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Molecular characterization of a bactericidal permeability-increasing protein/lipopolysaccharide-binding protein from black rockfish (*Sebastes schlegelii*): Deciphering its putative antibacterial role

Seongdo Lee [a, b, 1], Don Anushka Sandaruwan Elvitigala [a, b, c, 1], Sukkyoung Lee [a, b], Hyun Chul Kim [d], Hae-Chul Park [e, **], Jehee Lee [a, b, *]

[a] Department of Marine Life Sciences, Jeju National University, Jeju Self-Governing Province, 63243, Republic of Korea
[b] Fish Vaccine Research Center, Jeju National University, Jeju Self-Governing Province, 63243, Republic of Korea
[c] Department of Zoology, University of Sri Jayewardenepura, Gangodawila, Nugegoda, 10250, Sri Lanka
[d] Genetics & Breeding Research Center, National Institute of Fisheries Science, Geoje, 53334, Republic of Korea
[e] Graduate School of Medicine, Korea University, Ansan, Gyeonggido, 15355, Republic of Korea

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ABSTRACT

Bactericidal permeability-increasing protein (BPI)/lipopolysaccharide (LPS) binding proteins (LBPs) are well-known proteins that play an indispensable role in host antimicrobial defense. Herein, we report a homolog of BPI/LBP from black rockfish (*Sebastes schlegelii*) (designated as RfBPI/LBP) and characterize its structural and functional features at the molecular level. We identified the putative complete open reading frame (1422 bp) of *RfLBP* that encodes a 474 amino acid protein with a predicted molecular mass of ~51.5 kDa. The primary protein sequence of RfBPI/LBP contains domain features of BPI/LBP family proteins and shares significant sequence consistency with its homologs. Our phylogenetic analysis clearly demonstrated the vertebrate ancestral origin of RfBPI/LBP, further reinforcing its evolutionary relationship with teleostean homologs. Recombinant RfBPI/LBP demonstrated *in vitro* LPS-binding activity and antibacterial activity against *Escherichia coli*, but not against *Streptococcus iniae*. Moreover, *RfBPI/LBP* exhibited temporal transcriptional activation against pathogens and pathogen-associated molecular patterns. Collectively, our findings suggest that RfBPI/LBP plays an essential role in host antimicrobial defense, plausibly through selective eradication of invading bacteria.

1. Introduction

Pattern recognition receptors (PRRs) are a crucial component of the innate immune system, which is specialized to sense invading pathogens through conserved pathogen-associated molecular patterns (PAMPs) (Bayne and Gerwick, 2001). Among the wide array of PAMPs identified to date, lipopolysaccharide (LPS), a major constituent of the outer surface membrane of almost all Gram-negative bacteria, is one of the most potent immune stimulators (Alexander and Rietschel, 2001). LPS harbors an oligo or polysaccharide region bound to a lipid moiety, lipid A, which is generally involved in immune stimulation (Tobias et al., 1989). Toll-like receptor 4 (TLR4), membrane bound or soluble forms of CD14, and bactericidal permeability-increasing proteins (BPIs) or LPS-binding proteins (LBPs) are some of the common LPS sensors that activate the intracellular immune signaling network (Lee et al., 1992).

BPIs and LBPs have been categorized under a family of lipid transfer/LPS-binding proteins that also encompasses mammalian phospholipid transfer protein (PLTP) and cholesteryl ester protein (CEPT) (Bingle and Craven, 2004). These two proteins interact with CD14 and induce proinflammatory cytokines, including TNF-α (Gallay et al., 1993; Lee et al., 1992; Mathison et al., 1992; Tobias et al., 1989). BPI and LBP contain an N-terminal domain and a C-terminal domain that exert different functions. The N-terminal domain binds LPS and demonstrates antibacterial as well as LPS

* Corresponding author. Marine Molecular Genetics Lab, Department of Marine Life Sciences, College of Ocean Science, Jeju National University, 66 Jejudaehakno, Ara-Dong, Jeju, 690-756, Republic of Korea.
** Corresponding author. Graduate School of Medicine, Korea University, Ansan, Gyeonggido, 425-707, Republic of Korea.
E-mail addresses: hcpark67@korea.ac.kr (H.-C. Park), jehee@jejunu.ac.kr (J. Lee).
[1] These authors have contributed equally to this work.

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neutralizing activity (Beamer et al., 1997; Ooi et al., 1987). In contrast, the C-terminal domain delivers LPS to CD14 (Beamer et al., 1998). The synergistic action of both domains is important in opsonic activity and the promotion of bacterial phagocytosis (Iovine et al., 2002). LBPs primarily function as LPS transporters to CD14 and TLR complexes (Hailman et al., 1994; Kopp and Medzhitov, 1999; Means et al., 2000; Wright et al., 1990), whereas BPIs inhibit cellular responses triggered by LPS, mainly counteracting LBPs by reducing LPS availability for LBPs (Dentener et al., 1993; Wiese et al., 1997). In general, LBP and BPI are important in host antibacterial defense. As previously reported, LBPs mediate bacterial clearance in circulation (Jack et al., 1997). On the other hand, BPI cooperatively functions with defensin and the complement system, exerting potent bactericidal activity to eradicate invading bacteria at the site of inflammation (Levy et al., 1995).

Although BPI and LBP proteins found in higher vertebrates, such as mammals, are clearly distinguished from each other, those with teleostean origin cannot be clearly discerned from each other due to lack of information on the functional differences of these proteins. However, some orthologs of BPI/LBP have been identified and characterized in several teleosts, including olive flounder (*Paralichthys olivaceus*) (Nam et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Inagawa et al., 2002), Atlantic cod (*Gadus morhua* L.) (Stenvik et al., 2004), ayu (*Plecoglossus altivelis altivelis*) (Suzuki et al., 2009), and large yellow croaker (*Pseudosciaena crocea*) (Huang et al., 2008), demonstrating their transcriptional responses against LPS treatments or experimental pathogen infections, alongwith portraying their potent antimicrobial properties using the recombinantly expressed proteins or synthetic peptides. Interestingly, in the study of flounder PBI/LBP, significant antimicrobial activities with limited hemolytic activities were shown against several Gram-negative bacteria, Gram-positive bacteria, and the yeast *Candida albicans* by using five different synthetic peptides derived from the C-terminus of flounder LBP/BPI precursor protein. Moreover, further analysis showed that flounder LBP/PBI peptides could bind with DNA and some of them could inhibit the activity of DNA polymerases, suggesting their potential bactericidal mechanism.

Finfish mariculture is becoming a profitable industry worldwide, especially in the Asia Pacific region. Black rockfish (*Sebastes schlegelii*) is one of the most highly demanded cultured finfish delicacies in Northeastern Asia, especially in Korea. However, suboptimal culturing conditions on these farms facilitate the spread of lethal diseases caused by various viruses, bacteria, and parasites (Park, 2009). These diseases have detrimentally affected the quality and yield of this aqua-crop over time. Therefore, the development of a proper disease management system in rockfish mariculture farming is becoming increasingly crucial to increase the resistance of these fish to infections. Deciphering innate immune components at the molecular level is critical to develop modern molecular techniques to increase disease resistance and successfully combat pathogenic threats. The objective of this study was to identify and molecularly characterize a BPI/LBP homolog from black rockfish. We investigated the temporal transcriptional modulation of the protein in response to either pathogen infection or exposure to PAMPs. We also further analyzed BPI/LBP antimicrobial properties using its recombinantly expressed N-terminal domain. Moreover, as a novel finding compared to some of the known reports of rockfish BPI/LBP homologues, including flounder BPI/LBP, we have affirmed the in-vitro LPS binding ability of recombinant RfBPI/LBP.

2. Materials and methods

2.1. Identification and in silico characterization

A homologous sequence to known BPI/LBPs was identified from our previously constructed black rockfish cDNA database (Elvitigala et al., 2015) using the Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and was designated as RfBPI/LBP. The complete putative open reading frame (ORF) of the identified sequence was demarcated using DNAsist 2.2 software to derive the corresponding amino acid sequence. Conservation of the typical domain architecture of known BPI/LBPs in RfBPI/LBP was analyzed using the SMART online server (http://smart.embl-heidelberg.de/) and some physicochemical properties of the protein were determined using the ExPASy ProtParam tool (http://web.expasy.org/protparam). Sequence compatibility with known homologs was analyzed using a pairwise sequence alignment and multiple sequence alignment strategies by Matgat software (Campanella et al., 2003) and the ClustalW2 server (http://www.Ebi.ac.uk/Tools/clustalw2), respectively. The evolutionary position of RfBPI/LBP with other vertebrate and invertebrate homologs was determined by constructing a phylogenetic tree using Molecular Evolutionary Genetics Analysis (version 6.0) software (MEGA 6.0) (Tamura et al., 2013) under the neighbor-joining platform with the support of 1000 bootstrap replicates.

2.2. Construction of a recombinant plasmid

The coding sequence for the N-terminal domain of RfBPI/LBP (RfBPI/LBP-N) was PCR amplified and cloned into the pMAL-c2X vector, following the vendor's protocol (New England Biolabs, Ipswich, MA, USA). In brief, the coding sequence of RfBPI/LBP-N was PCR amplified using sequence-specific primers RfBPI/LBP-F and RfBPI/LBP-R (Table 1) that contained restriction enzyme sites for *Eco*RI and *Hin*dIII, respectively. PCR was carried out in a TaKaRa thermal cycler in a total volume of 50 μL with 5 U of Ex Taq™ Polymerase (TaKaRa, Japan), 5 μL of Ex Taq™ Buffer, 4 μL of 2.5 mM dNTPs, 80 ng of template DNA, and 40 pmol of each primer. Reaction conditions were as follows: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified fragment (675 bp) was then cloned into the pMAL-c2X plasmid after digesting the vector and the amplicon with the corresponding restriction enzymes, followed by overnight ligation at 4 °C using Mighty Mix (TaKaRa). The ligation mixture was transformed into *Escherichia coli* DH5α competent cells using standard molecular techniques and then the construct was sequenced. The sequence-

Table 1
Oligomers used in the study.

Name	Purpose	Sequence (5′ →3′)	Amplicon length (bp)
RfBPI/LBP-qF	qPCR of *RfLBP*	ACAAGCATCTCCCATCCGACTCAA	105
RfBPI/LBP-qR	qPCR of *RfLBP*	GCCGTCTTCACCAGCAGTTTCAT	
RfBPI/LBP-F	Amplification of coding region (*Eco*RI)	GAGAGAgaattcAGGCTAACAGGAAAAGGCCTT	690
RfBPI/LBP-R	Amplification of coding region (*Hin*dIII)	GAGAGAaagcttTCAATGCTTCCCGATGTTGTAAAATTCA	
RfEFA-F	qPCR for black rockfish EF1A	AACCTGACCACTGAGGTGAAGTCTG	107
RfEFA-R	qPCR for black rockfish EF1A	TCCTTGACGGACACGTTCTTGATGTT	

verified construct was then transformed into *E. coli* BL21 (DE3) cells for subsequent protein expression.

2.3. Overexpression and purification of recombinant RfBPI/LBP-N (rRfBPI/LBP-N)

rRfBPI/LBP-N was overexpressed in *E. coli* BL21 (DE3) as a fusion protein with maltose binding protein (MBP) according to the pMAL™ Protein Fusion and Purification protocol (New England Biolabs) with minor modifications. Briefly, *E. coli* BL21 (DE3) cells containing the sequence-confirmed RfBPI/LBP-N/pMAL-c5X construct were grown in 500 mL Luria broth (LB) supplemented with ampicillin (100 μg/mL) and glucose (0.2%) at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.6. Thereafter, the culture was treated with 0.5 mM isopropyl-β-D-galactopyranoside (IPTG) and induced at the same temperature for 3 h. Induced cells were then chilled on ice for 30 min and harvested by centrifugation at 2500 × *g* for 30 min at 4 °C. Harvested cells were resuspended in 20 mL of column buffer (20 mM Tris-HCl pH 7.4 and 200 mM NaCl) and were stored at −20 °C. The following day, *E. coli* cells were thawed and lysed in column buffer by cold sonication. Thereafter, the recombinant protein was purified using the pMAL™ Protein Fusion and Purification System. The purified protein was eluted using elution buffer (10 mM maltose in column buffer) and the concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard (Bradford, 1976). The antimicrobial activity of the purified fusion protein (rRfBPI/LBP-N) was then assayed. Samples collected at different steps of the rRfBPI/LBP-N purification were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using standard protein size markers (Enzynomics, Korea) under reducing conditions. The gel was stained with 0.05% Coomassie blue R-250 and observed following a standard destaining procedure.

2.4. LPS-binding activity assay

In order to analyze the LPS binding ability of rRfBPI/LBP-N, an enzyme-linked immunosorbent assay (ELISA) was carried out. Briefly, LPS (*E. coli* 055:B5, Sigma, St. Louis, MO, USA) was dissolved in coating buffer (30 mM Na_2CO_3 and 70 mM $NaHCO_3$; pH-9.6) to meet the concentration of 40 μg/mL. Subsequently, 50 μL of the obtained LPS solution was added into the wells of a 96 well ELISA plate and incubated at 37 °C for 3 h. After discarding the coating buffer, the plate was blocked with 200 μL of 1 mg/mL BSA in 0.1% TTBS solution (10% Tween 20, 20 mM Tris, and 137 mM NaCl; pH 7.7) at 37 °C for 3 h and washed four times with 200 μL of TTBS. Thereafter, wells of the plate were treated with 50 μL of purified rRfBPI/LBP-N or MBP (Control) prepared in TTBS containing 1% skim milk to meet different concentrations. After incubating for 3 h at room temperature, plates were again washed with TTBS as described, and treated with the mouse anti-MBP antibody (each well 50 μL; New England Biolabs; 1:5000 dilution) followed by an additional 3 h incubation at 37 °C. Plates were then rinsed four times with TTBS and 50 μL of horseradish peroxidase (HRP) conjugated rabbit anti-mouse antibodies (Thermo Fisher Scientific, USA; 1:3000 dilution) was added to each well followed by 2 h incubation at 37 °C. Finally, the wells were washed again with TTBS and HRP substrate (TMB: 3,3′,5,5′-tetramethylbenzidine) was added according to the vendor's protocols (Sigma). Optical density of the reaction mixture in each well was then measured by a Multiskan™ GO Microplate Spectrophotometer (Thermo scientific) at 450 nm and recorded as the means of triplicated assays.

2.5. Bactericidal activity assay

Potential bactericidal activity of rRfBPI/LBP-N was determined using *E. coli* as Gram-negative bacteria and *Streptococcus iniae* as Gram-positive bacteria. Briefly, *E. coli* and *S. iniae* bacteria were cultured in LB medium and Brain Heart Infusion (BHI) medium, respectively, to reach $OD_{600} \approx 0.8$. Cultures were subsequently centrifuged and pellets were resuspended in sterilized phosphate buffered saline (PBS) to reach $OD_{600} \approx 0.2$ after washing with sterilized PBS. Thereafter, 50 μL of each bacterial suspension was separately added into the wells of 96 well microplates and treated with 100 μg of the rRfBPI/LBP-N. For controls, equal volumes of cell suspensions were treated either with MBP or elution buffer. After 3 h incubation at 37 °C, each control and experimental *S. iniae* or *E. coli* culture in the microplate was serially diluted and plated (20 μL) on BHI-agar or LB-agar plates, respectively. Each plate was incubated overnight at the corresponding temperature (*S. iniae* at 28 °C and *E. coli* at 37 °C) and colonies were counted to calculate the colony forming units (CFU) in 1 mL of the originally treated samples or controls. This assay was repeated three times to ensure consistent outcomes.

2.6. Experimental fish and tissue sampling

Healthy rockfish that were acclimatized to laboratory conditions were obtained from the aquariums at the Marine Science Institute of Jeju National University, Jeju Self Governing Province, Republic of Korea, and were maintained in 400 L laboratory aquarium tanks filled with aerated seawater at 22 ± 1 °C. Five healthy fish with an average body weight of 200 ± 20 g were dissected for tissue collection. Before the dissection, approximately 1 mL of blood was collected from each fish using sterile syringes coated with 0.2% heparin sodium salt (USB, USA), and the peripheral blood cells were separated by immediate centrifugation at 3000 × *g* for 10 min at 4 °C. Other tissues, including head kidneys, spleen, liver, gills, intestines, entire kidney, brain, skeletal muscle, skin, heart, and stomach were excised, snap-frozen in liquid nitrogen, and stored at −80 °C.

2.7. Pathogen or chemical-mediated immune stimulation

Healthy rockfish with an average body weight of 200 ± 20 g were used in a time course immune stimulatory experiment to determine the transcriptional response of *RfBPI/LBP* under pathogenic stress. Viable Gram-positive *S. iniae* (10^5 CFU/μL), Gram-negative bacterial endotoxin LPS (*E. coli* 055:B5, Sigma), and polyinosinic:polycytidylic acid (150 μg/μL; Poly(I:C); Sigma), which resembles double-stranded viral RNA, were used as immune stimulants after resuspending or dissolving in PBS. Fish were intraperitoneally injected with each stimulant in a total volume of 200 μL. Separate group of fish were injected with 200 μL PBS as an injection control. Spleen and head kidney tissues were sampled from five individuals in each group at 3, 6, 12, 24, 48, and 72 h post-injection, as described in Section 2.6.

2.8. Total RNA extraction and cDNA synthesis

Pools of tissue samples (~40 mg from each fish) from five individual naïve or treated fish were used to extract total RNA using QIAzol® (Qiagen) reagent, according to the manufacturer's instructions. RNA quality was examined using 1.5% agarose gel electrophoresis, and spectrophotometrically by measuring the absorbance ratio at 260 and 280 nm. Concentration of RNA was determined at 260 nm using a μDrop Plate (Thermo Scientific). First strand cDNA was synthesized in a 20 μL reaction mixture using 2.5 μg of RNA from each sample as a template with the

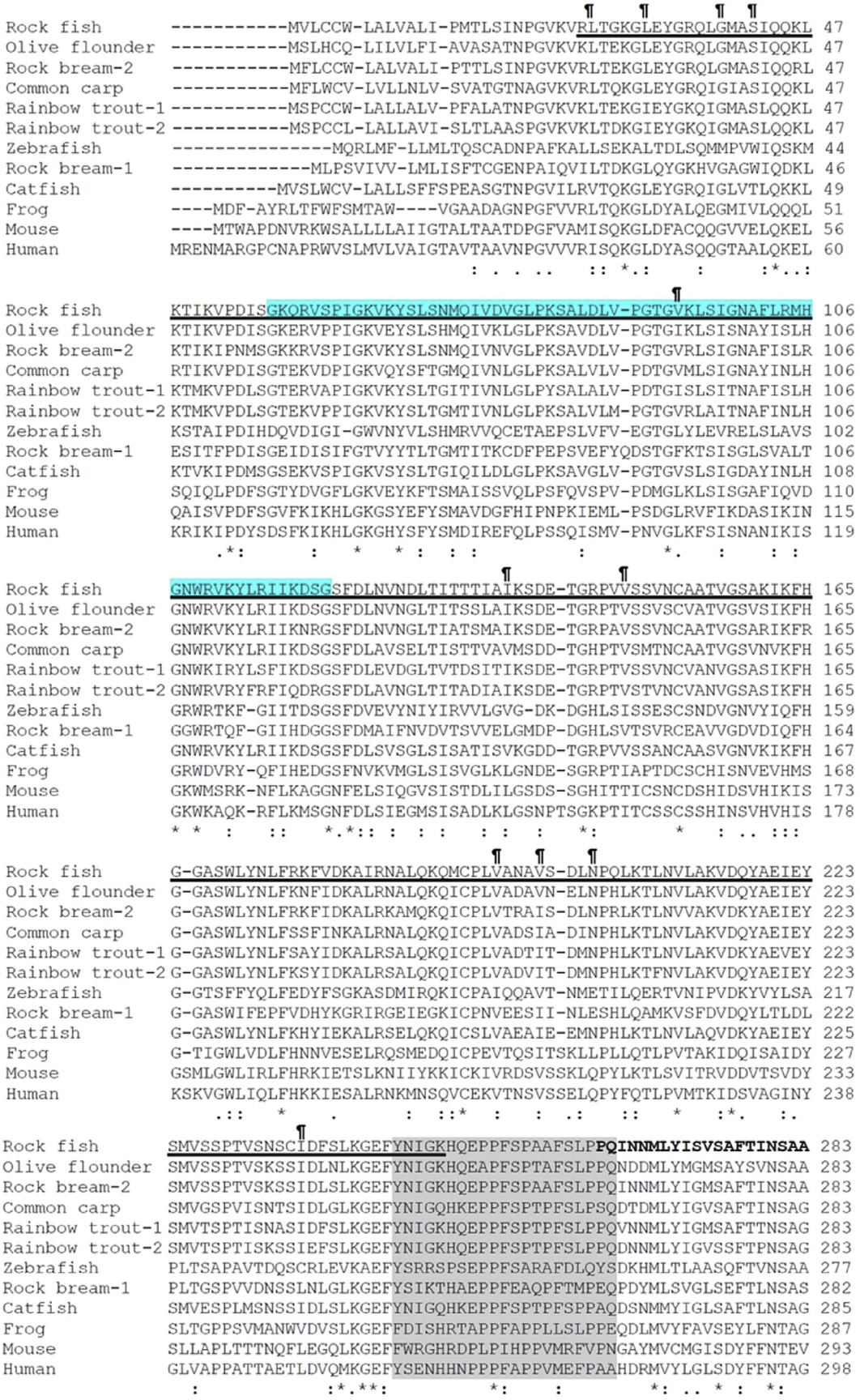

Fig. 1. Multiple sequence alignment of fish BPI/LBPs, including black rockfish BPI/LBP (RfBPI/LBP) with mammalian and amphibian BPI protein sequences. N-terminal and C-terminal domains of RfBPI/LBP are marked by underlining and boldface, respectively. The N-terminal signal peptide sequence of RfBPI/LBP is denoted by gray shading and the putative LPS-

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Rock fish       FVYNTAGALSLYITDDMIPQASPIRLNTRTFGAFIPQVAKRFPGLMMKLLVKTAKNPVVT 343
Olive flounder  FVYNKAGALSLYITDDMIPQSSPIRLNTRTFGTFIPEISKRFPGLMMKLLVQTVKTPVIT 343
Rock bream-2    FVYNKAGALSLSITDDMIPKRSPVRLNTRTFGVLIPQIAQRFPGLMMKLLVKTAKNPVMT 343
Common carp     FVYNRAGALSLYITDDMIPSGSPIRLNTKTFGAFIPQIEKMYPGLMMKLLVETVKEPIVT 343
Rainbow trout-1 FVYNNAGALSLYITDDMIPPSSPIRLNTRTFGAFIPEIAKRFPSMMMKLLVKTVKEPTIF 343
Rainbow trout-2 FVYNNAGALSLYVTDDMIPPSSPIRLNTGTFGVFIPEIAKRFPGMMMKLLVKTVKEPTIS 343
Zebrafish       FAYLRSGALQTNITDDMIPKGSPLHLNTSQFGVFIPQLRTLYPDMKMQVLLYASDMPLFS 347
Rock bream-1    YAYYSAGLFQALINDSMVPPSSPVHLNTSAMGPFIPQLPKMFPGLLMDLQVYAREVPLFS 342
Catfish         FVYNNAGVLSLYITDDMIPSSFPIRLNTKTFGTFIPQIAKQYPGLMMKLLVKAEKEPNVS 345
Frog            LVYQSAGALVFNLTDDMIPKESSVHLNTSSFGLLIPSVSKMYPNMLMKLEMSAASAPALN 347
Mouse           LAYQQSGTLKMTLGGQLLSNNGRFQLNTDFLRTFLPKVAKMFPSMGVQLLISAPVPVHLS 353
Human           LVYQEAGVLKMTLRDDMIPKESKFRLTTKFFGTFLPEVAKKFPNMKIQIHVSASTPPHLS 358
                 .*  :* :   :  .::     .:*.*  :  ::*.:   :* : :.: : :     .

Rock fish       FEPNKVTVQATSTVTAYAIQPNTTLSPLFVLNLE---TSVSARVFVSGMRLAGAVTLN-K 399
Olive flounder  FQPNNATTQASCTVTAYAIQPNSTLSPLFILNLE---TSVSAQVFISGMRVAGAVSLN-K 399
Rock bream-2    FEPNNMTVQATGTVTAYAIQPNATLSPLFILNLE---TSFSARVFVSGMRLAGSVDLN-R 399
Common carp     FEPNNMTVQASSTVTAYAIQPNSTLSPLFVLNLE---VSVSTHIYVTELKLAGNVTLN-K 399
Rainbow trout-1 FEPNNVTVQASGSVTAYAIQPNTTLSPLFVLNME---GSVSARLYVTGVRLAGAVTLN-K 399
Rainbow trout-2 LEPNNVTVQASGTVTAYAIQPNTTLSPLFVLNME---GSVSAQMNVTGVKLAGAITLN-K 399
Zebrafish       FTSGLMNIHVKMAAKFSAVKADDALVPLFTLNVD---SRFSGIAQISNQKLTGAFKVN-N 393
Rock bream-1    FQPGAVALGFQGAIKAFAIQPNGTQTPLFKLNVD---SKFSGKVWIADERLKGSMAMN-N 398
Catfish         FEPDNVTLQASSTVTAYAIQPNATLSPLFVLNVN---ASVSARIYVSGLTVAGNLSLD-E 401
Frog            INPGNLTLSPVGNIQAYAILPNSSLAPLFLLQMK---MNALAKVAVNSGKIVGSLELG-K 403
Mouse           IQPSGLSFNPKLETQAFVVLPNASLVPLFVLGMVRRKTNASLEVDAEENRLVGEMKLGSR 413
Human           VQPTGLTFYPAVDVQAFAVLPNSSLASLFLIGMH---TTGSMEVSAESNRLVGELKLD-R 414
                .                .:  : :   ** : :                 : * . :  .
Rock fish       MALTLGTSYVGEFQVRSLDSIFQVVLKVVVIPILNVQLAKGYPLPTLGKMKLVNTELQVL 459
Olive flounder  MDLTLRTSYVGEFKVRSLDNILQMVLKVVVIPKLNVQLAKGYPLPAIGKMKLVKTQLKVL 459
Rock bream-2    MDLTLGTSYVGEFQVKSLNNIIQVVLKLVVIPQLNVQLAKGYPLPTLGKMKLVNPQLQVL 459
Common carp     INMSLAKSYVGPFQVTSLDNIFTIVLKFAVIPKVNARLQEGYPLPAIGKMQLVNSQLKVL 459
Rainbow trout-1 IEMTLETSYVGQFQVRSLDNIFLMVLKVAVIPKVNARLEKGFPLPSIGKMNLVNTQLQVL 459
Rainbow trout-2 IEMTLGTSYVGQFQVQSLDNIFLMVLKVVVIPKVNARLEKGFPLPTIGKMNLINTQLQVL 459
Zebrafish       ITLTVGSSEIGDFKTDTIRQVLVIAVNTIILPKLNARLRSGFLLPTLQGFSLSNSQLLIK 453
Rock bream-1    LTLTLVSSEVGTFKTDAIEGLARMG-ATLAMARLNEKLGKGVVLPRMKDVQLVNTVLEMD 457
Catfish         MDMTLATSYVGPFQVKSLDSFLTMIMKAVVIPVVNALLRQGYPLPAIGNMNLVNTQLQIL 461
Frog            VEISLVHSDVGLFSVSVISMAVNYYVSATLLPRVNEILKNGFPLPLIDHIQLTDFVIETY 463
Mouse           WLLELKESKFGPFKVEYLEDVINYLVSTLVLPKINERLRRGFPLPLPAGIRFSHFTFYPY 473
Human           LLLELKHSNIGPFPVELLQDIMNYIVPILVLPRVNEKLQKGFPLPTPARVQLYNVVLQPH 474
                  : :   * .* * .  :             :  :*  *  *  **    . : .  :

Rock fish       KDYMLIGTDVQFTG----- 473
Olive flounder  KDYMLIGTDVQFTG----- 473
Rock bream-2    KDYMLIGTDVQFTG----- 473
Common carp     KDYLLIGTDVQFTG----- 473
Rainbow trout-1 KDYMLIGTDVQFTG----- 473
Rainbow trout-2 KDYMLIGTDVQFTG----- 473
Zebrafish       NGFVVIFTDIRLPDGLNAP 472
Rock bream-1    EGFIAISSDAQLITDRGFN 476
Catfish         KDYVLIGTDVQFTG----- 475
Frog            EHYLLFGANAHYE------ 476
Mouse           QNFLLLEADLHLI------ 486
Human           QNFLLFGADVVYK------ 487
                : :: : ::
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Fig. 1. (*continued*).

PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. The synthesized cDNA was diluted 40-fold in RNAse free water (Ambion, USA) and stored in a freezer at −80 °C until use.

2.9. Determination of mRNA expression

mRNA expression levels of *RfBPI/LBP* in tissues (Section 2.6) obtained from naïve fish and in head kidney and spleen tissues of immune stimulated animals at each time point following immune stimulations (Section 2.7) were quantified by quantitative real-time polymerase chain reaction (qPCR) using diluted cDNA samples as the templates. qPCR was performed using the Dice™ Real Time System thermal cycler (TP800; TaKaRa) in a 10 μL reaction volume, containing 3 μL of diluted cDNA from each tissue, 5 μL of 2 × TaKaRa Ex Taq™ SYBR premix, 4 pmol of each primer (RfBPI/LBP-qF and RfBPI/LBP-qR; Table 1), and 1.2 μL of ddH_2O, as per the essential MIQE guidelines (Bustin et al., 2009). PCR conditions were as follows: 95 °C for 10 s, 35 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s, and a final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Each assay was repeated three times. The baseline was set automatically by the Dice™ Real Time System software (version 2.00). Negative Controls (No template control and no RT-control experiments) were carried out to affirm that the PCR

binding domain is in blue color. Proline-rich central domains of different homologs are indicated by gray shading and residues in the apolar binding pocket are indicated by the '¶' symbol. Residues conserved in all aligned sequences are represented by asterisks (*), whereas partially conserved residues are marked using (.) or (:). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Percentage similarity and identity values of RfBPI/LBP with homologs.

Species name	Protein	GenBank accession number	Taxonomy	Length in amino acids	Identity (%)	Similarity (%)
1. *Oplegnathus fasciatus* (Rock bream)	BPI/LBP-2	BAM21038	Fish	473	85.4	93.9
2. *Paralichthys olivaceus* (Olive flounder)	BPI/LBP	ACV74252	Fish	473	80.1	91.8
3. *Plecoglossus altivelis altivelis* (Ayu)	BPI/LBP	BAG49475	Fish	471	75.5	88.8
4. *Osmerus mordax* (Rainbow smelt)	BPI/LBP	ACO09816	Fish	470	75.5	87.5
5. *Oncorhynchus mykiss* (Rainbow trout)	BPI/LBP-1	NP001118057	Fish	471	75.3	89
6. *Oncorhynchus mykiss* (Rainbow trout)	BPI/LBP-2	NM001124198	Fish	473	74.6	89.4
7. *Cyprinus carpio* (Common carp)	BPI/LBP	BAC56095	Fish	473	72.3	86.3
8. *Ictalurus punctatus* (Catfish)	BPI/LBP	NP001187129	Fish	475	68.2	86.1
9. *Gadus morhua* (Atlantic cod)	BPI/LBP-A	AAM52335	Fish	473	62.6	80.5
10. *Gadus morhua* (Atlantic cod)	BPI/LBP-B	AAM52336	Fish	473	62.6	80.5
11. *Oplegnathus fasciatus*(Rock bream)	BPI/LBP-1	BAB91243	Fish	473	37	60.1
12. *Xenopus tropicalis* (Frog)	BPI	NP001107736	Amphibia	476	36.6	61.1
13. *Macaca mulatta* (Monkey)	BPI	EHH19710	Mammalia	487	35.6	59.3
14. *Macaca fascicularis* (Macaque)	BPI	EHH65352	Mammalia	487	35.2	59.1
15. *Bos taurus* (Cattle)	BPI	NP776320	Mammalia	482	35.1	60.4
16. *Salmo salar* (Atlantic salmon)	BPI	NP001135199	Fish	485	35	58.1
17. *Homo sapiens* (Human)	BPI	ABD66755	Mammalia	487	35	59.8
18. *Larimichthys crocea* (Large yellow croaker)	BPI	ABO32254	Fish	472	34.7	58.6
19. *Sus scrofa* (Pig)	BPI	NP001152779	Mammalia	483	33.8	58.4
20. *Danio rerio* (Zebrafish)	LBP	XP001342762	Fish	472	32.6	56.4
21. *Bos taurus* (Cattle)	LBP	NP001033763	Mammalia	481	32.4	58.4
22. *Homo sapiens* (Human)	LBP	AAB31143	Mammalia	481	32.3	59.5
23. *Mus musculus* (Mouse)	LBP	NP32515	Mammalia	481	32.1	57
24. *Sus scrofa* (Pig)	LBP	NP001121907	Mammalia	481	31.7	56.5
25. *Mus musculus* (Mouse)	BPI	NP808518	Mammalia	486	28	55.6
26. *Crassostrea gigas* (Pacific oyster)	BPI/LBP	AAN84552	Mollusca	477	27.1	49.9
27. *Cricetulus griseus* (Hamster)	BPI	ERE71743	Mammalia	563	26.3	49.4

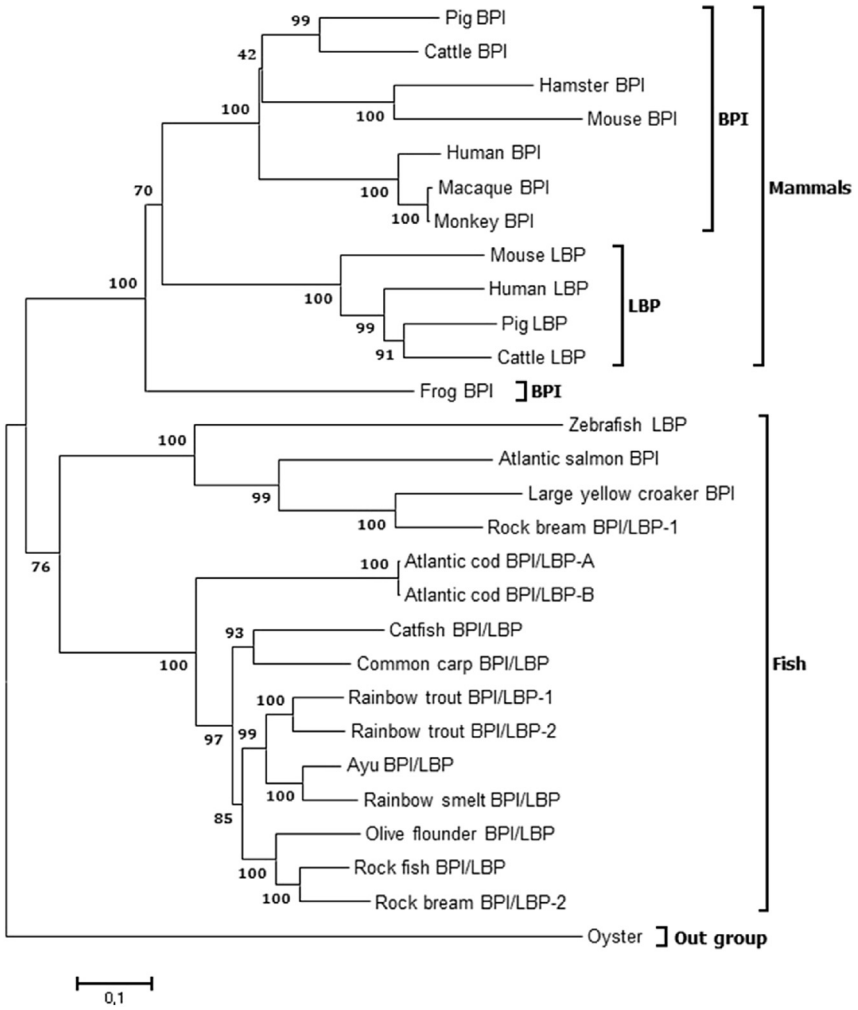

Fig. 2. Phylogenetic reconstruction of RfBPI/LBP generated based on ClustalW multiple sequence alignment with different vertebrate and invertebrate LBP or BPI homologs under the neighbor-joining platform using MEGA version 6.0. Bootstrap supporting values obtained by 1000 bootstrap replicates are denoted at the tree branches and NCBI-GenBank accession numbers of LBP or BPI homologs are mentioned in Table 2.

reagents were not contaminated with templates and there was no significant contamination of template cDNA with genomic DNA, respectively. Melting curves of each amplicon were analyzed to confirm the amplification of a single specific product with unique melting temperature. The relative *RfBPI/LBP* expression was determined using the Livak ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). The black rockfish elongation factor 1α (RfEF1A) gene was used as an internal reference (GenBank ID: KF430623), because it was previously validated as an appropriate internal control for qPCR in black rockfish gene expression studies (Liman et al., 2013). In order to apply the aforementioned Livak ($2^{-\Delta\Delta CT}$) method, amplification efficiencies for the internal control and the desired product (RfBPI/LBP) were determined and confirmed the consistency with substantial values (RfBPI/LBP: 107.7%, RfEF1A: 102.3%). Primers used for the internal reference are listed in Table 1. Data are presented as the mean ± standard deviation (SD) of the relative mRNA expression from three experiments. In the immune stimulation experiments, the level of *RfBPI/LBP* mRNA level was calculated relative to that of RfEF1A. The expression values were further normalized to the corresponding PBS-injected controls at each time point. The relative expression level in the un-injected control at 0 h was used as the baseline reference. One way ANOVA following Duncan's Multiple Range test was used to determine the statistical significance ($p < 0.05$) between each time point including un-injected control (0 h) group using SPSS (11.0) program.

3. Results and discussion

3.1. Sequence profile, homology, and evolutionary relationship

The identified RfBPI/LBP cDNA sequence (4152 bp) contains a putative ORF of 1422 bp that encodes a 474 amino acid protein with a predicted molecular mass of ~51.5 kDa and theoretical isoelectric point of 9.74. According to *in silico* predictions, RfBPI/LBP consists of two domains, an N-terminal (26–249 residues) and a C-terminal domain (264–467 residues), with an N-terminal signal peptide sequence (1–18 residues), resembling the typical characteristics of the conserved domain architecture of BPI/LBP proteins (Fig. 1). The secretory nature of the protein is reflected by the signal peptide sequence. In addition, a putative LPS-binding domain was also identified in RfBPI/LBP (57–121 residues; Fig. 1). As expected, RfBPI/LBP shared significant sequence similarity (60.1–93.9%) and identity (37–85.4%) with its teleostean homologs. It shared its highest sequence consistency with one variant of rock bream BPI/LBP (BPI/LBP 2–85.4% identity and 93.9% similarity; Table 2). However, the other variant (rock bream PBI/LBP2) only showed relatively very low sequence consistency with RfBPI/LBP 2. Moreover, RfBPI/LBP showed relatively low sequence consistency with mammalian or amphibian orthologs. Alignment of RfBPI/LBP with several other vertebrate orthologs in our multiple sequence alignment approach identified a conserved proline-rich central domain and partially conserved residues involved in apolar binding pocket formation in RfBPI/LBP as common features of vertebrate BPI/LBPs (Fig. 1). As expected, the phylogenetic reconstruction revealed that BPI and LBP homologs were separately clustered within the mammalian clade diverging from a common ancestral origin (Fig. 2). Intriguingly, two distinct clades were identified in the main fish cluster, where two rock bream BPI/LBP homologues were clustered separately in two clades, one (Rock bream BPI/LBP2) with very closer evolutionary proximity to RfBPI/LBP. The divergence of these two rock bream homologues may have occurred due to a gene duplication event. In the whole reconstruction, invertebrate BPI ortholog of oyster serves as an out-group. Together, our phylogenetic analysis ascertains the vertebrate ancestral origin of RfBPI/LBP further reinforcing its greater evolutionary proximity with teleostean homologs.

3.2. Integrity and purity of overexpressed protein

Overexpression and purification of rRfBPI/LBP-N as a fusion protein was monitored using SDS-PAGE. Successful overexpression of the MBP-tagged rRfBPI/LBP-N was confirmed by the intense band observed around 70 kDa, since the expected fusion protein size was ~66.5 kDa (RfBPI/LBP-N = 24 kDa and MBP = 42.5 kDa; Fig. 3, lane 2). Moreover, substantial purity and integrity of the finally eluted purified protein product was confirmed by the single band resolved on lane 3 with the same expected size (66.5 kDa).

3.3. In vitro LPS-binding activity of rRfBPI/LBP-N

As reported previously, animal BPI/LBPs are known to bind LPS (Gonzalez et al., 2007; Zhang et al., 2011). Thus, we investigated the

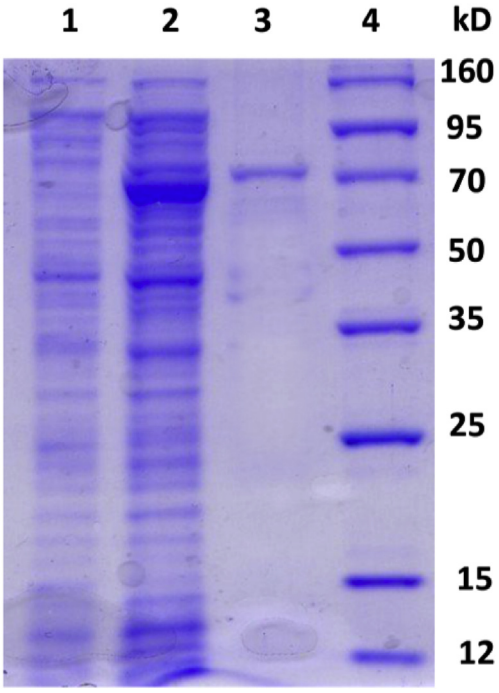

Fig. 3. SDS-PAGE analysis of purified recombinant RfBPI/LBP-N fusion protein collected during different steps of the rRfBPI/LBP-N purification. Lane 1, Total soluble cellular extract from *E. coli* BL21 (DE3) containing the rRfBPI/LBP-N-MBP fusion vector construct prior to IPTG induction; Lane 2, Crude extract of rRfBPI/LBP-N after IPTG induction; Lane 3, Purified RfBPI/LBP-N; Lane 4, Protein size marker.

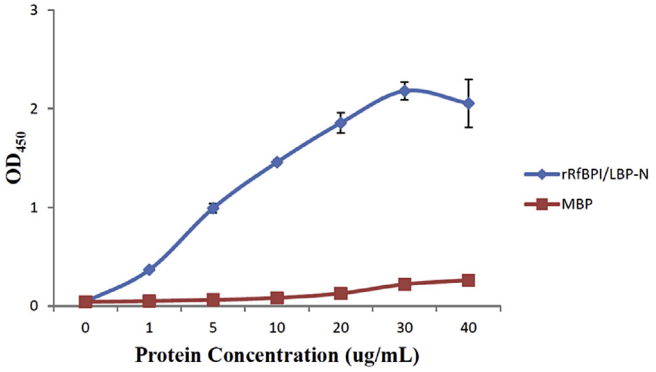

Fig. 4. LPS-binding activity of rRfBPI/LBP-N. Binding ability of rRfBPI/LBP-N or MBP (control) to immobilized LPS was determined by an indirect ELISA assay. Data represent the mean OD values of triplicated assays and error bars represent the SD.

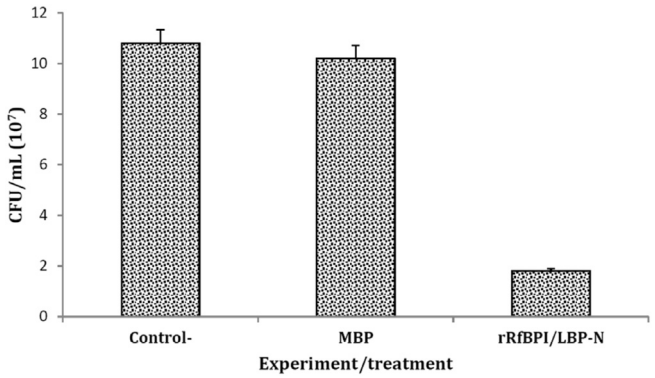

Fig. 5. Bactericidal activity of rRfBPI/LBP-N against *E. coli* as detected by reductions in cell viability. Cell viability was determined by CFU/mL in each culture after respective treatment. Data illustrate respective experiments. Error bars represent the SD (n = 3).

potential LPS-binding activity of our purified rRfBPI/LBP-N using standard indirect ELISA assay with immobilized LPS and anti-mouse MBP antibody. OD_{450} of the reaction mixture increased with the concentration of rRfBPI/LBP-N until 30 μg/mL, reflecting concentration-dependent LPS-binding of rRfBPI/LBP-N. The OD_{450} showed a slight drop at 40 μg/mL, suggesting saturation conditions (Fig. 4). As expected, OD for MBP was negligible relative to rRfBPI/LBP-N, possibly indicating non-specific binding. Overall, the results of this assay clearly confirm the potent LPS-binding ability of rRfBPI/LBP-N and ascertained the presence of the LPS-binding domain that was predicted by our *in silico* analysis of the protein sequence (Fig. 1).

3.4. In vitro antibacterial activity of rRfBPI/LBP-N

The N-terminal domain of BPIs is reportedly involved in antibacterial activities (Capodici and Weiss, 1996). Thus, we carried out an antibacterial assay using two different bacteria, *E. coli* and *S. iniae*, to analyze the potential antibacterial properties of rRfBPI/LBP-N, as previously reported for known BPI/LBPs (Zhang et al., 2011). After treating *E. coli* cultures with rRfBPI/LBP-N, significantly lower bacterial viability (~1.8×10^7 CFU/mL) was observed than in MBP-treated cultures (~10.2×10^7 CFU/mL) and control cultures (~10.8×10^7 CFU/mL), suggesting that rRfBPI/LBP-N treatment kills *E. coli* (Fig. 5). However, *S. iniae* cultures treated with the same amount of rRfBPI/LBP-N did not show any significant change in CFU count compared to the MBP or elution buffer-treated cultures, suggesting that there is no detectable effect of rRfBPI/LBP-N on their viability (data not shown). Collectively, these results suggest a selective bactericidal role of RfBPI/LBP in black rockfish immunity where it exclusively eradicates Gram-negative bacterial pathogens. Similar to our observations in this experimental approach, BPI paralogs from humans and the Pacific oyster (*Crassostrea gigas*) (Gonzalez et al., 2007; Weiss et al., 1978; Zhang et al., 2011) reportedly demonstrated potent bactericidal effects on Gram-negative bacteria, but not on Gram-positive bacteria.

3.5. Tissue-specific mRNA expression

Different abundances of basal *RfBPI/LBP* mRNA expression were detected in each examined tissue (Fig. 6). The most abundant *RfBPI/LBP* transcript levels were observed in spleen tissues, whereas the least abundant expression was detected in liver and muscle tissues. Spleens are one of the largest lymphoid organs in teleost fish (Zapata et al., 2006). These organs house cellular components involved in host innate immune responses, including aggregates of macrophages known as melanomacrophage centers, and splenic ellipsoids (Uribe et al., 2011). Thus, splenic tissues of teleosts likely express abundant levels of antimicrobial agents like BPI/LBP, which are known to trigger immune signaling cascades by activating proinflammatory cytokines. Similar to our observation, one variant of BPI/LBP of rock bream was highly expressed in spleen tissue, although another variant was highly expressed in peripheral blood leukocytes and kidney tissues (Kim et al., 2012). Moreover, expression of this variant was found to be much less abundant in liver, agreeing with our observations of RfBPI/LBP. On the other hand, the mRNA homolog found in Atlantic cod was prominently expressed in head kidneys and moderately expressed in the spleen (Stenvik et al., 2004).

3.6. Transcriptional responses to pathogen stress

Temporal regulation of *RfBPI/LBP* expression following *S. iniae* treatment and LPS or poly(I:C) exposure was analyzed using qPCR to investigate transient alterations in the mRNA expression of *RfBPI/*

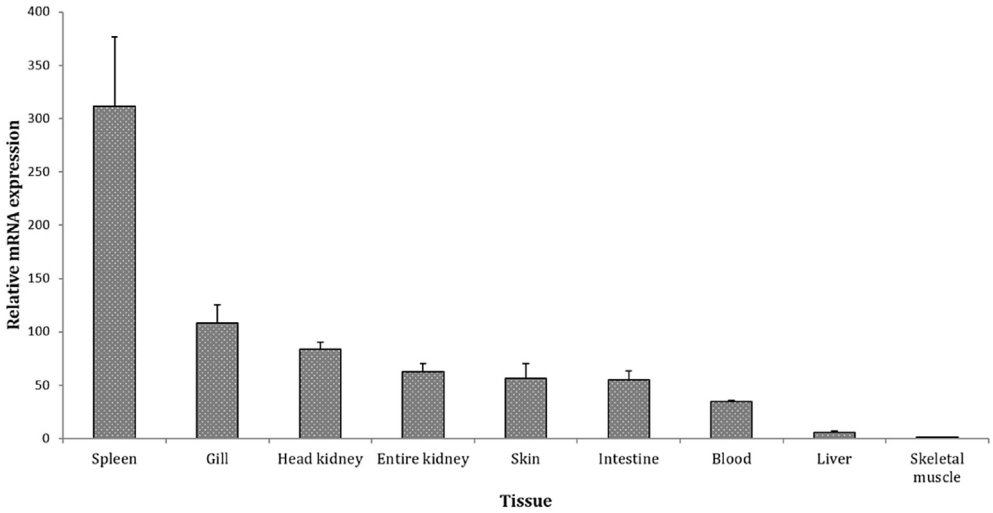

Fig. 6. Tissue-specific distribution of *RfBPI/LBP* expression in black rockfish measured using quantitative real-time polymerase chain reaction (qPCR). Fold-changes in expression are shown relative to the level of mRNA expression in liver tissue. Error bars represent SD (n = 3).

LBP in response to a pathogenic stimulus. *RfBPI/LBP* expression in head kidney tissues was markedly increased (~26 fold) at 12 h post-treatment (p.t.) with *S. iniae* (Fig. 7A). Transcription was upregulated, relative to the basal (0 h) level until 72 p.t. Similar to this observation, *S. iniae* treatment elicited an inductive transcriptional response in spleen tissues at the early phase (6 h p.t.) to late phase (48 h p.t.). *RfBPI/LBP* expression levels gradually decreased from 12 h p.t. to 48 h p.t. (Fig. 7A). These transcriptional responses might have been elicited to rapidly increase RfBPI/LBP during the early phase to trigger cytokine-mediated immune responses rather than to exert direct antibacterial activity against *S. iniae*, since RfBPI/LBP did not show any detectable antibacterial activity against this bacterium in our antibacterial activity assessment. The observed decreases in expression levels over time suggest that the need for high RfBPI/LBP concentrations in cells diminishes, plausibly because of successful clearance of the infecting pathogen. Consistent with our experimental outcomes, an BPI/LBP homolog in olive flounder reportedly demonstrated elevated transcription in spleen, head kidney, intestine, gill, and liver tissues after *S. iniae* treatment (Nam et al., 2010). Moreover, one of the two variants of rock bream BPI/LBP, which shared 36.9% identity to each other, was upregulated after experimental *S. iniae* infection, although the other variant showed no transcriptional response to the infection (Kim et al., 2012).

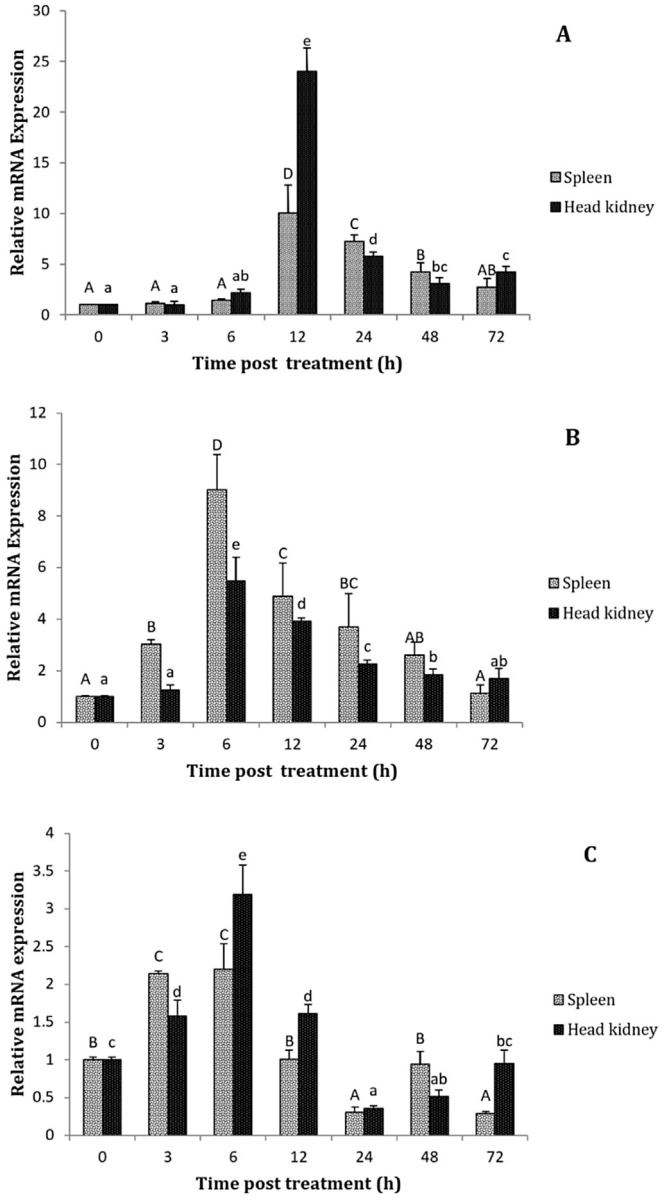

Fig. 7. Temporal regulation of *RfBPI/LBP* mRNA expression in spleen and head kidney tissues upon immune stimulation with (A) *S. iniae* (B) LPS and (C) poly(I:C) as determined by qPCR. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The black rockfish EF1A gene was used as the internal reference gene and mRNA expression was further normalized to the corresponding PBS-injected controls at each time point. The relative fold-change in expression at 0 h post-injection (un-injected control) was used as the baseline. Error bars represent SD (n = 3). Different letters on errer bars indicate significantly different expression levels ($p < 0,05$).

Upon treatment with a well-known Gram-negative bacterial endotoxin, LPS, *RfLBP* was upregulated in the early phase in head kidney and spleen tissues (6 h p.t. and 3 h p.t., respectively) through the late phase (48 h p.t. in both tissues) (Fig. 7B). This finding suggests that LPS recognition through increasing amounts of RfBPI/LBP triggers immune responses and this response weakens over time as PAMP recognition meets adequate levels to inactivate the immune responses. On the other hand, this observation may provide clues for the potential eradication of Gram-negative bacteria by RfBPI/LBP, after recognition of their PAMPs like LPS, which can be supported by the detected LPS-binding activity and antibacterial activity of its recombinant protein. Consistent with our observations, rainbow trout BPI/LBP-1 and BPI/LBP-2, which shared 87% of identity 93% of similarity to each other at amino acid level, were transcriptionally induced in head kidney tissues after LPS treatment. The observed initial increases in expression and later decreases in expression agree with our results (Inagawa et al., 2002). However, neither variant has shown significant transcriptional regulation in rainbow trout liver tissues in response to LPS treatment. On the other hand, inductive transcriptional responses were detected only at 3 h post-LPS stimulation in head kidney and liver tissues of common carp; although, expression levels at the late phase transcription were downregulated (Kono and Sakai, 2003).

RfBPI/LBP was noticeably upregulated in response to exposure to a synthetic viral PAMP, poly(I:C), at early time points post-treatment in both head kidney and spleen tissues, suggesting the potential sensitivity of teleostean BPI/LBP to viral infection (Fig. 7C). *RfBPI/LBP* was significantly downregulated at the late phase of the poly(I:C) experiment. However, further studies are required to validate this conclusion. The late phase downregulation may represent an RNA turnover event under the stress generated by poly(I:C) in spleen or head kidney cells. Although, no studies have yet reported evidence of the involvement of BPIs or LBPs in antiviral defense, mRNA levels of both rock bream BPI/LBP-1 and -2 were elevated after red sea bream iridoviral treatment (Kim et al., 2012), suggesting that those homologs may sense and respond to viral PAMPs.

Collectively, the outcomes of our pathogen or PAMP-mediated immune stimulations on antibacterial activity suggest that RfBPI/LBP plays an indispensable role in host antimicrobial defense.

4. Conclusion

We identified and molecularly characterized a BPI/LBP homolog from black rockfish investigating its putative importance in host antimicrobial defense. RfBPI/LBP contained most of the conserved domain features of BPI/LBP proteins and shared considerable sequence compatibility with known BPI/LBP orthologs. Recombinantly expressed RfBPI/LBP-N demonstrated selective antibacterial activity and potent LPS-binding activity. Moreover, the expression of *RfBPI/LBP* transcripts was found to be temporarily upregulated upon treatment with pathogens or PAMPs when compared to the basal expression level. Taken together, our results suggest a significant role of RfBPI/LBP in host antimicrobial defense.

Acknowledgments

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