DOI: 10.3724/SP.J.1035.2012.00009

MOLECULAR CLONING OF TRYPSIN AND THE EFFECT OF DIETARY PROTEIN LEVELS AND STARVATION ON TRYPSIN mRNA EXPRESSION AND ENZYME ACTIVITY IN JUVENILE CHINESE SUCKER (*MYXOCYPRINUS ASIATICUS* BLEEKER)

ZHENG Kai-Di [1, 2], FENG Bo [3], LI Yun[1] and LI Ying-Wen [4]

(1. *Key Laboratory of Freshwater Fish Reproduction and Development* (*Ministry of Education*), *College of Animal Science and Technology*, *Southwest University*, *Chongqing* 400716, *China*; 2. *Department of Biochemistry*, *Wenzhou Medical College*, *Wenzhou* 325035, *China*; 3. *Institute of Health and Environmental Ecology*, *Wenzhou Medical College*, *Wenzhou* 325035, *China*; 4. *College of Life Science*, *Chongqing Normal University*, *Chongqing* 400047, *China*)

Abstract: The effect of dietary protein and starvation on the expression of trypsin was evaluated in the Chinese sucker (*Myxocyprinus asiaticus* Bleeker). The complete trypsin cDNA was cloned from juvenile Chinese sucker pancreatic tissue by using RACE and PCR methods. We used semi-quantitative RT-PCR and enzymatic activity measurements to quantify mRNA expression and trypsin activity in fish that were either starved or fed differing levels of dietary casein (35%, 45% and 55%). The results showed that the Chinese sucker *trypsin* cDNA sequence was 912 bp in length. Trypsin activity and mRNA levels were higher in fish that were fed moderate (45% casein) levels of protein than those that were fed high or low levels. Starvation significantly decreased mRNA expression level and trypsin activity. The changes in trypsin activity tended to lag behind the changes in mRNA levels. There was no direct relationship between the trypsin activity and mRNA level. Given this, the trypsin synthesis is a complex process regulated by a balance of several factors in the Chinese sucker.

Key words: Chinese sucker (*Myxocyprinus asiaticus* Bleeker); Dietary protein; Enzymatic activity; Starvation; *Trypsin* mRNA

CLC number: Q344^{+}.1 **Document code:** A **Article ID:** 1000-3207(2012)01-0009-09

Trypsin (EC 3.4.21.4) is an enzyme secreted by the pancreas to digest dietary protein, which play a key role in growth regulation in Atlantic salmon (*Salmo salar*)[1] and Atlantic cod (*Gadus morhua*) [2]. Trypsin genes have been identified and characterized in many teleosts species, including Atlantic salmon[3], Japanese flounder (*Paralichthys olivaceus*) [4], Atlantic Cod [5], and winter flounder (*Pleuronectes americanus*) [6].

The measurement of digestive enzymes, such as trypsin, provides an insight into the nutritional status of teleosts[7, 8]. The effects of starvation and dietary protein levels on trypsin activity are variable in fish. Some studies have shown that trypsin activity was affected by starvation [9] and dietary protein levels [10], while others have found no effect [11]. It has been found that fasting induced a significant decrease in trypsin activity in Adriatic sturgeon (*Acipenser naccarii*) [12] and tilapia (*Oreochromis mossambicus*) [13]. In contrast, trypsin activity increased significantly in Atlantic salmon[14] and rainbow trout (*Oncorhynchus mykiss*)[12] during the early period of starvation.

The changes in trypsin activity are likely mediated at the gene transcriptional level. Researches in crustaceans have shown that dietary protein levels and starvation significantly alter *trypsin* mRNA levels[15, 16]. However, few studies have evaluated the effect of dietary protein levels and starvation on *trypsin* mRNA expression in fish. Wang, *et al*. [10] reported that *trypsin* mRNA levels were influenced by dietary protein in yellow catfish (*Pelteobagrus fulvidraco*). In contrast, dietary protein had no effect on *trypsin* mRNA levels in sea bass (*Dicentrarchus labrax*) larvae [11]. The effect of starva-

Received date: 2010-11-01; **Accepted date:** 2011-09-25

Foundation item: the National Key Scientific Program of China (2009CB941200); the National Natural Science Foundation of China (30670226); the Scientific Research Foundation for the Returned Overseas Chinese Scholars; Education Ministry of China (2006-331); Doctoral and Post-doctoral Research Foundation of Southwest University (2005-05)； China Three Gorges Corporation Research Project to Y. Li

Corresponding author: Li Yun, Tel: 86-2368251255; Fax: 86-2368251196; E-mail: aquatics@swu.edu.cn; Li Ying-Wen, Tel: 86-2365910316; Fax: 86-2365910312; E-mail: liyingwen@live.cn

tion on *trypsin* mRNA level has received some attention in marine fish, such as Atlantic salmon[14], Japanese flounder[17] and yellowtail [18]. However, there is little information on the response in freshwater fish.

The Chinese sucker (*Myxocyprinus asiaticus,* Bleeker) distributes in the upper reaches of the Yangtze River and is the only member of the family Catostomidae in Asia. The Chinese sucker is popular as commercial culture species and pet. Despite its potential importance, little is known about the nutritional physiology of this species.

Our objective was to determine the effects of dietary protein and starvation on the trypsin enzyme of the Chinese sucker. We cloned the complete *trypsin* cDNA from juvenile Chinese sucker pancreatic tissue. We then measured trypsin activity and mRNA levels in fish that were either starved or fed diets containing 35%, 45% or 55% casein.

1 Materials and methods

1.1 Experimental fish and diets

We obtained Chinese suckers with weight of (23.3 ± 2.1) g from the Chongqing Wanzhou Fisheries Research Institute. The fish were held in 15 rectangular glass tanks (1.1 m×0.5 m×0.55 m, 50 fish per tank). The tanks were persistently aerated using an air pump. The fish were held at 24–25°C under a 12h light: 12h dark photoperiod (light between 09:00 and 21:00) and fed a commercial diet (with 36% protein content, Tongwei Group Co., Ltd., China). After 4 weeks acclimation to the tanks, the fish were divided into five treatment groups. Each treatment group consisted of three replicate tanks. Three groups (T1–T3) were fed with diets that contained different levels of casein (55, 45, and 35%, respectively; Tab. 1). The fourth group (TF) was fasted and a control group (TC) was fed with the commercial formula. The fish were fed twice daily (08:00 and 17:00).

Tab. 1 Formulation and proximate analysis of the experimental diets

Ingredients (in %)[a]	T1	T2	T3
Casein [b]	55.0	45.0	35.0
α-Starch	17.0	17.0	17.0
Dextrin	16.0	16.0	16.0
Microcrystalline Cellulose	3.0	13.0	23.0
Colza oil	6.0	6.0	6.0
Vitamin mixture [c]	1.5	1.5	1.5
Mineral mixture [d]	1.5	1.5	1.5

a. Dietary ingredients were obtained from commercial suppliers. Casein, Starcand, dextrin, α-Starch and Microcrystalline Cellulose were from Si Chuang Xilong Chemical Co., Ltd (China). The colza oil was from Chongqing supermarket; b. casein was the only resource of protein; c. Per kg of vitamin mix: retinyl acetate 1g; cholecalciferol 2.5 mg; all-rac-a-tocopherol acetate 10 g; menadione 1 g; thiamin 1 g; riboflavine 0.4 g; D-calcium pantothenate 2 g; pyridoxine HCl 0.3 g; cyanocobalamin 1 g; niacin 1 g; choline chloride 200 g; ascorbic acid 20 g; folic acid 0.1 g; biotine 1 g; meso-inositol 30 g; d. Per kg of mineral mix[19]: KCl 90 g; KI 40 mg; $CaHPO_4{\cdot}2H_2O$ 500 g; NaCl 40 g; $CuSO_4{\cdot}5H_2O$ 3 g; $ZnSO_4{\cdot}7H_2O$ 4 g; $CoSO4{\cdot}_7H_2O$ 20 mg; $FeSO_4{\cdot}7H_2O$ 20 g; $MnSO_4{\cdot}H_2O$ 3 g; $CaCO_3$ 215 g; $MgSO_4{\cdot}7H_2O$ 124 g; NaF 1 g

1.2 Sampling

We randomly netted 15 fish from each group (5 fish per tank) prior to administering the morning food supply on days 0, 5, 10, 15, 20, 25 and 30. The fish were anesthetized and sacrificed with a sharp blow to the head immediately. The pancreatic tissue and the complete intestine were dissected from each fish. The pancreatic (≈50 mg) for cloning of *trypsin* cDNA sequences and RNA preparation was snap frozen in liquid nitrogen and the intestine with the remained pancreas for trypsin activity was washed in sodium chloride before frozen in liquid nitrogen. All samples were then stored at −80°C until being used.

1.3 Molecular cloning and analysis of trypsin cDNA sequencing

Total RNA was extracted by RNAiso Reagent (D9108B, TAKARA, Japan), quantified by a Smart Spec™ Plus Bioanalyzer (Bio-Rad, USA) and reversely transcribed using oligo $(dT)_{18}$ primers and RevertAid™ M-MuLV Reverse Transcriptase (MBI Fermentas, Canada, EP0442) following the manufacturer's instructions. A 750 bp core cDNA fragment was amplified by nested PCR (YPF2 and YPR2 for the nested amplification) in following cycles parameters: 94°C for 30s, 55°C for 45s, 72°C for 60s. PCR products were resolved on 1.0% agarose gel and stained with ethidium bromide to visualize the bands. Target fragments were purified using an Axygen Gel Extraction Kit (Axygen, USA), cloned into a pMD-19T vector (TAKARA, Japan), and sequenced by company (Shanghai Sangon, China). Gene-specific primers for 3′RACEand 5'RACE were designed based on obtained sequence. 5′ end was obtained using a 5′-full RACE Kit (TAKARA, Japan D6122) and semi-nested PCR (5′-YA1 and 5′-YS1 for the first amplification, 5′-YA1 and 5′-YS2 for the semi-nested amplification) in the cycles setting: 94°C for 30s, 57°C for 45s, 72°C for 60s. 3′ end was acquired using a 3′-full RACE Kit (TAKARA, Japan, D6121) and nested PCR (3′-F2 for the nested amplification) in following cycles profile: 94°C for 30s, 55°C for 45s, 72°C for 60s. Full-length cDNA amplification was performed using nested PCR (LF2 and LR2 for the nested amplification) in following cycles profile: 94°C for 30s, 60°C for 45s, 72°C for 90s. All PCR was carried out in a TECHNE thermal cycle (UK, TC-512), first denaturated at 94°C for 3min, amplified for 35 cycles and finally extended for 10min at 72°C. Tab. 2 contained a list of all the primers used in the study.

Tab. 2　Primers sequences used in the study

Primer name	Sequence	Purpose
YPF1	5′- GGCTTTCATTCTTCTGGCTCGTT -3′	cDNA fragment PCR
YPF2	5′- CTTCTGT (C) GGA (TG) GGCTCC (T) CTG -3′	
YPR1	5′- GCTTAGTTGGAGTTGATGGTGCT -3′	
YPR2	5′-CCAG (A) GAC (G) ACAAT (GC) ACCCTG-3′	
3′-F1	5′-TCTAATGTGCCTGTGGGCTCCTA-3′	3′-RACE
3′-F2	5′-TGGCTTCTTGGAGGGCGGCAAG-3′	
RTYP	5′- TAACTGTTCAGGGTG -3′	5′-RACE
5′-YA1	5′- AGCGAACAGAGCCAGAAGAA -3′	
5′-YS1	5′- CTCTGGGTCGTGTCTGCTGC -3′	
5′-YS2	5′- GCGTCTTGGTGAGCATAACA -3′	
LF1	5′-TTGACGTCTCTGGGGGCACCGAG-3′	Full-length cDNA amplification
LF2	5′-GGATCCATGAAGGCTCTCATTCTTCTGGC-3′	
LR1	5′-AGTTTTAACTCAAACATTTATTTG-3′	
LR2	5′-GCGGCCGCTTAATTGGAGTTAATGGTGTTT -3′	
SeniQ-TrypsinF1	5′-CATTCTTCTGGCTCTGTTCGC-3′	Semi-quantitative RT-PCR
SeniQ-TrypsinR1	5′-AGCCCACAGGCACATTAGACG-3′	
β-actin-F	5′-GCCCATCTATGAGGGTTAC-3′	Semi-quantitative RT-PCR
β-actin-R	5′- GAGGGCAAAGTGGTAAACG -3′	

1.4　Semi-quantitative RT-PCR analysis

Total RNA was extracted and quantified following the method described in 2.3 and treated with DNase I (RNase-free) (Promega, USA, M610A) to eliminate genomic DNA. Reverse transcription based on 5 μg of total RNA was performed using a RevertAid™ M-MuLV Reverse Transcriptase kit (MBI Ferments, Canada, EP0442) following the manufacturer's protocol and 0.5 μL cDNA was used as template for amplification. PCR was conducted with SeniQ-TrypsinF1 and SeniQ-TrypsinR1 primers (Tab. 2) and following parameters: initial denaturation at 94°C for 3min, followed by 35 cycles of amplification (denaturation for 30s at 94°C, annealing for 30s at 57°C, extension for 60s at 72°C) and a 10min final extension at 72°C. The housekeeping gene *β-actin* (30 cycles) was amplified to serve as a reference using 0.5 μL cDNA as templet and primers *β-actin*-F and *β-actin*-R (Tab. 2), which were designed based on the sequence for zebrafish (GenBank accession No. AF057040) in following thermal profile: denaturation at 94°C for 3min, followed by 30 cycles of amplification (94°C for 30s, 56°C for 30s, and 72°C for 60s) and a 10 min final extension at 72°C. 8 μL PCR products were resolved in 1.0% agarose gels and gels were stained with ethidium bromide to visualize the bands. The bands were analyzed using the image analysis software Quantity One (Bio-Rad, USA). Adjusted volume (measured volume of band minus the background volume) of every band (lane) in a gel picture was measured with an identical volume rectangle tool. Relative *trypsin* mRNA expression level was expressed as the adjusted volume ratio of *trypsin* / *β-actin*. All experiments were performed three times.

1.5　Determination of trypsin activity

Trypsin activity was measured following the description[20], using TAME as a substrate. The published enzyme solution (20 μL) was mixed with 3.0 mL TAME solution (1 mM TAME in 10 mM Tris–HCl buffer, pH 8.0). The mixture was then incubated at 30°C for 20min. We measured production of p-tosyl-arginine by monitoring the increase in absorption at 247 nm. One unit of activity was defined as the amount on trypsin required to generate an increase in absorption of 0.001 per minute at 247 nm. All experiments were performed in triplicate.

1.6　Data analysis

Data were expressed as the mean ± SD. We used ANOVA to compare mRNA levels and trypsin activity among the treatment groups. All statistical analysis were performed by using GraphPad Prism 5 (GraphPad Software, San Diego, USA). Differences were considered to be statistically significant when $P<0.05$.

2　Results

2.1　Trypsin cDNA sequence

The RACE and PCR yielded a 921 bp *trypsin* cDNA. The cDNA fragment contained a 47 bp 5′untranslated region, a 133 bp 3′untranslated region, and a 741 bp open reading frame (ORF). The initiation codon ATG was located at positions 48–50 and the stop codon TGA was at positions 788–790. The canonical polyadenylation signal (AATAAA) was located 16 bp upstream of the

poly (A) tail (GenBank accession No. EF493027) (Fig. 1).

2.2 Effect of dietary protein on trypsin activity and mRNA expression

There was no significant difference in trypsin activity among the treatment groups during the first two sampling periods (Fig. 2A). However, diet had a significant effect on trypsin activity during the last 20 days of the experiment (Fig. 2A). Trypsin activity was the lowest in the fish that were fed a diet containing 55% casein (T1 group) from day 10. Trypsin activity was significantly higher in T2 group than T3 group on days 20, 25, and 30.

The level of dietary protein also had a significant effect on trypsin mRNA levels in the Chinese sucker (Fig. 2B). Trypsin mRNA levels were significantly higher in T2 group than in those fish that were fed more (T1 group)

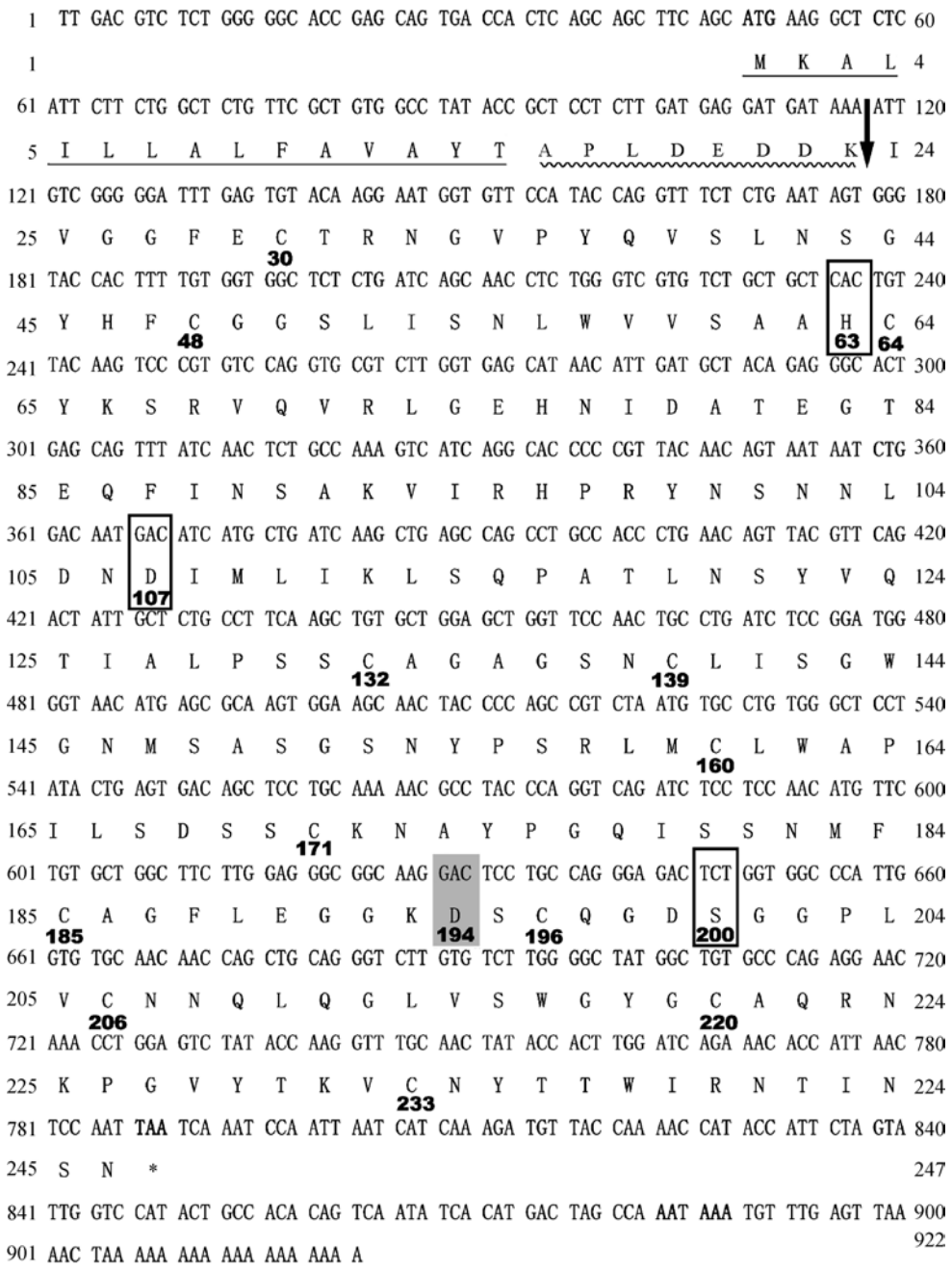

Fig. 1 Nucleotide sequence encoding the trypsin gene and the deduced amino acid sequence of Chinese sucker (*Chinese sucker*)

The amino acid sequence is given below the nucleotide sequence. The nucleotides and amino acids are numbered along the upper and lower margins, respectively. The start (ATG) and stop (TAA) codons are indicated by the overstrike. The signal peptide is underlined and the activation peptide is underlined with a wave line. The activation peptide cleavage site is marked with an arrowhead. Residues of the catalytic triad (His63, Asp107, Ser200) are indicated by the box. The amino acid (Asp194) at the bottom of the substrate pocked is shadowed. Cysteine residues are marked by numbers. The nucleotides and amino acids are numbered along the upper and lower margins, respectively

or less (T3 group) dietary protein. The trypsin mRNA level was significantly higher in T1 group than T3 group on day 5, but significantly lower on day 10. There was no significant difference in trypsin mRNA levels between T1 group and T3 group at any other time.

Trypsin activity changed significantly throughout the experiment in all treatment groups (Fig. 3A). Trypsin activities increased steadily in the T2 group but decreased in the T1 group. Conversely, trypsin activities increased in the first 20 days in the T3 group then decreased during the last 10 days.

Trypsin mRNA levels increased significantly in T2 group during the first 5 days, then stabilized and remained at a relatively high level for the duration of the experiment (Fig. 3B). Trypsin mRNA levels markedly decreased in both T1 and T3 groups during the first 10 days. Following this period, the changes in mRNA levels were less rapid. In the T1 group, the levels increased

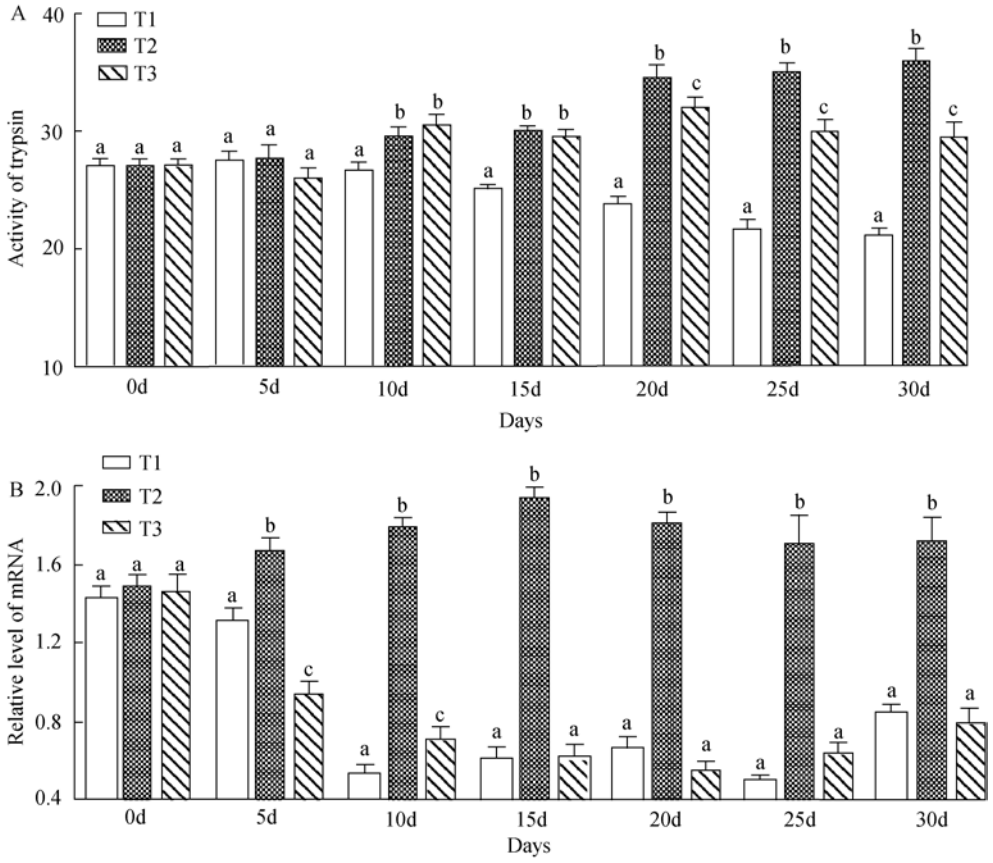

Fig. 2　A, Trypsin activity (△A247nm/min·mg) of juvenile Chinese sucker fed diets containing 55, 45, and 35% dietary casein (T1, T2 and T3, respectively). Each bar represents the mean ± SD of 3 fish. B, Trypsin mRNA expression of juvenile Chinese sucker fed diets containing 55%, 45%, and 35% dietary casein (T1, T2 and T3, respectively). Fifteen bar represents the mean ± SD of fifteen fish. Bars that have different lower-case superscripts in the same day are significantly different ($P < 0.05$)

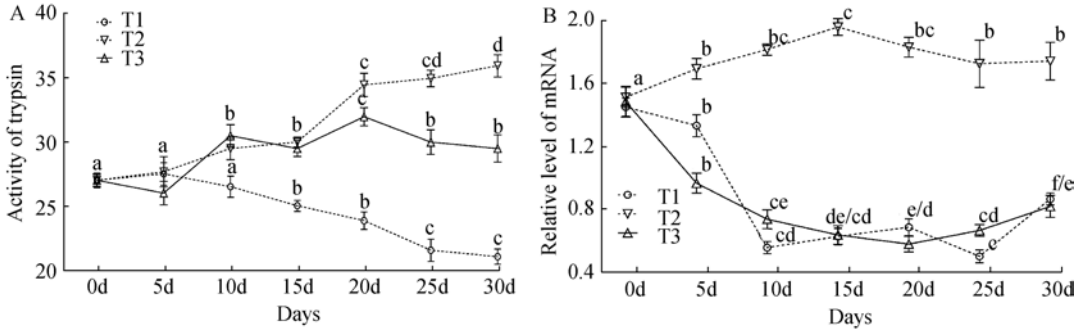

Fig. 3　A, Time-course of the changes in trypsin activity (△A247nm/min·mg) in juvenile Chinese suckers fed diets containing 55%, 45% and 35% dietary casein (T1, T2 and T3, respectively). B, Time-course of the changes in *trypsin* mRNA expression in juvenile Chinese suckers fed diets containing 55%, 45% and 35% dietary casein (T1, T2 and T3, respectively). Each point represents the mean ± SD of fifteen fish. Points that have different lower-case superscripts in the same diet (treatment) are significantly different ($P < 0.05$), the former lower-case separated by slash for T1 group and the later for T3 group

between days 10 and 20 then decreased between days 20 and 25. Conversely mRNA levels decreased between days 10 and 20 in T3 group before increasing after day 20. In both T1 and T3 groups, the mRNA levels increased significantly between days 25 and 30.

2.3 Trypsin mRNA expression and enzymatic activities under food deprivation

Trypsin mRNA level was significantly higher in the starved fish relative to the control group on day 5 (Fig. 4B). However, there was no significant difference in trypsin activity among these two groups at this time (Fig. 4A). In contrast, both trypsin activity and mRNA levels were significantly lower in the TF group relative to the controls at all time points after day 10.

Trypsin mRNA levels increased rapidly during the first 5 days in the fasted group. However, the levels decreased to half the starting value at day 10 and remained constant thereafter (Fig. 4B). Trypsin activity decreased significantly during the first fasted 15 days of the experiment (Fig. 4A). After this initial period, trypsin activity increased sharply between days 15 and 20, then decreased again during the remainder of the experiment.

3 Discussion

Trypsin activity and trypsin mRNA levels were significantly higher in Chinese sucker that were fed a moderate protein diet than those that were fed lower or higher levels of protein (Fig. 2A and B). Furthermore, the pattern of the response was similar for both indices, suggesting that trypsin gene expression is regulated by the level of dietary protein in certain extent in Chinese sucker, at least at the transcriptional level. Increased protease activity due to increases in the level of dietary protein has been reported in yellowtail (*Seriola quinqueradiata*) [21], catfish (*Clarias batrachus* × *Clarias gariepinus*) [22], and Asian sea bass (*Lates calcarifer*) [23]. The increase in enzyme specific activity was associated with an increase in mRNA leve1s in yellow catfish [10] and the Chinese mitten crab (*Eriocheir sinensis*) [15]. This regulatory mechanism allowed the organism to regulate trypsin synthesis at a level that was appropriate for the protein content of a meal, improving the efficiency of protein digestion.

Interestingly, trypsin activity and *trypsin* mRNA levels were significantly lower in the Chinese suckers that were fed a very high protein (55% casein) diet relative to those fed lower levels. This is consistent with the response in crustaceans, such as the white shrimp (*Penaeus vannamei*) [16]. Our results suggested that the inhibition of trypsin specific activity at high levels of dietary protein was likely related to inhibition of trypsin synthesis. This may be caused by an excess of amino acids. Alternatively, activity may be inhibited by the upregulation of pancreatic secretory trypsin inhibitors, which may also be controlled by dietary protein levels[24].

We did not observe any changes in trypsin activity associated with the treatments during the first five days of the experiment. This is consistent with reports in other aquatic animals, including Chinese mitten crab juveniles [15] and larval red drum (*Sciaenops ocellatus*) [25]. During the first days, the fish would be able to digest trial food with the same effectiveness as in a state of continual feeding. Our results suggested that the Chinese sucker may require several days to adapt to a new diet.

The timing of changes in trypsin activity tended to

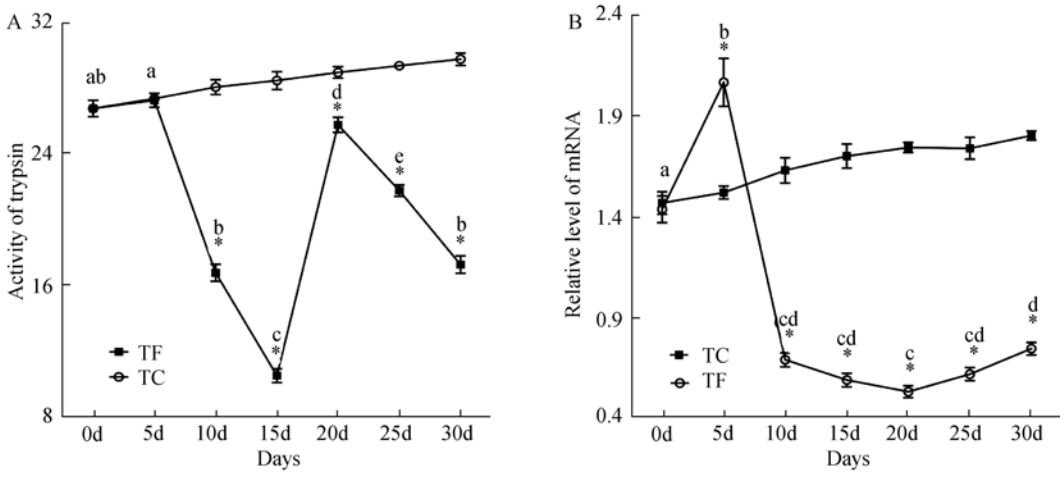

Fig. 4 A, Changes in trypsin activity (△A247nm/min·mg) in fasted (TF) or control (TC) groups of Chinese suckers. The control group was fed a commercial formula containing 36% protein. B, Changes in *trypsin* mRNA expression in fasted (TF) and control (TC) groups of Chinese suckers. The control group was fed a commercial formula containing 36% protein. Each point represents the mean ± SD of fifteen fish. Points within a treatment group that have different lower-case superscripts are significantly different ($P < 0.05$). Points that are significantly different ($P< 0.05$) between TF and TC are indicated with "*"

lag behind the changes in mRNA levels. This lag suggested that trypsin activity was regulated by multiple mechanisms. Trypsin synthesis was a complex process. Pretrypsinogen, precursor of trypsin, was synthesized in pancreatic acinar cell. After removal of the signal peptide, it became trypsinogen and being stored within secretory granules. It was secreted into the pancreatic duct and ultimately into the duodenum, where trypsin was activated into the mature form by enterokinase[26]. Pretrypsinogen synthesis may be regulated during pretranslation[27] or both transcription and translation[11, 19]. The regulation may occur at any of several stages, for example, during the synthesis or posttranslational modification of pretrypsinogen, or during the targeting, transport, or activation of trypsinogen. Moreover, some mRNA was never translated into protein. The translational efficiency of eukaryotic mRNAs was dependent on sequence characteristics[28]. Furthermore, the activity of trypsin may be regulated by activation of trypsin from trypsinogen by enterokinase.

Starvation significantly increased mRNA transcription during the first 5 days (Fig. 4B). Other researchers have reported similar results in yellowtail[18] and white shrimp[29]. An increase in mRNA levels in response to a protein-scarce diet was an adaptive process that was crucial to the survival of the organism during periods of protein deprivation. At beginning of starvation, Chinese sucker should keep highly alerted for efficient digestion of food at all times. To maintain digestion efficiency during this time, trypsin was accumulated in pancreatic tissue and trypsin mRNA levels increased to promote enzyme synthesis.

Trypsin mRNA levels decreased rapidly after day 5 before levelling and remained constant for the remainder of the starvation experiment (Fig. 4B). These changes most likely reflected the sensitivity of Trypsin mRNA transcription to substrate availability. The decline in Trypsin mRNA levels may also conserve energy that was formerly allocated to trypsin synthesis[29]. This was a useful strategy for minimizing the effects of starvation. In the other hand, at the first 5 days, excessive trypsin accumulated in the pancreatic tissue. Trypsin synthesis seemed to be accelerated after trypsin secretion from pancreatic tissue and decreased when trypsin accumulates in the pancreatic tissue[18]. Trypsin activity was the lowest on day 15, but was still measurable, suggesting that there was a basal level of secretion in the Chinese sucker. A small amount of stored trypsin was released into the lumen where it acted as a sensing mechanism. In the presence of a protein substrate, the enzyme, either directly or indirectly, produces a signal (possibly small peptides or free amino acids). This signal was then amplified, triggering a regulatory cascade which results in the activation of late trypsin gene(s) as well as other digestive enzymes, including aminopeptidase[30].

Interestingly, trypsin activity increased rapidly between days 15 and 20 in the starved groups. We hypothesized that this was caused by changes in substrate utilization. We speculated that the increase in trypsin activity was caused by the conversion from lipids to proteins during this period. Both total and specific protease activity was significantly higher in starved fish, suggesting that tissue protein was actively catabolised. The trend indicated that protein was the one of principal substances used to meet the energy requirements of starved fish during the period[31]. Enzymatic activity declined steadily after day 20, suggesting that the animals may not have recovered from the effects of starvation beyond this time.

In summary, trypsin activity and mRNA levels increased as the level of dietary casein level increased within the range from 35% to 45%. However, higher levels of protein (55% casein) inhibited trypsin activity and decreased mRNA levels. Trypsin activity and mRNA levels were also generally lower in Chinese sucker that were starved. The changes in trypsin activity and mRNA levels were similar, suggesting that trypsin gene expression was altered by dietary protein levels and starvation via transcriptional regulation. However, our results also suggested that trypsin gene expression was regulated by different ways including post-transcriptional regulation, translational regulation, and posttranslational regulation. Juvenile Chinese suckers were able to endure up to 2 weeks of nutritional deprivation without relying on endogenous tissue protein. However, it appeared that beyond this point the fish began to utilize endogenous tissue protein to meet their energy requirements. These data will be useful for the culture and management of Chinese suckers.

References:

[1] Torrissen K R, Lied E, Espe M. Differences in digestion and absorption of dietary protein in Atlantic salmon (*Salmo salar*) with genetically different trypsin isozymes [J]. *Journal of Fish Biology*, 1994, **45**(6): 1087—1104

[2] Lemieux H, Blier P, Dutil J D. Do digestive enzymes set a physiological limit on growth rate and food conversion efficiency in the Atlantic cod (*Gadus morhua*) [J]? *Fish Physiology and Biochemistry*, 1999, **20**(4): 293—303

[3] Male R, Lorens J B, Smalås A O*, et al.* Molecular cloning and characterization of anionic and cationic variants of trypsin from atlantic salmon [J]. *European Journal of Biochemistry*, 1995, **232**(2): 677—685

[4] Suzuki T, Srivastava A S, Kurokawa T. cDNA cloning and phylogenetic analysis of pancreatic serine proteases from Japanese flounder, *Paralichthys olivaceus* [J]. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 2002, **131**(1): 63—70

[5] GudmundsdÓTtir Á, GudmundsdÓTtir E, ÓSkarsson S, *et al.* Isolation and characterization of cDNAs from Atlantic cod encoding two different forms of trypsinogen [J]. *European Journal of Biochemistry*, 1993, **217**(3): 1091—1097

[6] Douglas S E, Gallant J W. Isolation of cDNAs for trypsinogen from the winter flounder, *Pleuronectes americanu*; Pleuronectes americanus</e7&gt [J]. *Journal of Marine Biotechnology*, 1998, **6**(4): 214—219

[7] Liu X D, Liang M Q, Zhang L M, *et al.* Effect of fish protein hydrolysate levels on growth performance and biological and physiological parameters in tongue sole (*Cynoglossus semilaevis* Günther, 1873) post-larvae [J]. *Acta Hydrobiologica Sinica*, 2010, **34**(2): 242—249 [柳旭东, 梁萌青, 张利民, 等. 饲料中添加水解鱼蛋白对半滑舌鳎稚鱼生长及生理生化指标的影响. 水生生物学报, 2010, **34**(2): 242—249]

[8] Ge H Y, Ding J H, Zhang W, *et al.* Tolerance and efficacy of cysteamine hydrochloride in the diets of largemouth bass (*Micropterus salmoides*) [J]. *Acta Hydrobiologica Sinica*, 2010, **34**(6): 1142—1149 [葛红云, 丁建华, 张伟, 等. 半胱胺酸盐在大口黑鲈饲料中的应用及耐受性评价. 水生生物学报, 2010, **34**(6): 1142—1149].

[9] Gildberg A. Digestive enzyme activities in starved pre-slaughter farmed and wild-captured, Atlantic cod (*Gadus morhua*) [J]. *Aquaculture*, 2004, **238**(1-4): 343—353

[10] Wang C, Xie S, Zhu X, *et al.* Effects of age and dietary protein level on digestive enzyme activity and gene expression of *Pelteobagrus fulvidraco* larvae [J]. *Aquaculture*, 2006, **254**(1-4): 554—562

[11] Péres A, Zambonino Infante J L, Cahu C. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae [J]. *Fish Physiology and Biochemistry*, 1998, **19**(2): 145—152

[12] Furne M, Garcia-Gallego M, Hidalgo M C, *et al.* Effect of starvation and refeeding on digestive enzyme activities in sturgeon (*Acipenser naccarii*) and trout (*Oncorhynchus mykiss*) [J]. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, 2008, **149**(4): 420—425

[13] Chan C R, Lee D N, Cheng Y H, *et al.* Feed deprivation and re-feeding on alterations of proteases in Tilapia *Oreochromis mossambicus* [J]. *Zoological Studies*, 2008, **47**(2): 207—214

[14] Rungruangsak-Torrissen K, Moss R, Andresen L, *et al.* Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L.) [J]. *Fish Physiology and Biochemistry*, 2006, **32**(1): 7—23

[15] Jiang H B, Chen L Q, Wang Q, *et al.* Effects of dietary protein on activities of digestive enzyme and trypsin mRNA abundance in *Eriocheir sinensis* juveniles [J]. *Journal of Fishery of China*, 2005, **29**(2): 216—221 [江洪波, 陈立侨, 王群, 等. 饵料蛋白质对中华绒螯蟹仔蟹消化酶活性及胰蛋白酶 mRNA 丰度的影响. 水产学报, 2005, **29**(2): 216—221]

[16] Muhlia-Almazan A, Garcia-Carreno F L, Sanchez-Paz J A, *et al.* Effects of dietary protein on the activity and mRNA level of trypsin in the midgut gland of the white shrimp *Penaeus vannamei* [J]. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 2003, **135**(2): 373—383

[17] Bolasina S, Perez A, Yamashita Y. Digestive enzymes activity during ontogenetic development and effect of starvation in Japanese flounder, *Paralichthys olivaceus* [J]. *Aquaculture*, 2006, **252**(2—4): 503—515

[18] Murashita K, Kubota S, Kofuji P Y M, *et al.* Trypsin restoration time in the pyloric ceca of yellowtail *Seriola quinqueradiata* [J]. *Fisheries Science*, 2005, **71**(6): 1274—1279

[19] Cahu C, Ronestad I, Grangier V, *et al.* Expression and activities of pancreatic enzymes in developing sea bass larvae (*Dicentrarchus labrax*) in relation to intact and hydrolyzed dietary protein; involvement of cholecystokinin [J]. *Aquaculture*, 2004, **238**(1-4): 295—308

[20] Hummel B C W. A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin [J]. *Canadian Journal of Biochemistry and Physiology*, 1959, **37**: 1393—1399

[21] Kofuji P Y M, Akimoto A, Hosokawa H, *et al.* Seasonal changes in proteolytic enzymes of yellowtail *Seriola quinqueradiata* (Temminck & Schlegel; Carangidae) fed extruded diets containing different protein and energy levels [J]. *Aquaculture Research*, 2005, **36**(7): 696—703

[22] Giri S S, Sahoo S K, Sahu A K, *et al.* Effect of dietary protein level on growth, survival, feed utilisation and body composition of hybrid clarias catfish (*Clarias batrachus* × *Clarias gariepinus*) [J]. *Animal Feed Science Technology*, 2003, **104**(1): 169—178

[23] Eusebio P S, Coloso R M. Proteolytic enzyme activity of juvenile Asian sea bass, *Lates calcarifer* (Bloch), is increased with protein intake [J]. *Aquaculture Research*, 2002, **33**(8): 569—574

[24] Carreira S M, Puigserver A J. Correlated regulation of the mRNAs encoding secretory trypsin inhibitors and anionic trypsinogen I in the rat pancreas depending on the dietary protein intake [J]. *The Journal of nutritional biochemistry*, 1996, **7**(4): 230—236

[25] Lazo, Holt, Arnold. Ontogeny of pancreatic enzymes in larval red drum *Sciaenops ocellatus* [J]. *Aquaculture Nutrition*, 2000, **6**(3): 183—192

[26] Chen J M, Kukor Z, Le Marechal C, *et al.* Evolution of trypsinogen activation peptides [J]. *Molecular Biology and Evolution*, 2003, **20**(11): 1767—1777

[27] Hara H, Hashimoto N, Akatsuka N, *et al.* Induction of pancreatic trypsin by dietary amino acids in rats: Four trypsinogen isozymes and cholecystokinin messenger RNA [J]. *The Journal of nutritional biochemistry*, 2000, **11**(1): 52—59

[28] Pain V M. Initiation of protein synthesis in eukaryotic cells [J]. *European Journal of Biochemistry*, 1996, **236**(3): 747—771

[29] Sanchez-Paz A, Garcia-Carreno F, Muhlia-Almazan A, *et al.* Differential expression of trypsin mRNA in the white shrimp (*Penaeus vannamei*) midgut gland under starvation conditions [J]. *Journal of Experimental Marine Biology and Ecology*, 2003, **292**(1): 1—17

[30] Lemos F J A, Cornel A J, Jacobs-Lorena M. Trypsin and aminopeptidase gene expression is affected by age and food composition in *Anopheles gambiae* [J]. *Insect Biochemistry and Molecular Biology*, 1996, **26**(7): 651—658

[31] Johnston D J, Ritar A J, Thomas C W. Digestive enzyme profiles reveal digestive capacity and potential energy sources in fed and starved spiny lobster *(Jasus edwardsii)* phyllosoma larvae [J]. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 2004, **138**(2): 137—144

胭脂鱼胰蛋白酶 cDNA 克隆以及不同蛋白含量日粮和饥饿对 mRNA 表达和酶活力的影响

郑凯迪[1, 2]　冯　波[3]　李　云[1]　李英文[4]

(1. 西南大学动物科技学院, 淡水鱼类资源与生殖发育教育部重点实验室, 重庆 400716; 2. 温州医学院生物化学教研室, 温州 325035; 3. 温州医学院健康与环境生态研究所, 温州 325035; 4. 重庆师范大学生命科学学院, 重庆 400047)

摘要: 为检测不同蛋白含量的日粮和饥饿对胭脂鱼(*Myxocyprinus asiaticus*)幼鱼胰蛋白酶活性和 mRNA 表达的影响, 首先用 RACE 和 PCR 的方法从胭脂鱼幼鱼的肝胰脏组织中克隆得到胰蛋白酶 cDNA 全长, 然后用半定量 RT-PCR 和酶活性检测方法分别检测了经不同蛋白含量日粮(酪蛋白含量分别为 35%、45% 和 55%)投喂和饥饿处理后的胭脂鱼幼鱼的胰蛋白酶 mRNA 表达水平和胰蛋白酶活力的变化。结果显示, 胭脂鱼胰蛋白酶 cDNA 全长为 912 bp。投喂蛋白质含量适中(45%酪蛋白)日粮组的试验鱼胰蛋白酶活性和 mRNA 水平高于投喂高蛋白水平日粮组(55%酪蛋白)和低蛋白水平日粮组(35% 酪蛋白); 饥饿明显降低 mRNA 水平和胰蛋白酶活性; 胰蛋白酶活性的变化滞后于 mRNA 水平的变化。胰蛋白酶活力的变化与 mRNA 水平的变化之间未呈现直接相关性。因此, 胭脂鱼胰蛋白酶合成可能是一个由多种因素共同调控的复杂过程。

关键词: 胭脂鱼; 蛋白水平; 胰蛋白酶活性; 饥饿; 胰蛋白酶 mRNA