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Functional analysis and gene expression profiling of extracellular cathepsin Z in red sea bream, *Pagrus major*

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ABSTRACT

Cathepsin Z (*CTSZ*) is a lysosomal cysteine protease that is known to be involved in the maintenance of homeostasis and the biological mechanisms of immune cells. In this study, we have confirmed the tissue specific expression of the cathepsin Z (*PmCTSZ*) gene in *Pagrus major*, and confirmed its biological function after producing recombinant protein using *Escherichia coli* (*E. coli*). Multiple sequence alignment analysis revealed that the active site of the cysteine proteases and three *N*-glycosylation sites of the deduced protein sequence were highly conserved among all of the organisms. Phylogenetic analysis revealed that *PmCTSZ* was included in the clusters of *CTSZ* and the cysteine proteases of other bony fish and is most closely related to Japanese flounder *CTSZ*. *PmCTSZ* was distributed in all of the tissues from healthy red sea bream that were used in the experiment and was most abundantly found in the spleen and gill. Analysis of mRNA expression after bacterial (*Edwardsiella piscicida*: *E. piscicida* and *Streptococcus iniae*: *S. iniae*) or viral (red seabream iridovirus: RSIV) challenge showed significant gene expression regulation in immune-related tissues, but they maintained relatively normal levels of expression. We produced recombinant *PmCTSZ* (*rPmCTSZ*) using an *E. coli* expression system and confirmed the biological function of extracellular *rPmCTSZ* in vitro. We found that bacterial proliferation was significantly inhibited by *rPmCTSZ*, and the leukocytes of red sea bream also induced apoptosis and viability reduction.

1. Introduction

The cathepsins are a group of lysosomal hydrolases that degrade a wide range of proteins and are ubiquitous in organisms. The cathepsins have been classified as follows based on sequence alignments and their traditional functions: serine protease cathepsins (cathepsin A and G), aspartic protease cathepsins (cathepsin D and E) and cysteine protease cathepsin (cathepsins B, C, F, H, K, L, O, S, V, X/Z and W) [1].

Cysteine cathepsin proteases, which are stable in acidic cellular compartments, are well known proteases of intra- and extracellular proteins [2–4]. These enzymes have important functions in tumour progression and metastasis [5–7]. In addition, they have also been reported to play an important role in physiological processes including antigen presentation, hormone activation, natural killer cell development, inflammatory processes, proliferation and migration of immune cells [8–10]. In the fish, cathepsins B and L are bacteriocytes that are involved in the nonspecific immunity of Japanese eels [11]. Cathepsin S has also been reported to have a possible role in antimicrobial immunity in the red drum [12].

Cathepsin Z (*CTSZ*), also known as cathepsin X and P, is a lysosomal cysteine protease. *CTSZ* shows the unique characteristics of an integrin binding motif of a pro-form (RGD: Arg-Gly-Asp) and a mature form (ECD: Glu-Cys-Asp), which is cleaved and removed after activation [13,14]. *CTSZ* has been reported to play a role in the progression and metastasis of tumours [15–17]. It is also involved in the proliferation, maturation, migration and adhesion of immune cells; for example, *CTSZ* activates the β2 integrin receptor, which is involved in the activation, proliferation and phagocytosis of T lymphocytes [18,19]. Moreover, previous studies have suggested that *CTSZ* influences haematopoietic stem and progenitor cell trafficking in the bone marrow [20].

This multifunctional enzyme, *CTSZ*, has been reported in a variety of ways, but information about it is still lacking in the fish. To date, the molecular characterization and functional analysis of *CTSZ* has been reported only in some teleosts, including carp and rainbow trout

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[21–23]. In addition, we still have no information about the sequence of *CTSZ* cDNA in the red sea bream (*PmCTSZ*).

The red sea bream (*Pagrus major*) is an economically important marine fish species in Japan and South Korea. Damage caused by disease and environmental-related problems leads to enormous economic losses due to death or growth delays, and various studies are needed to understand and control these.

The aims of this study were to clone and identify the *PmCTSZ* gene from red sea bream. The *PmCTSZ* mRNA expression was analysed under healthy conditions and in various tissues after pathogen challenge. Finally, based these results and information from previous studies, we demonstrated the biological and immunological activity of a recombinant *PmCTSZ* (*rPmCTSZ*).

2. Materials and methods

2.1. Experimental fish, virus and bacterial strain

The red sea breams (weight: 173.2 ± 31.1 g, body length: 22.4 ± 0.9 cm) were provided by Gyeongsangnam-do Fisheries Resources Research Institute (Tongyeong, Republic of Korea) and maintained at 21 ± 1 °C in aerated seawater. We confirmed the presence or absence of disease by clinical examination before use and euthanized the fish with benzocaine (Sigma-Aldrich, USA) before sample collection.

For the pathogen challenge test, *Streptococcus iniae* (*S. iniae*) FP5228, *Edwardsiella piscicida* (*E. piscicida*) FSW910410 and red sea bream iridovirus (RSIV) were obtained from the Fish Pathology Division of the National Institute of Fisheries Science (Busan, Republic of Korea).

2.2. Sequence analysis and phylogenetic analyses

An open reading frame (ORF) containing the sequence of *PmCTSZ* was identified from the liver samples from bacteria infected red sea bream by next generation sequencing (NGS) analysis. Sanger sequencing was performed to confirm the integrity of the cDNA sequence. The nucleotide sequences and predicted amino acid sequences of *PmCTSZ* were analysed by the BLAST algorithm of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Based on the amino acid sequences, the signal peptide, characteristic domain and *N*-glycosylation sites were predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), Expert Protein Analysis System PROSITE Scan tool (http://prosite.expasy.org) and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. The molecular mass and theoretical isoelectric point were calculated using the ProtParam tool of ExPASy (http://web.expasy.org/protparam/). Multiple sequence alignment was performed with the DNAMAN program version 10 (Lynnon Biosoft, Canada). A phylogenetic tree was constructed with molecular evolutionary genetics analysis (MEGA) version 6.0 using the neighbour-joining method by bootstrapping with 2,000 replicates.

2.3. Quantitative real-time PCR analysis

2.3.1. PmCTSZ expression analysis in various tissues

After acclimation of the fish, the total RNA was extracted from tissues including trunk kidney, head kidney, liver, stomach, spleen, skin, muscle, intestine, eye, brain, heart and gill with a TRIzol-based (RNAiso Plus) reagent (Takara, Japan) according to the manufacturer's instructions. Recombinant DNase I (Takara) was used to remove contaminating genomic DNA. The concentration and purity of the total RNA samples were calculated from measurements obtained by a NanoVue spectrophotometer (GE Healthcare, UK). The cDNA synthesis was carried out using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. The diluted cDNA was used as a template for quantitative real-time PCR (RT-qPCR) with a DICE Real-Time System Thermal Cycler (Takara) using SYBR premix Ex Taq™ (Takara) and specific primers (Table 1). The relative mRNA expression levels were calculated according to the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method and normalized to the elongation factor 1 alpha of red sea bream (*PmEF-1α*). The primer sequences of *PmEF-1α* are shown in Table 1.

Table 1
Primer sequences used in this study.

Usage	Primer name	Primer sequence (5′–3′)
RT-qPCR (control)	PmEF-1α (F)	CCTTCAAGTACGCCTGGGTG
	PmEF-1α (R)	CTGTGTCCAGGGGCATCAAT
RT-qPCR	PmCTSZ (F)	ACGTCGGACTGAAGACTGCT
	PmCTSZ (R)	TACTGGGGAATGTGCTGGTT
Recombinant protein	rPmCTSZ (F)	GGATCCGATGCTGCCCAAATCCTGGGACTG
	rPmCTSZ (R)	GTCGACCATGTAGGACTTGGGGACGATTGG

* The restriction enzyme sites are underlined.

2.3.2. PmCTSZ expression in tissues during pathogen infection

To investigate the expression profiles of *PmCTSZ* during the host defence against bacterial infection, the healthy red sea breams were randomly divided into three groups and injected intraperitoneally with pathogenic *S. iniae* (1×10^5 CFU/fish), *E. piscicida* (1×10^5 CFU/fish) or RSIV (1×10^6 copies/fish), respectively. The animals were maintained in aerated seawater at 23 ± 1 °C throughout the experiment. Five fish from each group were randomly selected at hours (0, 1 and 12) and days (1, 3, 5 and 7) post-infection (hpi and dpi), and then the fish were sacrificed using benzocaine. After sacrifice, the immune-related tissues (whole kidney, gills, liver and spleen) were taken from each individual fish. Total RNA extraction, cDNA synthesis and RT-qPCR were conducted as described above. All data are reported as the *PmCTSZ* mRNA levels relative to that of the *PmEF-1α* gene mRNA and are expressed as the means ± SDs.

2.4. Expression and purification of recombinant PmCTSZ (rPmCTSZ)

The ORF of *PmCTSZ* was amplified by PCR with specific primers (Table 1) and cloned into the pET-22b(+) vector (Novagen, Germany) via the restriction sites *Bam*HI and *Sal*I, which was then transformed into BL21 (DE3) *E. coli* (Novagen). The transformed cells were cultured in Luria Bertani (LB) broth supplemented with 100 μg/mL ampicillin at 37 °C with shaking at 150 rpm and grown until they reached an optical density of 1.8–2.0 at 600 nm (OD_{600}), at which point protein expression was induced using isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 0.5 mM at 20 °C. The induced cells were harvested by centrifugation, were resuspended in denaturation buffer (10 mM Tris–HCl, 8 M urea and 0.1% SDS, pH 8.0), and then were sonicated on ice. The lysate was loaded onto a Ni-NTA affinity chromatography column (QIAGEN, Germany) and finally washed with 40 mM imidazole buffer. For the elution of *rPmCTSZ*, the column was subjected to 500 mM imidazole buffer. The eluted *rPmCTSZ* was analysed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250 (Biosesang, Republic of Korea). Refolding of the *rPmCTSZ* was achieved by dialyzing against TGE buffer (0.15 M Tris–HCl, 0.02 M NaCl, 0.4 M L-arginine, 1 mM EDTA, 0.8 mM KCl, 2 mM reduced glutathione, 0.2 mM oxide glutathione and a 3–1 M urea gradient, pH 8.0) at 4 °C. The concentration of *rPmCTSZ* was measured by the Warburg-Christian assay using a NanoVue spectrophotometer (GE Healthcare).

2.5. Test of inhibition of bacterial growth

The bacteria (*E. piscicida* and *S. iniae*) were first cultured in BHI medium to an OD_{600} of 0.8–1.0 at 27 °C then diluted to 1×10^4 CFU/

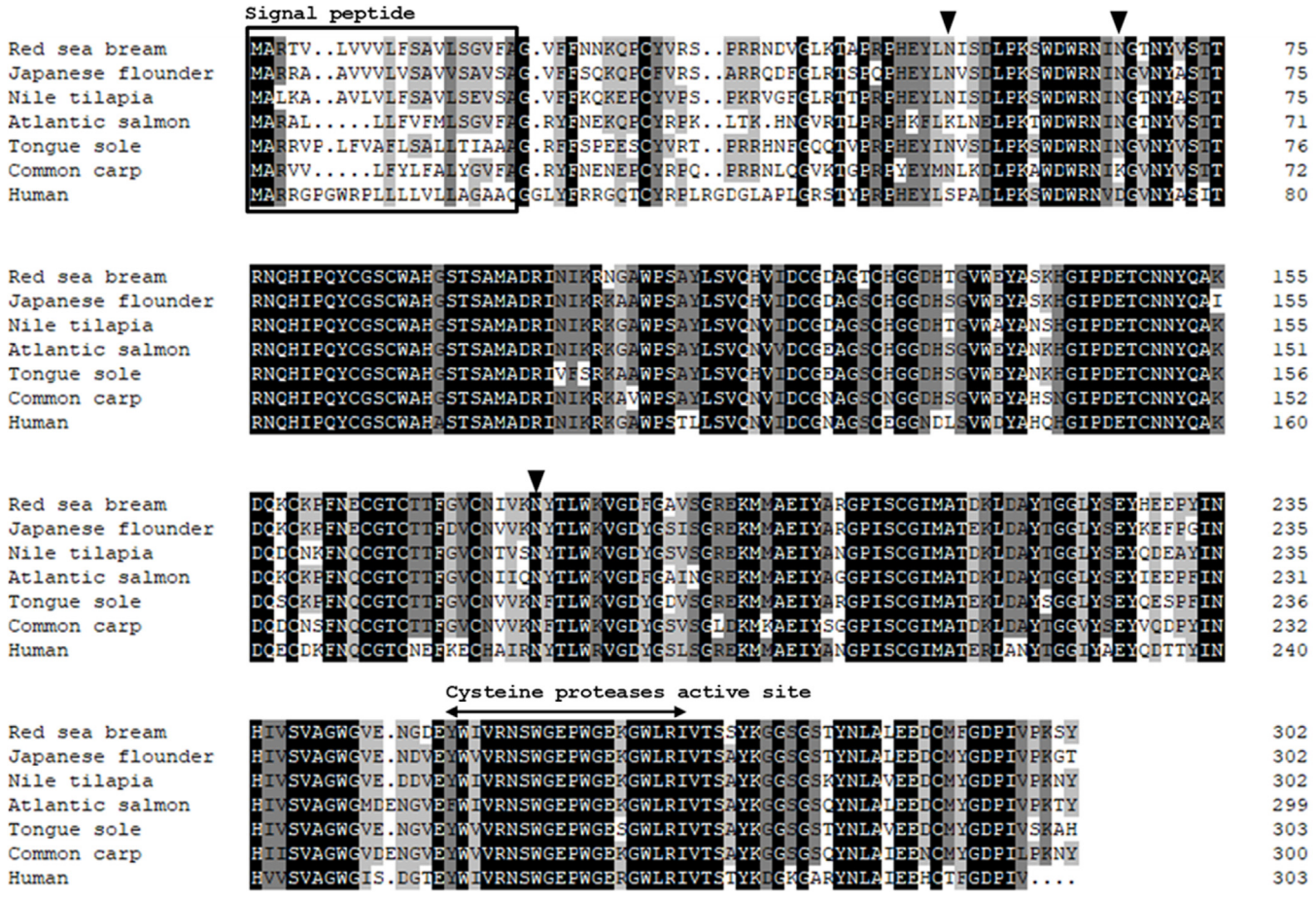

Fig. 1. Multiple alignments of deduced amino acid sequences of *PmCTSZ* with other known cathepsin Z sequences. This analysis is based on the following sequence data: Japanese flounder (XP_019942181), Nile tilapia (XP_003441550), Atlantic salmon (XP_014022687), tongue sole (XP_008318331), common carp (AAX51298) and human (AAH42168). The signal peptide, cysteine proteases active site and three predicted *N*-glycosylation sites are indicated by the box, arrow and a symbol (▼), respectively. Black boxes: identity = 100%; Grey boxes: 75% ≤ identity < 100%; Light grey boxes: 50% ≤ identity < 75%.

mL in BHI medium. One hundred microliters of the diluted bacterial inoculum were added to each well of a 96 well plate. Then, we added either 100 μL of *rPmCTSZ* (100, 50 or 25 μg/mL) or bovine serum albumin (BSA; 100, 50 or 25 μg/mL) diluted in PBS followed by incubation at 27 °C for 1 h, and aliquots of the dilutions were plated on BHI agar plates. The results were estimated by counting the CFUs on the BHI agar plates.

2.6. WST-1 leukocyte viability assay

The effect of *rPmCTSZ* on leukocyte viability was detected by WST-1 assay. The peripheral blood leukocytes (PBLs) were separated by gradient centrifugation using a gradient of 34% and 53% Percoll (Sigma-Aldrich) in whole blood, and then red blood cell lysis buffer (Sigma-Aldrich) was used to remove the remaining red blood cells. The prepared PBLs (10^6 cells/mL) were incubated with various concentrations of *rPmCTSZ* or BSA (25, 50 and 100 μg/mL) in 96 well plates at 5% CO_2 for 2 h. Then, 10 μL of Water-Soluble-Tetrazolium-salt (WST-1) reagent (QIAGEN) was added to each well and the cells were incubated for 1 h at 22 °C. The OD value of each well was measured using a Victor 3 microplate reader (PerkinElmer, USA) at a wavelength of 440 nm.

2.7. Cell cycle assay

The effect of *rPmCTSZ* on the cell cycle of leukocytes was confirmed using flow cytometry analysis of the cell cycle distribution based on DNA content. The prepared PBLs (10^6 cells/mL) were incubated with *rPmCTSZ* or BSA at 5% CO_2 for 2 h and, then fixed in 70% ethanol overnight at −20 °C. Cells were collected by centrifugation and the resuspended cells were incubated with an RNase A solution (100 μg/ml ribonuclease A (Sigma-Aldrich) in PBS) and the supernatant was discarded. The cells were resuspended in PBS and then incubated with a PI solution (100 μg propidium iodide (Sigma-Aldrich), 150 mM NaCl and 10 mM potassium phosphate buffer, pH 7.6) for 10 min in the dark at room temperature. The cell cycle distribution of the granulocytes was measured by an Accuri™ C6 flow cytometer (BD Biosciences, USA).

2.8. Statistical analysis

All samples were analysed in triplicate; the results are reported as the mean ± standard deviation (SD) and statistical analysis was performed using SPSS software version 19 (IBM, USA). *PmCTSZ* expression in tissues during pathogen infection were assessed using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. The bacterial growth test, WST-1 assay and cell cycle assay were analysed using the independent two-sample *t*-test (*$p < 0.05$ and **$p < 0.01$).

3. Results

3.1. Sequence characterization and identification of PmCTSZ

The *PmCTSZ* cDNA contained a 912 bp ORF (GenBank accession No. MK034467) that was predicted to encode a 303 amino acid (aa)

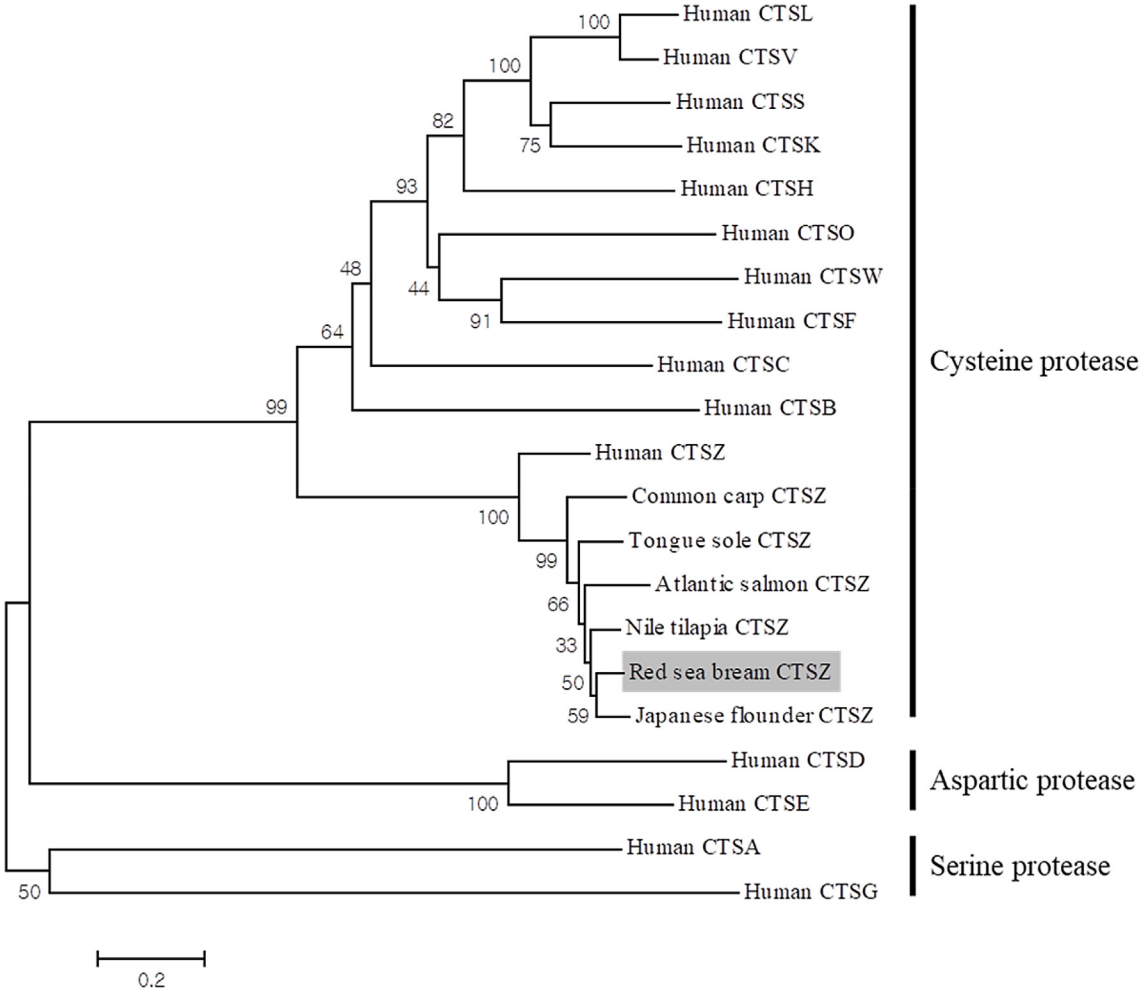

Fig. 2. The phylogenetic tree based on the neighbour-joining method of *PmCTSZ* and other known cathepsin homologues. The scale bar indicates a branch length of 0.2. Numbers were bootstrap percentile from 2,000 replicates. GenBank accession numbers are as follows: Human *CTSL* (AAI42984), Human *CTSV* (BAA25909), Human *CTSS* (AAC37592), Human *CTSK* (NP_000387), Human *CTSH* (EAW99143), Human *CTSO* (CAA54562), Human *CTSW* (AAC32181), Human *CTSF* (AAD41790), Human *CTSC* (AAL48195), Human *CTSB* (AAH95408), Human *CTSZ* (AAH42168), Common carp *CTSZ* (AAX51298), Tongue sole *CTSZ* (XP_008318331), Atlantic salmon *CTSZ* (XP_014022687), Nile tilapia *CTSZ* (XP_003441550), Japanese flounder *CTSZ* (XP_019942181), Human *CTSD* (AAA51922), Human *CTSE* (AAA52300), Human *CTSA* (AAH00597) and Human *CTSG* (EAW66006).

protein, with a predicted molecular weight of 33.7 kDa and a theoretical isoelectric point of 6.5. *PmCTSZ* has a predicted signal peptide, a cysteine protease active site and three *N*-glycosylation sites located at 1–20, 251–270 aa and Asn (53, 67 and 179), respectively. The multiple sequence alignment with *CTSZ* of different species showed that *PmCTSZ* shared the highest identities with Japanese flounder (86.5%), followed by identity with Nile tilapia (85.4%), Atlantic salmon (83.5%), tongue sole (81.6%), common carp (77.8%) and human (67.8%) (Fig. 1). A phylogenetic tree was further constructed based on the deduced amino acid sequences of the *CTS* genes of other species. The *CTS* genes were divided into three subgroups: a cysteine protease branch, an aspartic protease branch and a serine protease branch. The *PmCTSZ* was located in the *CTSZ* group and showed the closest relationship with the Japanese flounder (Fig. 2).

3.2. Tissue expression of PmCTSZ in healthy conditions and after pathogens challenge

In healthy red sea bream, *PmCTSZ* mRNA displayed the highest expression levels in the spleen (17.4-fold) and gill (16-fold) compared to the liver (Fig. 3).

When analysing the gene expression after the pathogen challenge, *PmCTSZ* expression was significantly regulated in the gills, kidney, liver and spleen after *S. iniae*, *E. piscicida* or RSIV infection (Fig. 4). After *S. iniae* challenge, it decreased significantly at 3 dpi and then significantly increased at 7 dpi in the gills. On the other hand, in the kidney, liver and spleen, it increased at 1 hpi and then decreased to maintain a steady expression level. After the *E. piscicida* challenge, it was significantly increased at 1 hpi and 7 dpi in the gills and decreased at 1 hpi in the kidney, liver and spleen. After the RSIV challenge, there was no significant expression change in the gills, but it was significantly higher at 7 dpi in the kidney. In the liver and spleen, at 1 hpi and 3 dpi it was significantly decreased, after which the level of expression returned to and remained at the baseline level of expression.

3.3. Antibacterial activity of rPmCTSZ

Recombinant proteins of cathepsin Z (*rPmCTSZ*) were prepared from *E. coli* to analyse the biological and immunological functions of the in red sea bream. The identity and purity of the recovered *rPmCTSZ* were confirmed by 15% SDS-PAGE analysis (Supplementary Fig. 1). The growth of both *E. piscicida* and *S. iniae* were significantly inhibited by 100 μg/mL *rPmCTSZ* in a dose dependant manner (Fig. 5).

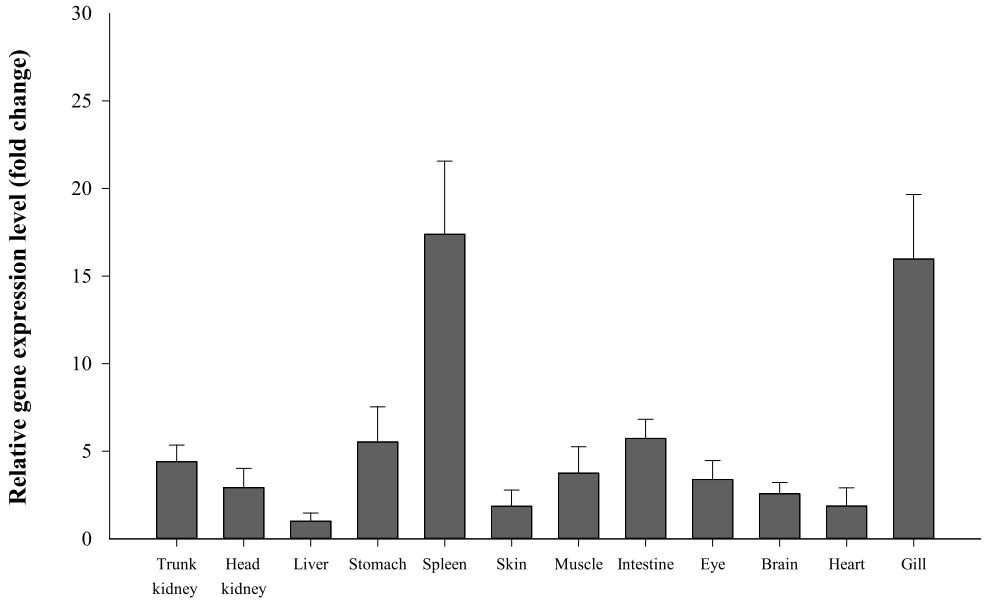

Fig. 3. Gene expression analysis of P*mCTSZ* gene by quantitative real-time PCR in various tissues of healthy red sea bream. The *PmEF-1α* gene was used to normalise the RT-qPCR results. Expression levels are shown as fold increases relative to the value in liver. All data are presented as the mean ± SD from five independent cDNA samples with three replicates per sample.

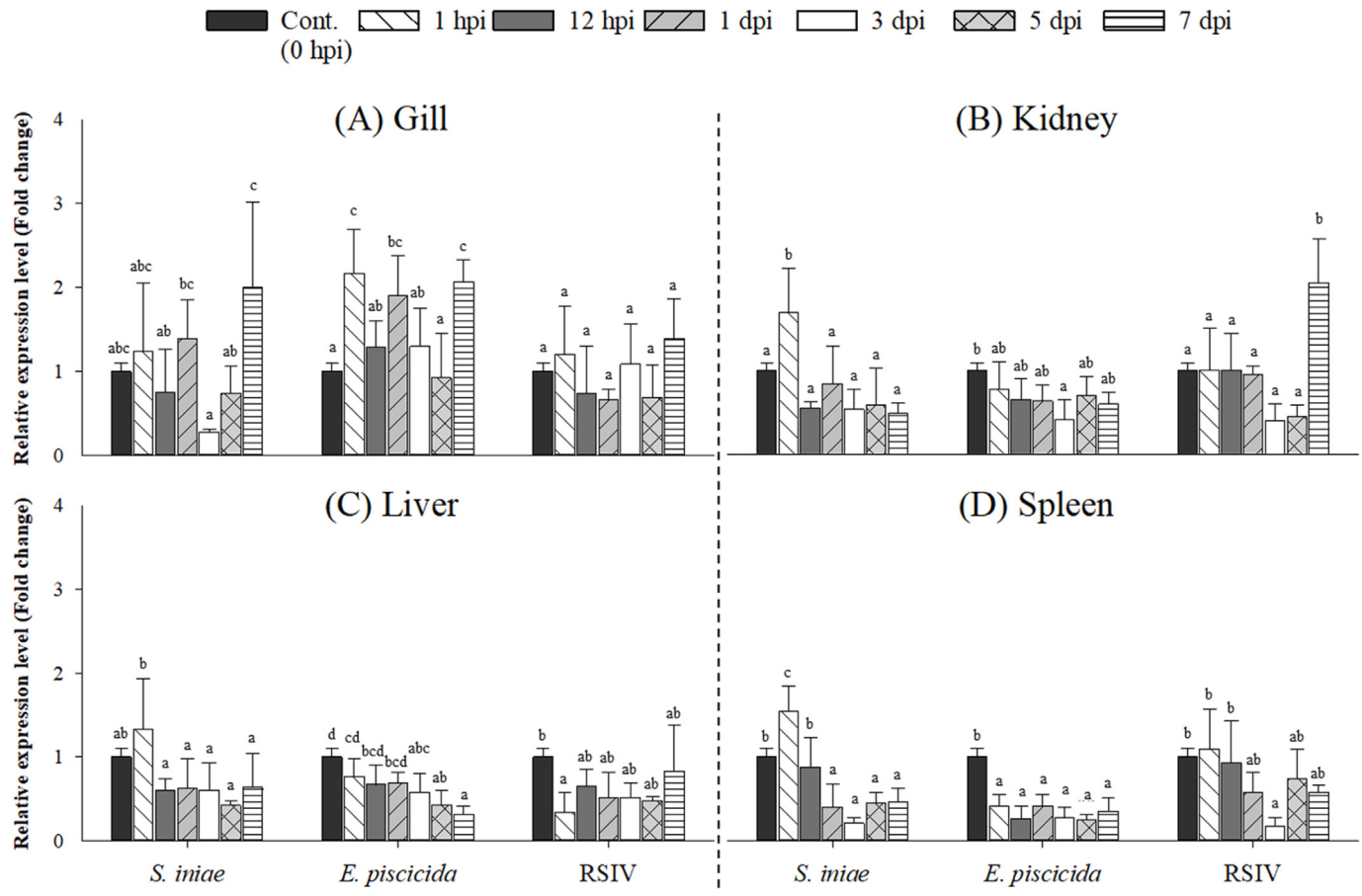

Fig. 4. Expression analysis using RT-qPCR of *PmCTSZ* mRNA in the gill, kidney, liver and spleen of red sea bream after infection with *Edwardsiella piscicida*, *Streptococcus iniae* or red sea bream iridovirus (RSIV). *PmCTSZ* was quantified relative to that of the *PmEF-1α* gene. Gene expression and its significance are represented as the mean ± SD (N = 5). Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) versus the control (0 h).

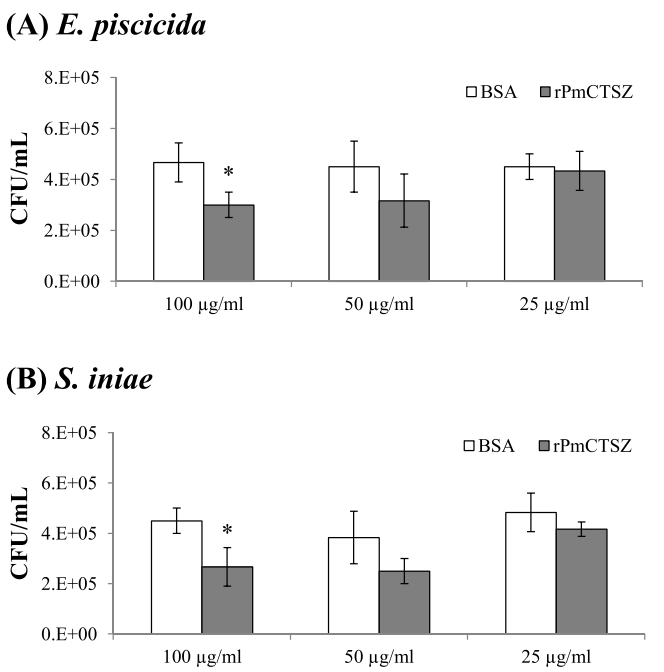

Fig. 5. Antibacterial activity of *rPmCTSZ* against (A) *Edwardsiella piscicida* and (B) *Streptococcus iniae*. Bacteria were incubated with different concentrations of *rPmCTSZ* or BSA at 27 °C. Data are the mean of three independent assays and are shown as the mean ± SD. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) compared to the control (BSA).

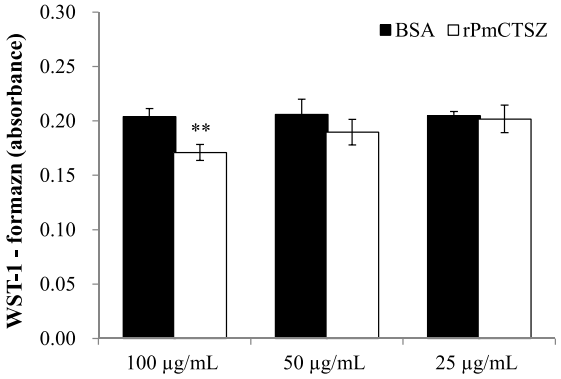

Fig. 6. Effect of *rPmCTSZ* on leukocyte viability as detected by WST-1 assay. Red sea bream leucocytes were treated with various concentrations of *rPmCTSZ* for 2 h. The results are the mean of three independent assays and are shown as the mean ± SD. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) versus the control (BSA).

3.4. Effect of rPmCTSZ on leukocyte viability

To assess the effect of *rPmCTSZ* on leukocyte viability, we examined cell growth using the WST-1 assay (Fig. 6). After 2 h of *rPmCTSZ* treatment, the formation of formazan was significantly reduced in the 100 μg/mL group compared to the control group (BSA), and it was confirmed that the cell activity of PBLs was reduced. These results also confirmed a concentration-dependent pattern.

3.5. Effect of rPmCTSZ on the cell cycle of leukocytes

To evaluate the efficacy of *rPmCTSZ* in affecting the cell cycle of leukocytes in vitro, PBLs were incubated with *rPmCTSZ* or BSA for 2 h (Fig. 7). As a result, the granulocytes in the sub-G1 phase were significantly increased in a concentration-dependent manner, and the cells in the G0/G1 phase were significantly decreased at high concentrations of 100 and 50 μg/mL *rPmCTSZ*. In addition, S phase granulocytes were significantly decreased in the group treated with 100 μg/mL *rPmCTSZ*.

4. Discussion

Cathepsin is classified into four categories based on the essential amino acid residues of the active site, the activity of the optimal pH range, amino acid sequence similarity, and similarity responses to inhibitors [1]. Cysteine cathepsin is synthesized as an inactive proenzyme, and mature cysteine proteases are regulated by the endogenous protein inhibitors, cystatin, tyropine and sppyrin and are physiologically maintained and delicately regulated [24].

Multiple alignment analysis showed that *PmCTSZ* has highly conserved three potential *N*-glycosylation sites across other species of *CTSZ* (Fig. 1). Glycosylation is an important post-translational modification in which the sugar moiety is attached to the protein and it is found in approximately 50% of all proteins. It is important in providing a recognition epitope that activates protein folding, cell-to-cell adhesion, stability, secretion and innate immune systems, and the *N*-glycosylation site is known to be important for the activation and regulation of cysteine protease [25–27]. However, our results are different from the *CTSZ* results for the previously reported carp (two *N*-glycosylation sites) [23], which may affect expression or functional differences.

Cysteine cathepsin protease efficiently degrades various substrates as a proteinase in acidic cell compartments such as lysosomes and endosomes but also performs various functions both inside and outside the cell [28–30]. In carp, *CTSZ* mRNA was the most highly expressed in the kidney and was also expressed in the testis, liver and spleen [23]. The expression level of *PmCTSZ* was also high in immune-related tissues, the spleen, gill, kidney and intestine (Fig. 3). *CTSZ* has been reported to be highly expressed in antigen presenting cells (APCs) [31,32], and has been reported to act as a lysosomal cysteine protease that hydrolyses self- and exogenous proteins in APCs [19]. These results suggest that *PmCTSZ* may play an important role in immune function and the maintenance of homeostasis.

The expression of *PmCTSZ* mRNA in the gills, kidneys, liver and spleen was confirmed after pathogen injection (Fig. 4). In previous studies, the expression and activity of *CTSZ* mRNA was increased in a dose- and time-dependent manner in lipopolysaccharide (LPS) -stimulated mouse-derived cells, and abalone *CTSZ* mRNA was upregulated by *V. parahaemolyticus*, *L. monocytogenes* or LPS [33,34]. However, the *CTSZ* mRNA of the olive flounder was maintained at a constant level in the kidney and spleen tissues after LPS stimulation [35]. Our results showed that expression of *PmCTSZ* mRNA was partially altered after a pathogen challenge, but no large expression changes were observed when comparing overall expression levels. It is thought that *PmCTSZ* is strictly maintained at a constant level in the red sea bream even though its expression can be affected by pathogen infection.

Previous reports have confirmed the antimicrobial activity of cysteine protease against *Bacillus* sp. and *Burkholderia* sp [36,37]. We confirmed the report of the limited function of *CTSZ* in teleosts and performed functional analyses by constructing *PmCTSZ* as a recombinant protein using an *E. coli* system. The addition of extracellular *rPmCTSZ* significantly inhibited the growth of fish pathogenic bacteria (Fig. 5). These results confirmed the possibility that *rPmCTSZ* plays an antimicrobial activity in the extracellular regions of the red sea bream.

A variety of tetrazolium compounds have been used for the detection and status determination of cells, among which WST-1 is reduced on the outside of the cell to measure cell viability and to test the cytotoxic effect of the compound [38]. We have shown that when extracellular *rPmCTSZ* was co-cultured after addition to PBLs of red sea bream, a significant cell activity decline in PBLs was confirmed by the indirect measurement method (the WST-1 method) (Fig. 6). We also measured the cell cycle of granulocytes by staining DNA with propidium iodide. As a result, sub-G1 phase cells were significantly increased in extracellular *rPmCTSZ* in a concentration-dependent fashion, and

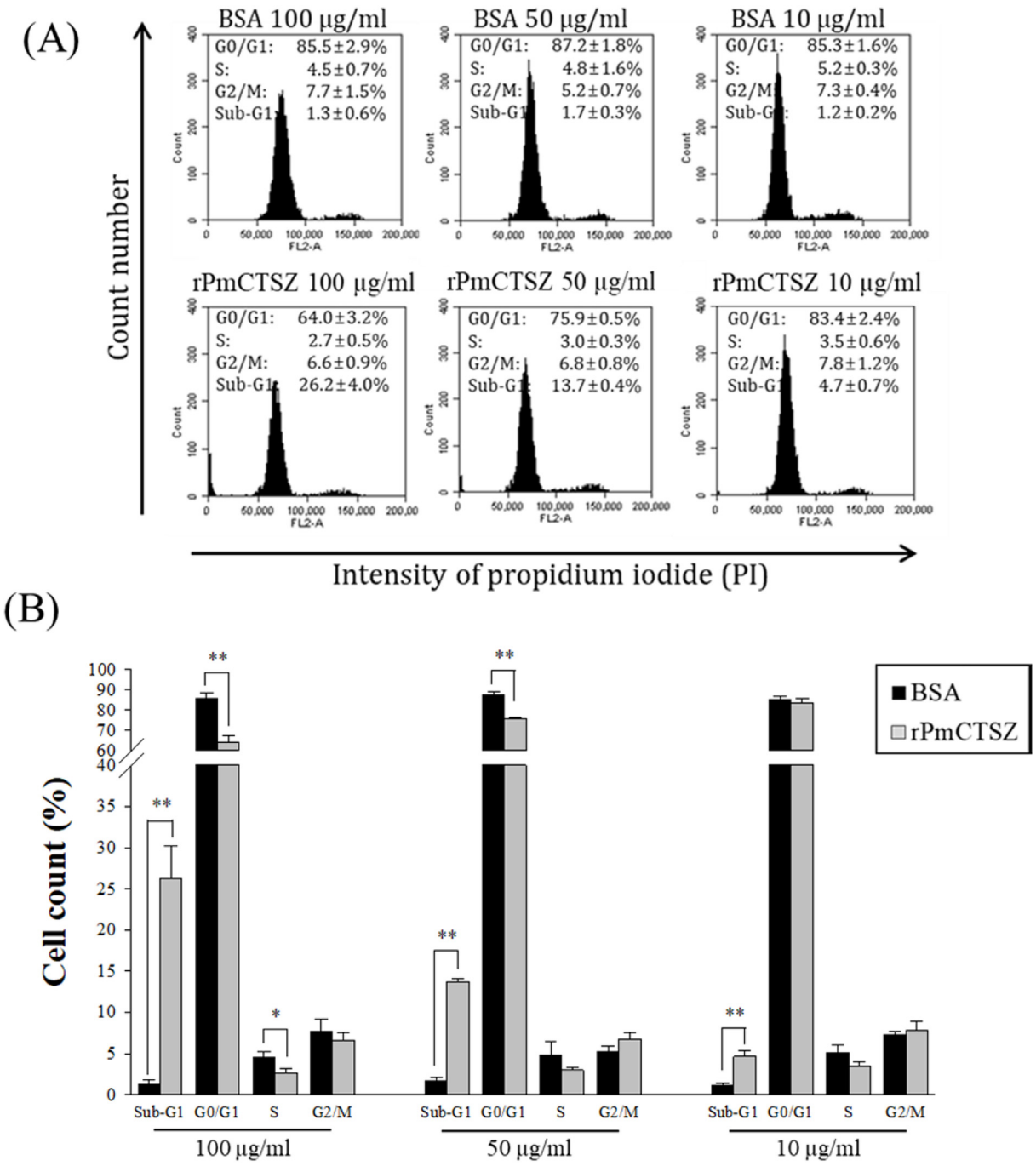

Fig. 7. Effect of *rPmCTSZ* on cell cycle analysis of leukocytes based on flow cytometry analysis. Red sea bream peripheral blood leukocytes (granulocyte) were incubated with *rPmCTSZ* or BSA for 2 h. (A): Flow cytometry histogram plots showing fluorescent intensity in granulocytes and (B): the data were transformed into a bar chart. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) versus the control (BSA).

G0/G1 and S phase cells were significantly decreased (Fig. 7). The increase of sub-G1 and the decrease of G0/G1 and S phase cells means increased cell death, which means that *rPmCTSZ* induces the death of red sea bream granulocytes in the extracellular space. Cysteine cathepsins not only directly affect extracellular proteolysis on the cell surface but also affect intracellular signalling pathways, and various functions besides extracellular proteolysis have been reported [39]. Furthermore, *CTSZ* is involved in the proliferation of T lymphocytes by activating the < beta > 2 integrin receptor (LFA-1) and inhibition of the proliferation of peripheral blood mononuclear cells by activating of the lymphocyte growth factor (Mac-1) [19,40].

In addition, *CTSZ* is known to regulate phagocytosis and T lymphocyte activity by interacting with lymphocytes in a β2 integrin-dependent manner [19,41,42]. In *CTSZ*-deficient mice, the efficiency of the antigen-presenting cells decreases and they secrete IL-1β, which could confirm the role of *CTSZ* in the propagation of IL-1β-induced neuroinflammation [43]. These results suggest that *CTSZ* is an immunologically important protein in teleosts.

Thus, we found that extracellular *rPmCTSZ* reduced bacterial growth and induced apoptosis of red sea bream PBLs, and we confirmed the antimicrobial activity of *rPmCTSZ* against fish pathogenic bacteria in vitro. In previous studies, lysosomal cysteine cathepsin was shown to be a non-specific enzyme with no definite substrate-recognition site [44], and this result also confirmed the character of the non-specific

enzyme activity of the cysteine cathepsin, *PmCTSZ*. It is also known that cathepsin plays an important role in the physiology and pathophysiology processes in lysosomes as well as in the extracellular, cytoplasmic and nuclear compartments. Our results also suggest that *PmCTSZ* plays an important physiological role as a protease both intracellularly and extracellularly in red sea breams.

Acknowledgements

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Appendix A. Supplementary data

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