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A cystatin F homologue from large yellow croaker (*Larimichthys crocea*) inhibits activity of multiple cysteine proteinases and Ii chain processing *in vitro*

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ABSTRACT

Cystatin F, a member of the family II cystatins, plays important roles in immune response-related processes through inhibiting specific enzyme targets. In this study, a cystatin F homologue, LycCysF, was identified and characterized from large yellow croaker (*Larimichthys crocea*). The deduced LycCysF protein exhibits a typical structural feature of type II cystatins, including three evolutionally conserved motifs, Gly^{35}, $QVVRG^{79-83}$ and $PW^{130-131}$. Tissue expression analysis showed that *LycCysF* mRNA was expressed in all tissues examined, albeit at different levels. Recombinant LycCysF (rLycCysF) produced in *Pichia pastoris* could inhibit the activity of multiple cysteine proteases, including papain, legumain and recombinant large yellow croaker cathepsin B, L and S. Moreover, rLycCysF could inhibit the Ii chain processing by recombinant cathepsin S *in vitro*. These data suggest that LycCysF may participate in regulation of cathepsins and MHC-II associated Ii chain processing. In addition, mammalian cystatin F is produced as an inactive dimer, becoming activated by proteolysis in the endo/lysosome of immune cells and then exerts its function of regulating downstream proteases activity. However, the N-terminal extension and two additional cysteine residues responsible for dimer formation are absent in LycCysF and cystatin F from other fish species, reptiles and Aves, indicating that these proteins can not form dimer and may regulate the proteases activity via an alternate pathway distinct from mammalian cystatin F. To our knowledge, this is the first report on molecular characteristics of a teleost cystatin F and its role in Ii chain processing.

1. Introduction

Cystatin superfamily is a group of reversible, tight-binding natural protein inhibitors, which regulate the activity of papain-like cysteine proteinases and C13 legumain peptidases [1,2]. These inhibitors are divided into three major types [3–5]. Type I cystatins, including cystatins A and B (also called stefins), are approximately 100 amino-acid long cytosolic proteins lacking signal peptides and disulfide bridges [6]. Type II cystatins, comprising at least 14 members [7], are secreted proteins approximately 120 amino acids long and contain at least two disulfide bridges [8]. Type III cystatins, the kininogens, are larger multifunctional plasma proteins containing three repeated type II-like cystatin domains [5,9]. Type II cystatins identified in mammals include the well-known cystatins C, D, E/M, S, SA, SN and an unusual cystatin F, which possesses a number of unique properties [10,11].

In humans, cystatin F shares relatively low sequence identity with other family members (~35%), containing distinct features including a 6-amino-acid N-terminal extended region, two additional cysteines (Cys^{26} and Cys^{63}) and several non-conservative substitutions in the putative protease-interacting domains [10,11]. Human cystatin F is produced as a disulfide-linked dimer, which is inactive until reduced to monomeric form [10,12,13]. Several factors have been implicated in this monomerization. For example, glycosylation at Asn^{62} could defend the intermolecular disulfide from reduction [13]. On the other hand, an N-terminal processing by a protease, probably cathepsin V, in the cytotoxic granules could significantly strengthen the monomerization of dimer [14]. Intact monomeric human cystatin F effectively inhibits cysteine

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peptidases, such as the cathepsins F, L, K and V, whereas it can only partially inhibit cathepsins S and H, and is unable to inhibit cathepsins B, X and C [10,11,15]. Mammalian legumain or asparaginyl endopeptidase (AEP), the C13 cysteine protease involved in antigen processing, is also known to be inhibited by human cystatin F, although this occurs with a reduced affinity as compared to cystatins C or E/M [5]. Cystatin F is expressed exclusively in immune cells, such as cytotoxic T cells, natural killer cells (NK cells), monocytes, and dendritic cells (DCs) [16,17]. Although it contains a signal peptide, only a small proportion is secreted predominantly as an inactive dimer, and the majority is retained within cells either as a monomer or dimer [13,18]. In human pro-monocyte U937 cells, a large proportion of cystatin F resides intracellularly in the endosome/lysosome-like vesicles, mainly as a truncated monomeric form [15,19], whereas in the endoplasmic reticulum and Golgi apparatus, it predominantly exists as an inactive dimer form [2]. In cytotoxic cells, such as T cells, NK cells, neutrophils and mast cells, truncated cystatin F has been suggested to inhibit cathepsin C [2,20], which plays an important role in the immune response via activation of the granzymes and effector proteases [20]. In immature DCs, human cystatin F colocalized with cathepsin S, indicating it may be involved in regulating the MHC II-associated invariant chain(Ii chain)processing, since cathepsin S has been shown to play important roles in the late stage of Ii chain processing [21,22]. However, in maturing adherent DCs, cystatin F encounters cathepsin L, suggesting that it may regulate the activity of this protein and thus may control the processing of procathepsin X, which promotes cell adhesion via activation of Mac-1 (CD11b/CD18) integrin receptor [23]. In a recent study, cystatin F was shown to regulate proteinase activity in IL-2-activated NK cells and was proposed to be involved in the regulation of split anergy in these cells [14,24].

Currently, there are only amino acid sequences of fish cystatin F deposited in GenBank, but none of them are molecularly or functionally characterized. In this study, a cystatin F homologue (LycCysF) from large yellow croaker (*Larimichthys crocea*), an economically important fish species in China, was identified and characterized. The LycCysF possesses unique structural and functional features as compared to mammalian cystatin F, suggesting they may regulate the cysteine proteases activity via distinct pathways. Recombinant LycCysF (rLycCysF) expressed in *Pichia pastoris* could inhibit the activity of multiple cysteine proteases including papain, legumain and recombinant large yellow croaker cathepsin B, L and S. Furthermore, the rLycCysF can inhibit the Ii chain processing by recombinant cathepsin S *in vitro*, suggesting that it may participate in the major histocompatibility complex (MHC)-II associated antigen processing and presentation. To our knowledge, this is the first report on the molecular characteristics of a teleost cystatin F and providing evidence for its function in antigen processing and presentation.

2. Materials and methods

2.1. Sequence cloning and analysis

A scaffold containing the full-length cDNA sequence of large yellow croaker cystatin F (*LycCysF*) was obtained when analysing the spleen transcriptome of large yellow croaker at 12 h after infection with *Aeromonas hydrophila* [25]. The primer set of CysF-F and CysF-R (sequences in Table 1) was designed based on the obtained scaffold and used in a PCR to validate the sequence. The resulted fragment was then sequenced and analysed by software as described in Ref. [26]. Phylogenetic tree was constructed with the Molecular Evolution Genetics Analysis (MEGA) software version 6.0 using the neighbour-joining method [27].

Table 1
Primers used in this study.

Name	Sequence (5′–3′)	Purpose
CysF-F	ATGGGAGTAAAGACGCTGTTGCCTGT	Sequence validation
CysF-R	TCAGGATTTACACAATAAAACCAGAG	
CysF-F1	CGCTGCCTACTCCTTCAACA	Tissue expression analysis
CysF-R1	GACGACTGTCGGACTGGAAG	
rCysF-F2	GCGAATTCAGACATCATGGCAC	Recombinant expression
rCysF-R2	GCTCTAGACCGGATTTACACAATAAA	
Actin-F1	GACCTGACAGACTACCTCATG	β-actin amplification
Actin-R1	AGTTGAAGGTGGTCTCGTGGA	

2.2. Tissue expression profile analysis of LycCysF by real-time PCR

Various tissues including brain, skin, spleen, intestine, gills, kidney, heart, blood, liver and muscle were collected from five healthy large yellow croakers (mean weight 100 g) at a mari-culture farm at Lianjiang county, Fuzhou city, China. Tissues were immediately frozen in liquid nitrogen until transferred to −80 °C for long term storage. Total RNA was isolated and transcribed into first strand cDNA as described in Ref. [26]. To examine the tissue expression profile of *LycCysF*, real-time PCR was performed with the primer set of CysF-F1 and CysF-R1 (Table 1). *β-actin* was amplified with the primer set of actin-F and actin-R (Table 1), as an internal control for normalization to determine the concentration of each template. The PCR conditions were 1 min at 95 °C, then 40 cycles of 95 °C for 5 s, 57 °C for 15 s and 72 °C for 20 s. All real-time PCR experiments were performed in three independent biological replicates. Data were collected, analysed using GraphPad Prism 5 software, and expressed as the mean ± standard error of the mean (S.E.M).

2.3. Expression, purification and deglycosylation analysis of recombinant LycCysF in P. pastoris

The LycCysF protein excluding the signal peptide was expressed as a recombinant 6 × His-tagged fusion protein in *P. pastoris* SMD1168. Briefly, a *LycCysF* gene fragment deleting the signal peptide encoding sequence was amplified with the primer set of rCysF-F2 and rCysF-R2 (Table 1), and subcloned into the *Eco*RI/*Xba*I digested *P. pastoris* expression vector pPICZαA. An engineered *P. pastoris* strain containing the recombinant expression vector, SMD1168/pPICZαA-*LycCysF* was constructed and the expression of rLycCysF was induced by methanol according to the manufacturer's instructions. Culture supernatant was collected at 72 h of induction. Then centrifuged at 12,000 rpm for 10 min and subjected to SDS-PAGE as well as western-blot analysis. The recombinant LycCysF (rLycCysF) protein was then purified by Ni Sepharose™ 6 Fast Flow chromatography (GE healthcare, UK) according to the manufacturer's protocol and quantified by spectrophotometry (Nanodrop 1000 spectrophotometer, Thermo Fisher Scientific, USA).

For deglycosylation analysis, 20 μg of purified rLycCysF, 10 × glycoprotein denaturing buffer and ddH_2O were mixed to make a 60 μL total reaction volume then incubated at 100 °C for 10 min. Subsequently, 10 μL of 10 × G5 reaction buffer, 1 μL of deglycosylase Endo H (NEB, England) and ddH_2O were added to bring the volume to 100 μL and incubated at 37 °C. At 5 min, 30 min, 1 h and 6 h after reaction started, 10 μL aliquots of reaction mixture were sampled and mixed with an equal volume of 2 × SDS-PAGE loading buffer, then subjected to SDS-PAGE analysis with silver staining.

2.4. Cysteine protease inhibition assay

To measure the cysteine protease inhibitory activity of rLycCysF,