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Effects of dietary size-fractionated fish hydrolysates on growth, activities of digestive enzymes and aminotransferases and expression of some protein metabolism related genes in large yellow croaker (*Larimichthys crocea*) larvae

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ABSTRACT

The present study was conducted to investigate the effects of size-fractionated fish hydrolysates on growth, activities of digestive enzymes and aminotransferases and expression of some protein metabolism related genes in large yellow croaker (*Larimichthys crocea*) larvae. Fish (initial body weight 3.15 ± 0.15 mg) were fed for 30 days with four diets: the control diet was produced using fish meal (FM) as the main protein source and other three diets were formulated with permeate after ultra-filtration of fish hydrolysate (PUFH), retentate after ultra-filtration of fish hydrolysate (RUFH) or non-ultrafiltered fish hydrolysate (NUFH) replacing approximately 40% FM. The results showed that specific growth rate and survival rate were significantly lower in fish fed diets with NUFH and RUFH than that of fish fed the control diet ($P < 0.05$) and there were no significant differences between PUFH-40 group and the control group ($P > 0.05$). Specific activities of digestive enzymes and the ratio "pancreatic enzyme in intestinal segment/pancreatic enzyme in pancreatic segment" were not significantly different between fish fed diets with PUFH and fish meal and they were both higher than that of fish fed diets with NUFH and RUFH ($P < 0.05$). Specific activities of both alanine and aspartate aminotransferases were significantly higher in fish fed the diet with PUFH than that of fish fed other diets ($P < 0.05$). Transcription of peptide transporter 1 (PepT1), cholecystokinin (CCK) and trypsin was significantly down-regulated in fish fed diets with NUFH and RUFH ($P < 0.05$). No significant differences were observed in the target of rapamycin (TOR) gene expression of fish among dietary treatments ($P > 0.05$). These results suggest that when replacing 40% FM, PUFH seems optimal for large yellow croaker larvae compared to NUFH and RUFH and size-fractionated fish hydrolysates could significantly affect digestion and absorption of protein in large yellow croaker larvae by altering the mRNA expression levels of CCK, PepT1 and trypsin.

1. Introduction

Marine fish larvae undergo major morphological and functional changes during the first weeks of life (Cahu et al., 2004). Before these salutatory changes, the altricial larvae have a very simple differentiation of the digestive tract with limited luminal protease activity. Hence, digestive processes, particularly protein digestion, have been pointed as a major limitation to the utilization of artificial diets by fish larvae (Cahu et al., 2004; Rønnestad et al., 2007). Fish hydrolysate is promising as a core material in microdiet since it typically consists of low molecular-weight peptides resulting from protein pre-digestion, which are more likely to be absorbed by enterocytes compared to high molecular-weight macromolecules (Önal and Langdon, 2009). The nutritional value of fish hydrolysate has been evaluated in diets for rearing marine fish larvae and improved growth and/or survival rate has been reported (Han et al., 2010; Kvåle et al., 2009; Liu et al., 2010). Furthermore, fish hydrolysate contains various molecular weight compounds ranging from dozens to thousands of Da which may affect the absorption capacity and rate of passage of the diet through the gastrointestinal tract (Espe and Lied, 1999). In the last decade, studies have been conducted to investigate the effects of size-fractionated fish hydrolysates on growth and feed utilization in a variety of juvenile fish including rainbow trout (*Oncorhynchus mykiss*), Japanese flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus* L.) and Atlantic cod (*Gadus morhua*) (Aksnes et al., 2006a,b; Zheng et al., 2012, 2013). These studies indicate that removal of small molecular weight

Abbreviations: PepT1, peptide transporter 1; CCK, cholecystokinin; TOR, target of rapamycin; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

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compounds from fish hydrolysate exerts deleterious effects on growth performance of fish. However, previous researches failed to elucidate the mechanisms related to the physiological effects of size-fractionated fish hydrolysates well. In addition, to our knowledge, no information is available about the effects of size-fractionated fish hydrolysates on growth in any fish larvae.

Fish show high amino acid demand to meet anabolic requirement during their larval stage (Dabrowski, 1986). Therefore, the availability of amino acid for protein synthesis becomes critical. Following feeding, digestion of protein is mainly mediated by trypsin in most marine fish larvae in which stomach is not differentiated well (Rønnestad et al., 2007). The digestive capability is influenced by many factors. Among them, cholecystokinin (CCK) is a crucial gastrointestinal hormone and plays a key role in the stimulation of pancreatic enzyme secretion (Liddle, 1997). The final stage of protein digestion is catalyzed by the brush border peptide hydrolases which results in free amino acids and di- and tri-peptides (Ganapathy et al., 2006). Di and tri-peptides are transported into the enterocytes by a low affinity and high capacity transporter, the peptide transporter 1 (PepT1) (Fei et al., 1994). Besides, in fish, as well as in mammals, the target of rapamycin (TOR) signaling pathway plays a crucial role in protein metabolism which coordinates the balance between protein synthesis and protein degradation in response to nutrient quality and quantity (Chen et al., 2012; Lansard et al., 2010; Wullschleger et al., 2006). Until now, none of the previous studies have provided any information about the mRNA expression of these protein metabolism related genes (CCK, trypsin, PepT1 and TOR genes) in response to size-fractionated fish hydrolysates.

Large yellow croaker (*Larimichthys crocea*) is a marine fish species that has been widely cultured in southern China due to its delicious taste and important commercial value. There have been a number of studies on the nutrition for large yellow croaker larvae (Ai et al., 2008; Li et al., 2013; Liu et al., 2006; Ma et al., 2005; Mai et al., 2005; Xie et al., 2011, 2012; Yu et al., 2012; Zhao et al., 2008). Especially, Liu et al. (2006) found that fish hydrolysate replacing 25% fish meal in the diet could benefit growth performance and development of large yellow croaker larvae. Unfortunately, little attention has been paid to the peptide profile of fish hydrolysate. To better reveal the effects of the experimental fish hydrolysate, diets in the present study were designed with a higher fish hydrolysate replacement level, which was expected not to be optimal for larvae growth and development. The main objective of this study was to determine whether and how size-fractionated fish hydrolysates by ultra-filtration (that essentially vary in the fractional amount of peptides of different molecular weights and amino acids) influence growth, activities of digestive enzymes and aminotransferases and expression of some protein metabolism related genes in large yellow croaker larvae and which the most appropriate type of fish hydrolysate is.

2. Materials and methods

2.1. Experimental diets

Fish hydrolysate was produced from the flesh of Pollock (*Theragra chalcogramma*) by enzymatic treatment. Alcalase and flavourzyme were used to hydrolyse fish meal (FM) protein in a 55 °C water bath for 3 h (pH = 8.5). At the end of the enzymatic treatment, the hydrolysate was kept at 90 °C for 20 min. Then bones and sludge were removed by a decanter, and oil was separated by centrifugation. Thereafter, the hydrolysate was obtained by filtration through Pellicon 2 Ultrafiltration Modules PLAC (Millipore, Billerica, MA, USA) with a filter of 1000 Da. The permeate and retentate after ultra-filtration as well as the non-ultrafiltered fish hydrolysate, named PUFH, RUFH and NUFH respectively, were frozen-dried and used as ingredients in the experimental feeds. The molecular weights of the three protein fractions (Table 1) are analyzed with the help of Analysis and Testing Center of Jiangnan University (Wuxi, China).

Table 1
Amino acid composition (g/100 g dry matter) and peptide molecular weight (Da) distribution of the soluble protein fraction.

	PUFH	NUFH	RUFH
Amino acids (AA)			
EAA[a]			
Valine	3.71	3.40	3.62
Methionine	2.48	2.19	2.23
Isoleucine	3.49	3.23	3.48
Leucine	6.65	5.76	5.93
Phenylalanine	3.63	3.21	3.19
Histidine	1.35	1.54	1.55
Lysine	7.26	6.64	7.31
Arginine	4.87	4.54	5.01
Threonine	3.18	2.83	3.05
Tryptophan	ND[b]	ND[b]	ND[b]
∑EAA	36.62	33.34	35.37
NEAA[c]			
Tyrosine	2.01	2.31	1.83
Proline	2.15	2.05	2.44
Aspartic acid	7.62	7.1	8.24
Serine	2.75	2.47	2.7
Glutamic acid	12.26	11.07	12.39
Glycine	3.6	3.25	3.86
Alanine	4.66	4.07	4.41
Cystine	0.17	0.24	0.21
∑NEAA	35.22	32.56	36.08
Peptide molecular weight (Da) distribution (% total protein)[d]			
>2000 Da	<1	<1	<1
2000–1000 Da	3.26	34.86	45.53
1000–500 Da	22.01	24.45	30.51
500–180 Da	45.26	24.12	17.93
<180 Da	29.15	16.46	5.68
Chemical composition (% dry matter)			
Crude protein	80.70	79.90	79.22
Crude lipid	2.37	3.21	3.53

[a] EAA: essential amino acids.
[b] ND: not determined.
[c] NEAA: nonessential amino acids.
[d] Analysed from Analysis and Testing Center of Jiangnan University (Wuxi, China).

Four diets were formulated: the control diet was produced using FM as the main protein source and other three diets were formulated with PUFH, RUFH or NUFH replacing approximately 40% fish meal, which were named PUFH-40, RUFH-40 or NUFH-40, respectively (Table 2). The amino acid compositions of the experimental diets are shown in Table 3.

Micro-diet (MD) was manufactured by micro-bonding technology. The particle size of the formulated diets ranged from 150 to 250 μm for larvae between 15 and 22 days after hatch (DAH), and 250 to 425 μm diets for larvae thereafter. All formulated diets were packed in double plastic bags to keep light off, and stored at −20 °C until use.

2.2. Experimental procedure

Large yellow croaker (*L. crocea*) larvae used in this study were obtained and reared at the hatchery of the Aquatic Technology Extension Station of Ningde (Fujian, China). Prior to the start of the experiment, all fish in the hatchery were fed with rotifers, *Brachionus plicatilis* ($0.5–1.5\cdot10^4$ ind L^{-1}) from 3 to 8 DAH, *Artemia nauplii* ($1.0–1.5\cdot10^4$ ind L^{-1}) from 6 to 11 DAH, and live copepods and a commercial pellet diet (RQ Com., manufactured by Marubeni Nisshin Feed Co., Ltd, Japan) from 10 to 14 DAH. From 15 DAH on, the fish were fed with experimental diets.

At the onset of the feeding trial, three hundred larvae at 15 DAH were randomly sampled to measure wet body weight. Fish with similar sizes (3.15 ± 0.15 mg) were randomly distributed into 12 fiberglass cylindrical tanks (water volume 500 L) and each tank was stocked with 5000 individuals. Each diet was randomly assigned to triplicate tanks. Fish were manually fed to apparent satiation five times daily

Table 2
Formulation and proximate analysis of the experimental diets (% dry weight).

	Diets			
	Control	PUFH-40	NUFH-40	RUFH-40
Ingredient				
LT[a]–white fish meal[b]	50.00	30.00	30.00	30.00
LT[a]–shrimp meal[b]	10.00	10.00	10.00	10.00
LT[a]–squid meal[b]	15.00	15.00	15.00	15.00
PUFH[c]	0	20.00	0	0
NUFH[c]	0	0	20.00	0
RUFH[c]	0	0	0	20.00
Sodium alginate	1.50	1.50	1.50	1.50
Yeast	3.00	3.00	3.00	3.00
α-Starch	6.00	7.00	6.80	6.50
Glycine	1.10	1.10	1.10	1.10
Glutamic	0.90	0.90	0.90	0.90
Vitamin premix[d]	1.70	1.70	1.70	1.70
Mineral premix[e]	1.50	1.50	1.50	1.50
Sodium benzoate	0.05	0.05	0.05	0.05
Antioxidant	0.05	0.05	0.05	0.05
Choline chloride	0.20	0.20	0.20	0.20
Fish oil	4.50	3.50	4.00	3.70
Soybeans lecithin	4.50	4.50	4.50	4.50
Proximate analysis				
Crude protein	56.73	61.13	60.83	60.57
Crude lipid	16.16	13.74	13.86	13.49
Ash	15.42	14.27	14.03	13.40

[a] LT: low temperature.
[b] White fish meal: crude protein 71.5% dry matter, crude lipid 6.89% dry matter; shrimp meal: crude protein 53% dry matter, crude lipid 3.5% dry matter; squid meal: crude protein 73% dry matter, crude lipid 3.7% dry matter (all were obtained from Cishan Fisheries, Shandong, China).
[c] PUFH: permeate after ultra-filtration of fish hydrolysate; NUFH: non-ultrafiltered fish hydrolysate; RUFH: retentate after ultra-filtration of fish hydrolysate.
[d] Vitamin premix (IU or g kg^{-1} premix): retinal palmitate, 3,000,000 IU; cholecalciferol, 1,200,000 IU; DL-α-tocopherol acetate, 40.0 g kg^{-1}; menadione, 8.0 g kg^{-1}; thiamin-HCl, 5.0 g kg^{-1}; riboflavin, 5.0 g kg^{-1}; D-calcium pantothenate, 16.0 g kg^{-1}; pyridoxine-HCl, 4.0 g kg^{-1}; meso-inositol, 200.0 g kg^{-1}; D-biotin, 8.0 g kg^{-1}; folic acid, 1.5 g kg^{-1}; para-aminobenzoic acid, 5.0 g kg^{-1}; niacin, 20.0 g kg^{-1}; cyanocobalamin, 0.01 g kg^{-1}; ascorbyl polyphosphate (contained 25% ascorbic acid), 100.0 g kg^{-1}.
[e] Mineral premix (g kg^{-1} premix): $Ca(H_2PO_4)_2 \cdot H_2O$, 675.0; $CoSO_4 \cdot 4H_2O$, 0.15; $CuSO_4 \cdot 5H_2O$, 5.0; $FeSO_4 \cdot 7H_2O$, 50.0; KCl, 50.0; KI, 0.1; $MgSO_4 \cdot 2H_2O$, 101.7; $MnSO_4 \cdot 4H_2O$, 18.0; NaCl, 80.0; $Na_2SeO_3 \cdot H_2O$, 0.05; $ZnSO_4 \cdot 7H_2O$, 20.0.

Table 3
Amino acid composition of the experimental diets (% dry matter).

AA/∑AA	Diets			
	Control	PUFH-40	NUFH-40	RUFH-40
EAA[a]				
Valine	4.60	4.89	4.64	4.77
Methionine	2.77	2.80	2.86	2.83
Isoleucine	4.21	4.27	4.26	4.27
Leucine	7.66	7.67	7.89	7.77
Phenylalanine	4.17	4.27	4.39	4.57
Histidine	1.97	2.04	2.04	2.13
Lysine	7.53	7.69	7.32	7.13
Arginine	7.68	7.44	7.76	7.47
Threonine	3.88	3.90	3.94	3.93
Tryptophan	ND[b]	ND[b]	ND[b]	ND[b]
∑EAA	44.47	44.98	45.11	44.87
NEAA[c]				
Tyrosine	2.98	3.01	2.88	3.26
Proline	4.45	4.39	4.52	4.66
Aspartic acid	9.71	9.92	9.63	9.60
Serine	3.61	3.67	3.52	3.76
Glutamic acid	16.79	16.95	16.92	16.42
Glycine	10.63	10.25	10.77	10.76
Alanine	6.18	6.21	6.41	6.20
Cystine	0.29	0.60	0.25	0.47
∑NEAA	54.65	55.00	54.91	55.13

[a] EAA: essential amino acids.
[b] ND: not determined.
[c] NEAA: nonessential amino acids.

(06:00, 08:30, 12:30, 14:30, and 17:00). The feeding trial lasted for 30 days. All tanks were supplied with seawater that had been filtered through two-grade sand filter. During the rearing period, water temperature was kept constant at 23 ± 1 °C; pH ranged from 7.8 to 8.2 and salinity from 22 to 26‰. About 200–300% of the water volume was renewed daily and all rearing tanks were provided with continuous aeration. Fish were reared under a 14:10 h, light/dark regime. The maximum light intensity was 8.5 W m^{-2} at the water surface during daytime. Undissolved surface materials were skimmed with a polyvinylchloride pipe in time and accumulations of feed and feces at the tank bottoms were siphoned twice daily.

2.3. Sampling and dissection

At the termination of the experiment, fish were fasted for 24 h before harvest. Then, fifty individuals were randomly sampled from each tank to be weighed in a microbalance and final fish number in each tank was recorded in order to determine the survival rate. Fifty individuals were randomly collected from each tank and immediately frozen in liquid nitrogen and then stored at −80 °C for enzymatic and PepT1, trypsin and TOR gene expression assays. Another fifteen individuals were randomly collected from each tank and cut off their head along with operculum and immediately frozen in liquid nitrogen and then stored at −80 °C for CCK mRNA expression assays. The remaining fish from each tank were collected and stored at −20 °C for subsequent analysis.

In order to separate pancreatic and intestinal segments, the fish (45 DAH) were dissected as described by Cahu and Zambonino-Infante (1994) and Ma et al. (2005). Dissection was conducted on a glass plate maintained at 0 °C.

2.4. Analytical methods

Chemical analysis of the experimental diets and whole fish was performed following the standard procedures (AOAC, 1995). The samples of diets and fish were dried to a constant weight at 105 °C to determine the dry matter content. Protein was determined by Kjeldahl method using a Kjeltec system (Kjeltec-2300, Sweden); lipid by ether extraction using Soxtec System HT apparatus (B-801, Switzerland); ash by combustion using a muffle furnace at 550 °C for 16 h.

Samples of fish hydrolysate and experimental diets were freeze-dried for AA analysis. The samples (0.02 g) were hydrolyzed with 15 mL of 6 N HCl at 110 °C under an atmosphere of nitrogen for 24 h, then filtered and added to ultrapure water (from Milli-Q system, Millipore, Billerica, MA, USA) in a 50 mL volumetric flask. A 2 mL solution was then transferred to a glass bottle and dried in a vacuum drying chamber (VD23, Germany). Thereafter, the bottle was added 2 mL of ultrapure water to adjust pH and dried in the vacuum drying chamber twice, and then 2 mL of ultrapure water was added to the bottle and was freeze-dried. And 2 mL of loading buffer was added to dissolve the remains. The supernatant was analyzed by the ninhydrin method with an automatic AA analyzer (Biochrom 30, GE, Biochrom Ltd, Cambridge, UK).

For fish at 45 DAH, the dissected samples, 0.2–0.3 g pancreatic segments, were homogenized in 2 mL cold (0 °C) ultrapure water (from Milli-Q system), then centrifuged at 3300 ×*g* for 3 min, and the supernatant was collected for further assays. In addition, 0.2–0.3 g intestinal segments were homogenized to purify brush border membrane (BBM) according to a method developed for intestinal scraping (Crane et al., 1979) and adapted to intestinal segments (Cahu and Zambonino-Infante, 1994). Before $CaCl_2$ solution addition, 1 mL homogenate was diverted for intestinal enzyme assays. This homogenate was then centrifuged at 3300 ×*g* for 3 min, and the supernatant was used for enzyme assays. Trypsin activity was assayed according to Holm et al. (1988). Leucine-aminopeptidase (LA) and alkaline phosphatase (AP) were assayed both in intestinal segment and BBM according to Maroux et al. (1973) and Bessey et al. (1946). Activities of alanine

Table 4
Sequence of the primers used for q-PCR in this study.

Target gene	Reference	Forward (5′–3′)	Reverse (5′–3′)	Annealing temperature (°C)
PepT1[a]	GenBank accession no. KC485004	CGTGTTCATTGTTGGTAGTGGTTTG	TTTCCTCAGCCCAGTCCATCC	58
CCK[b]	GenBank accession no. KF938690	GTGCTTTTGGCTGCTTTGTCC	AGGCTGGCTTGGTTGTAGTTAG	57
Trypsin	GenBank accession no. KF669617	GCCACCCTGAACCAGTATGT	TCCAGGTATCCAGCGCAGAA	56
TOR[c]	Li et al. (2013)	AGGCTGGCTTGGTTGTAGTTAG	GGACTGGATGCGGATGATGG	58
β-actin	Yao et al. (2009)	TTATGAAGGCTATGCCCTGCC	TGAAGGAGTAGCCACGCTCTGT	58

[a] PepT1: peptide transporter 1.
[b] CCK: cholecystokinin.
[c] TOR: target of rapamycin.

aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to the method described by Reitman and Frankel (1957). Enzyme activities are expressed as specific activity. Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA, A-2153, Sigma) as a standard.

2.5. RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA from larvae body was extracted using TRIzol Reagent (Invitrogen, USA) and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The RNA was treated with RNA-Free DNase (Takara, Japan) to remove DNA contaminant and reverse transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara, Japan) following the manufacturer's instructions. Real-time RT-PCR was carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany). The primer sequences for reference gene (β-actin), PepT1, CCK, trypsin and TOR gene were designed following the published sequences for large yellow croaker and were listed in Table 4. The amplification was performed in a total volume of 25 μL, containing 1 μL of each primer (10 μM), 1 μL of the diluted first strand cDNA product, 12.5 μL of 2× SYBR® Premix Ex Taq™ II (Takara, Japan) and 9.5 μL of sterilized double-distilled water. The real-time PCR program was as follows: 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s, an annealing step for 10 s, and 72 °C for 20 s. The annealing temperature was different for each primer pair (Table 4). At the end of each PCR reaction, melting curve analysis was performed to confirm that only one PCR product was present in these reactions. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analyzed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$. The primer amplification efficiency of PepT1, CCK, trypsin, TOR and β-actin was 0.830, 1.002, 0.942, 0.849 and 0.831, respectively. The ΔC_T ($C_{T,\text{ target genes}} - C_{T,\text{ reference gene}}$) was determined for each cDNA dilution. Then, a plot of the log cDNA dilution versus ΔC_T was made. The absolute value of the slope was less than 0.1, which was close to zero and indicated that $\Delta\Delta C_T$ calculation for the relative quantification of target genes could be use. To calculate the expression of PepT1, CCK, trypsin and TOR, the comparative CT method ($2^{-\Delta\Delta Ct}$ method) was used as described by Yao et al. (2009).

2.6. Calculations and statistical analysis

The following calculations were performed:

Specific growth rate (SGR, $\%day^{-1}$) = $((\text{Ln}W_f - \text{Ln}W_i)/d) \times 100$

where W_f is the final wet body weight, W_i is the initial wet body weight and d is the experimental period in days.

Survival rate (SG, %) = $N_f/N_i \times 100$

where N_f is the number of fish larvae in each tank at the end of the experiment and N_i is the number of fish larvae in each tank at the beginning of the experiment.

Results are given as mean ± S.E. Data from each treatment were subjected to a one-way analysis of variance (ANOVA) using the software program SPSS 16.0. Tukey's honest significant difference test (Tukey's HSD test) was chosen as a multiple comparison test and the significance level of 5% was used.

3. Results

3.1. Survival and growth

Survival rate was significantly lower in fish fed the diet with non-ultrafiltration of fish hydrolysate than that of fish fed other diets ($P < 0.05$). However, there were no significant differences in survival among fish in PUFH-40 group, RUFH-40 group and the control group ($P > 0.05$, Table 5).

Growth of larvae, expressed as specific growth rate (SGR), was significantly lower in fish fed the diet with retentate after ultra-filtration of fish hydrolysate than that of fish fed other diets ($P < 0.05$). However, there were no significant differences in SGR among fish in PUFH-40 group, NUFH-40 group and the control group ($P > 0.05$, Table 5).

3.2. Body composition

There were no significant differences in whole-body protein, lipid and moisture content among dietary treatments ($P > 0.05$, Table 6).

3.3. Specific activities of digestive and AA catabolism enzymes

Specific activity of trypsin (in intestinal segment) in fish fed the diet with permeate after ultra-filtration of fish hydrolysate was significantly higher than that of fish fed other diets ($P < 0.05$). Both the specific

Table 5
Effects of different size-fractionated fish hydrolysates on growth and survival of large yellow croaker larvae (45 DAH, mean ± S.E., n = 3)[a].

Index	Control	PUFH-40	NUFH-40	RUFH-40	*P*-value
Initial number	5000	5000	5000	5000	
Survival number	878 ± 13[a]	861 ± 8[a]	677 ± 33[b]	794 ± 77[ab]	
Survival rate (%)	17.6 ± 0.27[a]	17.21 ± 0.16[a]	13.55 ± 0.66[b]	15.89 ± 1.55[ab]	<0.01
Initial weight (mg)	3.15 ± 0.15	3.15 ± 0.15	3.15 ± 0.15	3.15 ± 0.15	
Final weight (mg)	90.2 ± 5.75[ab]	88.3 ± 5.05[ab]	81.3 ± 2.67[bc]	66.7 ± 4.13[c]	<0.01
SGR[b] ($\%\ day^{-1}$)	11.2 ± 0.22[a]	11.1 ± 0.19[a]	10.8 ± 0.11[ab]	10.2 ± 0.20[b]	<0.01

[a] Values in the same row with the same superscripts are not significantly different ($P > 0.05$).
[b] SGR: specific growth rate.

Table 6
Effects of dietary size fractionated fish hydrolysates on body composition of large yellow croaker larvae (45 DAH, mean ± S.E., n = 3)[a].

Treatments	Protein (%)	Lipid (%)	Moisture (%)
Control	9.77 ± 0.20	2.08 ± 0.04	84.96 ± 0.97
PUFH-40	10.27 ± 0.03	2.09 ± 0.02	84.31 ± 0.26
NUFH-40	9.92 ± 0.39	2.07 ± 0.03	84.99 ± 0.02
RUFH-40	9.56 ± 0.05	2.05 ± 0.01	85.02 ± 0.63
P-value	$P > 0.05$	$P > 0.05$	$P > 0.05$

[a] Values in the same row with the same superscripts are not significantly different ($P > 0.05$).

activity of trypsin (in pancreatic segment) and the ratio of specific activity of trypsin in intestinal segment and that in pancreatic segment (trypsin (I)/trypsin (P)) were significantly higher in fish fed the diet with fish meal than that of fish fed diets with retentate after ultra-filtration of fish hydrolysate and non-ultrafiltration of fish hydrolysate ($P < 0.05$), but were not significantly different from that of fish in PUFH-40 group ($P > 0.05$). Specific activities of both leucine-aminopeptidase (LA) and alkaline phosphatase (AP) were significantly higher in fish fed the diet with permeate after ultra-filtration of fish hydrolysate compared with that of fish fed diets with retentate after ultra-filtration of fish hydrolysate and non-ultrafiltration of fish hydrolysate ($P < 0.05$), but were not significantly different from that of fish fed the diet with fish meal ($P > 0.05$, Table 7).

Specific activities of alanine aminotransferase (ALT) and aspartate aminotransferases (AST) were significantly higher in fish fed the diet with permeate after ultra-filtration of fish hydrolysate than that of fish in other treatments ($P < 0.05$, Table 7).

3.4. The expression of PepT1 gene

The PepT1 expression level in fish fed the diet with retentate after ultra-filtration of fish hydrolysate was significantly lower than that of fish fed other diets ($P < 0.05$). However, there were no significant differences among fish in PUFH-40 group, NUFH-40 group and the control group ($P > 0.05$). The transcript level of PepT1 was up-regulated by 0.58-fold in the PUFH-40 group compared with the control group. The expression levels of PepT1 transcript were down-regulated by 0.10-fold and 0.43-fold in the NUFH-40 group and RUFH-40 group compared with the control group, respectively (Fig. 1).

3.5. The expression of CCK gene

The results showed that the mRNA expression of CCK gene of fish fed the diet with fish meal was significantly higher than that of fish fed diets with NUFH and RUFH ($P < 0.05$), but was not significantly different from that of fish fed the diet with PUFH ($P > 0.05$). The transcript level of CCK gene in PUFH-40 group was up-regulated by about 0.72-fold than that of fish in the control group. The transcript levels of CCK gene in NUFH-40 group and RUFH-40 group was down-regulated by about 0.60-fold compared to that of fish in the control group (Fig. 2).

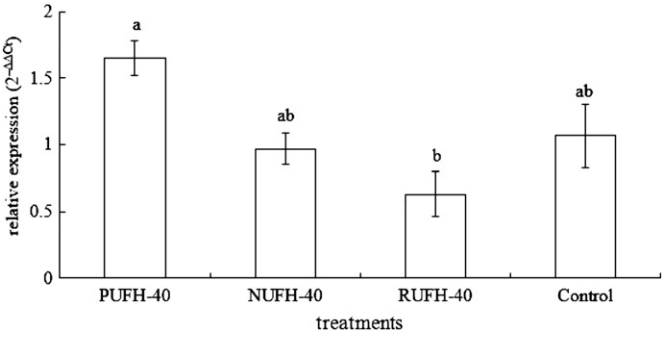

Fig. 1. Effects of dietary fish protein hydrolysates on the relative expression level of PepT1 gene in large yellow croaker (*Larimichthys crocea*) larvae. Values are means ± S.E. (n = 3). Bars of the same gene bearing with same letters are not significantly different by Tukey's test ($P > 0.05$). PepT1: peptide transporter 1.

3.6. The expression of trypsin gene

The results showed that the mRNA expression of trypsin gene of fish fed the diet with permeate after ultra-filtration of fish hydrolysate was not significantly different from that in the control group ($P > 0.05$) and they were both significantly higher than that in RUFH-40 group and NUFH-40 group ($P < 0.05$) (Fig. 3). The transcriptional level of trypsin gene in NUFH-40 group and RUFH-40 group was decreased by approximately 0.80-fold compared to that in the control group.

3.7. The expression of TOR gene

At the end of the experiment, no significant differences were observed in TOR gene expression of larvae among dietary treatments ($P > 0.05$, Fig. 4). Compared to the control group, the mRNA levels of TOR gene were increased by about 0.31-fold, 1.43-fold and 1.65-fold in NUFH-40 group, RUFH-40 group and PUFH-40 group, respectively.

4. Discussion

In the current study, three diets containing different size-fractionated fish hydrolysates only differed in molecular weight distribution of their peptides. Permeate after ultra-filtered fraction of fish hydrolysate used in this experiment was mainly small peptides and free amino acids in which molecular weight was below 1000 Da. In the present study, SGR and survival rate of fish were significantly lower in NUFH-40 group and RUFH-40 group than

Table 7
The effects of different size fractionated fish hydrolysates on specific activities of digestive enzymes and amino acid metabolic enzymes of large yellow croaker larvae (45 DAH, mean ± S.E., n = 3)[a].

Index	Control	PUFH-40	NUFH-40	RUFH-40	*P*-value
PS[b] trypsin (mU mg^{-1}·protein)	98.67 ± 5.7[ab]	112.04 ± 6.12[a]	84.38 ± 4.06[bc]	66.49 ± 7.68[c]	<0.01
IS[b] trypsin (mU mg^{-1}·protein)	82.84 ± 1.23[b]	102.30 ± 3.05[a]	58.51 ± 4.61[c]	40.78 ± 7.41[d]	<0.01
Trypsin (I)/trypsin (P)[c]	0.84 ± 0.01[ab]	0.91 ± 0.03[a]	0.69 ± 0.05[bc]	0.61 ± 0.11[c]	<0.01
Specific activities of digestive enzymes in purified brush border membrane of intestine					
Leucine-aminopeptidase (mU mg^{-1}·protein)	159.73 ± 3.84[a]	192.31 ± 8.35[a]	111.46 ± 9.51[b]	67.03 ± 4.30[c]	<0.01
Alkaline-phosphatase (U mg^{-1}·protein)	453.75 ± 33.65[bc]	642.23 ± 60.54[a]	363.04 ± 14.22[c]	321.91 ± 10.34[c]	<0.01
Specific activities of amino acid metabolism enzymes in whole-body of larvae					
Aspartate aminotransferases (U mg^{-1}·protein)	47.55 ± 1.52[b]	63.85 ± 0.95[a]	34.26 ± 1.26[c]	24.74 ± 1.91[d]	<0.01
Alanine aminotransferase (U mg^{-1}·protein)	62.98 ± 0.86[bc]	69.18 ± 1.34[a]	57.39 ± 1.33[c]	42.19 ± 1.66[d]	<0.01

[a] Values in the same row with the same superscripts are not significantly different ($P > 0.05$).
[b] PS: pancreatic segments; IS: intestinal segments.
[c] Trypsin (I): trypsin of intestinal segment; trypsin (P): trypsin of pancreatic segment.

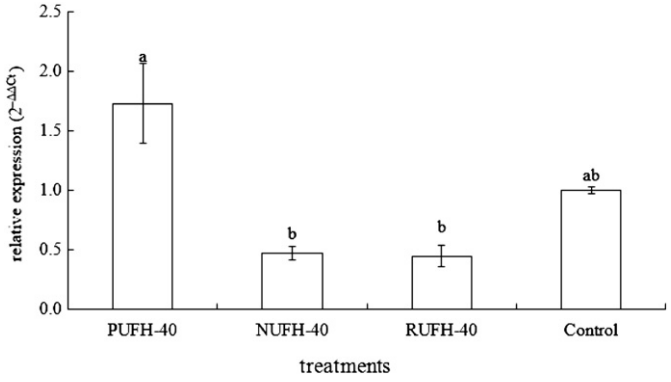

Fig. 2. Effects of dietary fish protein hydrolysates on the relative expression level of CCK gene in large yellow croaker (*Larimichthys crocea*) larvae. Values are means ± S.E. (n = 3). Bars of the same gene bearing with same letters are not significantly different by Tukey's test ($P > 0.05$). CCK: cholecystokinin.

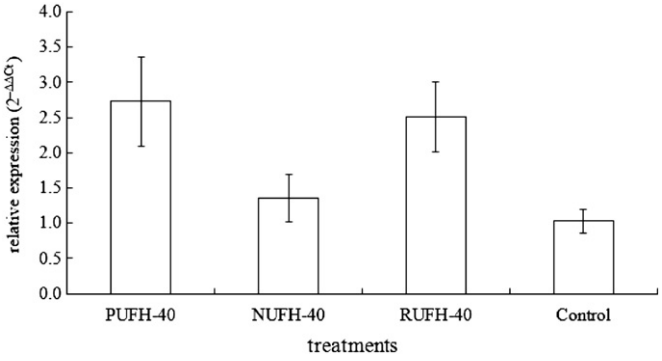

Fig. 4. Effects of dietary fish protein hydrolysates on the relative expression level of TOR gene in large yellow croaker (*Larimichthys crocea*) larvae. Values are means ± S.E. (n = 3). Bars of the same gene bearing with same letters are not significantly different by Tukey's test ($P > 0.05$). TOR: target of rapamycin.

that of fish in the control group, while no significant differences were observed between PUFH-40 group and the control group, which suggests that compared to NUFH and RUFH, only PUFH could replace 40% fish meal without compromising the growth performance of large yellow croaker larvae. PUFH is therefore equivalent to fish meal as protein source for large yellow croaker larvae. This is in agreement with the findings in Japanese flounder (*P. olivaceus*), turbot (*S. maximus* L.), rainbow trout (*O. mykiss*) and Atlantic cod (*G. morhua*) (Aksnes et al., 2006a, b; Zheng et al., 2012, 2013).

The ratio of trypsin (I)/trypsin (P) reflects the secretion level of pancreatic enzymes (Zambonino-Infante et al., 1996). In the present study, this ratio was significantly higher in fish fed the diet with fish meal than that of fish in NUFH-40 group and RUFH-40 group and there were no significant differences between PUFH-40 group and the control group. Indeed, better secretory function of pancreas of fish in the control group than NUFH-40 group and RUFH-40 group could be due to relatively higher expression of CCK gene. Previous study has demonstrated that in fish, as well as in mammals, CCK is critical in the stimulation of pancreatic enzyme secretion and can affect digestion by altering the expression of trypsin (Cahu et al., 2004; Furutani et al., 2013; Murashita et al., 2007; Rojas-García and Rønnestad, 2002; Tillner et al., 2013). In the current study, mRNA levels of CCK and trypsin correlated well with the change of the ratio of trypsin (I)/trypsin (P). Therefore, these results together suggest that CCK gene could promote secretory function of pancreas of fish in treatment with fish meal by inducing the expression of trypsin gene. Furthermore, it was noted that the mRNA expression of CCK and trypsin was no significantly different in fish between PUFH-40 group and the control group, higher than that of fish in NUFH-40 group and RUFH-40 group, thus PUFH might exert beneficial effect on pancreatic secretory function in fish larvae through the mechanism identical to fish meal. Besides the achievement of pancreas secretion function, the onset of brush border membrane (BBM) enzymes is one of two stages crucial in the maturation process of the digestive function in intestine (Ma et al., 2005). LA and AP are regarded as markers for a well-differentiated intestinal BBM and have previously been found to exhibit high activities in fish larvae fed optimal diets (Cahu et al., 1999; Moren et al., 2004; Zambonino-Infante and Cahu, 2001). Compared to NUFH and RUFH, better development of intestinal BBM in fish by dietary inclusion of PUFH could be characterized by higher specific activities of LA and AP. In addition, based on higher activities of protease in fish fed the diet with PUFH, which showed no significant differences with fish meal, it could be concluded that fish hydrolysate rich in small molecular weight compounds replacing 40% fish meal is optimal to protein digestion in large yellow croaker larvae compared to NUFH and RUFH.

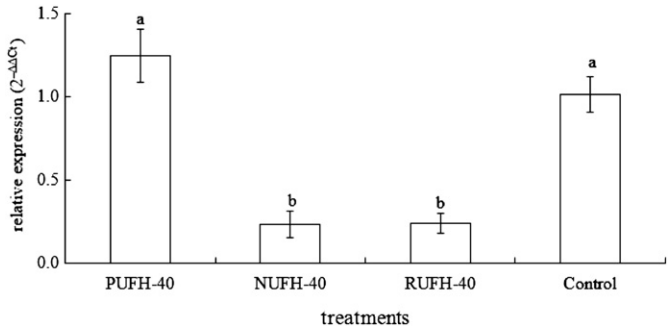

Fig. 3. Effects of dietary fish protein hydrolysates on the relative expression level of trypsin gene in large yellow croaker (*Larimichthys crocea*) larvae. Values are means ± S.E. (n = 3). Bars of the same gene bearing with same letters are not significantly different by Tukey's test ($P > 0.05$).

The ultimate products of protein digestion in intestine are free amino acids and di- and tri-peptides (Ganapathy et al., 2006). In addition, di and tri-peptides are transported into the enterocytes by the peptide transporter PepT1 (Fei et al., 1994). In this study, the expression level of PepT1 was significantly lower in fish fed the diet with RUFH than that of fish fed the diet with fish meal, while no significant differences were observed among PUFH-40 group, NUFH-40 group and the control group. This implies that in fish hydrolysate, some small molecular weight compounds are essential for maintaining normal short peptides absorption in intestine. Since the expression of free amino acid transporters is not included in the present study, it is still unknown whether free amino acid absorption by intestine in large yellow croaker larvae is affected by different diet compositions in the present study. However, it should be admitted that some small molecular weight compounds contained in fish hydrolysate might play a crucial role in amino acid absorption at least from the present study. On the contrary, Bakke et al. (2010) found that the change in diet composition (substitution of intact protein by inclusion of fractions of peptides or FAAs) has played an irrelevant role in the regulation of PepT1 expression in juvenile Atlantic cod. Different fish species, developmental stages and diet formulations could account for the inconsistent results.

ALT and AST are the most important aminotransferases in fish livers (Cowey and Walton, 1989; Fynn-Aikins et al., 1995). Moreover, the activity of AST or ALT is closely related to amino acid metabolism in fish and the transaminase activity enhanced with the increase of amino acid metabolism (Cheng et al., 2010; Deng et al., 2009; Feng et al., 2012; Luo et al., 2012). In this study, the activities of ALT and AST were significantly higher in fish in PUFH-40 group than that of fish in other groups. This suggests that some small molecular weight compounds contained in fish hydrolysate could enhance the amino acid metabolism of large yellow croaker larvae.

TOR signaling pathway is a key regulator of balance between protein synthesis and degradation in response to nutrition quality and quantity and the central in TOR signaling pathway is TOR protein (Wullschleger et al., 2006). In the present study, the mRNA expression of TOR gene in fish was not significantly affected by different diets. Indeed, Li et al. (2013) and Luo et al. (2012) have found that nutritional status had no effect on transcription level of TOR gene in fish. This is probably because the regulation of TOR gene expression is controlled by the modulation of translation and/or post translation level rather than transcription level. However, no direct evidence was obtained in the present study and investigations are needed to verify the hypothesis in further studies. Furthermore, previous studies have demonstrated that diet composition could influence TOR signaling pathway via the target genes of TOR protein, such as eukaryotic translation initiation factor 4E binding protein (4E-BP) (Chen et al., 2012; Tang et al., 2013; Zhao et al., 2012). Thus, whether different diet compositions in the present study could affect the expression of target genes of TOR protein is needed to be investigated in future studies.

In conclusion, the present study mainly provides evidence of the significance of the molecular weight distribution of peptides in fish hydrolysate for large yellow croaker larvae. In the present study, when replacing 40% fish meal, PUFH which contains mainly small molecular weight compounds seems optimal for large yellow croaker larvae compared to NUFH and RUFH. Furthermore, we have also demonstrated that size-fractionated fish hydrolysates could significantly affect digestion and absorption of protein in large yellow croaker larvae by modulating the mRNA expression levels of CCK, PepT1 and trypsin.

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