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Research paper

Molecular characterization and dietary regulation of aminopeptidase N (APN) in the grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Aminopeptidase N (APN) is a member of the peptidase M1 family and plays an important role in protein digestion. In the present study, an APN gene was cloned from the intestine of Ctenopharyngodon idellus. The full-length cDNA sequence of APN encodes an 892-amino-acid peptide that includes one helix transmembrane region. Phylogenetic analysis showed that the APN sequence clustered with *Danio rerio* as its closest neighbor, sharing a sequence similarity of 81.5%. APN mRNA was differentially expressed in different tissues, with a gradient expression from high to low in the tissues of the fore-intestine, hind-intestine, liver, mid-intestine, kidney, muscle, spleen and heart. APN expression in grass carp had a circadian pattern, showing time-dependent higher expression between 06:00 and 18:00 and lower expression between 18:00 and 06:00. In addition, the protein levels and resource in the diet-regulated APN expression suggested that low crude protein (CP) level and fish meal stimulated APN gene expression. Furthermore, the mRNA expression of APN in the intestine was significantly suppressed by high concentrations of glutamine and glutamine dipeptides, respectively. This study may provide valuable knowledge on the regulation of APN expression in teleost, which has potential applications for improving fish dietary formulations.

1. Introduction

The teleost is a special vertebrate that requires a high protein diet for protein synthesis and energy metabolism. In the intestine of fish, the digestive system metabolizes protein to generate a mixture of free amino acids and small peptides which are mainly absorbed by transporters in the intestine (Wilfart et al., 2008; Moharrery et al., 2014). Aminopeptidase N (APN), a member of the M1 metalloprotease family, acts as a zinc-dependent membrane-bound proteolytic enzyme and plays an essential role in the digestion of proteins and metabolic balance by selectively removing amino acids from the N-terminus of peptides (Firla et al., 2002). Beyond its key role in protein metabolism, APN has also been shown to function as a regulator of inflammatory responses. For example, it is believed that APN can be used as a pathologic indicator of cholelithiasis, biliary atresia, cytomegalovirus infection, hepatocellular carcinoma and intestinal lesions (de la Porte et al., 1996; Kim et al., 2012). Some studies have shown that APN can trigger inflammatory responses via the regulation of chemokine levels. The previous research has demonstrated that the APN gene is widely expressed in different tissues, including intestinal epithelial, kidney, liver, blood monocytes, granulocytes and lung (Liu et al., 2009; Gabrilovac et al., 2011; Ningshen et al., 2013; Lin et al., 2014). To date, several studies have identified APN in different teleosts, including zebrafish (*Danio rerio*) (Park et al., 2014), Japanese flounder (*Paralichthys olivaceus*) (Kurokawa and Suzuki, 1998) and common carp (*Cyprinus carpio*) (Liu et al., 2008). In these studies of different teleosts, the APN protein was purified and its molecular function characterized. However, the expression patterns of APN and the dietary mechanisms that regulate APN in teleosts are still unknown.

Grass carp (*Ctenopharyngodon idella*) is a native Chinese species that is distributed from the catchment area of the Pearl River in southern China to the Heilongjiang River in northern China. Because of its high economic relevance and grass feeding attribute, grass carp has been introduced to over 40 countries. Like other breeding fishes, grass carp are fed special diets in aquaculture. Because the grass carp is a herbivore, the dietary protein levels and protein sources are different from other breeding species, as most breeding species are omnivorous and carnivorous. In previous studies, we showed that the dietary protein level and

Abbreviations: APN, aminopeptidase N; CP, crude protein; RACE, rapid-amplification of cDNA ends; bp, base pair; UTR, untranslated region; aa, amino acids; d, day; Fig., Figure.

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source had a significant effect on protein absorption related genes in the intestines, including PepT1 and GDH (Liu et al., 2012; Liu et al., 2013). Additionally, different feed additives affected the expression levels of these genes. These results provided new insight on the influences of diet on protein digestion and transport in intestines. As a key enzyme for protein excision, APN is an important target and indicator for formulating diets. Additional studies with APN would be beneficial for designing more appropriate dietary protein compositions for better absorption. In this regard, we cloned the full-length cDNA of APN from the intestine of grass carp and characterized its expression pattern in different tissues, at different developmental stages and at different times of the day using quantitative real-time PCR. Furthermore, we extended our study to determine the dietary protein and feed additives that affected APN expression. Data from this study may provide valuable knowledge on the dietary regulation mechanisms of APN expression in grass carp.

2. Materials and methods

2.1. Animals and tissue preparation

All of the grass carp in the present study were provided by the Hunan Institute of Aquatic Science. After anaesthetization of the fish with 2-phenoxyethanol (Sigma), tissue samples from the healthy adult fish were collected and stored at −80 °C until further analysis.

2.2. RNA isolation and cDNA synthesis

The total RNAs were isolated from tissues using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Genomic DNA was removed from the RNA samples via DNase I treatment (Promega, USA). The integrality and quantity of the extracted RNA were confirmed by agarose gel electrophoresis and spectrophotometry (BioPhotometer Eppendorf). Subsequently, cDNA was synthesized using 1 μg of total RNA and the AMV reverse transcriptase (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions.

2.3. Cloning of the full-length cDNA of APN and sequence analysis

To obtain the full-length coding sequence of APN, degenerated primers were designed based on conserved sequences in teleosts (Table 1). The PCR amplification conditions were denaturalization at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 90 s. After obtaining a central core sequence of APN, full-length cDNA was obtained using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). The nested PCR primers for RACE were designed according to the partial sequences (Table 1). For both 3′ and 5′ RACE, the amplification conditions were denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. All the specific PCR products were size-separated on 1% agarose gels and then purified with a TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa, Japan). After purification, the DNA fragments were ligated into the pMD18-T vector (TaKaRa) and transformed into competent *Escherichia coli* DH5α cells. Randomly selected clones were sequenced on a 3730 Applied Biosystems (ABI) DNA sequencer using the universal primers M13-47 and RV-M.

Table 1
Primers used for APN cloning and quantitative real-time PCR.

Primer names	Sequences	Usage
P+	5′-GTCGCCACAACTCAGATG-3′	CDS
P−	5′-GTAAACCTCBGWGCGRTC-3′	CDS
GSPP1+	5′-CCGTTATTAACCGGGCACAG-3′	3′ RACE
GSPP2−	5′-TGAGCCACAGGTCATTCC-3′	5′ RACE
NGSPP1+	5′-GTTTGATCGCTCGGAGGTT-3′	3′ RACE
NGSPP2-	5′-GCATCTGAGTTGTGGCGAC-3′	5′ RACE
APN rt+	5′-GCCCAAAACTGTGCAAGAAAT-3′	Real-time PCR
APN rt−	5′-CTCTGATGCGTGTCTGGTTTAGT-3′	Real-time PCR
β-actin+	5′-GAACACTGTGCTGTCTGGAGGTA-3′	Real-time PCR
β-actin−	5′-CTTGGGTTGGTCGTTTGAATC-3′	Real-time PCR

The cDNA and deduced amino acid sequences of APN were analyzed using the BLAST tool available from the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid sequence was deduced using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). A neighbor-joining (NJ) phylogenetic tree was constructed based on the multiple sequence alignment using the MEGA 4.0 package with 1000 bootstrap repetitions.

2.4. Quantitative real-time PCR

The APN mRNA levels were determined by quantitative real-time PCR in a Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers were designed using Primer Express 3.0 software (Table 1). β-Actin was used as an endogenous control. For each sample, three replicates were performed with the conditions of 95 °C for 30 s followed by 40 cycles at 95 °C for 3 s, 55 °C for 25 s and 72 °C for 11 s. The corresponding real-time PCR efficiency was calculated using the equation, PCR efficiency $= (10^{-1/\text{slope}} - 1) \times 100\%$. The values detected from different amounts of template RNA (5-fold series dilution) from the representative samples were parallel with the respective standard curve. Under these conditions, the mean PCR efficiencies were 102.3% and 101.8%, respectively, for APN and β-actin. Following the amplification, a dissociation curve was performed to verify that a single product was generated. Negative RT controls and negative no template controls (NTC) were performed to rule out the possibility of DNA and/or dimmer contamination. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method with SDS software v1.3.1.

2.5. APN expression patterns in grass carp

For the tissue distribution analysis of APN transcripts, total RNAs were isolated from 8 different tissues (foregut, midgut, hindgut, heart, liver, spleen, kidney and muscle) in adult grass carp. For expression analysis at different developmental stages, the samples were collected from blastocyst, gastrul stage, nerve stage, organ stage, pre-hatching, hatching stage, 1d posthatch, 2d posthatch, 3d posthatch, 4d posthatch, 7d posthatch, 14d posthatch, 21d posthatch, 28d posthatch and 35d posthatch (for each stage n = 5). To determinate the diurnal rhythm of APN transcript expression in grass carp intestine, the intestine was divided into three sections, the foregut, midgut and hindgut. The RNAs were isolated from all of the intestinal tissues in grass carp (n = 5), and cDNAs were synthesized as described above. APN mRNA expression levels were assayed by quantitative real-time PCR.

2.6. Dietary protein level and source on APN regulation

To determine the effects of dietary protein levels on APN expression in grass carp, 22%, 32% and 42% crude protein (CP) diets were formulated (Table 2). 90 juvenile grass carp (0.5 years old) were randomly divided into three fiberglass tanks (n = 30 for each fiberglass tank). First, the fish were adapted to the experimental diets for 1 week. Second, the three groups of grass carp were fed with diets of 22%, 32% and 42% CP for 30 days. The intestines were collected on day 7, day 14, day 21 and day 28 (n = 5 for each group). The mRNA expression levels of APN were detected by real-time PCR.

The effect of the protein source on APN expression was also analyzed using real-time RT-PCR. Two diets with the same amount of protein were formulated with fish meal or soybean meal (Table 3) for 0.5 year-old fishes that were breeding in two fiberglass tanks (n = 30