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Characterization and dietary regulation of oligopeptide transporter (PepT1) in different ploidy fishes

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ABSTRACT

The oligopeptide transporter (PepT1) is located on the brush-border membrane of the intestinal epithelium which has been regarded as a mediator of protein absorption. Here, we cloned and characterized PepT1 genes from diploid (red crucian carp), triploid and tetraploid fish. Then, the PepT1 expression pattern in different tissues and embryogenesis were assayed. Meanwhile, using real-time PCR and western blotting, we showed the expression profiles of diets with different protein levels, protein sources and additives (sodium butyrate) in triploids. The cDNAs of the three different ploidy fishes have a high sequence similarity of PepT1 among vertebrates. PepT1 mRNA expression was also developmentally regulated and showed the strongest expression around the 2-cell and 4-cell stage in all three kinds of fishes. The maternal transcripts were first detected in eggs and dropped from blastula stage to muscle contraction stage. Tissue expression studies showed higher expression of PepT1 genes in the intestines of fishes compared with other tissues. In adults, triploids showed significantly higher expression levels of PepT1 in the intestines of the three kinds of ploidy fishes during breeding season and non-breeding season. In addition, high or low protein level diets both promote PepT1 expression in the intestine. We also confirmed that fish meal showed a significant increase in PepT1 expression than soybean meal in triploid intestines. Furthermore, sodium butyrate additives induce PepT1 expression that may be mediated by CDX2 and CREB. This research provides a new insight into protein absorption and its regulation in triploid fish.

1. Introduction

Peptide transporter 1 (PepT1) is primarily localized at the epithelial cells of brush-border membranes in the intestine and plays a crucial role in protein nutrition by mediating the uptake of dietary amino acids in di- and tripeptide form [9]. PepT1 genes have been characterized in many species of vertebrates, including mammals and aves [1,10,13,17,38,41]. In teleosts, PepT1 studies have been reported in zebrafish (*Danio rerio*) [37], common carp (*Cyprinus carpio*) [26], grass carp (*Ctenopharyngodon idella*) [21], Atlantic salmon (*Salmo salar*) [29], mummichog (*Fundulus heteroclitus*) [4], sea bass (*Dicentrarchus labrax*) [36] and rainbow trout (*Oncorhynchus mykiss*) [25]. These publications focus on PepT1 expression patterns and function in transporting di- and tripeptide and provide details of fish feeding protein utilization. By transporting bioactive molecules, such as Lys-Pro-Val, PepT1 plays an important anti-inflammatory role which could be a useful potential target for anti-inflammatory therapies [5,7]. In contrast with the well-studied PepT1 function, knowledge about regulation of PepT1 by feeding is scarce in fish. We still lack information regarding the dietary regulation of PepT1 gene expression to help us design more appropriate diets for better protein absorption. In addition, further understanding of the mechanisms in this process and potential improvement of fish diets with different formulations is required.

Protein is an important component of the fish diet. However, because of the scarcity of protein resources, enhancement of protein utilization efficiency is a universal method for feeding in aquaculture. Thus, researchers can help to improve feed protein formulation by regulating the dietary protein level and composition, as well as through the use of feed additives. Research into PepT1 offers a better understanding of protein absorption and potential improvements in fish diets with different formulations. Shiraga and colleagues reported that transcriptional activation of PepT1 could be induced by dietary protein [34]. Meanwhile, PepT1 gene expression was significantly enhanced under malnourished conditions

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[17]. These publications elucidated the dietary regulation of PepT1 in mammals.

Butyrate, a short-chain fatty acid, is easily produced by bacterial fermentation of carbohydrates and dietary fiber [40]. Supplementation with sodium butyrate increases growth performance and intestinal integrity in piglets [3,12]. Sodium butyrate has been used as feed additive for pigs [23], and cows [2] and is a potential feed additive for use in the aquaculture industry. Further knowledge of nutrient transport is regarded as important information for growth rate increases and the formulation of commercial diets [12,14,16]. Dalmasso and colleagues validated that butyrate enhances PepT1 expression and activity via transcription factor CDX2 and CREB in human intestinal epithelial Caco-2BBE cells [8]. Shimakura and colleagues also reported that CDX2 plays a key role in the transcriptional regulation of the intestine-specific expression of PepT1 [32,33]. These findings suggest that by stimulating PepT1 expression and activity via sodium butyrate we may improve growth and amino acid absorption for farmed creatures.

Studies on PepT1 would help researchers design novel diets with more appropriate protein compositions for better absorption. In our previous study, different ploidy fish lines, including diploid, triploid and tetraploid, from the red crucian carp × common carp have been obtained by interspecies crossing and reverse crossing technologies [20]. The triploid population is of high value in aquaculture because of its fast growth rate, good resilience and sterile appearances. These different ploidy fish lines provide a biological platform for comparative studies of ploidy fishes and aquatic application [19]. Although sterile appearance and genome variation has been reported in triploids, little attention has been paid to understanding their mechanism of growth rate and protein metabolism. We still lack information on absorption of protein and regulation in different ploidy fishes. Meanwhile, PepT1 regulation by different protein levels, protein sources and butyrate in triploid fishes is still unknown. Hence, we cloned PepT1 cDNAs from the intestine and studied their expression pattern in tissues of different ploidy fishes. Furthermore, we analyzed the regulation of PepT1 by dietary protein levels and composition, as well as feed additives (sodium butyrate) in triploid fish.

2. Materials and methods

2.1. Fish

The allotetraploid hybrids ($4n = 200$) were produced by interspecific cross of female red crucian carp and male common carp. The triploid hybrids ($3n = 150$) were obtained by intercrossing of female red crucian carp and male allotetraploid. The diploid red crucian carp, triploid hybrids and tetraploid hybrids were sourced from the Engineering Centre of Polyploidy Fish Breeding of the National Education Ministry at Hunan Normal University.

2.2. Cloning of full length cDNA of PepT1 and phylogenetic analysis

Total RNAs were extracted from tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was carried out from 1 μg total RNA using AMV reverse transcriptase (Fermentas, Vilnius, Lithuania) with oligo $(dT)_{12-18}$ primers. The degenerated primers were designed based on conserved sequences of PepT1 in other teleosts (Table 1). Polymerase chain reaction (PCR) amplifications were done for 35 cycles with denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and elongation at 72 °C for 30 s. The products were separated with 2% agarose gel and then cloned and sequenced. Subsequently, the full-length cDNA was amplified using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Specific nested PCR primers were designed based on the obtained sequences (Table 1). For 3′RACE, the amplifications were done under the conditions: 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min with 30 cycles. For 5′RACE, the amplification conditions were: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min with 30 cycles.

Table 1
Primers used in this work.

Primer name	Primer sequence (from 5′–3′)	Purpose
PepT1-S	TCCATTAACGCTGGCAGT	CDS
PepT1-A	AACATTGGCAGAGGGATA	CDS
PepT1-3-1	TGGTGGTTGCCCTCATTGTGTTCATC	3′RACE
PepT1-3-2	GAGCACTGGATGGACTGGGCGGAAG	3′RACE
PepT1-5-1	ATTTCTCTTCCGCCCAGTCCATCCAG	5′RACE
PepT1-5-2	AGCAGGATGTTGCCTTTGGGGGATTC	5′RACE
PepT1-RT-S	CAGGTTTGTGAATGGCTTTACC	Real-time PCR
PepT1-RT-A	TGTGGATGTCGTTCGGGTTA	Real-time PCR
CDX2-RT-S	GAACCCTCAGAATTTTGTACCCG	Real-time PCR
CDX2-RT-A	GGTAATTCCAGGGACGTGATG	Real-time PCR
CREB-RT-S	TCAATCAGTCCCACGGATAGAA	Real-time PCR
CREB-RT-A	GTCTGTAGCCCTTGAACTCCAT	Real-time PCR
β-Actin-S	GCTCTTCCCCATGCAATCCT	Real-time PCR
β-Actin-A	GGTTCCCATCTCCTGCTCAA	Real-time PCR

Phylogenetic analysis was conducted to compare with other vertebrates. Amino acids sequences were aligned by ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analysis was performed using Neighbor-Joining method with 1000 bootstrap in Mega version 4.1 [18].

2.3. Quantitative real-time PCR

PepT1, CDX2 and CREB mRNA expression were performed using quantitative real-time PCR in ABI PRISM 7500 Real Time PCR System (Applied Biosystems). The primers were designed based on the cDNA sequences (Table 1) and β-actin was used as an internal control. To eliminate endogenous DNA contamination, 1 μg RNA was digested by DNase I prior to the cDNA synthesis. 5 μL of first-strand cDNAs (in a dilution of 1:20) as a template was added to 20 μL PCR solution containing 20 nmol/L primers and SYBR Green PCR Master Mix (ABI). The procedure was repeated three times for each sample: 50 °C for 5 min, 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. The dissociation curve was used to verify single product generation at the end of the assay and the analysis of relative mRNA expression was performed using the $2^{-\Delta\Delta Ct}$ method.

2.4. Preparation of antibodies

Recombinant red crucian carp PepT1 (GenBank accession code: JQ411137) was prepared in *Escherichia coli* and purified with a His-tag. Polyclonal PepT1 antibodies were raised against purified recombinant PepT1 in female rabbits (White New Zealand). The titer of the antibodies was >1:5000.

2.5. Western blot analysis

The tissues were homogenated with RIPA buffer (Pierce) according to the manufacturer's instruction. The proteins were measured by Bradford way and adding the SDS-PAGE loading buffer into the prototype we gathered. Following SDS-PAGE, the gels were transferred onto PVDF membranes. After blocking the membranes with the albuminous coat and incubation with the primary antibody (β-actin antibody 1:2000 dilution, Sigma–Aldrich; Polyclonal PepT1 antibody 1:1000 dilution) and secondary antibody (IgG antibody 1:20,000, Millipore), the signals were detected using ECL Reagent (Bio-Rad). For an internal control, β-actin was used to normalize the target bands.