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Molecular identification and function analysis of bactericidal permeability-increasing protein/LPS-binding protein 1 (*BPI/LBP1*) from turbot (*Scophthalmus maximus*)

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ABSTRACT

Bactericidal permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP) play important roles in host antimicrobial defense. In the present study, we identified one isoform of *BPI/LBP* gene from turbot (*Scophthalmus maximus*), designated as *SmBPI/LBP1*. The full-length cDNA sequence of *SmBPI/LBP1* was 1826 bp, which encoding one secreted protein with 480 amino acid residues. Structurally, the *SmBPI/LBP1* showed high similarity to its homologs from other vertebrates or invertebrates, which all contained a signal peptide, a BPI/LBP/CETP N-terminal with a LPS-binding domain, and a BPI/LBP/CETP C-terminal domain. The deduced amino acid sequences of *SmBPI/LBP1* shared significant similarity to *BPI/LBP* of *Seriola lalandi dorsalis* (71%) and *Paralichthys olivaceus* (69%). Phylogentic analysis further supported that *SmBPI/LBP1* act as a new member of vertebrate *BPI/LBP* family. *SmBPI/LBP1* was ubiquitously expressed in all tested tissues, with the highest expression level in spleen tissue. The mRNA expression of *SmBPI/LBP1* in spleen and kidney were significantly up-regulated after *Vibrio vulnificus* challenge. Finally, the recombinant *SmBPI/LBP1* showed high affinity to lipopolysaccharide, followed by peptidoglycan and lipoteichoic acid, which is the ubiquitous component of Gram-negative or Gram-positive bacteria. These results indicated that *SmBPI/LBP1* probably played important roles in immune response against bacteria infection.

1. Introduction

The vertebrate organisms contain two types of immune system, innate and adaptive immunity, which play different roles against the infection of pathogens [1]. Among them, the innate immune system is the body's first line of defense against foreign pathogens. The initial sensing of infection is mediated by pattern recognition receptors (PRRs) [2], which can recognize the conserved pathogen-associated molecular patterns (PAMPs) from microorganisms [3]. Lipopolysaccharides (LPS), a well-characterized PAMP, is the major components of the outer surface membrane of Gram-negative bacteria. It can serve as an early warning signal of bacterial infection and then initiate strong innate immune responses [4,5]. Gram-positive bacteria, which lack LPS, are also responsible for a series of fish diseases. Peptidoglycan (PGN) and lipoteichoic acid (LTA), the major cell wall of gram-positive bacteria, are thought to contribute to its infection process [6].

The Bactericidal permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP), two crucial lip transfer/LPS-binding proteins, play crucial roles in responding to Gram-negative bacteria infection by mediating the signal transmission of LPS. However, BPI and LBP play different biological roles as agonist and antagonist, respectively [7]. On the one hand, LBP combines with LPS and stimulates induction of proinflammatory cytokines [8,9]. On the other hand, BPI counteracts LBPs by competitive combination with LPS, and then inhibit cellular responses triggered by LPS [9]. Furthermore, BPI is not just a LPS-neutralizing protein, but also a protein with diverse physiological functions, such as opsonization, antiangiogenesis, reproduction, and inhibition of dendritic cells maturation [10–14].

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Table 1
Primers used in this research.

Primer	Sequence (5′ – 3′)
SmBPI/LBP1-5′RACE-R1	GGAGGGTGACGTGCTCCAACATCTTC
SmBPI/LBP1-3′RACE-F1	GTGAAGTGGGAAGCTTCGGGACCG
SmBPI/LBP1-RT-F1	GAGGAATTGATTGCAAGCGTAGAG
SmBPI/LBP1-RT-R1	GTAGGTGAGGTGCTGAGAGAGAGG
18SrRNA-RT-F	ATGGCCGTTCTTAGTTGGTG
18SrRNA-RT-R	CTCAATCTCGTGTGGCTGAA
SmBPI/LBP1-eF	CCGGAATTCGAAAATCCTGCAATACAAGTCATCCT
SmBPI/LBP1-eR	CCGCTCGAGTCAGTTCTGTTGGTTGAAGCCT

Structurely, BPI and LBP share two conserved domains, one BPI/LBP/CETP N-terminal domain which binds the lipid A moiety of LPS and one BPI/LBP/CETP C-terminal domain which mediates the delivery of LPS to downstream immune molecules [10,15].

However, BPI and LBP homologs does not distinguish from each other in teleosts, in which they are deemed as single BPI/LBP. To date, some orthologs of BPI/LBP have been identified and characterized in several teleosts, such as tongue sole (*Cynoglossus semilaevis*) [16], black rockfish (*Sebastes schlegelii*) [17], blunt snout bream (*Megalobrama amblycephala*) [18], rock bream (*Oplegnathus fasciatus*) [19], and so on. It was identified that *BPI/LBPs* exhibited temporal transcriptional activation when against Gram-negative bacteria, Gram-positive bacteria, viral and pathogen-associated molecular patterns, which suggest that BPI/LBPs may play an essential role in host antimicrobial defense. However, the molecular characterization and expression patterns of BPI/LBP have not been well known in turbot (*Scophthalmus maximus*).

Turbot (*S. maximus*), native to Europe, is a species of farmed flatfish with high economic value. The species was introduced to China in the 1990s [20]. However, with the speedy development of aquaculture, different kinds of diseases, especially bacterial diseases, have caused considerable death and economic damage. *Vibrio vulnificus*, most commonly zoonotic bacteria found in marine waters and estuarine [21,22], which would induce the intestines problem even serious septicemia through the wound [23]. Therefore, the researches on immune-related genes not only contribute to elucidate the defense mechanisms of *S. maximus*, but could give new insights into its health management, diseases control and molecular breeding.

Hence, in the present study, we intend to describe molecular characteristics of one putative *BPI/LBP* homolog from *S. maximus*. We elucidate its tissue distributions and relative expression profile post *V. vulnificus* stimulation. Besides, the affinity of *rSmBPI/LBP1* to LPS, PGN and LTA was also researched. Based on above researches, we have understand its roles during the innate immune responses of *S. maximus*.

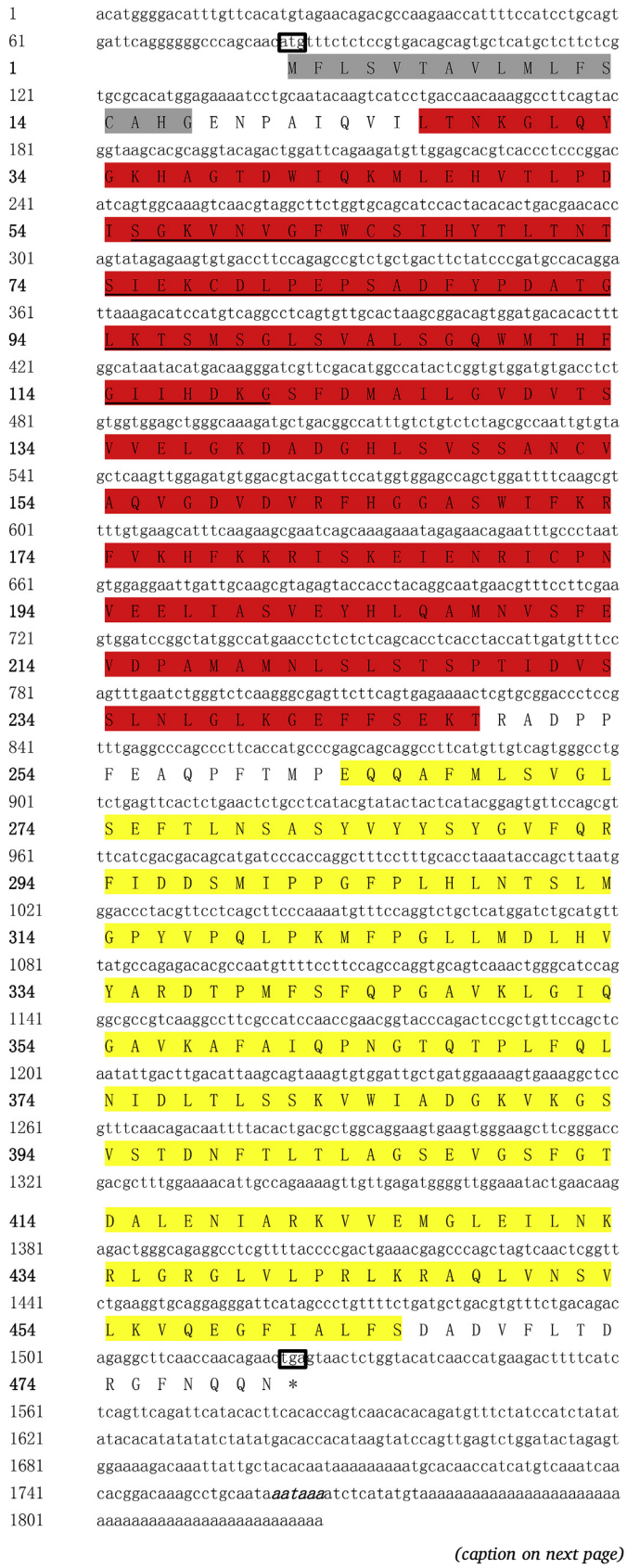
2. Materials and methods

2.1. Animal and RNA extraction

The normal and healthy turbot samples used in this research were collected from the fish farm (Laizhou, Shandong, China). The fish (with an average weight of 15.6 g and an average length of 5.5 cm) were acclimated in aquariums with aerated flow-through water (with an average temperature of 18 °C) for one week in the laboratory before the experiments. The total RNA of eight normal tissues (liver, spleen, kidney, brain, gill, intestine, skin and muscle) were extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was assessed by 1.2% agarose gel electrophoresis. The concentration of RNA was measured by spectrophotometer at 260 nm absorbance.

2.2. Obtain the full-length of SmBPI/LBP1

The complete cDNA sequence was obtained with the Rapid amplification of the cDNA ends (RACE) method. 5′-RACE-ready and 3′-RACE-ready were synthesized from the total RNA using a SMART™ RACE cDNA amplification kit (Clontech). Then, gene specific primers were designed based on the corresponding EST sequences. For the 5′-



(caption on next page)

Fig. 1. The cDNA and deduced amino acid sequences of *SmBPI/LBP1*. The numbers on the left are for the nucleotide and deduced amino acid sequences. The letters in boxes indicate the start codon (atg) and the stop codon (tga). The signal peptides are indicated in gray shadow. Predicted BPI/LBP/CETP N-terminal and BPI/LBP/CETP C-terminal domain sequences are indicated in red and yellow shadow, respectively. The LPS-binding domain are underlined and the polyadenylation signal (AATAAA) are indicated by bold italics. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

RACE, the PCR reactions were performed with *SmBPI/LBP1*-5′-RACE-R1 and Universal Primer A Mix (UPM) (Table 1). For the 3′-RACE, the PCR reactions were performed with *SmBPI/LBP1*-3′RACE-F1 and UPM (Table 1). The PCR reactions were carried out with total volumes of 25 μL, containing 16 μL ddH_2O, 2.5 μL 10X Advantage 2 PCR buffer, 2 μL dNTP Mix (2.5 mM each), 0.5 μL 50X Advantage 2 polymerase mix, 1 μL RACE-Ready cDNA template, 2.5 μL 10X Universal Primer A mix (10 μM), and 0.5 μL gene-specific primer (10 μM). PCR was performed under the following conditions: 5 cycles of 94 °C for 30 s and 72 °C for 2 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 2 min; and 25 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 2 min; and 72 °C for 10 min. The PCR fragments were cloned into pEASY-T1 vector (Trans, China) and sequenced by Personalbio.

2.3. Sequence analysis

The full-length cDNA sequence of *SmBPI/LBP1* was blasted against NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder/) was used to identify the open reading frame. Structure domains of *SmBPI/LBP1* were predicted by Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). Multiple sequences alignment was performed with the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). In addition, a neighbor-joining (NJ) phylogenetic tree was constructed based on the amino acid sequences alignment by MEGA 5 (Molecular Evolutionary Genetics Analysis 5.0) program.

2.4. Quantitative real-time PCR analysis

The cDNA was synthesized using the Takara PrimerScript™ First Strand cDNA Synthesis kit (TaKaRa, China) according to the manufacturer's instructions. Then, the mRNA expression level of *SmBPI/LBP1* in different tissues were determined by qRT-PCR with the 7500 Real-Time PCR System (Applied Biosystems, USA). For quantification of the *SmBPI/LBP1* expression, a pair of gene specific primers (*SmBPI/LBP1*-RT-F1, *SmBPI/LBP1*-RT-R1) was used, and the primers *18SrRNA*-RT-F and *18SrRNA*-RT-R were used to amplify the internal control. The qRT-PCR amplification was performed as previously [24]. All experiments were conducted with three replicates. The relative expression levels of *SmBPI/LBP1* were calculated according to the $2^{-\triangle\triangle Ct}$ method. A statistical analysis described compared expression in different tissues using SPSS18.0 (http://www-01.ibm.com/software/analytics/spss/). Data significance was determined by one-way ANOVA. Significance was set at $P < 0.05$.

2.5. Expression patterns after bacterial challenge

In this experiment, the bacteria *V. vulnificus* were isolated from diseased fish and kept in our laboratory. In brief, the bacteria were incubated to mid-logarithmic stage at 28 °C in LB broth medium, collected by centrifugation and re-suspended in PBS. To determine the expression profiles of *SmBPI/LBP1* following an immune challenge, fish were intraperitoneally injected individually with 100 μL live *V. vulnificus* suspension (1.0×10^6 CFU/ml). Spleen and kidney tissue samples from 6 fish were isolated at 0, 3, 12, 24, 48 and 72 h post-injection.

2.6. Expression and purification of recombinant SmBPI/LBP1

The pair of primers, *SmBPI/LBP1*-eF and *SmBPI/LBP1*-eR (Table 1), were used to amplify the fragment encoding the mature *SmBPI/LBP1*. The cDNA fragments were subcloned into the pGEX4T-1 plasmid. The cDNA fragment and pGEX4T-1 were firstly digested with the corresponding restriction enzymes (EcoRI and XhoI) and then ligated by T4 DNA ligase. The recombinant plasmid pGEX4T-1-*SmBPI/LBP1* was transformed into E. coli BL21 (DE3) cells for IPTG-induced recombinant expression. The recombinant *SmBPI/LBP1* was induced at 23 °C for overnight with 0.5 mM IPTG. Then, the recombinant *SmBPI/LBP1* was purified by Glutathione Sepharose 4B (GE Healthcare), and analyzed by 12% SDS-PAGE. The concentration of the recombinant protein was determined using Bradford's method.

2.7. Affinity of recombinant SmBPI/LBP1 to LPS, PGN and LTA

The binding ability of *rSmBPI/LBP1* with LPS, PGN and LTA was detected in vitro by ELISA method. The ELISA experiment was conducted as previously described, but with some modification [25]. Briefly, LPS, PGN or LTA (0.1 μg/well) were coated to 96 microtitor plate at 4 °C overnight, respectively. The wells were blocked with 100 μL/well of BSA (5%) at 4 °C for 1 h. 100 μL of the increasing concentrations of purified recombinant *SmBPI/LBP1* (0, 0.1, 0.5, 1, 5 and 10 μg/mL) were added into each ligand-coated well, with three replicates for each concentration, and incubated at 37 °C for 1.5 h. The wells were incubated with 100 μL mouse Anti-GST Mouse Monoclonal Antibody (Trans, China) (diluted 1:5000 in 5% BSA) at 37 °C for 1 h. HRP conjugated Goat Anti-Mouse IgG secondary antibodies (100 μL, 1:10000 dilution) was added into each well and incubated at 37 °C for 40 min. During the whole process, the plates were washed four times with 300 μL/well of PBST after each step. After the last wash, the color was developed by adding 100 μL of TMB Solution buffer (Solarbio) for 5 min. Finally, the reaction was stopped by adding 0.5 M sulfate, and absorbance was measured at 450 nm.

3. Results

3.1. Cloning and characterizations of SmBPI/LBP1 cDNAs

The full length of *SmBPI/LBP1* cDNA (GeneBank accession number: MH085222) was 1826 bp, including a 81 bp 5′-UTR, a 302 bp 3′-UTR with a polyadenylation signal (AATAAA) and a polyadenylation site, a 1443 bp open reading frame (ORF) that encoded a predicted product of 480 amino acid residues. The predicted *SmBPI/LBP1* protein had a calculated molecular weight of 52.6 kDa and theoretical isoelectric point (pI) of 5.95, which had a predicted signal peptide with a cleavage site located between amino acid positions 17 and 18. Amino acid sequence analysis indicated that *SmBPI/LBP1* possessed several characteristic features, including the BPI/LBP/CETP N-terminal domain (aa 26–248), BPI/LBP/CETP C-terminal domain (aa 263–465), and the LPS-binding domain (aa 55–120) (Fig. 1).

3.2. Homology and phylogenetic analysis

Blastp analysis indicated that the deduced amino acid sequences of *SmBPI/LBP1* shared high homology with counterparts from other animals. For example, *SmBPI/LBP1* exhibited 71% identity with *BPI/LBP* from *Seriola lalandi dorsalis* (XP_023283225.1) and *Seriola dumerili* (XP_022597564.1). In order to compare the amino acid sequences of *SmBPI/LBP1* with other vertebrates, multiple sequences alignment was conducted and it showed that the predicted LPS binding domain was very conservative (Fig. 2).

A phylogenetic tree was established to explain the phylogenetic relationships between *SmBPI/LBP1* with other counterparts. As shown in Fig. 3, BPI/LBPs from fish and mammal were gathered together

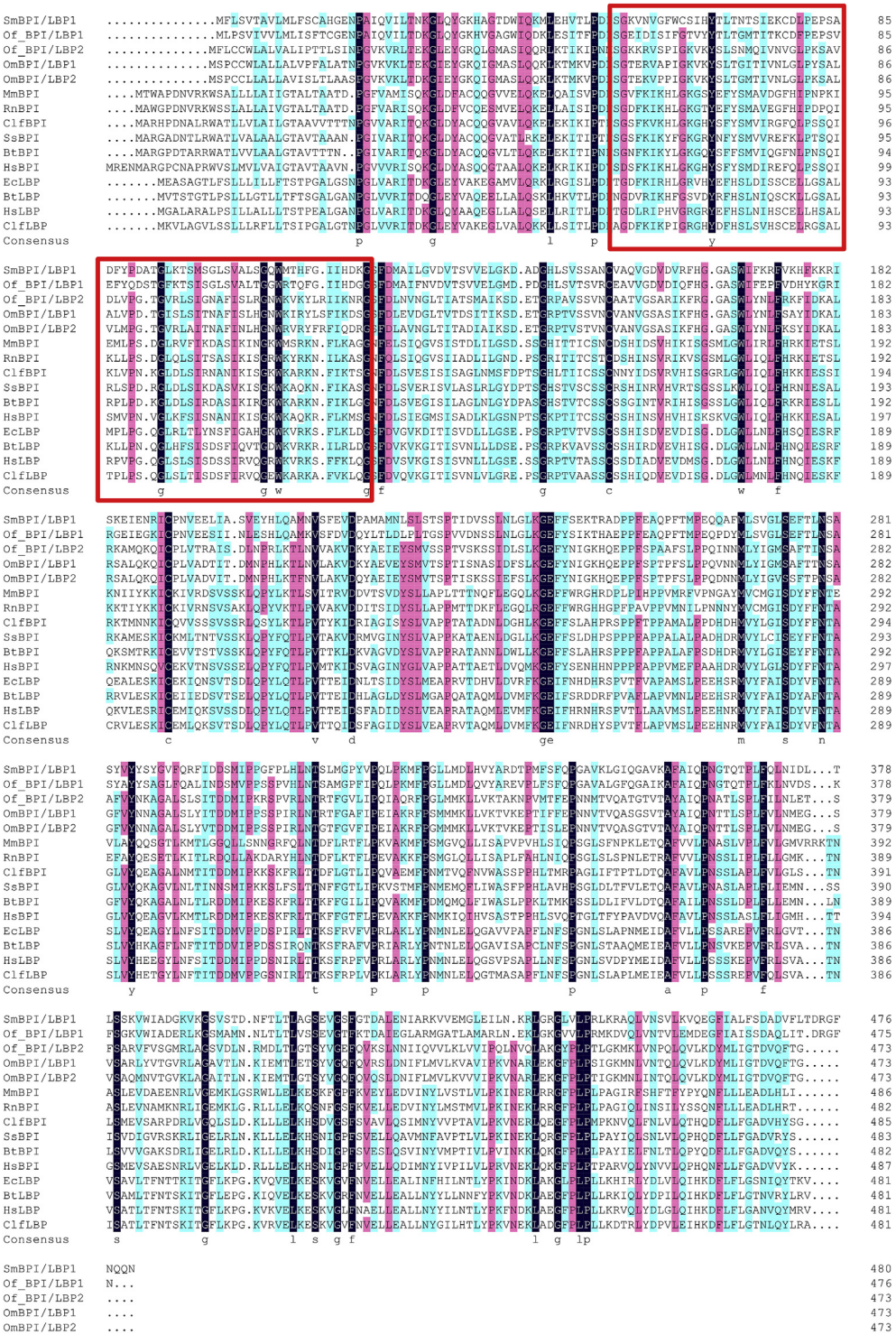


Fig. 2. Multiple sequence alignment of *SmBPI/LBP1* with other known *LBP*, *BPI* and *BPI/LBP* protein sequences. Alignment of the deduced amino acid sequence of *SmBPI/LBP1* with those of other species, constructed with Clustal-W. Similar amino acid residues are marked with lower-case letters. The predicted LPS binding domain is indicated by a red box. Protein sequences used in this analysis: OfBPI/LBP1 (*Oplegnathus fasciatus* BPI/LBP-1, BAM21037), OfBPI/LBP2 (*Oplegnathus fasciatus* BPI/LBP-2, BAM21038), OmBPI/LBP1 (*Oncorhynchus mykiss* LBP/BPI-1, AB042025), OmBPI/LBP2 (*Oncorhynchus mykiss* LBP/BPI-2, AB042026), MmBPI (*Mus musculus* BPI, NP_808518), RnBPI (*Rattus norvegicus* BPI, AAH79318), ClfBPI (*Canis lupus familiaris* BPI, XP_534417), SsBPI (*Sus scrofa* BPI, ABO34136), BtBPI (*Bos taurus* BPI, NP_776320), HsBPI (*Homo sapiens* BPI, ABD66755), EcLBP (*Equus caballus* LBP, XP_001499834), BtLBP (*Bos taurus* LBP, NP_001033763), HsLBP (*Homo sapiens* LBP, AAD21962), ClfLBP (*Canis lupus familiaris* LBP, XP_542993). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

within vertebrate cluster, but invertebrate BPI/LBPs were aggregated into another cluster. Specifically, vertebrate cluster could be subgroup into three groups, including fish BPI/LBPs, mammal BPIs, and mammal LBPs.

3.3. The tissues distribution

The expression of *SmBPI/LBP1* was analyzed in extensive range of tissues, e.g. liver, spleen, kidney, brain, intestine, gill, skin and muscle.

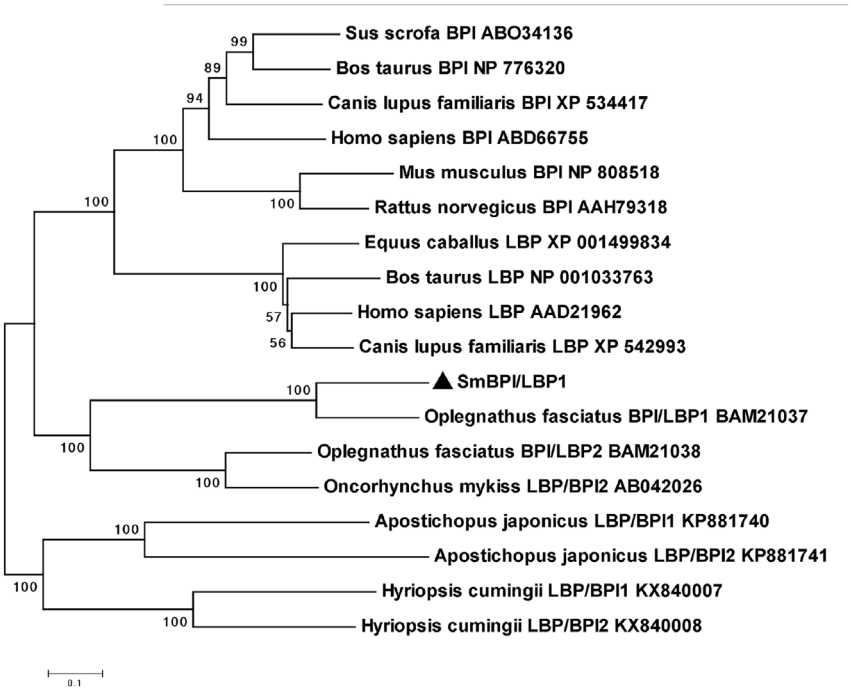


Fig. 3. The phylogenetic tree showing the relationship between *SmBPI/LBP1* with other *BPI/LBPs* protein. A phylogram was constructed using neighbor-joining algorithm in MEGA 4.0. The relative genetic distances were indicated by the scale bar and the branch lengths. Protein sequences used in this analysis: *Oplegnathus fasciatus* BPI/LBP-1 (BAM21037), *Oplegnathus fasciatus* BPI/LBP-2 (BAM21038), *Oncorhynchus mykiss* LBP/BPI-2 (AB042026), *Mus musculus* BPI (NP_808518), *Rattus norvegicus* BPI (AAH79318), *Canis lupus familiaris* BPI (XP_534417), *Sus scrofa* BPI (ABO34136), *Bos taurus* BPI (NP_776320), *Homo sapiens* BPI (ABD66755), *Equus caballus* LBP (XP_001499834), *Bos taurus* LBP (NP_001033763), *Homo sapiens* LBP(AAD21962), *Canis lupus familiaris* LBP(XP_542993).

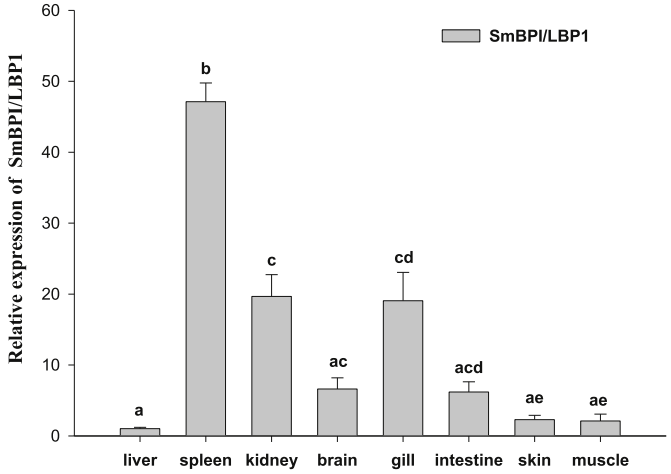


Fig. 4. Expression levels of *SmBPI/LBP1* mRNAs in *S. maximus* tissues evaluated by qRT-PCR. The expression level of *SmBPI/LBP1* in various tissues were normalized using the internal control genes (*18SrRNA*). The mean ± SEM values from three separate individuals (n = 3) are shown. Bars with different lowercase letters indicate statistically significant differences ($P < 0.05$).

qRT-PCR results showed *SmBPI/LBP1* was expressed in all detected tissues (Fig. 4). *SmBPI/LBP1* showed significantly highest expression in spleen, relatively high in kidney and gill, and slight expression in brain, skin, muscle and liver.

3.4. Temporal expression of SmBPI/LBP1 in spleen and kidney post V. vulnificus challenge

The temporal expression of *SmBPI/LBP1* in spleen and kidney of turbot post *V. vulnificus* challenge were shown in Fig. 5. In general, *SmBPI/LBP1* was significantly up-regulated in spleen and kidney tissues. In spleen, the expression of *SmBPI/LBP1* was markedly increased at 24 h (2.85-fold, $P < 0.05$). In kidney, *SmBPI/LBP1* was firstly up-regulated from 0 h to 24 h, and then gradually decreased from 24 h to 72 h. Comparison with the basal (0 h) level, the expression of *SmBPI/LBP1* was significantly higher at 24 h (7.01-fold, $P < 0.05$).

3.5. LPS, PGN and LTA binding capacity of rSmBPI/LBP1

The recombinant *rSmBPI/LBP1* protein were successfully expressed in E. coli BL21 (DE3). The molecular weight of *rSmBPI/LBP1* was approximately 76 kDa with a GST-tag at the N-terminal (Fig. 6). In order to investigate the binding ability of *rSmBPI/LBP1* on pathogen-associated molecular patterns, an ELISA experiment was performed. As shown in Fig. 7, the binding capacity of *rSmBPI/LBP1* to LPS, PGN or LTA significantly increased with the increasing concentrations of purified recombinant protein. Furthermore, *rSmBPI/LBP1* exhibited higher affinity to LPS, moderate affinity to LTA and lower affinity to PGN.

4. Discussion

The innate immune system of vertebrate recognizes PAMPs via PRRs, and then initiate a well orchestrated immune response [3,26]. Therefore, identification of PRRs and understanding their mechanisms of recognizing pathogen and stimulating immune response is significantly important for both basic research and fishery industry. PRRs can be broadly categorized into three groups based on their cellular location, including serum or tissue fluid, membrane, and cytoplasmic [27]. Bactericidal permeability inducing protein (*BPI*) and lipopolysaccharide binding protein (*LBP*), one of serum or tissue fluid PRPs, are members of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) family of proteins. It has been reported that *LBP*, *BPI* and *BPI/LBP* play crucial roles in the innate immune response to Gram-negative bacteria [28].

In our study, one isoform of *BPI/LBP* was identified by RACE techniques. The full-length of *SmBPI/LBP1* cDNA, including the ORF, 5′-UTR and 3′-UTR, was cloned and characterized. On one hand, the complete ORF laid the foundation for its functional study. On the other hand, the complete UTR provide important information on the regulation of gene expression. For example, mRNA stability, localization, and translation are largely determined by sequences in the 3′-UTR. Besides, recent studies have shown that miRNAs can also target the 5′-UTR [29]. By summarizing the previous reports, we identified that mammalian *LBP* and *BPI* showed different theoretical pI. Specifically, pI of *BPI* ranges from 9.51 to 10.16 whereas *LBP* shows a pI value of around 6.00 except in rats [30]. In this research, *SmBPI/LBP1* displayed

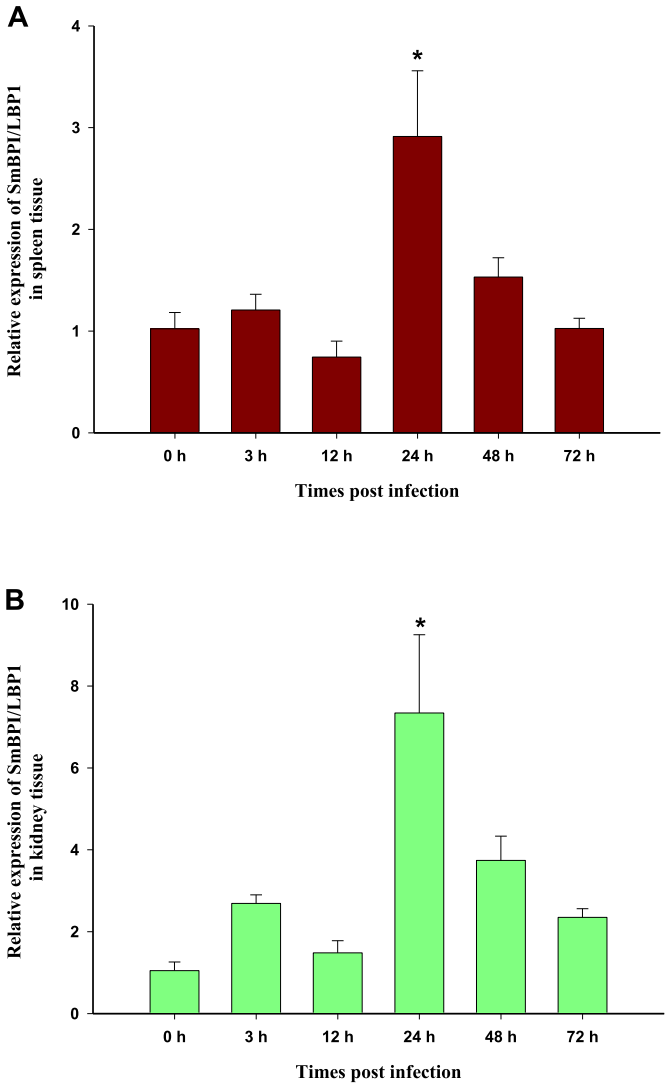


Fig. 5. Expression profile of *SmBPI/LBP1* in the spleen (A) and kidney (B) under the bacteria *V. vulnificus*-infected. The data determined by qRT-PCR at different time points (0, 3, 12, 24, 48, 72 h) compared with 0 h time point respectively. The mean ± SEM values from three separate individuals (n = 3) are shown. The symbol * represent $P < 0.05$.

a theoretical pI of 5.95, which was similar to the value of mammalian LBP. In terms of protein structure, *SmBPI/LBP1* shared high similarity with its homologs from other teleosts or vertebrates, all of them including a putative signal peptide, a BPI/LBP/CETP N-terminal domain contained a LPS-binding, and a BPI/LBP/CETP C-terminal domain. It was identified that *SmBPI/LBP1* had numbers of positively charged amino acids (15 lysines and 4 arginines) in the N-terminal portion. Similar findings have been reported in *O. fasciatus*, where *RbBPI/LBP1* has a lower number of positively charged amino acids (10 lysines and 4 arginines) in the N-terminal portion than the *RbBPI/LBP2* counterpart (23 lysines and 15 arginines) [19]. Previous researches have been proved that the bactericidal activity of *BPI* was related to the high positive charge of its N-terminal domain than *LBP* in mammalian [15]. The positively charged amino acids in the N-terminal domain of *LBP* and *BPI* can bind to the anionic portion of lipid A [15,31]. Above all, it was suggested that *SmBPI/LBP1* was more like mammalian LBP.

The phylogenetic analysis showed that BPI/LBPs from teleosts and mammals were gathered together within vertebrate cluster, impling that they may have a common ancestor. The divergence of these two homologues may occurred due to the genome duplications, which promoted the evolution of biology. Although, *BPI* or *LBP* homologues have not been made a distinction between each other in teleost, this phylogenetic analysis proved that there are also two branches for BPI/LBPs of teleost. Therefore, we suggest that the *BPI/LBP* genes should be redefined in the future according to their theoretical pI, positive charge of its N-terminal domain, the phylogenetic analysis, and functional properties.

In this study, *SmBPI/LBP1* was expressed ubiquitously in all examined tissues. Specifically, *SmBPI/LBP1* showed highest expression level in spleen and kidney. As previously reported, BPI/LBP was widely expressed in almost all tissues, e.g. kidney, head kidney, liver, gill, spleen and heart [32–34], even in gonads of *Crassostrea gigas* and *Scapharca broughtonii* [35,36]. Similarity to our observation, *BPI/LBPs* were highly expressed in spleen and kidney tissues, e.g. *RbBPI/LBP-1* and *RbBPI/LBP-2* from *O. fasciatus* [19], *SsBPI/LBP* from *S. schlegelii* [17], and *MaBPI/LBP* from *Megalobrama amblycephala* [18]. Different from that of mammals, the kidney was usually a major lymphoid organ in fish [37]. Besides, fish possess a thymus, spleen, head kidney and mucosa-associated lymphoid tissues (MALT, i.e. the gills and intestine), which contain lymphocytes, macrophages and many types of granulocytes [38]. Here, the predominant expression of *SmBPI/LBP1* in immune organ (spleen, kidney and gill) indicated that it may play a crucial role in preventing invasion by microorganisms in the aquatic environment.

During the challenge process of *V. vulnificus*, *SmBPI/LBP1* transcripts were significantly up-regulated in spleen and kidney. Consistently, most of *BPI/LBPs* from teleosts are significantly induced by bacterial challenge, viral infection or LPS stimulation, such as *C. semilaevis* [38], *S. schlegelii* [17], *M. amblycephala* [18], *O. fasciatus* [19] and so on. Besides, the transcription of *LBPs* and *BPIs* in mammals also remarkably increased in response to LPS or Gram-negative bacteria [39–42]. It was speculated that *SmBPI/LBP1* could recognize LPS as PRPs, consequently activate the innate immunity of turbot. It was reported that *RbBPI/LBP-1* and *RbBPI/LBP-2* were up-regulated post bacterial challenge, but exist distinct and complementary expression patterns [19]. Probably, the differential expression patterns predicted that they may play different roles during infection process.

In order to confirm *SmBPI/LBP1* is involved in innate immunity by binding PAMPs, we expressed and purified recombinant *SmBPI/LBP1*. Furthermore, affinity of recombinant *SmBPI/LBP1* to LPS, PGN and LTA was researched and it was showed that *rSmBPI/LBP1* could bind all examined PAMPs tightly. Consistently, most of *BPI/LBPs* from teleosts showed potent LPS-binding ability, such as *S. schlegelii BPI/LBP* [17] and *Plecoglossus altivelis BPI/LBP* [43]. LPS is a major constituent of the outer membrane of Gram-negative bacteria, and it was speculated that *rSmBPI/LBP1* bind the lipid A component of LPS. In agreement with these results, nearly all recombinant *BPI/LBPs* from teleosts were able to bind a number of Gram-negative bacteria [16–18]. Besides, *rSmBPI/LBP1* also showed affinity to PGN and LTA, which is consist with the results that analogs of the olive flounder *BPI/LBP* homolog showed strong antimicrobial activity against Gram-positive bacteria including *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus* [44]. It has been proved that recombinant *C. semilaevis BPI* could bind with bacteria which will lead to bacterial death through membrane permeabilization and structural destruction [16].

5. Conclusion

In conclusion, we identified and characterized one *BPI/LBP* homolog from *S. maximus*. *SmBPI/LBP1* showed homologous with other teleost *BPI/LBPs*. *SmBPI/LBP1* were more similar to the value of mammalian *LBP*. *SmBPI/LBP1* was diffusely expressed in immune-related tissues, and temporarily up-regulated during challenged with *V. vulnificus*. Furthermore, PAMPs-binding experiment revealed that *rSmBPI/LBP1* could bind LPS, PGN and LTA tightly. In short, our study suggested *SmBPI/LBP1* may play significant roles in host antimicrobial

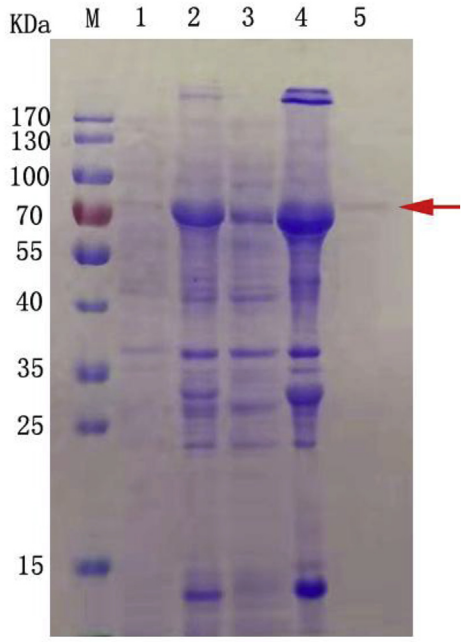


Fig. 6. SDS-PAGE analysis of purified recombinant *SmBPI/LBP1*. Lane M: molecular weight marker; lane 1: crude extract of negative control (without IPTG induction); lane 2: crude extract of experimental group (with IPTG induction); Lane 3, total soluble cellular extract from experimental group; Lane 4, total precipitation cellular extract from experimental group; lane 5, purified recombinant *SmBPI/LBP1*.

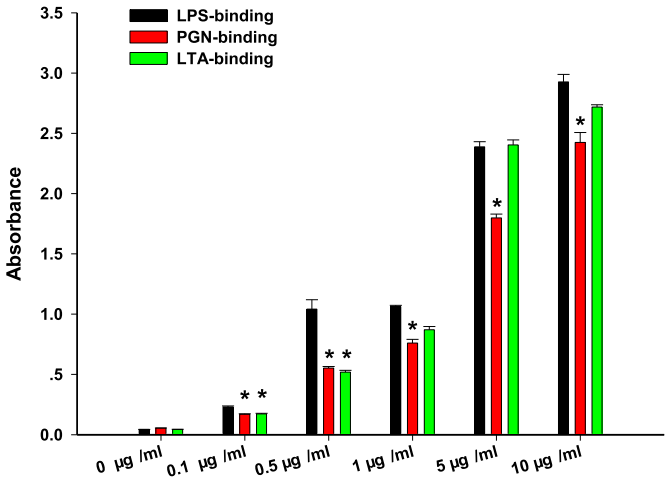


Fig. 7. Results of the in vitro binding assay. The abscissa represents different protein concentrations, and the ordinate represents the absorbance value of the product. * indicate a significant difference between three PAMPs under the same concentration ($p < 0.05$).

defense.

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Ethical statement

All experimental animal protocols weress approved by Qingdao Agricultural University Fisheries Research Institute's animal care and use committee. All tissues were removed under MS222 anesthesia, and all efforts were made to minimize fish suffering.

Conflicts of interest

All authors have no competing interest to declare.

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