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The effect of peptide absorption on PepT1 gene expression and digestive system hormones in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The present study evaluates the effect of protein source (dipeptides, free amino acids, and intact protein) on development and growth of Salmonid fish alevin. Specifically, we follow the expression of oligopeptide transporter protein PepT1 in the intestine of rainbow trout (*Oncorhynchus mykiss*). Fish were fed exogenously one of four diets: three formulated (lysyl–glycine dipeptide supplemented diet — PP, free lysine and glycine supplemented diet — AA, control diet with no lysine — CON) or commercial starter (Aller Futura — AF). Fish increased mean body weight 8 fold with PP- and AA-supplemented diets resulting in significantly higher weight gain than fish fed CON. Statistical analysis revealed a significant increase in relative PepT1 expression of fish fed experimental diets. Immunohistochemical staining with PepT1 antibody showed the presence of the transporter protein in the brush border membrane of the proximal intestinal enterocytes of fish from all experimental groups. Leptin immunoreactivity occurred not only in the gastric glands but also in proximal intestine and pyloric caeca of fish fed PP, AA and AF diets. Leptin immunoreactivity was also observed in hepatocyte cytoplasm and pancreatic acinar cells. Gastrin/CCK immunoreactive cells were present in the proximal intestine and pyloric caeca.

1. Introduction

Morphological, physiological and biochemical adaptation of the digestive system, as well as regulation of metabolic rate maximizes feeding rate and efficiency in juvenile fish (Dabrowski et al., 2003). When larval tissues exceed 50% protein content, they show high amino acid demand to cover anabolic requirement (Dabrowski, 1986). Therefore, the availability of amino acids for protein synthesis becomes critical.

An accelerated rate of amino acid absorption occurs when short peptides are transported by PepT1, present in the apical membrane of enterocytes (Daniel, 2004). Expression of this transporter protein is regulated by amino acids and peptides (Shiraga et al., 1999), as well as growth factors (Nielsen et al., 2001).

Peptide transporters are membrane proteins responsible for selective transport of dipeptides and tripeptides across the enterocyte membrane (Chen et al., 2005). One of these proteins, the PepT1, occurs in the small intestine epithelium. The PepT1 gene was described for the first time in rat (Fei et al., 1994), and 1 year later PepT1 was also sequenced in human (Liang et al., 1995). Sequences are also available for zebrafish *Danio rerio* and *Chionodraco hamatus* (Verri et al., 2003; Maffia et al., 2003) and cod (*Gadus morhua*) (Amberg et al. 2008). The PepT1 protein in higher vertebrates is consistent in length, approximately 700 amino acids, while in most lower vertebrates it is usually longer (Liang et al., 1995; Miyamoto et al., 1996; Fei et al., 2000; Rønnestad et al., 2007). In comparison to higher vertebrates both the protein and nucleotide sequence of the PepT1 transporter protein in fish are conserved (Ostaszewska et al., 2009).

Oligopeptide carrier proteins are a large family of peptide transporters (PTR) (Steiner et al., 1995). They are less studied in lower vertebrates, including fish, than in mammals. Studies of peptide transporters may provide information about their molecular structure, function, and the process of peptide transport in vertebrates. This group of proteins include PepT1 showing high expression in vertebrate intestine (Daniel, 2004; Rønnestad et al., 2007). In teleost fish, and in higher vertebrates peptide transport is strongly stimulated by the inside the negative transmembrane electric potential (Thamotharan et al., 1996; Maffia et al., 1997). Similarly seen in mammals (Daniel, 2004), the structure and function of PepT1 in the teleost fish *D. rerio* revealed the protein to be active, under conditions of low-affinity/high-capacity, but it also depends on the extracellular pH (Verri et al., 2003).

In mammals expression of PepT1 can be regulated by hormones like insulin (Meredith and Boyd, 2000), epidermal growth factor (Nielsen

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et al., 2001), leptin and thyroid hormone (Buyse et al., 2001; Ashida et al., 2002). Similarly in fish leptin, cholecystokinin and gastrin may have regulatory effects on the developmental regulation of intestinal transport.

The aim of the present study was to evaluate the effect of protein sources (dipeptides, free amino acids and intact protein) on growth, development and expression of PepT1 oligopeptide transporter in the intestine of rainbow trout juveniles. Additionally the location and expression of PepT1, leptin and gastrin/CCK in fish digestive tract (tissues, cells and subcellular compartments) were evaluated.

2. Materials and methods

2.1. Experimental design

Rainbow trout alevins (average body mass: 0.13 ± 0.02 g; total body length: 24.42 ± 1.67 mm) were obtained after complete yolk sac resorption from the Department of Salmonid Fish Culture of Inland Fisheries Institute in Rutki. The experiment was carried out in the Division of Ichthyobiology and Fisheries, Warsaw University of Life Sciences. The fish were stocked (50 fish/tank) into 16 tanks (four experimental groups, each in four replicates) of 20 L in volume. The recirculation system incorporated mechanical and biological filtration as well as UV irradiation. The average water temperature was 17.5 ± 1.4 °C, pH 6.9 ± 0.06, dissolved oxygen concentration 8.27 ± 0.39 mg/L, the level of NH_4^+ 0.24 ± 0.08 mg/L, and PO_4^{3-} 0.49 ± 0.5 mg/L. The fish were maintained in 12 h light:12 h dark photoperiod regime.

The fish were fed four diets of different composition: three formulated diets (Lys-Gly), dipeptide based diet (PP), free lysine and glycine based diet (AA), control diet without lysine supplement (CON), and a commercial salmonid starter Aller Futura (AF) (Aller Aqua, Poland). The formulated diets were developed and made in the Aquaculture Laboratory, Ohio State University (Table 1). The total fish biomass in each tank was evaluated weekly. The following feeding regime was applied during 12 h light: days 1–7: every hour (12 h biomass), days 8–14: every 2 h (12 h) (3% biomass), days 15–28: every 2 h (12 h) (5% biomass).

Table 1
Composition of feeds used in this experimental study.

Diet					
Lys-Gly dipeptide based diet (PP)		Free lysine and glycine based diet (AA)		Control diet (CON)	
Ingredient	% in diet	Ingredient	% in diet	Ingredient	% in diet
Fish meal	12.4	Fish meal	12.4	Fish meal	12.4
Wheat gluten[a]	37	Wheat gluten[a]	37	Wheat gluten[a]	37
Wheat	17.18	Wheat	17.18	Wheat	17.18
Fish	5	Fish	5	Fish	5
Lecithin	15	Lecithin	15	Lecithin	15
Mineral mix	3	Mineral mix	3	Mineral mix	3
Vitamin mix	4	Vitamin mix	4	Vitamin mix	4
Lysine–gycine[d]	3.4	Lysine[e]	2.1	Lysine[e]	0
Cysteine[b]	0.2	Glycine[e]	1.3	Glycine[e]	1.3
Arginine	0.65	Cysteine	0.2	Glutamate[c]	2.1
Methionine	0.33	Arginine	0.65	Cysteine	0.2
Threonine	0.29	Methionine	0.33	Arginine	0.65
Ca-mono P	1.5	Threonine	0.29	Methionine	0.33
Vitamin C	0.05	Ca-mono P	1.5	Threonine	0.29
		Vitamin C	0.05	Ca-mono P	1.5
				Vitamin C	0.05

[a] MP Biomedicals-Solon-OH.
[b] Hara (2006); most potent olfactory stimulating amino acid.
[c] Hughes (1985); non-toxic level of glutamic acid.
[d] MP Biomedicals.
[e] Bachem, NY.

2.2. Tissue collection and preparation for relative expression measurement

Intestinal samples for gene expression evaluation were collected before the first feeding (0), and then on days 7, 14, 21 and 28 of the experiment. The intestines were dissected from three fish for each experimental group ($n = 3 \times 4$). The tissue samples were washed in 1.1% NaCl, and stored at −80 °C in RNA*later* (Ambion, Austin, TX, USA), according to the producer's instruction, until RNA isolation.

2.3. Total RNA isolation and cDNA synthesis

Total RNA was isolated using a microcolumn kit, according to the recommended procedure (A&A Biotechnology, Gdynia, Poland). To eliminate contamination of RNA with DNA, the samples were digested with DNase I, according to the manufacturer's instruction (AppliChem, Darmstadt, Germany). Concentration (ng/μL) and purity ($A_{260\ nm}/A_{280\ nm}$) of the isolated RNA was measured using Nanodrop® (Thermo Scientific, Wilmington, DE, USA). Additionally, the level of RNA degradation was evaluated in 1% denaturing agarose gel (AppliChem, Darmstadt, Germany). Total RNA (1 μg) was then used for the reverse transcriptase, followed by polymerase chain reaction (RT-PCR) to synthesize cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The RT-PCR reaction was carried out according to the manufacturer's recommendation.

2.4. Quantitative real time-PCR analysis

Quantitative real time polymerase chain reaction (qRT-PCR) was carried out using Kappa® SYBR® Green QRT-PCR Master Mix Kit (Kappa Laboratories, Boston, MA, USA), in the Opticon Mini thermocycler (Bio-Rad Laboratories). The qRT-PCR was started with a preliminary matrix denaturation for 5 min at 94 °C, followed by 40 cycles of: denaturation at 95 °C for 15 s, primers binding at 60 °C for 20 s, and synthesis of qRT-PCR specific product at 72 °C for 40 s. All samples were processed in three replicates. Additionally, each plate contained control samples (without matrix). Specificity of qRT-PCR products was verified using the melting curve.

Primers for the rainbow trout PepT1 gene amplification were designed according to a sequence of 384 base pairs (bp) in length obtained from degenerated primers. The sequence was deposited in the GenBank (access no. EU853718). We surveyed β-actin (access no. AJ438158), Elongation Factor 1-α (access no. AF498320.1), and 18 S rRNA (access no. AF308735) as potential internal control genes for RT-PCR (data not shown).

The rainbow trout β-actin gene (access no. AJ438158) was used as the reference. The sequence of the primers used in the present experiment is shown in Table 2. The relative expression was calculated according to Pfaffl's method (Pfaffl, 2001). The C_T value obtained for "0" samples (collected before first feeding) was used as the reference.

2.5. Immunohistochemistry analysis for PepT1, leptin, and gastrin/CCK

Eight fish were collected from each experimental group on day 28 of the experiment for immunohistochemical analysis. Fish were anesthetized using 1:5000 MS-222 solution (pH 7.5 adjusted with $NaHCO_3$), and preserved in Bouin's solution and 4% buffered formaldehyde. The

Table 2
Sequence of the primers used for qRT-PCR.

Gene	Primer	Sequence (5′→3′)	Primers length (bp)	Product length (bp)
PepT1	PepT1 F	CCTGTCAATCAACGCTGGT	19	161
	PepT1 R	CACTGCCCATAATGAACACG	20	
β-actin	β-actin F	ACTGGGACGACATGGAGAAG	20	190
	β-actin R	GAGGCGTACAGGGACAACAC	20	