Genetics and Molecular Research

Molecular cloning and gene expression analysis of cystatin C-like proteins in spinyhead croaker *Collichthys lucidus*

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Genet. Mol. Res. 15 (1): gmr.15017417
Received August 11, 2015
Accepted November 16, 2015
Published March 24, 2016
DOI http://dx.doi.org/10.4238/gmr.15017417

ABSTRACT. Cystatins are natural tight-binding reversible inhibitors of cysteine proteases. In this study, a cDNA library was constructed from *Collichthys lucidus* using the SMART technique. A complete cDNA sequence with high identity to the conserved sequence of the cystatin C gene was cloned from the library using EST analysis and rapid amplification of cDNA ends (RACE), then subjected to further investigation. The full-length cDNA of cystatin C from *C. lucidus* (*Clcys*) was 699 bp long, including a 5'-terminal untranslated region (5'-UTR) of 52 bp, a 3'-UTR of 290 bp, and an open-reading frame of 357 bp. The gene encoded a polypeptide of 118 amino acids, constituting a predicted molecular weight of 12.875 kDa and a theoretical isoelectric point of 8.81. The amino acid sequence of *Clcys* possessed typical features of type II cystatins and had the highest identity with cystatin C of *Pseudosciaena crocea* (89%); therefore, it clustered with the cystatin C group in the UPGMA phylogenetic tree. Quantitative real-time reverse transcription analysis revealed that the highest expression was found in the kidney, followed by the liver, heart, and testis, with the lowest expression in muscle. Interestingly, *Clcys* had relatively low identity

with cystatin C genes from other fish and mammals, and its expression pattern did not possess features of a housekeeping gene. Based on these findings, we suspect that the classification of cystatins in fish is somewhat confusing, and the identification of more cystatin gene sequences is needed before a definite conclusion can be drawn.

Key words: Cystatin C; *Collichthys lucidus*; Gene cloning; Expression; Quantitative real-time PCR

INTRODUCTION

Cysteine proteases exist in all living organisms and are involved in various biological and pathological processes, including protein catabolism, antigen processing, inflammation, dystrophy, and metastasis (Vray et al., 2002). The protein from chicken egg white that inhibits cysteine proteinases, known as 'cystatin', has been purified by ovomucin precipitation and affinity chromatography (Anastasi et al., 1983). Following this, other cystatins were found in succession (Grubb et al., 1984; Yamada et al., 2000; Sadaf et al., 2005). Studies on cystatins have commonly focused on tumorigenesis, stabilization of matrix metalloproteinases, glomerular filtration rate (GFR), immunomodulation, and neurodegenerative diseases in mammals (Herget-Rosenthal et al., 2004; Filler et al., 2005; Ochieng and Chaudhuri, 2010; Asnani and Reid, 2015).

Based on the location, molecular weight, and complexity of the peptides, cystatin can be categorized into three major families. Stefins, including stefin A and B, also known as cystatins A and B, belong to Family 1. These are unglycosylated cysteine cathepsin inhibitors of ~11 kDa, which lack a signal sequence and disulfide bonds, and are generally expressed intracellularly. The lack of cystatin B results in progressive myoclonus epilepsy (Maher et al., 2014). Those belonging to Family 2 have a molecular mass of 13-14 kDa and contain a signal sequence and disulfide bonds at the carboxy terminus (C-terminus) of the molecule. Cystatins C, D, S, SA, and SN are representative of Family 2, and some members in this family are glycosylated (Ni et al., 1997; Cornwall and Hsia, 2003). Human serum cystatin C has been suggested to be a simple, accurate, and rapid endogenous marker of GFR (Coll et al., 2000; Dharnidharka et al., 2002; Calero-Paniagua et al., 2014). Kininogens are representative of Family 3, and have molecular weights ranging from 88 to 114 kDa. These proteins are glycosylated and contain three Family 2 cystatin domains, including two (domains 2 and 3) that have protease-inhibitory activities (Ochieng and Chaudhuri, 2010).

Unlike mammals, studies investigating cystatins in fish have mainly focused on their immune role and their application in food engineering. Many cystatin-like proteins have been characterized, including those from rock bream (Premachandra et al., 2012), *Pseudosciaena crocea* (Li et al., 2009), *Clarias batrachus* Linnaeus (Mohindra et al., 2013), chum salmon (Yamashita and Konagaya, 1996), sturgeons (Bai et al., 2002), rainbow trout (Li et al., 1998), and olive flounder (Ahn et al., 2013a,b). Some of these have been shown to respond to bacterial challenge or to be involved in other immune processes (Li et al., 2009; Xiao et al., 2010; Premachandra et al., 2012), while some recombinant cystatins have been shown to have potential use in food engineering, where they may be used to prevent gel weakening by addition to surimi (Nakamura et al., 1998; Kang and Lanier, 2005; Jiang 2006; Li et al., 2007). In addition, cystatin has been identified as a component of carp chorion and is an important compound for preventing polyspermy (Chang et

al., 1998; Wang and Huang, 2002; Su et al., 2005). In *C. batrachus*, a novel cystatin-like gene clustering in Family 2 has been cloned and its expression was found to respond to both short and long periods of hypoxia, which suggests that *C. batrachus* cystatin may be involved in tolerance to hypoxia (Mohindra et al., 2013). Taken together, these findings highlight the importance of studies investigating cystatin in fish.

Collichtys lucidus Richardson (Perciformes, Sciaenidae, *Collichthys*) is a commercially important near-shore species widely distributed in the South China and East China Seas (Cheng et al., 2012). Although great progress has been made in artificial propagation of *C. lucidus* since 2014, studies on this species have mainly focused on the mitogenome, microsatellites, and auxology (Cheng et al., 2012; Zhao et al., 2015). The aim of the present study was to characterize *C. lucidus* cystatin C (*Clcys*) mRNA sequences, analyze one sequence using bioinformatics and to clarify the pattern of tissue expression in adult *C. lucidus*, in an attempt to lay a preliminary foundation for the further study of *C. lucidus*.

MATERIAL AND METHODS

Construction of a cDNA library and cloning of *Clcys* cDNA from *C. lucidus*

Healthy fish weighing 35 ± 5 g were captured from the East China Sea area near Shanghai, China. Tissues weighing 200 ± 50 mg for RNA extraction were obtained through vivisection on fishing boats and stored in 1.5 mL RNA fixer (Bioteke Corporation, Beijing) at -20°C. Total RNA was isolated from the liver, gill, heart, muscle, and blood of *C. lucidus* using TRIpure Reagent (Aidlab, Beijing) following the manufacturer protocol. RNA quality and concentrations were determined by agarose gel electrophoresis (Universal Hood II, Bio-Rad Laboratories Inc., Hercules, CA, USA) and spectrophotometry (DU800 Nucleic acid/Protein Analyzer, Beckman Coulter). The RNA was stored at -80°C until use.

A cDNA library was constructed successfully using SMART™ cDNA Library Construction Kit (TaKaRa, Dalian, China), followed by transformation, screening, and sequencing. Random sequencing was performed after cDNA library construction. All the expressed sequence tags were subjected to BLAST analysis.

A cDNA sequence comprising a *Clcys* domain was obtained and subjected to further investigation. Next, 3'-rapid amlification of cDNA ends (RACE) was carried out following the manufacturer protocol using the 3'-full RACE Core Set (TaKaRa) to obtain the full-length cDNA sequence. Two gene specific-primers, namely CL-cys-outer (5'-AAGCGTCGAGCTGTTGTT-3', as 3'-RACE amplification outer primer) and CL-cys-inner (5'-TGTCCCAGCGAAGTGTTA-3', as the nest PCR primer) were designed according to the obtained sequence and used for 3'-RACE. PCR followed the manufacturer instructions. The PCR products were checked by 1.0% agarose gel and purified by the Agarose Gel DNA Purification kit Ver. 2. 0. (TaKaRa Biotechnology), and subsequently cloned into the pMD19-T vector (TaKaRa) for sequencing.

Bioinformatic analysis

General features of *Clcys* full-length cDNA were detected by Vector NTI Advance 11.5, and the peptide sequence was deduced and confirmed according to other cystatins using NCBI ORF Finder (available online at http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The biochemical features of

the deduced protein were calculated with the Expert Protein Analysis System (http://web.expasy.org/). Signal peptide cleavage sites were predicted by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Amino acid sequences from various species were obtained from NCBI GenBank and analyzed using the MEGA software version 5.0. A UPGMA tree was constructed using the MEGA software version 5.0 and the confidence level in the generated tree was obtained using 1000 bootstraps.

Tissue distribution analysis of the mRNA of *Clcys* in adult *C. lucidus*

Tissues including the liver, gill, heart, muscle, brain, testis, cholecyst, spleen, blood, and kidney were obtained using the same method described above. Total RNA (~1 μg) was extracted and reverse transcribed using a quantitative real-time PCR (qRT-PCR) Kit (Toyobo, Japan) to generate first-strand cDNA. The products were diluted 10X and stored at -20°C until qRT-PCR analysis.

The qRT-PCR assay was carried out in a detection system (StepOne Plus, Applied Biosystems) and the transcript expression profiles of *Clcys* in eight different tissues of *C. lucidus* were detected by qRT-PCR using a pair of *Clcys*-specific primers (CL-cys-RT-F: 5'-ACAATTACGTCATAACCACCAAG-3' and CL-cys-RT-R: 5'-CGTCACCGATATTTCATTCAGC-3'), and a pair of 18S rRNA primers (18S-RT-F: 5'-GCCTGAATACCGCAGCTAGGAATAA-3' and 18S-RT-R: 5'-TTTCACCTCTAGCGGCACAATACG-3') designed from the 18S rRNA sequence deposited in NCBI GenBank (accession No. JN211725.1) was used as an internal control. Levels of *Clcys* expression were calculated by the standard curve method. Amplifications were performed on a 96-well plate in a 20-μL reaction volume containing 10 μL 2X Power SYBR Green PCR Master Mix (TaKaRa, Applied Biosystems), 1.0 μL PCR Forward Primer (10 μM), 1.0 μL PCR Reverse Primer (10 μM), 2.0 μL cDNA temple, and 6 μL diethylpyrocarbonate-treated water. The reaction process for qRT-PCR was 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min.

RESULTS AND DISCUSSION

Cloning of *Clcys* cDNA

The full-length cDNA of *Clcys* was 699 bp long (GenBank accession No. KP771714), containing a 357-bp ORF encoding a protein of 118 amino acids, and the *Clcys* sequence included a 5'-UTR of 52 bp and a 3'-UTR of 290 bp including a poly(A) tail (Figure 1). The predicted molecular weight of *Clcys* was 12.875 kDa with an isoelectric point of 8.81. A 21-amino acid signal peptide (amino acid residues 1-21) was found in the N-terminal region, suggesting that *Clcys* is secreted. In addition, *Clcys* contains structural features of Family 2 cystatins, including three evolutionally conserved motifs that are known to interact with the active sites of cysteine peptidases: Gly at the N-terminus (Gly25), Gln-X-Val-X-Gly motif (Q69LVAG73), and Pro-Try pair at the C-terminus (P106W107) (Dieckmann et al., 1993; Li et al., 2009). These findings suggest that *Clcys* may serve as a cysteine protease inhibitor that is involved in physiological processes of *C. lucidus*.

Human cystatin C, a powerful physiological protein inhibitor of cathepsins B, H, and L, contains two disulfide bridges (Cys73-Cys83 and Cys97-Cys117), at least one of which is essential for its inhibitory activity (Grubb et al., 1984). However, Cys83 seemed to be deleted in *C. lucidus*

and an alternative Cys residue (Cys56 in *Clcys*) was found, which may cause the first disulfide bond of *Clcys* to move towards the N-terminus. This has previously been found in *P. crocea*, and does not appear to have a significant effect on the biological activity of cystatin C of *P. crocea* (Lyccys), since rLyccys produced in *Escherichia coli* exhibited obvious protease-inhibitory activity and affinity binding to papain with a K_i of 1.3 x 10^{-13} M, which is in the pM-fM range of K_i determined as it is in mammalian and avian cystatin C (Li et al., 2009).

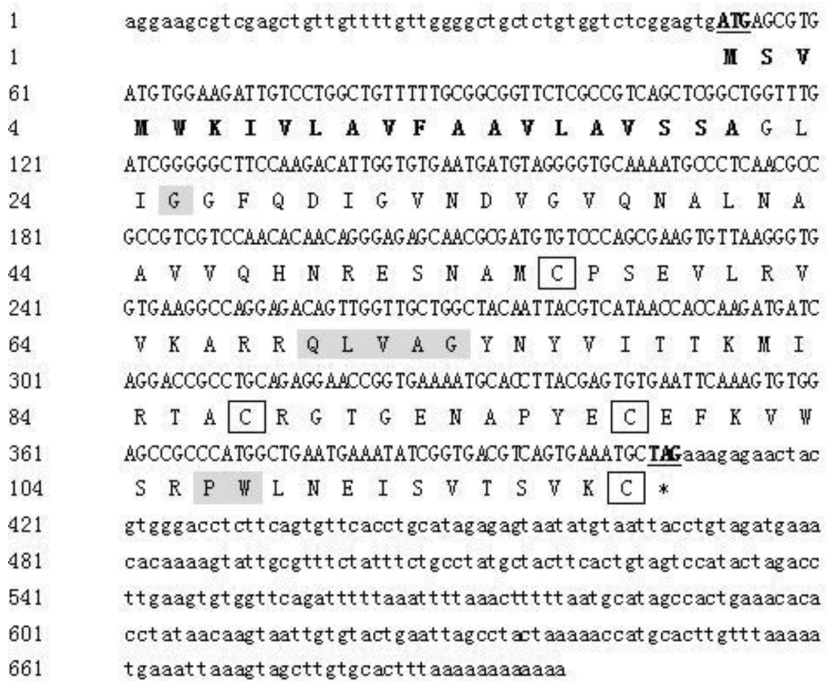

Figure 1. Nucleotide sequence of cDNA and predicted amino acid sequences of the spinyhead croaker *Collichthys lucidus* cystatin C (*Clcys*). The deduced amino acid sequences are translated. Signal peptide sequences are bolded, Gly25, Q69LVAG73, P106W107, are shadowed, and four cys residues are boxed. The start codon (ATG) and stop codon (TGA) are bolded and underlined.

Sequence analysis

Forty-three amino acid sequences were obtained using “cystatins A, B, C, D, F” and “kininogen” as the key word when searching in the protein database (http://www.ncbi.nlm.nih.gov/guide/proteins/#databases). All sequences were aligned by the ClustalW in MEGA 5.0 and a UPGMA tree was constructed using the MEGA software version 5.0. The confidence level in the generated tree was obtained using 1000 bootstraps. According to the tree, all cystatins were split between three branches (type I, type II, and type III), *Clcys* belongs to the type II of cystatin family, and is clustered with cystatin C of *P. crocea* (XP_010728274.1), *Paralichthys olivaceus* (ACC86115.1), and *Danio rerio* (NP_001026843.2) (Figure 2). Among the cystatins, all those from mammals were clustered, as were those from fish. *Clcys* is most closely related to *P. crocea*, thus supporting traditional taxonomic relationships.

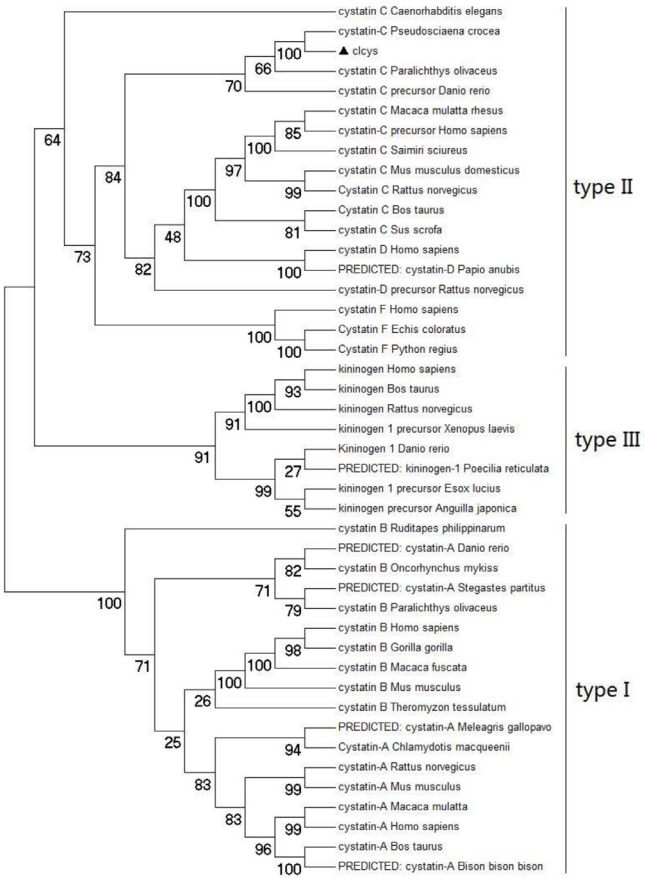

Figure 2. Evolutionary tree of *Collichthys lucidus* cystatin C (*Clcys*). The UPGMA tree is constructed using the MEGA 5.1 program based on multiple-sequence alignment by Clustal W. The scale bar corresponds to estimated amino acid substitutions per site. The protein sequences used in this analysis were obtained from GenBank as follows: cystatin-A *Macaca mulatta* (NP_001245071.1), cystatin-A *Homo sapiens* (NP_005204.1), cystatin-A *Rattus norvegicus* (NP_001099346.1), cystatin-A *Bos taurus* (NP_001161296.1), PREDICTED: cystatin-A *Bison bison* (XP_010832555.1), cystatin-A *Mus musculus* (NP_001028411.1), PREDICTED: cystatin-A *Danio rerio* (XP_698401.1), PREDICTED: cystatin-A *Stegastes partitus* (XP_008295700.1), PREDICTED: cystatin-A *Meleagris gallopavo* (XP_003202689.1), cystatin-A *Chlamydotis macqueenii* (KFP39525.1), cystatin B *Homo sapiens* (AAF44059.1), cystatin B *Mus musculus* (AAC52851.1), cystatin B *Paralichthys olivaceus* (ACC86114.1), cystatin B *Theromyzon tessulatum* (AAN28679.1), cystatin B *Ruditapes philippinarum* (AFP50149.1), cystatin B *Macaca fuscata* (BAC21008.1), cystatin B *Gorilla_gorilla* (BAC20307.1), cystatin C *Mus musculus* (AAA63298.1), cystatin C *Rattus norvegicus* (AAH87591.1), cystatin C *Bos taurus* (AAI09630.1), cystatin C *Saimiri sciureus* (AAB64051.1), cystatin C *Macaca mulatta* (AAB49517.1), cystatin C *Sus scrofa* (ABG48755.1), cystatin C *Caenorhabditis elegans* (CAC33822.1), cystatin D *Homo sapiens* (CAA49838.1), cystatin-D precursor *Rattus norvegicus* (NP_001102431.1), PREDICTED: cystatin-D *Papio anubis* (XP_009214933.1), cystatin F *Homo sapiens* (CAD52872.1), cystatin F *Echis coloratus* (JAC96569.1), cystatin F *Python regius* (JAC94922.1), cystatin C precursor *Danio rerio* (NP_001026843.2), cystatin-C precursor *Homo sapiens* (NP_000090.1), kininogen *Homo sapiens* (AAB59551.1), kininogen *Rattus norvegicus* (AAA41486.1), kininogen *Bos taurus* (CAA24736.1), kininogen 1 precursor *Xenopus laevis* (NP_001088128.1), PREDICTED: cystatin-C *Pseudosciaena crocea* (XP_010728274.1), cystatin C *Paralichthys olivaceus* (ACC86115.1), kininogen 1 *Danio rerio* (AAH83429.1), kininogen 1 precursor *Esox lucius* (NP_001290822.1), kininogen precursor *Anguilla japonica* (AHV79431.1), PREDICTED: kininogen-1 *Poecilia reticulata* (XP_008404505.1).

Eleven cystatin C amino acid sequences including *Clcys* were aligned by ClustalW in MEGA 5.0 (Figure 3). The results revealed that the typical features of Family 2 (G25, Q69LVAG73, and P106W107 in *Clcys*) were conserved. Among these sequences, *Clcys* had the highest identity with *P. crocea* (89%), followed by *D. rerio* (40%), *P. olivaceus* (38%), and *Homo sapiens* (35%). Interestingly, cystatin C sequences were relatively conserved among all mammal species under investigation, with the lowest identity of 68% found between *H. sapiens* and *Rattus norvegicus*. Two hypotheses can explain this; first, cystatin C may not be as conserved in fish species as it is in mammals, and second, the classification of cystatin C in fish might differ from that in mammals. Considering that *Clcys* has high identity with *P. crocea*, which has a closely genetic relationship with *C. lucidus* (Qu et al., 2012), we tend to support the second hypothesis, although more cystatin C sequences need to be identified from fish species to confirm this hypothesis.

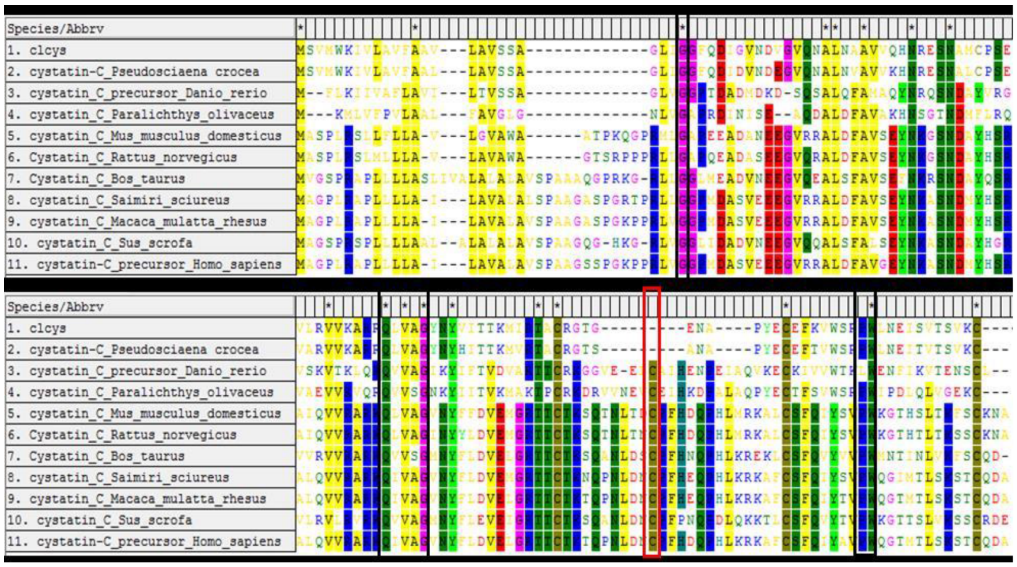

Figure 3. ClustalW alignment and comparison of the amino acid sequences deduced from *Collichtys lucidus* cystatin C cDNA with similar protein sequences of cystatins. Similarities more than 60% are labeled in different colors. The amino acid sequence deduced from spinyhead croaker is marked as *Clcys*. The typical feature of type II cystatins are in a black box, the deleted cys residue are in a red box. Other similar proteins are predicted and obtained from GenBank as follows: PREDICTED: cystatin-C *Pseudosciaena crocea* (XP_010728274.1), cystatin C *Paralichthys olivaceus* (ACC86115.1), cystatin C precursor *Danio rerio* (NP_001026843.2), cystatin C *Mus musculus* (AAA63298.1), cystatin C *Rattus norvegicus* (AAH87591.1), cystatin C *Bos taurus* (AAI09630.1), cystatin C *Saimiri sciureus* (AAB64051.1), cystatin C *Macaca mulatta* (AAB49517.1), cystatin C *Sus scrofa* (ABG48755.1), cystatin-C precursor *Homo sapiens* (NP_000090.1).

Analysis of *Clcys* expression in various tissues

The expression of *Clcys* in 10 tissues from adult *C. lucidus* was examined using qRT-PCR. *Clcys* expression was found in all tissues, with the highest expression detected in the kidney, followed by the liver, heart, and testis, and the weakest expression in muscle (Figure 4). *Lyccys* expression was high in the spleen, kidney, and liver of *P. crocea*. There was a significant upregulation of Lyccys transcript in the spleen and kidney in response to poly (I:C) or an inactivated

trivalent bacterial vaccine, and mRNA levels of *LycTNF-2* and *LycIL-10* in these two tissues were also significantly up-regulated by rLyccys (Li et al., 2009), suggesting that *Clcys* may also play an important role in immune processes of *C. lucidus*. However, the cystatin C gene in *P. olivaceus* seems to be of the housekeeping type, and the *Paralichthys olivaceus* cystatin C gene was ubiquitously expressed throughout healthy and LPS-stimulated individuals (Ahn et al., 2013a). The expression pattern of cystatin C as a "housekeeping" gene also appears in diverse species, including humans (Abrahamson et al., 1990), mice (Huh et al., 1995), dogs (Sekine and Poulik, 1982), chickens (Colella et al., 1991), and salmon (Yamashita and Konagaya, 1996). The differential expression of *Clcys* in various tissues suggests that it is not a housekeeping gene; however, whether there is another cystatin C gene that serves as a housekeeping gene warrants further studied.

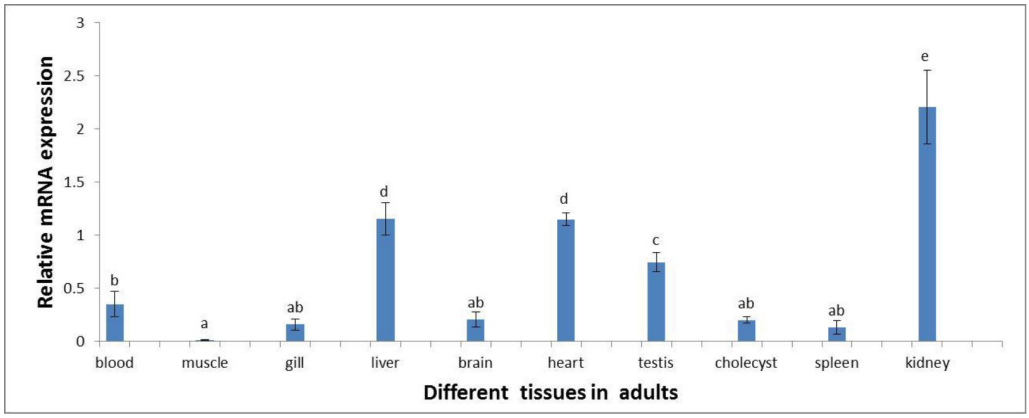

Figure 4. Expression profiles of *Clcys* in different tissues. *Clcys* mRNA was normalized to the 18S rRNA transcript level. Data are reported as means ± SD of three independently repeated experiments. The letter shows that there are significant differences during these stages ($P < 0.05$). The y-axis represents the relative ratio of expression levels of *Clcys*/18S rRNA mRNA.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Basic Research Fund for State-Level Nonprofit Research Institutes of ESCFRI, CAFS (#Dong2015M08).

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