25 °C for 3 h, the binding of rBjHsp5 and rBjHsp90α to the ligands were measured as described above.

2.11. Assay for bacterial agglutination

Pilot experiments showed that neither rBjHsp5 nor rBjHsp90α had antibacterial activity (data not shown), thus we tested their agglutination activity towards the bacteria. Both the Gram-negative bacterium *E. coli* (ATCC 25922) and the Gram-positive bacterium *S. aureus* (ATCC 25923) were cultured to mid-logarithmic phase and harvested by centrifugation at 6000*g* for 5 min. The bacteria were washed twice in PBS and re-suspended in PBS yielding a density of 2×10^8 cells/ml. Aliquots of 25 μl bacterial suspensions were mixed with 25 μl of 100 μg/ml rBjHsp5, rBjHsp90α or rTRX (control) in the presence or absence of 10 mM $CaCl_2$, incubated at 25 °C for 1 h, and observed under a microscope.

2.12. Statistical analysis

The experiments were performed in triplicate, and repeated three times. Data were subjected to statistical evaluation with unpaired *t*-test, and the value $p < 0.05$ was considered as significant. All the data were expressed as mean ± SEM.

3. Results

3.1. Sequence characteristics, phylogeny and genomic organization of BjHsp5 and BjHsp90α

The open reading frame (ORF) of *Bjhsp5* cDNA we obtained (GenBank accession number: MN218730) was 2001 bp in length, and coded for a deduced protein of 666 amino acids with a calculated molecular mass of ~73.8 kDa and an isoelectric point (pI) of 4.72 (Supplementary Figure 1). BjHsp5 possessed an N-terminal signal peptide of 22 amino acids and a HSP5 domain of 606 amino acids (Fig. 1A). The ORF of *Bjhsp90α* cDNA obtained (GenBank accession number: MN218731) was 2196 bp long, and encoded a deduced protein of 732 amino acids with a calculated molecular mass of ~84.1 kDa and a pI of 4.72 (Supplementary Figure 1). BjHsp90α had a HATPase_c domain of 155 amino acids and an HSP90α domain of 530 amino acids (Fig. 1B). Phylogenetic tree constructed using the amino acid sequences of Hsp5 and Hsp90α homologues available showed that BjHsp5 and BjHsp90α were both positioned at the base of vertebrate Hsp5 and Hsp90α proteins (Fig. 1C and D), well reflecting the phylogeny of chosen organisms.

A search of the completed draft assembly and automated annotation of *B. floridae* and *B. belcheri* genome both revealed the presence of a single *hsp5* cDNA and its genomic DNA sequence (transcript id: XP_002588195.1 for *B. floridae*; XP_019640840.1 for *B. belcheri*). The single *hsp5* cDNAs from *B. floridae* and *B. belcheri* both encoded a protein with approximately 97% identity to BjHsp5, suggesting that amphioxus Hsp5 is highly conserved in interspecies. Analysis of the genomic structure exhibited that human, mouse and spider *hsp5* genes possessed 8 exons interspaced by 7 introns, while rabbit, jungle fowl, caecilian, pupfish, zebrafish, amphioxus and snail *hsp5* genes had 9 exons interspaced by 8 introns, suggesting that the genomic organization of *hsp5* genes remained largely unchanged during evolution (Fig. 1E). Similarly, we identified only one *hsp90α* cDNA and its genomic DNA sequence in both *B. floridae* and *B. belcheri* genomes (transcript id: XP_019643040.1 for *B. floridae*; XP_019643040.1 for *B. belcheri*). The single *hsp90α* cDNAs from *B. floridae* and *B. belcheri* encoded a protein with 96% identity to BjHsp90α, suggesting that amphioxus Hsp90α is also highly conserved in interspecies. Analysis of the genomic structures revealed that *Bjhsp90α* gene consisted of 7 exons and 6 introns, and *hsp90α* genes of jawed vertebrates, including human, mouse, jungle fowl, boa, frog, pupfish and zebrafish, contained 11 exons and 10 introns. By contrast, fruit fly *hsp90α* gene only had 2 exons and 1 intron, and sea snail *hsp90α* gene comprised 6 exons and 5 introns. Sequence comparison revealed that the exon 2 in *Bjhsp90α* was split into the exons 2, 3, 4, 5, 6 and 7 in

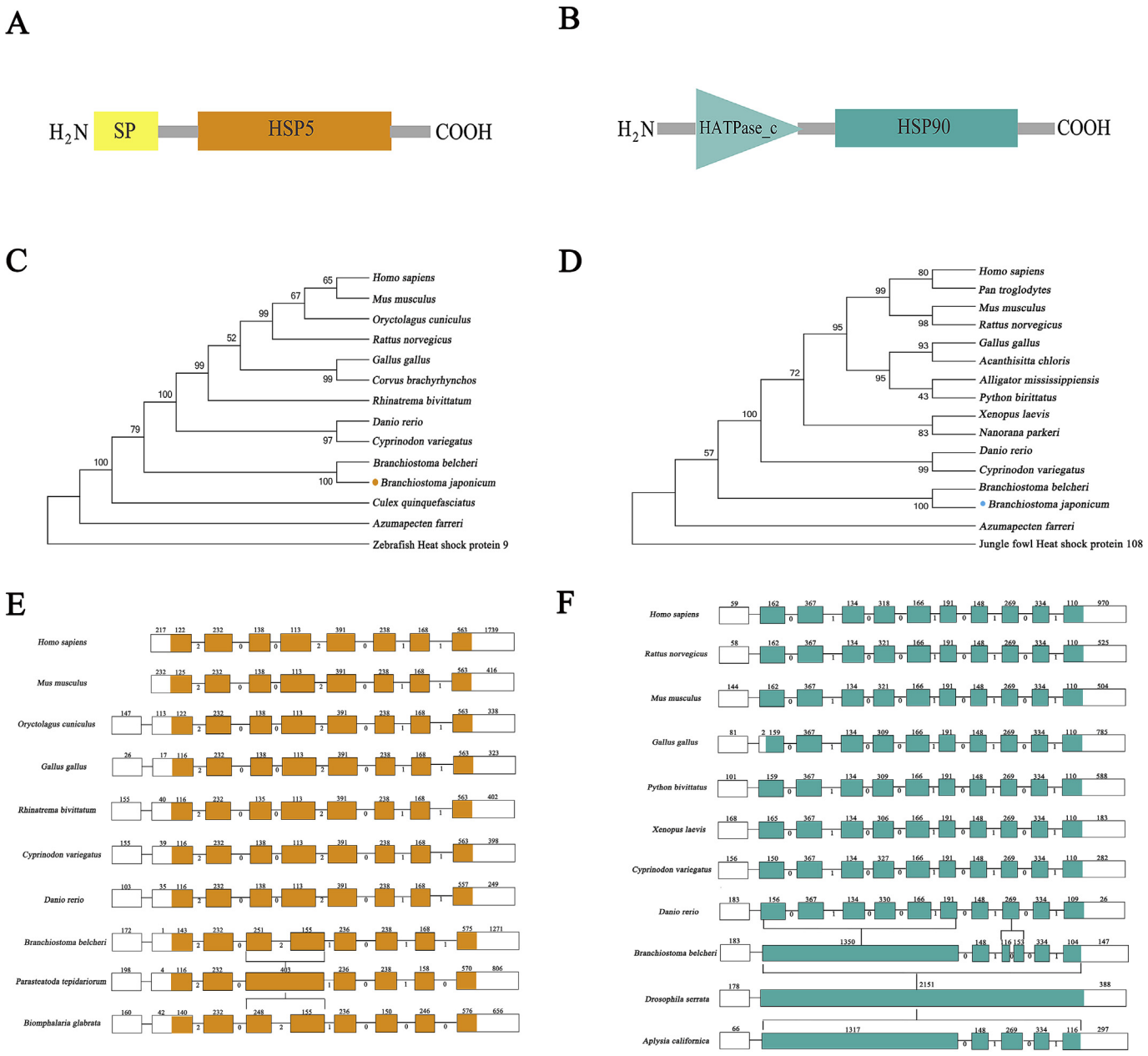

Fig. 1. Domain architecture, phylogenetic tree analysis and genomic organization of BjHsp5 and BjHsp90α.
(A) Domain architecture of BjHSP5 and BjHSP90α. (B) Phylogenetic tree of Hsp5 and Hsp90α proteins from various species. The phylogenetic tree was drawn by MEGA (version 6.0) based on multiple sequence alignment by Clustal W using the neighbor-joining method. The reliability of each node was estimated by bootstrapping with 1000 replications. The numbers shown at each node indicate the bootstrap values (%).The accession numbers of Hsp5 and Hsp90α used were listed in Supplementary Table 3 and Supplementary Table 4. (C) The diagrams showing the genomic structure, length, and organization of *hsp5* genes from human, mouse, rabbit, jungle fowl, caecilian, pupfish, zebrafish and amphioxus, and *hsp90α* genes from human, mouse, jungle fowl, boa, frog, pupfish, zebrafish and amphioxus. Boxes represent the exons of the gene (coding region in colored box; non-coding region in open box), and thin lines indicate the introns. The length of exons and the phases of introns are shown.

jawed vertebrate *hsp90α* genes, and the exons 4 and 5 in *Bjhsp90α* combined into the exon 9 in the vertebrate *hsp90α* genes (Fig. 1F). In addition, the exons 2, 3, 4, 5, 6 and 7 in *Bjhsp90α* matched the exon 2 in fruit fly *hps90α* gene and the exons 2, 3, 4, 5 and 6 in sea snail *hsp90α* gene. In spite of these differences, each exon of *Bjhsp90α* possessed an identical sequence in the vertebrate and invertebrate *hsp90α* genes, suggesting that the general genomic sequence of *hsp90α* genes remains fairly stable throughout evolution.

3.2. Expression profiles of Bjhsp5 and Bjhsp90α

qRT-PCR was employed to detect the transcriptional profiles of *Bjhsp5* and *Bjhsp90α* in the different tissues of *B. japonicum*. As shown in Fig. 2, both *Bjhsp5* and *Bjhsp90α* were ubiquitously expressed in all the tissues examined, including the notochord, testis, muscle, hind-gut, hepatic caecum, gill and ovary. Notably, they both showed highest expression in the ovary. These indicated that both *Bjhsp5* and *Bjhsp90α* had a distinct tissue expression pattern.

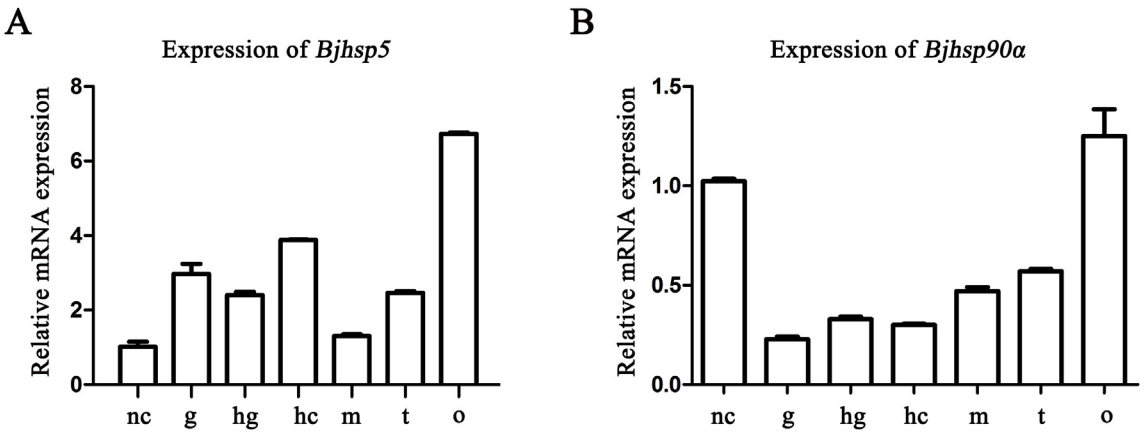

Fig. 2. Tissue-specific expression of *Bjhsp5* and *Bjhsp90α*.
Total RNAs were extracted from various tissues of *B. japonicum*, and the expression profiles of *Bjhsp5* (A) and *Bjhsp90α* (B) were determined in the different tissues by qRT-PCR. nc, notochord; g, gill; hg, hind-gut; hc, hepatic caecum; m, muscle; t, testis; o, ovary. The results shown are mean values ± S.E.M (n = 3).

3.3. Expression of Bjhsp5 and Bjhsp90α in response to LPS and LTA

The challenge with LPS induced a significant increase in the expression of *Bjhsp5* in amphioxus at 8–72 h, and the challenge with LTA induced an upregulation of *Bjhsp5* expression at 48–72 h (Fig. 3A). Similarly, LPS treatment caused a marked increase in the expression of *Bjhsp90α* at 2–72 h, and LTA treatment caused an upregulation of *BjHsp90α* expression at 4–8 h (Fig. 3B). Apparently, the challenge with LPS and LTA both resulted in a considerable increase in the expression of *Bjhsp5* and *Bjhsp90α* in amphioxus, hinting at the clue that BjHsp5 and BjHsp90α may both be involved in the anti-infectious response in amphioxus.

3.4. Binding of rBjHsp5 and rBjHsp90α to bacteria and ligands

The recombinant proteins rBjHsp5 and BjHsp90α expressed in *E. coli* were purified by chromatography on a Ni-NTA resin column. SDS-PAGE analysis showed that purified rBjHsp5 and rBjHsp90α both yielded a single band of approximately 75.3 kDa and 87.9 kDa, respectively, well matching the expected sizes. Western blotting showed that rBjHsp5 and rBjHsp90α were reacted with anti-His tag antibody, indicating that they were correctly expressed (Fig. 4A).

Western blotting analysis revealed that both rBjHsp5 and rBjHsp90α showed an affinity to the Gram-negative bacteria *E. coli* and *A. hydrophila* as well as the Gram-positive bacteria *S. aureus* and *B. subtilis* (Fig. 4B). By contrast, rTRX used as control showed little affinity to the same bacteria examined. These indicated that both rBjHsp5 and rBjHsp90α could specifically bind to the Gram-negative and -positive bacteria.

Next, we examined the ligand binding activity of rBjHsp5 and rBjHsp90α. As shown in Fig. 4C, both rBjHsp5 and rBjHsp90α were able to bind to LPS, LTA and PGN in a dose-dependent manner. By contrast, rTRX displayed little binding to the ligands. These indicated that rBjHsp5 and rBjHsp90α could specifically interact with the bacterial signature molecules LPS, LTA and PGN.

3.5. Inhibition of binding of rBjHsp5 and rBjHsp90α to ligands by sugars

Fig. 5 shows the effects of various sugars on the binding of rBjHsp5 and rBjHsp90α to the ligands. Among the six kinds of sugars tested, we found that only D-mannose significantly inhibited the bindings of both rBjHsp5 and rBjHsp90α to LPS at the concentration of 100 mM (resulting in an approximate 60% reduction). Interestingly, the bindings of rBjHsp5 and rBjHsp90α to LTA were markedly inhibited by GlcNAc, GluNAc and ManNAc at the same concentrations. In addition, GlcNAc and D-glucose also inhibited the interaction of rBjHsp5 and rBjHsp90α

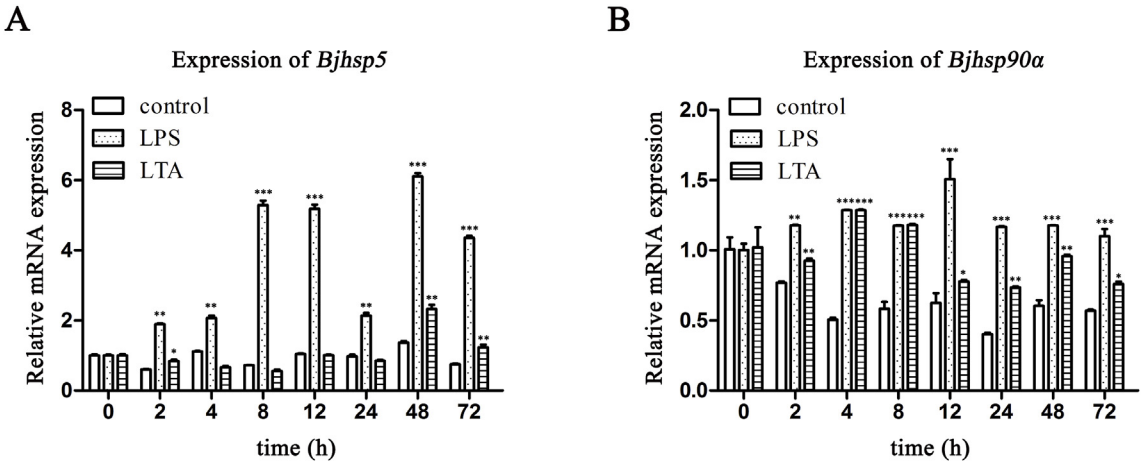

Fig. 3. LPS- and LTA-induced expression of *Bjhsp5* and *Bjhsp90α*.
B. japonicum were sampled at 0, 2, 4, 8, 12, 24, 48 and 72 h after exposure to sterilized seawater containing LPS (10 mg/ml), LTA (10 mg/ml), or sterilized seawater as a control, and total RNA was extracted from whole animals. The expression profiles of *Bjhsp5* (A) and *Bjhsp90α* (B) were determined by qRT-PCR. Datas were normalized to the *EF1α* gene as internal control. The results shown are mean ± S.E.M (n = 3). Asterisks indicate statistically different (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared to the control.

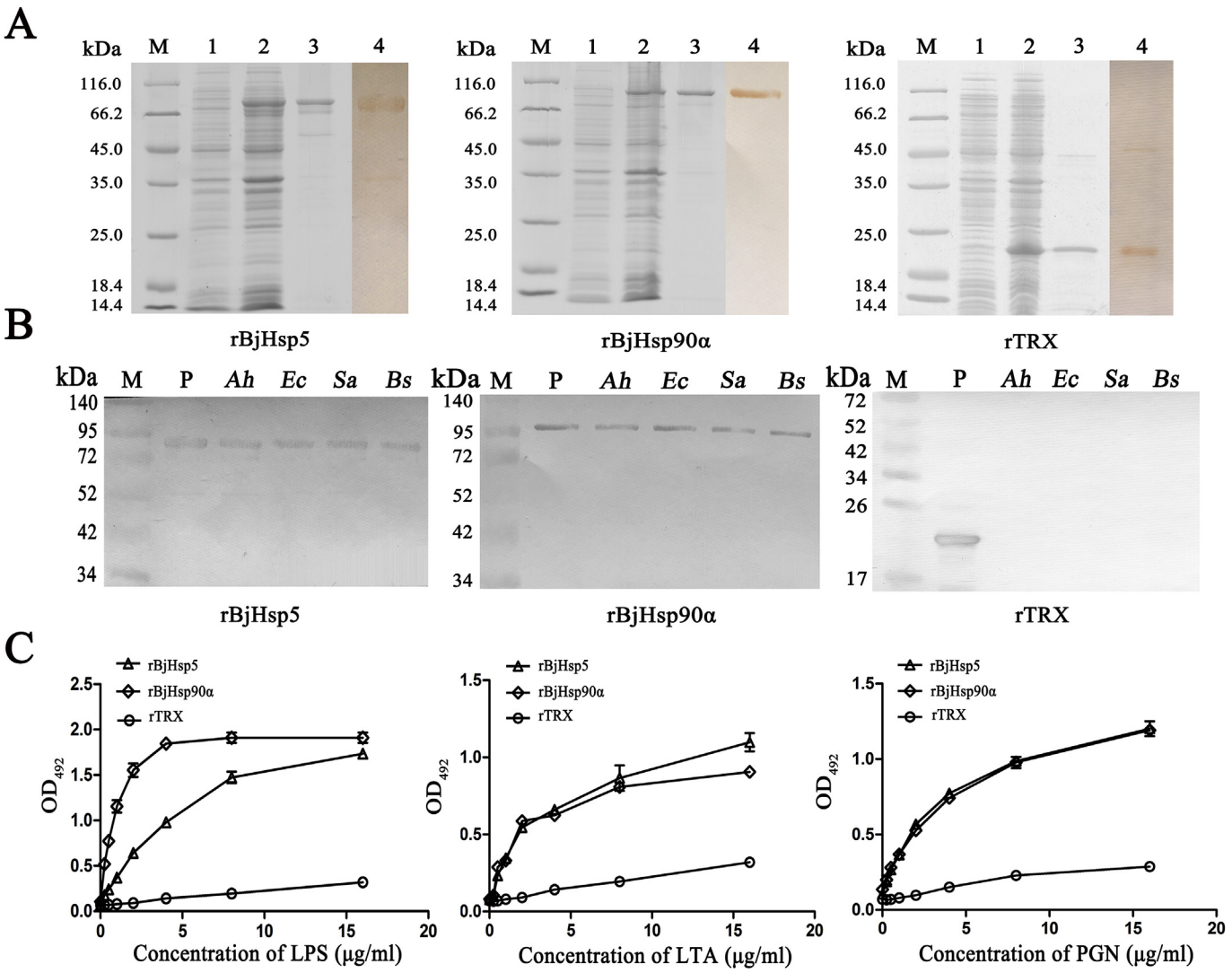

Fig. 4. SDS-PAGE and Western blotting of recombinant proteins and bacterial and ligand binding activity of rBjHsp5 and rBjHsp90α.
(A) SDS-PAGE and Western blotting of recombinant proteins rBjHsp5, rBjHsp90α and rTRX. Lane M, marker; lane 1, total cellular extracts from *E. coli* transetta (DE3) containing expression vector before induction; lane 2, total cellular extracts from IPTG induced *E. coli* transetta (DE3) containing expression vector; lane 3, purified recombinant proteins; lane 4, Western blotting of purified recombinant proteins. (B) Binding of rBjHsp5 and rBjHsp90α to the bacterial cells was revealed by Western blotting. rTRX was employed as control. M, molecular mass standards; P, purified recombinant proteins; *Ah, A. hydrophila* incubated with recombinant proteins; *Ec, E. coli* incubated with recombinant proteins; *Sa, S. aureus* incubated with recombinant proteins; *Bs, B. subtilis* incubated with recombinant proteins. (C) ELISA analysis of the affinity of rBjHsp5 and rBjHsp90α to the ligands LPS, LTA and PGN. rTRX was used as control. The results shown are mean ± S.E.M (n = 3).

with PGN, though their inhibitory activities were apparently weaker (resulting in 20–30% reduction). Together, these data suggested that both rBjHsp5 and rBjHsp90α had lectin-like property.

3.6. Agglutinating activity of rBjHsp5 and rBjHsp90α

As pilot experiments showed that neither rBjHsp5 nor rBjHsp90α showed any antibacterial activity against *E. coli* and *S. aureus* (data not shown), we thus tested if rBjHsp5 and rBjHsp90α had any bacterial agglutination activity. As shown in Fig. 6, both rBjHsp5 and rBjHsp90α showed a conspicuous agglutinating activity towards *E. coli* and *S. aureus* in the presence of Ca^{2+}, but they did not in the absence of Ca^{2+}. By contrast, rTRX (control) showed little agglutinating activity towards *E. coli* and *S. aureus* even in the presence of Ca^{2+}. These indicated that both rBjHsp5 and rBjHsp90α had bacterial agglutinating activity in a Ca^{2+}-dependent fashion.

4. Discussion

Hsps are virtually present in all organisms from prokaryotes to eukaryotes including mammals. In this study we have cloned and characterized the cDNAs of two Hsp genes, *hsp5* and *hsp90α*, in *B. japonicum*, the first data as such in the cephalochordate amphioxus. Both the genes show distinct tissue expression patterns, and are inducible by challenge with LPS and LTA, in amphioxus, generally consistent with those of *hsp90β* and *hsp27* in the vertebrates like fish [15,24]. The basal position of BjHsp5 and BjHsp90α on the phylogenetic tree constructed suggests that they may both represent the archytype of respective Hsps in the vertebrates. Both Hsp5 and Hsp90α are highly conserved in both *B. japonicum* and *B. floridae* as well as *B. belcheri*, thus the genomic structures of *B. belcheri* Hsp5 and Hsp90α can allows us to compare the amphioxus gene structure with that of vertebrate and invertebrate Hsp5 and Hsp90α genes. The comparison clearly demonstrates that the general genomic organization of both Hsp5 and Hsp90α genes is highly conserved throughout evolution in terms of both exon-intron structure and sequence homology, hinting at the clue that both the gene

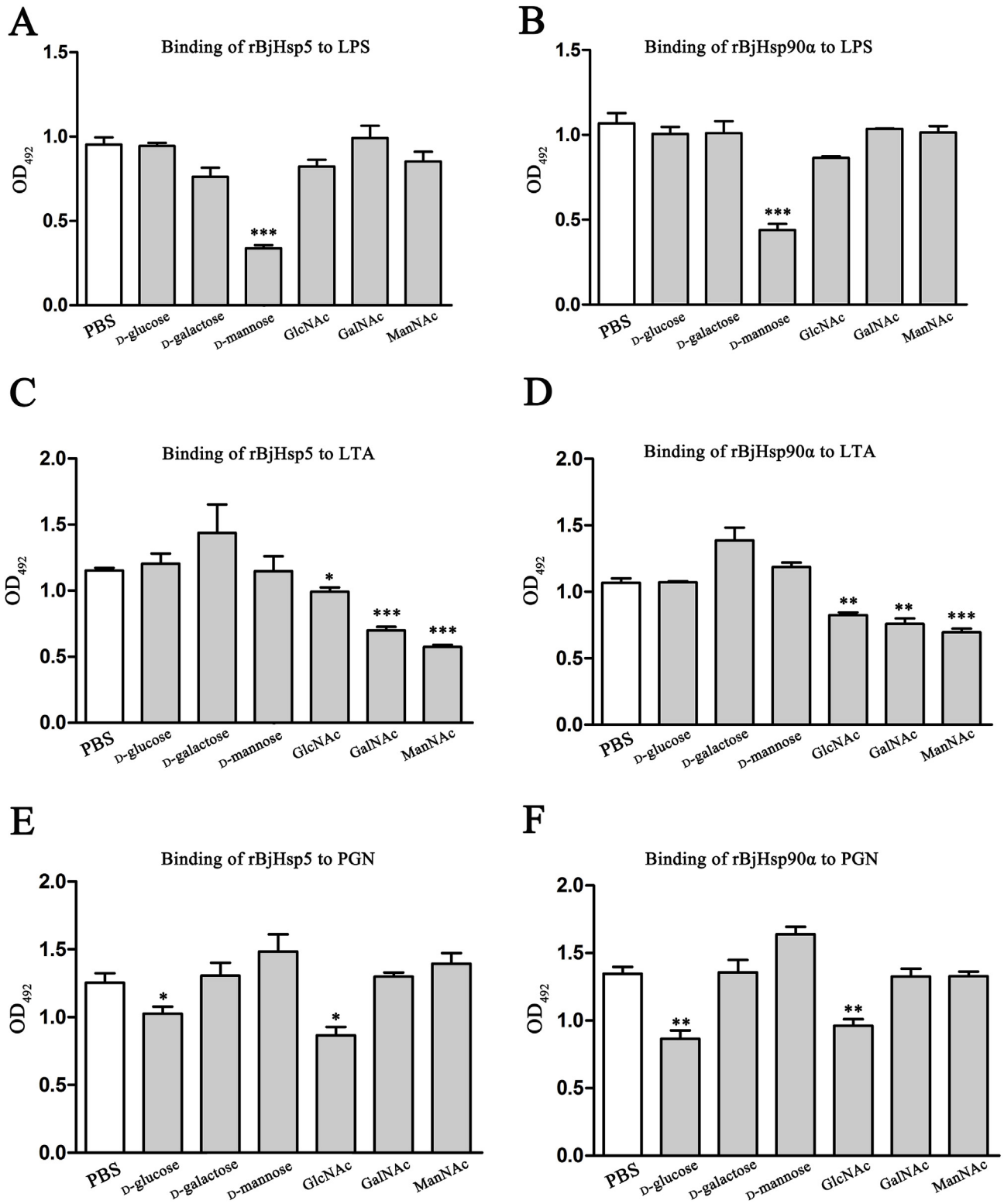

Fig. 5. Inhibitory effects of D-glucose, D-galactose, D-mannose, GlcNAc, GalNAc and ManNAc on the binding of rBjHsp5 and rBjHsp90α to the ligands. The results shown are mean ± S.E.M (n = 3). Asterisks indicate statistically different (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared to the control.

transcription is regulated similarly in amphioxus, vertebrates and invertebrates.

Hsps such as Hsp90, Hsp70, Hsp40 and Hsp27 have been reported to exhibit a variety of functions in immune response [8,11,46]. However, it remains unclear about the role of Hsps during a bacterial infection. We show here that both rBjHsp5 and rBjHsp90α exhibit lectin-like activities with apparent affinity to the Gram-negative and -positive bacteria as well as their signature molecules LPS and LTA. This suggests that BjHsp5 and BjHsp90α may both function as a pattern recognition receptor in the host, capable of recognizing the invading pathogens. We also show that rBjHsp5 and rBjHsp90α both possess the ability to agglutinate the Gram-negative and -positive bacteria in the presence of

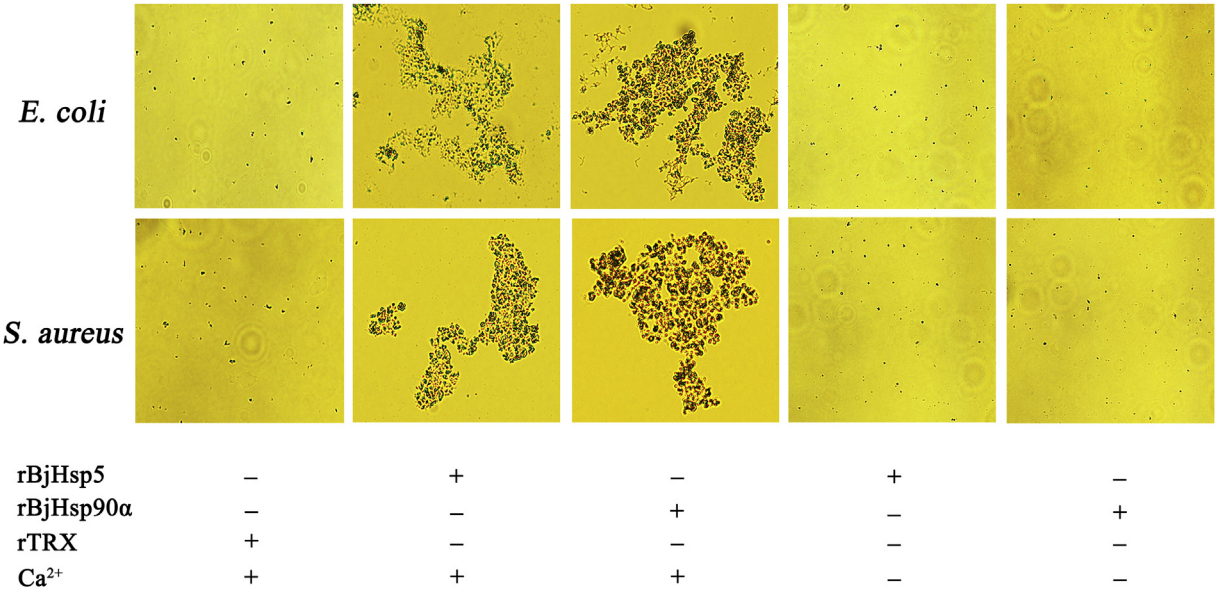

Fig. 6. Bacterial agglutination activity of rBjHsp5 and rBjHsp90α.
Agglutination of *E. coli* and *S.aureus* by rBjHsp5 and rBjHsp90α in the presence or absence of Ca^{2+}.

Ca^{2+}. This may have an important physiological significance that BjHsp5 and BjHsp90α may trap the invading pathogens together, avoiding them moving around, thereby protecting the host from pathogenic assault.

In summary, this study reports the first identification and characterization of Hsp5 and Hsp90α in the basal chordate amphioxus. It also shows for the first time that both Hsp5 and Hsp90α have lectin-like and bacterial-agglutinating activities, providing a new angle to the role of Hsps in immune defense.

Declaration of competing interest

None.

Acknowledgements

This work was supported by the National Key Research and Development Project of the Ministry of Science and Technology [grant numbers 2018YFD0900505] and the Fundamental Research Funds for Central Universities [grant numbers 201822016, 201841005].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.074.

References

[1] C. Garrido, C. Paul, R. Seigneuric, H.H. Kampinga, The small heat shock proteins family: the long forgotten chaperones, Int. J. Biochem. Cell Biol. 44 (2012) 1588–1592, https://doi.org/10.1016/j.biocel.2012.02.022.
[2] K.K. Kim, R. Kim, S.H. Kim, Crystal structure of a small heat-shock protein, Nature 394 (1998) 595–599, https://doi.org/10.1038/29106.
[3] Q. Xu, Q. Zou, H. Zheng, F. Zhang, B. Tang, S. Wang, Three heat shock proteins from Spodoptera exigua: gene cloning, characterization and comparative stress response during heat and cold shocks, Comp. Biochem. Physiol. B 159 (2011) 92–102, https://doi.org/10.1016/j.cbpb.2011.02.005.
[4] W. Sang, W.H. Ma, L. Qiu, Z.H. Zhu, C.L. Lei, The involvement of heat shock protein and cytochrome P450 genes in response to UV-A exposure in the beetle Tribolium castaneum, J. Insect Physiol. 58 (2012) 830–836, https://doi.org/10.1016/j.jinsphys.2012.03.007.
[5] Y. Zhang, Y. Liu, J. Zhang, Y. Guo, E. Ma, Molecular cloning and mRNA expression of heat shock protein genes and their response to cadmium stress in the grasshopper Oxya chinensis, PLoS One 10 (2015) 1–16, https://doi.org/10.1371/journal.pone.0131244.
[6] H. Wang, K. Li, J.Y. Zhu, Q. Fang, G.Y. Ye, H. Wang, K. Li, J.Y. Zhu, Cloning and expression pattern of heat shock protein genes from the endoparasitoid wasp, pteromalus puparum in response to environmental stresses, Arch. Insect Biochem. Physiol. 79 (2012) 247–263, https://doi.org/10.1002/arch.21013.
[7] R. Planelló, O. Herrero, J.L. Martínez-Guitarte, G. Morcillo, Comparative effects of butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) on the aquatic larvae of Chironomus riparius based on gene expression assays related to the endocrine system, the stress response and ribosomes, Aquat. Toxicol. 105 (2011) 62–70, https://doi.org/10.1016/j.aquatox.2011.05.011.
[8] S.K. Calderwood, J. Gong, A. Murshid, Extracellular HSPs: the complicated roles of extracellular HSPs in immunity, Front. Immunol. 7 (2016), https://doi.org/10.3389/fimmu.2016.00159.
[9] G. Multhoff, L.E. Hightower, Distinguishing integral and receptor-bound heat shock protein 70 (Hsp70) on the cell surface by Hsp70-specific antibodies, Cell Stress Chaperones 16 (2011) 251–255, https://doi.org/10.1007/s12192-010-0247-1.
[10] B. Bukau, J. Weissman, A. Horwich, Molecular chaperones and protein quality control, Cell 125 (2006) 443–451, https://doi.org/10.1016/j.cell.2006.04.014.
[11] T. Zininga, L. Ramatsui, A. Shonhai, Heat shock proteins as immunomodulants, Molecules 23 (2018), https://doi.org/10.3390/molecules23112846.
[12] M. Manchado, E. Salas-Leiton, C. Infante, M. Ponce, E. Asensio, A. Crespo, E. Zuasti, J.P. Cañavate, Molecular characterization, gene expression and transcriptional regulation of cytosolic HSP90 genes in the flatfish Senegalese sole (Solea senegalensis Kaup), Gene 416 (2008) 77–84, https://doi.org/10.1016/j.gene.2008.03.007.
[13] E. Hermesz, M. Ábrahám, J. Nemcsók, Identification of two hsp90 genes in carp, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 129 (2001) 397–407, https://doi.org/10.1016/S1532-0456(01)00216-2.
[14] L. Song, C. Li, Y. Xie, S. Liu, J. Zhang, J. Yao, C. Jiang, Y. Li, Z. Liu, Genome-wide identification of Hsp70 genes in channel catfish and their regulated expression after bacterial infection, Fish Shellfish Immunol. 49 (2016) 154–162, https://doi.org/10.1016/j.fsi.2015.12.009.
[15] T. Wei, Y. Gao, R. Wang, T. Xu, A heat shock protein 90 β isoform involved in immune response to bacteria challenge and heat shock from Miichthys miiuy, Fish Shellfish Immunol. 35 (2013) 429–437, https://doi.org/10.1016/j.fsi.2013.04.045.
[16] Y. Xie, L. Song, Z. Weng, S. Liu, Z. Liu, Hsp90, Hsp60 and sHsp families of heat shock protein genes in channel catfish and their expression after bacterial infections, Fish Shellfish Immunol. 44 (2015) 642–651, https://doi.org/10.1016/j.fsi.2015.03.027.
[17] Q. Gao, J. Zhao, L. Song, L. Qiu, Y. Yu, H. Zhang, D. Ni, Molecular cloning, characterization and expression of heat shock protein 90 gene in the haemocytes of bay scallop Argopecten irradians, Fish Shellfish Immunol. 24 (2008) 379–385, https://doi.org/10.1016/j.fsi.2007.08.008.
[18] L. Song, L. Wu, D. Ni, Y. Chang, W. Xu, K. Xing, The cDNA cloning and mRNA expression of heat shock protein 70 gene in the haemocytes of bay scallop (Argopecten irradians, Lamarck 1819) responding to bacteria challenge and naphthalin stress, Fish Shellfish Immunol. 21 (2006) 335–345, https://doi.org/10.1016/j.fsi.2005.12.011.
[19] W. Junprung, P. Supungul, A. Tassanakajon, Litopenaeus vannamei heat shock protein 70 (LvHSP70) enhances resistance to a strain of Vibrio parahaemolyticus, which can cause acute hepatopancreatic necrosis disease (AHPND), by activating shrimp immunity, Dev. Comp. Immunol. 90 (2019) 138–146, https://doi.org/10.1016/j.dci.2018.09.011.
[20] W. Rungrassamee, R. Leelatanawit, P. Jiravanichpaisal, S. Klinbunga,

N. Karoonuthaisiri, Expression and distribution of three heat shock protein genes under heat shock stress and under exposure to Vibrio harveyi in Penaeus monodon, Dev. Comp. Immunol. 34 (2010) 1082–1089, https://doi.org/10.1016/j.dci.2010.05.012.

[21] J. Zhou, W.N. Wang, W.Y. He, Y. Zheng, L. Wang, Y. Xin, Y. Liu, A.L. Wang, Expression of HSP60 and HSP70 in white shrimp, Litopenaeus vannamei in response to bacterial challenge, J. Invertebr. Pathol. 103 (2010) 170–178, https://doi.org/10.1016/j.jip.2009.12.006.

[22] L.P. Shan, X.H. Chen, F. Ling, B. Zhu, G.X. Wang, Targeting Heat Shock Protein 70 as an antiviral strategy against grass carp reovirus infection, Virus Res. 247 (2018) 1–9, https://doi.org/10.1016/j.virusres.2018.01.005.

[23] K. Yuan, F.H. Yuan, H.H. He, H.T. Bi, S.P. Weng, J.G. He, Y.H. Chen, Heat shock 70 kDa protein cognate 5 involved in WSSV toleration of Litopenaeus vannamei, Dev. Comp. Immunol. 72 (2017) 9–20, https://doi.org/10.1016/j.dci.2017.02.003.

[24] Y. Le, P. Jia, Y. Jin, W. Liu, K. Jia, M. Yi, The antiviral role of heat shock protein 27 against red spotted grouper nervous necrosis virus infection in sea perch, Fish Shellfish Immunol. 70 (2017) 185–194, https://doi.org/10.1016/j.fsi.2017.08.032.

[25] K. Kumada, N. Fuse, T. Tamura, C. Okamori, S. Kurata, HSP70/DNAJA3 chaperone/cochaperone regulates NF-κB activity in immune responses, Biochem. Biophys. Res. Commun. 513 (2019) 947–951, https://doi.org/10.1016/j.bbrc.2019.04.077.

[26] N.H. Putnam, T. Butts, D.E.K. Ferrier, R.F. Furlong, U. Hellsten, T. Kawashima, M. Robinson-Rechavi, E. Shoguchi, A. Terry, K. Yu, È. Benito-Gutiérrez, I. Dubchak, J. Garcia-Fernàndez, J.J. Gibson-Brown, I.V. Grigoriev, A.C. Horton, P.J. De Jong, J. Jurka, V.V. Kapitonov, Y. Kohara, Y. Kuroki, E. Lindquist, S. Lucas, K. Osoegawa, L.A. Pennacchio, A.A. Salamov, Y. Satou, T. Sauka-Spengler, J. Schmutz, T. Shin-I, A. Toyoda, M. Bronner-Fraser, A. Fujiyama, L.Z. Holland, P.W.H. Holland, N. Satoh, D.S. Rokhsar, The amphioxus genome and the evolution of the chordate karyotype, Nature 453 (2008) 1064–1071, https://doi.org/10.1038/nature06967.

[27] S. Zhang, C. Wang, Y. Wang, R. Wei, G. Jiang, H. Ju, Presence and characterization of complement-like activity in the amphioxus Branchiostoma belcheri tsingtauense, Zool. Sci. 20 (2004) 1207–1214, https://doi.org/10.2108/zsj.20.1207.

[28] M.M. Suzuki, N. Satoh, M. Nonaka, C6-like and C3-like molecules from the cephalochordate, amphioxus, suggest a cytolytic complement system in invertebrates, J. Mol. Evol. 54 (2002) 671–679, https://doi.org/10.1007/s00239-001-0068-z.

[29] Y. Endo, M. Nonaka, H. Saiga, Y. Kakinuma, A. Matsushita, M. Takahashi, M. Matsushita, T. Fujita, Origin of mannose-binding lectin-associated serine protease (MASP)-1 and MASP-3 involved in the lectin complement pathway traced back to the invertebrate, amphioxus, J. Immunol. 170 (2003) 4701–4707, https://doi.org/10.4049/jimmunol.170.9.4701.

[30] Z. Li, S. Zhang, C. Wang, Q. Pang, Complement-mediated killing of Vibrio species by the humoral fluids of amphioxus Branchiostoma belcheri: implications for a dual role of O-antigens in the resistance to bactericidal activity, Fish Shellfish Immunol. 24 (2008) 215–222, https://doi.org/10.1016/j.fsi.2007.10.016.

[31] Y. Liang, S. Zhang, L. Lun, L. Han, Presence and localization of antithrombin and its regulation after acute lipopolysaccharide exposure in amphioxus, with implications for the origin of vertebrate liver, Cell Tissue Res. 323 (2006) 537–541, https://doi.org/10.1007/s00441-005-0088-x.

[32] L. Lun, S. Zhang, Y. Liang, Alanine aminotransferase in amphioxus: presence, localization and up-regulation after acute lipopolysaccharide exposure, BMB Rep. 39 (2006) 511–515, https://doi.org/10.5483/bmbrep.2006.39.5.511.

[33] L. Ju, S. Zhang, Y. Liang, X. Sun, Identification, expression and antibacterial activity of a tachylectin-related homolog in amphioxus Branchiostoma belcheri with implications for involvement of the digestive system in acute phase response, Fish Shellfish Immunol. 26 (2009) 235–242, https://doi.org/10.1016/j.fsi.2008.10.015.

[34] C. Yu, M. Dong, X. Wu, S. Li, S. Huang, J. Su, J. Wei, Y. Shen, C. Mou, X. Xie, J. Lin, S. Yuan, X. Yu, Y. Yu, J. Du, S. Zhang, X. Peng, M. Xiang, A. Xu, Genes "Waiting" for recruitment by the adaptive immune system: the insights from amphioxus, J. Immunol. 174 (2005) 3493–3500, https://doi.org/10.4049/jimmunol.174.6.3493.

[35] A. Sato, W.E. Mayer, J. Sato, A molecule bearing an immunoglobulin-like V region of the CTX subfamily in amphioxus, Immunogenetics 55 (2003) 423–427, https://doi.org/10.1007/s00251-003-0589-2.

[36] J.A. Hernández Prada, R.N. Haire, J.P. Cannon, G.W. Litman, D.A. Ostrov, Crystallization and preliminary X-ray analysis of VCBP3 from Branchiostoma floridae, Acta Crystallogr. Sect. D Biol. Crystallogr. 60 (2004) 2022–2024, https://doi.org/10.1107/S0907444904020827.

[37] L.F.C. Castro, R.F. Furlong, P.W.H. Holland, An antecedent of the MHC-linked genomic region in amphioxus, Immunogenetics 55 (2004) 782–784, https://doi.org/10.1007/s00251-004-0642-9.

[38] D. Li, G. Li, K. Wang, X. Liu, W. Li, X. Chen, Y. Wang, Isolation and functional analysis of the promoter of the amphioxus Hsp70a gene, Gene 510 (2012) 39–46, https://doi.org/10.1016/j.gene.2012.08.031.

[39] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729, https://doi.org/10.1093/molbev/mst197.

[40] G. Wang, S. Zhang, Z. Wang, Responses of alternative complement expression to challenge with different combinations of Vibrio anguillarum, Escherichia coli and Staphylococcus aureus: evidence for specific immune priming in amphioxus Branchiostoma belcheri, Fish Shellfish Immunol. 26 (2009) 33–39, https://doi.org/10.1016/j.fsi.2008.09.018.

[41] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[42] T. Li, H. Li, S.Q. Peng, F.M. Zhang, L.G. An, G.W. Yang, Molecular characterization and expression pattern of X box-binding protein-1 (XBP1) in common carp (Cyprinus carpio L.): indications for a role of XBP1 in antibacterial and antiviral immunity, Fish Shellfish Immunol. 67 (2017) 667–674, https://doi.org/10.1016/j.fsi.2017.06.055.

[43] H. Li, F.M. Zhang, H.Y. Guo, Y.Y. Zhu, J.D. Yuan, G.W. Yang, L.G. An, Molecular characterization of hepcidin gene in common carp (Cyprinus carpio L.) and its expression pattern responding to bacterial challenge, Fish Shellfish Immunol. 35 (2013) 1030–1038, https://doi.org/10.1016/j.fsi.2013.07.001.

[44] S. Wang, Y. Wang, J. Ma, Y. Ding, S. Zhang, Phosvitin plays a critical role in the immunity of zebrafish embryos via acting as a pattern recognition receptor and an antimicrobial effector, J. Biol. Chem. 286 (2011) 22653–22664, https://doi.org/10.1074/jbc.M111.247635.

[45] Z. Gao, M. Li, J. Ma, S. Zhang, An amphioxus gC1q protein binds human IgG and initiates the classical pathway: implications for a C1q-mediated complement system in the basal chordate, Eur. J. Immunol. 44 (2014) 3680–3695, https://doi.org/10.1002/eji.201444734.

[46] A.G. Pockley, B. Henderson, Extracellular cell stress (Heat shock) proteins—immune responses and disease: an overview, Philos. Trans. R. Soc. Biol. Sci. 373 (2018), https://doi.org/10.1098/rstb.2016.0522.