The Potential Role of Marine Protein Hydrolyzates in Elevating Nutritive Values of Diets for Largemouth Bass, *Micropterus salmoides*

Min Dai¹, Songlin Li¹,²,³*, Cefeng Fu¹, Hongjie Qiu¹ and Naisong Chen¹,³*

¹ Research Centre of the Agriculture Ministry on Environmental Ecology and Fish Nutrition, Shanghai Ocean University, Shanghai, China, ² Guangxi Key Laboratory of Marine Natural Products and Combinatorial Biosynthesis Chemistry, Guangxi Beibu Gulf Marine Research Center, Guangxi Academy of Sciences, Nanning, China, ³ National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Lingang New City, China

The study was conducted to explore the improvement function of marine protein hydrolyzates on nutritive values of diets for largemouth bass. The diet with no inclusion of marine protein hydrolyzate was considered as the control (NC), and four other diets were formulated with fish soluble (FS), squid paste (SqP), shrimp paste (ShP), or a mixture of FS, SqP, and ShP (Mix). Triplicate tanks of fish were fed to apparent satiation twice daily for 81 days. Results showed that the growth performance was elevated due to the inclusion of marine protein hydrolyzates along with significantly elevated feed intake and protein digestibility. Thereinto, the inclusion of FS and ShP significantly improved the growth performance compared to NC. The supplementation of marine protein hydrolyzates elevated the protein content and lysozyme activity of serum, but significantly decreased the activity of liver alanine transaminase and aspartate transaminase. The gene expression analysis revealed that marine protein hydrolyzate inclusion up-regulated the expression of insulin-like growth factor I (IGF-I) with increased expression of TOR pathway-related genes including protein kinase B (AKT) 1 and ribosomal protein S6. However, the expression of activating transcription factor 4 (ATF4) and regulated in development and DNA damage responses 1 (REDD1) involved in the amino acids response (AAR) pathway was depressed with the addition of marine protein hydrolyzates. The activation of the TOR pathway and depression of the AAR pathway may be beneficial for the improved performance of fish. In the above, the marine protein hydrolyzate, especially FS and ShP, can elevate nutritive values of diets for largemouth bass.

Keywords: marine protein hydrolyzates, growth performance, gene expression, nutrient-sensing pathway, largemouth bass

INTRODUCTION

Fish meal has been recognized as an indispensable protein source in the feed for carnivorous fish with high protein requirement because of its abundant protein content, excellent amino acid profile, and high palatability (Tacon and Metian, 2008). However, the reduced resource and rising price of fish meal seriously limit its use (FAO, 2016), and therefore, alternative protein sources have been
widely explored to reduce the use of fish meal in aquafeed (Gatlin et al., 2007; Lim and Webster, 2008).

In general, high inclusion of plant protein in aquafeed commonly produces negative effects on digestive physiology and further reduces the growth performance of farmed fish, mainly due to their shortcomings in enriched anti-nutritional factors, unbalanced amino acid composition, and poor palatability (Burel et al., 2000; Barros et al., 2002; Colburn et al., 2012; Song et al., 2014). The marine by-products, such as the by-products of shrimp, squid, scallop, and fish, could be effectively converted to biologically functional products via enzymatic hydrolysis (Shahidi, 1994; Gildberg and Stenberg, 2001). The study in red sea bream (Pagrus major) has demonstrated the improving nutritive values of low fish meal diet with the inclusion of small amounts (10%) of some crude ingredients including fish solubles, krill meal, and squid meal (Kader et al., 2010). In addition, the mixture of seafood by-products and soybean proteins realizes the complete substitution of fish meal in the diet for red sea bream (Kader et al., 2012). Accordingly, marine protein hydrolyzates, containing balanced amino acids and high trace elements including taurine and free amino acids similar to fish meal, have been confirmed to improve the growth performance of farmed fish fed with high plant protein diets (Cahu et al., 2004; Kader et al., 2010, 2012; Kader and Koshio, 2012).

The nutritional status effectively affects the growth hormone (GH)/insulin-like growth factor (IGF) system to regulate the development and growth of vertebrates (Thissen et al., 1994; Duan, 1998; Reinecke et al., 2005). In general, high replacement of fish meal commonly produces negative effects on growth performance of fish species with depressed expression of IGF-I (Gomez-Requeni et al., 2004; Luo et al., 2012; Men et al., 2014; Wang et al., 2017). The mechanistic target of rapamycin (TOR) pathway and amino acids response (AAR) pathway act as the main cellular nutrient-sensing pathway to regulate protein synthesis and downstream metabolism (Kimball and Jefferson, 2002). The activation of the TOR pathway promotes protein synthesis and inhibits autophagy to regulate organismal growth (Laplante and Sabatini, 2012), while the AAR pathway could be activated by amino acid deficiency to repress global protein synthesis (Kilberg et al., 2005). In the study of teleost, the replacement of fish meal with soybean meal or meat and bone meal resulted in the down-regulation of the TOR pathway and activation of the AAR pathway, and this accounted for the reduced growth performance of turbot, Scophthalmus maximus (Song et al., 2016; Xu et al., 2016). Available literatures have reported that the marine protein hydrolyzates act as attractants in high plant protein-based diets for maintaining normal feeding behavior and growth performance (Hidaka et al., 2000; Toften et al., 2003; Kofuji et al., 2006; Kousoulaki et al., 2009; Kader et al., 2010, 2012), while it is unclear whether the improved growth performance is mediated by the TOR pathway and AAR pathways.

Largemouth bass (Micropterus salmoides) has been widely cultured in China and its production is approximately 432,000 tons in 2018 in China (Yearbook, 2019). This carnivorous fish possesses high dietary protein requirement (48%) with fish meal as major protein source (Huang et al., 2017). A previous study in our lab has demonstrated that soybean meal could reduce the use of fish meal in the diet for largemouth bass to 35% without negative effect on growth performance (Chen et al., 2013). The present study was conducted to evaluate the effect of marine protein hydrolyzate inclusion, fish solubles (FS), squid paste (SqP), and shrimp paste (ShP), on the growth performance and feed utilization of largemouth bass fed the diet with relative low fishmeal. In addition, the response of IGF-1 expression, TOR pathway, and AAR pathway was investigated at the transcription level in the present study.

MATERIALS AND METHODS

Experimental Diets and Experimental Procedure

Five isonitrogenous (crude protein 48%) and isolipidic (12%) diets were formulated, where diet was with no inclusion of marine protein hydrolysate (NC) or with 10% FS, SqP, ShP, or a mixture of the above three protein hydrolyzate each at the 3.33% (Mix) (Table 1). The contents of amino acids and free amino acids in experimental diets were presented in Tables 2, respectively. The marine protein hydrolyzate, FS, SqP, and ShP, used in the present study was supplied by Zhejiang Yifeng Marine Biological Products Co., Ltd (Zhejiang, China). The addition of marine protein hydrolyzate was conducted at the expense of fermented soybean meal and corn gluten meal to maintain consistent crude protein content. Fish oil content was changed to adjust the lipid content caused by the addition of marine by-product, and soybean oil was used to maintain the consistent of crude lipid content. CaSO₄ was added as an indicator to evaluate the apparent digestibility coefficients (ADCs) of nutrients. The formulated process of experimental diets was conducted following the description of Li et al. (2019). Briefly, the low-lipid ingredients, after being grounded through 75-µm mesh, were blended thoroughly, followed by the mixture with lipid ingredients. Then, water was added to make a stiff dough, which was then extruded by a pelleting machine. The extruded pellets were stored at −20°C after being cooked for starch gelatinization and dried in a ventilated oven until used.

The animal study was reviewed and approved by the Animal Care and Use Committee of Shanghai Ocean University. The experimental largemouth bass were obtained from a commercial hatchery (Suzhou, China) and then reared in an indoor temperature-controlled recirculating freshwater system (Shanghai, China) for 4 weeks’ domestication with commercial feed. After acclimatization, the fish with uniform size (initial body weight, 10.68 ± 0.01 g), after being fasted for 24 h, were selected and allocated into 15 glass reinforced plastic tanks with a body weight, 10.68 ± 0.01 g, after being fasted for 24 h, were selected and allocated into 15 glass reinforced plastic tanks with a 3.33% (Mix) (Table 1). The contents of amino acids and free amino acids in experimental diets were presented in Tables 2, respectively. The marine protein hydrolyzate, FS, SqP, and ShP, used in the present study was supplied by Zhejiang Yifeng Marine Biological Products Co., Ltd (Zhejiang, China). The addition of marine protein hydrolyzate was conducted at the expense of fermented soybean meal and corn gluten meal to maintain consistent crude protein content. Fish oil content was changed to adjust the lipid content caused by the addition of marine by-product, and soybean oil was used to maintain the consistent of crude lipid content. CaSO₄ was added as an indicator to evaluate the apparent digestibility coefficients (ADCs) of nutrients. The formulated process of experimental diets was conducted following the description of Li et al. (2019). Briefly, the low-lipid ingredients, after being grounded through 75-µm mesh, were blended thoroughly, followed by the mixture with lipid ingredients. Then, water was added to make a stiff dough, which was then extruded by a pelleting machine. The extruded pellets were stored at −20°C after being cooked for starch gelatinization and dried in a ventilated oven until used.

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TABLE 1 | Ingredients composition and proximate analysis of the experimental diet (% dry matter).

| Ingredients                  | Marine protein hydrolyzates |
|------------------------------|-----------------------------|
|                             | NC  | FS  | SqP | ShP | Mix |
| Fish meala                  | 35  | 35  | 35  | 35  | 35  |
| Fermented soybean mealb     | 12.5| 7   | 8.5 | 6.5 | 7   |
| Corn gluten mealc           | 17.5| 11  | 13.5| 11.5| 12.5|
| Blood meal                  | 5   | 5   | 5   | 5   | 5   |
| Wheat gluten meal           | 2   | 2   | 2   | 2   | 2   |
| Beer yeast                  | 2   | 2   | 2   | 2   | 2   |
| α-Starch                    | 5   | 5   | 5   | 5   | 5   |
| Soybean oil                 | 1.8 | 2.4 | 2.2 | 2.4 | 0.8 |
| Fish oil                    | 3.6 | 2.6 | 0   | 3   | 3.4 |
| Lecithin oil                | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Vitamin mixtured            | 1   | 1   | 1   | 1   | 1   |
| Mineral mixture              | 1   | 1   | 1   | 1   | 1   |
| Ca(H₂PO₄)₂                  | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Cr₂O₃                       | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Carboxymethyl cellulose     | 7.8 | 10.2| 9   | 9.8 | 9.6 |
| Zeolite powder              | 0   | 0   | 0   | 0   | 0   |
| Fish solubilde              | 0   | 0   | 0   | 0   | 0   |
| Squid paste                 | 0   | 0   | 0   | 0   | 0   |
| Shrimp paste                 | 0   | 0   | 0   | 0   | 0   |
| Phytase                     | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |

Proximate composition (% dry matter basis)

- Crude protein: 49.74 49.71 49.66 49.71 49.74
- Crude lipid: 12.21 12.11 12.19 12.48 12.29
- Ash: 19.36 20.77 19.53 20.96 20.51
- Gross energy (kJ g⁻¹): 18.82 18.44 18.58 18.79 18.62

a Supplied by Zhejiang Xinxin Tianen Aquatic Feed Corporation (Jiaxing, China): white fish meal, crude protein, 67.44%, crude lipid, 8.83%; wheat gluten meal, crude protein, 76.56%; fermented soybean meal, crude protein, 51.62%, crude lipid, 0.54%; corn gluten meal, crude protein, 63.01%, crude lipid, 7.57%; blood meal, crude protein, 78.12%, crude lipid, 1.53%; brewer’s yeast meal, crude protein, 31.22%, crude lipid, 0.40%.
b Supplied by Zhejiang Yifeng Marine Biological Products Co., Ltd (Zhejiang, China): fish soluble, crude protein, 31.43%, crude lipid, 4.51%; squid paste, crude protein, 29.54%, crude lipid, 2.43%.
c Supplied by Zhejiang Huarun Marine Biological Products Co., Ltd (Zhoushan, Zhejiang, China): beer yeast, crude protein, 78.12%, crude lipid, 1.53%; brewer’s yeast meal, crude protein, 76.56%; fermented soybean meal, crude protein, 51.62%, crude lipid, 0.54%.
d Supplied by Zhejiang Nanyang Marine Biological Products Co., Ltd (Zhoushan, Zhejiang, China): fish hydrolysate, crude protein, 31.22%, crude lipid, 4.51%; squid paste, crude protein, 29.54%, crude lipid, 2.43%.

Sample Collection

Fifteen juveniles were randomly collected prior to the feeding trial for the analysis of initial body composition. Feces samples were collected with the collection column fixed to the bottom of experimental tanks following the description of Lee (2002) after 4 weeks’ feeding. When the feeding trial was finished, fish number per tank was counted and final body weight was recorded after fish being fasted for 24 h. Then, five fish per tank were randomly picked for whole body composition analysis, and another 12 fish of each tank were randomly selected for the measurement of body length and weight to calculate condition factor (CF) followed by the collection of blood samples with syringes.

TABLE 2 | Amino acid content of the diets (g 100 g⁻¹ dry matter)*

| Amino acids                  | NC  | FS  | SqP | ShP | Mix |
|------------------------------|-----|-----|-----|-----|-----|
| Threonine                    | 2.11| 2.21| 2.13| 2.19| 2.16|
| Valine                       | 2.56| 2.63| 2.56| 2.66| 2.59|
| Methionine                   | 1.16| 1.25| 1.17| 1.17| 1.16|
| Isoleucine                   | 1.81| 1.88| 1.99| 1.90| 1.77|
| Leucine                      | 4.60| 4.66| 4.70| 4.40| 4.45|
| Phenylalanine                | 2.18| 2.23| 2.26| 2.19| 2.14|
| Histidine                    | 1.73| 1.73| 1.73| 1.71| 1.74|
| Lysine                       | 3.09| 3.46| 3.12| 3.30| 3.26|
| Arginine                     | 2.50| 2.50| 2.76| 2.45| 2.49|
| Total EAA                     | 21.72| 22.26| 22.42| 21.97| 21.75|

Non-essential amino acid (NEAA)

- Proline: 3.09 3.04 3.3 3.17 3.04
- Aspartic acid: 4.53 4.72 4.66 4.55 4.69
- Glutamic acid: 8.35 8.33 8.39 8.08 8.53
- Serine: 2.52 2.45 2.60 2.43 2.54
- Glycine: 2.32 2.60 2.28 2.58 2.59
- Alanine: 3.14 3.22 3.02 3.12 3.20
- Cystine: 0.51 0.49 0.49 0.45 0.49
- Tyrosine: 1.48 1.46 1.56 1.42 1.45
- Total NEAA: 25.94 26.31 26.29 25.79 26.54
- Total AA: 47.66 48.57 48.71 47.76 48.29

*Tryptophan was not determined in the present study.

TABLE 3 | Free amino acid content of the diets (g 100 g⁻¹ dry matter)*

| Amino acids                  | NC  | FS  | SqP | ShP | Mix |
|------------------------------|-----|-----|-----|-----|-----|
| Threonine                    | 0.03| 0.12| 0.06| 0.12| 0.10|
| Valine                       | 0.04| 0.17| 0.08| 0.15| 0.13|
| Methionine                   | 0.01| 0.09| 0.02| 0.07| 0.06|
| Isoleucine                   | 0.03| 0.16| 0.06| 0.14| 0.12|
| Leucine                      | 0.07| 0.31| 0.13| 0.24| 0.23|
| Phenylalanine                | 0.05| 0.21| 0.08| 0.16| 0.14|
| Histidine                    | 0.16| 0.15| 0.16| 0.15| 0.15|
| Lysine                       | 0.06| 0.18| 0.1  | 0.14| 0.15|
| Arginine                     | 0.05| 0.05| 0.08| 0.05| 0.06|
| Total EAA                     | 0.50| 1.44| 0.76| 1.22| 1.13|

Non-essential amino acid (NEAA)

- Proline: 0.24 0.42 0.33 0.45 0.43
- Aspartic acid: 0.04 0.19 0.07 0.15 0.13
- Glutamic acid: 0.07 0.31 0.11 0.18 0.19
- Serine: 0.03 0.03 0.06 0.07 0.06
- Glycine: 0.03 0.17 0.05 0.19 0.14
- Alanine: 0.12 0.22 0.15 0.21 0.22
- Cystine: 0.01 0.02 0.01 0.01 0.01
- Tyrosine: 0.04 0.16 0.05 0.13 0.11
- Total NEAA: 0.65 1.66 0.97 1.63 1.45
- Total AA: 1.15 3.10 1.73 2.85 2.58

* Tryptophan was not determined in the present study.
blood samples were then centrifuged at 4000 × g for 10 min (4°C) to separate the serum to evaluate the non-specific immune response. After that, liver samples of six fish were sampled for gene expression analysis and enzymatic activity analysis. Another six fish were sampled to dissect and weight the viscera and liver to calculate viscerosomatic index (VSI) and hepatosomatic index (HSI), respectively, and the liver and muscle samples were obtained for proximate composition analysis.

**Chemical Analysis**

The moisture, ash, and crude protein content were detected following the method described by AOAC (2003). Briefly, moisture was measured by the weight loss method through drying samples to constant weight at 105°C, and ash was measured by the burning method with muffle furnace (AOAC, 2003). Crude protein was determined by the Kjeldahl method (N × 6.25) (Kjeltec 2200, FOSS, Denmark) (AOAC, 2003). Crude lipid was measured with the chloroform–methanol extraction method ( Folch et al., 1957). Oxygen bomb calorimeter (6200, Parr, United States) was used to determine the gross energy of diets. The CₚO₅ content of diet and feces was analyzed with the wet-ash digestion method based on the description of Divakaran et al. (2002). Amino acid content was determined by an automatic amino acid analyzer (S-433D, Sykam, Germany) following the method of Llames and Fontaine (1994), and free amino acid content was analyzed using the o-phthaldialdehyde precolumn derivatization method (Antoine et al., 1999).

**Non-specific Immune Response and Transaminase Activities**

The activity of serum lysozyme was evaluated following the method of Ellis (1990), and serum protein content was determined with the Coomassie Brilliant Blue method (Bradford, 1976). The obtained liver simples were homogenized in ice-cold phosphate buffer (1:9, w/v) and then centrifuged (3000 rpm, 10 min, 4°C) to separate the supernatant. Then, the isolated supernatant was maintained at −20°C until it is used for the measurement of transaminase activities. The activity of alanine transaminase (ALT) and aspartate transaminase (AST) was measured with the Reitman–Frankel method (Reitman and Frankel, 1957).

**RNA Extraction and Real-Time Quantitative PCR**

The total RNA of liver samples was extracted using Trizol Reagent (Takara, Japan) followed by quality measurement with denaturing agarose gel. Then, first-strand cDNA was synthesized through reverse transcription reaction with Prime Script™ RT kit (Takara, Japan). Specific primers used in the present study were designed based on the obtained nucleotide sequences (Table 4). The β-actin was used as housekeeping gene following the verification of its stability, and the amplification was conducted in a quantitative thermal cycler (Mastercycler EP Realplex, Eppendorf, Germany) with the following procedure: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 57°C for 10 s, and 72°C for 20 s. Melting curve analysis was conducted, after the reaction, to confirm the single PCR product in the reactions. The relative gene expressions were calculated with the 2−ΔΔCt method (Livak and Schmittgen, 2001).

| Gene            | Forward sequence (5’-3’) | Reverse sequence (5’-3’) |
|-----------------|-------------------------|-------------------------|
| IGF-I           | CTTCAAGAGGTGGATGTGC     | GCCATAGCGTGGTTTACTG     |
| TOR             | TCAGGACCTTTCTCCATGGG    | CTCCTCCACCAGTTTCTCTT    |
| AKT1            | CACCATGAGAACGGAGCCCCTGT | GGCCATGAAAGATCCTAAGAGA |  S6    | CCGATCAGCCCTTCAAC   | CGCTGCTAACATCATCCTCCTT |
| IGF-I           | CTTCAAGAGTGCGATGTGC     | GCCATAGCGTGGTTTACTG     |
| S6              | CCGATCAGCCCTTCAAC       | CGCTGCTAACATCATCCTCCTT |
| elf2a           | TAAGTCGAGCCATCCAAAG     | ACCGGGAGGAGGCCATCAAG    |
| ATF4            | GGAGACCAAGAAAGATGCGTAG  | TGTCGAGCAAGATGCGTACA    |
| REDD1           | TGACCTGTCGCCCTCTTAAGTA  | ATGGCTGTCAGAAGTCTTCTGA  |
| β-Actin         | ATGGGCGGACGCTTGTGTGGAC  | CCTGTTGCGTTGGGGTTC      |
| IGF-I, insulin-like growth factor I; TOR, target of rapamycin; AKT, protein kinase B; S6, ribosomal protein S6; elf2a, elongation initiation factor 2a; ATF4, activating transcription factor 4; REDD1, regulated in development and DNA damage responses 1. |

**Calculations and Statistical Methods**

The following variables were calculated:

- **Survival rate (SR,%):** final fish number/initial fish number × 100.
- **Specific growth rate (SGR,%/day):** (Ln final body weight − Ln initial body weight) × 100/days.
- **Condition factor (CF):** final body weight (g)/length (cm)³ × 100.
- **Hepatosomatic index (HSI, %):** liver weight/final body weight × 100.
- **Viscerosomatic index (VSI, %):** viscera weight/final body weight × 100.
- **Feed efficiency ratio (FER):** Increased body weight/dry feed consumed.
- **Protein efficiency ratio (PER):** Increased body weight/protein intake.
- **Feed intake (g/day):** (Feed offered − feed discarded or uneaten feed)/days.
- **Nutrient retention rate (NRR, %):** Nutrient retention/nutrient intake × 100.
- **Apparent digestibility coefficient (ADC) of nutrient (%):** 100 − 100 × (tracer in diet/tracer in feces) × (nutrient in feces/nutrient in diet).

The results were expressed as the mean ± standard error of the mean (SEM). All data were compared with one-way analysis of variance (ANOVA) using SPSS. Turkey’s multiple range test was chosen as multiple comparison test, and P < 0.05 was considered significantly.

**RESULTS**

**Growth Performance and Feed Utilization**

No significant difference was observed in survival rate (SR) with the inclusion of marine protein hydrolyzates (P > 0.05) (Table 5).
Table 5 | Growth performance and feed utilization of largemouth bass fed the experimental diets for 81 days*

| Parameters          | Marine protein hydrolyzates |
|---------------------|-----------------------------|
|                     | NC  | FS  | SqP | ShP | Mix |
| IBW (g)             | 10.68 ± 0.01 | 10.68 ± 0.01 | 10.68 ± 0.01 | 10.68 ± 0.01 | 10.68 ± 0.01 |
| FBW (g)             | 100.52 ± 2.41 | 107.84 ± 0.29 | 106.24 ± 0.87 | 110.06 ± 0.31 | 104.3 ± 0.62 |
| SR (%)              | 96.67 ± 2.20 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| SGR (%/day)         | 2.80 ± 0.02 | 2.89 ± 0.00 | 2.87 ± 0.01 | 2.92 ± 0.00 | 2.85 ± 0.01 |
| CF                  | 2.11 ± 0.02 | 2.13 ± 0.02 | 2.16 ± 0.03 | 2.08 ± 0.02 | 2.07 ± 0.01 |
| HSI (%)             | 2.49 ± 0.20 | 2.63 ± 0.28 | 2.37 ± 0.05 | 2.72 ± 0.14 | 2.79 ± 0.01 |
| VSI (%)             | 8.96 ± 0.52 | 8.10 ± 0.18 | 8.43 ± 0.12 | 8.78 ± 0.40 | 8.84 ± 0.41 |
| FI (g/fish/day)     | 0.98 ± 0.01 | 1.14 ± 0.01 | 1.07 ± 0.00 | 1.14 ± 0.01 | 1.05 ± 0.01 |
| FER                 | 1.14 ± 0.01 | 1.07 ± 0.01 | 1.11 ± 0.01 | 1.08 ± 0.01 | 1.11 ± 0.01 |
| PER                 | 2.15 ± 0.06 | 2.04 ± 0.01 | 2.14 ± 0.02 | 2.07 ± 0.02 | 2.12 ± 0.02 |
| PRR (%)             | 38.73 ± 1.08 | 38.73 ± 0.20 | 39.14 ± 0.44 | 38.45 ± 0.45 | 39.63 ± 0.21 |
| LRR (%)             | 67.75 ± 2.26 | 70.24 ± 2.08 | 66.95 ± 1.92 | 67.52 ± 0.87 | 70.70 ± 0.34 |
| ADC of protein (%)  | 19.40 ± 0.18 | 93.62 ± 0.20 | 91.34 ± 0.24 | 92.57 ± 0.19 | 92.67 ± 0.25 |
| ADC of lipid (%)    | 92.57 ± 0.16 | 94.32 ± 0.18 | 93.54 ± 0.18 | 93.79 ± 0.16 | 91.78 ± 0.28 |

*Values (mean ± SEM, N = 3) within a row with a common superscript letter are not significantly different from the other dietary groups (P > 0.05). IBW, initial body weight; FBW, final body weight; SR, survival rate; SGR, specific growth rate; CF, condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index; FI, feed intake; FER, feed efficiency ratio; PER, protein efficiency ratio; PRR, protein retention rate; LRR, lipid retention rate; ADC, apparent digestibility coefficient.

Meanwhile, the addition of marine protein hydrolyzates elevated growth of largemouth bass, and the final body weight (FBW) and specific growth rate (SGR) in fish fed the diets with FS and ShP were significantly higher than that of NC (P < 0.05) (Table 5). However, marine protein hydrolyzates produced no significant influence on morphological index including CF, HSI, and VSI (P > 0.05) (Table 5). The inclusion of marine protein hydrolyzates significantly elevated the feed intake (FI) (P < 0.05), and meanwhile, the value in fish fed the diet with FS and ShP were remarkably higher than that of SqP and Mix (P < 0.05) (Table 5). However, the feed efficiency ratio (FER) was significantly reduced due to the inclusion of FS and ShP compared to NS (P < 0.05) (Table 5). The marine protein hydrolyzates did not cause any significant difference on protein efficiency ratio (PER) and protein retention rate (PRR), which was consistent with the variation of lipid retention rate (LRR) (P > 0.05) (Table 5). However, the ADC of protein in fish fed the diet with FS, ShP, and Mix was significantly higher than that of NC with the highest value in FS group (P < 0.05), while the inclusion of SqP did not lead to any significant difference on ADC of protein (P > 0.05) (Table 5). The fish fed the diet with FS, SqP, and ShP obtained significantly higher ADC of lipid than the other two groups (P < 0.05) (Table 5).

### Body Composition Analysis

The inclusion of protein hydrolyzates caused no significant difference on the proximate composition except the ash content (P > 0.05) (Table 6). The ash content in fish fed the diet with FS, ShP, and Mix was significantly higher than that of NC (P < 0.05) (Table 6). However, the inclusion of marine protein hydrolyzates significantly decreased the hepatic lipid content without any significant difference on the content of moisture, protein, and ash in liver (P < 0.05) (Table 6). The moisture, protein, and lipid content of muscle in fish fed the diet with marine protein hydrolyzates showed no significant difference than that of NC (P > 0.05) (Table 6). However, the ash content in muscle of fish fed the diet with Mix was significantly higher than that of ShP (P < 0.05) (Table 6).

### Non-specific Immune Response and Transaminase Activities

The activity of serum lysozyme was elevated with the inclusion of marine protein hydrolyzates, and fish fed the diet with FS and Mix significantly improved its activity compared to that of NS (P < 0.05) (Table 7). Meanwhile, the marine protein hydrolyzates increased the serum protein content, and the inclusion of Mix resulted in a significant serum protein content compared to that of NC (P < 0.05) (Table 7). The activity of hepatic ALT was significantly decreased with the inclusion of marine protein hydrolyzates (P < 0.05) (Table 7), and the variation of AST activity followed a similar pattern with that of ALT (P < 0.05) (Table 7).

### Gene Expression Analysis

The inclusion of marine protein hydrolyzates elevated the expression of IGF-I, and its expression in fish fed the diet with FS was significantly higher than that of NS (P < 0.05) (Figure 1). No significant difference was observed in the expression of TOR with the inclusion of marine protein hydrolyzates (P < 0.05) (Figure 2). However, the supplementation of marine protein hydrolyzates significantly up-regulated the expression of protein kinase B (AKT) 1 (P < 0.05), and meanwhile, the expression of AKT1 in the FS group was significantly higher than that of SqP and Mix (P < 0.05) (Figure 2). The marine protein hydrolyzates including FS, SqP, and ShP elevated the expression of ribosomal protein S6 (S6), and the inclusion of FS significantly elevated its expression compared to that of NC (P < 0.05) (Figure 2).
### TABLE 6 | Whole-body, liver proximate composition, and muscle proximate composition of largemouth bass fed the experimental diets for 81 days.

| Marine protein hydrolyzates | NC | FS | SqP | ShP | Mix |
|------------------------------|----|----|-----|-----|-----|
| **Whole body**              |    |    |     |     |     |
| Moisture (%)                | 69.30 ± 0.32 | 69.39 ± 0.33 | 69.59 ± 0.61 | 68.97 ± 0.42 | 68.39 ± 0.31 |
| Crude protein (%)           | 18.01 ± 0.16 | 18.02 ± 0.20 | 18.30 ± 0.38 | 18.55 ± 0.37 | 18.72 ± 0.25 |
| Crude lipid (%)             | 7.73 ± 0.01  | 8.16 ± 0.18  | 7.67 ± 0.15  | 8.17 ± 0.13  | 8.25 ± 0.12  |
| Ash (%)                     | 3.87 ± 0.05c | 4.58 ± 0.17abc | 4.21 ± 0.06bc | 4.79 ± 0.05a | 4.56 ± 0.04abc |
| **Liver**                   |    |    |     |     |     |
| Moisture (%)                | 68.35 ± 0.32 | 68.85 ± 0.36 | 68.19 ± 0.27 | 68.23 ± 0.43 | 67.51 ± 0.41 |
| Crude protein (%)           | 9.58 ± 0.16  | 9.26 ± 0.11  | 9.28 ± 0.06  | 9.59 ± 0.07  | 9.76 ± 0.27  |
| Crude lipid (%)             | 3.32 ± 0.09b | 2.57 ± 0.13abc | 2.77 ± 0.07b | 2.1 ± 0.14abc | 1.97 ± 0.1f  |
| Ash (%)                     | 0.94 ± 0.08  | 1.04 ± 0.03  | 1.04 ± 0.01  | 1.00 ± 0.03  | 1.00 ± 0.04  |
| **Muscle**                  |    |    |     |     |     |
| Moisture (%)                | 76.50 ± 0.27 | 76.47 ± 0.19 | 76.82 ± 0.23 | 77.04 ± 0.13 | 77.21 ± 0.03 |
| Crude protein (%)           | 21.22 ± 0.11 | 21.19 ± 0.18 | 20.71 ± 0.21 | 20.51 ± 0.08 | 20.76 ± 0.04 |
| Lipid (%)                   | 1.53 ± 0.07  | 1.49 ± 0.03  | 1.47 ± 0.04  | 1.43 ± 0.03  | 1.55 ± 0.06  |
| Ash (%)                     | 1.63 ± 0.06abc | 1.51 ± 0.03abc | 1.72 ± 0.07abc | 1.51 ± 0.07bc | 1.73 ± 0.02a |

Values (means ± SEM, N = 3) within a row with a common superscript letter are not significantly different from the other dietary groups (P > 0.05).

### TABLE 7 | Non-specific immune response and hepatic transaminase activities of largemouth bass fed the experimental diets for 81 days.

| Marine protein hydrolyzates | NC | FS | SqP | ShP | Mix |
|-----------------------------|----|----|-----|-----|-----|
| Lysozyme activity (U/µl)    | 4.69 ± 0.37b | 7.57 ± 0.70a | 7.00 ± 0.35ab | 7.07 ± 0.76ab | 7.40 ± 0.46ab |
| Serum protein content (mg/ml) | 40.71 ± 0.46b | 41.67 ± 0.63abc | 41.35 ± 0.66ab | 41.73 ± 0.23abc | 43.83 ± 0.73abc |
| ALT (U/gprot)               | 15.92 ± 0.32a | 10.27 ± 0.56b | 10.16 ± 0.35b | 11.32 ± 0.08b | 11.7 ± 0.45b |
| AST (U/gprot)               | 27.06 ± 0.91a | 18.47 ± 0.44c | 21.94 ± 0.41b | 22.57 ± 0.56b | 22.71 ± 0.23b |

Values (means ± SEM, N = 3) within a row with a common superscript letter are not significantly different from the other dietary groups (P > 0.05). ALT, alanine transaminase; AST, aspartate transaminase.

### DISCUSSION

The replacement of fish meal with alternative protein sources is essential for sustainable aquaculture due to the limited fish meal resources. The fish meal content could be reduced to 35% with plant protein source in the diet for largemouth bass without negative effects on growth performance (Chen et al., 2013). Previous studies have demonstrated that the supplementation of some marine protein hydrolyzates could benefit for improving nutritive values of low fish meal diet (Toften et al., 2003; Kououlaki et al., 2009; Kader et al., 2010, 2012; Kader and Koshio, 2012; Bui et al., 2014; Wu et al., 2018). In the present study, inclusion of marine protein hydrolyzates did not result in any significant difference on the expression of elongation initiation factor 2α (eIF2α) (P > 0.05) (Figure 3). However, the expression of the activating transcription factor 4 (ATF4) was decreased with the supplementation of marine protein hydrolyzates, and ATF4 expression of fish fed the diet with ShP was significantly lower than that of NC (P < 0.05) (Figure 3). Additionally, the expression of regulated in development and DNA damage responses 1 (REDD1) was significantly down-regulated with the inclusion of marine protein hydrolyzates (P < 0.05), and SqP and Mix supplementation significantly reduced the expression of REDD1 compared to the FS and ShP (P < 0.05) (Figure 3).
The growth performance was elevated with the inclusion of marine protein hydrolyzates in the present study. The promotion effect of marine protein hydrolyzates on growth performance has also been observed in red sea bream (Kader et al., 2010, 2012; Bui et al., 2014), rainbow trout *Oncorhynchus mykiss* (Toften et al., 2003), Atlantic salmon *Salmo salar* (Kousoulaki et al., 2009), and yellow catfish *Pelteobagrus fulvidraco* (Wu et al., 2018). Meanwhile, the inclusion of marine by-products in the present significantly elevated the FI in accordance as described previously (Toften et al., 2003; Kofuji et al., 2006; Kousoulaki et al., 2009; Kader et al., 2010, 2012), and this mainly related to the increased SGR. Some free amino acids, such as alanine and glycine, have been considered as feeding stimulant to enhance the appetite of fish (Shamushaki et al., 2007). Additionally, taurine exhibits...
the function of promoting feeding in fish (Kim et al., 2005; Chatzifotis et al., 2008). Therefore, the elevated free amino acids and taurine may partly account for the elevated FI and SGR with the inclusion of marine protein hydrolyzates. Meanwhile, fish fed the diet with FS and ShP showed relative higher growth and feed intake than that of SqP and Mix, which also followed a similar pattern with the content of taurine and free amino acids in diets.

The improvement in the non-specific immune response has been reported with the inclusion of marine protein hydrolyzates in some previous studies (Bui et al., 2014; Khosravi et al., 2015; Siddik et al., 2019). Lysozyme is an essential component of innate immune system, and its activity effectively reflects the innate immune response to pathogen infection (Saurabh and Sahoo, 2008). The activity of lysozyme was significantly elevated with the inclusion of marine protein hydrolyzates in red sea bream (Bui et al., 2014; Khosravi et al., 2015) and barramundi Lates calcarifer (Siddik et al., 2019). Similarly, in the present study, the inclusion of marine protein hydrolyzates significantly elevated the serum lysozyme activity. The major components of serum protein are albumin and immunoglobulin, and the supplementation of marine protein increased the immunoglobulin content in red sea bream (Bui et al., 2014; Khosravi et al., 2015). Meanwhile, the serum protein content was significantly increased with the marine protein hydrolyzate inclusion in the present study, indicating the immunostimulatory effect of marine protein hydrolyzates. Aminotransferases, such as AST and ALT, are capable of catabolizing amino acids and transferring amino to α-keto acids, commonly used for the detection of liver damage or organ dysfunction in fish species (Lin and Luo, 2011; Luo et al., 2012; Li et al., 2014; Lu et al., 2017). In general, high fish meal replacement with plant protein significantly decreased the activity of AST and ALT with negative effects on growth performance (Lin and Luo, 2011; Luo et al., 2012; Li et al., 2014). However, in the present study, the inclusion of marine protein hydrolyzates significantly decreased the activity of AST and ALT, which seemed to be in conflict with the improved performance with the marine protein hydrolyzate inclusion. The marine protein hydrolyzates are also rich in soluble protein, peptides, and low-molecular-weight components (Harnedy and FitzGerald, 2012), and the more digestible protein hydrolyzates may account for the decreased transaminase activities and the elevated ADC of protein in fish fed the diet with marine protein hydrolyzates.

It is well known that IGF-I is essential in regulating the development and growth of vertebrates (Moriyama et al., 2000). In general, the expression of IGF-I could partly illustrate the variation in growth performance induced by feed intake (Duan, 1998), and meanwhile, a positive relationship between IGF-I and growth has been observed in Japanese flounder Paralichthys olivaceus (Zheng et al., 2012), gilthead seabream Sparus aurata (Gómez-Requeni et al., 2004), Japanese seabass Lateolabrax japonicus (Men et al., 2014), and hybrid grouper Epinephelus lanceolatus × E. fuscoguttatus (Luo et al., 2016). The inclusion of fish protein hydrolyzates has been confirmed to promote liver IGF-I mRNA expression and serum IGF-I content (Zheng et al., 2012). Similarly, in the present study, the expression of IGF-I was improved with the inclusion of marine protein hydrolyzates, and the elevated IGF-I expression could partly account for the better growth performance in those groups.

The IGF system has been considered as the main nutrient-sensing pathway, which develops its function in regulating protein synthesis through the integration of phosphatidylinositol 3-kinase (PI3K)/AKT/TOR pathways (Fuentes et al., 2013). In the present study, the expression of AKT1 was elevated with the inclusion of marine protein hydrolyzates, which may be associated with the increased expression of IGF-1. Meanwhile, the activation of TOR directly induces the expression of translation regulators eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1) and S6 to promote protein synthesis (Ma and Blenis, 2009). The up-regulation of S6 in the present study revealed the activation of the TOR pathway to some extent. Meanwhile, the TOR pathway is necessary to perceive the amino acid abundance at the cellular level (Gallinetti et al., 2013). The significantly enhanced ADC of protein in the marine protein hydrolyzate groups may produce the variation in amino acid abundance, which partly accounted for the activation of the TOR pathway. Additionally, in mammals, the inclusion of taurine could also activate the TOR pathway (Li et al., 2012; Solon et al., 2012), and therefore, the increased taurine content caused by the inclusion of marine protein hydrolyzates may also be responsible for the activation of the TOR pathway. The AAR pathway commonly functions in opposition to the TOR pathway, and the scarce or imbalanced amino acids commonly leads to the phosphorylation of eIF2α to reduce protein synthesis and the promotion of translation of ATF4 (Kilberg et al., 2009; Castilho et al., 2014). Previous studies in turbot suggest that the activation of the AAR pathway partly accounts for the protein synthesis with high replacement of fish meal (Song et al., 2016; Xu et al., 2016). In the present study, the inclusion of marine protein hydrolyzates significantly decreased the expression of ATF4, which indicated the inhibition of the AAR pathway to some extent. However, no significant difference was observed in the expression of eIF2α, which may be regulated at the phosphorylation level as in mammals. The phosphorylation of eIF2α induces the expression of REDD1 in an ATF4-dependent manner (Jin et al., 2009; Whitney et al., 2009). In the present study, the expression of REDD1 was significantly decreased with the inclusion of marine protein hydrolyzates, and this confirmed the down-regulation of the AAR pathway in those groups. In addition, REDD1 acts as a negative regulator of the TOR pathway in mammals (Ma and Blenis, 2009), and the down-regulation of REDD1 and activation of the TOR pathway indicated the existence of similar regulation mechanism in fish species.

In the above, the inclusion of marine protein hydrolyzates improved the growth performance of largemouth bass with enhanced non-specific immunity, and fish fed the diet with FS and ShP obtained significantly higher growth than that of NC. The promotion of growth was partly associated with the elevated feed intake and protein digestibility. In addition, with the supplementation of marine protein hydrolyzates, the expression of IGF-1 was up-regulated along with the activation of the TOR pathway and depression of the AAR pathway. Further studies would be conducted to replace the use of fish meal with...
suitable marine protein hydrolyzates, FS and ShP, in the diet of largemouth bass.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/supplementary material.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the Animal Care and Use Committee of the Shanghai Ocean University.

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**AUTHOR CONTRIBUTIONS**

MD conducted most of the experiment, analyzed the data, and wrote the manuscript under the guidance of SL and NC. CF and HQ offered their help in the feeding trial. All authors read and approved the final manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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