Chinese Journal of Oceanology and Limnology
Vol. 33 No. 2, P. 291-298, 2015
http://dx.doi.org/10.1007/s00343-015-4047-4

Molecular cloning, tissue expression of gene *Muc*2 in blunt snout bream *Megalobrama amblycephala* and regulation after re-feeding*

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Received Mar. 31, 2014; accepted in principle May 28, 2014; accepted for publication Jun. 28, 2014

Abstract Mucins are important components of mucus, which form a natural, physical, biochemical and semipermeable mucosal layer on the epidermis of fish gills, skin, and the gastrointestinal tract. As the first step towards characterizing the function of Muc2, we cloned a partial *Megalobrama amblycephala Muc2* cDNA of 2 175 bp, and analyzed its tissue-specific expression pattern by quantitative real-time PCR (qPCR). The obtained sequence comprised 41 bp 5′-untranslated region (5′-UTR), 2 134 bp open reading frame encoding a protein of 711 amino acids. BLAST searching and phylogenetic analysis showed that the predicted protein contained several common secreted mucin-module domains (VWD-C8-TIL-VWD-C8) and had high homology with mucins from other vertebrates. Among four candidate reference genes (*β-Actin*, *RPI13α*, *RPII*, *18S*) for the qPCR, *RPII* was chosen as an appropriate reference gene because of its lowest variation in different tissues. *M. amblycephala Muc2* was mainly expressed in the intestine, in the order (highest to lowest) middle-intestine > fore-intestine > hind-intestine. *Muc2* was expressed relatively poorly in other organs (brain, liver, kidney, spleen, skin and gill). Furthermore, after 20-days of starvation, *M. amblycephala Muc2* expressions after refeeding for 0 h, 3 h, 16 h, 3 d, and 10 d were significantly decreased in the three intestinal segments (P<0.05) at 16 h, and were then upregulated to near the initial level at 10 d.

Keyword: *Megalobrama amblycephala*; *Muc2*; tissue expression; re-feeding

1 INTRODUCTION

Compared with terrestrial vertebrates, fishes are exposed in a more complicated aquatic environment, and are more susceptible to contact with pathogenic organisms, such as bacteria, viruses, and parasites. To inhibit the invasion of pathogens, the epithelium of the skin, gill and gastrointestinal tract are covered with a thin mucus layer, which plays a critical role in the defense mechanism and acts as a biological barrier (Esteban, 2012). The mucus contains various protective and antimicrobial substances secreted by epithelial cells, including mucins, immunoglobulins, complement components, proteases, lectins, piscidins and defensins (Lang et al., 2007; Neuhaus et al., 2007; Van der Marel et al., 2010; Esteban, 2012). Mucins are very important components of mucus, and are characterized by one long mucin domain that is rich in amino acids Ser, Thr, and Pro (PTS domain). Until now, at least 14 mucin-type glycoproteins have been assigned to the *MUC* gene family, and may be roughly divided into two groups: membrane-bound mucins (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, MUC17, MUC20), and secreted gel-forming mucins (MUC2, MUC5B, MUC5AC, MUC6, and MUC19)

* Supported by the National Natural Science Foundation of China (No. 31302222), the Earmarked Fund for China Agriculture Research System (No. CARS-46), the Freshwater Fisheries Research Center, CAFS Grant (Nos. 2013JBFM10, 2013JBFM03), and the Natural Sciences Foundation of Jiangsu Province (No. BK2011182)
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(Lang et al., 2007). The SEA domain (first found in Sea urchin sperm protein, Enterokinase and Agrin), and Transmembrane (TM) domains characterize membrane-bound mucins. Secreted gel-forming mucins contain 3–4 von Willebrand factor (VWF), a cysteine rich domain (CysD), and a cysteine knot (CT). In mammals, MUC2 is the primary gel-forming mucin in the small and large intestine, and forms the fundamental architecture of the mucus-gel on the intestinal surface (Hansson, 2012). Deficiency of *Muc2* in mice caused the epithelial barrier to become permeable to bacteria and led to colonic inflammation and spontaneous colitis (Bergstrom et al., 2010). The importance of the mucus has led to the piscine mucins receiving extensive research interest in recent years. The expression of *Muc2* of gilthead sea bream (*Sparus aurata*) infected by myxosporean *Enterromyxum leei* was significantly reduced (Pérez-Sánchez et al., 2013), and after being challenged with enteric pathogen *Edwardsiella ictalur*, *Muc2* of channel catfish (*Ictalurus punctatus*) was markedly upregulated (Li et al., 2012). Further, diet components and additives significantly affected fish *Muc2* expression (Van der Marel et al., 2012; Sahlmann et al., 2013).

Blunt snout bream (*Megalobrama amblycephala*), also known as Wuchang fish, is an indigenous cyprinid fish in China, and is an important farmed species. Culture of this fish has expanded rapidly during the last decade because of increasing consumption, and the total output reached 677 887 ton in 2011 (Ministry of Agriculture of the People's Republic of China's, 2012). However, recently, the issue of mucus deficiency in blunt snout bream skin and gastrointestinal tract, considerably affected its culture. Mucus deficiency make the fish particularly susceptible to pathogenic bacteria (*Aeromonas hydrophila*) infection, and reduces the survival ratio after transportation. To explore the underlying cause of the mucus deficiency and to find a solution to this issue, as a first step, this study cloned the secreted gel-forming mucin gene *Muc2* of *M. amblycephala* and characterized its expression patterns in different tissues, and detected the impact on *Muc2* expression of re-feeding after starvation.

2 MATERIAL AND METHOD

2.1 Experimental fish and sampling

For molecular cloning and expression, blunt snout bream (56.5±6.6 g, n=12) were collected from the Nanquan Experimental Station of Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, and acclimatized in laboratory aquaria for 2 weeks. Feeding of the fish was stopped 24 h before sampling. The brain, liver, kidney, spleen, skin, gills, fore-intestine (anterior to the first fold of the intestine), middle-intestine (the middle segment of the intestine) and the hind-intestine (posterior to the last fold) were collected separately, immersed in a 2 mL-tube with 1 mL RNAiso Plus (TaKaRa, Dalian, China), and then immediately stored at -80°C until use.

For the re-feeding experiment, healthy fish (50.3±5.0 g, n=60) were transferred to an indoor thermo-regulated recirculating water system. The water temperature was kept at 25±0.5°C. After an acclimatization period, the fish were starved for 20 days, and then fed a basal diet (32.3% crude protein, 6.2% crude lipid). At 0 h, 3 h, 16 h, 3 d, and 10 d of re-feeding, six fish were randomly sampled from the tanks, and anaesthetized with tricaine methane sulfonate (MS-222, 200 mg/L). The fore-intestine, middle-intestine and hind-intestine were collected, and immediately immersed in RNAiso Plus.

2.2 cDNA production

Total RNA was extracted from the collected samples according to the RNAiso Plus manufacturer's instructions (TaKaRa, Dalian). Agarose-gel electrophoresis (1.2%) and spectrophotometric analysis on a UV spectrophotometer 1800 (Shimadzu, Japan) determined the RNA quality and quantity. For the tissue expression analysis, 12 fish were dissected; tissue samples of each three fishes were mixed together and considered as one biological sample. For gene regulation after re-feeding analysis, tissue samples of the six fish from the same sampling time-point were assigned as three biological samples (2×3). For every biological sample, RNA were pooled together, diluted to a final concentration 400 ng/μL, and then the first strand cDNA was reverse-transcribed using a PrimeScript™ RT reagent Kit with gDNA eraser (TaKaRa, Dalian).

2.3 Partial *Muc2* sequence cloning

At the first step, partial *Muc2* gene fragments were amplified with primers muc2-f183 and muc2-r920 (Table 1), which were designed based on the conserved regions of *Muc2* sequences in common carp (JF343440.1), zebrafish (XM_002667544.3), fugu (XP_003967809), human (Q02817.2), mouse (XP_003945756), and then amplified toward the 3′

Table 1 Primers used for cloning and expression analysis

Primer	Primer sequence	Gene	Usage
Muc2-f183	CCAAAATGGGCATCACTGTC	*Muc2*	Endpoint PCR
Muc2-r920	ATAGCCAACCTTCCGTCCG		
VWD1-2 For	GTGGTCTTCTTGTAGCTGGGTT	*Muc2*	Endpoint PCR
VWD1-2 Rev	AGAGTCCTTCTGGACATGCG		
GSP1	CTGACACCCTGCTGAT	*Muc2*	5' RACE
GSP2	TGGCGAATGAGGCTTTGGCA	*Muc2*	5' RACE
GSP3	TGCCTGGGAACTGATAAACG	*Muc2*	5' RACE
Muc2-3f	CTGCCAAAGCCTCATTCAC	*Muc2*	qPCR
Muc2-3r	TGATACTAACTGACACCCTGCTGA		
18S_qF1	AAACGGCTACCACATCCAA	*18S*	qPCR
18S_qR1	TTACAGGGCCTCGAAAGAGA		
RPII-F	CGCGAGTCATTCCTGTAACATC	*RPII*	qPCR
RPII-R	TGACCCTTCCTCAGCTTTACCA		
Rpl13a-F	TCTGGAGGACTGTAAGAGGTATGC	*RPI13α*	qPCR
Rpl13a-R	AGACGCACAATCTTGAGAGCAG		
Actb-F	CCTGAGCGTAAATACTCCGTCTG	*β-Actin*	qPCR
Actb-R	AAGCACTTGCGGTGGACGAT		

The primers for the housekeeping (HK) genes were according to the published articles. *18S* (Van der Marel et al., 2012), *RPII* (Zhao et al., 2011), *Rpl13α* (Tang et al., 2007), and *β-Actin* (Ming et al., 2009).

end of the cDNA with primers VWD1-2 For (designed based on the obtained fragment) and VWD1-2 Rev (designed based on a small *Muc2*-like fragment in the Blunt Snout Bream transcriptome data, NCBI SRA045792, kindly offered by Prof. Wei-Min Wang). The 5′ end of the open reading frame (ORF) was amplified using 5′ rapid amplification of cDNA ends (RACE) system kit (Invitrogen) and GSPs primers (Table 1), according to the manufacturer's instructions.

A QIAquick agarose Gel Extraction Kit (Qiagen, GmbH) purified the amplified target products, which were cloned into pMD-18T vector system (TaKaRa, Dalian). Positive clones were screened and sequenced (Sangon Biotech, China). Sequences were checked for homologs in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Software Clustal X (www.clustal.org/clustal2) and DNAMAN 7.0 (Lynnon Corporation) were used to align the putative amino acid sequences, and conserved mucin domains were predicted according to the human MUC2 sequence (Q02817.2).

2.4 Expression analysis

Four candidate reference genes, *β-Actin*, *RPI13α* (ribosomal protein L13a), *RPII* (RNA polymerase II), *18S* (40S ribosomal protein S18) and their designed primers (Table 1) from the available literature were selected to determine the most stable gene between tissues (n=7). The quantitative real-time PCR (qPCR) were performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) Kit (TaKaRa) on an ABI 7500 Real-time PCR System. Briefly, the reaction mixture was prepared as follows: 10 μL of 1× SYBR Premix Ex Taq™ II (Tli RNaseH Plus), 0.4 μL of ROX Reference Dye II (50×), 0.4 μmol/L of each primer, 2 μL of 10× diluted cDNA, and nuclease free water to a final volume of 20 μL. The amplification program included an initial denaturation at 95°C for 3 min; and then 40 cycles of 95°C for 5 s and 62°C for 30 s. At the end of the run, a dissociation curve of the PCR product was recorded.

The two-standard curve method was implemented for *Muc2* expression analysis. Serial dilutions of cDNA generated from a pool of samples were used to calculate standard curves for the target gene *Muc2* and the most stable reference gene. The cDNA of the test samples were diluted six times. The specific primers for *Muc2* for qPCR were designed based on the obtained sequence (Table 1), and the qPCR program was the same as above. Denaturing curve analysis was used to verify the PCR specificity. Each sample in *Muc2* expression analysis was performed in triplicate.

2.5 Data analysis

The relative stability of the four candidate reference gene expression levels was analyzed by comparing the standard deviation (SD) of the cycle threshold (Ct) values in different tissues (n=7). *Muc2* expression was normalized against the reference gene, and the differences in expression were tested by Student t-test in software SPSS 13.0 (SPSS Inc. USA).

3 RESULT

3.1 Molecular cloning and sequence analysis of *Muc2*

The partial *Muc2* cDNA sequence of *M. amblycephala* was 2175 bp and has been deposited in GenBank (accession No. KJ152145). The cDNA comprises a 41 bp 5′-untranslated region (5′-UTR) and a 2 134 bp ORF encoding a protein of 711 amino acids. BlastX analysis showed that the nucleotide sequence was 90% identical with *Cyprinus carpio Muc 2-like* (JF343440.1) and 89% with *Danio rerio Muc 2* (XM_002667544.3). The deduced amino acid sequence was 88% identical to common carp and

87% identical to zebrafish Muc2, respectively (Fig.1b). Several conserved domains were detected: von Willebrand factor D domain (Pfam00094), C8 domain (Pfam08742) and Trypsin Inhibitor-Like (TIL) cysteine rich domain (Pfam01826) (Fig.1a).

3.2 Validation of a reference gene for *M. amblycephala*

Expression variations of the four candidate reference genes in different tissues were determined (Table 2), and the C_t values ranged from 14.61 to 26.73. According to the C_t values, 18S rRNA was the most highly expressed gene in all tissues, and other reference genes were expressed in the order (highest to lowest) *RPI13α>β-Actin>RP II*. The standard deviation (SD) value of the four candidate reference genes in different tissues were *RP II<RPI13α<18S<β-Actin* (Table 2). Thus, *RP II* showed the most stable expression in the different tissues of *M. amblycephala* and was selected as the reference gene.

3.3 Tissue specific expression of *M. amblycephala Muc2*

The PCR amplification efficiency of the reference gene *RP II* and the objective gene *Muc2* were 104 and 99.9%, and the correlation coefficient (R^2) were above 0.99 (Table 3), which indicated that the designed primers were suitable for the subsequent relative qPCR analysis. The results of tissue expression analysis showed that *Muc2* was highly expressed in the intestine samples, in the order (highest to lowest) middle-intestine > fore-intestine > hind-intestine (Fig.2). However, the C_t values of other six tissues were all above 30, and the normalized values were extreme low (<0.02). Thus, *Muc2* expression was tissue-specific in the intestine with remarkably low expression level in the brain, liver, kidney, spleen, skin and gill.

Table 2 The mean cycle threshold (C_t) ±SD of the candidate reference genes expressed in different tissues of *Megalobrama amblycephala* (*n*=7)

	18S	*RP II*	*Rpl13a*	*β-Actin*
Mean±SD	14.61±1.36	26.73±0.70	20.40±1.12	21.95±1.80

Table 3 PCR amplification efficiency of *Muc2* and *RP II*

Target gene	PCR amplification efficiency (%)	R^2	Slope
Muc2	99.9	0.994	-3.325
RP II	104	0.999	-3.229

3.4 Variation of *Muc2* expression in the intestinal tract after re-feeding

Re-feeding significantly affected the *Muc2* expression in the intestine (Fig.3). The *Muc2* expression levels in the three segments presented similar variation curves, which showed significantly reduced expression before 16 h after re-feeding, and a gradual increase thereafter. Expression of *Muc2* in the fore-intestine was downregulated before 16 h, but the subsequent increase was not significant (P>0.05). In contrast, *Muc2* expressions in the middle-intestine and hind-intestine after 16 h significantly increased (P<0.05). After 20-d of starvation (0 h), the *Muc2* expression pattern in the intestine segments (fore-intestine > middle-intestine > hind-intestine) was different from that in normal fish (Figs.2 & 3). At 10 d after re-feeding, the expression in the middle-intestine was non-significantly higher than that in the fore-intestine.

4 DISCUSSION

4.1 Identification of *M. amblycephala Muc2* partial sequence

Most secreted mucin mRNAs are higher than 10 kbp, and contain large repetitive units (PTS domains), which pose a challenge for new gene discovery and classification (Lang et al., 2007). Until now, no fish full-length mucin genes have been cloned and sequenced. However, the predicted amino acid sequence of *M. amblycephala Muc2* with its common VWD-C8-TIL-VWD-C8 secreted mucin module domains, and high homology with other vertebrates,

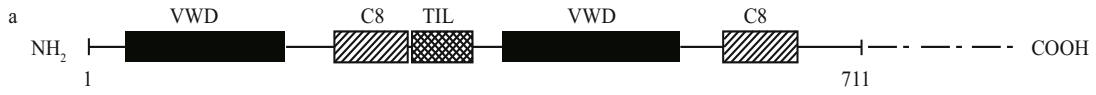


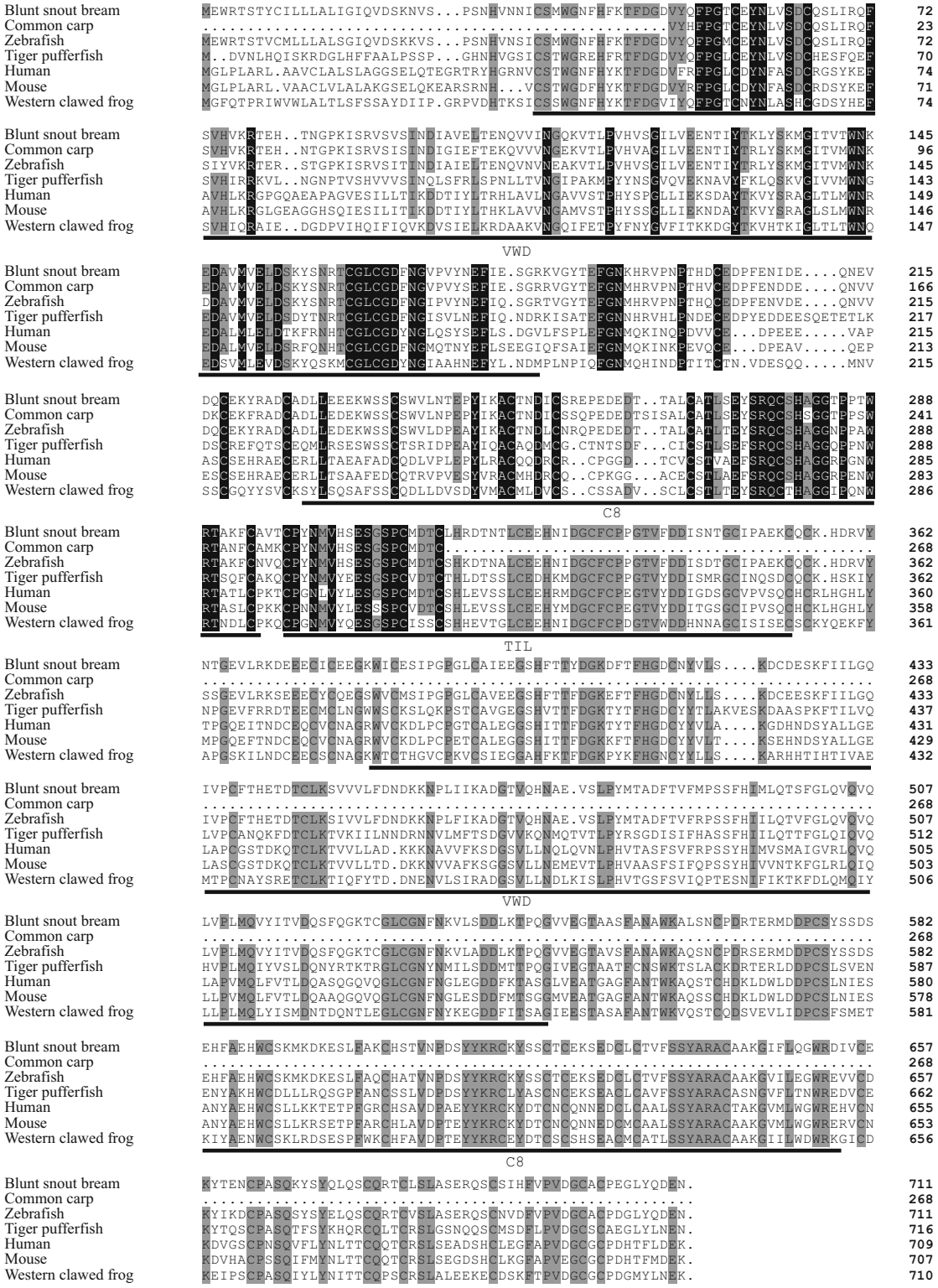
Fig.1 Sequence analysis of Muc2 segment of blunt snout bream

a. Schematic representation of the domain structure of the determined Muc2 segment of blunt snout bream;

To be continued

Fig.1 Continued

b



b. Multiple alignment of the amino acid (aa) sequences of blunt snout bream *Muc2* with six other vertebrate *Muc2* (or-like) genes retrieved from GenBank. Common carp (*Cyprinus carpio*) (JF343440), zebrafish (*Danio rerio*) (XP_002667590.3), tiger pufferfish (*Takifugu rubripes*) (XP_003967809), human (*Homo sapiens*) (Q02817.2), mouse (*Mus musculus*) (XP_003945756) and western clawed frog (*Xenopus tropicalis*) (XP_002936089). Identical residues are shaded in dark gray and bars (-) indicate gaps inserted for maximal similarity. The predicted domains are underlined at the bottom. Abbreviations: VWD: von Willebrand factor D domain (dark box); C8: cysteine rich domain (italic line box); TIL: trypsin inhibitor-like cysteine-rich domain.

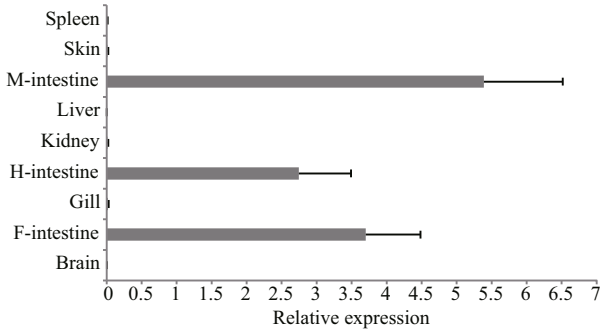


Fig.2 Expression of *Megalobrama amblycephala Muc2* in brain, liver, kidney, spleen, skin, gills, fore-intestine, middle-intestine, and hind-intestine

Data are presented as relative copy numbers of *Muc2* mRNA normalized against *RP II* mRNA from the same sample.

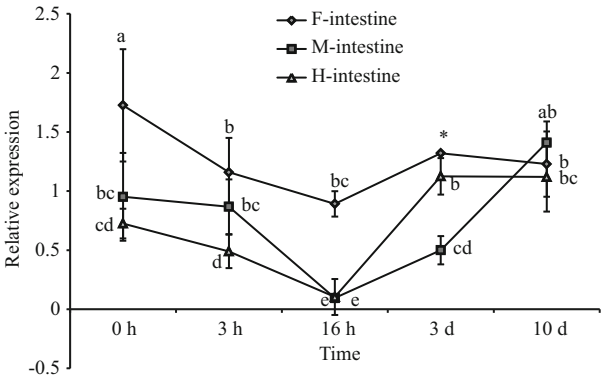


Fig.3 Variation of *Muc2* expression in the intestinal tract during re-feeding after starvation

Data are presented as relative copy numbers of *Muc2* mRNA normalized against *RP II* mRNA from the same sample. * indicates only one sample of front-intestine at 3d was tested (P<0.05)

demonstrated that the cloned gene encoded a Mucin. Gel forming mucins share strong structural similarities: the categorization of the molecular identities of mucins was unequivocally established on the basis of BLAST searches (Pérez-Sánchez et al., 2013). BlastX analysis showed that the nucleotide sequence was most similar to common carp *Muc2*, and the deduced amino acid sequence had high homology with Mucin 2 of common carp (Van der Marel et al., 2012) and Zebra fish. The above results indicated the cloned gene should be *Muc2*. According to the previous reports, *Muc2* is the only mucin gene that is highly expressed in intestine tissue of fish and mammals (Van Klinken et al., 1999; Roussel and Delmotte, 2004; Van der Marel et al., 2012). The cloned gene in this study was also mainly expressed in the intestines, which further supported the assertion that it was *M. amblycephala Muc2*.

4.2 Expression stability of reference genes in *M. amblycephala* tissues

Among the four candidate reference genes, the expression level of *18S* in different tissues of *M. amblycephala* was the highest, but *RP II* was the lowest, which was consistent with the results in Jian carp (Tang et al., 2012) and Atlantic salmon tissues (Jorgensen et al., 2006). *β-Actin* is the most frequently used reference gene for normalizing qPCR data; however, in the present study, it showed much higher variation than the *RP II* gene. However, Zhao et al. (2011) showed that the gene expressions in young *M. amblycephala* (about 100 g) were *β-Actin*> *RPI13α*>*18S*>*RP II*. This difference may reflect the different tissues tested or the sizes of the fish. It has been observed that the gene expression stability at different stages of *M. amblycephala* was inconsistent (Zhao et al., 2011).

4.3 Tissue specific expression

Muc2 was highly expressed in whole intestine of *M. amblycephala*, which was consistent with the *Muc2* expression pattern in common carp (Van der Marel et al., 2012) and the gilthead sea bream (Pérez-Sánchez et al., 2013). However, *Muc2* expression in the intestine of different fish is inconsistent. The *Muc2* expression in *M. amblycephala* followed the pattern (high to low) middle-intestine > fore-intestine > hind-intestine, while it was fore-intestine > middle-intestine > hind-intestine in gilthead sea bream.

The gilthead sea bream is a marine fish with an extensible stomach; however, the *Muc2* expression in its stomach is extremely low relative to the intestine, which suggested that *Muc2* plays an important role in the posterior intestine, but not in the stomach. We detected almost no *Muc2* expression in the brain, liver, kidney, spleen, skin or gills, which was consistent with results obtained in common carp (Van der Marel et al., 2012). Thus, we demonstrated that *Muc2* is specifically expressed in fish intestines. Recent research indicated that *Muc2* expression in the intestine was affected by bacterial or parasitic infection (Li et al., 2012; Pérez-Sánchez et al., 2013).

4.4 The effect of re-feeding on *M. amblycephala Muc2* expression in the intestine.

After re-feeding, *M. amblycephala Muc2* expression in the intestine sharply decreased. The expression in different intestinal segments reached a

minimum at 16 h, and then increased to near the initial level at 10 d. In this report, after 20-days of starvation stress, the metabolism of fishes' digestive system acclimatized to the starvation conditions (Zeng et al., 2012). Sudden re-feeding would represent a kind of stimulation to the digestive tract. The stimulation might reduce gastrointestinal mucous gene expression. A similar phenomenon was also reported for the specific activity of juvenile roach chymotrypsin, which was significantly decreased after re-feeding (Abolfathi et al., 2012). Like other animals, fish exhibit various behaviors to maintain their metabolism during starvation (Wang et al., 2006). The digestive system of fish is very adaptable (Hofer, 1979), and gradually recovers to fit the situation after re-feeding for several days; thus, this phenomenon might explain the *Muc2* expression tendency in the present report.

The mucous layer in the fish digestive tract is important to prevent bacterial infection (Corfield et al., 2001; Mayer, 2003), and has important roles in gastrointestinal digestion and absorption. Re-feeding after long-time starvation could cause temporary dysfunction in the digestive tract, and increase the risk of disease. Proper feeding management in spring is important for the health and disease resistance of *M. amblycephala*.

5 CONCLUSION

In this study, the N-terminal conserved domains of *M. amblycephala* Muc2 were successfully cloned, which had an open reading frame of 2 134-bp encoding 711 amino acid residues. Tissue expression pattern analysis showed *M. amblycephala* Muc2 transcripts were significantly expressed in intestine, and the re-feeding after starvation considerably affected the *Muc2* expression.

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