Immunolocalization of Jian Carp (*Cyprinus Carpio* Var. Jian) Cathepsin B: Cloning, Expression, Characterization, and Antibody Preparation

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Abstract: Cathepsin B (CatB) cDNA of 759 bp from Jian carp (*Cyprinus carpio* var. Jian) with amino acid similarity of 99.6% to common carp was cloned. The mature CatB was expressed in *Escherichia coli* BL21 transferred with vector CatB-pET-30a. It was purified and identified as a single band (29 kDa) on SDS-PAGE. Optimum CatB activity was observed at 40 °C and pH 5.5. Mouse anti-CatB polyclonal antibody with a high titer of 1:256000 was prepared successfully and shown to specifically recognize the antigen both in prokaryotic cells and in the tissues of Jian carp according to western blotting and immunohistochemistry results. Immunolocation analysis showed that CatB distribution at protein level varied among the tested tissues. The results presented in this study may provide a significant reference for future research on the inherent relationship between CatB and the quality of fish or fish products at both the gene and protein levels.

Keywords: Cathepsin B, cloning, expression, polyclonal antibody, tissue distribution

Practical Application: The investigation on the cDNA cloning and biochemical characteristics of Jian carp CatB and its immunolocalization is essential for utilizing piscine Cathepsins, or researching the inherent relationship between CatB and the quality of fish or fish products.

Introduction

Cathepsins mainly exist in lysosome and include numerous members such as CatB, C, F, H, K, and L. Among them, CatB is the only member of the CatB-like subfamily in the family of papain-like cysteine proteases. It has been well recognized for its role in protein degradation. CatB also participates in various physiological processes including protein turnover, immunity, osteolysis, and cell apoptosis (Conus and Simon 2008; Turk and Turk 2008); moreover, it is involved in several pathological phenomena such as tumor (Kolwijck and others 2010), osteoporosis (Gray and others 2002), and rheumatoid arthritis (Berdowska 2004) and has been explored as a cancer target in recent studies (Gondi and Rao 2013). There has been relatively little research on piscine CatB, though some studies have indicated that CatB functions in the immune response of fish (Whang and others 2010; Li and others 2014, 2015; Zhou and others 2015).

In the meat processing research, it is commonly accepted that CatB plays an important and positive role in the processes of postmortem aging (Delbarre-Ladrat and others 2006) and tenderization (Sentandreu and others 2002). The allele genotype of porcine CatB in particular is related with back-fat thickness, which influences the quality of dry-cured ham (Russo and others 2002). Furthermore, CatB activity level shows a moderate heritability in Italian Large White pigs (Russo and others 2000); the residual enzyme activity of CatB is also regarded as a reliable indicator for the textural defects associated with excessive proteolysis in Spanish dry-cured ham (Garc A-Garrido and others 2000). In short, CatB is likely closely related to the quality of processed meat products.

To date, research on the potential relationship between CatB and the quality of fish fillets or fish products has been mainly focused on the postmortem softening or textural quality of surimi products. In these studies, higher levels of protein expression and CatB activity are typically regarded as key adverse factors due to their associated destruction of the structural integrity of muscle proteins (Chéret and others 2007; Godiksen and others 2009) and degradation of the pivotal protein, myosin, in the gel network of surimi products (Liu and others 2008).

In addition, CatB might indirectly affect the quality of fish muscle texture to some degree, as its expression level in tissues vary considerably during certain physiological periods or under certain stress conditions. CatB is thought to participate in the embryonic development of juvenile fish. During the oocyte growth period of scad (*Decapterus maruadsi*), for example, high-level expression of CatB activity characterizes one of the main proteases involved in hydrolyzing yolk protein; the extent of hydrolysis influences the seasonal yield of fish eggs and quality of juvenile fish (Aranishi 2000). To this effect, observing the expression of CatB during the development of juvenile fish may be significant for evaluating the yield and quality of fish—this is of considerable importance for commercial fishes, as well. In a feeding experiment, after being starved for 4 d and exposed to crowding stress for 24 h, a muscle softening phenomenon occurs in Atlantic salmon (*Salmo salar*) accompanying increasing gene expression and enzyme activities of CatB in the muscle (Bahuaud and others 2010). In addition, the type and content of dietary fatty acids appears to impact the activity and gene expression of CatB in Atlantic salmon (*Salmo salar*), resulting in a significant detachment between myofibers (Bahuaud and others 2009).

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By far, most reports on the potential relation of CatB and the quality of meat or fillets and processed products have focused on the gene expression and activity levels of CatB. There have been few studies on the level of protein expression via immunological testing, especially in fish, due to the lack of readily available CatB-antibodies in fish. Most of the CatB-antibodies in commercial sales are for common terricolous mammals. Considering the low homology of CatB between aquatic animals and terricolous mammals, however, it is necessary to prepare the specific piscine CatB antibody for detecting the expression level of protein in fish. As in similar study, the CatB antibody of clam (*Meretrix meretrix*) was prepared from recombinant antigen protein in one study (Yao and others 2011) and used to analyze CatB expression at the protein level in different tissues and its dynamic variations after starvation, then accordingly was supposed to implicate in the quality of clams due to its regulatory effects on nutrition metabolism. Jian carp (*Cyprinus carpio* var. Jian), an economic species of freshwater fish, is absolutely dominant in aquaculture production in China and has been developed into a number of processed aquatic food products. The quality of the Jian carp itself, in addition to features related to harvest, is quite a crucial indicator of the quality of the resulting products. In view of the latent effects of CatB on the quality of fish or processed fish products, there undoubtedly is important theoretical and practical significance in studying the bioactivity of Jian carp CatB and analyzing the expression patterns both at the gene level and protein level. The cathepsin gene of some fish has been cloned, but there have been no reports on gene cloning or antibody preparation of Jian carp CatB. Moreover, the by-products or offcut generated during processing such as dark flesh, skin, and viscera are typically discarded, which will bring a serious pollution to the environment; it will be beneficial to detect the distribution of CatB in the abandoned tissues of Jian carp in terms of making full use of piscine cathepsin resources.

The primary goal of this study was to obtain the cDNA sequence of mature Jian carp CatB and its active protein to facilitate further investigation of the expression patterns and tissue distribution. We 1st cloned the CatB cDNA of Jian Carp, followed by prokaryotic expression, purification, and characterization of the expressed CatB protein. The specific CatB-antibody was prepared and its recognition capability to antigen was detected by western blotting in the prokaryotic expression system, and by immunohistochemical analysis of multiple tissues of Jian carp.

Materials and Methods

Vectors, strains, and chemicals

Vector pMD19-T was purchased from Takara Biochemical Co. Ltd. (Dalian, Liaoning, China) and vector pET-30a from Novagen (Madison, Wis., U.S.A.). *Escherichia coli (E. coli)* strains DH5α and BL21 (DE3) were offered by the aquatic products processing laboratory of Sichuan Agriculture Univ. Primer synthesis and DNA sequencing were conducted by Introvigen Biotechnology (Shanghai, China).

Standard DNA marker (III, IV) and protein marker (14.4 to 94 kDa) were obtained from Tiangen Biochemical Co. Ltd. (Beijing, China). 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and benzyloxycarbonyl-arginylarginine-4-methyl-7-coumarylamide (Z-Arg-Arg-MCA) were obtained from Sigma-Aldrich (St.Louis, Mo., U.S.A.), and isopropyl-β-thiogalactopyranoside (IPTG) was contributed by Solarbio Science and Technology (Beijing, China). All other chemicals used were of analytical grade.

Animal materials

Ten healthy Jian carps (*Cyprinus carpio* var. Jian, about 500 g per fish) were collected alive randomly in the Tong Wei Hatchery (Sichuan, China). All Jian carps collected were acclimatized to the laboratory environments for 2 wk. During this period, dissolved oxygen was not less than 5 mg/L, and water temperature and pH were maintained at 25 ± 1 °C and 7.0 ± 0.5, respectively. Prior to sacrifice, the Jian carps were anaesthetized by being immersed in 4% urethane solution (pH 7.0 ± 0.4, 20 ± 0.5 °C) for 20 min.

Muscles for RNA extraction were sampled from 5 fishes and stored in liquid nitrogen. Target tissues for immunohistochemical experiment (heart, hepatopancreas, spleen, small intestine, and muscle) were respectively collected from another 5 fishes, and analyzed by the procedures described in "Immunohistochemical staining of CatB protein in Jian carp."

Specific pathogen-free mouse (SPF) BALB/c mice (Male, 20 ± 2 g per mouse) were provided by the animal center of Jilin Univ. (numbers of Production License: SCXK Ji 2013-0001).

All animal experiments were undertaken according to the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments. All procedures were approved by the Animal Care Advisory Committee of Sichuan Agricultural Univ.

Total RNA extraction and cDNA synthesis

The total RNA was extracted from the muscle of Jian carp using a RN15 reagent Kit (Biofit Technology, Chengdu, China) according to the manufacturer's instructions. The single-strand cDNA was synthesized by RT-PCR (reverse transcription polymerase chain reaction = reverse transcription PCR) from the total RNA, oligo-dT primed with a PrimeScript® RT Reagent Kit (Takara Biotechnology, Dalian, China). All cDNA obtained was stored at −80 °C for subsequent molecular cloning.

Gene cloning for the mature peptide DNA of Jian carp CatB

Specific primers *Nde* I/CatBF (5′-CGCATATGCTCCCC ACTAACTTTGATGCCAG-3′) and *Xho*I/CatBR (5′-AA CTCGAGCTGTGGGATTCCAG-3′; the underline denotes restriction enzyme recognition sites) were designed according to the previously published sequence (Tan and others 2006; GenBank accession: AB215097.1). The CatB gene was amplified under the following PCR conditions: 94 °C for 3 min followed by 35 cycles of 94 °C for 40 s, 53 °C for 30 s, and 72 °C for 40 s with an additional final elongation step of 72 °C for 10 min. The PCR product was retrieved with a Universal DNA Purification Kit (Tiangen Biotechnology, Beijing, China) and ligated into the pMD19-T vector. The recombinant vector was transformed into *E. coli* DH5α competent cells. The recombined plasmid CatB-pMD19-T from several positive strains were chosen for gene sequencing (Invitrogen Biotechnology, Shanghai, China) after a blue/white-screen and confirmation by restrictive double-enzyme digestion; DNAMAN 8.0 and MEGA 6.0 software were respectively adopted to analyze the sequencing results and construct a phylogenetic tree via neighbor-joining method.

Expression and purification

To construct the prokaryotic expression vector CatB-pET-30a, CatB-pMD19-T and pET-30a were double-digested by *Nde*I/*Xho*I respectively, then CatB gene fragment was ligated into pET-30a with T4 DNA ligase (16 °C, 16 h). After selection by colony PCR and identification through *Nde* I/*Xho* I double digestion, the correctly restructured CatB-pET-30a was transformed

into an expression strain of *E. coli* BL21 (DE3). The recombinant protein of CatB was efficiently expressed in the form of an inclusion body after induction by 1 mM/L IPTG for 3 h at 37 °C. The inclusion body was washed with gradient urea solution and dissolved in 8 M urea, and finally purified by Ni^{2+}-NTA agarose affinity chromatography (1.6 × 5 cm) after dialysis. SDS-PAGE (Laemmli 1970) was used to analyze the expression and purification of the recombinant CatB. The renaturation of the purified CatB was performed with a Protein Refolding (dialysis) Kit (GeneMed Science, Mass., U.S.A.) according to the manufacturer's instructions, and then the activated enzyme was used for subsequent enzyme activity assays.

Enzyme activity assay of recombinant CatB

CatB activity was routinely assayed with Z-Arg-Arg-MCA by the method from (Barrett and Kirschke 1981). Fluorescence intensity was measured in a Multiskan Spectrum Fluorescence Microplate Spectrophotometer (Varioskan Flash 3001, Thermo Scientific, Mass., U.S.A.) with excitation and emission wavelengths of 380 and 460 nm, respectively. One unit of enzyme activity was defined as the amount of activity that released 1 nmol of AMC per min under the assay conditions.

The effects of temperature and pH on CatB were measured as reported previously (Li and others 2008). The optimum temperature of CatB activity was assayed in the range of 20 to 80 °C and the optimum pH was detected in McILvaine's buffer with a pH range of 3.0 to 8.0; thermal stability was assayed by incubating CatB at a variety of temperatures from 20 to 80 °C at pH 6.0 for 1 h and then cooling it to 4 °C; pH stability was evaluated after incubating the enzyme in McIlvaine's buffer (pH 3.0 to 8.0) at 4 °C for 1 h then regulating it to pH 6.0. The relative activity of CatB was expressed as a percentage of the activities measured under the test conditions above to the maximum.

Preparation of polyclonal antibody against CatB

Quick Antibody-Mouse5W adjuvant (KangBiQuan Biotechnology, Beijing, China) was used for polyclonal antibody preparation following the manufacturer's instructions. In brief, 2 male BALB/c mice were immunized twice by way of subcutaneous injections at the hind leg during a 5-wk period, and serum prior to immunization was collected as a control. For the 1st injection, the recombinant CatB (0.4 mg/mL) and adjuvant were aseptically mixed at an equal volume proportion of 50 μL for each mouse; the same dose of the booster injection was then given 21 d later. On the 35th d, a small volume of tail blood was collected to detect the titer by enzyme-linked immunosorbent assay (ELISA), then the whole blood was collected to isolate antisera once the titer reached a ratio equal to or greater than 1:10000.

Characterization of polyclonal antibody

Indirect ELISA was conducted to identify the titer of CatB polyclonal antibody. The recombinant CatB at the final concentration of 20 μg/mL in 0.05 mol/L sodium carbonate buffer (pH 9.6) was incubated at 4 °C overnight in a 96-well plate (100 μL each well). On the 2nd d, the plate was washed with PBST 3 times (5 min each time) and then blocked for 2 h with 2% BSA at room temperature (RT) after removing the sodium carbonate buffer supernatant. Multiple proportion dilutions (1:1000 to 1:1024000) of antisera were added into the plate (100 μL each well, and incubated at 37 °C for 1 h); the negative control group of preimmune sera was tested under the same protocol. The plate was again washed 3 times (5 min each time), then the 2nd antibody of goat anti-mouse IgG conjugated to horse radish peroxidase (HRP; Dingguo, Beijing, China) was added to the plate. After 37 °C incubation for 1 h, the 2nd antibody was removed and the plate was washed again. A SuperSignal West Pico chemiluminescence substrate (Thermo Scientific) was applied for 5 to 10 min chromogenic reaction, and then the reaction was ended with 2 mol/L H_2SO_4. Finally, optical density (OD) values were measured at 450 nm.

Western blotting was performed to detect the prokaryotic expression of CatB and identify the specificity of the CatB polyclonal antibody. Samples were separated on 14% SDS-PAGE and electro-transferred onto the polyvinylidene fluoride membrane (PVDF membranes, 0.45 μm, Milipore, U.S.A.) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, California, U.S.A.) at 100 V for 30 min. The PVDF membrane was developed by chromogenic reaction with SuperSignal West Pico chemiluminescence substrate (Thermo Scientific) as the following steps: blocking with TBST buffer containing 5% BSA (RT, 2 h), incubation with primary antibody (1:500 diluted, 4 °C, overnight), then incubation again with goat anti-mouse IgG (H+L) conjugated to HRP (1:5000 diluted, 37 °C, 2 h). The membrane was photographed with an ELC chemiluminescence detector (Image-Quant, GE, U.S.A.).

Immunohistochemical staining of CatB protein in Jian carp

The target tissues for immunohistochemisty were fixed in 4% paraformaldehyde for 24 h and dehydrated in serial concentrations of ethanol. The tissues were then embedded in optimal cutting temperature compound (OCT compound, Sakura, California, U.S.A.), and sectioned into 10 μm slides with a Cryostat Microtome (CM1900-1-1, Leica, Germany).

Immunohistochemical staining was performed with an Ultra-Sensitive SP (Mouse/Rabbit) IHC Kit (Maixin Biotechnology, Fujian, China) in a humid box. All procedures were carried out at RT unless otherwise specified. Slides were treated with 0.3% Triton X-100 for 30 min to implement cell perforation, followed by washing in PBS 3 times (5 min each), then were incubated in 50 μL blocking solution for 10 min to block endogenous peroxidase. Next, normal nonimmune goat serum was added on the slides and held for 30 min to avoid nonspecific staining. The slides were in order incubated in CatB antibody (1:500 diluted) and then the secondary antibody, biotinylated goat anti-mouse IgG respectively, each for 1 h at 37 °C. After incubation, a routine procedure of washing in PBS was implemented. Afterwards, the streptavidin–peroxidase complex was applied for 10 min, and then the slides were washed routinely and stained with an incubation of freshly prepared 3,3N-diaminobenzidine tetrahydrochloride (DAB, Maixin Biotechnology, Fujian, China) for 3 to 10 min and observed under an optical microscope. Finally, after dehydration in gradient concentration ethanol (70%, 80%, 90%, 100% I, 100% II) and clearing in xylene, the slides were sealed with neutral gum. Negative control group was conducted with the same manner except the substitute of CatB antibody by PBS. The stained sections were observed and photographed through fluorescence microscope (IX71, Olympus, Japan). OD was analyzed by IPP6.0 (Image-pro plus 6.0).

Results and Discussion

Cloning and sequence analysis of CatB

As shown in Figure 1, the 759-bp gene fragment of Jian carp CatB was amplified by PCR and the recombinant plasmid

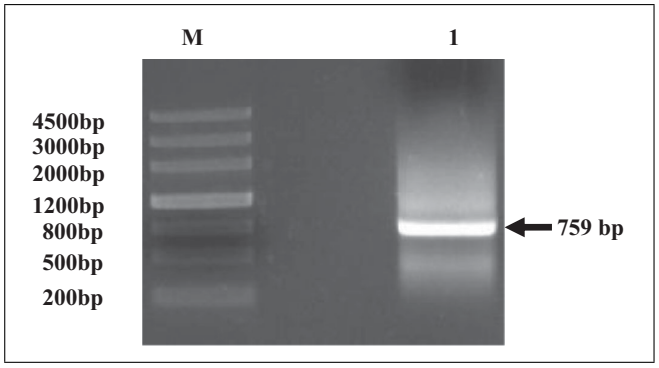

Figure 1–Electrophoresis of Jian carp CatB gene coding sequence. M, DNA Marker III; lane 1, amplification of CatB.


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1    ATGCTCCCCACTAACTTTGATGCCAGAGAGCAGTGGCCCAACTGCCCCACTCTTAAAGAG
1     M  L  P  T  N  F  D  A  R  E  Q  W  P  N  C  P  T  L  K  E
61   ATCAGAGACCAGGGTTCTTGCGGCTCATGCTGGGCATTTGGGGCTGCTGAAGCCATATCT
21    I  R  D  Q  G  S  C  G  S  C  W  A  F  G  A  A  E  A  I  S
121  GACAGAGTATGCATCCACAGCAATGCCAAAGTGAGTGTGGAGATCTCCGCTCAGGACCTG
41    D  R  V  C  I  H  S  N  A  K  V  S  V  E  I  S  A  Q  D  L
181  CTTACCTGCTGTGATGGCTGTGGTATGGGATGTAACGGTGGATACCCTTCTGCTGCTTGG
61    L  T  C  C  D  G  C  G  M  G  C  N  G  G  Y  P  S  A  A  W
241  GACTTCTGGAGCTCAGATGGTCTGGTCACCGGTGGCCTGTATAACTCTCATATTGGCTGT
81    D  F  W  S  S  D  G  L  V  T  G  G  L  Y  N  S  H  I  G  C
301  CGTCCATACACTATTGAACCCTGTGAGCATCATGTGAATGGCAGTCGCCCTCCTTGTACC
101   R  P  Y  T  I  E  P  C  E  H  H  V  N  G  S  R  P  P  C  T
361  GGAGAGGGTGGAGATACTCCTAACTGTGATATGTCCTGTGAGCCTGGCTACAGCCCCTCT
121   G  E  G  G  D  T  P  N  C  D  M  S  C  E  P  G  Y  S  P  S
421  TACAAACAGGACAAACACTTTGGAAAGACGTCCTATAGTGTCCCATCTAATCAGAAGGAC
141   Y  K  Q  D  K  H  F  G  K  T  S  Y  S  V  P  S  N  Q  K  D
481  ATTATGAAAGAGCTCTACAAGAATGGCCCAGTAGAGGGAGCTTTCACTGTCTATGAGGAC
161   I  M  K  E  L  Y  K  N  G  P  V  E  G  A  F  T  V  Y  E  D
541  TTCCTGTCATATAAATCTGGTGTATATCAGCATGTGAGTGGACCTGCACTAGGTGGTCAT
181   F  L  S  Y  K  S  G  V  Y  Q  H  V  S  G  P  A  L  G  G  H
601  GCCATTAAGATCCTGGGCTGGGGAGAAGAGAATGGTGTCCCCTACTGGCTTGCTGCTAAC
201   A  I  K  I  L  G  W  G  E  E  N  G  V  P  Y  W  L  A  A  N
661  TCCTGGAACACTGACTGGGGTGATAATGGATATTTCAAGATTCTCCGAGGTGAGGACCAC
221   S  W  N  T  D  W  G  D  N  G  Y  F  K  I  L  R  G  E  D  H
721  TGTGGCATTGAATCTGAAATTGTGGCTGGAATCCCACAG
241   C  G  I  E  S  E  I  V  A  G  I  P  Q
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Figure 2–Nucleotide and deduced amino acid sequences of Jian Carp CatB cDNA.

CatB-pMD19-T was constructed accordingly. The sequencing data demonstrated that nucleotide sequences consisted of 759 bases (Figure 2). The deduced mature peptide sequences from the cDNA contained 253 amino acid residues with a calculated molecular weight of 27467.4 Da and a theoretical p*I* of 4.62.

According to homology analysis of the mature peptide sequence, Jian carp CatB shared 74.81% to 77.52% similarity to that of terrestrial animals and 78.35% to 99.6% to the teleosts, respectively (Figure 3A). The highest similarity of 99.6% was obtained with comparison to common carp CatB, followed by zebrafish (92.09%), which also belonged to Cyprinidae; thus, Jian carp CatB might be highly homologous within Cyprinidae. Furthermore, as shown in Figure 3A, there existed an oxyanion hole (Gln^{23}), 3 predicted active sites (Cys^{29}, His^{119}, Asn^{219}), and a conserved fragment of occluding loop (Pro^{101}-Glu^{122}) in the mature peptide sequence, which formed the typical domain architecture of cysteine proteinase. In addition, a putative N-glycosylation site was found at Asn^{112}. The phylogenetic tree constructed on the basis of the amino acid sequences revealed that Jian carp CatB formed a cluster with the known fish CatB, exhibiting a high degree of evolutionary relationship to carp CatB (Figure 3B).

CatB protein has been purified from several types of fish, but most purified protein only contains the heavy chain (Bonete and others 1984; Matsumiya and others 1989; Sherekar and others 2006) or segment (Tan and others 2006) of this protease. However, more comprehensive information about CatB structural features and chemical nature can be obtained by the research of gene cloning and recombinant expression. As stated in the introduction, there has been relatively little research on the gene cloning, protein expression, or analysis of the structural properties of piscine CatB. To date, in the existing studies reported, the gene sequences of piscine CatB are only cloned from the fish such as barred knifejaw (*Oplegnathus fasciatus*; GenBank accession: AEA48884.1), orange-spotted grouper (*Epinephelus coioides*; GenBank accession: AHF27212.1), Japanese flounder (*Paralichthys olivaceus*; GenBank accession: ABM47001.1), large yellow croaker (*Pseudosciaena crocea*; GenBank accession: AIR07786.1), and turbot (*Scophthalmus maximus*; GenBank accession: AKE47503.1). This study marks the 1st such systematic exploration of Jian carp CatB.

Expression and purification of CatB

The inclusion body of CatB obtained from the prokaryotic expression system was further purified by affinity chromatography after being dissolved in urea (Figure 4). The samples from each step were analyzed on SDS-PAGE, and the ultimate acquisition of CatB with high purity appeared as a single band of about 29 kDa (Figure 5)—fairly close to the DNAMAN results. Similarly, the deduced molecular weight of carp CatB mature peptide was 28.3 kDa (Tan and others 2006). The recombinant mature form of turbot (*Scophthalmus maximus*) CatB also exhibited a single band (27 kDa) on reducing SDS-PAGE as opposed to double bands (Zhou and others 2015).

The active mature enzyme of CatB is thought to work in double-chain form *in vivo* (that is, heavy chain and light chain cross-linked by disulfide bond) in mammals (Wiederanders and others 1991), but it is likely very readily cleaved into 2 individual chains under reducing condition due to the breakage of disulfide bond. So, generally 2 separated bands corresponding to the heavy and light chains respectively were observed on SDS-PAGE. The carp CatB processes the analogical cleavage site (Lys^{49}-Val^{50}) for forming the double-chain structure similarly to terrestrial animal CatB (Arg^{49}-Val^{50} of mouse and bovine, Lys^{49}-Val^{50} of chicken, seen in Figure 3A), and therefore is suggested to be quite liable to be broken at this cleavage site *in vivo* (Tan and others 2006). The Jian carp CatB also possesses the predicted cleavage site (Lys^{49}-Val^{50}). We thus conclude that Jian carp CatB presumably functions in double-chain form *in vivo*. The natural CatB purified from the muscle of horse mackerel (*Trachurus japonicus*) has been definitely proven to form 2 chains via the *N*-terminal sequencing of 2 bands (28 and 6 kDa) on reducing SDS-PAGE (Yoshida and others 2015). However, the recombinant mature CatB obtained in the study presented only one band on reducing SDS-PAGE and a similar phenomenon was also observed for the recombinant mature turbot (Zhou and others 2015). This might be attributed to the recombinant mature CatB has not be processed into double-chain form *in vitro* as what has been done for the natural enzyme *in vivo*.

It is also worth noting that the molecular weight of the intact mature peptide of Jian carp CatB is a little higher than those purified from mullet (*Mugil auratus*; 24 kDa; Bonete and others 1984), tilapia (*Tilapia mossambica*; 23.5 kDa; Sherekar and others 2006), or common mackerel (*Scomber japonicas*; 23 kDa; Matsumiya

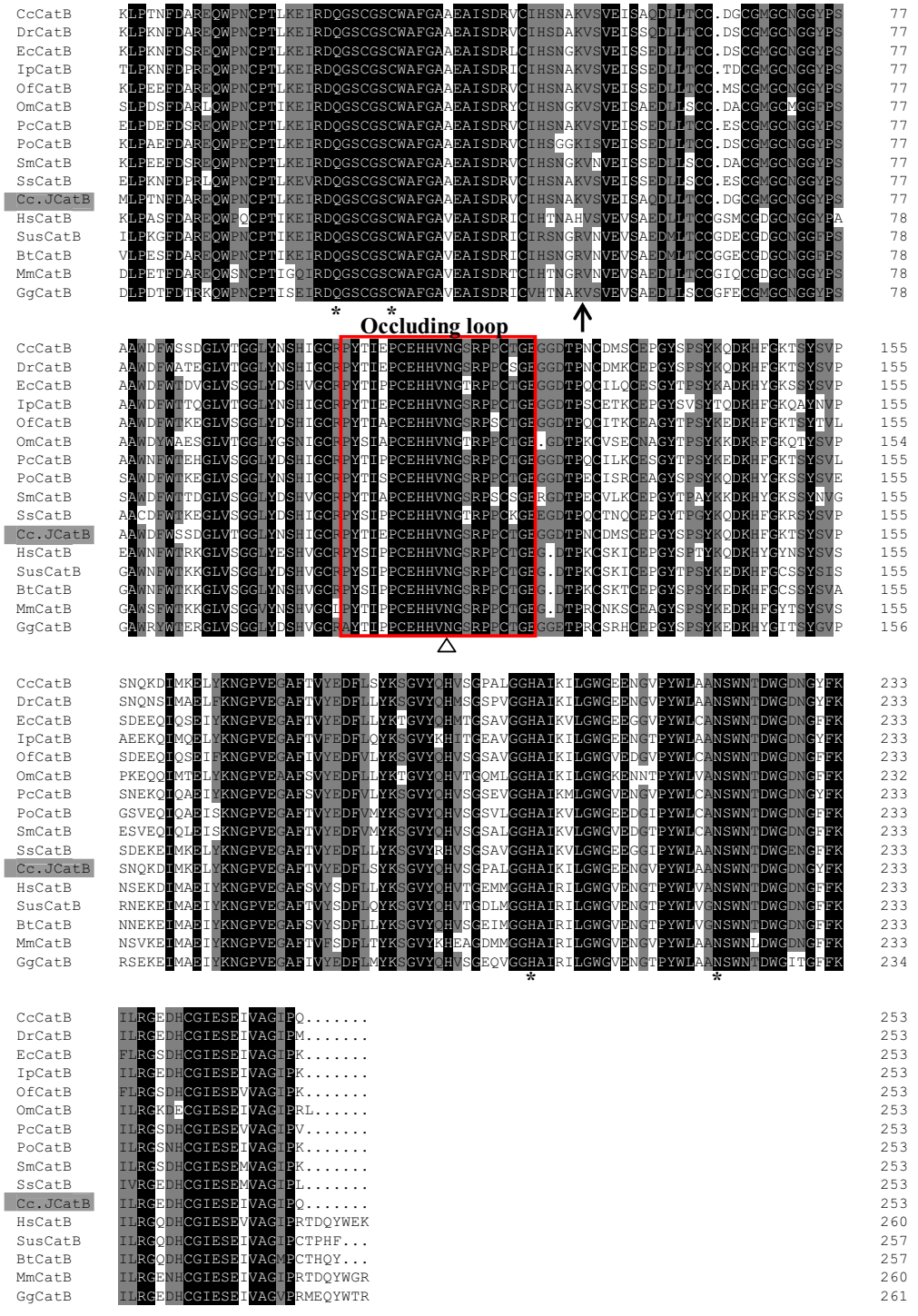

Figure 3–(A) Alignment of amino acid sequence of Jian carp CatB (Cc.JCatB, Cyprinus carpio var. Jian CatB) with CatB from other species. Identical amino acid residues are darkly shaded, similar amino acids are lightly shaded, unrelated residues have a white background, and amino acid numbers are shown on the right. Arrowheads indicate the predicted cleavage site. The conservative active sites (C^{29}, H^{199}, and N^{219}) and oxyanion hole (Q^{23}) of cysteine proteinase are marked by asterisks (*); the N-glycosylation site (N^{112}) is marked by a hollow triangle. The occluding loop is boxed. The data of similarity and GenBank accession numbers used in the alignment are as follows: CcCatB, *Cyprinus carpio* CatB (BAE44111), 99.6%; DrCatB, *Danio rerio* CatB (AAQ97764), 92.09%; EcCatB, *Epinephelus coioides* CatB (AHF27212), 82.21%; IpCatB, *Ictalurus punctatus* CatB (AHH43031.1), 86.56%; OfCatB, *Oplegnathus fasciatus* CatB (AEA48884), 82.61%; OmCatB, *Oncorhynchus mykiss* CatB (NP_001117776), 78.35%; PcCatB, *Pseudosciaena crocea* CatB (AIR07786), 84.19%; PoCatB, *Paralichthys olivaceus* CatB (ABM47001), 82.21%; SmCatB, *Scophthalmus maximus* (AKE47503.1), 79.45%; SsCatB, *Salmo salar* CatB (NP_001133994), 83.00%; HsCatB, *Homo sapiens* CatB (AAH10240), 75.57%; SusCatB, *Sus scrofa* (ACB59245), 76.36%; BtCatB, *Bos taurus* (AAA03064), 77.52%; MmCatB, *Mus musculus* (AAH06656), 74.81%; GgCatB, *Gallus gallus* (AAA87075), 75.57%. (B) Phylogenetic relationships of Jian carp CatB with other piscine CatB and representative mammalian groups. The phylogenetic tree is constructed with MEGA 6.0 using the neighbor-joining method. Bootstrap values of 1000 replications (%) are indicated on the branches. Jian carp CatB is darkly shaded.

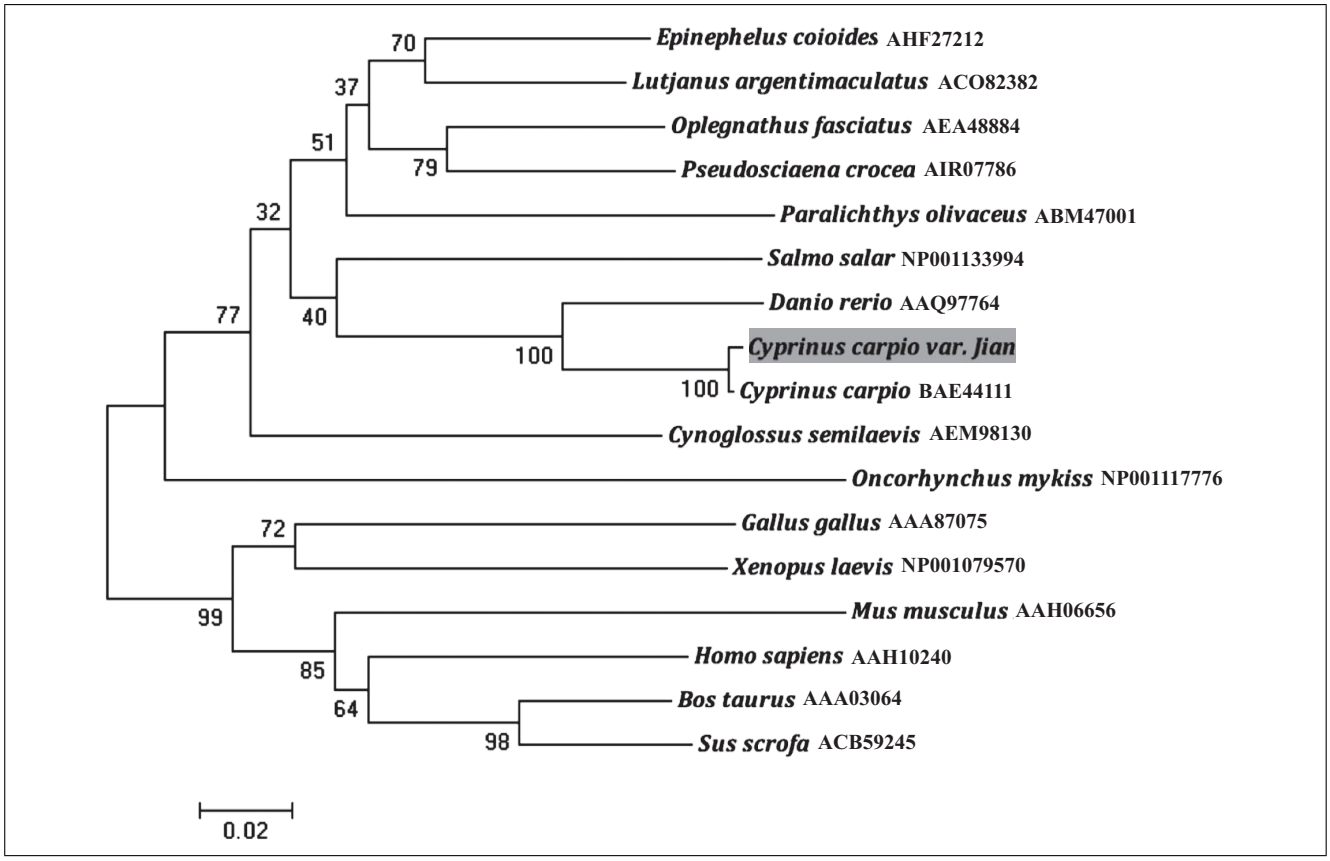

Figure 3–*Continued.*

and others 1989). This may be due to the species differences, or the purified CatB with lower molecular weight may be in the form of heavy chain or peptide segment. As reported for a kindred species previously, the unbridged mature peptide of silver carp Cat B has a molecular weight about 29 kDa as high as that of Jian carp (Liu and others 2008).

Effects of pH and temperature on CatB

After being renatured, Jian carp Cat B was assayed for the enzyme activity. The optimum activity appeared at 40 °C (Figure 6A) and pH 5.5 (Figure 6B), respectively, and it was stable to heating since it still retained partial activities at temperature of 50 °C (Figure 6A). The protease presented a rather broad range of pH stability from 3.0 to 7.0 (Figure 6B). Notably, it still

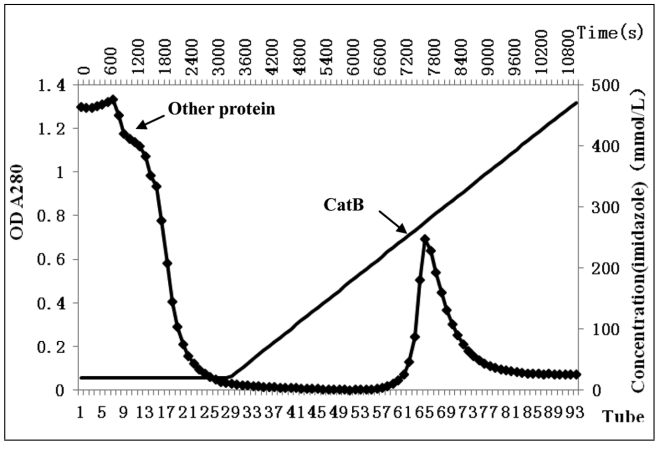

Figure 4–Chromatogram of CatB purification. The concentration of imidazole is indicated by a solid line (—); UV value is indicated by a rhombus (♦).

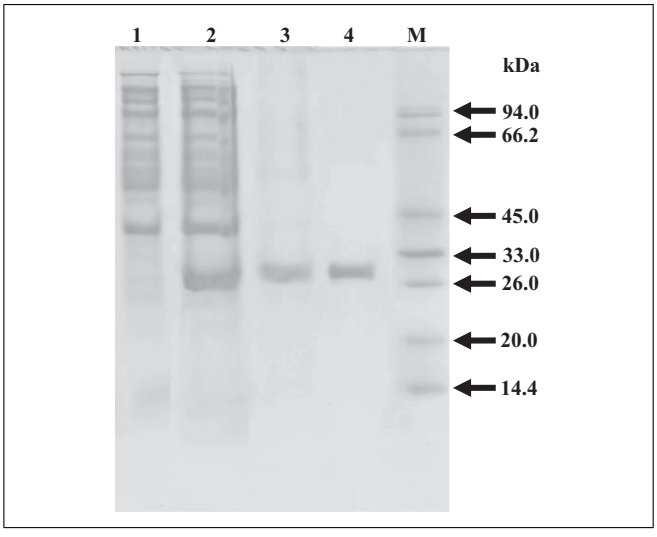

Figure 5–Electrophoregram of expression and purification of recombinant CatB detected by SDS-PAGE. M, protein marker (14.4-94 kDa); lane 1, total expressed protein of *E. coli* BL21 (DE3) before induction; lane 2, total expressed protein of *E. coli* BL21 (DE3) after induction; lane 3, the protein dissolved in 8 M urea; lane 4, purified CatB after Ni^{2+}-NTA agarose affinity chromatography.

maintained 40% residual activity when pH increased to 8.0. The optimum temperature and pH of Jian carp CatB was similar to that of common carp CatB purified from the hepatopancreas (45 °C, pH 6.0; Aranishi and others 1997), likely due to their similar amino acid sequence and domain architecture.

Surimi product processed from Jian carp has shown very attractive market prospect in China. The residual CatB activity in surimi is regarded to be involved in the deterioration of texture quality of the products after washing, as it can degrade actomyosin, the main structural protein of the surimi (Liu and others 2008; Martín-Sánchez and others 2009). In this study, the optimum temperature of Jian carp CatB was exactly approximate to the optimal gelation temperature range for surimi, 30 to 40 °C. It also retained about 60% of the hydrolysis activity at pH 6.5 to 7.5, which is a suitable pH for the gelation of surimi—thus, it is highly possible that CatB participates in the destruction of texture quality during the gelation process. Therefore, it is yet necessary to conduct further analysis on the properties of Jian carp CatB to avoid these adverse phenomena more efficiently during industrial production.

Characterization of polyclonal antibody

ELISA was conducted to characterize the activity of the CatB polyclonal antibody from the serum of mice immunized by purified CatB. The titer of CatB antisera was more than 1:256000 (Table 1).

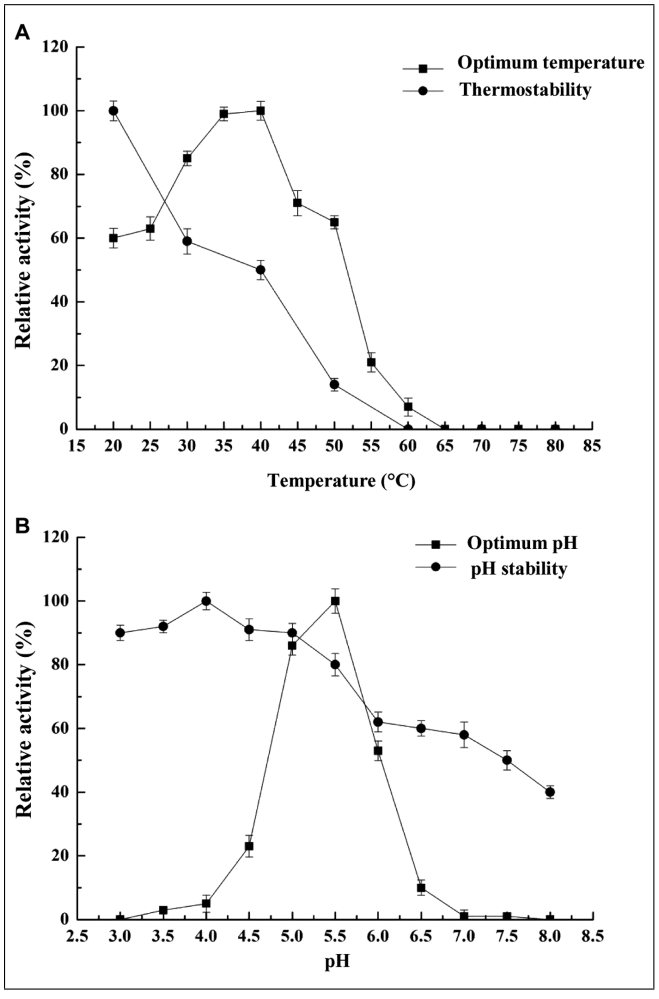

Figure 6–Activity profiles of recombinant Jian carp CatB. (A) Optimum temperature and thermostability. (B) Optimum pH and pH stability.

We also detected the recognition capability to antigens of the CatB polyclonal antibody by western blotting (Figure 7). In the *E. coli* BL21 (DE3) sample with pET-30a-CatB, CatB was detected as a single band on the PVDF membrane, whereas it was negative in the expression strain without the fragment CatB. Moreover, strong signals were detected in both the samples dissolved in urea and from chromatography. Although this polyclonal antibody was prepared using recombinant CatB expressed in prokaryotic cells, it showed excellent detection-specificity for recognizing the native antigen protein of CatB in the tissues of Jian carp (Figure 8). Similarly, Ahn and others (2008) proved that the CatX-antibody of olive flounder (*Paralichthys olivaceus*) can react with both natural and recombinant CatX. These results support our observation that the CatB antibody has very favorable sensitivity and specificity.

As mentioned above, the cathepsin antibodies available in the commercial market are mammalian-derived products, so their application in fish studies is limited due to the distinct differences in CatB homology between fish and mammals. Studies on CatB antibodies with suitably high sensitivity for the piscine antigen are rather scarce. It is reported previously that the antibody prepared from the purified CatB of common carp cannot recognize the rat CatB (Aranishi and others 1996). In this study, we prepared the specific antibody of piscine CatB using the recombinant protein as antigen to investigate the target at the protein level. The recombinant CatB is more available than the purified, natural CatB, which makes it feasible to produce the specific antibody of piscine CatB at large scale.

Localization and expression of CatB in Jian carp tissues

The immunohistochemical localizations of CatB in different tissues of Jian carp were detected as shown in Figure 8. CatB protein exhibited a wide distribution pattern. Varying intensity of positive staining was observed in hepatopancreas, muscle, small intestine, heart, and spleen; specifically, CatB was located in the small intestine muscular layer and mucosa (mainly in epithelium, lamina propria) and in the splenic cord of the spleen; there was also positive staining in hepatopancreas, heart, and skeletal muscle cells. Subsequent OD analysis results showed that CatB is most abundant in the spleen, and least abundant in the skeletal muscle (Figure 9).

At present, qRT-PCR or enzymatic activity assay are most often employed in studies on the distribution of cathepsins in fish. However, considering the existence of inactive CatB, immunological methods can be considered superior to these assay methods. Immunohistochemical analysis shows the location of the CatB protein, whether it is active or not, in human colorectal mucosa, for example (Bleeker and others 2000). Although the expression and distribution of CatB in channel catfish (*Ictalurus punctatus*) was

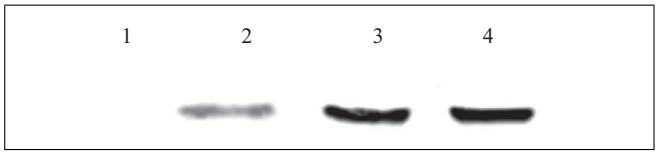

Figure 7–CatB antibody recognition assay by western blotting. 1, Total expressed protein from *E. coli* BL21 (DE3) with plasmid pET-30a; 2, total expressed protein from *E. coli* BL21 (DE3) with plasmid pET-30a-CatB; 3, recombinant CatB dissolved in 8 M urea; 4, purified CatB after Ni^{2+}-NTA agarose affinity chromatography.

Table 1–Titer detection of anti-CAT-B serum with indirect ELISA.

Dilution ratio ($\times 10^3$)	1	2	4	8	16	32	64	128	256	512	1024
Antibody serum (sample)	3.3430	3.1950	2.9655	2.8329	2.4210	2.0760	1.9520	1.7210	1.4740	1.1580	1.1080
Serum before immunizing (negative)	0.7498	0.7500	0.7521	0.7374	0.7213	0.6900	0.6434	0.6450	0.6610	0.6830	0.6515
Control	0.154	0.154	0.154	0.154	0.154	0.154	0.154	0.154	0.154	0.154	0.154
P(+)/N(−)	+	+	+	+	+	+	+	+	+	−	−

Optical density (OD) was detected via microplate reader at 450 nm. All data listed are the mean value ($n = 3$).
(Sample-control)/(negative-control)>2.1 was considered to be positive (+), otherwise negative (−).

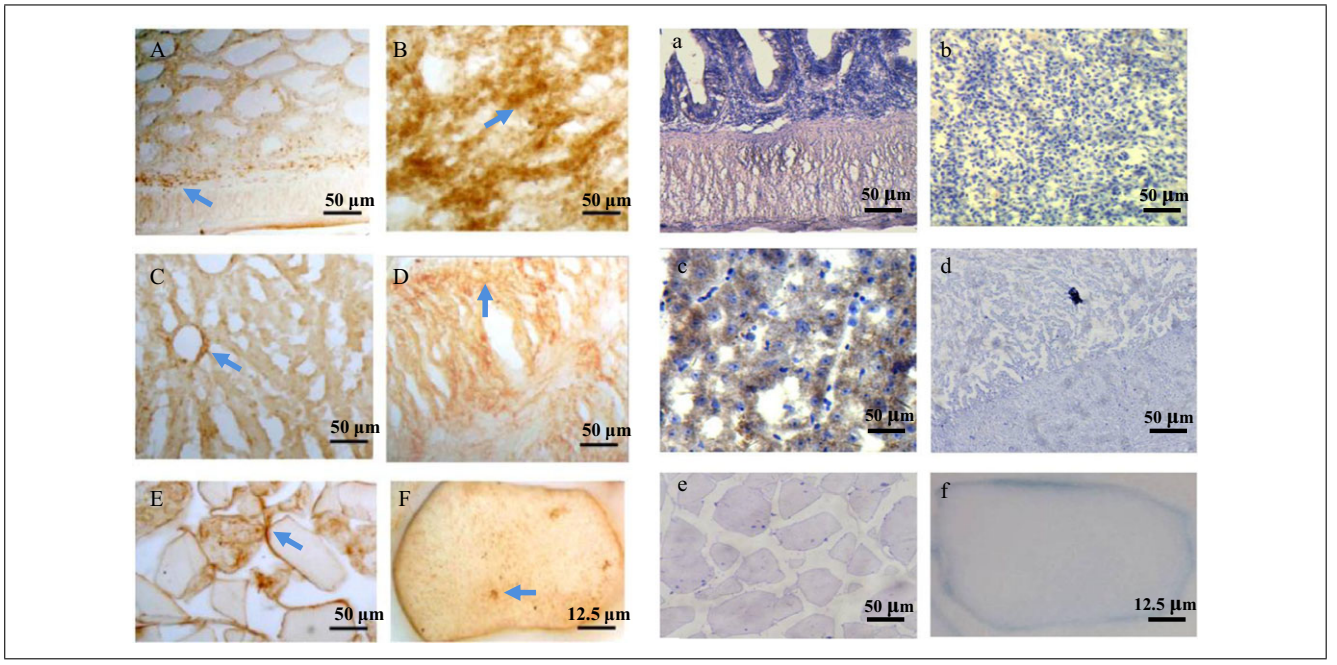

Figure 8–Immunolocalization of Jian carp CatB detected by immunohistochemistry. Microscopic observations of the tissues of Jian carp, A–F: positive group, a–f: negative control group. (A, a) small intestine; (B, b) spleen; (C, c) hepatopancreas; (D, d) heart; (E, e) muscle, magnification 100×, scale bar = 50 μm; (F, f) muscle, magnification 400×, scale bar = 12.5 μm.

demonstrated in a previous study at the mRNA level by qRT-PCR, the author emphasized the importance of exploring the additional physiological and biological functions of CatB in piscine tissues at the protein level (Li and others 2015).

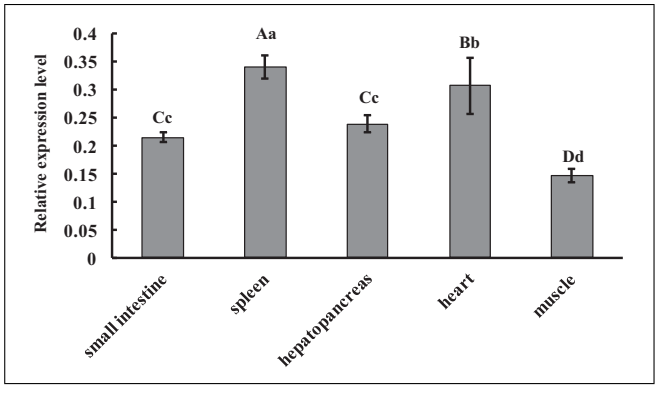

Figure 9–Protein quantification of CatB in the tissues of Jian carp. Integral optical density (IOD) and staining areas (A) of positive group and negative control group were detected by IPP 6.0. Relative expression level was defined as IOD/A (positive group) − IOD/A (negative control group). One-way ANOVA method was performed by SPSS 22.0 to analyze the significance of difference between 5 groups. The significant differences were indicated with different lowercase letters ($0.01 < P < 0.05$) or capital letters ($P < 0.01$).

In addition, as mentioned in the literature review, CatB may be closely related to the quality of fish and processed fish products, but few studies have been performed at the protein level in effort to elucidate the intrinsic mechanism. One such study on the sea cucumber (*Stichopus japonicus*) is of certain significance in this regard, as it established a clear correlation between the distribution of cathepsin L-like proteinase in the body wall and the texture quality caused by autolysis by means of immunohistochemical assay (Zhou and others 2014).

Conclusion

In this study, a 759-bp Jian carp CatB coding sequence was cloned and expressed as a 29 kDa recombinant protein. The renatured CatB exhibited an optimum activity at 40 °C and pH 5.5; highly specific CatB antibody was also prepared with a titer greater than 1:256000 which was shown to specifically recognize the antigen protein existing in the prokaryotic expression system and in several tissues of Jian carp. Immunohistochemical staining not only revealed a wide distribution and expression level of Jian carp CatB, but also demonstrated its detailed location in the tissues.

We hope that the results presented here lay the foundation for future research on exploring the inherent relationship between CatB and the quality of fish or fish products at both the gene and protein levels. We also hope that the data we gathered regarding the character and distribution of Jian carp CatB will be significant in making full use of cathepsin resources in the future.

Acknowledgment

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Authors' Contributions

Shu-Hong Li and Ran Li designed the experimentation, performed 80% of the experimentation, and wrote the manuscript; Hai-Xia Zhong and Xiu-Hua Chen contributed to the analysis of bioinformatics; Ai-Ping Liu, Juan Yang, and Qiang Hu executed 20% of the experimentation; Qin-Qin Ke and Mei-Liang Li performed and processed activity detection data.

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