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RESEARCH PAPER

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# Identification and Expression of Cathepsin B from Yellow Catfish (*Pelteobagrusfulvidraco*) in Response to Bacteria and Poly (I:C)

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## Abstract

Cathepsin B, a major lysosomal cysteine protease of the papain-like superfamily, plays an important role in host immune response. To study the immune response of yellow catfish (*Pelteobagrus fulvidraco*) to pathogens, a 1297- bp cDNA of cathepsin B (*PfCTSB*) from yellow catfish was cloned. It contained a 993-bp ORF flanked by a 25-bp 5'-untranslated region (UTR) and a 279- bp 3'-UTR. The ORF encoded a 36.1 kDa cysteine protease with its deduced amino acid sharing a 90% sequence identity with that of *Ictalurus punctatus*. Besides, the predicted PfCTSB was a precursor, including a signal peptide, a propeptide, and a mature peptide. The mature peptide was predicted to be both an endopeptidase based on a catalytic triad (Cys107, His277 and Asn297) and an exopeptidase based on an occluding loop. Furthermore, *PfCTSB* mRNA was constitutively expressed in all examined tissues, with the highest level seen in liver. Yellow catfish were then injected with inactivated *Aeromonas hydrophila*or poly (I:C), and *PfCTSB* mRNA remarkably increased in the liver, spleen and head kidney when compared with the PBS control. It can be speculated that the identified cathepsin B from yellow catfish was involved in host d-efense against infection.

## Introduction

Cathepsin B is a major lysosomal cysteine protease belonging to the C1 family of papain-like superfamily (Turk *et al.*, 2012). Its cysteine protease activity is exerted by a catalytic triad (Cys, His and Asn) (Turk *et al.*, 2012). Besides, cathepsin B also acts as an exopeptidase (dipeptidyl-carboxypeptidase). This activity is catalyzed by a 20-residue occluding loop (Illy *et al.*, 1997), mainly through two sequential histidines within the loop (Musil *et al.*, 1991; Krupa *et al.*, 2002).

It is inactive when cathepsin B is first synthesized as a prepro cathepsin, including a signal peptide, a propeptide, and a mature peptide, since the propeptide physically blocks the active sites of the mature peptide (Turk *et al.*, 2000). After the signal peptide being hydrolyzed, the remaining procathepsin is modified with a mannose-6-phosphate tag. This tag is then recognized by a lysosome receptor. After translocation to the lysosome, the acidic environment facilitates the excision of the pro-domain by an autocatalytic mechanism or by other proteases, thus resulting in the release of the active mature peptidase. So, a slightly acidic pH is optimal for the activity of cathepsin B, as identified in that of *Cynoglossus semilaevis* (Chen & Sun, 2012), *Pseudosciaena crocea* (Li *et al.*, 2014), and *Scophthalmus maximus* (Zhou *et al.*, 2015b).

Cathepsin B plays not only physiological function in intracellular protein turnover and extracellular matrix degration (Cavallo-Medved *et al.*, 2009), but also pathological mechanism in Alzheimer's disease (Hook *et al.*, 2014; Na *et al.*, 2016), rheumatoid arthritis (Trabandt *et al.*, 1991; Hashimoto *et al.*, 2001) and cancer (Aggarwal & Sloane, 2014). Besides, it is also

necessary for the host immune response to pathogens. For instance, cathepsin B of *Paralichthys olivaceus*is induced in response to virus and LPS (Zhang *et al.*, 2008), and is one of the dominant lysosomal proteases in the immune system against Gram-positive ($G^+$) bacteria (Cha *et al.*, 2012). Cathepsin B of *Epinephelus coioides* plays a functional role in Singapore Grouper Iridovirus (SGIV) infection (Wei *et al.*, 2014). Cathepsin B of *Oplegnathus fasciatus* is involved in the immune response to Gram-negative ($G^-$) bacteria (Whang *et al.*, 2011). Cathepsin B (*ctsba*) of *I. punctatus* is important for mucosal immunity (Li *et al.*, 2015). These results indicate that cathepsin B is necessary when fishes encounter bacteria or virus infection.

Yellow catfish, an important commercial fish in China, often suffers bacteria or virus infection in summer due to intensive culture, leading to huge loss annually (Zhou *et al.*, 2015a; Wang *et al.*, 2016a). So far, most studies of yellow catfish focus on its feeding (Dan *et al.*, 2013), development (Jing *et al.*, 2014), and pathogen isolation (Ke *et al.*, 2010). Little is known about its immune response to bacteria or virus. So, a full-length cDNA of cathepsin B from yellow catfish was cloned. Its expression in the healthy and challenged specimen was analyzed.

## Materials and Methods

In all assays, yellow catfish, weighting about 100 g, were obtained from the Pingxi Lake in Pingdingshan city, China. They were maintained in a 500-L tank with aeration and filtration systems at 25°C for 2 weeks. The fishes were fed with commercial pellets twice a day at a 2% feeding rate. The water within the tank was half refreshed daily. The "Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research" were followed when the fishes were sacrificed.

The *A. hydrophila* (GIM1.172) was obtained from the Guangdong Microbiology Culture Center, cultured in LB medium at 28°C, and inactivated by using formalin (Wang *et al.*, 2016b).

The 5′-end cDNA sequence of *PfCTSB* was obtained by sequencing a cDNA library of yellow catfish constructed by our laboratory (Ke *et al.*, 2015). Its 3′-end sequence was amplified by 3′-RACE with the following primers (CTSB-3′: 5′-CACTATGGAAAACAAGCCTACAGTG-3′, CTSB-3′-1: 5′-CATCTGATGAGA AGCAGATCATG-3′) as previously described (Ke *et al.*, 2015).The ORF was analyzed by software DNA star. Its deduced amino acid sequence was identified by BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with its homologues by ClustalW. Based on alignment, a phylogenic tree was constructed by using neighbor-joining algorithm with a bootstrap of 1,000 in MEGA 6.0. Signal peptide and conserved domains of PfCTSB were analyzed by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP) and the SMART (http://smart.embl-heidelberg.de). The N-glycosylation sites were predicted by NetNglyc 1.0 Server (http://www.cbs.dtu.dk/ services/NetNGlyc).

To analyze the tissue distribution of *PfCTSB* mRNA in the yellow fish, eleven kinds of tissues (trunk kidney, intestine, blood, spleen, muscle, skin, liver, head kidney, fin, gill and heart) were collected from the healthy specimen (n=3). Then, total RNA was extracted and transcribed into cDNA (Wang *et al.*, 2016a). Last, the cDNA was used as template to detect the *PfCTSB* expression via quantitative PCR (qPCR) by using the specific primers (qCTSB-F: 5′-TGTGACAAACAATGCGAACCC-3′, qCTSB-R: 5′-TGTGACAAACAATGCGAACCC-3′). The 18S rRNA was served as a reference gene (q18S-F: 5′-GGACACGGAAAGGATTG ACAGA-3′, q18S-R: 5′-GTTCGCTATCGGAATTAACCAGA-3′). The relative fold was calculated with $2^{-\Delta\Delta CT}$ method.

To detect the temporal expression of *PfCTSB* post challenge, the healthy fish were intraperitoneally injected with 200-μl PBS (control), or inactivated *A. hydrophila* ($1\times10^7$ cells/ml), or poly (I:C) (1 μg/μl, Sigma-Aldrich). After injection, the liver, spleen and head kidney were collected at 0, 6, 12, 24, 72, 120, or 168 h from the control and the treated groups(n=3). The *PfCTSB* expression was quantified via qPCR. The significance between samples was determined by performing an ANOVA in SPSS 19.0, with significance defined as (*P<0.05, or **P<0.01).

## Results

The obtained 1297-bp *PfCTSB* cDNA contained a 25-bp 5′-UTR, a 993-bp ORF, and a 279-bp 3′-UTR with a polyadenylation signal-AATAAA located 18-bp upstream of the 21-bp poly(A) tail (GenBank accession number: KX914677). The ORF encoded a 330-aa precursor with a predicted molecular mass of 36.1 kDa and a theoretical is oelectric point of 5.80. This precursor contained a signal peptide (1-18aa), a propeptide (19-78aa), and a mature peptide (79-330 aa) (Figure 1). Acatalytic triad (Cys107, His277 and Asn297) and an oxyanion hole Gln101 are located in the mature peptide. Besides, a segment of ''occluding loop'' of cathepsin B family is also situated in the mature peptide, which endows PfCTSB with the unique dipeptidyl-carboxypeptidase activity. Two putative N-glycosylation sites were predicted in Asn37 and Asn190 of PfCTSB (Figure 1). The Asn37 and Asn190 are separately situated in the propeptide and the occluding loop.

The deduced PfCTSB amino acid sequence showed the highest identity 90% with that of the *I. punctatus* (Figure 1). It also demonstrated 69-84% identities with cathepsin B of other fishes, mammals, avifauna, and amphibians. The phylogenic analysis also showed that PfCTSB falls into the cathepsin B family with high bootstrap values in branching nodes (Figure 2). In detail, the cathepsin B family are divided into two clusters: one for the fishes, and the other for the mammals, avifauna

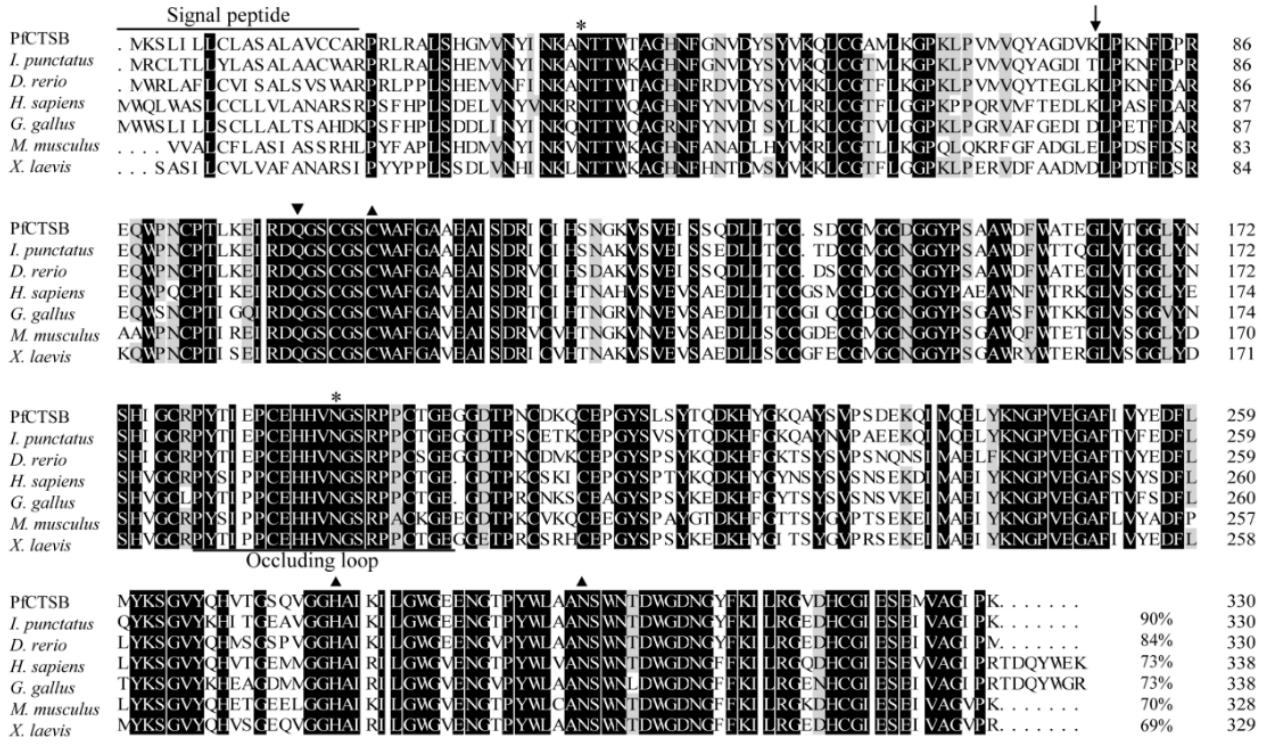


**Figure 1.** Alignment of the deduced PfCTSB amino acid sequence with its homologues from other species. Their sequence identities with PfCTSB are presented in the bottom right. Dots (.) denote gaps introduced for maximum matching. Consensus sequences are shaded dark; and residues identities≧75% are shade gray. The signal peptide is marked with overline. The propeptide and the mature peptide are divided by using an arrow. Residues of the catalytic triad (Cys107, His277, and Asn297) and the oxyanion Gln101 are showed with equilateral triangles and inverted triangle. Two N-glycosylation sites (Asn37 and Asn190) are indicated with asterisks (*). GenBank accession numbers of the aligned sequences are as follows: *I. punctatus*, JT419332; *D. rerio*, NP_998501.1; *H. sapiens*, NP_001899.1; *G. gallus*, NP_990702.2; *M. musculus*, NP_031824.1; *X. laevis*, NP_001079570.

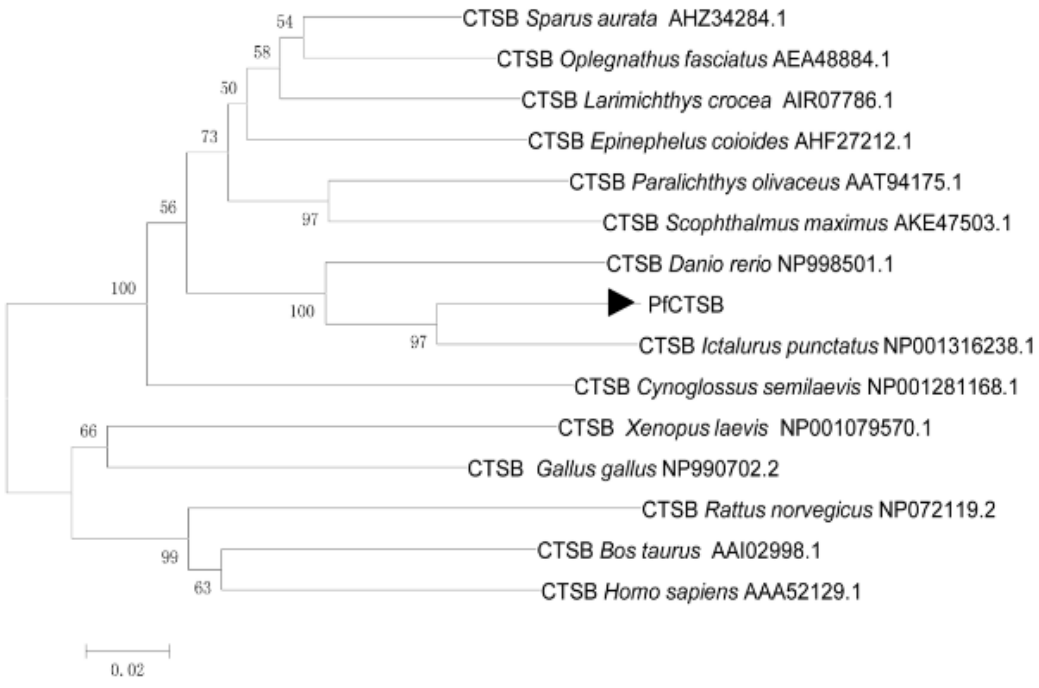


**Figure 2.** Phylogenic analysis of the deduced PfCTSB amino acid sequence and its homologues. Sequences were aligned by ClustalW, and a phylogenic tree was constructed by using a neighbor-joining algorithm with a bootstrap of 1,000based on MEGA6.0. The bar (0.02) represents the genetic distance.

and amphibians. PfCTSB groups most closely with that of *I. punctatus* among the fishes.

The *PfCTSB* mRNA was quantified via qPCR, with 11 tissue types from healthy fish examined and normalized to the blood level (Figure 3). *PfCTSB* was ubiquitously expressed in all examined tissues. The highest expression level was seen in liver (5.9-fold> blood).

The *PfCTSB* expression patterns were also checked in liver, spleen and head kidney after inactivated *A. hydrophila* stimuli by running qPCR (Figure 4). *PfCTSB* mRNA remarkably increased in the three tested tissues compared with the control. *PfCTSB* mRNA in the liver and head kidney begins to elevate at 6 h and reaches the highest level at 12 h (Figure 4a and Figure 4c). Differently, *PfCTSB* mRNA in the spleen shows no difference from the control until the 7 d (Figure 4b).

Cathepsin B was involved in not only the antibacterial response, but also the antiviral response. *PfCTSB* mRNA remarkably accumulated in three examined tissues after poly (I:C) injection (Figure 5). Moreover, *PfCTSB* mRNA has significantly increased at the early 6 h in the three tested tissues.

## Discussion

Cysteine proteases are the major proteases of the lysosome. They all have conserved Cys, His, Asn and Gln in their active sites (Lecaille *et al.*, 2002). These four key amino acids are also found in the PfCTSB. Any amino acid mutation of the active sites can cause a dramatical reduction in activity (Chen & Sun, 2012). Like its homologues, PfCTSB shares a typical architecture: a N-terminal signal peptide, a middle propeptide, and a C-terminal mature peptide of a papain family. In addition, the segment of ''occluding loop'' located in the mature peptide is also conserved among different species, which endows PfCTSB with dipeptidyl-carboxy peptidase activity and is used to identify the enzyme previously (Illy *et al.*, 1997; Eykelbosh & Van Der Kraak, 2010).

Besides its typical architecture, the amino acid sequence of cathepsin B is also highly conserved among different species. The PfCTSB amino acid sequence showed the highest identity 90% with that of the *I. punctatus*, which suggests their evolutional relationship. This result is consistent with our previous study (Wang *et al.*, 2016b, 2017). PfCTSB also shared 69-84% identities with cathepsin Bs of fishes, amphibians and mammals. Such high degree of amino-acid-sequence identity hints its fundamental function in physiological process.

Cathepsin B also play an important role in the host immune system. To study the function of *PfCTSB*, its expression in different tissues was first quantified via qPCR. Its mRNA was distributed in all tested tissues, thus indicating its multifunctionality. Cathepsins B and L can destroy the network structure of silver carp surimigels, leading to gel softening (Liu *et al.*, 2008). In gentamicin-induced acute kidney injury in rats, the amount of cathepsin B decrease along the increased severity of the histopathological lesions of the proximal convoluted tubules, and increase in the well preserved proximal straight tubules (Svara *et al.*, 2010). In human hearts, cathepsin B is expressed and linked to myocardial infarction, the deficiency of which can attenuate cardiac remodeling in response to pressure overload (Wu *et al.*, 2015). Recombinant *Trichinella spiralis* cathepsin B-like protein can promote STAT6-dependent M1 to M2

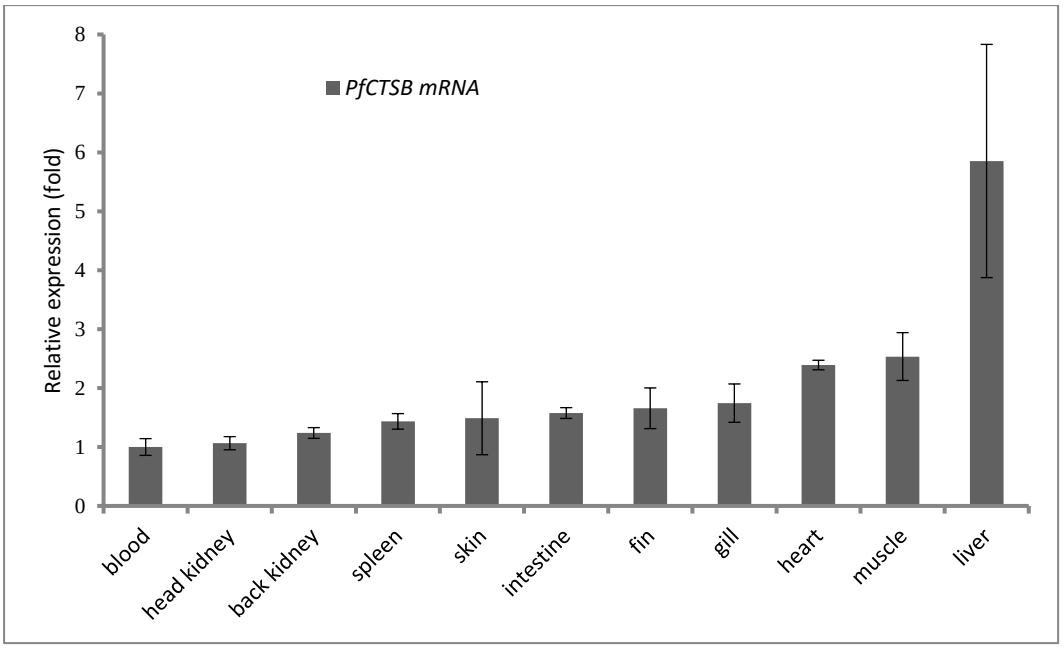


**Figure 3.** Quantification of the relative expression of *PfCTSB* mRNA in 11 tissues from healthy yellow catfish via quantitative PCR. The 18S rRNA was served as a reference gene, and samples were calibrated against the blood. The relative fold change was calculated with $2^{-\Delta\Delta CT}$method.

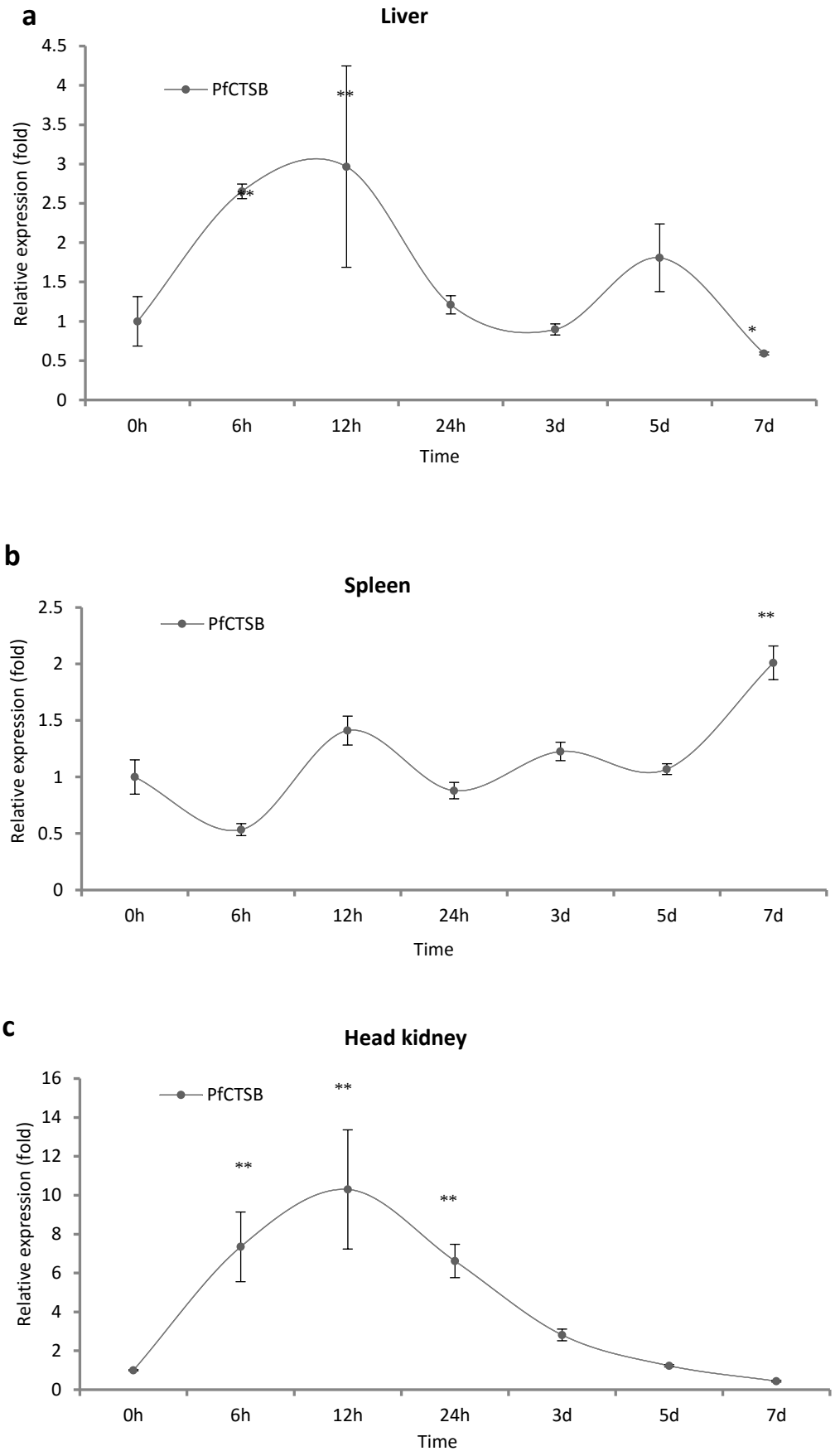


**Figure 4.** The temporal expression of *PfCTSB* mRNA in liver (a), spleen (b), and head kidney (c) post bacteria injection compared with the PBS control. The healthy fishes were injected with 200-µl inactivated *A. hydrophila* suspended in PBS ($1\times10^7$ cells/ml) or PBS (control). The *PfCTSB* mRNA were quantified via qPCR, with the18S rRNA employed as an internal reference. Values were from three replicates and expressed as a mean ± SD; *P<0.05, **P<0.01.

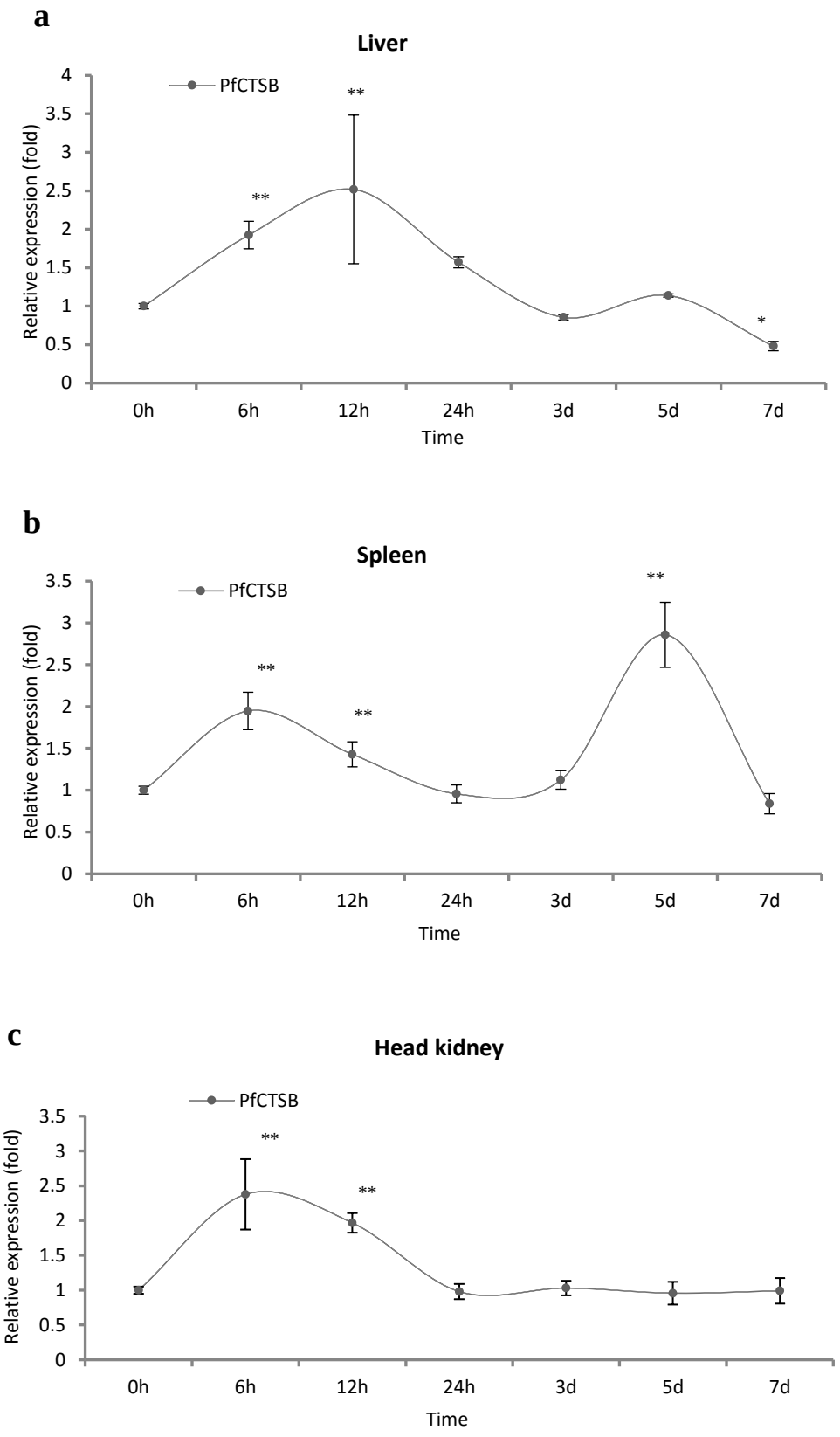


**Figure 5.** The temporal expression of *PfCTSB* mRNA in liver (a), spleen (b), and head kidney (c) post poly (I:C) injection relative to the PBS control. The healthy fishes were injected with 200-µlpoly (I:C) suspended in PBS (1 µg/µl) or PBS (control). *PfCTSB* relative expression levels were detected by using qPCR. The 18S rRNA was served as a housekeeping gene. Values were from three replicates and expressed as a mean ± SD; *P<0.05, **P<0.01

transition, thus ameliorates intestinal injury (Liu *et al.*, 2015). Increased liver CatB expression in the nonalcoholic steatohepatitis mice, and CatB deficiency can ameliorate liver lipid deposition, inflammatory cell infiltration, and fibrosis (Fang *et al.*, 2020).

The highest expression level was seen in liver (5.9-fold > blood), analogous to the *RbCathepsin B* from *Oplegnathus fasciatus* (Whang *et al.*, 2011). Since liver is an important immune organ for yellow catfish (Patman, 2014), the highest expression level of *PfCTSB* in liver suggests its importance in the immune system. Differently, the *LycCatB* (Li *et al.*, 2014) and *SmCatB* (Zhou *et al.*, 2015b) are abundant in spleen. *Fc-CB* (Li *et al.*, 2013) and *PcCTSB* (Dai *et al.*, 2016) are highly expressed in hepatopancreas. The *ctsba* is abundant in trunk kidney (Li *et al.*, 2015). Cathepsin B of *Miiuy croaker* is mainly distributed in intestine (Che *et al.*, 2014). These studies present that cathepsin B may be specific in tissue distribution in different species.

Further study revealed that the *PfCTSB* mRNA was remarkably increased in liver, spleen and head kidney, when the healthy fish were injected within activated *A. hydrophila*, Liver, spleen and head kidney are important immune organs of fish, so the elevated *PfCTSB* mRNA may be helpful in fending off infection (Cha *et al.*, 2012; Li *et al.*, 2015).The same induction was seen in *CsCatB* expression by bacterial infection (Chen & Sun, 2012). Analogously, *PcCTSB* expression in the hepatopancreas was induced following LPS stimuli (Dai *et al.*, 2016). The cathepsin B gene in the kidney of olive flounder is significantly upregulated at the early stage when fish body was infected $G^+$ *S. parauberis* (Cha *et al.*, 2012). These results indicate that cathepsin B in fish could be induced by $G^+$, $G^-$ bacteria and their pathogenic agent, LPS. However, the temporal expression patterns of *PfCTSB* in different tissues are divergent. The *PfCTSB* mRNA in the liver increased within the early 12 h and decreased in the 7 d. The *PfCTSB* mRNA in the spleen only remarkably responded in the later period. The *PfCTSB* mRNA in the head kidney significantly elevated within the 24 h. These results indicated that *PfCTSB* may work in different ways in different organs. Moreover, LycCatB also participates in processing MHC class II-associated invariant (Ii) chain, which is necessary for antigen presentation to $CD4^+$ T-cells, suggesting its importance in adaptive immunity (Li *et al.*, 2014). To detect whether PfCTSB could process Ii chain, we had overexpressed PfCTSB in *E. coli*, however, resulting in inclusion body. Further study is needed to obtain the active protease.

Cathepsin B also plays an important role in the antiviral response. *PfCTSB* mRNA remarkably accumulated in liver, spleen and head kidney after poly (I:C) injection. Poly (I:C) is a synthetic analog of double strand RNA (dsRNA), which is produced by most viruses during their replication (Norval, 2012). So, the accumulation of *PfCTSB* mRNA suggested that it might be involved in viral infection (Zhang *et al.*, 2008; Wei *et al.*, 2014). Moreover, *PfCTSB* mRNA significantly increased at early 6 h in the three tested tissues post stimuli. Similarly, *PcCatB* mRNA remarkably goes up at 0.5 or 1 h after poly (I:C) challenge (Zhang *et al.*, 2008). These results suggest that cathepsin B may respond in the acute stage of virus infection.

Cathepsin B was also involved in the antiviral response of yellow catfish. PfCTSB mRNA remarkably accumulated in liver, spleen and head kidney after poly (I:C) injection. Poly (I:C), long strands of inosine poly(I) homopolymer annealed to strands of cytidinepoly(C) homopolymer, is a synthetic analog of double strand RNA (dsRNA) which is produced by most viruses during their replication (Norval, 2012). Therefore, it is usually used to model the actions of extracellular dsRNA of infected virus, and the increase of PfCTSB mRNA after stimuli suggested its involvement in viral infection (Zhang *et al.*, 2008; Wei *et al.*, 2014). Moreover, PfCTSB mRNA accumulated as early as 6 h after injection in all three examined tissues. Similarly, PcCatB mRNA remarkably goes up at 0.5 or 1 h after poly (I:C) challenge (Zhang *et al.*, 2008). These results suggest that cathepsin B may respond at the early stage of virus infection.

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