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Biochemical and molecular identification of a novel hepcidin type 2-like antimicrobial peptide in the skin mucus of the pufferfish *Takifugu pardalis*

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ABSTRACT

Fish skin mucus is considered to act as the first line of defense against waterborne pathogens and to be potential source of novel antimicrobial components. Here we report the purification and characterization of a novel hepcidin type 2-like antimicrobial peptide (*Tp*HAMP2) from the skin mucus of the pufferfish *Takifugu pardalis*. The purified *Tp*HAMP2 comprised of 23 amino acids (AAs) with eight Cys residues that form four intramolecular disulfide bonds. The *Tp*HAMP2 gene shared overall structural characteristics with all known hepcidins, which have a tripartite exon-intron gene organization and three structural signatures in the precursor protein. Phylogenetically, *Tp*HAMP2 was classified as HAMP2 class in acanthopterygian fish. Interestingly, the AA sequence of *Tp*HAMP2 did not contain a proprotein cleavage site (RXXR motif) that conserved in most hepcidins and showed a highly positive charged (RKR-) short N-terminus and Val^{18} and Gly^{22} residues, which are distinctive structures compared to other known active hepcidins. Recombinant *Tp*HAMP2 identical to the native form exhibited a broad spectrum and potent antimicrobial activity against tested gram-positive and -negative bacteria. Expression of *Tp*HAMP2 mRNA was predominant in the liver and was upregulated in the liver, the spleen, the intestine, and the skin of *T. pardalis* post immune challenge. Thus, our findings suggests that *Tp*HAMP2 might be of importance in the framework of discovering the fish hepcidins, especially type 2s, and provide noteworthy insight into its gene structure and expression and in the innate immunity as well as the mucosal immunity in regard to hepcidins' evolutionary history in fish species.

1. Introduction

Antimicrobial peptides (AMPs) are crucial components of the innate immune system and display a broad-spectrum of antimicrobial activity *in vitro* and *in vivo* against bacteria, fungi, yeast, protozoa, and viruses [1,2]. These molecules share common features, such as small size (typically less than 100 amino acids, AAs) with cationic and hydrophobic properties within a linear or cyclic structure, while displaying considerable diversity in their AA sequence. The sequence diversity of AMP families might have arisen from co-evolutionary arm race between host and pathogens, which might be manifested in neo- or sub-functionalization processes, and enabled the host to survive in different microbial environments, such as high concentration of potential pathogens, as observed in various aquatic conditions [3,4]. Thus, it is not surprising that the aquatic environment in which fish live necessitates the presence of a more diverse and potent suite of AMPs.

Mucosal surfaces of fish are continuously in intimate contact with an aquatic environment harboring waterborne pathogens. Therefore, the mucosal surfaces of fish play a crucial role in the first line of defense system against invading pathogens that could enter their body through mucosal surfaces of the skin, the gill, the intestine, and the urogenital system [5–7]. Among these surface, the skin mucus, which is secreted by goblet cells and is considered one of the distinctive features in fish, contains a variety of immune-relevant factors including lectins, lysozymes, calmodulin, immunoglobulins, complement, C-reactive proteins, proteolytic enzymes, and AMPs [8] and provides fish with the

Abbreviations: AMPs, antimicrobial peptides; CFU, colony forming unit; MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; ORF, open reading frame; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-qPCR, real-time quantitative PCR; UTR, untranslated region

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first line of protection against waterborne pathogens. Fish skin mucus could be easily obtained by gently scraping the skin surface, avoiding a contamination of other tissues, which would provide nonlethal alternatives for both detecting and monitoring the pathogenic bacteria infection in fish.

So far, several AMPs have been identified from fish skin mucus using biochemical purification and antimicrobial activity as a screening method; pleurocidin from winter flounder, *Pleuronectes americanus* [9], parasin 1 from *Parasilurus asotus* [10], hipposin from *Hippoglossus hippoglossus* [11], oncorhyncin II and III from *Oncorhynchus mykiss* [12,13], SAMP-H1 from *Salmo salar* [14], histone H2B and ribosomal protein L40, L36A, and L35 from *Gadus morhua* [15], myxinidin from *Myxine glutinosa L.* [16], and pelteobagrin from *Pelteobagrus fulvidraco* [17]. These biochemical purification of AMPs have allowed the identification of even more peptides in skin or skin mucus as the increased use of omics techniques [8,18]. The structural characteristics of AMPs from skin mucus appear to have a high degree similarity to their homologues in terrestrial animals, nonetheless, these AMPs showed a broad spectrum of activity that was approximately 10–100 times more potent than that of the homologues derived from terrestrial animals against various fish and human pathogens [10,19]. Moreover, unlike most AMPs sensitive to high salt concentration, some of the fish mucus AMPs retained its activity even at extremely high salt concentrations such as those found in the marine environment [9,20,21]. These suggest that fish skin mucus is a rich source of potent AMPs that are good potential targets for development as therapeutic antimicrobials for infectious diseases of human and fish. The stimulation of their gene expression by exogenous factors could be useful in preventing the growth of pathogenic microbes in aquaculture.

Pufferfish, which is a teleost fish belonging to the order Tetraodontiformes, is considered as a good model organism due to its extraordinarily compact genome that has a similar gene repertoire to human [22]. Recent studies revealed that several immune components including lectins, immunoglobulins, and trypsin inhibitors were identified from the skin mucus of pufferfish species [23–26], suggesting that the skin mucus of pufferfish also serves as a repository of immune components including AMPs. Here, we report the biochemical purification of a novel hepcidin type 2-like AMP from the skin mucus extract of the pufferfish *Takifugu pardalis*, designated as *Tp*HAMP2. Genomic DNA (gDNA) and full-length complementary DNA (cDNA) sequence encoding the *Tp*HAMP2 precursor protein were cloned and sequenced, enabling investigation of its mRNA expression levels in various tissues and in response to *in vivo* exposure of *Vibrio anguillarum*. We also evaluated the antimicrobial activity using the recombinant mature *Tp*HAMP2 that had biochemically identical properties to the native peptide produced in a heterologous bacterial-expression system against various bacteria and the effect of salt concentrations on the antimicrobial activity.

2. Materials and methods

2.1. Animals and sample extraction

The live pufferfish (*Takifugu pardalis*, 10–13 cm in total length) of both male and female were purchased from a local fish market in Daeyeon-dong, Busan, South Korea. All specimens were transported alive to the laboratory of Pukyong National University and were placed in a recirculating aquarium tanks equipped with sand-filtered and UV-sterilized seawater at 13 ± 1 °C. This study was approved by the Animal Ethics Committee of Pukyong National University (Approval #2017–38) and was performed according to the guideline for the care and use of laboratory animals. Skin mucus were collected by lightly scraping the dorsal-lateral surface of 30 fishes with a plastic spatula. The mucus harvest was immediately soaked in 4 vol of pre-heated distilled water containing 1% acetic acid in a water bath for 5 min to inhibit proteolytic enzyme activity. The boiled sample was cooled on ice and then completely homogenized (Ultra-Turrax T10 basic, IKA, Staufen, Germany). The homogenate was centrifuged (20,000 x g, 30 min, 4 °C) and then the supernatant (75 mL) was subjected to solid phase extraction (SPE) using a C18 cartridge (Sep-pak C18; 20 cc, Waters Corp, USA). The cartridge was washed with water/0.1% trifluoroacetic acid (TFA) and retained materials were then eluted with 60% methanol/0.1% TFA. The eluate was concentrated up to 3 mL using a speed vacuum concentrator (MaxiDry Plus, Heto, Allered, Denmark) and its antibacterial activity was evaluated as described below in the materials and methods section for antimicrobial activity assay.

2.2. HPLC purification of antimicrobial peptide

The 60% methanol eluate on the C18 cartridge was applied to a cation exchange high performance liquid chromatography (HPLC) column (TSKgel SP-5PW, 7.5 × 7.5 mm; Tosoh Corp., Minatoku, Tokyo, Japan). The elution was performed with a linear gradient of 0–1.0 M sodium chloride in 10 mM phosphate buffer (PB, pH 6.0) at a flow rate of 1.0 mL/min for 100 min. Active fractions responsible for antibacterial activity, which were eluted between 0.8 and 0.9 M sodium chloride, were pooled and subjected to a reversed phase (RP)-HPLC column (Capcell-Pak C18, 5 μm, 4.6 mm × 250 mm, Shisheido Co., Tokyo, Japan) with a linear gradient of 5–65% acetonitrile/0.1% TFA for 60 min at a flow rate of 1 mL/min. A single absorbance peak with antibacterial activity was eluted at 25% acetonitrile/0.1% TFA. Finally, the active peak was re-chromatographed for primary structure determination using the same column as the previous step, but with an isocratic elution of 25% acetonitrile/0.1% TFA at a flow rate 0.5 mL/min. An aliquot of each fraction or peak during the purification steps was used to test antibacterial activity against *Escherichia coli* D31.

2.3. Primary structure determination of the purified peptide

The molecular mass and amino acid (AA) sequence of the purified peptide were analyzed using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE^{TM} PRO spectrometer, Perseptive Biosystems, USA) with α-cyano-4-hydroxycinamic acid matrix and an automated N-terminal AA gas-phase sequencer (PPSQ-31A/33A protein sequencers; Shimadzu Co., Kyoto, Japan), respectively.

2.4. Cloning of cDNA and gDNA sequence encoding precursor protein of the purified peptide

Cloning of the full-length cDNA encoding the purified peptide was performed by 3′ and 5′ rapid amplification of cDNA ends (RACE). Total RNA was extracted from 100 mg of skin tissue using the Hybrid-R kit (GeneAll, Seoul, Korea), and the mRNA was re-purified using an Oligotex mRNA mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. The synthesis of RACE-ready cDNA templates was performed using the GeneRacer kit (RLM-RACE; Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. Base on the obtained AA sequence from N-terminal sequencing in which Xs were replaced with cysteine residues, two degenerate primers were designed for 3′ RACE (primer sequences used for RACE are listed in Supplementary Table S1). The first and the second nested 3′ RACE were performed in two sets of primer pairs, *Tphamp2*-deg-F1 and GeneRacer 3′ primer pair, and *Tphamp2*-deg-F2 and GeneRacer 3′ nested primer pair, respectively. Both the first and the second nested 3′ RACE was conducted with following conditions: initial denaturation at 95 °C for 5 min, 5 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, 5 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, 25 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. The

secondary nested 3′ RACE product was introduced into the pGEM-T easy vector system (Promega, Madison, WI, USA) and verified by sequencing. Two gene-specific primers (*Tphamp2*-gsp-R1 and -gsp-R2) for 5′ RACE were designed according to the partial nucleotide sequences acquired from the 3′ RACE (Supplementary Table S1). The first 5′ RACE was conducted using the nTaq Hot polymerase (Enzynomics, Daejeon, Korea) with following conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The second nested 5′ RACE was included initial denaturation at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The nested 5' RACE products was also verified by sequencing. To determine the gDNA sequence of the purified peptide, gDNA was extracted from the liver using the gDNA isolation kit (GeneAll, Seoul, Korea). The gene specific primer sets for gDNA cloning were designed based on the full-length cDNA sequence obtained (Supplementary Table S1). PCR was performed using high-fidelity DNA polymerase (*n*Pfu-Forte, Enzynomics) with following condition: initial denaturation at 95 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 10 min, with a final extension at 72 °C for 5 min. The amplified PCR products were verified by sequencing of both directions. Exon-intron organization in the gDNA sequence was determined by comparison with the sequence of cDNA obtained.

2.5. In silico analysis and phylogenetic tree construction

The full-length cDNA sequence was translated into protein sequence using ExPASy (https://web.expasy.org/translate/). A putative signal peptide of translated precursor proteins were predicted using the SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and the ProP 1.0 (http://www.cbs.dtu.dk/services/ProP/), respectively. Theoretical isoelectric points (*p*Is) and molecular mass of peptides/proteins were computed using ExPASy *p*I/Mw tool (https://web.expasy.org?compute_pi/). The exon-intron organization of the purified peptide gene was determined by aligning between cDNA and gDNA sequence. Homology searches of nucleotide and protein sequences were performed using BLAST algorithm against National Centre for Biotechnological Information (NCBI) non-redundant (NR) databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment of the precursor protein of the purified AMP with vertebrate orthologous, which were retrieved from NCBI GenBank databases, was conducted using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and refined manually. Sequences and GeneBank accession numbers of hepcidin employed for the alignment and phylogenetic analysis were shown in Supplementary Table S2. A phylogenetic tree was constructed using Maximum-Likelihood (ML) method with Jones-Taylor-Thornton (JTT) model as a substitution model, Nearest-Neighbor-Interchange heuristic model, and complete deletion of gap using MEGA6 program [27]. The confidence of branch topology was accessed with 1000 bootstrap replications. According to the result of homology search and phylogenetic analysis, the purified peptide was designated as *T. pardalis* hepcidin type 2-like AMP (*Tp*HAMP2).

2.6. Recombinant production of mature TpHAMP2

The recombinant *Tp*HAMP2 (r*Tp*HAMP2) was produced using a slightly modified pET-28a(+)-*TrxA* fusion vector system, which is introduced by our laboratory as described previously [28]. Briefly, the *Tp*HAMP2 coding cDNA sequence was amplified by PCR using the forward primer *Tphamp2-BamH*I-Met-F denoted by a Met codon to incorporate a cyanogen bromide (CNBr)-cleavage site immediately upstream of the N-terminus of the purified *Tp*HAMP2 and the reverse primer *Tphamp2-Xho*I-R with a stop codon (primer sequences are listed in Supplementary Table S1). The PCR product was cloned in-frame with the N-terminal His6 tag using the *BamHI*/*Xho*I sites of the pET28a-*TrxA* fusion vector, which is comprised of an N-terminal His6-tag fused with thioredoxin A (TrxA) protein improving disulfide formation, and verified by sequencing. The pET28a-*TrxA/TpHAMP2* plasmid was transformed into *Escherichia coli* BL21(DE) cells (Novagen, Madison, WI, USA) for expression of the His6-tagged fusion protein. The cells were grown at 37 °C to an optical density at 600 nm (OD_{600}) of 0.6 in Luria-Bertani medium supplemented with 30 μg/mL kanamycin. Expression of the fusion protein was induced with 1.0 mM isopropyl β-$_D$-1-thio-galactopyranoside (IPTG) at 37 °C for 4 h, and bacterial cells were harvested by centrifugation. The cells were washed with 1 × phosphate-buffered saline (PBS; pH 7.4) three times and lysed by re-suspending bacterial pellets in 1 × PBS, followed by three sonication steps at 40% amplitude for 20 s using a Sonifier 250 (Branson Ultrasons, Annemasse, France). The lysate was centrifuged (20,000 × *g*, 20 min, 4 °C), and the precipitate was dissolved in 1 × PBS containing 8 M urea and 5 mM imidazole prior to His-tag-affinity purification. His-tagged *Tp*HAMP2 fusion protein was purified using affinity chromatography by incubating the re-suspended precipitate with nickel-nitrilotriacetic acid (Ni-NTA) resin (Novagen) at a ratio of 30:1 (v/v) for 1 h at room temperature and then eluted with two column volumes of 1 × PBS (pH 7.4) containing 8 M urea and 0.5 M imidazole. Eluted fusion protein was dialyzed against 5% acetic acid using SpectraPor dialysis membranes (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and lyophilized. The methionine residue between TrxA fusion protein and mature *Tp*HAMP2 was cleaved by 10 mg/mL of CNBr (final concentration) in 50% formic acid for 8 h in darkness at room temperature. The cleavage reaction was terminated by adding 10 vol of water, followed by lyophilization. The cleaved fusion-peptide mixture was dissolved in 3 mL of 50% formic acid and purified to homogeneity by RP-HPLC (Capcellpak C18; 4.6 × 250 mm; Shisheido). A peak predicted to be r*Tp*HAMP2 was collected and lyophilized. The r*Tp*HAMP2 were confirmed by analyzing the first three AA residues using automated an N-terminal AA gas-phase sequencer (Shimadzu Co.) and the molecular mass using a MALDI-TOF MS. Moreover, retention time of the r*Tp*HAMP2 on RP-HPLC was compared with that of native peptide under an isocratic 23% acetonitrile/0.1%TFA elution at a flow rate of 1 mL/min.

2.7. Microbial strain and antimicrobial activity assay

All microbial strains used in this study were grown overnight in the appropriated broth and temperature listed in Supplementary Table S3. Monitoring antimicrobial activity during purification of the *Tp*HAMP2 was tested against *E. coli* D31, which was highly susceptible to the eluate of 60% methanol/0.1% TFA through the SPE. Ultrasensitive radial diffusion assay (URDA) was employed to determine the antimicrobial activity of r*Tp*HAMP2 as described previously [29,30]. Briefly, the grown microbial suspensions were diluted to concentration of 10^8 colony forming unit (CFU)/ml for bacteria and 10^6 CFU/mL for fungus with 0.5 of a McFarland turbidity standard (Vitek Colorimeter #52–1210, Hach, Loveland, Colorado) using 20 mM phosphate buffer (PB, pH 6.57). One-half milliliter of diluted microbial suspension was mixed with 9.5 mL of underlay gel containing 0.03% TSB and 1% Type I (low EEO) agarose in 20 mM PB (pH 6.6), followed by transfer to a square petri dish with grids making the concentration of the underlay gel into 5 × 10^6 CFU/mL for bacteria and 5 × 10^4 CFU/mL for fungus. Each sample in 5 μL of 0.01% acetic acid was added to 2.5 mm diameter wells made in approximately 1 mm thick underlay gel. After complete diffusion of each sample for 3 h at room temperature, underlay gels containing bacterial strains were overlaid with 10 mL of double-strength overlay gel containing 6% TSB with 2% sodium chloride for *V. anguillarum* or 6% TSB for other microbial strains and 1% agarose in 20 mM PB (pH 6.6). The effect of supplementation with sodium chloride (0, 1, 2 and 3%) on the activity of r*Tp*HAMP was evaluated as described previously [30]. Plates were incubated for 18–24 h for microbial strains growth at an appropriate temperature, and the clear zone were measured. After subtracting the diameter of the well, the clear zone

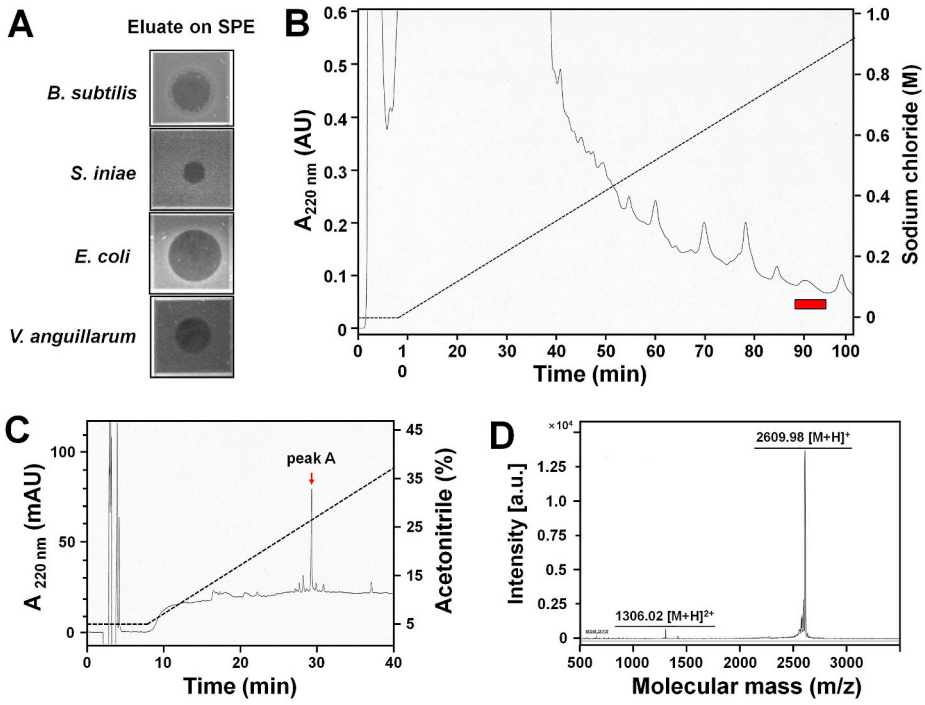

Fig. 1. Purification of the antibacterial peptide from the skin mucus of the pufferfish *T. pardalis*. (A) An aliquot of the eluate through SPE displayed antibacterial activity against *B. subtilis*, *S. iniae*, *E. coli*, and *V. anguillarum* conducted by URDA (B) Fractionation of the eluate on SPE by cation-exchange HPLC reveals an active fraction (red box) was eluted between 0.8 and 0.9 M sodium chloride showed the antimicrobial activity against *E. coli*. A single absorbance peak (peak A) responsible for the antibacterial activity was obtained in the second RP-HPLC step. (D) The molecular mass of peaks A as determined by MALDI-TOF MS was 2609.98 Da according to protonated molecular ions $(M+H)^+$. (E) The N-terminal AA sequences of the peak A was obtained by automated Edman degradation, where X represents unidentified residues returning blank cycles sequencing. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diameter was expressed in units (0.1 mm = 1 U). Minimal effective concentration (MEC, μg/mL) of the r*Tp*HAMP2 was calculated as the X-intercept of a plot of units against the $\log_{10}$ of the peptide concentration [31]. Synthetic piscidin-1, an α-helical AMP isolated from hybrid striped bass, was used as a positive control [32].

2.8. In vivo exposure to V. anguillarum

The pufferfish *T. pardalis* was acclimated in three of 300 L seawater tanks with a stocking density of 30 fish per each tank for one week, which were fed one a day with commercial fish pellets. The seawater tanks were closed-recirculation system of a flow rate of 900 L/h seawater, which was sand-filtered and UV-sterilized, with a salinity of 31 ppt and a temperature 13 ± 1 °C. Cultured *V. anguillarum* was washed with 1 × PBS (pH 7.4) three times and re-suspended to 10^8 CFU/mL with 1 × PBS (pH 7.4). In order to test for bacterial infection *in vivo*, *T. pardalis* was exposed by inoculating 30 mL of 10^8 CFU/mL *V. anguillarum* into the seawater tanks during which the seawater was stopped circulating for one hour and then restarted. An unexposed control group was performed under the same conditions by adding the same volume of PBS. Three individuals were randomly collected from each thank at 0, 16, and 32 h post infection (hpi).

2.9. Real time-quantitative PCR (RT-qPCR) for TpHAMP2 mRNA

Total RNA was extracted from the collected tissues, including the skin, gill, intestine, spleen, liver, head-kidney, and body-kidney, using Hybrid-R (GeneAll, Seoul, Korea), and RNA quality was assessed by 1.0% agarose gel electrophoresis and then quantified spectrophotometrically using a NanoDrop Lite (Thermo Fisher Scientific, Wilmington, MA, USA). cDNA was synthesized using the TOPscript cDNA synthesis kit with oligo dT (dT18; Enzynomics, Daejeon, Korea) according to manufacturer instructions. RT-qPCR was employed using a CFX Connect real-time PCR detection system (Bio-Rad Laboratories) as previously described, with slight modifications [29]. Briefly, amplification was performed in 20 μL volume reactions containing 10 μL of 2 × SYBR Green premix (TOPreal qPCR 2 × PreMix; Enzynomics), 1 μL (10 pmoL/μL) of each gene-specific forward and reverse primer, 1 μL of 10-fold-diluted cDNA template, and nuclease-free water added to make a final volume of 20 μL. Primer pairs used for amplifying *Tp*HAMP2 mRNA, with the pufferfish *T. rubripes* β-actin (accession no.: P68142) as an internal control for normalization [33], were *Tphamp2*-qPCR-F and -R and *Trβactin*-qPCR-F and -R, respectively (Supplementary Table S1). The thermal-cycling profile was 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 15 s, with fluorescence recording at the end of each cycle. Melting-curve analysis was performed to ensure product specificity over the temperature range 60 °C–90 °C. Amplicons were analyzed on agarose gels to confirm product size. The relative expression levels of *Tp*HAMP2 mRNA in each tissue and at each time point were normalized against the level of β-actin using the comparative CT method ($2^{-\Delta\Delta CT}$) [34]. Triplicate cDNA-sample amplifications were performed independently, and the results were analyzed in statistic. For statistical analysis, one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test for the basal expression or two-way ANOVA with Dunnett's multiple comparison test for *V. anguillarum* infection was performed using GraphPad Prism (v7.0 for Windows; GraphPad Software). Relative expression and fold change of *Tp*HAMP2 mRNA expression in response to the infection was presented as the mean ± standard deviation. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Biochemical identification of AMP from the skin mucus of T. pardalis

The skin mucus extract after SPE revealed the antibacterial activity against tested bacteria including gram-positive bacteria, *B. subtilis* and *S. iniae*, and gram-negative bacteria, *E. coli* and *V. anguillarum*, indicating that it was an appropriate source to isolate mucosal immune components (Fig. 1A). In the initial purification step using cation-exchange HPLC, fractions eluted between 0.8 and 0.9 M sodium chloride showed the antibacterial activity against *E. coli* (Fig. 1B). This fraction

was subjected to the next step purification of an RP-HPLC, resulting a single absorbance peak (peak A) was eluted with 25% acetonitrile/0.1% TFA showing antibacterial activity against *E. coli* (Fig. 1C). The active peak was re-chromatographed using RP-HPLC for further analysis to determine its primary structure. Purified peak A was identified with the molecular mass 2609.98 Da according to protonated molecular ions $(M+H)^+$ (Fig. 1D) and as the peptide composed of 23 AAs with the sequence RKRXRFXXNXXPGKQGXVFXXGF (X represents unidentified residues returning blank cycles during Edman sequencing) (Fig. 1E). Unidentified AAs, such as Cys residues, emerged as blank cycles during AA-sequencing procedures in the absence of the reduction and alkylation steps, with these cysteine residues commonly forming disulfide bonds in AMPs to enhance folding and stability. Accordingly, a theoretically molecular mass of the purified peptide, where eight unidentified residues were replaced with cysteine residue, was calculated as 2617.07 Da of the monoisotopic mass, which was increased by approximately 8 Da compared with the molecular mass obtained experimentally. The results suggest that the purified peptide contains eight cysteine residues that are likely to form four intramolecular disulfide bonds. Subsequently, homology search revealed that the purified peptide was aligned with numerous hepcidin-like antimicrobial peptides (HAMPs) with an identity range of 62–79% in fish species, which were also conserved with eight cysteine residues in their sequences. Therefore, the purified peptide was named *T. pardalis* hepcidin-like AMP (*Tp*HAMP2), and it was found to belong to hepcidin type 2 (HAMP2) class according to the results of sequence comparison and phylogenetic analysis described below.

3.2. Cloning of cDNA and gDNA encoding TpHAMP2 precursor protein

To confirm the predicted primary structure of the purified AMP, the gDNA and full-length cDNA encoding a precursor protein of the purified AMP were cloned and sequenced, the resulting nucleotide sequence and the deduced protein sequence are shown in Fig. 2A. The sequences of *Tp*HAMP2 cDNA and gDNA were submitted to the GenBank database with accession numbers MK728734 and MK728735, respectively. The full-length cDNA sequence encoding the purified AMP precursor protein comprised 575 bp, starting with a 5′-untranslated region (UTR) of 90 bp, followed by an open reading frame (ORF) of 267 bp, and a 3′-UTR of 218 bp containing a polyadenylation consensus sequence (AATAAA) located 15-bp upstream of the poly(A)$^+$ tail (Fig. 2A and C). The deduced precursor ORF sequence encodes a protein comprising 88 AA residues with a 24-AA residue signal peptide, which was predicted to be cleaved by a signal peptidase between Ala^{24} and Leu^{25} (SSA/LP) using SignalP 4.1, followed by two peptide fragments: a 41 AA residues of N-terminal prodomain (Leu^{25}-Leu^{65}) containing several acidic AAs and the mature peptide comprising 23 AA residues (Arg^{66}-Phe^{88}), which had eight cysteine residues in the sequence, a predicted the monoisotopic molecular mass of 2617.1 Da, and the *p*I of 8.97 (Fig. 2D). Collectively, the purified *Tp*HAMP2 was 23 AA residues AMP with the sequence RKRCRFCCNCCPGKQGCVFCCGF, indicating the purified *Tp*HAMP2 is derived from a precursor protein, which underwent post-translational modifications such as formation of intramolecular disulfide bonds to produce its active *Tp*HAMP2.

The gDNA sequence encoding *Tp*HAMP2 gene was represented a tripartite exon-intron organization, which has been known to be a common structure of vertebrate hepcidin genes (Fig. 2A and B) [35]. Exon-1 encoded a 5′ UTR (90 bp) followed by the signal peptide (72 bp, 24 AAs) and the C-terminus of the proprotein (15 bp, 5AAs), exon-2 encoded the middle region of the prodomain (78 bp, 26 AAs), and exon-3 encoded the N-terminus of the prodomain (30 bp, 10 AAs) and the active *Tp*HAMP2 (69 bp, 23 AAs) with a stop codon followed by 3' UTR (218 bp). Each exon was connected by two introns, intron-1 (105 bp) and intron-2 (108 bp). The classical canonical splicing recognition sequence GT/AG was present at the exon-intron junction.

3.3. Sequence comparison of TpHAMP2 with other fish hepcidins

The deduced AA sequence of the *Tp*HAMP2 precursor protein was aligned with several other known and predicted hepcidin-like peptides from fishes (Fig. 3A). Sequence comparison revealed that *Tp*HAMP2 precursor protein shared several features in the sequence and the length of three regions (signal peptide, prosequence, and mature peptide) with acanthopterygian fish hepcidin type 2s, while they were slightly

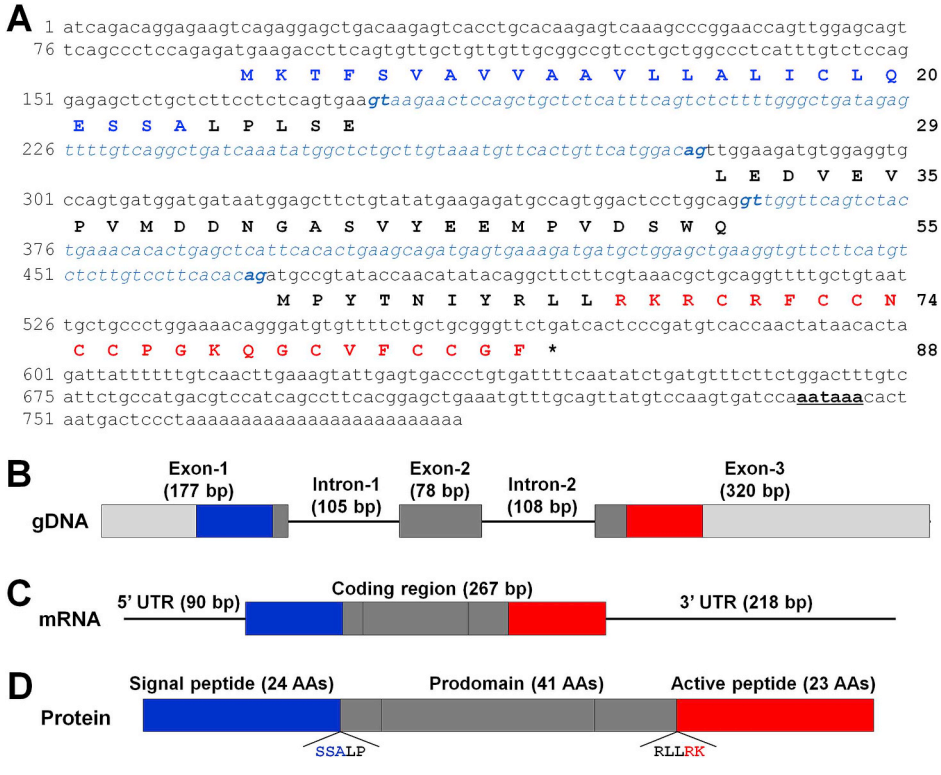

Fig. 2. Sequence of gDNA and full-length cDNA encoding *Tp*HAMP2 precursor protein. (A) Genomic (lowercase, 788 bp) and protein (uppercase 88 AAs) sequence of *Tp*HMP2. In the DNA sequence, three exons (black letters) are separated by two introns (blue letters). The canonical splicing recognition sequence gt/ag and the polyadenylation signal site are italic blue (*gt/ag*) and underlined, respectively. The amino acid sequence of the precursor is shown below the coding sequence, with the predicted signal peptide, anionic prodomain and the purified active *Tp*HAMP2 shown in blue, black, and red, respectively. The asterisk shows the position of the stop codon. Schematic showing the structure of gDNA (B) and cDNA (C) encoding the TpHAMP2 precursor protein (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

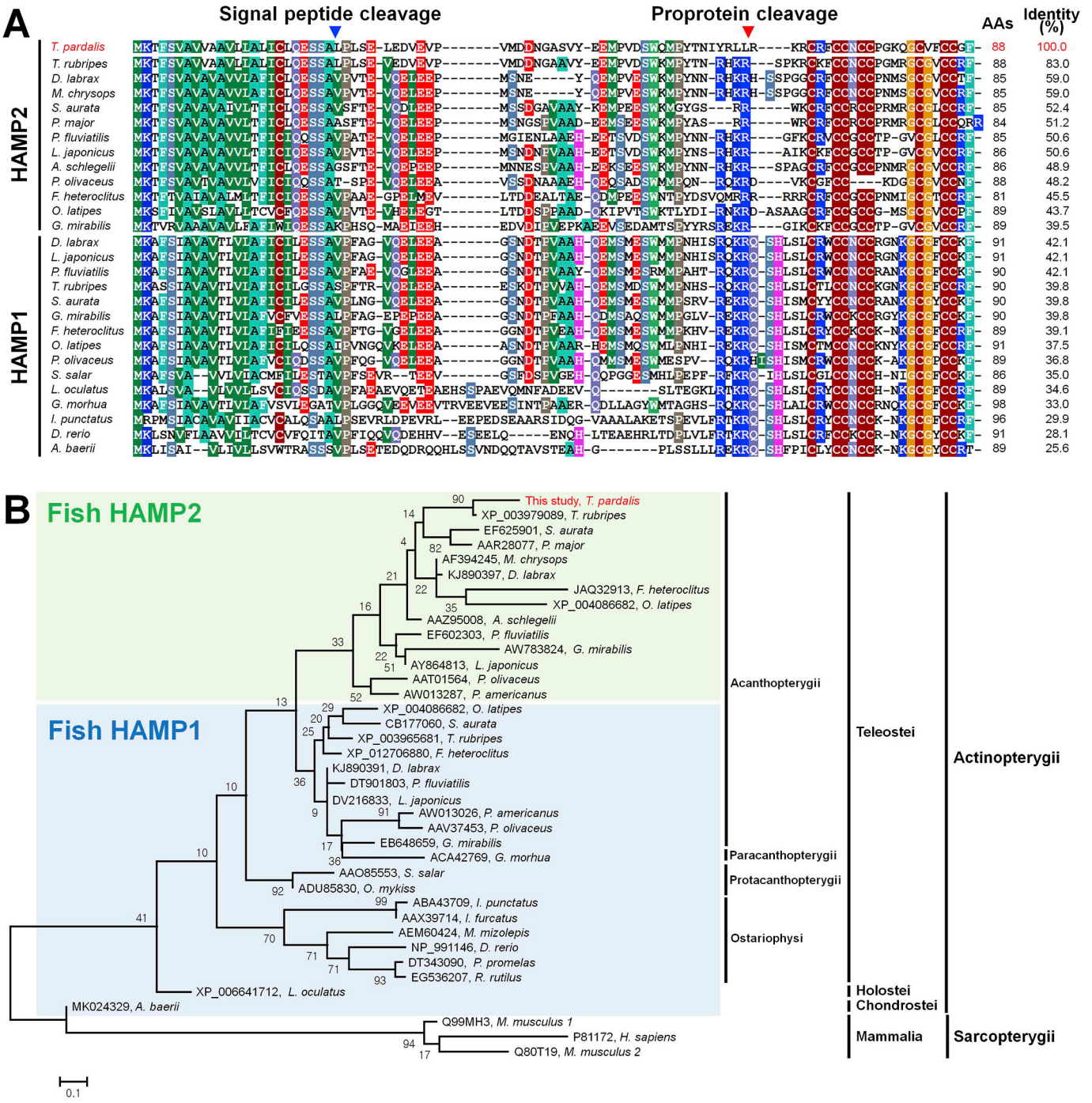

Fig. 3. Sequence comparison and phylogenetic analysis of *Tp*HAMP2 precursor protein with other know hepcidins. (A) Multiple sequence alignment of the *Tp*HAMP2 precursor protein with other fish hepcidins. (B) Phylogenetic analysis of the *Tp*HAMP2 precursor protein with other mammals and fish hepcidins. Phylogenetic tree was constructed by ML method based on the JTT matrix-based model using MEGA6 program [27]. The confidence in the phylogenetic tree branch topology was accessed with bootstrap using 1000 replications. Hepcidins for the alignment and the phylogenetic analysis include 38 AAs sequences listed in Supplementary Table S3.

different in non-acanthopterygian fish hepcidin type 1s. The highest identity was found in HAMP2 from the Japanese pufferfish *T. rubripes* (83.0%), while the lowest identity was found in a hepcidin from the Siberian sturgeon *Acipenser baerii* (25.6%), which belonged to hepcidin type1 (HAMP1) [35]. In addition, the mature *Tp*HAMP2, did not possess the hypothetical iron regulatory sequence (Q-S/I-H-L/I/F/-S/A/P-L/M/I) present in the N-terminus of the mature fish HAMP1s. Moreover, the number of residues prior to the first Cys residue was 3 AAs in *Tp*HAMP2, which corresponded to the length observed in fish HAMP2 (3–6 AAs), but not to the length found in fish HAMP1 (at least 6AAs). Thus, the *Tp*HAMP2 was more similar to fish HAMP2 than fish HAMP1. Nevertheless, eight Cys residues that likely form four intramolecular disulfide bonds (except for *Paralichthys olivaceus* HAMP2 possessing six Cys residues) and a Phe residue at the end of C-terminus (except for *A. baerii* and *Pagrus major*) were clearly conserved in all the mature hepcidins aligned. Interestingly, in the C-terminus, a Gly residue conserved in all fish hepcidins and a basic AA residue (Arg or Lys) present in most fish were replaced with Val and Gly residues in *Tp*HAMP2, respectively (Fig. 3A). In addition, a typical RXXR motif for recognition and cleavage by proprotein convertases (PCs) was not detected in *Tp*HAMP2

precursor protein [36,37]. However, it is clear that the mature *Tp*HAMP2 consisting of 23 AAs, which was biochemically purified, was produced from its precursor protein cleaved at the Leu residue that placed at the end of the prosequence.

3.4. Phylogenetic analysis

To study the phylogenetic relationship of *Tp*HAMP2 and those of other fish and mammal, 37 hepcidin precursor protein sequences (34 from fish and 3 from mammal) were utilized to construct phylogenetic tree using ML method with JTT model (Fig. 3B and Supplementary Table S2). Phylogenetic tree showed that hepcidins were clustered into two main clades: one comprising hepcidins from mammals and chondrostean (*A. baerii*) as described previously [35] and the other comprising neopterygian fish hepcidins, in which a holostean (*Lepisosteus oculatus*) hepcidin held the basal position followed by various teleostean hepcidins. In the teleostean lineage, branching patterns of subclade were consistent with typical taxonomic classification including the separations of early teleostean groups, such as ostariopysian, protacanthopterygian, and paracanthopterygian fish, from the recently evolved acanthopterygian group, in which HAMP2s were represented as a monophyletic group. In this context of the phylogenetic analysis of hepcidins, *Tp*HAMP2 belonged to the HAMP2 class and was closely related to the *T. rubripes* HAMP2. Together with sequence comparison, *Tp*HAMP2 is a novel isoform as an orthologue of HAMP2, which would have diverged to acanthopterygian fish species.

3.5. Recombinant production of TpHAMP2 (rTpHAMP2)

In order to obtain sufficient amount to investigate biological activity, *Tp*HAMP2 was produced using a heterologous expression system according to a slightly modified version of previously described methods [28]. The constructed pET28a-*TrxA*-fused *TpHAMP*2 plasmid was transformed into *E. coli* BL21(DE3) cells (Fig. 4A) and His6-tagged TrxA fused r*Tp*HAMP2 protein was overexpressed as an insoluble protein and purified by affinity chromatography under denaturing condition (Fig. 4B). The affinity purified TrxA fused r*Tp*HAMP2 protein was cleaved by CNBr to generate active r*Tp*HAMP2 and the cleaved mixture was purified by RP-HPLC (Fig. 4C). The first three AA residues and the molecular mass of the r*Tp*HAMP2 were RKR by the N-terminal sequence analysis and 2611.19 Da $(M+H)^+$ by MALDI-TOF MS, respectively (Fig. 4C and D). Existence of disulfide bonds in the r*Tp*HAMP2 was confirmed by 100 mM 1,4-dithiothreitol treatment and molecular mass measuring. The retention time and the molecular mass of reduced form of r*Tp*HAMP2 shifted to the right from 15.9 min to 26.2 min (Fig. 4E) and from 2611.19 Da $(M+H)^+$ to 2618.10 Da $(M+H)^+$ (Fig. 4F), indicating the r*Tp*HAMP2 should contain four intramolecular disulfide bonds. Furthermore, the mixture of recombinant and native *Tp*HAMP2 was co-eluted and merged as a single absorbance peak via RP-HPLC (Fig. 4G). This shows that rTpHAMP had the same biochemical properties with the native *Tp*HAMP2 suggesting the 8 cysteine residues of r*Tp*HAMP2 were properly paired and, consequently, r*Tp*HAMP2 would be biologically active. Thus, the TrxA fusion protein approach was found to be excellent for the expression of r*Tp*HAMP2 without the need of reduction-oxidation step (often present in other protocols for cysteine-rich AMPs preparation) [28,38].

3.6. Antimicrobial activities of rTpHAMP2

Antimicrobial activity of r*Tp*HAMP2 was tested against various microorganisms including five gram-positive, nine gram-negative bacteria, and one fungus to determine the MEC (Table 1). The results showed that r*Tp*HAMP2 displayed potent antimicrobial activity against all tested bacteria and fungus with the MECs ranging from 1.7 to 39.4 μg/mL and was even more potent than piscidin-1 (a reference peptide) against *B. subtilis*, *E. coli*, *S. enterica*, *Sh. sonnei*, *Sh. flexneri*, *P. aeruginosa*, and fungus *C. albicans*. Moreover, r*Tp*HAMP2 showed the potent antibacterial activity (MEC; 4.8 μg/mL) against a fish pathogen, *V. anguillarum*, which causes an infamous fish disease in aquaculture known as Vibriosis [39]. Meanwhile, the antimicrobial activity of r*Tp*HAMP was abolished in the medium containing 1% sodium chloride against *B. subtilis* and 2% sodium chloride against *E. coli* and fungus *C. albicans*. (Supplementary Table S4). Collectively, these results suggest that antimicrobial activity of *Tp*HAMP2 is broad to both gram-positive and gram-negative bacteria as well as fungus and is specifically potent to certain microorganisms.

3.7. Expression analysis of TpHAMP2 mRNA

The relative expression levels of *Tp*HAMP2 mRNA were determined by RT-qPCR. The basal expression levels of *Tp*HAMP2 mRNA in seven tissues showed that significantly high expression level ($p < 0.0001$) was detected in the liver, while the expression in other tissue (body kidney, gill, head kidney, intestine, skin, and spleen) was only minute compared to that of liver, indicating that the liver is a major organ for basal expression of *Tp*HAMP2 mRNA (Fig. 5A). In response to *in vivo* exposure of *V. anguillarum*, *Tp*HAMP2 mRNA expression increased by approximately 2.5-fold in the liver and by 4.4-fold in the spleen at 16 hpi, then decreased to the basal level at 32 hpi, and increased by 1.5-fold in the skin and by 3.6-fold in the intestine at 32 hpi (Fig. 5B). Meanwhile, the expression of *Tp*HAMP2 mRNA not only was not affected in the gill but also decreased in the body kidney and the head kidney at 36 hpi. Accordingly, the expression level of *Tp*HMAP2 mRNA in response to *in vivo* exposure of *V. anguillarum* was significantly upregulated early in the liver and the spleen, and then statistically upregulated later in the skin and the intestine. Overall, these results suggest that *Tp*HAMP2 would be involved in the first-line defense, including mucosal innate immune system that responds to invading pathogens from an aquatic environment.

4. Discussion

Fish comprise the greatest group of vertebrate species, even though their adaptive immune system is less developed than that of higher vertebrates. Therefore, a potent innate immune system is essential to survive in most extreme aquatic habitats, in which arises frequent and high levels of exposure to a large number of various pathogens. Fish skin mucus is believed to act as the first line of defense against a wide variety of pathogenic infections and is considered to be a potential source of novel antimicrobial components [8]. Among these components, hepcidin is a small and cationic cysteine-rich AMP that was originally identified from human urine as an AMP (originally named LEAP-1, liver-expressed antimicrobial peptide-1) and later known to play a key role in iron homeostasis as a regulatory hormone [40,41]. Fish hepcidin was first purified from the hybrid striped bass using a bioassay monitoring antibacterial activity [42]. In this study, we report the purification and characterization of a novel hepcidin type 2-like AMP (*Tp*HAMP2) from the skin mucus in the pufferfish *T. pardalis*. Though a series of purification procedures, the antibacterial peptide with a single absorbance peak was obtained from acidic extract of the skin mucus. The complete primary structure of the native *Tp*HAMP2 was obtained by a combination of AA sequencing, MALDI-TOF-MS, and sequence analyses of cDNA and gDNA. The purified native *Tp*HAMP2 comprised 23 AAs containing eight cysteine residues that formed four intramolecular disulfide bonds. The disulfide bonds in hepcidin molecules have been proposed to be one vicinal and three inter-strands disulfide bonds and to be important in the structural integrity and the proper antimicrobial activity [43,44].

Sequence comparison revealed that not only was the sequences of the signal peptide and the prodomain in the *Tp*HAMp2 precursor protein were highly similar to those of acanthopterygian fish, but also the mature *Tp*HAMP2 shared the eight cysteine residues, the Gly residue

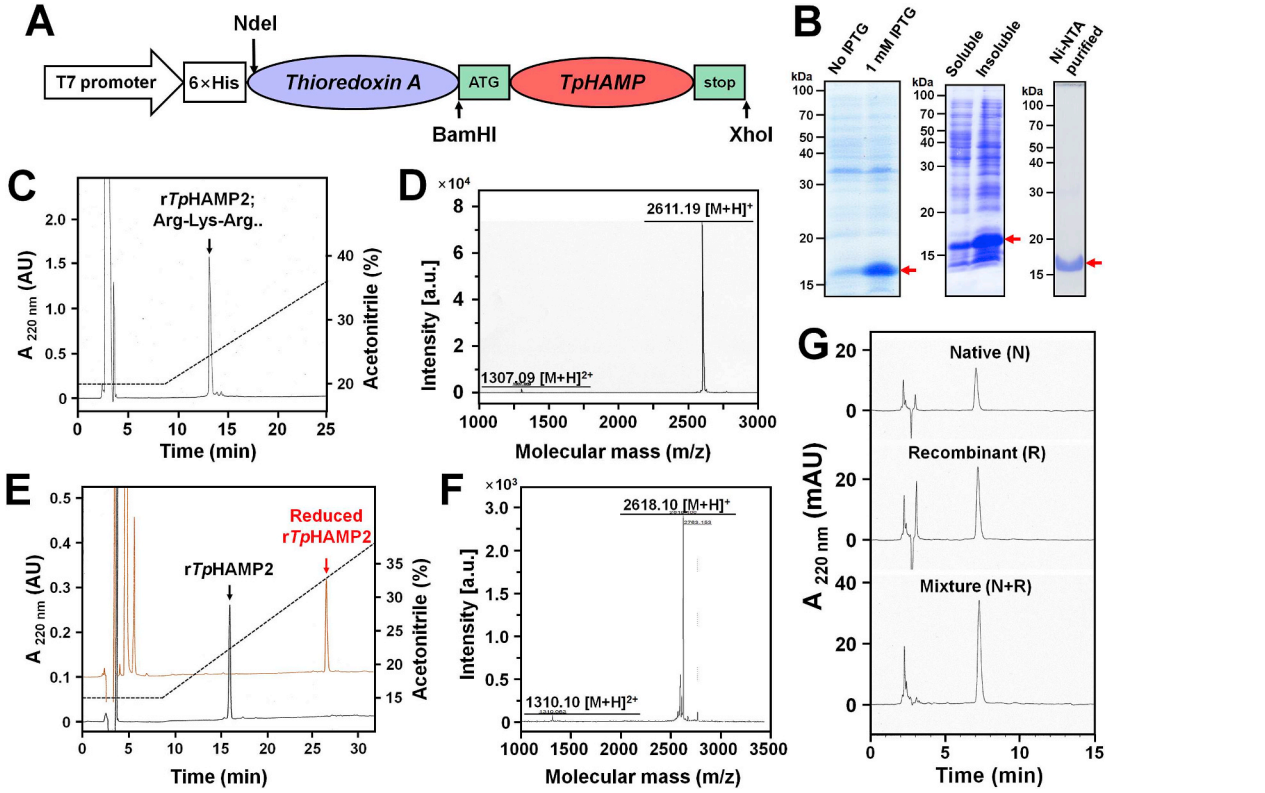

Fig. 4. r*Tp*HAMP2 production using the pET28a-TrxA/*Tp*HAMP2 construct in *E. coli* BL21(DE3) cells. (A) Schematic representation of the pET28a-TrxA fusion vector. TrxA-fused *Tp*HAMP2 protein was induced by 1.0 mM IPTG (B, left), and the single band migrated to ~ 17 kDa represents the TrxA-fused TpHAMP2 (B, centre) purified by His-affinity chromatography under denaturing conditions (B, right). (C) The mature *Tp*HAMP2 peptide was purified using RP-HPLC after CNBr cleavage and (D) its protonated molecular mass (three AAs at N-terminus of purified peptide are shown in the chromatogram). (E) Retention time of reduced r*Tp*HAMP2 was right shifted about 11 min and (F) molecular mass was increased approximately 7 Da. (G) Comparison of chromatographic properties between native and recombinant *Tp*HAMP2 on RP-HPLC. The mixture of native and rTpHAMP2 was co-eluted on RP-HPLC.

Table 1
Antimicrobial activities of r*Tp*HAMP2 and piscidin-1 against various microbial strains.

Microorganisms	Gram	[a] Minimal effective concentration (μg/mL)	
		r*Tp*HAMP2	Piscidin-1
B. subtilis KCTC 1021	+	3.5	4.4
S. auresus KCTC 1621	+	28.1	4.2
S. iniae FD 5228	+	39.4	5.0
M. luteus KCTC1071	+	24.72	4.5
L. garvieae ATCC49156	+	23.02	4.47
E. coli D 31	–	1.7	4.1
A. hydrophila KCTC 2358	–	36.0	4.7
E. tarda KCTC 12267	–	17.6	15.6
S. enterica ATCC 13311	–	2.4	5.5
V. anguillarum KCTC2711	–	4.8	3.8
Sh. sonnei KCTC2009	–	3.64	4.37
Sh. flexneri KCTC2517	–	2.89	3.69
P. aeruginosa KCTC2004	–	4.47	5.33
E. cloacea KCTC1685	–	13.68	6.15
C. albicans KCTC 7965	Fugus	3.02	3.91

[a] The values were calculated in the average activities from three separated experiments.

prior to the sixth Cys residue, and the Phe residue at C-terminus, which were found in the most mature fish hepcidins. Nevertheless, the *Tp*HAMP2 proprotein and its active form possessed a number of distinctive features; 1) no the PCs cleavage site, 2) three positively charged residues (RKR-) prior to the first Cys residue, 3) Val^{18} residue (numbering from the mature *Tp*HAMP2 sequence) replaced from the conserved Gly, 4) Gly^{22} residue replaced from the mostly conserved a basic residue (K or R). It is proposed that the prevalence of mutations is usually most elevated in the sequence of mature peptide compared to those of signal peptide and prodomain [3]. It is clear that the purified active *Tp*HAMP2, similar to other hepcidins, was generated from the precursor protein, which underwent two cleavages of the signal peptide and the prodomain. However, the cleavage motif recognized by PCs was not observed in the AA sequence of *Tp*HAMP2. Many secretory proteins/peptides are synthesized as inactive precursors that, in addition to signal peptide cleavage, undergo post-translational processing to become biological active proteins/peptides [45]. The activation of proproteins is controlled by furin and related PCs, which generally cleave at the consensus recognition motif (K/R)-(X)n- (K/R), where n = 0, 2, 4, or 6, and X can be any AA residues with the exception of cysteine or proline residue, immediately preceding the N-terminal bioactive peptide [41,46]. Although molecular identification of many hepcidins from various vertebrates has been suggested the consensus motif RXXR is highly conserved to be cleaved by PCs to produce the active hepcidins, it would be presumptuous to conclude that the consensus motif to be the true cleavage site for fish hepcidins and synthesize the active form accordingly for the investigation of *in vitro* and *in vivo* activity. There still remains a risk of utilizing fish hepcidins that might be different from the native, because fish possess multiple copies, structural diversity, and putative different function of hepcidins. Strikingly, biochemical identification of human hepcidins (25 AAs) and its truncated isoforms (22 and 20 AAs) and of bass hepcidin revealed that hepcidins could be cleaved by a different process than by the PCs, even though the biological mechanism and purpose of the process are still unclear (Fig. 6) [41,42,47]. Previous study on hepcidin structure in relation to its function have demonstrated that 5 AA residues at N-terminus of mature hepcidin, DTHFP- in human HAMP and QSHLS- in zebrafish HAMP, are essential for its iron-regulatory activity [43,48].

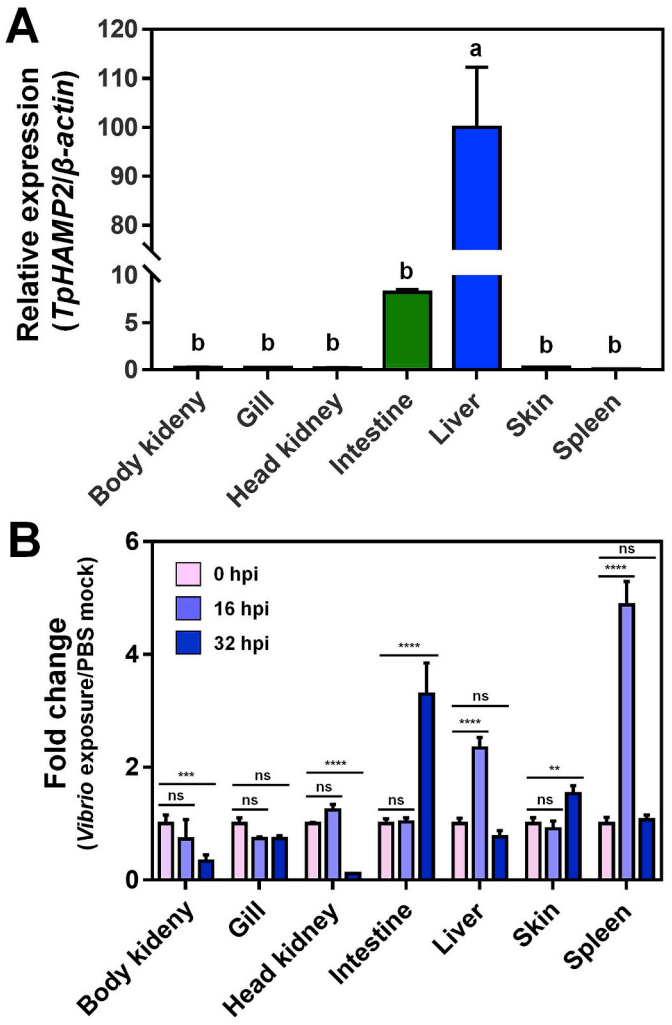

Fig. 5. Expression analysis of *Tp*HAMP2 mRNA. (A) Relative expression of *Tp*HAMP2 mRNA in seven tissues (i.e., body kidney, gill, head kidney, intestine, liver, skin, and spleen). Means ± standard deviation ($n = 3$) are shown. Means denoted by the same letter did not differ significantly ($p > 0.05$) while different letters (a, b) at the top of the bars indicate statistically significant differences ($p < 0.05$) between tissues determined by one-way ANOVA followed by Bonferroni's multiple comparison test. (B) Fold change of *Tp*HAMP2 mRNA in response to *in vivo V. anguillarum* exposure in different times (0, 16, and 32 hpi). Means denoted by "n.s." (no significance) or the asterisks (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$) at the top of the bars indicate statistically significant differences between control (PBS treatment) and *in vivo V. anguillarum* exposure. All statistical significances of *Tp*HAMP2 mRNA expression were determined by two-way ANOVA followed by Dunnett's multiple comparison test.

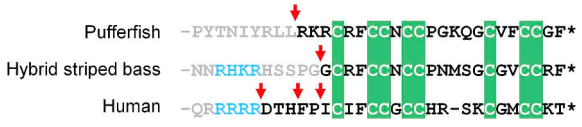

Fig. 6. The cleavage sites for mature hepcidins of bass, human, and pufferfish that were biochemically identified using antibacterial activity as monitoring method are shown in red down arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

These sequences are highly conserved in all fish HAMP1s, but not in fish HAMP2s. Thus, it could be assumed that the antimicrobial activity of fish HAMP2 is due to direct bacterial death rather than through an indirect iron regulatory activity, which limits the iron mobilization and availability in bacteria, thereby resulting in an attenuation of viability and pathogenicity of bacteria [49]. Consistently, due to the motif of numerous cationic residues and amphipathic structure similar to other well-known AMPs, it has been hypothesized that the positive charge or *p*I of the active hepcidins may have the ability to interact with the negatively charged microbial components such as lipopolysaccharide or phospholipids to disrupt the cell membrane, thereby causing bacterial death directly or increasing the bacterial membrane permeability, or to target the internal structures such as DNA or RNA by entering into the bacteria to kill the bacteria [50]. A study on action mechanism of a synthetic hep-1 (fish HAMP2 class) from *D. labrax* suggest that hep-1 may have an intracellular target, which would affect the replication in *V. anguillarum* [51]. Furthermore, recent studies using scanning electron microscopy have shown that synthetic peptides for fish HAMP1 and HAMP2 led to disruption of the bacterial outer membrane, demonstrating that fish hepcidins might be directly interact with the cell membrane to release the cytoplasmic content leading to cell death [52,53]. In this context, the purified active *Tp*HAMP2 displayed conserved structures that are considered to be important for antimicrobial activity [49,52,53]. Meanwhile, the active *Tp*HAMP2 also possessed distinctive sequences (RKR-at N-terminus, Val^{18}, and Gly^{22}) compared with other fish hepcidins, which will affect the antimicrobial activity of *Tp*HAMP2 similar to those seen in other AMPs [54,55]. Although the structure-activity relationship of *Tp*HAMP2 should be further investigated in detail, the active r*Tp*HAMP2, which was produced in heterologous system and identical to the native form, exhibited a broad spectrum of antibacterial activity against all tested microorganisms including gram-positive, gram-negative bacteria, and a fungus. In addition, potent antibacterial activity that is comparable to Piscidin-1, the positive control used in this study, against *B. subtilis*, *E. coli*, *S. enterica*, *V. anguillarum*, *Sh. sonnei*, *Sh. flexneri*, *P. aeruginosa*, and *C. albicans* was observed. Although the effect of salt on the activity of r*Tp*HAMP was more sensitive than Psicidin-1, the activity still remained against *E. coli* and *C. albicans* until 1% (171.1 mM) sodium chloride concentration that was higher than salt concentrations (121 ± 4 mM of Na and 129 ± 5 mM of Cl concentration) in bronchopulmonary fluids in cystic fibrosis patients [56]. The Piscidin AMP family plays an important role in innate immunity in fish and have potent activity against a variety of microbes including fungi, yeast, and gram-positive and -negative and their resistant versions, therefore, the Piscidin family are a potent candidate for development as an antimicrobial agent [32]. Collectively, the potent antimicrobial activity of r*Tp*HAMP2 comparable to Psicidin-1 and its microorganism specificity may be due to the conserved structures in *Tp*HAMP2 with other known hepcidins and its unique structure, which make r*Tp*HAMP2 is a good candidate for development as an antimicrobial agent.

Phylogenetic analysis showed that *Tp*HAMP2 precursor protein from *T. pardalis* was classified into fish HAMP2 clustered together with a putative hepcidin from *T. rubripes* belonging to Tetraodontiformes order in acanthopterygian fishes. This finding is consistent with previously reported results that acanthopterygians fish have acquired additional HAMP2 copies by recent gene duplication and positive Darwinian selection [35,57], suggesting *T. pardalis* might have other hepcidin genes copies, one of which belong to HAMP1 class. Gene duplication and further positive Darwinian selection have been suggested as a molecular strategy in the evolution of hepcidins [58,59]. Mammals (except for mouse) have a single hepcidin gene that act both as an AMPs and an iron regulator, while many teleost fish species have been reported to possess multiple copies of hepcidin genes with certain distinctive characteristics [57,60]. The *Tp*HAMP2 gene and its precursor protein have several distinctive AA sequences, even though they share overall feature with other fish hepcidins, particularly with fish HAMP2s. The genomic structure and organization of *Tp*HAMP2 gene consisting three exons and two introns were highly conserved in its orthologues in mammals and fish [61]. Meanwhile, the small size of intron-2 in *Tp*HAMP2 gene would be responsible for representing the

small *Tp*HAMP2 gene in length (788 bp), which is comparable to other fish HAMP2 genes identified from *A. schlegelii* (790 bp referred to as As-hep2), *E. coioides* (762 and 822 bp for Ec-hepcidin 1 and 2), *Micorpterus salmoides* (766 bp for hep-2), *P. auriga* (599 and 831 bp for PauHepc3 and 4, respectively), and *Scatophagus maximus* (472 bp, no UTRs information available, for hepcidin-2) [62–65]. Although the functional roles of hepcidin introns have not been clearly elucidated, the variation of intron size in genome may reflect the regulation of gene expression and the divergent of gene function [66,67]. Together with sequence comparison as highlighted above, phylogenetic analysis implies that the sequence of *Tp*HAMP2 gene and its deduced protein, especially the active peptide, might have evolved through an accelerated rate of AAs substitution to effectively combat various aquatic pathogens.

So far, many hepcidin isoform mRNAs have been identified from various fish species using molecular approach [35,63,68]. In most of the reported fish species, the basal expression of fish HAMP2 mRNA was predominant in the liver, as in mammals, but fish HAMP2s were also expressed in various other tissues including the spleen and the kidney as well as mucosa-associated tissues including the skin, the gill, and the intestine [42,60,64,69–71]. In addition, the expressions were also upregulated by immune challenge. These finding suggest that fish HAMP2 might play an important role in mucosal immune tissues, fish innate immune responses. As assuming the high antimicrobial potential of fish HAMP2 mentioned above [61], we speculated that the *Tp*HAMP2 mRNA would be expressed widely in various tissues including mucosa-associated tissues, gill, intestine, and skin, of the pufferfish *T. pardalis*. However, we found that the basal expression of *Tp*HAMP2 mRNA was notably robust in the liver, but minute in the intestine, and barely detectable in other tissues, which was a different from our speculation and was only consistent with certain fish HAMP2s from *E. coioides*, *M. salmoides*, *P. auriga S. argus*, and *S. maximus* [63–65,72]. Although, further research on the relationship of the gene length and expression patterns in regard to evolution is necessary in various fish, surprisingly, the expression pattern of *Tp*HAMP2 mRNA seem to follow the gene length, suggesting that expression pattern of fish HAMP2 genes is closely related to the evolutionary change in the gene length. We also evaluated the modulation of *Tp*HAMP2 mRNA expression by *in vivo* exposure of *V. anguillarum* that invaded into the body of fish through various mucosal immune tissues. We found that *Tp*HAMP2 mRNA in response to *V. anguillarum* infection was upregulated in two systemic immune organs (the liver and the spleen) and then in two mucosal immune organs (the intestine and the skin). Although the changes in the expressions of fish HAMP2s in response to immune challenge are still unclear because of different on their expression tissues, levels, time and even bacteria, which are quite specific to fish species, our finding suggests *Tp*HAMP2 gene might be involved in both systemic and mucosal innate immunity in *T. pardalis*.

In summary, we purified and characterized a novel hepcidin type 2-like AMP (*Tp*HAMP2) from the skin mucus and characterized its gene encoding the *Tp*HAMP2 precursor protein. Sequence comparison and phylogenetic analysis showed the *Tp*HAMP2 was classified into fish HAMP2 class, nevertheless the peptide had distinctive structures including the cleavage site between the proprotein and the active peptide and some AA sequences in the mature peptides. r*Tp*HMAMP2 produced in heterologous expression system displayed a broad spectrum and potent antibacterial activity, which reflect its distinctive AA sequence in the mature peptide. Thus, identification of *Tp*HAMP2 might be of importance in the framework of discovering the fish hepcidins, especially type 2s, and in providing noteworthy insight into its gene and its expression and into the innate immunity as well as mucosal immunity associated with their evolutionary history in fish species.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.017.

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