The Smallest Vertebrate, Teleost Fish, Can Utilize Synthetic Dipeptide-Based Diets

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ABSTRACT In vitro studies of brush-border intestinal transport of dipeptides and cytoplasmic hydrolysis in fish suggest that these processes could be key mechanisms in the absorption and utilization of nutrients for growth. However, in vivo experimentation to study the nutritional importance of these processes was needed. We compared three dietary formulations based on free, peptide and protein sources of amino acids. Our results were the first to show that a synthetic dipeptide (PP)-based diet could support growth in the early stages of ontogenesis of a teleost fish, rainbow trout (Oncorhynchus mykiss), whereas a free amino acid (FAA)-based diet failed. We found that fish fed an FAA-based diet had an increased rate of ammonia excretion [1.78 ± 0.19 mmol NH₃-N/(kg body wt·h)], compared with fish fed a PP-based diet [1.25 ± 0.07 mmol NH₃-N/(kg body wt·h)], suggesting that deamination is involved in the metabolism of dietary FAA. Teleost fish are known to obtain a high proportion of total energy from protein, compared with higher vertebrates. However, we found that feeding trout alevins a PP-based diet increased postprandial oxygen consumption for 2 to 24 h, whereas other treatments decreased 24-h postprandial metabolism. This may indicate that peptide metabolism is less efficient than protein metabolism. Juvenile rainbow trout differed from alevins in their response to FAA- and PP-based diets. These observations strongly suggest that intestinal dietary peptide transport and hydrolysis could support protein synthesis and growth in vertebrates that respond poorly to FAA-based diets. We conclude that nutrient administration may be improved by manipulating dietary peptide composition and peptide/protein ratios, leading to better utilization of synthetic peptides, with nutritional and therapeutic implications for all vertebrates.

KEY WORDS: ● peptides ● free amino acids ● rainbow trout

Morphological, physiological and biochemical adaptations of the digestive tract and the general metabolism of larval fish maximize nutrient uptake and transfer efficiency. Teleost fish larvae, considered to be the smallest vertebrates on earth (1), are extremely well adapted for nutrient utilization (digestion and absorption) and have a correspondingly fast growth rate [150 to 300 g/(kg body wt·d)] (2,3). This contradicts the frequent claims in the literature that larval fish are “morphologically and functionally incomplete” (4). We submit that studies on nutrient accretion in larval fish should focus on differentiating protein, peptide and free amino acid transport, metabolism and utilization for growth. Teleost fish larvae provide a uniquely suited model organism because of their size and the similarity of the intestinal processes of peptide and amino acid uptake to those in higher vertebrates, including humans (5,6).

Intestinal transepithelial transport of intact peptides is frequently observed to be of greater quantitative significance in vertebrates than transport of free amino acids (7,8), and fish adhere to this model (6,9). Studies of nutrient absorption and transfer to the blood using radiolabelled intact dipeptides (6,9) and studies of dipeptide hydrolysis in intestinal epithelial cell cytoplasm (10) provide evidence for this model of nutrient accretion. Intact peptide uptake in concert with the hydrolytic capacity of the intestinal mucosa [i.e., brush-border dipeptidas (11,12)] may explain how a mixture of dietary peptides functions as a major source of amino acids, absorbed in the digestive tract without the need for proteases secreted by the stomach and pancreas. Studies demonstrating that diets including concentrates of protein hydrolysates (random peptide mixtures) sometimes caused enhanced growth rates in larval fish (13–15) provided some evidence for this model. However, these experiments did not use an appropriate control (i.e., they neglected to include the same source of protein in both its intact and hydrolyzed forms). Therefore, reports of enhanced growth as a result of using two different fish meals (intact or hydrolyzate) may not differ from earlier observations with salmonid fish (16). It is recognized that free amino acid–based diets are inferior for rainbow trout, compared with intact protein–based diets (17) although some authors have disagreed (18).

The present study aimed to determine whether a diet composed exclusively of synthetic dipeptides of known composition is adequate for growth in vertebrates. The formulation of amino acids and the choice of peptides in such a diet can be
completely controlled, compared with a protein hydrolyzate-based diet. We carried out a series of feeding trials with alevis and larger juvenile rainbow trout. Diets were composed of identical quantities of indispensable amino acids provided in the form of free amino acids (FAA), 3 dipeptides (PP) or intact proteins from casein-gelatin (CA) and casein-gelatin with maca meal (CA-M). We hypothesized that the incorporation of dietary peptides would provide an in vivo test for intestinal transporters as well as for the efficiency of protein synthesis, facilitating the ultimate determination of balanced proportions of all indispensable amino acids. We suggest that juvenile fish be used as an animal model for other vertebrates because intestinal absorption processes in fish resemble the transport paradigm proposed in studies with mammals (7,8).

MATERIALS AND METHODS

The compositions of the experimental diets and their nutrient contents are listed in Table 1. Diets were isonitrogenous (58.6 ± 1.0%) and isolipidic (16.2 ± 1.0%) and contained casein-gelatin as the protein source (CA and CA-M), a mixture of synthetic dipeptides (PP) or a mixture of synthetic L-amino acids (FAA) that matched the amino acid composition of the protein-based diets (CA and CA-M). All diets covered the amino acid requirements for rainbow trout (19), and the proportions of amino acids in the peptide formulations (Table 1) prevented amino acid imbalances. Maca (Lepidium meyenii) meal was substituted for wheat meal in the CA-M, FAA and PP diets. This new ingredient has been proven to increase feed acceptance and growth rates in rainbow trout fed CA-based diets (20). Maca meal contains ~12% protein and is a negligible source of protein in the final formulation (2% out of 55%; Table 1). All diets were mixed, pelleted and freeze-dried to prepare pellets of the desired sizes (0.5 to 1.5 mm). Pellet size was gradually increased over the course of both trials as the fish grew.

In the first experiment with alevis, rainbow trout of the local registered strain (London, OH) were hatched and randomly distributed at the first feeding stage into twelve 40-L tanks (40 fish per tank). Three tanks were used per dietary treatment; tanks were supplied with 4 L/min of partly recirculated water at 14 to 16°C. Dechlorinated city water contained the following elements: K, 4.2 mg/L; Na, 16.1 mg/L and Ca, 27 mg/L. The mean weight of alevis before the first feeding was 114 ± 16 mg. Fish were fed ad libitum during wk 1, then the daily ration was adjusted to 4% body weight. Fish were initially fed 8×/d; fish were then fed 4×/d from wk 3 until completion of the first experiment. In addition, groups of 10 fish were fed the FAA diet were separated into three tanks at the completion of wk 3 and fed a “recovery” CA-M diet during wk 4 and 5. Growth and survival rates of these groups were monitored along with those of the other treatment groups.

In a second experiment, fish from the first trial’s CA-M group (mean weight 0.78 ± 0.08 g) were distributed into groups of 20 fish per treatment and stocked into nine separate tanks (n = 6 to 7). Three diets were tested, FAA, PP and CA. At the completion of each experiment, wk 5 and 2, respectively, oxygen consumption and ammonia and urea excretion were measured in 120-ml closed metabolic chambers. All fish were fed equal rations per body weight prior to the metabolic measurement. Metabolites were measured 2 and 24 h after feeding as previously described (21,22). These times were considered characteristic for postprandial and basal levels (23,24). Histological sections were made of the median and posterior intestine of fish fed the CA (control) diet, fish fed the FAA diet for 3 wk and fish fed the FAA diet for 3 wk, then switched to the CA diet. Methods for tissue preparation and staining were as described by Rinhardt et al. (25).

Three groups of fish were fed each experimental diet, using a completely randomized design. All data were subjected to one-way ANOVA (final weight, survival and specific growth rate), two-way ANOVA (time × treatment) and least significant difference multiple comparison testing using SPSS version 10.0 statistical software (SPSS, Chicago, IL). Percentages were arcsine transformed before analysis. Student’s t test was used to compare the mean difference in oxygen consumption and ammonia and urea excretion at 2 and 24 h after feeding. Differences were considered significant at P < 0.05.

RESULTS

In wk 1 of the study, when fish were fed ad libitum, alevis trout consumed the FAA-based diet most willingly (aggressively). Minimal loss of dietary ingredients was anticipated in this case, because the feed particles were ingested directly from the surface of the water (26). Other experimental diets were not equally accepted, and some of the mortality may be related to an overall delay in first feed intake (Table 2, Fig. 1). The PP- and protein (CA and CA-M)-based diets were completely accepted within 2 to 3 d. The recovery-phase growth rate of trout fed the FAA-based diet for 3 wk, then transferred to the

<table>
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<th>Component</th>
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<th>CA-M diet</th>
<th>FAA diet</th>
<th>PP diet</th>
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1 Free amino acid mixture composition (g/446 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine hydrochloride, 15; lysine hydrochloride, 18 (ICN Biomedicals, Costa Mesa, CA); histidine, 7; isoleucine, 9; leucine, 14; methionine, 10; phenylalanine, 18; threonine, 8; tryptophan, 2; valine, 12; proline, 111; serine, 111 and D,L-alanine, 111 (Sigma Chemicals, St. Louis, MO).

2 Dipeptide mixture composition (g/446 g dry weight mixture; arginine-valine acetate salt, 28.7; histidine-leucine, 12.1; glycine-isoleucine, 14.1; glycine-methionine, 13.8; threonine-leucine, 15.6; valine-leucine hydrochloride, 4.5; alanine-glycine, 100; alanine-glycine, 150; glycyl-tyrosine, 49.5 (Sigma Chemicals); lysine-glycine hydrochloride, 29.6; phenylalanine-leucine hydrolyte, 30.3; glycyl-tryptophan hydrolyte, 2.6 (ICN Biomedicals).

3 Soluble fish protein hydrolysate (Soprophure, Boulogne Sur Mer, France).

4 Roche Performance Premix composition (g/kg mixture); vitamin A acetate, 7.56; cholecalciferol, 0.0055; α-tocopherol acetate, 66.1; cobalamin, 0.0013; riboflavin, 13.2; niacin, 61.7; D-pantothenic acid, 22.1; pyridoxine, 4.42; thiamin, 7.95; o-biotin, 0.31 (Hoffman-La Roche, Nutley, NY).

5 Selenium from Bernhart Tomarelli salt mixture (5 g Na2Se-Se/kg mixture; ICN Pharmaceuticals, Costa Mesa, CA).

6 Mg-L-ascorbyl-phosphate (Showa Denko, Tokyo, Japan).

7 Carboxymethylcellulose (ICN Biomedicals, Costa Mesa, CA).

Abbreviations used: CA, casein-gelatin; CA-M, casein-gelatin with maca; FAA, free amino acid; PP, dipeptide.
CA-based diet, was comparable to that of fish fed intact protein–based diets. Fish fed the PP-based diet more than doubled in weight over 5 wk; however, this growth rate was significantly lower than that of fish fed CA-based diets (Table 2, Fig. 1). In the second experiment with juvenile trout, the growth rate of fish fed the PP-based diet was threefold greater than that of fish fed the FFA-based diet; however, both rates were lower than that of fish fed a CA-based diet. The interaction of time and dietary treatment was significant in both alevins and juveniles (two-way ANOVA; \(P < 0.05\) and \(P < 0.01\), respectively).

Characteristically, fish fed the PP-based diet exhibited elevated oxygen consumption levels for 24 h after feeding, resulting in high metabolic maintenance requirements (16% higher than those of fish fed the CA-M diet; Fig. 2). This finding corresponds with our observation of a slower feed

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evacuation rate in fish fed a PP-based diet. Major differences in digestive physiology and metabolism development between the alevin and juvenile stages caused absent or inferior growth rates in fish fed the FAA-based diet in experiments 1 and 2, respectively. For instance, we observed increased deamination of dietary FAA in alevins \(0.17 \pm 0.18 \text{ mmol NH}_3\text{-N/(kg body wt h)}\) compared with fish fed the CA-based diet \(0.56 \pm 0.04\) and \(0.59 \pm 0.19 \text{ mmol NH}_3\text{-N/(kg body wt h)}\), for FAA- and CA-based diets, respectively. This observation supports a metabolic basis for the ability or inability of fish to utilize dietary FAA at the first feeding stage. Increased urea synthesis and excretion continued for more than 24 h after food ingestion in juvenile trout fed an FAA-based diet \(0.39 \pm 0.05 \text{ mmol urea/(kg body wt h)}\), which differed markedly from the pattern observed in faster-growing fish fed CA-based \(0.16 \pm 0.03 \text{ mmol urea/(kg body wt h)}\) and PP-based \(0.19 \pm 0.05 \text{ mmol urea/(kg body wt h)}\) diets.

Histological analysis of the intestinal epithelium revealed that fish fed an FAA-based diet had significantly smaller and less differentiated intestinal folds (Fig. 3). The marked reduction in the thickness of the intestinal wall and presence of numerous goblet cells were similar in appearance to signs of starvation in fish (27–29). These differences were not the result of physiological aging, because fish subsequently fed an intact protein–based diet began to gain weight within 1 wk. However, the microvillous processes, noticeable on the intestinal epithelium, were not fundamentally changed, suggesting that the functional integrity of the absorptive surface was intact. Only 11% of fish fed the FAA-based diet survived for 5 wk. Undernourishment at the intestinal level did not cause the arrest in epithelial cell growth. The negative protein synthesis/degradation ratio was the most likely cause of the failure to gain weight.

**DISCUSSION**

The first advantage of including PP in fish diets is that PP acts as a fish attractant (30), thus enhancing acceptance of the feed. Indeed, we observed that rainbow trout juveniles immediately ingested the PP-based diet. This was particularly evident when we replaced 50% of the dietary protein in CA-based diets with PP (unpublished data). Growth rates of rainbow trout alevins in the present study (2 to 5%/d) were comparable with or higher than those reported in the literature (31,32).

Infusion of FAA into the vascular system of rainbow trout caused a proportional increase in oxygen consumption equivalent to 15 to 32% of gross FAA energy (33). An unbalanced proportion of indispensable amino acids causes higher postprandial oxygen consumption. We hypothesize that we observed a similar phenomenon in alevins in the present study (Fig. 2), and this suggests that considerable improvements can be made in the composition and proportion of dietary peptides to enhance their hydrolysis, absorption and transport (bioavailability).

Rainbow trout that are prevented from feeding or fed an FAA-based diet may maintain absorptive functioning for several weeks, and the rate of FAA uptake may in fact increase (34). However, the present study suggests that salmonid alevins are unable to utilize FAA for growth. This may require a reshaping of current theory concerning the formulation of FAA-based diets in marine and freshwater larval fish nutrition (35–37).

The concept that small peptides are absorbed in the vertebrate intestine has been reviewed in depth (8,38,39); however, the nutritional and metabolic significance of the mixture of peptides remains unclear. Evidence that vertebrates can grow on diets composed exclusively of synthetic PP of known composition has never been reported. There is evidence that a single peptide can be more efficiently absorbed than a mixture of the identical amino acids in both fish (40,41) and mammals (8,38,42). The present study provides the first in vivo evidence of the higher efficiency of peptide utilization for growth in vertebrates, compared with FAA utilization.

Information on brush-border aminopeptidase activity in the digestive tract of fish in the early larval stages is somewhat contradictory (43,44). However, both cytosolic and brush-border aminopeptidases may be involved in the utilization of dietary peptides (44,45). Aminopeptidase activity in the larval fish intestine has a specific ontogenetic profile. The dietary source of the substrate markedly affects the activity (45). How specific peptide transporters in the intestinal brush-border epithelium (9) of larval fish are mediated by dietary concentrations of substrates and varying compositions of dipeptides remains to be addressed.

Halver (46) first reported on the growth of salmonid juveniles fed FAA-based diets; however, the growth rate was slow \((0.46%/d; ninefold less than in the present study; Table 2)\) because the fish were reared at very low water temperatures. Similarly, the growth rate Ace et al. (18) reported for rainbow trout juveniles (mean weight 8.3 g) fed an FAA-based diet was inferior compared with that for fish fed protein-based diets [see review in Dabrowski and Guderley (47)]. Therefore, studies of optimum environmental conditions for attaining maximum growth rates in fish can be considered representative when estimating quantitative requirements of indispensable amino...
acids. Peptide-based diets have the potential to meet these needs in fish at the first feeding stage.

Reports have asserted that peptides are superior to proteins in larval fish diets. However, the peptides used as supplements were not hydrolyzates of the proteins they replaced, and the amino acid compositions of both were unknown (14). In other words, there were two amino acid compositions obtained from two entirely different protein sources. Therefore, we interpret the results of experiments by Zambonino et al. (14,15) and others (48) as inconclusive regarding the role of fish protein hydrolyzates in fish diets. It was demonstrated that using two fish meals in combination affected fish growth synergistically, compared with using each of them separately (16). Some studies of such experimental design, conducted without appropriate controls, reported detrimental effects of protein hydrolyzates on fish growth (13,14).

The present study indicates the need to validate the nutrient requirements obtained from studies of diets based on FAA mixtures and to test diets based exclusively on PP in larger fish and other vertebrates, including humans. Moreover, PP-based diets require quantitative evaluation of different PP as carriers of the same indispensable amino acids to improve bioavailability, and optimization of the ideal proportions of peptides, protein and amino acids to support maximum growth. It is imperative that the observations presented here be used to modify current strategies for estimating the nutritional requirements of vertebrates, thus contributing to better understanding of the process of nutrient accretion and growth.

LITERATURE CITED