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Short communication

Molecular characterization, expression and functional analysis of cystatin C in Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

Cystatins are natural tight-binding reversible inhibitors of cysteine proteases found in a wide arrange of organisms. Studies have shown that cystatins play important roles under both physiological and pathological conditions in mammals. However, much less is known about fish cystatins. In this study, we described the identification and analysis of the gene encoding cystatin C in Japanese flounder (*Paralichthys olivaceus*). This gene had a high homology with the sequence of cystatin C in many fish species and had a signal peptide and three conserved functional sites. The results of qRT-PCR showed that the gene was highly expressed in the liver. Lipopolysaccharide, peptidoglycan and polyinosinic-polycytidylic acid all increased its expression after stimulation. Functional analysis showed that the recombinant *P. olivaceus* cystatin C purified from *Escherichia coli* had cysteine protease inhibitory activity and could inhibit bacterial growth by binding to bacteria. Meanwhile, rPocystatin C could up-regulate the expression of cytokines tumor necrosis factor α and interleukin 10. These results indicated that cystatin C of *P. olivaceus* might be considered to have the similar immunomodulatory function to mammalian cystatin.

1. Introduction

Protease inhibitors have inhibitory effects on proteolytic enzymes and play crucial roles in the innate immune response of vertebrates. Cystatins are a family of cysteine protease inhibitors that contain at least one cystatin domain. They interact with and neutralize cathepsin cysteine proteases, hence possessing pivotal functions in various physiological processes, such as protein degradation, arthritis, tumor invasion, metastasis and antigen presentation [1–3]. Based on structural differences, cystatins are classified into several major types. Type 1 cystatins (cystatin A and B) are cytoplasmic proteins composed of ~100 amino acid residues that are ~11 kDa in size. They lack signal peptide, disulfide bonds and carbohydrates. Type 2 cystatins, including cystatins C, D, E/M, F, G, S, SA and SN, are extracellular polypeptides of ~120 amino acid residues. They contain two conserved disulfide bonds and localize mainly to the extracellular matrix [4,5]. One of the features shared by these two types of cystatins is the tertiary structure of a "cystatin fold" formed by a five-stranded β-sheet wrapped around a central α-helix [6,7].

Up to now, many studies on fish cystatins have been performed in *Oncorhynchus keta* [8], *Cyprinus carpio* [9], *Oncorhynchus mykiss* [10], *Acipenser sinensis* [11], *Pseudosciaena crocea* [12], *Scophthalmus maximus* [13], *Oplegnathus fasciatus* [14] and *Clarias batrachus* [15], revealing valuable results: Some of these cystatin-like proteins may respond to bacterial challenge or are involved in other immune processes [12–14]. Despite of all these efforts, the expression patterns and potential functions of teleost cystatins remain limited.

Japanese flounder (*Paralichthys olivaceus*) is an important cultured fish in China. Along with the deterioration of the culture environment, the cultured population is susceptible to several kinds of virus, bacteria and pathogens, which leads to high mortalities and poor benefits [16]. All these disadvantages let researchers to develop stocks with higher resistance to diseases. In this paper, we aimed to analyze the expression and function of *P. olivaceus cystatin C* (*Pocystatin C*). LPS, peptidoglycan (PGN) and polyinosinic-polycytidylic acid (poly I:C) stimulation was performed to detect the expression changes of *Pocystatin C* mRNA. The antibacterial effect of recombinant *P. olivaceus* cystatin C protein (rPocystatin C) was tested. Furthermore, we examined the effects of rPo-cystatin C on cytokines tumor necrosis factor α (TNF-α) and interleukin 10 (IL-10). By these experiments, we could acquire a deeper understanding about the role of cystatin C in teleost immune system.

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2. Materials and methods

2.1. Fish and cell line

P. olivaceus individuals of one-year-old were obtained from a fish farm in Yantai, Shandong Province, China, and kept at 17 °C. Three male and three female individuals were anesthetized by MS-222 (30 mg/mL) and then killed by severing the spinal cord. Brain, heart, intestine, head kidney, liver, spleen, gill and muscle of specimens were collected from each individual, immediately frozen in liquid nitrogen and stored at −80 °C for further use.

P. olivaceus gill cell line (FG-9307) was derived from gill tissue of *P. olivaceus* in 1997 by Tong et al. [17]. Cells was cultured in Eagle's minimal essential medium (EMEM, BI, Israel) supplemented with 10% (*V/V*) fetal bovine serum (BI, Israel), 1% (*V/V*) non-essential amino acids (NEAA, Gibco, US), and 100 U/mL penicillin at 24 °C.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol, treated with RNase-free DNase I (TaKaRa, Dalian, China) to degrade genomic DNA, and then frozen at −80 °C. Reverse transcription and cDNA synthesis were performed with 1 μg of total RNA and random primers using the Reverse Transcriptase M-MLV Kit (TaKaRa, Dalian, China) in accordance with the manufacturer's instructions. Quality and quantity of the cDNA were evaluated via 1.5% agarose gel electrophoresis and spectrophotometry using NanoPhotometer Pearl (Implen, Germany).

2.3. Identification of Pocystatin C cDNA and bioinformatic analysis

To acquire the *Pocystatin C* gene, the available sequences of teleosts and mammals *cystatin C* genes were obtained from NCBI and Ensembl. Then, the retrieved sequences were used as query sequences in BLAST searches. The mRNA sequence of *Pocystatin C* gene was identified using tBLASTn analysis from *P. olivaceus* transcriptome previously sequenced by our laboratory [18]. A pair of specific primers (*PocystatinC*-Fw1/Rv1, Table 1) was designed to amplify the gene. The PCR products were purified, sub-cloned and sequenced.

Multiple alignment analysis of the protein sequences was performed using MUSCLE3.8.31. The domains in Pocystatin C protein structure were predicted by SMART software, and SignalP 4.1 server was used to search canonical signal peptides. The phylogenetic tree was constructed based on the deduced amino acid sequences by using MEGA 7.0. The reliability of each node was estimated by bootstrapping with 1000 replications.

2.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was conducted on LightCycler 480 (Roche, Forrentrasse, Switzerland). The SYBR Green Master Mix (Roche, Basel, Switzerland) was used in the PCR detection system. Thermocycling consisted of an initial polymerase activation of 2 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. A pair of specific primers *PocystatinC*-RT-Fw/Rv (Table 1) was used for qRT-PCR. The *β-actin* gene was selected to normalize relative gene expression for its convincing and statistically repeatable results. All qRT-PCR experiments were performed in triplicate. Data were analyzed through the $2^{-\Delta\Delta Ct}$ method. The expression level of genes (*Pocystatin C*, *TNF-α* and *IL-10*) relative to that of β-actin was calculated. Fold changes represented the ratio of relative expression levels between experimental and control groups.

2.5. Immune challenge assay

Monolayer-cultured gill cells with good growth state were transferred to 12-well plates and cultured for 24 h. When the rate of cell coverage reached 90%–95%, LPS, PGN and poly I:C were added to each well of the experimental group to a final concentration of 50 μg/mL (the concentration was determined based on pre-experiments). The control group was added with the same concentration of PBS. Four wells of each group were sampled at every time point (0, 1, 2, 4, 8 and 12 h after stimulation). After washed 3 times in PBS, cells were sampled and stored in TRIzol at −80 °C until further use.

2.6. Construction of expression vector

The cDNA sequence (the sequence encoding the signal peptide and the transmembrane region was not included) of *Pocystatin C* was amplified by PCR with the specific primers *PocystatinC*-Fw2/Rv2 (Table 1). The reaction was performed under the following conditions: an initial denaturation step at 94 °C for 5 min, a step containing 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 68 °C and extension at 72 °C for 1 min, and an additional extension step at 72 °C for 7 min. The PCR product was digested with *Bam*HI (TaKaRa, Dalian, China) and *hind*III (TaKaRa, Dalian, China), and sub-cloned into the expression vector pET-32a (Novagen, USA) treated with the same restriction enzymes. The correct recombinant plasmid was screened by sequencing and named pET32a-Pocystatin C.

2.7. Expression and purification of rPocystatin C

The constructed plasmid pET32a-Pocystatin C was transformed into *E. coli* BL21 (DE3) and cultured overnight in Luria-Bertani (LB) broth containing kanamycin (100 mg/mL). The culture was then diluted 1:100 with LB and incubated at 37 °C for 4 h. The expression of rPocystatin C was induced by isopropyl-β-*D*-thiogalactoside (IPTG,

Table 1
List of primer sequences used in the study.

Primers	Sequence (5′–3′)	T_m (°C)	Usage
PocystatinC -Fw1	CTGCAGCCGTGCGTATATCA	58	Degenerate PCR
PocystatinC -Rv1	GAGTCCAGTACTTGTTAAAT	58	Degenerate PCR
PocystatinC -RT-Fw	AAGGACCGTGTTGTGAATG	60	qRT-PCR
PocystatinC -RT-Rv	CTCTCGCAGTTCTCTGTCTA	60	qRT-PCR
PocystatinC -Fw2	CGCGGATCCAATTTGGTGGGGGCGCCG	68	Plasmid construction
PocystatinC -Rv2	CCCAAGCTTTCTCCCCCACCAGCTGGAGGT	68	Plasmid construction
TNF-α-Fw	GTCCTGGCGTTTTCTTGGTA	58	qRT-PCR
TNF-α- Rv	CTTGGCTCTGCTGCTGATTT	58	qRT-PCR
IL-10-Fw	TTTCAAAAGCCCGTTTGCGT	60	qRT-PCR
IL-10-Rv	TTGGTTTCCTCCGTCACTCC	60	qRT-PCR
β-actin-Fw	GGTATCCTGACCCTGAAGTA	60	qRT-PCR
β-actin-Rv	CTTCTCCCTGTTGGCTTTAG	60	qRT-PCR

The restriction enzyme sites are underlined.

0.01 mmol/L). After incubation at 19 °C overnight, the bacterial cells were harvested by centrifugation at 5000 g at 4 °C for 10 min, re-suspended in PBS (30 mmol/L, pH 7.3) and disintegrated by ultrasonication on ice. The cell debris was removed by centrifugation at 12 000 g at 4 °C for 30 min, and the supernatant was loaded onto a Ni-NTA resin column (Novagen, USA). The column was successively washed with TBS (pH 7.3) containing 0, 10, 20, 30 and 40 mmol/L imidazole, respectively, and then eluted with TBS containing 200 mmol/L imidazole. The purity of recombinant protein was analyzed by SDS-PAGE on a 12% gel and stained with Coomassie Brilliant Blue R-250. To express the TRX-His-tag peptide as control, *E. coli* BL21 (DE3) cells were transformed by plasmid pET-32a (Novagen, USA) and induced with IPTG (0.1 mmol/L) at 28 °C for 8 h. The peptide was purified and processed as described above. Vivaspin® ultrafiltration spin columns (Sartorius, Germany) were used to concentrate the protein. The protein concentration was detected by BCA Protein Assay Kit (CWBIO, Beijing, China) using bovine serum albumin as standards.

2.8. Protease inhibition assay

To determine the inhibition activity of rPocystatin C, 90 μL Tris buffer (50 mM, pH 7.4) containing 0, 0.1, 0.5, 1.0, 5.0, 10.0 or 20.0 μg of rPocystatin C was incubated with 10 μL papain (0.1 μg/μL, Sigma, USA) at 28 °C for 30 min in a flat-bottomed microtiter plate. The reaction was initiated by 200 μL 0.2% (*W/V*) azocasein (Sigma, USA), and conducted at 37 °C for 2 h. The reaction was terminated by 200 μL 10% trichloroacetic acid and chilled on ice for 15 min. The precipitate was separated by centrifugation, and the absorbance (*A*) at 440 nm was measured using a spectrophotometer. Negative control data were obtained with the TRX-His-tag peptide. Each single experiment was performed in triplicate for statistical analysis. The relative activity of rPocystatin C was calculated as follows: $100 \times (1 - (A_{440}$ of rPocystatin C)/(A_{440} of control)).

2.9. Antibacterial activity assay

Oxford cup method was used to identify the antibacterial activity of rPocystatin C. *E. coli* and *Staphylococcus aureus* were inoculated in LB medium, and *Edwardsiella tarda*, *Bacillus subtilis*, *Vibrio anguillarum* and *Aeromonas hydrophila* were inoculated in MA medium. When A_{600} = 1100 μL, bacterial liquid was evenly spread on the surface of a 90-mm plate. Three Oxford cups were placed and added with 200 μL rPocystatin C and equal volumes of PBS, or TRX-His-tag peptide which served as a control. The appearance of the inhibition zone was observed to determine the antibacterial activity of the rPocystatin C after the incubation at 37 °C or 28 °C for 16–18 h.

2.10. Bacteria-binding assay

E. coli, *S. aureus*, *E. tarda*, *B. subtilis*, *V. anguillarum* and *A. hydrophila* were respectively incubated with rPocystatin C to measure the binding activity according to the procedures proposed by Chen et al. [19]. Briefly, a total of 1×10^7 bacteria were incubated with 10 mg rPocystatin C in PBS by gentle orbital rotation for 2 h at room temperature. Bacterial cells were pelleted and washed five times with 1 mL PBS buffer. The pellets were then suspended in a 100 mL reducing sample buffer (250 mmol/L Tris-HCl, pH 6.8, 10% (*W/V*) SDS, 2.5% bromophenol blue, 50% glycerol, and 5% β-mercaptoethanol) and analyzed by western blotting using rabbit anti-His tag antibody. The band was visualized using ECL Plus Kit (Beyotime, Shanghai, China), according to the manufacturer's instructions. The TRX-His-tag peptide incubated with the above bacteria was used as a negative control, while the recombinant protein without incubation was used as a positive control.

2.11. Detection of inflammatory factors

The gill cell line was used to detect the inflammatory factors after rPocystatin C stimulation. The experimental group was added with rPocystatin C and the control group was added with TRX-His-tag peptide in each well to a final concentration of 50 μg/mL. Four wells of each group were sampled at every time point (0, 1, 2, 4, 8 and 12 h after stimulation). Then cells were sampled and washed 3 times in PBS. Total RNA was extracted for cDNA synthesis. qRT-PCR was performed to detected the relative expression of *TNF-α* and *IL-10* using primers *TNF-α*-Fw/Rv and *IL-10*-Fw/Rv (Table 1) according to the procedures described above.

2.12. Statistical analysis

Data analyzed using one-way ANOVA followed by LSD test using SPSS 20.0, and $P < 0.05$ indicated the statistical significance. All data were expressed as mean ± standard error of the mean (SEM).

3. Results and discussion

3.1. Conserved functional domains in vertebrate cystatin C

The gene sequence *Pocystatin C* was identified from *P. olivaceus* transcriptome and verified by PCR. The *Pocystatin C* cDNA sequence (GenBank accession number: MH432648) consisted of a 165-nt 5′-UTR, a 790-nt 3′-UTR and an ORF of 381 nt encoding a polypeptide of 126 amino acids with a predicted molecular weight of 14.03 kDa and a theoretical isoelectric point of 8.40 (Fig. 1). The deduced amino acid sequence of Pocystatin C contained a typical cystatin domain and an N-terminal signal peptide of 18 amino acids. Additionally, Pocystatin C protein contained three conserved domains: an N-terminal G22, a Gln-X-Val-X-Gly motif (Gln64-Val-Val-Ser-Gly68) and a C-terminal PW motif (Pro114-Trp115). The three evolutionally conserved motifs are known to interact with the active sites of cysteine peptidase [20]. It was found that the major domains were conserved in Pocystatin C protein and its homologs. Four cysteine amino acids were the most conserved, which participated in the formation of disulfide bonds. Besides, three cysteine protease inhibitory sites were conserved (Fig. 1). To explore the evolutionary position of Pocystatin C, a phylogenetic tree was constructed by MEGA 7.0 based on the amino acid sequences (Fig. 2).

Fig. 1. Multiple alignment of the deduced amino acid sequences of Pocystatin C and cystatin C proteins in other vertebrates. The black and gray shadows indicate the amino acids with similarity more than 75%. Characteristic and conserved glycine (G) residues are denoted by the "G" letter. The QXVXG domain is marked with box. Asterisk indicate the cysteine residues.

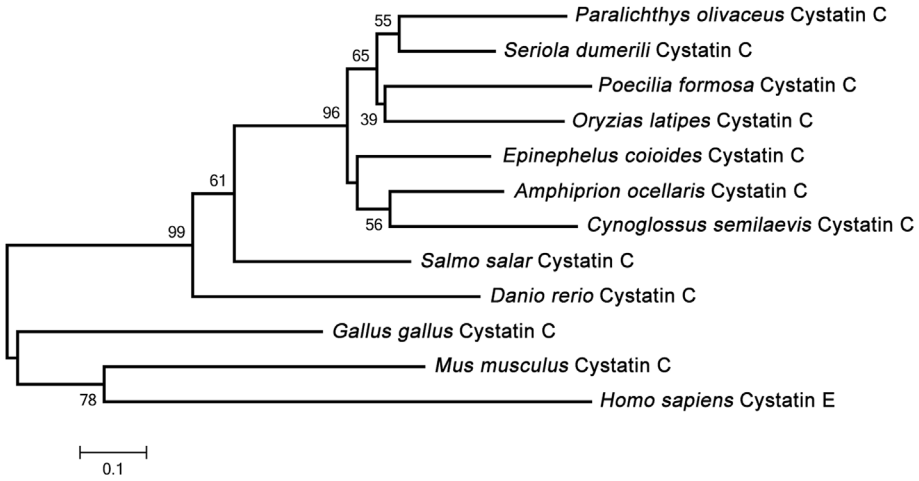

Fig. 2. Phylogenetic tree showing the relationship between Pocystatin C and other cystatin C proteins. The species and GenBank accession numbers were as follows: *M. musculus* AAB41056.1; *G. gallus* NP_990831.2; *D. rerio* NP_001026843.2; *S. dumerili* XP_022599426.1; *A. ocellaris* XP_023150415.1; *E. coioides* AMM45488.1; *S. salar* XP_014067438.1; *C. semilaevis* XP_008320464.1; *P. Formosa* XP_007546358.1; *O. latipes* XP_004077513.1; *H. sapiens* Cystatin E AAB61305.1.

Results showed that teleost cystatin C proteins, including Pocystatin C, were clustered, supporting the evolutional conservation of cystatin C proteins, which might imply their similar functions in immune responses. Compared with homologs in other vertebrates, Pocystatin C had more conserved functional domains, indicating that *Pocystatin C* might also resist the irruption of pathogenic microorganism in innate immunity.

3.2. Tissue distribution of Pocystatin C transcripts

qRT-PCR was employed to detect the expression profile of *Pocystatin C* in different tissues of *P. olivaceus*. The dissociation curve of PCR-amplified products in all the cases showed a single peak (data not shown), indicating that the amplification was specific. As shown in Fig. 3A, *Pocystatin C* mRNA was constitutively expressed at different levels in various tissues, which was consistent with previous studies in *Homo sapiens* [21], *O. keta* [8], *O. mykiss* [10] and *Collichthys lucidus* [22]. Previous research has shown that *H. sapiens* cystatin C is ubiquitously expressed in various tissues and body fluids, and plays roles in proteolysis-related biological processes [21,23,24]. Therefore the broad expression of *Pocystatin C* might be required for the regulation of proteolysis-related physiological processes in *P. olivaceus*. In addition, the transcripts of *Pocystatin C* were more abundant in liver than in other tissues, showing a tissue-differential manner. Meanwhile, it was observed that the profile of *Pocystatin C* in different tissues was different with other species reported in previous study [22]. The disparity might be caused by the various functions of cystatin C among different species and different tissues. Furthermore, the higher level of *Pocystatin C* in liver was speculated to be related with its function, since liver was believed to be an important defense organ of fish against invading microbes.

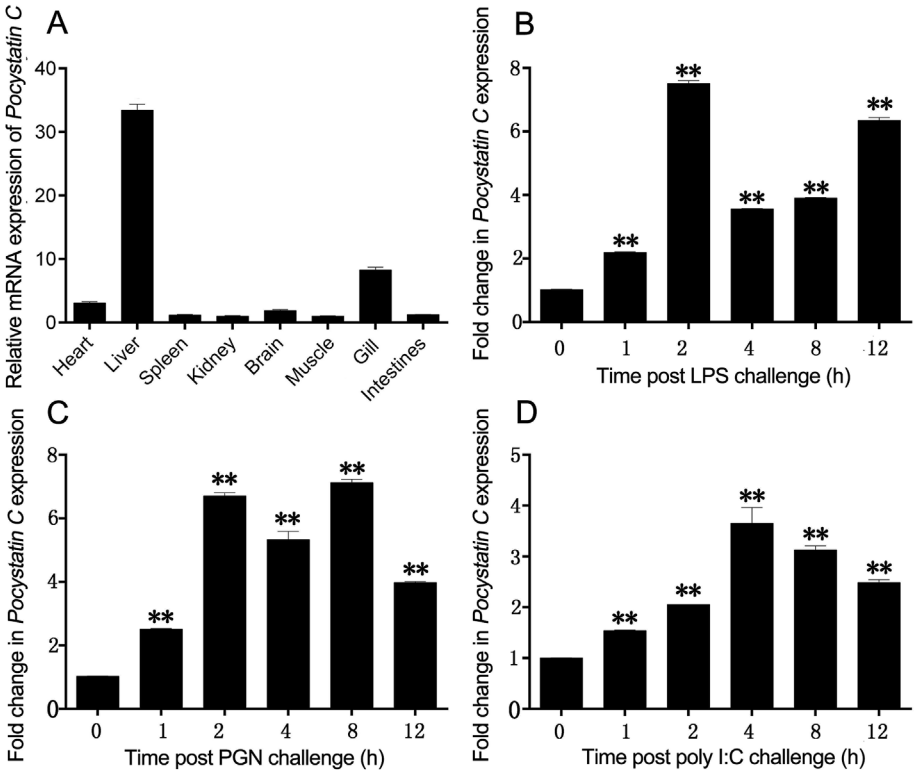

Fig. 3. Quantitative expression profiles of Pocystatin C transcript. (A) Relative expression levels of Pocystatin C in adult tissues. (B–D) Relative expression levels of Pocystatin C in FG cells following LPS, PGN or poly I:C challenge. The control group at 0 h was used as the reference sample. Data were normalized to the β-actin gene as internal control. The results are shown as mean ± SEM ($n = 3$). Asterisks indicate statistically different significance ($^*P < 0.05$, $^{**}P < 0.01$) compared with the control.

3.3. Expression changes of Pocystatin C in response to LPS, PGN and poly I:C

In order to better explore the regulation mechanism of *Pocystatin C* in innate immunity, we used LPS from Gram-negative bacteria, PGN from Gram-positive bacteria and poly I:C (a double-stranded RNA viral mimic) to stimulate the FG-9307 cells, and examined changes in *Pocystatin C* mRNA levels using qRT-PCR analysis. The challenge with LPS, PGN and poly I:C all resulted in a significant increase in the expression of *Pocystatin C* transcript. This result suggested that *Pocystatin C* gene not only was sensitive to bacterial infections but also had a certain immune response to viral infection, which was consistent with several reports on the potential immune responses of cystatin against invading microbial pathogens [25–27]. In particular, the stimulation effect of LPS (Fig. 3B) and PGN (Fig. 3C) was significant. The expression of *Pocystatin C* mRNA was significantly increased from 1 h to 12 h post stimulation ($P < 0.01$), and the peak value appeared in 2 h/12 h and 2 h/8 h post stimulation, respectively. In addition, the challenge with poly I:C also induced a significant increase in *Pocystatin C* at 1 h–12 h post stimulation ($P < 0.05$, Fig. 3D). The *Pocystatin C* level reached a peak at 4 h post stimulation, but the increase was not as obvious as that in the LPS and PGN groups. Cystatins have been described to inhibit the replication of certain viruses [28–30], although it has not yet been directly demonstrated that these effects are due to the protease-inhibitory capacity of the cystatins [31]. Our results revealed that bacterial infections might trigger a quicker and stronger *Pocystatin C* immune response than viral infection, but the antiviral mechanism remained to be further studied.

3.4. Inhibitory activities of rPocystatin C on the proteolytic activity of protease

To characterize the biochemical properties of Pocystatin C protein, a recombinant protein rPocystatin C was expressed in *E. coli*, and then purified by chromatography on a Ni-NTA resin column. SDS-PAGE analysis showed that the purified rPocystatin C yielded a single band of approximately 24 kDa (Fig. 4), matching well with the expected size. Western blot analysis showed that rPocystatin C was recognized by the anti-His tag antibody, indicating that it was correctly expressed (data now shown). According to the BCA assay, the concentration of the purified rPocystatin C was 214 μg/mL. As shown in Fig. 5, rPocystatin C was capable of inhibiting the proteolytic activity of papain in a dose-dependent manner. The inhibitory effects were enhanced with the increase of rPocystatin C concentration. It has been reported that cystatin

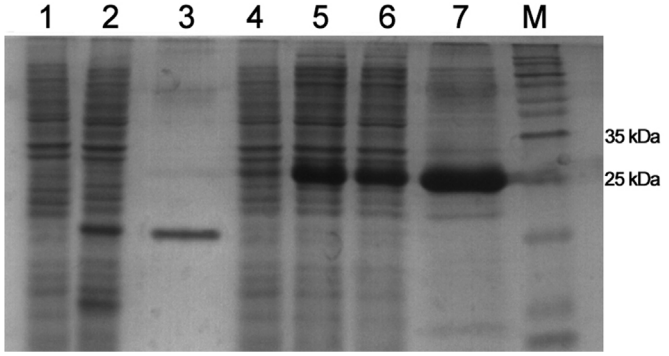

Fig. 4. Expression and purification of rPocystatin C. Lane M: molecular mass standards. Lane 1: negative control for recombinant TRX-His-tag peptide (without induction). Lane 2: total cellular extracts from IPTG-induced *E. coli* BL21 (DE3)-containing plasmid pET-32a. Lane 3: purified recombinant TRX-His-tag peptide. Lane 4: negative control for recombinant Pocystatin C (without induction). Lane 5: total cellular extracts from IPTG-induced *E. coli* BL21 (DE3)-containing expression vector. Lane 6: supernatant fluid of cellular extracts. Lane 7: purified Pocystatin C.

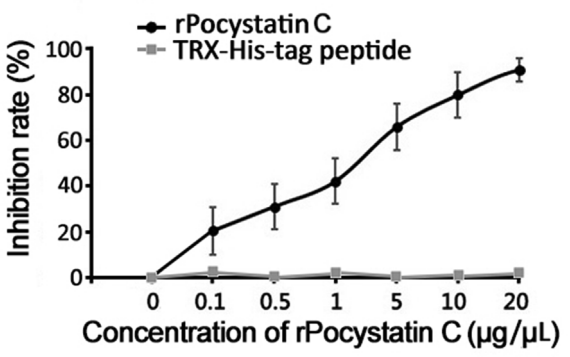

Fig. 5. Dose-dependent inhibition activity of rPocystatin C toward papain. TRX-His-tag peptide was used as negative control. The values are shown as mean ± SEM ($n = 3$).

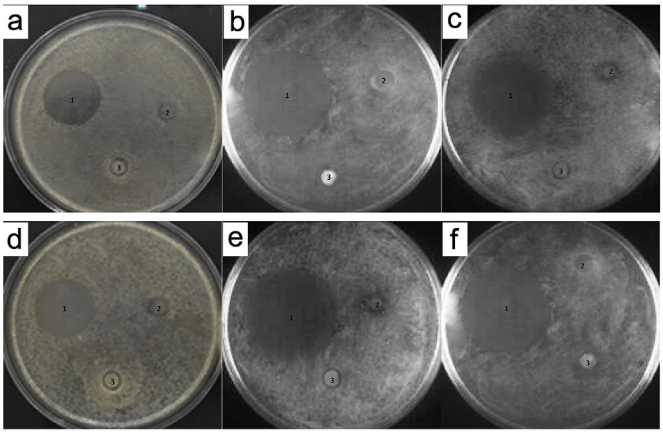

Fig. 6. Bacteriostatic action of rPocystatin C against (a) *E. coli*, (b) *E. tarda*, (c) *A. hydrophila*, (d) *S. aureus*, (e) *B. subtilis* and (f) *V. anguillarum*. rPocystatin C was added in Oxford cup 1. Recombinant TRX-His-tag peptide and PBS were respectively added in Oxford cups 2 and 3 as negative controls.

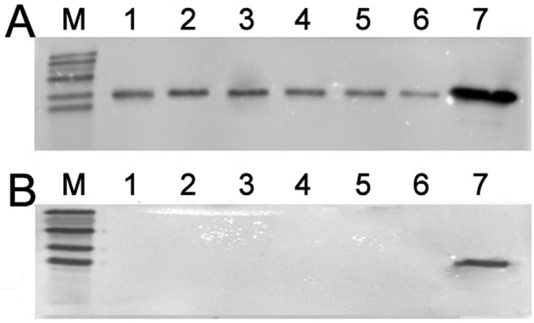

Fig. 7. Western blot analysis of rPocystatin C (A) and TRX-His-tag peptide (B) binding to bacteria. Lane M: molecular mass standards. Lanes 1–6: *E. coli*, *E. tarda*, *A. hydrophila*, *S. aureus*, *B. subtilis* and *V. anguillarum* incubated with recombined protein, respectively. Lane 7: recombined protein without incubation as a positive control.

plays an antibacterial role through the inhibition of the extracellular cysteine proteinase secreted by bacteria [25]. Taken together, it could be deduced that Pocystatin C might inhibit the activity of cysteine protease secreted by pathogenic bacteria after secreted into the extracellular matrix, thus achieving the bacteriostatic effect.

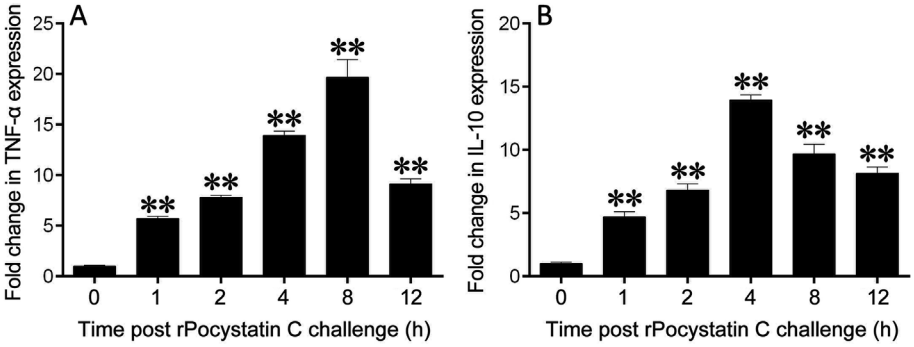

Fig. 8. mRNA expression of TNF-α (A) and IL-10 (B) in FG cells detected by qRT-PCR after rPocystatin C challenges. The control group at 0 h was used as a reference sample. Data were normalized to the β-actin gene as an internal control. The results are shown as mean ± SEM ($n = 3$). Asterisks indicate statistically significant differences ($^*P < 0.05$, $^{**}P < 0.01$) compared with the control.

3.5. The antibacterial activity of rPocystatin C

Oxford cup method was adopted to identify the antibacterial activity of rPocystatin C. As shown in Fig. 6, larger inhibition zones could be observed around the Oxford cup of rPocystatin C compared with the PBS control and TRX-His-tag peptide in the six plates, suggesting that rPocystatin C possessed an appreciable antibacterial effect against both Gram-negative bacteria (*E. coli*, *E. tarda, V. anguillarum* and *A. hydrophila*) and Gram-positive bacteria (*S. aureus* and *B. subtilis*).

3.6. Bacterial binding of rPocystatin C

To further explore the immunological functions of rPocystatin C, the protein was incubated with bacteria to measure its binding activity. As shown in Fig. 7A, rPocystatin C incubated with *E. coli*, *S. aureus*, *E. tarda*, *B. subtilis*, *V. anguillarum* and *A. hydrophila* all yielded one clear band, while no band could be detected in the negative control (Fig. 7B). The results suggested that rPocystatin C could bind to both Gram-negative and Gram-positive bacteria. Previous studies have shown that the conservative N terminal sequence of cystatins not only interacts with cysteine protease, but combines with the negative charge on the bacterial membrane [26], and further penetrates the bacterial cell membrane into the inside and inhibits the activity of cysteine protease in bacteria [32]. In this study, the immune challenge, protease inhibition, antibacterial activity and bacterial binding assays revealed that rPocystatin C recognized LPS or PGN, the chief cell wall components of bacteria, and then induced direct bacterial binding to execute its antibacterial functions.

3.7. rPocystatin C regulates the expression of inflammatory factors

Two inflammatory factors were detected after rPocystatin C stimulation in the FG cells. The result of qRT-PCR showed that the transcript level of *TNF-α* was significantly ($P < 0.01$) increased at 1 h–12 h post stimulation compared with the control group, and reached a peak at 8 h post stimulation (Fig. 8A). Meanwhile, an extremely significant increase of *IL-10* expression was also detected at 1 h–12 h compared with the PBS control group, and the peak value appeared at 4 h ($P < 0.01$, Fig. 8B). These results showed that rPocystatin C could up-regulate the expression of inflammatory factors. TNF-α is a classical pro-inflammatory cytokine, mainly produced by T-cells, mast cells and macrophages when they respond to parasites, viruses and other stimulants [33]. Contrarily, IL-10 is a classical anti-inflammatory cytokine, which suppresses gene expression and synthesis of pro-inflammatory cytokines [34,35]. Here, the significant up-regulation of *TNF-α* expression supported a pro-inflammatory function of rPocystatin C. At the same time, a coordinated up-regulation of *IL-10* expression which correlates with inhibition of pro-inflammatory TNF-α, was also observed, strongly suggesting that rPocystatin C might have an immunomodulatory role in *P. olivaceus* inflammatory response.

In summary, *Pocystatin C* has a conserved structure like its homologs in other vertebrates, and expressed in all the examined tissues of *P. olivaceus*. Its expression level is up-regulated after LPS, PGN or poly I:C stimulation. The recombinant Pocystatin C has cysteine protease inhibitory activities and inhibits bacterial growth. It also causes the increase of cytokines TNF-α and IL-10. Therefore, *Pocystatin C* may have the immunomodulatory functions similar to mammalian cystatin.

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References

[1] V. Turk, W. Bode, The cystatins: protein inhibitors of cysteine proteinases, FEBS Lett. 285 (1991) 213–219.
[2] K. Sol-Church, G.N. Picerno, D.L. Stabley, J. Frenck, S. Xing, G.P. Bertenshaw, et al., Evolution of placentally expressed cathepsins, Biochem. Biophys. Res. Commun. 293 (2002) 23–29.
[3] G.N. Rudenskaya, D.V. Pupov, Cysteine proteinases of microorganisms and viruses, Biochemistry Biokhimiia 73 (2008) 1–13.
[4] N. Kopitar-Jerala, The role of cystatins in cells of the immune system, FEBS Lett. 580 (2006) 6295–6301.
[5] V. Turk, V. Stoka, D. Turk, Cystatins: biochemical and structural properties, and medical relevance, Front. Biosci. 13 (2008) 5406–5420.
[6] W. Bode, R. Engh, D. Musil, U. Thiele, R. Huber, A. Karshikov, et al., The 2.0 A X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases, EMBO J. 7 (1988) 2593.
[7] M.T. Stubbs, B. Laber, W. Bode, R. Huber, R. Jerala, B. Lenarcic, et al., The refined 2.4 A X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction, EMBO J. 9 (1990) 1939–1947.
[8] M. Yamashita, S. Konagaya, Molecular cloning and gene expression of Chum Salmon cystatin, J. Biochem. 120 (1996) 483–487.
[9] Y.J. Tsai, G.D. Chang, C.J. Huang, Y.S. Chang, F.L. Huang, Purification and molecular cloning of carp ovarian cystatin, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 113 (1996) 573–580.
[10] F. Li, H. An, T.A. Seymour, C.S. Bradford, M.T. Morrissey, G.S. Bailey, et al., Molecular cloning, sequence analysis and expression distribution of rainbow trout (*Oncorhynchus mykiss*) cystatin C, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 121 (1998) 135–143.
[11] J. Bai, D. Ma, H. Lao, Q. Jian, X. Ye, J. Luo, et al., Molecular cloning, sequencing, expression of Chinese sturgeon cystatin in yeast *Pichia pastoris* and its proteinase inhibitory activity, J. Biotechnol. 125 (2006) 231–241.
[12] S. Li, J. Ao, X. Chen, Molecular and functional characterization of a cystatin analogue in large yellow croaker (*Pseudosciaena crocea*), Mol. Immunol. 46 (2009) 1638–1646.
[13] P.P. Xiao, Y.H. Hu, S. Li, Scophthalmus maximus cystatin B enhances head kidney macrophage-mediated bacterial killing, Dev. Comp. Immunol. 34 (2010) 1237–1241.
[14] H.K. Premachandra, I. Whang, Y.D. Lee, S. Lee, Z.M. De, M.J. Oh, et al., Cystatin B homolog from rock bream *Oplegnathus fasciatus*: genomic characterization, transcriptional profiling and protease-inhibitory activity of recombinant protein, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 163 (2012) 138–146.
[15] Vindhya Mohindra, K.R. Tripathi, Akanksha Singh, et al., Molecular characterization and expression analysis of a novel cystatin-like gene in a hypoxia-tolerant Indian catfish, *Clarias batrachus* [Linnaeus, 1758], Fish Shellfish Immunol. 34 (2013) 683–687.
[16] E.M. Moustafa, M. Naota, T. Morita, N. Tange, A. Shimada, Pathological study on the scuticociliatosis affecting farmed Japanese flounder (*Paralichthys olivaceus*) in Japan, J. Vet. Med. Sci. 72 (2010) 1359–1362.
[17] S.L. Tong, H. Li, H.Z. Miao, The establishment and partial characterization of a

continuous fish cell line FG-9307 from the gill of flounder *Paralichthys olivaceus*, Aquaculture 156 (1997) 327–333.
[18] W. Wang, J. Wang, F. You, L. Ma, X. Yang, J. Gao, et al., Detection of alternative splice and gene duplication by RNA sequencing in Japanese flounder, *Paralichthys olivaceus*, G3 (Bethesda) 4 (2014) 2419–2424.
[19] L. Chen, J. Yan, W. Sun, C. Sui, J. Qi, Y. Du, et al., A zebrafish intelectin ortholog agglutinates both Gram-negative and Gram-positive bacteria with binding capacity to bacterial polysaccharide, Fish Shellfish Immunol. 55 (2016) 729–736.
[20] T. Dieckmann, L. Mitschang, M. Hofmann, J. Kos, V. Turk, E.A. Auerswald, et al., The structures of native phosphorylated chicken cystatin and of a recombinant unphosphorylated variant in solution, J. Mol. Biol. 234 (1993) 1048–1059.
[21] M. Abrahamson, I. Olafsson, A. Palsdottir, M. Ulvsbäck, A. Lundwall, O. Jensson, et al., Structure and expression of the human cystatin C gene, Biochem. J. 268 (1990) 287.
[22] W. Song, K.J. Jiang, F.Y. Zhang, M. Zhao, L.B. Ma, Molecular cloning and gene expression analysis of cystatin C-like proteins in spinyhead croaker *Collichthys lucidus*, Genet. Mol. Res. 15 (2016).
[23] M. Abrahamson, A. Barrett, G. Salvesen, A. Grubb, Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids, J. Biol. Chem. 261 (1986) 11282–11289.
[24] L.A. Bobek, M.J. Levine, Cystatins–inhibitors of cysteine proteinases, Crit. Rev. Oral Biol. Med. Off. Publ. Am. Assoc. Oral Biol. 3 (1992) 307.
[25] E. Wesierska, Y. Saleh, T. Trziszka, W. Kopec, M. Siewinski, K. Korzekwa, Antimicrobial activity of chicken egg white cystatin, World J. Microbiol. Biotechnol. 21 (2005) 59–64.
[26] I. Björk, E. Pol, E. Raubsegall, M. Abrahamson, A.D. Rowan, J.S. Mort, Differential changes in the association and dissociation rate constants for binding of cystatins to target proteinases occurring on N-terminal truncation of the inhibitors indicate that the interaction mechanism varies with different enzymes, Biochem. J. 299 (Pt 1) (1994) 219–225.
[27] B. Korant, T. Towatari, M. Kelley, J. Brzin, B. Lenarcic, V. Turk, Interactions between a viral protease and cystatins, Biol. Chem. Hoppe Seyler 369 (Suppl) (1988) 281.
[28] L. Björck, P. Åkesson, M. Bohus, J. Trojnar, M. Abrahamson, I. Olafsson, et al., Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor, Nature 337 (1989) 385–386.
[29] L. Björck, A. Grubb, L. Kjellén, Cystatin C, a human proteinase inhibitor, blocks replication of herpes simplex virus, J. Virol. 64 (1990) 941.
[30] A.R. Collins, A. Grubb, Cystatin D, a natural salivary cysteine protease inhibitor, inhibits coronavirus replication at its physiologic concentration, Oral Microbiol. Immunol. 13 (2010) 59–61.
[31] J. Travis, J. Potempa, H. Maeda, Are bacterial proteinases pathogenic factors? Trends Microbiol. 3 (1995) 405–407.
[32] L. Wang, Q. Yuan, S. Chen, H. Cai, M. Lu, Y. Liu, et al., Antimicrobial Activity and Molecular Mechanism of the CRES Protein, PLoS One 7 (2012) e48368.
[33] B. Beutler, C.V. Huffel, Unraveling function in the TNF ligand and receptor families, Science 264 (1994) 667–668.
[34] C.A. Dinarello, Role of pro- and anti-inflammatory cytokines during inflammation: experimental and clinical findings, J. Biol. Regul. Homeost. Agents 11 (1997) 91.
[35] A. Koj, Termination of acute-phase response : role of Some cytokines and anti-inflammatory drugs, Gen. Pharmacol. Vasc. Syst. 31 (1998) 9–18.