JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

Cloning, Expression, Characterization, and Tissue Distribution of Cystatin C from Silver Carp (*Hypophthalmichthys molitrix*)

Ran Li,[‖] Xiaoqian Tan,[‖] Shuhong Li,[*] Yu Jin, Song Li, Shulei Li, Timo M. Takala, and Per. E. J. Saris

Cite This: *J. Agric. Food Chem.* 2021, 69, 5144−5154 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Cystatins are proteins, which inhibit cysteine proteases, such as papain. In this study, the 336-bp *cystatin C* gene (family II, Hm*CysC*) of silver carp (*Hypophthalmichthys molitrix*) was cloned and expressed in *Escherichia coli* BL21 (DE3). Hm*CysC* encodes the mature peptide of cystatin C (HmCystatin C), with 111 amino acids. A typical QXXXG motif was found in HmCystatin C and it formed a cluster with *Cyprinus carpio* and *Danio rerio* cystatin C in the phylogenetic tree. Quantitative real-time polymerase chain reaction analysis indicated that Hm*CysC* was transcribed at different levels in five tested tissues of silver carp. Following purification with Ni^{2+}− nitrilotriacetic acid agarose affinity chromatography, HmCystatin C displayed a molecular weight of 20 kDa in sodium dodecyl sulfate polyacrylamide gel electrophoresis. Purified HmCystatin C had strong inhibitory effects toward the proteolytic activity of papain. Immunochemical staining with anti-HmCystatin C antibody showed that HmCystatin C was widely distributed in silver carp tissues. These results collectively demonstrated the properties of HmCystatin C, providing information for further studies of cystatins from fish organisms.

KEYWORDS: *silver carp (Hypophthalmichthys molitrix), cystatin C, cloning, transcription level, tissue distribution, protein quantification*

1. INTRODUCTION

Cysteine proteases (e.g., papain, cathepsin B, and cathepsin L) are found in various organisms, and they play a crucial role in numerous physiological or pathological processes, such as protein turnover, immunity, and osteolysis.[1,2] As the endogenous inhibitors of cysteine proteases, the cystatins superfamily mediates specific inhibition effects against the proteolytic activity of cysteine proteases. Generally, the cystatins superfamily consists of three categories: family I stefins, family II cystatins, and family III kininogens.[3] Family II cystatins are secretory proteins with a theoretical molecular weight of 13−14 kDa (approximately 120 amino acids).[4] Typical members of family II cystatins are cystatin C, D, S, SN, SA, and chicken egg white cystatin. Family II cystatins are reversible inhibitors of cysteine proteases, with the signal peptide and disulfide bonds found at their C-terminus.[5]

Due to their unique relationships with cysteine proteases, studies about the promising functions of cystatins are now emerging. Previous studies on terrestrial animals have demonstrated that family II cystatins are involved in antibacterial process,[6] immune regulation,[7] cancer inhibition,[8] and embryonic development.[9] However, only a few studies have investigated the activity or function of piscine cystatins (family II). Wang and Huang (2002) found that the cystatin of carp (*Cyprinus carpio*) ovary bound and agglutinated spermatozoa through electrostatic interaction to prevent polyspermy.[10] Li et al. (2009) found that the cystatin analogue from the large yellow croaker (*Pseudosciaena crocea*) may function in the inflammatory response since they observed that the gene transcription level of cystatin was upregulated after polyinosinic/polycytidylic acid (poly I/C) and bacterial vaccine stimulation.[11] Moreover, *in vivo* administration of cystatin to the large yellow croaker upregulated the gene transcription levels of TNF-α2 and interleukin-10.

In the field of food science, the cysteine proteases cathepsin B and cathepsin L have been studied to illuminate their roles in deteriorating the integrity of fish muscle[12,13] and the texture of postmortem fish fillets[14,15] and surimi products.[16,17] By using cystatins to inhibit cathepsins B/L, scientists have demonstrated the potential of cystatins as antisoftening agents. Chinese sturgeon (*Acipenser sinensis*) cystatin could prevent fish muscle softening by inhibiting the proteolytic activity of cathepsin B.[12] For postmortem fish fillets, Carvajal-Rondanelli and Lanier (2010) found that recombinant soya cystatin could diffuse into the muscle cells of Pacific halibut (*Hippoglossus stenolepis*) and thus reduce proteolytic activity and prevent fillet softening.[18] The 18 kDa stefin purified from glassfish (*Liparis tanakai*) eggs inhibits cathepsins B/L more effectively than egg white cystatin (family II), and the results indicated that this glassfish stefin may be more suitable in preventing the deterioration of the surimi-gelling process.[19] Akpinar and An (2005) reported that recombinant cystatin from soya inhibits cathepsin L activity and the autolytic activity of Pacific whiting (*Merluccius productus*) surimi.[20] More robust evidence has been presented in our previous work: the gel-breaking force and gel strength of silver carp surimi are significantly enhanced

Received: January 18, 2021
Revised: April 9, 2021
Accepted: April 13, 2021
Published: April 21, 2021

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154

after adding recombinant silver carp cystatin (60 μg/g) (unpublished data).

The aforementioned studies have provided basic information for the application of piscine cystatins in fish muscle, fish fillets, and byproduct processing after clarifying their properties and distribution. However, at this point, only fish-origin cystatins (family II) of chum salmon (*Oncorhynchus keta*),[21] common carp,[22,23] rainbow trout (*Oncorhynchus mykiss*),[24] Chinese sturgeon,[12] olive flounder (*Paralichthys olivaceus*),[4] and large yellow croaker[11] have been cloned or expressed. No studies on silver carp cystatins have been performed, and their biochemical properties need to be investigated. Silver carp is one of the major freshwater fish in China with an economic importance. The yield of silver carp is the second highest in China (3.81 million tons in 2019).[25] The price of silver carp is relatively low, and its fillet contains abundant proteins and tastes well after cooking. Due to all these advantages, silver carp is the ideal aquatic processing material and consumed largely as a chilled fresh-cut fish fillet and frozen surimi or its derivatives. However, issues caused by endogenous cysteine proteases are still the obstacle to silver carp processing. Liu et al. (2008) revealed that the endogenous heat-stable cathepsins B/L degrade the abundant myofibrillar protein in silver carp surimi upon heating (50−70 °C);[26] thus, the gel structure of surimi is irreversibly damaged. The activities of cathepsins B/L remain high even after washing treatment of the surimi raw material.[26] Moreover, it has been well accepted that cathepsins contribute to the early postmortem disintegration of fish fillet myofibrils,[27] and Zhang et al. (2019) found the myofibril segmentation and increased cathepsins B/L activities, exacerbating the softening of silver carp fillet.[28] For both the processing industry and consumers, such softening is not desirable since the firm texture is of great importance to fish fillet and surimi products.[15,17]

In this study, the Hm*CysC* gene encoding the mature peptide of silver carp cystatin C (HmCystatin C) was cloned and analyzed. Subsequently, expression, purification, and characterization were performed. Polyclonal antibody against HmCystatin C was prepared and used in immunohistochemistry and Western blot. We also investigated the expression pattern of HmCystatin C at both the gene transcription and protein expression levels, aiming to elucidate its amount and distribution in silver carp tissues.

2. MATERIALS AND METHODS

2.1. Primers, Vectors, and Strains. The primers used in this study are listed in Table S1 (Supporting Information). Cystatin F/R primers with *Bam*HI/*Xho*I restriction sites were used for Hm*CysC* gene amplification and qCystatin-F/R primers were used in quantitative real-time polymerase chain reaction (qRT-PCR) analysis. A pMD19-T vector purchased from Takara Bio Inc. (Shiga, Japan) was applied in the TA cloning of Hm*CysC*. The vector for protein expression was pET-30a (Novagen, Madison, WI, USA), in which genetic tools such as the start codon, 6× His-tag, and selection marker were provided. The bacterial hosts *E. coli* DH5α and *E. coli* BL21 (DE3) were grown in an LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) at 37 °C. For transformant selection, the medium was supplemented with either ampicillin (100 μg/mL) or kanamycin (50 μg/mL).

2.2. Fish Sacrificing and Tissues Sampling. Silver carps (1591 ± 86 g, $n = 6$) were purchased from a fish farming company, and half of them were females carrying eggs. Before sacrifice, silver carps were kept for 20 min in 4% urethane solution (Sigma-Aldrich, St. Louis, MO, USA) for anaesthetization. Once the fish had lost consciousness, the concentration of the anesthetic was increased to an overdose level to euthanize them. Six types of tissues, including eggs, heart, muscle, spleen, small intestine, and hepatopancreas, were immediately collected and stored in liquid nitrogen.

2.3. Cloning of the Hm*CysC* Gene. The cloning strategy was to obtain the target gene fragment amplified from the cDNA of silver carp, after which TA cloning would be performed. Total RNA was first isolated from the eggs of silver carp and then reverse-transcribed into double-stranded cDNA using the PrimeScript RT Reagent Kit (Takara Bio Inc.). Using Cystatin F/R primers, PCR was conducted following this thermal cycling profile: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 40 s, and a final extension step at 72 °C for 5 min. After PCR, the fragment size was initially visualized in 1.5% agarose gel. Before ligation, the PCR product was purified using a DNA Purification Kit (Tiangen Biotechnology, Beijing, China). TA cloning was then performed to join the Hm*CysC* gene fragment and the pMD19-T vector. The ligation mixture was transferred into *E. coli* DH5α, and recombinants were screened with 100 μg/mL ampicillin in the LB medium. Next day, colonies were picked and verified by PCR for *E. coli* DH5α carrying the pMD19-T-Hm*CysC* plasmid. Positive transformants were sent for gene sequencing (Invitrogen Biotechnology Co., Ltd., Shanghai, China).

2.4. Sequence Analysis. The DNA sequence obtained was analyzed by DNAMAN 8.0 (Lynnon Biosoft, San Ramon, CA, USA) for its amino acid translation, physicochemical property prediction, and sequence alignment. A phylogenetic tree was constructed using MEGA 7.0 via the neighbor-joining method.[29] Using the amino acid sequence, the secondary structure of HmCystatin C was predicted by the online tool PSIPRED 4.0 (http://bioinf.cs.ucl.ac.uk/psipred/), and the tertiary structure was predicted by the online tool SWISS-MODEL (http://swissmodel.expasy.org/).

2.5. QRT-PCR Detection of Hm*CysC* Gene Transcription in Silver Carp Tissues. The gene transcription level of Hm*CysC* in different tissues of silver carp was determined by qRT-PCR. The qCystatin-F/R primers were designed for the Hm*CysC* gene, and 18S rRNA-F/R primers[30] were used for the internal control gene. Total RNA was isolated from the heart, muscle, spleen, small intestine, and hepatopancreas of silver carp, and cDNA was then synthesized as described in Section 2.3. Following the protocol of the Real Master Mix SYBR Green I kit (Tiangen Biotechnology), primers and cDNA of each tissue were mixed and used for qRT-PCR. Data were collected using a CFX96 Touch RT-PCR Detection System (Bio-Rad, Hercules, CA, USA) and analyzed by the $2^{-\Delta\Delta CT}$ method.[31] Briefly, the transcription ratio of Hm*CysC* was calculated using the 18S *rRNA* gene as the baseline. The final transcription levels of Hm*CysC* in all tissues were indicated as fold changes.

2.6. Heterologous Expression and Purification of HmCystatin C. Recombinant plasmid pMD19-T-Hm*CysC* was digested with *Bam*HI/*Xho*I restriction enzymes and the enriched gene fragment of Hm*CysC* was then recovered from the agarose gel. The same procedures were applied on the expression vector pET-30a. Ligation was performed between linearized pET-30a and Hm*CysC* for 16 h at 16 °C. The purified ligation product was transformed into *E. coli* BL21 (DE3) cells, which were screened with 50 μg/mL kanamycin in the LB medium. Transformants containing the plasmid pET-30a-Hm*CysC* were validated by plasmid isolation and double-enzyme digestion. Once the correct transformants were identified, expression and purification of sliver carp cystatin were initiated following the protocols from Sambrook et al. (1989).[32] In brief, *E. coli* BL21 (DE3) cells carrying pET-30a-Hm*CysC* were incubated in a selection broth supplemented with 1 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) (37 °C, 6 h). Afterward, cells were collected by centrifugation (5000×*g*, 5 min) and treated with lysozyme (33 μg/mL, 37 °C, 30 min) (Sigma-Aldrich). Freezing/thawing was then applied to lyse the cells. Hereafter, gradient urea solutions (1, 5, 8 mol/L) were applied to dissolve HmCystatin C. The last step was to purify HmCystatin C by Ni^{2+}−nitrilotriacetic acid (NTA) agarose affinity chromatography (1.6 × 5 cm, Qiagen, Hilden, Germany), and eluted samples were concentrated by the Amicon Stirred Cells system (Merck Millipore, Burlington, MA, USA). Samples collected during the processes of

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154

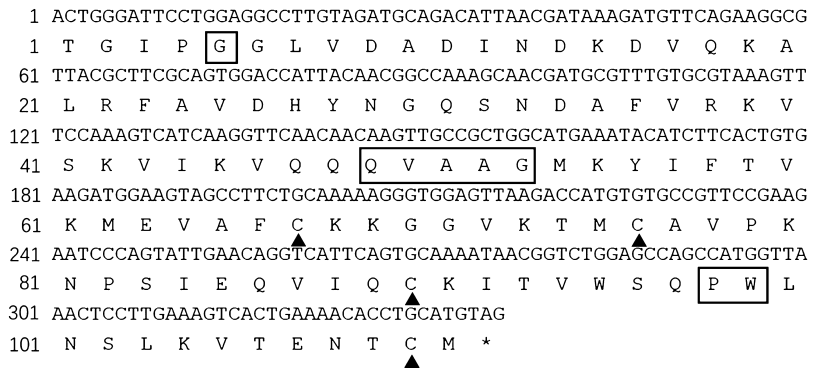

Figure 1. DNA sequence of Hm*CysC* and its deduced amino acid sequence. The gene is 336 bp in size (including stop codon TAG, marked with an asterisk), encoding a protein of 111 amino acid residues. The conserved domains: active site residue G (Gly^5), QXXXG motif (QVAAG in HmCystatin C), and PW residues (Pro^{98}–Trp^{99}) are marked with black rectangles. Four cysteine residues at C-terminus are indicated by black triangles.

expression and purification were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Purified HmCystatin C was later subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) for evaluating its purity. Prior to loading the Jupiter C18 chromatographic column (4.6 × 150 mm, 5 μm, 300 Å, Phenomenex, Torrance, CA, USA), HmCystatin C was treated with dialysis and microfiltration. Acetonitrile with 0.1% trifluoroacetic acid was used as the elution buffer, and the ratio of the elution buffer was adjusted following a gradient elution program: 0–30 min, 0–60%; 30–31 min, 60–100%; and 31–50 min, 100%. Other parameters of RP-HPLC were as follows: flow rate 0.2 mL/min, column temperature 25 °C, and detection wavelength 280 nm. The purity of the protein was indicated by its peak area proportion to the total peak areas detected in the chromatogram.

2.7. Inhibition Activity Characterization of HmCystatin C. The protease inhibition activity of recombinant HmCystatin C against papain (Sigma-Aldrich) was determined by using benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide (Z-Phe-Arg-MCA, Sigma-Aldrich) as a substrate according to the methods of Anastasi et al. (1983)[33] and Barrett et al. (1981).[34] The incubation conditions for proteases, substrates, and HmCystatin C were 40 °C and pH 6.8 in the reaction buffer (4 mmol/L EDTA, 4 mmol/L DTT, and 200 mmol/L K_2HPO_4–KH_2PO_4). The fluorescence intensity of 7-amido-4-methylcoumarin released after the enzymatic cleavage was measured using a Multiskan Spectrum Fluorescence Microplate Spectrophotometer (Varioskan Flash 3001, Thermo Fisher Scientific, Waltham, MA, USA) with excitation and emission wavelengths of 380 and 460 nm, respectively.

2.8. Antibody Preparation. To observe the tissue distribution of HmCystatin C using immunolocalization and to quantify its protein expression level using Western blot, a polyclonal antibody against HmCystatin C was prepared from mouse. The Quick Antibody-Mouse 5W Adjuvant (Kang Bi Quan Biotechnology, Beijing, China) was used according to the manufacturer's instructions. Briefly, two male BALB/c mice (specific pathogen-free, 20 ± 2 g) were injected with HmCystatin C at the hind leg to trigger an immune response. Serum was collected as a control before subcutaneous injection. On the 1st day, 50 μL of recombinant HmCystatin C (0.4 mg/mL) was aseptically mixed with an equal volume of adjuvant and then injected into each mouse. On the 21st day, the same dosage of booster injection was given. On the 35th day, tail blood from mice was collected. Briefly, the mouse was warmed and placed in an immobilizing tube. A sterile needle was used to puncture the tail blood vessel, and after bleeding, the blood was immediately collected in a capillary tube. Around 20 μL of blood samples was collected, and then the bleeding was stopped by applying finger pressure. ELISA assay was conducted to determine the titer. Once the titer reached a ratio that was equal to or greater than 1:10 000, up to 500 μL of blood was collected from each mouse during each sampling using the retro-orbital bleeding (ROB) technique.[35] Before ROB, the mouse was anesthetized using isoflurane (2–5%) in an anesthesia induction chamber and then placed on a heating mat to maintain the body temperature. The blood was collected from the retro-orbital sinus using a Pasteur pipette after the eyeball of the mouse was protruded out. The blood collected was then used to isolate antisera against HmCystatin C. After finishing all the blood collecting procedures and obtaining the qualified antisera, the mice were euthanized by cervical dislocation after anesthesia.[36]

2.9. Immunolocalization to Analyze the Tissue Distribution of HmCystatin C. Immunohistochemical staining based on the reaction between specific antibody and antigen was applied to visualize the distribution of HmCystatin C in silver carp tissues. Selected tissues (n = 6 for each type tissues including spleen, muscle, hepatopancreas, small intestine, heart, and fish eggs) were first fixed in 4% paraformaldehyde for 24 h and dehydrated in increasing concentrations of ethanol. Then, Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek, Torrance, CA, USA) was used to embed pretreated tissues. Embedded tissues were finally sectioned into 10 μm sections (three sections for each embedded tissue) with a Cryostat Microtome (CM1900-1-1, Leica Biosystems, Wetzlar, Germany). Immunohistochemical staining was then conducted as described in our previous research.[37,38] The first antibody was the HmCystatin C antibody (1:500 diluted), and the secondary antibody was a biotinylated antimouse IgG from goat (1:5 000 diluted; Beyotime Biotechnology, Shanghai, China). Endogenous peroxidase deactivation, blocking, and washing were routinely performed following the instructions of the Ultrasensitive SP (Mouse/Rabbit) IHC Kit (Maixin Biotechnology, Fujian, China). After antibody incubation, visualization was realized by adding 3,3′-diaminobenzidine tetrahydrochloride (Maixin Biotechnology), and the samples were incubated for 3–10 min. The same procedures were performed on the negative control except that the first antibody was replaced by phosphate-buffered saline.

2.10. Western Blot. The last step was to quantify the protein expression of HmCystatin C in different tissues using Western blot. Total proteins of silver carp tissues were extracted following the protocol below: 40 mg of tissues was manually ground into a powder in liquid nitrogen, and then 300 μL of cell extraction buffer (Invitrogen Biotechnology Co., Ltd.) supplemented with 3 μL of 100× Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) was added. Tissues were lysed on ice for 30 min with vortexing every 10 min for 5–10 s. Subsequently, the mixture was centrifuged at 14 000 ×g for 5 min, and the supernatant was collected. Protein concentration was determined by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). All total protein samples from each tissue were diluted into the same concentration and run on SDS-PAGE. Western blot was then performed routinely using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Poly(vinylidene difluoride) membrane was used, and blotting was conducted at 100 V for 30 min.

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144–5154

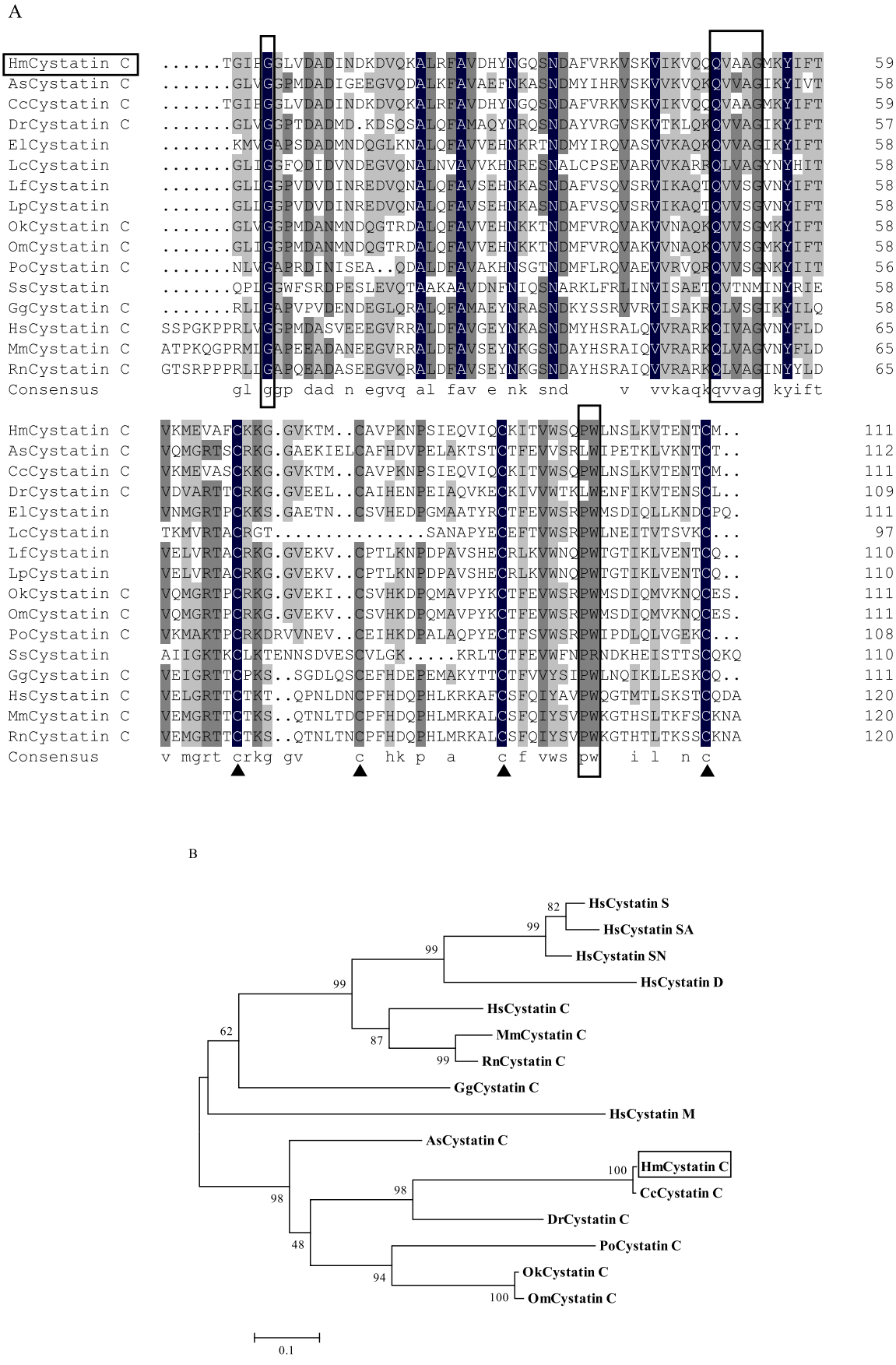

Figure 2. (A) Alignment of amino acid sequences of family II cystatins from different species. Identical amino acid residues are shown in black, similar amino acids in gray, and unrelated residues in white. Active site residue G, QXXXG motif, and PW residues are marked with black rectangles. Four cysteine residues at the C-terminus are indicated by black triangles. The sequences used and their similarities to HmCystatin C are

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154

Figure 2. continued

as follows: AsCystatin C, *Acipenser sinensis* cystatin C (AAK16731.1), 44.25%; CcCystatin C, *Cyprinus carpio* cystatin C (AAB48011.1), 99.10%; DrCystatin C, *Danio rerio* cystatin C (NP001026843.2), 56.76%; ElCystatin, *Esox lucius* cystatin (ACO14463), 41.07%; LcCystatin, *Larimichthys crocea* cystatin (ACF54620.1), 37.84%; LfCystatin, *Lctalurus furcatus* cystatin (ADO27871.1), 51.35%; LpCystatin, *Lctalurus punctatus* cystatin (NP001187957.1), 51.35%; OkCystatin C, *Oncorhynchus keta* cystatin C (BAA13149.1), 44.64%; OmCystatin C, *Oncorhynchus mykiss* cystatin C (AAA82049.1), 43.75%; PoCystatin C, *Paralichthys olivaceus* cystatin C (ACC86115.1), 39.29%; SsCystatin, *Salmo salar* cystatin (ACI69506.1), 22.81%; GgCystatin C, *Gallus gallus* cystatin C (NP990831.2), 34.82%; HsCystatin C, *Homo sapiens* cystatin C (NP000090.1), 25.00%; MmCystatin C, *Mus musculus* cystatin C (NP034106.2), 28.33%; and RnCystatin C, *Rattus norvegicus* cystatin C (NP036969.1), 28.33%. (B) Phylogenetic tree of family II cystatins from different species. The phylogenetic tree was generated by MEGA 7.0 using the neighbor-joining method. Bootstrap values of 1000 replications (%) are indicated on the branches. Besides the cystatin C mentioned in (A), information about other family II cystatin subtypes used for the phylogenetic tree is as follows: HsCystatin S, *Homo sapiens* cystatin S (NP001890.1); HsCystatin SA, *Homo sapiens* cystatin SA (NP001313.1); HsCystatin SN, *Homo sapiens* cystatin SN (NP001889.2); HsCystatin D, *Homo sapiens* cystatin D (CAA49838.1); and HsCystatin M, *Homo sapiens* cystatin M (NP001314.1).

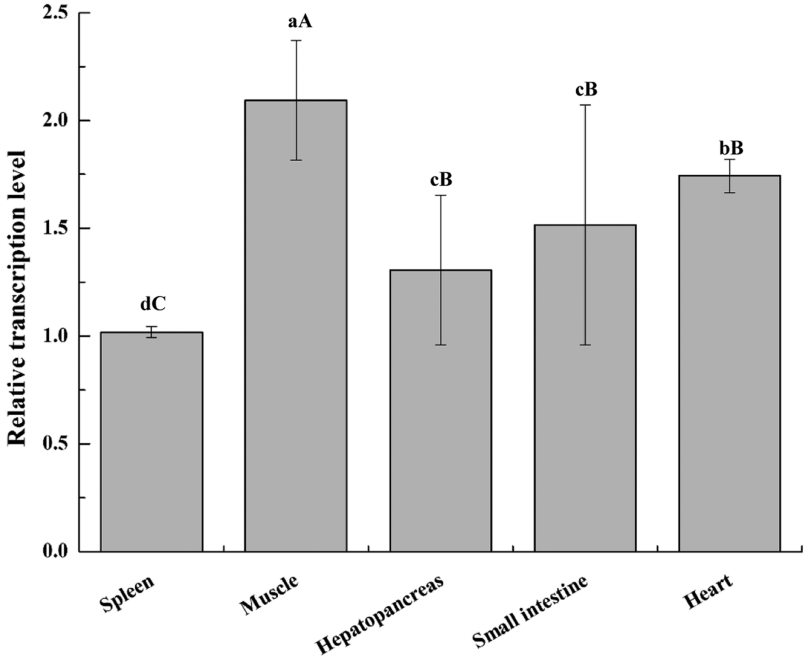

Figure 3. Relative gene transcription levels of Hm*CysC* in different tissues of silver carp. 18S *rRNA* gene was used to normalize the transcription level. All experiments were performed in triplicate. Data are presented as mean value with standard deviation. The differences are indicated with different lowercase letters ($0.01 < p < 0.05$) or capital letters ($p < 0.01$).

The first antibody was the HmCystatin C antibody (1:500 diluted), and the secondary antibody was a biotinylated anti-mouse IgG from goat (1:5 000 diluted). A highly sensitive chemiluminescence method was used for visualization. Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) was mixed at the ratio of 1:1 and added on the membrane for a 5 min reaction. Images were then captured by a ChemiDoc Imaging System (Bio-Rad) and analyzed by Quantity One 1-D Analysis Software (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the internal control, and the corresponding antibody from Beyotime Biotechnology was applied with the same procedures.

2.11. Data Analysis. Data were analyzed by SPSS Statistics 22.0 (IBM, Armonk, NY, USA). The mean values and standard deviations of three biological replicates were calculated. One-way analysis of variance was applied to analyze the significances between tested groups, and a significant difference was considered when the p value was less than 0.05.

3. RESULTS AND DISCUSSION

3.1. Sequence Analysis of the Hm*CysC* Gene. The cDNA of the Hm*CysC* gene from silver carp was amplified by PCR, and then the gene was cloned in the pMD19-T vector in *E. coli* DH5α and sequenced. The Hm*CysC* gene sequence and its deduced amino acid sequence are shown in Figure 1. The gene was found to be 336 base pairs, encoding 111 amino acid residues. The predicted molecular weight and theoretical isoelectric point (pI) of HmCystatin C were 12.3 kDa and 9.64, respectively. The molecular weight of HmCystatin C was similar to the molecular weights of chum salmon cystatin C (13 kDa),[21] common carp cystatin C (12 kDa),[22] and Chinese sturgeon cystatin C (14 kDa).[12] Following analysis using DNAMAN 8.0 and comparing the Hm*CysC* gene sequence with other piscine cystatins, we found that there was no signal peptide in the amino acid sequence of HmCystatin C. This analysis indicated that the Hm*CysC* fragment we cloned encoded the mature peptide of HmCystatin C. A previous study on chicken egg white cystatin revealed that there are three conserved domains in the amino acid sequence: the active site residue G (Gly at N-terminus), the QXXXG domain (X referring to any amino acid), and the PW residues (Pro−Trp at C-terminus).[39] The same pattern was found in the HmCystatin C sequence, and all the conserved domains are marked with black rectangles in Figure 1. Four cysteine residues at the C-terminus are marked by triangles, indicating the typical structure of family II cystatins: two disulfide bonds

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154

in the peptide chain.[3] The Hm*CysC* DNA sequence was submitted and deposited in GenBank (accession number: KF181446.1).

Similarities between HmCystatin C and cystatins (family II) from other species were analyzed. Mature peptide sequences, including cystatin C of teleosts and human, rat, mouse, chicken, and other undefined piscine cystatins, were collected from the NCBI database and used for multiple sequence alignment. As shown in Figure 2A, HmCystatin C shared the highest similarity of 99.10% with common carp cystatin C (*C. carpio*, AAB48011.1) and the lowest similarity of 39.29% with olive flounder cystatin C (*P. olivaceus*, ACC86115.1). The similarities between HmCystatin C and undefined piscine cystatin ranged from 22.81 to 51.35%, indicating that these cystatins were possibly other subtypes of family II cystatins. HmCystatin C displayed lower similarities (25.00−34.82%) with cystatin C from other vertebrates. When we constructed a phylogenetic tree (Figure 2B) using different subtypes from family II cystatins, HmCystatin C formed a cluster with piscine cystatin C. Notably, HmCystatin C exhibited a high-degree evolutionary relationship with the common carp and zebrafish cystatin C (*Danio rerio*, NP001026843.2), which both belonged to the Cyprinidae family. As for the results of structure simulation, only one α-helix was predicted in the secondary structure of HmCystatin C (Figure S1A, Supporting Information), which was consistent with its 3D model (Figure S1B, Supporting Information). In the tertiary structure of HmCystatin C, five β-sheets are shown based on the template of the chicken egg white cystatin (Protein Data Bank ID: 1CEW.1.A, sequence similarity: 39%).

3.2. Transcription Levels of the Hm*CysC* Gene in Different Tissues of Silver Carp. Piscine cystatin C was reported to present a nontissue-specific transcription in previous studies.[21,24] In this study, the gene transcription of Hm*CysC* was detected in all tested tissues at different levels (Figure 3). From high to low, the order of transcription level in the five tissues was muscle (2.094 ± 0.278), heart (1.743 ± 0.078), small intestine (1.515 ± 0.558), hepatopancreas (1.305 ± 0.347), and spleen (1.018 ± 0.026). The transcription level in muscle was significantly higher than those of the other four tissues ($p < 0.01$). Conversely, the transcription levels of cystatin analogue in large yellow croaker (*P. crocea*)[11] and cystatin C in chum salmon (*O. keta*)[21] and rainbow trout (*O. mykiss*)[24] were found to be the lowest in muscle. These differences might be caused by the methods used for investigation. Compared to the qRT-PCR technique, Northern blot was used in the research of chum salmon and rainbow trout, and standard PCR was used in the research of large yellow croaker, both of which are less sensitive than qRT-PCR. Moreover, species gap also matters. Reflected by the amino acid sequence alignment, the similarities of cystatins between these fish species were low.[24,40−42] For example, the similarity between cystatin C of silver carp and the cystatin analogue of large yellow croaker was only 37.84%, and the transcription patterns differed. Similar phenomena were found in other studies, for example, the similarity of stefin from large yellow croaker (GenBank accession number: ACJ24348.1) and turbot (GenBank accession number: ADM61584.1) was 42.30%. The stefin transcription levels in the muscle of large yellow croaker showed no differences compared to heart, spleen, and liver.[43] However, in turbot (*Scophthalmus maximus*), stefin B transcription in muscle was 3-, 5.7-, and 7-folds higher than that in spleen, liver, and heart, respectively.[44] The low similarities in the amino acid sequence revealed a low gene homology, which caused the differences of mRNA transcription in the same tissues. Nevertheless, Hm*CysC* gene displayed a wide transcription in all five tissues, and the transcription level varied.

3.3. Analysis of the Expression and Purification for Recombinant HmCystatin C. After cloning Hm*CysC* and analyzing its gene transcription pattern, an expression plasmid pET-30a-Hm*CysC* was constructed. The expression of HmCystatin C in host *E. coli* BL21 (DE3) cells was induced by IPTG at 37 °C for 6 h, and the total proteins of induced and noninduced hosts were analyzed by SDS-PAGE. When comparing with the protein profile from noninduced host cells (Figure 4, lane 1), a much stronger band appeared at approximately 20 kDa in the sample from induced host cells (Figure 4, lane 2, black arrow). The host *E. coli* BL21 (DE3) might produce some native proteins which owned similar molecular weight with HmCystatin C. This inference was later explained by gradient urea washing and Ni^{2+}−NTA agarose affinity chromatography. As shown in lanes 3 and 4 of Figure 4, HmCystatin C appeared as a single band on the SDS-PAGE gel, and the sample after chromatography purification (lane 4) carried fewer impurities than the sample after urea washing (lane 3). Due to the specificity of these two purification techniques, mainly the inclusion body and recombinant protein labeled with His-tag would be purified. Thus, the protein band at 20 kDa in lane 1 represented the native protein from the host. These results indicated that HmCystatin C was successfully expressed in host *E. coli* BL21 (DE3) cells. The apparent relative molecular weight of HmCystatin C was larger than its predicted size of 12.3 kDa. One potential reason was that the DNA fragment of linker sequences and the His-tag were added to HmCysC from the vector, with a molecular weight of around 5.46 kDa. Besides, the migration of proteins on the SDS-PAGE gel depends on several factors such as size, shape, and charges of proteins, leading to only a rough estimation of the molecular weight. Additionally, we characterized the purity of HmCystatin C. The results of RP-HPLC (Figure S2, Supporting Information) showed that the purified HmCystatin C was eluted at 14.2 min as a single peak, and its purity was 95.3% according to the proportion of

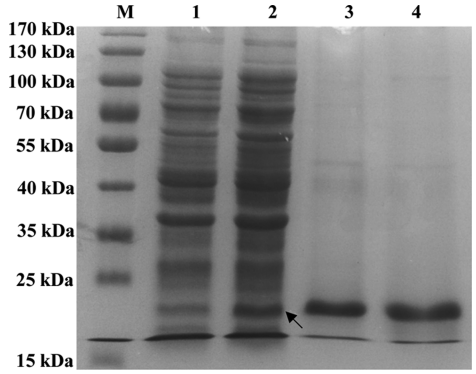

Figure 4. Expression and purification of recombinant HmCystatin C analyzed by SDS-PAGE. M: protein marker, 15−170 kDa; (1) total proteins of *E. coli* BL21 (DE3) which carries plasmid pET-30a-Hm*CysC* before IPTG induction; (2) total proteins of *E. coli* BL21 (DE3) which carries plasmid pET-30a-Hm*CysC* after IPTG induction; (3) the proteins after urea washing; and (4) the purified HmCystatin C after Ni^{2+}−NTA agarose affinity chromatography.

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154

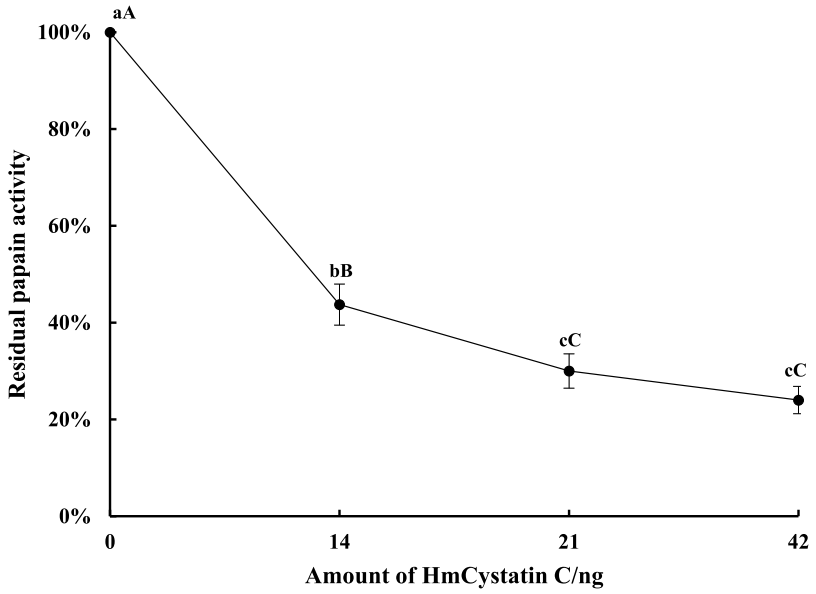

Figure 5. Activity inhibition of papain by different amounts of HmCystatin C. The curve is displayed as the change of the residual activity of papain along with the addition of HmCystatinC (ng). All experiments were performed in triplicate. Data are presented as mean value with standard deviation. The differences are indicated with different lowercase letters ($0.01 < p < 0.05$) or capital letters ($p < 0.01$).

peak area. The purified HmCystatin C was used for activity characterization and antibody preparation.

3.4. Inhibition Activity of HmCystatin C toward Papain. As a family II cysteine protease inhibitor, the most typical property of HmCystatin C was its inhibition effect against cysteine protease. Indeed, HmCystatin C presented a characteristic mode of inhibiting the cysteine protease papain (Figure 5). The residual activities of papain decreased along with the increase of the HmCystatin C amount, indicating the suppression of the proteolytic activity of papain. Notably, with a small amount of HmCystatin C (14 ng), the proteolytic activity of papain decreased from 100 to 43.73% ($p < 0.01$), and with adding of 21 ng the activity further dropped down to 27.98% ($p < 0.01$). Doubling the amount of HmCystatin C up to 42 ng decreased the activity only to 24.30% ($p > 0.05$), which is a hint for the saturation of papain by HmCystatin C. This phenomenon might be caused by the completely binding of HmCystatin C to the active site of papain. This result could also demonstrate that the activity inhibition of papain by HmCystatin C was a kind of reversible reaction, that is, the residual activities of proteases were no longer representative when a maximum dose of cystatin was used.

3.5. Distribution of HmCystatin C in Silver Carp Tissues. Before immunolocalization, a polyclonal antibody of HmCystatin C was prepared. This step was based on the consideration that the similarities of cystatins between terrestrial animals and aquatic animals were low.[24,40−42] For example, the similarities of the cystatin C sequences between silver carp and human (*Homo sapiens*, NP000090.1), mouse (*Mus musculus*, NP034106.2), and chicken (*Gallus gallus*, NP990831.2) are 25.00, 28.33, and 34.82%, respectively. However, most of the antibodies available now were produced for terrestrial animals and might not bind with piscine cystatins. In this study, we prepared a sensitive and reliable antibody using purified HmCystatin C for implementation in immunolocalization and Western blot. The titer of the antibody was 1:512 000. The specificity of the antibody was confirmed by Western blot (Figure S3, Supporting Information), and the results showed that this antibody could identify HmCystatin C from protein samples of HmCystatin C expressing *E. coli* cells with a low background.

Immunolocalization was then performed to investigate the tissue distribution of HmCystatin C. According to the image of stained tissues after immunohistochemistry (Figure 6), HmCystatin C was widely observed in all tested tissues, including spleen, muscle, hepatopancreas, small intestine, heart, and eggs. In the spleen of silver carp, HmCystatin C was localized in the macrophages, implying that HmCystatin C might play a role in the innate immunity of silver carp. In small intestine, HmCystatin C was localized at the edge of the intestinal villi and was probably related to the movement of the intestinal villi. Positive results were obtained from the yolk granule and oolemma (inner layer) in the fish egg cells. By far, most studies regarding cystatins at the protein level have been concentrated on purification and property characterization; only a few studies have investigated the tissue distribution. Chang et al. (1998) found similar localization patterns of carp cystatin in oolemma, cortical granules, yolk granules, oocytes, and follicular cells using immunochemistry techniques.[45] Tsai et al. (1996) purified cystatin from carp ovary and confirmed by Western blot that it was expressed only in ovary.[46] The tissue-specific distribution pattern was also obtained in the research for human cystatin S,[47] SA,[48] and SN.[49] However, human cystatin C was widely distributed in various tissues and body fluids,[50] which was in accord with the conclusions of our research. The different distribution patterns reveal that each type of family II cystatins might have its unique physiological properties.

3.6. Semiquantification of HmCystatin C Protein Expression in Silver Carp Tissues. Even though the gene encoding HmCystatin C was transcribed in several tissues of fish,[24] its protein expression level was still unclear. We performed a semiquantification for HmCystatin C, aiming to investigate its protein expression level in eukaryotic cells. As shown in Figure 7, HmCystatin C expression in the protein extracts from each tissue was detected using the antibody we

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154

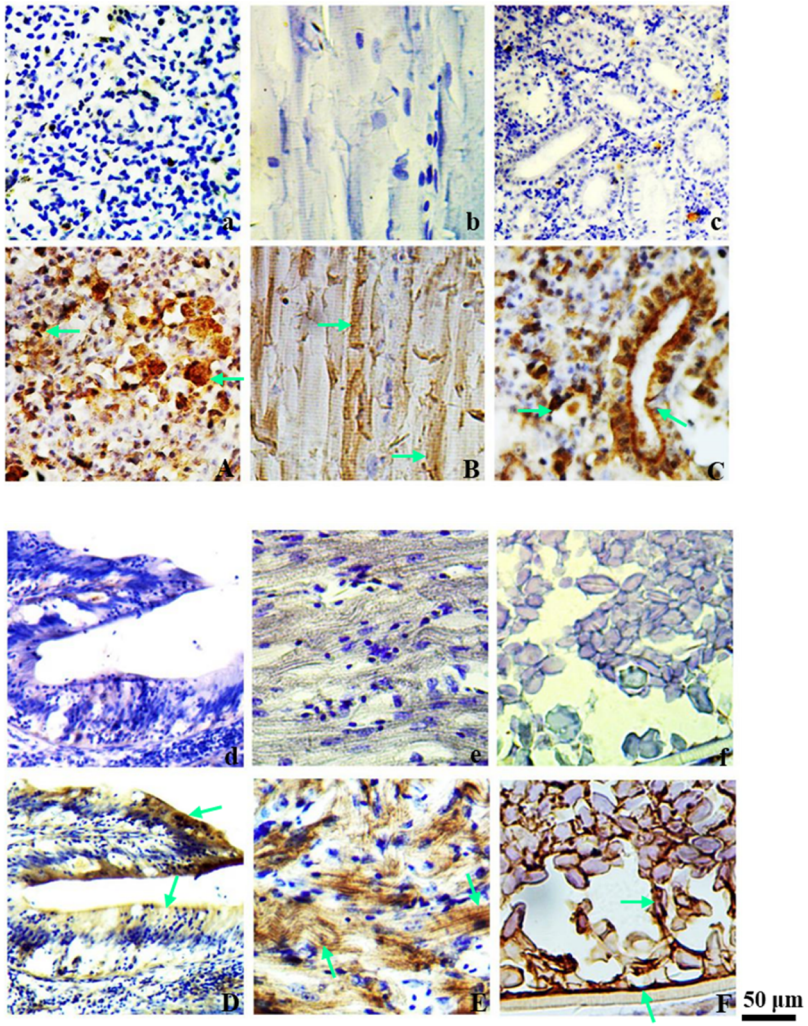

Figure 6. Immunostaining of HmCystatin C in the tissues of silver carp. (A−F) spleen, muscle, hepatopancreas, small intestine, heart, and fish eggs, respectively, treatment groups; (a−f): spleen, muscle, hepatopancreas, small intestine, heart, and fish eggs, respectively, negative control. HmCystatin C was stained in brown, and typical positive areas are arrowed in green. Each picture is magnified for 200 times. In spleen, HmCystatin C was observed in spleen cell (SNC) and macrophage (MP). The striated muscle was compact and its myofiber texture was clear, and positive staining was observed. In the hepatopancreas of silver carp, the positive staining was mainly located in the hepatopancreas cell and blood vessel of vein (BV). In the small intestine, HmCystatin C was mainly located at the edge of the intestinal villi. The striated muscle of heart was visible and enriched HmCystatin C. Different levels of positive staining were also observed in the yolk granule and oolemma (inner layer) of silver carp spawn. No positive staining was observed in the tissues of negative controls.

prepared. The areas and the grayscale values of the bands indicated varied expression levels of HmCystatin C, while the internal control GAPDH expressed quite evenly in the tissues of silver carp. We further quantified the expression level using Quantity One 1-D Analysis Software. From high to low, the order of protein expression level in the five tissues was muscle (2.343 ± 0.143), heart (1.929 ± 0.110), small intestine (1.727 ± 0.144), hepatopancreas (1.409 ± 0.197), and spleen (1.158 ± 0.138). The protein expression level in the muscle was significantly higher than in the other four tissues ($p < 0.01$). These results indicated that these five tissues contain abundant levels of HmCystatin C, consistent with the trends of tissue distribution and gene transcription. Our research results could provide a reference in practice. During the slaughtering and processing of silver carp, massive amounts of wasted tissues, such as skin and hepatopancreas, will be generated and abandoned. First, the highly sensitive antibody we prepared is a valid tool for detecting the cystatins from abandoned tissues. Moreover, the quantification results provide an important guidance for the recovery and utilization of cystatins from fish byproducts.

In summary, Hm*CysC*, the gene encoding the mature peptide of silver carp cystatin C, was cloned and expressed in this work. Thereafter, characterization on its sequence property, inhibition activity, and expression pattern at both gene and protein levels was performed. Prior to going into the deeper study or utilization of HmCystatin C, clarifying its basic properties is needed. The results collected in our study could provide some solid information such as the sequence, biochemical properties, and tissue distribution of HmCystatin C for further exploring its application potentials. Particularly, the result of immunochemistry indicated an extensive tissue distribution of silver carp cystatin C, and it may be used as a guide for recycling and utilizing cystatins in wasted tissues. In further studies, exploring the functions and mechanisms of cystatins in preventing quality deterioration of fish fillet and surimi would be ideal options. To realize this purpose, our

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154

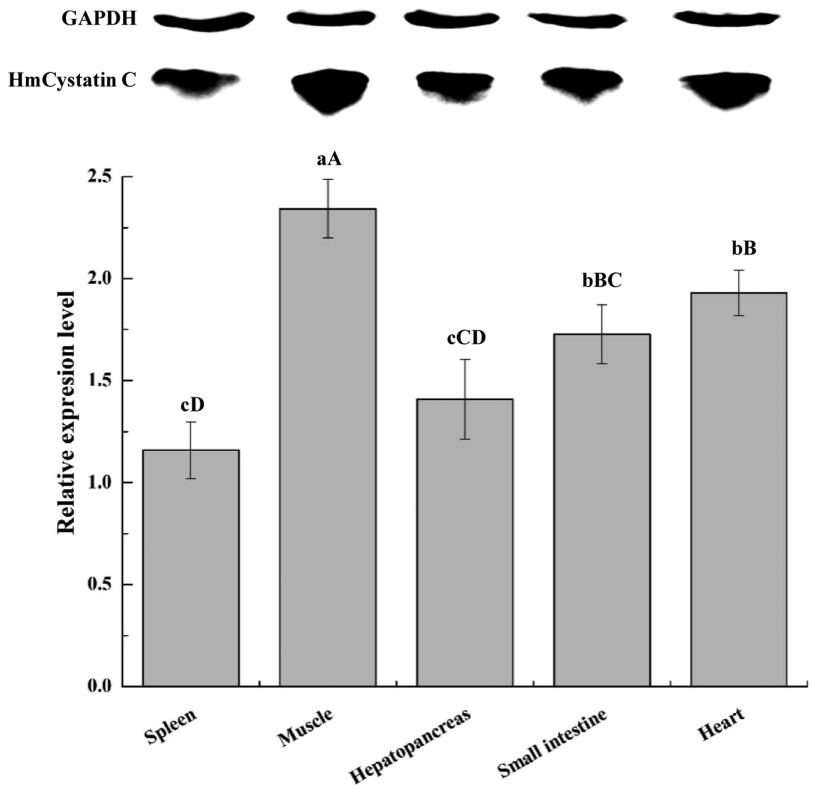

Figure 7. Western blot and protein quantification of HmCystatin C in the tissues of silver carp. Column chart indicates the quantification results of HmCystatin C analyzed from the gray scale using Quantity One 1-D Analysis Software (Bio-Rad). The results of Western blot in corresponding tissue are shown at the top of each column. GAPDH was used as the internal control. All experiments were performed in triplicate. Data are presented as mean value with standard deviation. The differences are indicated with different lowercase letters ($0.01 < p < 0.05$) or capital letters ($p < 0.01$).

proposed method can be used for detecting the dynamic changes of cystatins over time and at different locations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c00345.

Primers, structure simulation of HmCystatin C, RP-HPLC characterization of HmCystatin C, and antibody specificity identification (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Shuhong Li − *College of Food Science, Sichuan Agricultural University, Ya'an, Sichuan Province 625014, China;* orcid.org/0000-0002-2657-1747; Email: lish@sicau.edu.cn

Authors

Ran Li − *College of Food Science, Sichuan Agricultural University, Ya'an, Sichuan Province 625014, China; Department of Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Helsinki 00014, Finland*

Xiaoqian Tan − *College of Food Science, Sichuan Agricultural University, Ya'an, Sichuan Province 625014, China*

Yu Jin − *College of Food Science, Sichuan Agricultural University, Ya'an, Sichuan Province 625014, China*

Song Li − *College of Food Science, Sichuan Agricultural University, Ya'an, Sichuan Province 625014, China*

Shulei Li − *Department of Histology and Embryology, College of Basic Medical Sciences, Norman Bethune Health Science Center, Jilin University, Changchun, Jilin Province 130021, China*

Timo M. Takala − *Department of Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Helsinki 00014, Finland*

Per. E. J. Saris − *Department of Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Helsinki 00014, Finland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.1c00345

Author Contributions

‖R.L. and X.Q.T. made equal contributions to this work.

Funding

This work was supported by the Natural Science Foundation of Sichuan Province (no. 10ZA052).

Notes

The authors declare no competing financial interest.

All protocols regarding silver carps were approved by the Animal Care and Use Committee of the Sichuan Agricultural University (Project license: DY-S20141707). All procedures regarding mice were approved by the Animal Care Advisory Committee of the Jilin University (Project license: SCXK Ji 2013-0001).

ABBREVIATIONS

qRT-PCR, quantitative real time polymerase chain reaction; LB, lysogeny broth; cDNA, complementary DNA; MEGA 7.0, molecular evolutionary genetics analysis; Ni^{2+}−NTA, Ni^{2+} nitrilotriacetic acid; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; Z-Phe-Arg-MCA, benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; AMC, 7-amido-4-methylcoumarin; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; kDa, kilodalton

REFERENCES

(1) Vidak, E.; Javoršek, U.; Vizovišek, M.; Turk, B. Cysteine Cathepsins and Their Extracellular Roles: Shaping the Microenvironment. *Cells* **2019**, *8*, 264.

(2) Shamsi, T. N.; Parveen, R.; Fatima, S. Characterization, Biomedical and Agricultural Applications of Protease Inhibitors: A Review. *Int. J. Biol. Macromol.* **2016**, *91*, 1120−1133.

(3) Kordis, D.; Turk, V. Phylogenomic Analysis of the Cystatin Superfamily in Eukaryotes and Prokaryotes. *BMC Evol. Biol.* **2009**, *9*, 266.

(4) Ahn, S. J.; Bak, H. J.; Park, J. H.; Lee, J. Y.; Kim, N. Y.; Han, J. W.; Jo, H. I.; Chung, J. K.; Lee, H. H. Olive flounder (*Paralichthys olivaceus*) Cystatin C: Cloning, mRNA Expression, and Enzymatic Characterization of Olive flounder Cystatin C. *Appl. Biochem. Biotechnol.* **2013**, *170*, 1216−1228.

(5) Turk, V.; Stoka, V.; Turk, D. Cystatins: Biochemical and Structural Properties, and Medical Relevance. *Front. Biosci.* **2008**, *13*, 5406−5420.

(6) Eaves-Pyles, T.; Patel, J.; Arigi, E.; Cong, Y.; Cao, A.; Garg, N.; Dhiman, M.; Pyles, R. B.; Arulanandam, B.; Miller, A. L.; Popov, V. L.; Soong, L.; Carlsen, E. D.; Coletta, C.; Szabo, C.; Almeida, I. C. Immunomodulatory and Antibacterial Effects of Cystatin 9 against *Francisella tularensis*. *Mol. Med.* **2013**, *19*, 263−275.

(7) Magister, Š.; Kos, J. Cystatins in Immune System. *J. Canc.* **2013**, *4*, 45−56.

(8) Breznik, B.; Mitrović, A.; Lah, T. T.; Kos, J. Cystatins in Cancer Progression: More Than Just Cathepsin Inhibitors. *Biochimie* **2019**, *166*, 233−250.

(9) Parent, A. D.; Cornwall, G. A.; Liu, L. Y.; Smith, C. E.; Hermo, L. Alterations in the Testis and Epididymis Associated with Loss of Function of the Cystatin-Related Epididymal Spermatogenic (CRES) Protein. *J. Androl.* **2011**, *32*, 444−463.

(10) Wang, S.-C.; Huang, F.-L. Carp Ovarian Cystatin Binds and Agglutinates Spermatozoa via Electrostatic Interaction. *Biol. Reprod.* **2002**, *66*, 1318−1327.

(11) Li, S.; Ao, J.; Chen, X. Molecular and Functional Characterization of a Cystatin Analogue in Large Yellow Croaker (*Pseudosciaena crocea*). *Mol. Immunol.* **2009**, *46*, 1638−1646.

(12) Bai, J.; Ma, D.; Lao, H.; Jian, Q.; Ye, X.; Luo, J.; Xong, X.; Li, Y.; Liang, X. Molecular Cloning, Sequencing, Expression of Chinese Sturgeon Cystatin in Yeast *Pichia pastoris* and its Proteinase Inhibitory Activity. *J. Biotechnol.* **2006**, *125*, 231−241.

(13) Bahuaud, D.; Mørkøre, T.; Østbye, T.-K.; Veiseth-Kent, E.; Thomassen, M. S.; Ofstad, R. Muscle Structure Responses and Lysosomal Cathepsins B and L in Farmed Atlantic salmon (*Salmo Salar* L.) Pre- and Post-Rigor Fillets Exposed to Short and Long-Term Crowding Stress. *Food Chem.* **2010**, *118*, 602−615.

(14) Caballero, M. J.; Betancor, M.; Escrig, J. C.; Montero, D.; Espinosa de los Monteros, A.; Castro, P.; Ginés, R.; Izquierdo, M. Post Mortem Changes Produced in the Muscle of Sea Bream (*Sparus aurata*) During Ice Storage. *Aquaculture* **2009**, *291*, 210−216.

(15) Godiksen, H.; Morzel, M.; Hyldig, G.; Jessen, F. Contribution of Cathepsins B, L and D to Muscle Protein Profiles Correlated with Texture in Rainbow trout (*Oncorhynchus mykiss*). *Food Chem.* **2009**, *113*, 889−896.

(16) Liu, H.; Yin, L.; Zhang, N.; Li, S.; Ma, C. Isolation of Cathepsin B from the Muscle of Silver carp (*Hypophthalmichthys molitrix*) and Comparison of Cathepsins B and L Actions on Surimi Gel Softening. *Food Chem.* **2008**, *110*, 310−318.

(17) Hu, Y.; Ji, R.; Jiang, H.; Zhang, J.; Chen, J.; Ye, X. Participation of Cathepsin L in Modori Phenomenon in Carp (*Cyprinus carpio*) Surimi Gel. *Food Chem.* **2012**, *134*, 2014−2020.

(18) Carvajal-Rondanelli, P. A.; Lanier, T. C. Diffusion of Active Proteins into Fish Meat to Minimize Proteolytic Degradation. *J. Agric. Food Chem.* **2010**, *58*, 5300−5307.

(19) Ustadi; Kim, K. Y.; Kim, S. M. Purification and Identification of a Protease Inhibitor from Glassfish (*Liparis tanakai*) Eggs. *J. Agric. Food Chem.* **2005**, *53*, 7667−7672.

(20) Akpinar, O.; An, H. Purification and Determination of Inhibitory Activity of Recombinant Soyacystatin for Surimi Application. *Mol. Nutr. Food Res.* **2005**, *49*, 247−255.

(21) Yamashita, M.; Konagaya, S. Molecular Cloning and Gene Expression of Chum salmon Cystatin. *J. Biochem.* **1996**, *120*, 483−487.

(22) Tzeng, S.-S.; Chen, G.-H.; Chung, Y.-C.; Jiang, S.-T. Expression of Soluble Form Carp (*Cyprinus carpio*) Ovarian Cystatin in Escherichia coli and its Purification. *J. Agric. Food Chem.* **2001**, *49*, 4224−4230.

(23) Tzeng, S.-S.; Chen, G.-H.; Jiang, S.-T. Expression of Soluble Thioredoxin Fused-Carp (*Cyprinus carpio*) Ovarian Cystatin in Escherichia coli. *J. Food Sci.* **2002**, *67*, 2309−2316.

(24) Li, F.; An, H.; Seymour, T. A.; Bradford, C. S.; Morrissey, M. T.; Bailey, G. S.; Helmrich, A.; Barnes, D. W. Molecular Cloning, Sequence Analysis and Expression Distribution of Rainbow trout (*Oncorhynchus mykiss*) Cystatin C. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1998**, *121*, 135−143.

(25) China Society of Fisheries. *China Fishery Statistical Yearbook 2020*; China Agricultural Press: Beijing, China, 2020; pp 25.

(26) Liu, H.; Yin, L.; Li, S.; Zhang, N.; Ma, C. Effects of Endogenous Cathepsins B and L on Degradation of Silver Carp (*Hypophthalmichthys molitrix*) Myofibrillar Proteins. *J. Muscle Foods* **2008**, *19*, 125−139.

(27) Delbarre-Ladrat, C.; Chéret, R.; Taylor, R.; Verrez-Bagnis, V. Trends in Postmortem Aging in Fish: Understanding of Proteolysis and Disorganization of the Myofibrillar Structure. *Crit. Rev. Food Sci.* **2006**, *46*, 409−421.

(28) Zhang, L.; Zhang, Y.; Jia, S.; Li, Y.; Li, Q.; Li, K.; Hong, H.; Luo, Y. Stunning Stress-Induced Textural Softening in Silver Carp (*Hypophthalmichthys molitrix*) Fillets and Underlying Mechanisms. *Food Chem.* **2019**, *295*, 520−529.

(29) Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870−1874.

(30) Tang, Y.-K.; Yu, J.-H.; Xu, P.; Li, J.-L.; Li, H.-X.; Ren, H.-T. Identification of Housekeeping Genes Suitable for Gene Expression Analysis in Jian Carp (*Cyprinus carpio* var. Jian). *Fish Shellfish Immunol.* **2012**, *33*, 775−779.

(31) Liu, J.; Cao, D.; Liu, Y.; Li, Z.; Si, Y.; Wang, Z.; Zhang, Q.; Yu, H.; Wang, X. Expression and Functional Analysis of Receptor-Interacting Serine/Threonine Kinase 2 (RIP2) in Japanese flounder (*Paralichthys olivaceus*). *Fish Shellfish Immunol.* **2018**, *75*, 327−335.

(32) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: New York, USA, 1989.

(33) Anastasi, A.; Brown, M. A.; Kembhavi, A. A.; Nicklin, M. J. H.; Sayers, C. A.; Sunter, D. C.; Barrett, A. J. Cystatin, a Protein Inhibitor of Cysteine Proteinases. Improved Purification from Egg White, Characterization, and Detection in Chicken Serum. *Biochem. J.* **1983**, *211*, 129−138.

(34) Barrett, A. J.; Kirschke, H. [41] Cathepsin B, cathepsin H, and cathepsin L. *Methods Enzymol.* **1981**, *80*, 535−561.

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154

(35) Hoff, J. Methods of Blood Collection in the Mouse. *Lab. Anim.* **2000**, *29*, 47−53.
(36) American Veterinary Medical Association. AVMA Guidelines for the Euthanasia of Animals: 2020 Edition. https://www.avma.org/sites/default/files/2020-01/2020_Euthanasia_Final_1-15-20.pdf (accessed April 07, 2021).
(37) Li, S.-H.; Li, R.; Zhong, H.-X.; Chen, X.-H.; Liu, A.-P.; Yang, J.; Hu, Q.; Ke, Q.-Q.; Li, M.-L. Immunolocalization of Jian Carp (*Cyprinus Carpio* var. Jian) Cathepsin B: Cloning, Expression, Characterization, and Antibody Preparation. *J. Food Sci.* **2017**, *82*, 1092−1100.
(38) Li, R.; Li, S.-H; Chen, Z.-G; Jin, Y.; Li, S.-L; Li, S.; Bai, Z.-Z. Grass Carp (*Ctenopharyngodon idella*) stefin A: Systematic Research on its Cloning, Expression, Characterization and Tissue Distribution. *Food Chem.* **2021**, *335*, 127564.
(39) Bode, W.; Engh, R.; Musil, D.; Thiele, U.; Huber, R.; Karshikov, A.; Brzin, J.; Kos, J.; Turk, V. The 2.0 A X-ray Crystal Structure of Chicken Egg White Cystatin and its Possible Mode of Interaction With Cysteine Proteinases. *EMBO J.* **1988**, *7*, 2593−2599.
(40) Koide, Y.; Noso, T. The Complete Amino Acid Sequence of Pituitary Cystatin from Chum Salmon. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 164−169.
(41) Zhou, L.; Li-Ling, J.; Huang, H.; Ma, F.; Li, Q. Phylogenetic Analysis of Vertebrate Kininogen Genes. *Genomics* **2008**, *91*, 129−141.
(42) Wong, M. K.-S.; Takei, Y. Lack of Plasma Kallikrein-Kinin System Cascade in Teleosts. *PLoS One* **2013**, *8*, No. e81057.
(43) Li, S.; Yang, Z.; Ao, J.; Chen, X. Molecular and Functional Characterization of a Novel Stefin Analogue in Large Yellow Croaker (*Pseudosciaena Crocea*). *Dev. Comp. Immunol.* **2009**, *33*, 1268−1277.
(44) Xiao, P.-P.; Hu, Y.-H.; Sun, L. *Scophthalmus maximus* Cystatin B Enhances Head Kidney Macrophage-Mediated Bacterial Killing. *Dev. Comp. Immunol.* **2010**, *34*, 1237−1241.
(45) Chang, Y. S.; Weng, J. W.; Li, C. C.; Huang, F. L. Identification of Cystatin as a Component of Carp Chorion. *Mol. Reprod. Dev.* **1998**, *51*, 430−435.
(46) Tsai, Y.-J.; Chang, G.-D.; Huang, C.-J.; Chang, Y.-S.; Huang, F.-L. Purification and Molecular Cloning of Carp Ovarian Cystatin. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1996**, *113*, 573−580.
(47) Bobek, L. A.; Aguirre, A.; Levine, M. J. Human Salivary Cystatin-S - Cloning, Sequence Analysis, Hybridization *in situ* and Immunocytochemistry. *Biochem. J.* **1991**, *278*, 627−635.
(48) Isemura, S.; Saitoh, E.; Sanada, K. Characterization and Amino Acid Sequence of a New Acidic Cysteine Proteinase Inhibitor (Cystatin SA) Structurally Closely Related to Cystatin S, from Human Whole Saliva1. *J. Biochem.* **1987**, *102*, 693−704.
(49) Isemura, S.; Saitoh, E.; Sanada, K. Characterization of a new cysteine proteinase inhibitor of human saliva, cystatin SN, which is immunologically related to cystatin S. *FEBS Lett.* **1986**, *198*, 145−149.
(50) Abrahamson, M.; Olafsson, I.; Palsdottir, A.; Ulvsbäck, M.; Lundwall, Å.; Jensson, O.; Grubb, A. Structure and Expression of the Human Cystatin C Gene. *Biochem. J.* **1990**, *268*, 287−294.

https://doi.org/10.1021/acs.jafc.1c00345
J. Agric. Food Chem. 2021, 69, 5144−5154