Comparative Biochemistry and Physiology, Part B 155 (2010) 77–85

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Molecular characterization of trypsinogens and development of *trypsinogen* gene expression and tryptic activities in grass carp (*Ctenopharyngodon idellus*) and topmouth culter (*Culter alburnus*)

Guo-Liang Ruan [a,b], Yang Li [a], Ze-Xia Gao [a], Huan-Ling Wang [a], Wei-Min Wang [a,*]

[a] College of Fisheries, Key Lab of Freshwater Biodiversity Conservation and Utilization, Ministry of Agriculture, Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, Huazhong Agricultural University, Wuhan 430070, PR China
[b] College of Animal Science, Yangtze University, Jingzhou 434025, PR China

ARTICLE INFO

Article history:
Received 30 July 2009
Received in revised form 13 October 2009
Accepted 14 October 2009
Available online 23 October 2009

Keywords:
cDNA sequence
Ctenopharyngodon idellus
Culter alburnus
Gene expression
Ontogeny
Trypsin-specific activity
Trypsinogen

ABSTRACT

This study examined the gene structures and expression of *trypsinogens*, as well as the trypsin activities of the grass carp *Ctenopharyngodon idellus* (herbivorous) and the topmouth culter *Culter alburnus* (carnivorous), which are commercially important freshwater species of the family Cyprinidae in China. Isolated full-length trypsinogen cDNA clones were 869 bp and 857 bp. The deduced amino acid sequences were 242 aa and 247 aa long, both containing the highly conserved residues essential for serine protease catalytic and conformational maintenance. The results from isoelectric and phylogenetic analyses suggest that grass carp trypsinogen is grouped with teleost trypsinogen group I, while topmouth culter trypsinogen is grouped with group II. The expression pattern of trypsinogen mRNA was similar between these two species, appearing 2 days post-hatching (dph) and reaching peaks at 11 and 23 dph. The trypsin-specific activities in both species were detected 2 dph and reached the major peaks at 8 dph, however the minor peaks were observed at 20 dph in the grass carp and 17 dph in the topmouth culter. The trypsin-specific activity was significantly higher in the grass carp than in the topmouth culter, which may be attributed to the nature of their different nutritional habits.

1. Introduction

As one member of the serine protease family, trypsin (EC 3.4.21.4) is a well-characterized endopeptidase that is synthesized as a pre-proenzyme by acinar cells in the pancreas and secreted into the intestinal lumen as proenzyme trypsinogen (Halfon et al., 2004; Psochiou et al., 2007). The precursor of trypsinogen is activated by enterokinase or activated trypsin through the specific cleavage of trypsinogen and removal of a short activation peptide. Furthermore, trypsin can activate other digestive enzymes including chymotrypsin, elastase, collagenase, and lipase (Rust, 2002).

During the ontogenetic development stage of fish larvae, digestion is initiated in an alkaline environment (Yufera and Darias, 2007) and the achievement of functional pancreatic secretion constitutes the first step of the maturation process for digestive functionality (Ma et al., 2005). Many marine fish exhibit high trypsin activities at their first feeding, or even before the mouth opening forms (Zambonino-Infante and Cahu, 2001; Yufera and Darias, 2007; Gisbert et al., 2009). Consequently, trypsin may play an important role in protein digestion, as it is available in the early developmental stages of marine fish (García-Gasca et al., 2006; Perez-Casanova et al., 2006; Manchado et al., 2008). Moreover, tryptic enzyme activity was shown to relate to diets and environmental conditions in herring (*Clupea harengus*), European sea bass (*Dicentrarchus labrax*) and Nile tilapia (*Oreochromis niloticus*) larvae. Therefore, it was recognized as a useful and reliable indicator of nutritional conditions, feeding success and digestibility of food in marine fish larvae (Ueberschar and Clemmesen, 1992; Nolting et al., 1999; Drossou et al., 2006).

In order to better understand the physiological properties and potential of proteinic digestion, as well as develop compound diets that can substitute live prey and improve growth and survival rates of fish larvae, the temporal changes of trypsin activities was detected in many marine fish species during their early developmental stages (Ribeiro et al., 1999; Bolasina et al., 2006; Chen et al., 2006; García-Gasca et al., 2006; Perez-Casanova et al., 2006; Gisbert et al., 2009). The complete or partial cDNAs of trypsinogen have been cloned in many marine fish (Gudmundsdottir et al., 1993; Male et al., 1995; Genicot et al., 1996; Douglas and Gallant, 1998; Ahsan et al., 2001; Kurokawa et al., 2002; Psochiou et al., 2007), and the spatiotemporal expression of trypsinogen mRNA has been examined (García-Gasca et al., 2006; Wang et al., 2006; Darias et al., 2007; Psochiou et al., 2007; Manchado et al., 2008). However, few studies have examined the

* Corresponding author. Tel.: +86 27 87282113; fax: +86 27 87282114.
E-mail address: wangwm@mail.hzau.edu.cn (W.-M. Wang).

1096-4959/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2009.10.005

ontogeny of trypsin in freshwater fish, especially the family Cyprinidae (Rathore, et al., 2005; Chakrabarti et al., 2006), with even less information being reported on the appearance and spatiotemporal expression patterns of trypsinogen in these species.

The grass carp (*Ctenopharyngodon idellus*) is one of the"four major cultured fish species"in China with typical herbivorous habits. It has been considered one of the most popular freshwater fish species and became well accepted by consumers, due to its moderate price (Wang et al., 2008). The annual production of this species was 3.96 million tons in 2006, which was the highest among all the Chinese freshwater fish species (http://www.fjof.gov.cn). The topmouth culter (*Culter alburnus*) also belongs to the family Cyprinidae and is a ferocious carnivorous freshwater fish with a delicious taste and abundant nutrients (Chen, 1998). With the increasing annual cultured production, it has already become one of the most important commercial freshwater fish in China and holds the potential to be a candidate aquaculture species (Wang et al., 2007).

The grass carp and topmouth culter are stomachless species of the family Cyprinidae, thus they may be good teleost models for investigating and comparing the ontogeny of trypsin during the early developmental stages in fish with totally different feeding habits. In this study, the complete cDNA sequences of trypsinogens in these two cultured freshwater cyprinids were cloned as an essential molecular tool for the study of fish nutrition physiology. On this basis, the temporal expression patterns of trypsinogen, as well as trypsin activities, were examined in these two species. The results obtained from this study may help to gain further insight into the physiological and molecular differences in trypsin development between carnivorous and herbivorous teleosts.

2. Materials and methods

2.1. Rearing and sampling of larvae

Fertilized eggs of grass carp and topmouth culter were collected and transferred to the laboratory from a nearby hatchery in May, 2008. They were reared in plastic round aquaria (1.8 m diameter and 1.5 m height) with the density of about 5000 larvae per aquarium. Three replications were assigned for each species. After mouth opening formation, larvae were fed twice each day with rotifers (mainly *Brachionus angularis* and *Keratella cochlearis*) and cladocerans (mainly *Moina micrura* and *Diaphanosoma brachyurum*). The duration of the experiment was from hatching to 28 days post-hatching (dph). The temperature was set at 22 to 25 °C throughout the experiment. Dissolved oxygen was maintained above 5 mg/L by constant aeration. Fish from each aquarium were sampled each morning for RNA isolation and trypsin activity analysis, before food distribution. According to morphological characteristics, the early development of grass carp and topmouth culter are divided into three stages: yolk-sac larva stages, between the hatching and first feeding (3 dph for grass carp and 5 dph for topmouth culter); late-stage larva stages, between the first feeding and acquisition of minimum adult fin ray complement (8 dph for grass carp and 10 dph for topmouth culter); juvenile larva stages, characterized by the formation of scales and the assumption of adult body form. Samples for these three developmental stages were collected. The samples for the trypsin activity assays were carefully collected, rinsed with distilled water, and transferred into 2.0 mL microtubes on ice. They were immediately frozen in liquid nitrogen and stored at −80 °C. RNA isolation and enzymatic activity assays were conducted using the whole larvae when younger than 7 dph, or just the dissected abdominal portion in older fish.

2.2. Cloning cDNA sequence by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)

Total RNA from the hepatopancreases of grass carp and topmouth culter adults were extracted using TRIzol reagent (Tiangen, Beijing, China). Genomic DNA contamination was removed with RNase-free Dnase I (Fermantas, Burlington, Iowa, IA, USA). Approximately 50 μg total RNA was obtained and 5 μg total RNA was reverse-transcribed with reverse transcriptase (Fermantas) according to standard protocols. Based on the highly conserved coding region from trypsinogen deposited in the NCBI database, PCR primers were designed to amplify the conserved internal regions of trypsinogen of these two species (Table 1).

The PCR cycling conditions were 1 cycle of 94 °C for 5 min, 32 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 40 s, followed by 1 cycle of 72 °C for 10 min. The resultant products were isolated using an agarose purification kit (Omega Bio-Tek, Inc., Norcross, GA, USA), and cloned into the pGEM-T vector (Tiangen, Beijing, China), following the manufacturer's instruction. Putative clones were screened by PCR using the above primers under the same cycle conditions, and the selected clones were sequenced.

To recover the full-length cDNA sequence, 3′ RACE and 5′ RACE were performed using the gene-specific primers and adaptor primers (ROPs and RIPs) shown in Table 1. The PCR cycling conditions were 1 cycle of 94 °C for 5 min, 5 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 90 s, 5 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 90 s,

Table 1
Primers used for *trypsinogen* gene cloning and expression analysis.

Name	Sequence (5′–3′)	Application
TryF	CTGGGTTGTGTCTGCTGCTCA	Conserved region clonings; 3′ RACE first round PCR for grass carp
TryR	CTCAGCTTGATCAGCATGACGTC	Conserved region clonings; 5′ RACE first round PCR for grass carp
GIF	CCTCTGAAAAGGTCATTCGCCA	3′ RACE second round PCR for grass carp
GIR	CTGTCAATGGTCCAGGAGTTAT	5′ RACE second round PCR for grass carp
TIF	CTGCTGCTCACTGCTACAAGTCA	3′ RACE second round PCR for topmouth culter
TIR	TGACCTTAGAGGAGTCGATGAACTG	5′ RACE second round PCR for topmouth culter
3′ROP	TACCGTCGTTCCACTAGTGATTT	Outer reversed complement to the adaptor for 3′ RACE
3′RIP	CGCGGATCCTCCACTAGTGATTTCACTATAGG	Inner reversed complement to the adaptor for 3′ RACE
5′ROP	CATGGCTACATGCTGACAGCCTA	Outer reversed complement to the adaptor for 5′ RACE
5′RIP	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	Inner reversed complement to the adaptor for 5′ RACE
RT-GF	TCTGCTGCTCACTGCTACAAG	Real-time PCR primer used in expression study for grass carp
RT-GR	CTGTCAATGGTCCAGGAGTTAT	
RT-TF	CTGCTGCTCACTGCTACAAGTCA	Real-time PCR primer used in expression study for topmouth culter
RT-TR	TGACCTTAGAGGAGTCGATGAACTG	
Actin-F	CCTTCTTGGGTATGGAGTCTTG	Real-time PCR controls
Actin-R	AGAGTATTTACGCTCAGGTGGG	Real-time PCR controls
TFG-F	CTTCGCTCTGGATGATGACA	Confirmation of full-length trypsinogen cDNA of grass carp
TFG-R	GCAGATTTGGAGGGTGTATG	
TFT-F	TCTTCTGGCTCTATTCGCTG	Confirmation of full-length trypsinogen cDNA of topmouth culter
TFT-R	GGACTCTAAAAACTCGGGGA	