Characterization and expression analysis of bactericidal permeability-increasing protein (BPI) antimicrobial peptide gene from channel catfish *Ictalurus punctatus*

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Abstract

Antimicrobial peptides are important components of host defenses against microbial invasions. Bactericidal permeability-increasing protein (BPI) is an antimicrobial peptide belonging to the lipid transfer/LPS-binding protein family. It serves important roles in defending against Gram-negative bacteria in the innate immune system. Here we report cloning of complete BPI cDNA from channel catfish (*Ictalurus punctatus*) by 5′ RACE after obtaining the partial BPI cDNA sequence from EST analysis. The channel catfish BPI cDNA is 1640 bp in length with a 1428-bp open reading frame that encodes a protein of 475 amino acids. Catfish BPI gene shows high similarity with the BPI/LBP gene isolated from other teleost fish. As part of ongoing efforts in comparative genome analysis, we have assigned the catfish BPI gene to bacterial artificial chromosome (BAC) clones. Southern blot analysis on multiple BPI BAC clones indicated the presence of a single copy of the BPI gene in the catfish genome. Reverse transcription-polymerase chain reaction (RT-PCR) analysis on healthy tissues showed that BPI was expressed in a wide range of tissues including head kidney, gill, skin, trunk kidney, brain, intestine, liver, muscle, ovary, spleen and stomach. The BPI gene was not developmentally expressed until 48 h after fertilization. Quantitative real time PCR (QRT-PCR) analysis indicated that the BPI gene expression was induced after challenge with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC). BPI upregulation peaked 3 days after challenge, mirroring the expression pattern of inflammatory chemokines in catfish, suggesting that it plays a role in the innate defense response.
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1. Introduction

The innate immune system has the potential for quick, powerful, and non-specific responses to a wide

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range of pathogens. An effective innate immune response to an acute disease is often the difference between life and death for the host organism. Important questions remain as to how the innate immune system recognizes pathogens for eradication. A complex glycolipid in the outer membrane of Gram-negative bacteria known as lipopolysaccharide (LPS) plays an important role in the activation of the innate immune system [1,2]. The cellular component of innate immunity is activated upon recognition of pathogen associated molecular patterns (PAMPs) [3,4] including LPS and double-stranded RNA as well as host-derived cytokines. Upon breaching of the physical barrier, the invading pathogen faces a whole array of soluble factors including antimicrobial peptides, proteases, lysozyme, complement factors and acute-phase proteins [5,6].

The aqueous environment, vital to fish survival, also facilitates transmission of many water-borne pathogens. Although the innate immune system in teleost fish is still not completely understood, rapid progresses have been made in recent years towards the identification of many of its components including chemokines [7–11], complement factors [12–15], and antimicrobial peptides [16–20]. A number of anti-microbial peptides have been identified and their genes cloned from teleost fish including bactericidal permeability-increasing protein (BPI).

The antimicrobial peptide bactericidal per-meability-increasing protein (BPI) and lipopoly-saccharide (LPS) binding protein (LBP) are two related LPS-interactive proteins initially identified from mammals. Both belong to a family of lipid transfer/LPS-binding proteins, which also includes the mammalian phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) [21]. They respond to bacterial infection and modulate the inflammatory cascade triggered by the LPS [22]. BPI and LBP are thought to play a significant role in signal transduction from LPS [23,24]. They insert their functions through interaction with CD14 and induc-tion of proinflammatory cytokines such as tumor necrosis factor-α [23,25–28].

Both BPI and LBP have two domains, N-terminal domain and C-terminal domain, which serve distinct functions: binding and transferring of LPS, respect-ively, [29,30]. Both proteins bind LPS via their N-terminal domains but produce different effects: BPI

has full antibacterial and LPS-neutralizing activity whereas LBP is not toxic to Gram-negative bacteria and potentiates LBP bioactivity [31]. The C-terminal domains of BPI and LBP serve to transport LPS. In BPI, the C-terminal domain promotes bacterial attachment to neutrophils and monocytes, leading to phagocytosis. A similar role of the C-terminal domain of LBP is the delivery of LPS to CD14 on both polymorphonuclear leukocytes and monocytes result-ing in cell activation [31]. Through interaction with CD14, LPS activates cells via a transmembrane receptor capable of promoting signal transduction. This recognition/response cascade includes the Toll-like receptor family of proteins, most notably Toll-like receptor 4, which serves as the primary mediator of endotoxin signaling [32].

The complete cDNA sequences of BPI and LBP have been cloned from human [33], bovine [34], rabbit [35,36], mouse [37,38] and rat [39]. In fish, several BPI and LBP-like cDNAs have been isolated, but their orthologies were not established. Therefore, they have been referred to as BPI/LBP proteins [40–42]. BPI/LBP cDNA was recently cloned and characterized from rainbow trout [40], Atlantic cod [41] and common carp [42]. As a part of the effort to characterize the catfish innate immune system, here we report molecular cloning, characterization, and expression analysis of BPI/LBP gene in channel catfish (*Ictalurus punctatus*).

2. Materials and methods

2.1. 5′-RACE to obtain full-length BPI cDNA

The channel catfish BPI cDNA was initially identified from EST analysis of catfish [43–45] using a head kidney cDNA library [46]. The cDNA contained an 887-bp insert. Sequence analysis of this clone indicated that the BPI cDNA was incomplete at the 5′-end, missing approximately 660 bp. In order to obtain full-length cDNA of BPI, 5′-RACE (rapid amplification of cDNA ends) was conducted. The 5′-RACE system was purchased from Invitrogen (Carlsbad, CA) and RACE was performed following manufacturer's instructions. The 5′-RACE products were separated by electrophoresis using 1% agarose gel and purified by using QIAQuick Gel Extraction

Table 1
Primers and their sequences used in this study

Primer name	Sequences (5′–3′)	Location
Gene-specific primer GSP1 for 5′-RACE	TCTCAGCCTTCACCAGGAGC	1104–1123
Gene-specific primer GSP2 for 5′-RACE	ATGTAGAGGCTGAGCACTCC	979–998
Gene-specific primer GSP3 for 5′-RACE	CTATGGCTTCAGCCACCAGAG	689–709
Gene-specific primer GSP4 for 5′-RACE	ACATTGCCAACAGAGGCTGC	571–590
cDNA probe forward	CATCACAAGGTCAGAGTGGAC	85–105
cDNA probe reverse	AGATTCATGTTTCCGATGGCAG	1442–1463
Beta actin RT-PCR forward primer	AGAGAGAAATTGTCCGTGACATC	
Beta actin RT-PCR reverse primer	CTCCGATCCAGACAGAGTATTTG	
Overgo primer 1/RT forward primer	TATCAGCCTTCACCCTGAACTCAG	932–955
Overgo primer 2	TTGTACACGAATCCGGCTGAGTTC	940–963

Nucleotide positions used the coordinates of the cDNA sequence (accession number AY816351).

Kit (QIAGEN, Valencia, CA). The purified PCR product was cloned into pGEM-T vector (Promega, Madison, WI) and sequenced using an ABI 3100 automated DNA sequencer (ABI, Foster City, CA). Initial analysis indicated production of yet another incomplete cDNA, though extended almost 200 additional base pairs toward the upstream. A second round of 5′-RACE was conducted by designing gene-specific primer 3, GSP3 (Table 1), and amplified by using gene-specific nested primer GSP4. The RACE product was once again cloned into pGEM-T vector and sequenced. In order to obtain a long piece of cDNA to be used as probes for Southern blot hybridization, a new set of PCR primers were designed and RT-PCR conducted.

2.2. Fish tissue sampling and RNA extraction

Channel catfish larvae were reared at the hatchery of the Auburn University Fish Genetics Research Unit. Challenge experiments were conducted as previously described [47] with modifications [11,48]. Eleven tissues were collected from healthy channel catfish including brain, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, stomach, and trunk kidney. Head kidney was collected from challenged fish. Samples were collected from 10 fish at each time point including control (before challenge), and 4, 24 h, 3 days, and 7 days after challenge. Samples of each tissue from 10 fish were pooled. The experimental fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg per liter before tissues were collected. Tissues were quick frozen in liquid nitrogen and kept in a −80 °C ultra-low freezer until preparation of RNA. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powder and thoroughly mixed. A fraction of the tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method [49] using the Trizol reagents kit from Invitrogen following manufacturer's instructions. Extracted RNA was stored in a −80 °C freezer until used as template for reverse transcriptase PCR (RT-PCR).

2.3. RT-PCR

RT-PCR reactions were conducted using Super-Script™ III One Step RT-PCR System (Invitrogen). Detailed procedures followed the instructions of the manufacturer. The reaction included the primers of β-actin (Table 1), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: 45 °C for 15 min for one cycle (reverse transcription reaction), the samples were pre-denatured at 94 °C for 2 min, then the samples were amplified for 40 cycles with 94 °C for 15 s, 45 °C for 30 s, 68 °C for 1 min. Upon the completion of PCR, the reaction was incubated at 68 °C for an additional 5 min. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel and documented with a Gel Documentation System (Nucleotech Corp., San Mateo, CA).

2.4. Assigning BPI gene to BAC clones

An expressed sequence tag (EST) clone containing a partial BPI was identified during EST analysis [50].

The cDNA sequences were used to design overgo probes [51] for hybridization to BAC filters. High-density filters of channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA). Each set of filters contained 10-genome coverage of the channel catfish BAC clones from BAC library CHORI 212 (http://bacpac.chori.org/library. php?id=103). Sequences of the overgo primers are shown in Table 1 and their positions within BPI cDNA are shown in Table 1. Overgo hybridization method was adapted from a web protocol (http://www.tree.caltech.edu/). Briefly, overgo primers were selected following a BLAST search against GenBank to screen out repeated sequences and then purchased from Sigma Genosys (Woodlands, TX). Two hundred nanograms of overgo primers each were labeled with 40 µl of a freshly prepared master mix composed of 14.0 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.02 mM dGTP, 0.02 mM dTTP, 20 µCi [α-^{33}P]dCTP, 20 µCi [α-^{33}P]dATP (3000 Ci/mmol, Amersham, Piscataway, NJ), and 5 units of Klenow enzyme (Invitrogen). Labeling reactions were carried out at room temperature for 2 h. After removal of unincorporated nucleotides using a Sephadex G50 spin column, probes were denatured at 95 °C for 10 min and added to the hybridization tubes containing high-density BAC filters. Hybridization was performed at 54 °C for 18 h in hybridization solution (50 ml of 1% BSA, 1 mM EDTA at pH 8.0, 7% SDS, 0.5 mM sodium phosphate, pH 7.2). BAC filters were washed with 2× SSC at room temperature for 15 min, and exposed to X-ray film at −80 °C for 2 days. Positive clones were identified according to the clone distribution instructions from CHORI, and picked out from the channel catfish BAC library.

2.5. Preparation of BAC DNA and Southern blot analysis

After assigning of BPI to BACs, the putative BPI-positive BAC DNA was isolated with the Perfect-prep® BAC 96 BAC DNA isolation kit (Brinkmann Instruments, Inc., Westbury, NY). The isolated BAC DNA was subjected to Southern blot analysis to determine genomic copy numbers. The rationale was that if the clones represented a single copy, the same hybridization pattern would be expected.

Alternatively, if the clones were from different genomic copies (more than one gene), then variation in the hybridization pattern would be expected, independent of the number of bands. Briefly, BAC DNA was first digested with restriction endonuclease *Eco*RI, *Hin*dIII, and *Pst*I separately, and electrophoresed on a 1% agarose gel. The DNA was transferred to a piece of Immobilon nylon membrane (Millipore, Bedford, MA) by capillary transfer with 0.4 M NaOH overnight. DNA was fixed to the membrane by UV cross-linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) with the auto crosslink function. The membrane was washed in 0.5% SDS (w/v) at 65 °C for 15 min and then pre-hybridized in 50% formamide, 5× SSC [52], 0.1% SDS (w/v), 5× Denhardt's and 100 µg/ml sonicated and denatured Atlantic salmon sperm DNA (100 µg/ml) overnight. Hybridization was conducted overnight at 42 °C in the same solution with probes added. The BPI cDNA was used as the probe. The probe was prepared using the random primer labeling method [52] with a labeling kit from Roche Diagnostics (Indianapolis, IN). The nylon membranes were washed first in 500 ml of 2× SSC for 10 min, followed by three washes in 0.2× SSC with SDS at 0.2% (w/v) at 65 °C for 15 min each. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography.

2.6. DNA sequencing and sequence analysis

Plasmid DNA containing the BPI cDNA from RACE and from RT-PCR was prepared using the alkaline lysis method [52] using the Qiagen Spin Column Mini-plasmid kits (Qiagen, Valencia, CA). Sequencing reactions were conducted as we previously described [45]. Bioinformatic analysis of sequences was conducted by using BLAST and DNASTAR software package [53]. BLAST searches [54–55] were conducted to determine gene identities, and to determine if the cDNA contained a full open reading frame. DNASTAR software package was used for sequence analysis. MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTALX. For the analysis of potential cleavage site(s) of the signal peptide, SignalP (http://www.cbs.dtu.dk/services/SignalP/) software was used [56].

2.7. Phylogenetic analysis

The relevant sequences were retrieved from GenBank for multiple sequence alignments using CLUSTALX [57]. Percentage of amino acid identities were recorded after all multiple alignments. Phylogenetic trees were drawn by the neighbor-joining method [58] in PAUP (version 4b) [59] using amino acid sequence p-distances. The topological stability of the neighbor joining trees was evaluated by 10,000 bootstrapping replications.

2.8. Quantitative real time PCR (QRT-PCR)

Total RNA was used for QRT-PCR using a LightCycler (Roche Applied Science, Indianapolis, IN). Concentration and quality of total RNA was determined by spectrophotometry (optical density 260/280 ratio) and electrophoresis. Primers used in QRT-PCR are shown in Table 1. β-Actin was used as an internal control for real time RT-PCR. A standard curve was constructed by using various copy numbers of a plasmid containing BPI cDNA. QRT-PCR reactions of the standard curves were included in all runs in order to provide quantitative data. One-step QRT-PCR was carried out in the LightCycler using a Fast Start RNA Master SYBR Green I reagents kit (Roche Applied Science) following manufacturer's instructions with modifications as we previously described [11].

3. Results

3.1. Sequence analysis of BPI from channel catfish

The BPI cDNA was completely sequenced and its sequences have been deposited to GenBank with an accession number of AY816351. The channel catfish BPI cDNA has an open reading frame of 1428 bp encoding a protein of 475 amino acids. It contains a 5′-untranslated region (UTR) of 97 bp and a 3′-UTR of 115 bp. A typical poly $(A)^+$ signal AATAAA existed 12 bp upstream of poly A tails. However, no ATTTA sequences were found from the 3′-UTR, a motif identified from a number of inflammatory mediators such as cytokines [60–62,42].

The channel catfish BPI sequences were highly conserved with BPI sequences from other species (Fig. 1). It was most similar to the carp BPI with 72.9% amino acid identity, followed by rainbow trout BPI-1 (69.1%), rainbow trout BPI-2 (68.3%), Atlantic Cod A (57.1%) and Atlantic Cod B (57.1%). The teleost BPI genes were all similar sharing 57–73% similarities. The fish BPI sequences shared 32–37% amino acid identities with the mammalian BPI sequences (Table 2). Analysis of cleavage sites of the signal peptide using the SignalP program indicated the presence of a cleavage site between amino acids 20 and 21 (Fig. 1). This is similar to the cleavage sites in the rainbow trout and carp BPI genes (between amino acids 18 and 19). The LPS-binding domain, spanning a region of 65 amino acids, found in other organisms is well conserved in the catfish BPI gene (Fig. 1).

The channel catfish BPI cDNA was initially identified from expressed sequence tags (ESTs). BLAST searches provided a putative identity to an EST clone, AUF_IpHdk_44_g06, as being most similar to bactericidal permeability increasing protein. However, BLAST searches indicated that the cDNA was incomplete. The cDNA clone harbored only 882 bp, missing almost 220 amino acids or 660 bp as compared to the carp BPI sequences. The 5′-RACE was conducted to obtain the complete BPI cDNA. The first round of 5′-RACE produced a product of approximately 750 bp, predicted to be still incomplete. Sequencing analysis confirmed that the RACE product was still incomplete missing about 370 bp to the protein initiation codon ATG. The second round of RACE was conducted and the RACE product was sequenced. Sequences confirmed the amplified products being BPI-related sequences containing the translation initiation codon AUG. However, sequencing analysis of the RACE product indicated a deletion of 79 bp in the middle of the sequences. Therefore, RT-PCR was conducted to determine if the RACE product represented the complete BPI transcript, or part of it was deleted due to an artifact. Sequencing of the RT-PCR product revealed that the 79-bp catfish BPI sequences was, for some reason (see Section 4), skipped during the RACE procedure.

Signal peptide   **LPS-binding domain**

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  catfish  ------MVSLWCVLALLSFFSPEASGTNPGVILRVTQKGLEYGRQIGLVTLQKKLKTVKIPDMSGSEKVSPIGKVSYSLTGIQI    78
    cod_a  --------MFPWRWLALLALIP-LALSANAGVKVKLTQKGLDFGRELAVASLQKKLMTIKVPDISGKERVKPIGKVRYSVTEMRV    76
    cod_b  --------MFPWRWLALLALIP-LALSANAGVKVKLTQKGLDFGRELAVASLQKKLMTIKVPDISGKERVKPIGKVRYSVTEMRV    76
     carp  --------MFLWCVLVLLNLVS-VATGTNAGVKVRLTQKGLEYGRQIGIASIQQKLRTIKVPDISGTEKVDPIGKVQYSFTGMQI    76
  trout_1  --------MSPCCWLALLALVP-FALATNVGVKVKLTEKGIEYGKQIGMASLQQKLKTMKVPDLSGTERVAPIGKVKYSLTGITI    76
  trout_2  --------MSPCCLLALLAVIS-LTLAASPGVKVKLTDKGIEYGKQIGMASLQQKLKTMKVPDLSGTEKVPPIGKVKYSLTGMTI    76
bovine_BPI  MARGPDTARRWATLVVLAALGTAVTT-TNPGIVARITQKGLDYACQQGVLTLQKELEKITIPNFSGNFKIKYLGKGQYSFFSMVI    84
 human_BPI  MARGPCNAPRWVSLMVLVAIGTAVTAAVNPGVVVRISQKGLDYASQQGTAALQKELKRIKIPDYSDSFKIKHLGKGHYSFYSMDI    85
  RAT_LBP  --MKSATGPLLPTLLGLLLLSIPRTQGVNPAMVVRITDKGLEYAAKEGLLSLQRELYKITLPDFSGDFKIKAVGRGQYEFHSLEI    83
mouse_LBP  --MKAGTGPLLSTLLGLLFLSIQGTGGVNPGVVARITDKGLAYAAKEGLVALQRELYKITLPDFSGDFKIKAVGRGQYEFHSLEI    83
human_LBP  --MGALARALPSILLALLLTSTPEALGANPGLVARITDKGLQYAAQEGLLALQSELLRITLPDFTGDLRIPHVGRGRYEFHSLNI    83
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LPS-binding domain

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  catfish  LDLGLPKSAVGLVPGTGVSLSIGDAYINLHGNWRVKYLRIIKDSGSFDLSVSGLSISATISVKGDD-TGRPVVSSANCAASVGNV   162
    cod_a  VKLGLPSSAIGLVPGTGFSLTITNAFLSLHGRWRVKYLRFIKDRGSFDLAIKSLSITTSISLRSDN-MGLPAVAMASCTTTLGGV   160
    cod_b  VKLGLPSSAIGLVPGTGFSLTITNAFLSLHGRWRVKYLRFIKDRGSFDLAIKSLSITTSISLRSDN-MGLPAVAMASCTTTLGGV   160
     carp  VNLGLPKSVLVPDTGVMLSIGNAYINLHGNWRVKYLRIIKDSGSFDLAVSELTISTTVAVMSDD-TGHPTVSMTNCAATVGSV   160
  trout_1  VNLGLPYSALALVPDTGISLSITNAFISLHGNWKIRYLSFIKDSGSFDLEVDGLTVTDSITIKSDE-TGRPTVSSVNCVANVGSA   160
  trout_2  VNLGLPKSALVLMPGTGVRLAITNAFINLHGNWRVRYFRFIQDRGSFDLAVNGLTITADIAIKSDE-TGRPTVSTVNCVANVGSA   160
bovine_BPI  QGFNLPNSQIRPLPDKGLDLSIRDSSIKIRGKWKARKN-FIKLGGNFDLSVEGISILAGLNLGYDPASGHSTVTCSSCSSGINTV   168
 human_BPI  REFQLPSSQISMVPNVGLKFSISNANIKISGKWKAQKR-FLKMSGNFDLSIEGMSISADLKLGSNPTSGKPTITCSSCSSHINSV   169
  RAT_LBP  QSCQLRGSSLKPLPGRGLSLSISDSSISVRGKWKVRRS-FVKLHGSFDLDVKSVTISVDLLLGVDP-SERPTVTASGCSNRIRDL   166
mouse_LBP  QNCELRGSSLKLLPGQGLSLAISDSSIGVRGKWKVKRS-FLKLHGSFDLDVKGVTISVDLLLGMDP-SGRPTVSASGCSSRICDL   166
human_LBP  HSCELLHSALRPVPGQGLSLSISDSSIRVQGRWKVRKS-FFKLQGSFDVSVKGISISVNLLLGSES-SGRPTVTASSCSSDIADV   166
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  catfish  KIKFHG-GASWLYNLFKHYIEKALRSELQKQICSLVAEAIE-EMNPHLKTLNVLAQVDKYAEIEYSMVESPLMSNSSIDLSLKGE   245
    cod_a  SVKLHG-GASWLYNLFRRFIEKGLQSQLKKLCPLVAESVS-SMNQFLKTVKVMAPVDRYAEIAYPMVSSPDISSSSIGLNLKGE   243
    cod_b  SVKLHG-GASWLYNLFRRFIEKGLQSQLKKLCPLVAESVS-SMNQFLKTVNVMAPVDRYAEIAYPMVSSPDISSSSIGLNLKGE   243
     carp  NVKFHG-GASWLYNLFSSFINKALRNALQKQICPLVADSIA-DINPHLKTLNVLAKVDQYAEIEYSMVGSPVISNTSIDLGLKGE   243
  trout_1  SIKFHG-GASWLYNLFSAYIDKALRSALQKQICPLVADTIT-DMNPHLKTLNVLAKVDKYAEVEYSMVTSPTISNASIDFSLKGE   243
  trout_2  SIKFHG-GASWLYNLFKSYIDKALRSALQKQICPLVADVIT-DMNPHLKTFNVLAKVDQYAEIEYSMVTSPTISKSSIEFSLKGE   243
bovine_BPI  RIHISGSSLGWLIQLFRKRIESLLQKSMTRKICEVVTSTVSSKLQPYFQTLPVTTKLDKVAGVDYSLVAPPRATANNLDWLLKGE   253
 human_BPI  HVHISKSKVGWLIQLFHKKIESALRNKMNSQVCEKVTNSVSSKLQPYFQTLPVMTKIDSVAGINYGLVAPPATTAETLDVQMKGE   254
  RAT_LBP  ELHVSG-NVGWLLNLFHNQIESKLQKVLESKICEMIQKSVTSDLQPYLQTLPVTADIDTIILGIDYSLVAAPQAKAQTLDVMFKGE   250
mouse_LBP  DVHISG-NVGWLLNLFHNQIESKLQKVLENKVCEMIQKSVTSDLQPYLQTLPVTAEIDNVLGIDYSLVAAPQAKAQVLDVMFKGE   250
human_LBP  EVDMSG-DLGWLLNLFHNQIESKFQKVLESRICEMIQKSVSSDLQPYLQTLPVTTEIDSFADIDYSLVEAPRATAQMLEVMFKGE   250
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  catfish  FYNIGQHKEPPFSPTPFSPPAQDSNMMYIGLSAFTLNSAGFVYNNAGVLSLYITDDMIPSSFPIRLNTKTFGTFIPQIAKQYPGL   330
    cod_a  FYNIGNHMEPPFSPAPFFLPNQEQSMLYIGLSAFTANSASFVYNKAGTLSLKITDDMVPRSSPIRLTTNTFGVLIPQIAKLFPGL   328
    cod_b  FYNIGNHMEPPFSPAPFFLPNQEQSMLYIGLSAFTANSASFVYNKAGTLSLKITDDMVPRSSPIRLTTNTFGVLIPQIAKLFPGL   328
     carp  FYNIGQHKEPPFSPTPFSLPSQDTDMLYIGVSAFTINSAGFVYNRAGALSLYITDDMIPSGSPIRLNTKTFGAFIPQIEKMYPGL   328
  trout_1  FYNIGKHQEPPFSPTPFSLPPQVNNMLYIGMSAFTTNSAGFVYNNAGALSLYITDDMIPPSSPIRLNTRTFGAFIPEIAKRFPSM   328
  trout_2  FYNIGKHQEPPFSPTPFSLPPQDNNMLYIGVSSFTPNSAGFSLYVTDDMIPPSSPIRLNTGTFGVFIPEIAKRFPGM   328
bovine_BPI  FFSLAHRSPPPFAPPALAFPSDHDRMVYLGISEYFFNTAGFVYQKAGALNLTLRDDMIPKESKFRLTTKFFGILIPQVAKMFPDM   338
 human_BPI  FYSENHHNPPPFAPPVMEFPAAHDRMVYLGLSDYFFNTAGLVYQEAGVLKMTLRDDMIPKESKFRLTTKFFGTFLPEVAKKFPNM   339
  RAT_LBP  IFNRNHRSPVTTPTPTMSLPEDSKQMVYFAISDQAFNIATRVYHQAGYLNFTITDDMLPPDSNIRLNTKAFRPFTPLITRKYPDM   335
mouse_LBP  IFNRNHRSPVATPTPTMSLPEDSKQMVYFSISDHAFNIASRVYHQAGYLNFSITDDMLPPDSSIRLNTKAFRPFTPQIYKKYPDM   335
human_LBP  IFHRNHRSPVTLLAAVMSLPEEHNKMVYFAISDYVFNTASLVYHEEGYLNFSITDDMIPPDSNIRLTTKSFRPFVPRLARLYPNM   335
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  catfish  MMKLLVKAEKEPNVSFEPDNVTLQASSTVTAYAIQPNATLSPLFVLNVNASVSARIYVSGLTVAGNLSLDEMDMTLATSYVGPFQ   415
    cod_a  MMVLQLKMTKDPLSSIEANKVTVDAKSSLTAYAILANTKLAPLFVINLDFSVSARVNVVGNKLAGAVTLNRLDMSLGKSYVGDFK   413
    cod_b  MMVLQLKMTNDPLSSIEANKVTVDAKSSLTAYAILANTKLAPLFVINLDFSVSARVNVVGNKLAGAVTLNRLDMSLGKSYVGDFK   413
     carp  MMKLLVETVKEPIVTFEPNNMTVQASSTVTAYAIQPNSTLSPLFVNLEVSVSTHIYVTELKLAGNVTLNKINMSLAKSYVGPFQ   413
  trout_1  MMKLLVKTVKEPTIFFEPNNVTVQASGSVTAYAIQPNTTLSPLFVINMEGSVSARLYVTGVRLAGAVTLNKIEMTLETSYVGQFQ   413
  trout_2  MMKLLVKTVKEPTISLEPNNVTVQASSVTSSFTPNSAGFVTLSPLFVINMEGSVSAQMNVTGVKLAGAITLNKIEMTLGTSYVGQFQ   413
bovine_BPI  QMQLFIWASLPPKLTMKPSSLDLIFVLDTQAFAILPNSSLDPLFLLEMNLNLSVVVGAKSDRLIGELRLDKLLLELKHSDIGPFS   423
 human_BPI  KIQIHVSASTPPHLSVQPTGLTFYPAVDVQAFAVLPNSSLASLFLIGMHTTGSMEVSAESNRLVGELKLDRLLLELKHSNIGPFP   424
  RAT_LBP  NLELLGTVVSAPLLNVSPGNLSLAPQMEIEGFVILPSSARESVFRLGVVTNVFVSLTFDNSKVTGMLHPEKAQVRLIESKVGMFN   420
mouse_LBP  KLELLGTVVSAPILNVSPGNLSLAPQMEIEGFVILPTSAREPVFRLGVVTNVFASLTFNNSKVTGMLHPDKAQVRLIESKVGMFN   420
human_LBP  NLELQGSVPSAPLLNFSPGNLSVDPYMEIDAFVLLPSSSKEPVFRLSVATNVSATLTFNTSKITGFLKPGKVKVELKESKVGLFN   420
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  catfish  VKSLDSFLTMIMKAVVIPVVNALLRQGYPLPAIGNMNLVNTQLQILKDYVLIGTDVQFTG-   475
    cod_a  VGSLSNIFQIVLKWVVLPTVNVRLKEGFPLPSLGKINLKNTQLQILKGYIMIGTDLQNSG-   473
    cod_b  VGSLSNIFQIVLKWVVLPTVNVRLKEGFPLPSLGKINLKNTQLQILKGYIMIGTDLQNSG-   473
     carp  VTSLDNIFTIVLKFAVIPKVNARLQEGYPLPAIGKMQLVNSQLKVLKDYLLIGTDVQFTG-   473
  trout_1  VRSLDNIFLMVLKVAVIPKVNARLEKGFPLPSIGKMNLVNTQLQVLKDYMLIGTDVQFTG-   473
  trout_2  VQSLDNIFLMVLKVVVIPKVNARLEKGFPLPTIGKMNLINTQLQVLKDYMLIGTDVQFTG-   473
bovine_BPI  VESLQSVINYVMPTIVLPVINKKLQKGFPLPAYIELFNLTLQPYQDFLLFGADVQYS--   482
 human_BPI  VELLQDIMNYIVPILVLPRVNEKLQKGFPLPTPARVQLYNVVLQPHQNFLLFGADVVYK--   483
  RAT_LBP  VNLFQAFLNYYLINSLYPDVNDELAKGFPLPLPRRIKLHDLDFQIHKNFLYLGANVQYMRV   481
mouse_LBP  VNLFQAFLNYYLINSLYPDVNAELAQGFPLPLPRHIQLHDLDFQIRKDFLYLGANVQYMRV   481
human_LBP  AELLEALLNYYILNTLYPKFNDKLAEGFPLPLLKRVQLYDLGLQIHKDFLFLGANVQYMRV   481
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Fig. 1. ClustalX alignment of amino acid sequences of BPI and LBP. The line above the alignment indicates strongly conserved positions: '*' indicates a conserved residue, ':' indicate a conserved strong residue and '.' indicate a conserved weak residue, according to the Gonnet protein weight matrix. Predicted signal peptide and LPS-binding domain are shown.

Table 2
Pairwise similarities of selected BPI/LBP proteins

	Channel catfish	Carp	Trout	Trout BPI2	Atlantic Cod A	Atlantic Cod B	Bovine	Human	Mouse LBP	Rat LBP	Human LBP
Channel catfish	***	72.9	69.1	68.3	57.1	57.1	37.1	35.4	33.9	32.0	32.8
Carp		***	73.6	73.2	61.3	61.3	36.6	35.1	33.4	32.1	32.1
Trout			***	86.7	61.7	61.7	35.7	33.8	31.7	31.9	32.3
Trout BPI2				***	62.2	62.2	34.2	34.0	32.8	32.1	31.7
Atlantic Cod A					***	99.6	34.9	34.0	28.8	29.2	31.7
Atlantic Cod B						***	34.9	34.0	28.8	29.2	31.7
Bovine							***	63.9	44.1	43.2	44.5
Human								***	41.0	40.5	43.0
Mouse LBP									***	86.3	66.7
Rat LBP										***	67.4
Human LBP											***

3.2. Phylogenetic analysis of BPI sequences

Phylogenetic analysis of all published BPI/LBP sequences indicated that the BPI and LBP gene duplications were recent events. While all the mammalian BPI and LBP sequences clustered into distinct clades, demonstrating separate orthologies, the majority of fish BPI/LBP sequences clustered based on the species of origin, demonstrating paralogies (Fig. 2). However, two BPI-related sequences from fugu were placed in distinct clades. It is not clear at this time if the cloned BPI/LBP genes from fish are truly orthologues for BPI or for LBP.

BPI and LBP are highly related. In the human genome, BPI and LBP are both on chromosome 20 in the same location of 20q11.23-q12. The chromosome region also includes LOC388796, KIAA1755, and TGM2, with all three genes transcribed from the opposite strand of DNA as compared to BPI and LBP. Our initial attempts to establish syntenies in this chromosomal region failed to find the similar genes included in the contig of fugu containing its BPI gene. However, BLAST searches of the fugu genome with the channel catfish BPI sequences revealed presence of two types of homologous sequences, the first type is highly similar to the catfish BPI located in *Fugu rubripes* whole genome shotgun assembly SCAF-FOLD_65 (accession number CAAB01000065). The second type of sequences is less similar to the catfish BPI sequences located in the *Fugu rubripes* whole genome shotgun assembly SCAFFOLD_1448 (accession number CAAB01001448.1). Subsequently, part of the sequences of SCAFFOLD_1448 containing

the second BPI-related genes was used to search NR databases. The most homologous sequence was identified from *Tetraodon nigroviridis*. Phylogenetic analysis of all BPI-related sequences indicated the presence of a very distinct BPI-related gene in the Fugu genome that is highly related to two sequences from *Tetraodon nigroviridis*, CAG03233 and CR731335. These three sequences belong to a distinct clade that is more distant to any other fish BPI than to the mammalian BPI or LBP (Fig. 2).

3.3. Assigning BPI to BACs and copy number of channel catfish BPI gene

Initial analysis using Southern blot hybridization indicated the presence of more than one band with restriction endonuclease digestion using *Eco*RI, *Hin*dIII, and *Pst*I (data not shown). Conventionally, this would have been interpreted as presence of more than one gene copy in the catfish genome. However, a search of the human genome sequence revealed that the locus of BPI is large in size because of the presence of many large introns. It has 15 exons and 14 introns spanning over 31.5 kb. Similarly, the highly conserved human LBP gene also included many introns [63]. The large size of the BPI/LBP genes provided the possibility that most restriction enzymes would have recognition sites within the gene, resulting in presence of multiple bands after restriction enzyme digestion. Therefore, we took a BAC-based approach to gene copy number determination [64].
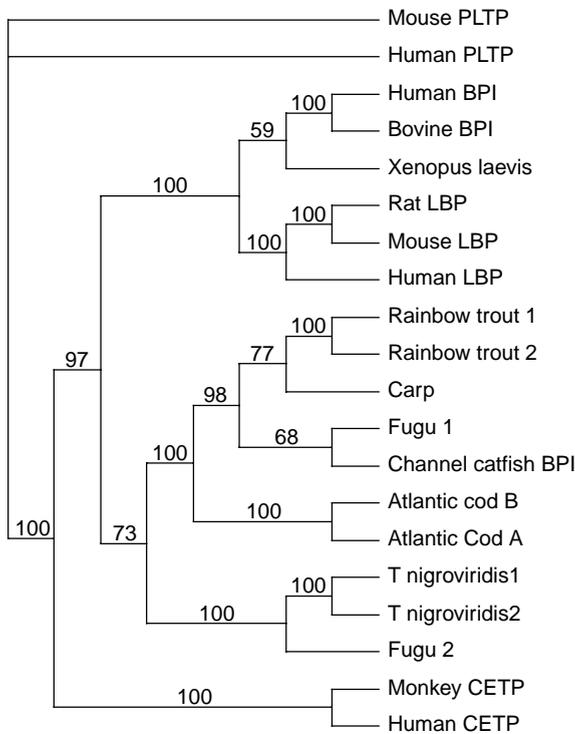
Fig. 2. Phylogenetic analysis of channel catfish BPI gene with lipid-interactive protein genes from other vertebrates. Phylogenetic tree is constructed with ClustalX and PAUP by NJ method. Bootstrap values represent the frequency (percent) of appearance of each clade in 10,000 bootstrap replicates. Accession numbers: *Xenopus laevis*(MGC84153), *T. nigroviridis* 1(CR731335), *T. nigroviridis* 2(CAG03233), Rat LBP(NP_058904), Rainbow trout 1(AB042025), Rainbow trout 2(AB042026), Mouse PLTP(AAH03782), Mouse LBP(NP_032515), Monkey CETP(P47896), Human PLTP(AAH19847), Human LBP(NP_004130), Human CETP(AAA51978), Human BPI(AAG42844), Fugu 1(CAAB01000065), Fugu 2(CAAB01001448), Carp(AU279378), Bovine BPI(NP_776320), Atlantic Cod A(AAM52335), Atlantic Cod B(AAM52336), Channel catfish BPI(AY816351).

First, overgo probes were used to assign BPI gene to BAC clones. Hybridization of the BPI overgo probes resulted in identification of 10 positive BAC clones, and subsequent Southern blot analysis confirmed nine BPI containing BAC clones (Table 3). Analysis of hybridization using high-density BAC

Table 3
Positive clones of BPI in BAC library CHORI 212

BPI positive BAC clones	5_J4, 13_K5, 17_C13, 30_J23, 77_M19, 104_A4, 107_P23, 126_H3, 174_H2

filters allowed initial estimation of gene copy numbers. If the BPI gene is present in multiple copies, then the number of clones positive to the BPI probe should be significantly larger than the genome coverage on the BAC filters (10×). Alternatively, if only a single copy exists in the channel catfish genome, then the expected positive clones should be approximately equivalent to the genome coverage on the BAC filters (10×). If multiple copies exist in the genome in a tandem arrangement in the same BAC clones, then the hybridization signal should be proportional to the genome copies. In this case, the analysis of BAC filters with 10× genome coverage resulted in nine positive clones, indicating the presence of a single gene copy.

In order to determine the copy numbers, BAC DNA from the positive clones was isolated and subjected to Southern blot analysis after digestion with three restriction enzymes, *Eco*RI, *Hin*dIII, and *Pst*I. All three restriction enzymes resulted in a single restriction pattern. Restriction digestion using *Eco*RI produced four bands (Fig. 3A); restriction digest with *Hin*dIII produced two bands (Fig. 3B), while restriction digest using *Pst*I produced multiple bands (not shown). Clearly, a single restriction pattern was produced regardless of the restriction enzyme used, demonstrating the presence of only one locus of BPI in the catfish genome. Because the hybridization intensity is comparable to the signals from those of single copy genes, the catfish genome contains only a single copy of BPI gene.

3.4. Expression of BPI in normal channel catfish tissues

RT-PCR analysis using total RNA from various normal tissues of channel catfish indicated that the BPI gene was expressed in a wide range of tissues. It was detected in all tested tissues including brain, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, stomach, and trunk kidney. It was expressed highly in most tissues, but its expression appeared to be slightly lower in the brain and muscle (Fig. 4).

The channel catfish BPI gene was expressed early during development. Although no BPI RNA was detected in fertilized egg, 8 or 24 h after fertilization, its expression was detected at 48 h after fertilization,
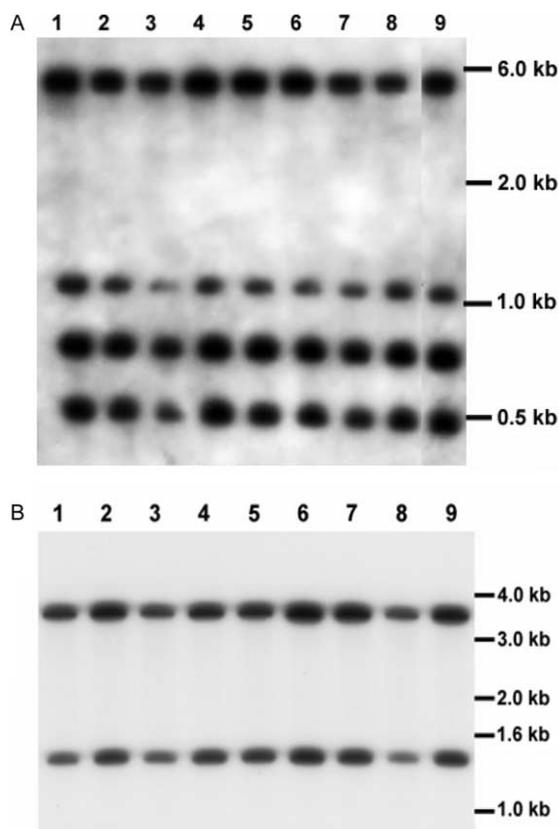
Fig. 3. Southern blot analysis of channel catfish BPI containing BAC DNA hybridized with a BPI probe. The probe spans almost the whole coding region. Digestions were performed with *Eco*RI(A), *Hin*dIII(B). Lanes 1–9 were individual BAC DNA digested with restriction endonuclease as specified. Molecular weight standards are indicated on the right margin.
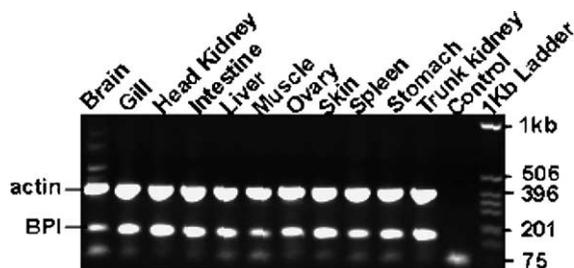
Fig. 4. Expression analysis of the channel catfish BPI gene in various tissues by RT-PCR. Tissues are indicated on the top of the gel. The RT-PCR products of BPI gene are 193 bp, β-actin RT-PCR products (396 bp) serve as an internal control. Molecular weight standards with the sizes indicated on the right margin in base pairs (bp).

Fig. 5. Expression analysis of the channel catfish BPI gene in developmental samples by RT-PCR. Developmental samples were from fertilized egg to 14 day post hatching larvae. The RT-PCR products of BPI gene are 193 bp, β-actin RT-PCR products (396 bp) serve as an internal control. MW, molecular weight standards with the sizes indicated on the right margin in base pairs (bp). Note that β-actin transcripts were not detected in fertilized eggs, suggesting its absence from the eggs; the samples were taken immediately after fertilization of the eggs perhaps before the onset of β-actin transcription.

after which its expression was sustained at high levels (Fig. 5). Like several other genes related to the innate immune system [65], BPI expression appeared to be low at the time of hatching (5 days after fertilization, Fig. 5).

3.5. The channel catfish BPI gene expression was induced by bacterial infection

In order to assess potential roles of BPI during bacterial infection, RT-PCR was conducted using RNA isolated from head kidney tissues of channel catfish at several time points after bacterial challenge. As shown in Fig. 6, BPI expression was significantly increased after challenge with *E. ictaluri*, the causative agent of enteric septicemia of catfish (ESC). The BPI expression was highly induced 3 days after challenge. At 7 days after challenge, the expression was still higher than base level expression without infection, but was not as high as the expression 3 days after challenge (Fig. 6). This pattern of expression profiles after ESC challenge mirrors those of many genes involved in the innate immune responses of catfish such as chemokines [11, 48], and several other antimicrobial peptides [65], suggesting its functions in the inflammatory response to infection with *E. ictaluri*.

4. Discussion

In this study, we have characterized a BPI-like gene from channel catfish. The gene encodes a protein
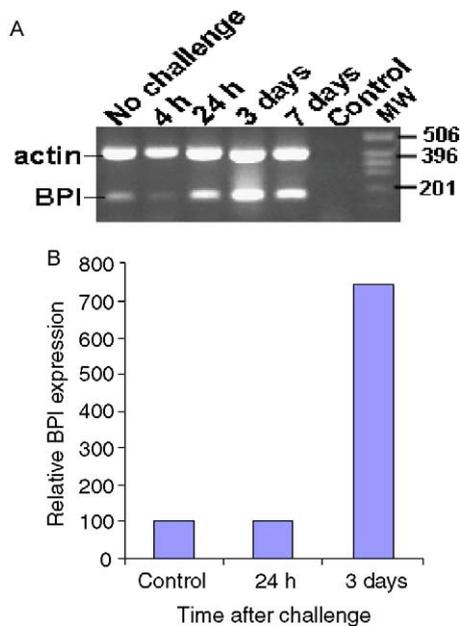
Fig. 6. (A) Expression analysis of the channel catfish BPI gene in head kidney of channel catfish infected with ESC. The time points of tissue collection were 4, 24 h, 3 and 7 day. The RT-PCR products of BPI gene are 193 bp, β-actin RT-PCR products (396 bp) serve as an internal control. MW, molecular weight standards with the sizes indicated on the right margin in base pairs (bp). (B) Changes in BPI mRNA levels in head kidney of channel catfish as analyzed by QRT-PCR. Data is expressed as the ratio of BPI RNA to β-actin RNA at various times: control, 24 h and 3 days after challenge.

of 475 amino acids that is similar to the carp BPI/LBP proteins. The catfish BPI exhibited at least 57% amino acid identities to BPI isolated from teleost fish. A single copy of the BPI gene exists in the catfish genome. The BPI gene is constitutively expressed in a wide range of tissues, and expressed early during development (Fig. 5). The catfish BPI gene was readily induced by challenge with *E. ictaluri*, a Gram-negative bacteria causing ESC disease. The induced expression profile mirrors those of other genes involved in the innate immune system such as chemokines and other antimicrobial peptides, suggesting its functions in the inflammatory response.

The catfish BPI RNA may form strong secondary structures in solution. Our large-scale EST analysis using high quality cDNA libraries [43–46] produced only a partial cDNA sequence. The first round of 5′-RACE extended another almost 200 bp, but still ended with a partial cDNA. When the second round

RACE was conducted, though the extension reaction resulted in production of a RACE-PCR product containing the complete 5′ end of the mRNA of BPI, a 79-bp segment was skipped during RACE. The entire cDNA was finally obtained by RT-PCR using primers designed from the upstream and downstream of the cDNA. An analysis using GeneBee software package (http://www.genebee.msu.su/services/rna2_reduced.html) for prediction of RNA secondary structures revealed a strong secondary structure at the region where the segment was skipped during RACE (Fig. 7), suggesting that the secondary structure existed in BPI RNA.

The catfish genome contains only a single copy of BPI, in contrast to two copies of LBP/BPI genes found in rainbow trout. However, it is widely believed that the salmonid fishes went through a process of tetraploidization [65,66]. A tBLASTn search of the draft zebrafish genome (http://www.ensembl.org/Danio_rerio/) indicated that zebrafish BPI gene is also present as a single copy gene on chromosome 3. We used a BAC-based approach for the analysis of copy numbers. This procedure, referred to as BAC-based Southern blot, provided much more accurate determination of copy numbers. Genomic Southern analysis relies on the assumption that most 6-bp cutters do not have recognition sites within genes and therefore, multiple bands should be produced when more than one copy exist in the genome. However, that assumption is problematic with genes of large sizes. When a gene spans for tens of kilo base pairs, it is likely that most 6-bp cutters have sites within the gene and should result in multiple bands. However, with the BAC-based Southern analysis, the number of bands is only a secondary consideration. Instead, the number of restriction patterns defines the number of genes. With the exception of instances when the gene segment is near the end of the insert, the same restriction pattern is expected for all clones. When a large number of BAC clones are used, e.g. 10 BACs, the chances of obtaining only one copy of the gene when multiple copies exist in the genome is small.

Our results using RT-PCR analysis indicated that BPI is expressed in most tissues although levels of expression appeared to be lower in the muscle and brain. This expression pattern is similar to what was found in carp [42], but were different from what was found from Atlantic cod [41]. In spite of detection of
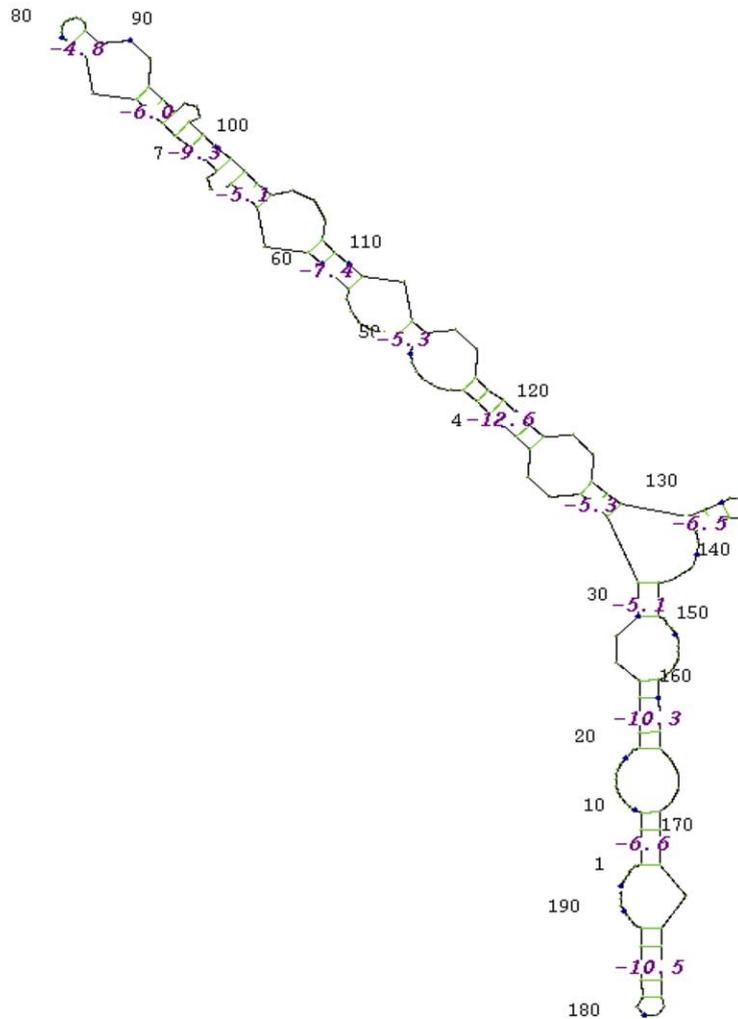
Fig. 7. Analysis of secondary structure of BPI RNA using GeneBee software, the segment from 70 to 148 bp in the figure was skipped during RACE.

BPI transcripts in many tissues in Atlantic cod, its RNA was very low in the brain, intestine, skin, and gill [41]. We believe that gene expression patterns could be different between the cold water ocean fish as compared to catfish or carp. In addition, the method of analysis could have contributed to some of the differences. In Atlantic cod, gene expression was analyzed by Northern blot hybridization that is not as sensitive as RT-PCR as conducted in catfish here and in carp [42]. Nonetheless, in all three cases, BPI

was expressed highest in head kidney, an organ in teleost believed to be equivalent to the mammalian bone marrow [67]. BPI was also expressed in the skin and intestine, suggesting it is expressed in epithelial cells as well. We do not believe the detected BPI expression was from plasma contamination in other tissues because plasma contamination was minimal, if not none, in the skin samples where BPI was found to be quite high. Expression of BPI in many tissues could suggest additional functions other than just

immune-related functions [24]. Future functional analysis of BPI in teleosts would provide an answer to this speculation.

The most interesting observation was the induced expression of BPI after bacterial infection as demonstrated by RT-PCR and QRT-PCR. The brief time course study indicated that induced gene expression of BPI peaked at day 3 after challenge. Although the exact functions of BPI during bacterial infection is currently unknown, its enhanced expression and the timing of its upregulation suggest a significant role in host defense. As BPI has been well characterized as an antimicrobial peptide through the mechanism of LPS neutralization, its upregulation after challenge with ESC bacteria could reflect its function in 'braking' the rapid inflammatory responses elicited by ESC infection.

Acknowledgements

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