Effects of Enzymatically Hydrolyzed Poultry By-Product Peptides on Growth Performance, Digestive Indices, and Non-specific Immune Indices of Litopenaeus vannamei (Postprint)

Authors: Lu Jingjing, Guo Ran, Xia Hui, Xie Wei, Wang Meixue, Yang Pin-Xian, Li Na

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Abstract

This study evaluated the feasibility of using enzymatically hydrolyzed poultry by-product peptides as a protein source to replace partial fish meal in Litopenaeus vannamei feed by assessing growth performance, digestive indices, and non-specific immune parameters. Five experimental diets were formulated by replacing 0% (control), 2%, 4%, 6%, and 8% of fish meal in the basal diet with premixed enzymatically hydrolyzed poultry by-product peptides (coated methionine was added to match the methionine content of domestic fish meal), designated as T0, T1, T2, T3, and T4, respectively. Nine hundred Litopenaeus vannamei with an average body weight of (0.31±0.01) g were randomly divided into 5 groups, each with 3 replicates of 60 shrimp, and reared for 8 weeks in an indoor culture trial. The results showed that replacement levels of 2%–6% did not significantly affect weight gain rate, specific growth rate, protein efficiency ratio, or feed conversion ratio compared to the control group ($P>0.05$). When replacement levels exceeded 2%, whole-body crude lipid content was significantly lower ($P<0.05$) and whole-body crude ash content was significantly higher than the control group ($P<0.05$). At the 2% replacement level, hepatopancreatic lipase activity was highest, plasma glucose and triglyceride concentrations were significantly reduced ($P<0.05$), and plasma lysozyme activity was significantly increased ($P<0.05$). Plasma peroxidase activity was significantly lower than the control group when replacement levels exceeded 2% ($P<0.05$). No significant differences were observed among groups in apparent digestibility of crude protein, apparent digestibility of crude lipid, or hepatopancreatic lysozyme activity, peroxidase activity, and total antioxidant capacity ($P>0.05$). It was concluded that under the experimental conditions, the optimal replacement level of fish meal by enzymatically hydrolyzed poultry by-product peptides in Litopenaeus

vannamei feed was 2% based on growth performance and comprehensive consideration of digestive and non-specific immune indices.

Full Text

Effects of Peptides Hydrolyzed from Poultry By-Products on Growth Performance, Digestive Indices and Non-Specific Immune Indices of *Litopenaeus vannamei*

LU Jingjing[1], GUO Ran[1]*, XIA Hui[1], XIE Wei[1], WANG Meixue[1], YANG Pinxian[1], LI Na[2]
(1. Ocean College of Hebei Agricultural University, Qinhuangdao 066000, China;
2. Gaotang First Middle School in Shandong Province, Liaocheng 252000, China)

Abstract: This study evaluated the feasibility of using peptides hydrolyzed from poultry by-products (PHFP) as a protein source to partially replace fish meal (FM) in the diet of *Litopenaeus vannamei* by assessing growth performance, digestive indices, and non-specific immune indices. Five experimental diets were formulated in which premixed PHFP (PHFP supplemented with coated methionine to match the methionine content of domestic FM) replaced 0% (control), 2%, 4%, 6%, and 8% of FM in the basal diet, designated as T0, T1, T2, T3, and T4, respectively. A total of 900 *L. vannamei* with an average body weight of (0.31±0.01) g were randomly allocated into five groups with three replicates per group and 60 shrimps per replicate for an 8-week indoor culture trial. The results showed that replacement of FM with PHFP at 2–6% did not significantly affect weight gain rate, specific growth rate, protein efficiency ratio, or feed conversion ratio compared with the control group ($P>0.05$). When the replacement level exceeded 2%, whole-body crude fat content was significantly lower ($P<0.05$) and whole-body ash content was significantly higher ($P<0.05$) than in the control group. At the 2% replacement level, hepatopancreatic lipase activity reached its maximum, while plasma glucose and triglyceride contents were significantly reduced ($P<0.05$) and plasma lysozyme activity was significantly increased ($P<0.05$). When the replacement level exceeded 2%, plasma peroxidase activity was significantly lower than in the control group ($P<0.05$). No significant differences were observed among groups in apparent digestibility of crude protein and crude fat, or in hepatopancreatic lysozyme, peroxidase activities, and total antioxidant capacity ($P>0.05$). Under the conditions of this experiment, the optimal replacement level of FM with PHFP in *L. vannamei* diets is 2% based on growth performance and considering digestive and non-specific immune indices comprehensively.

Keywords: PHFP; *Litopenaeus vannamei*; growth performance; digestive indices; non-specific immune indices

Introduction

In recent years, with the rapid development of aquaculture, intensive farming of *Litopenaeus vannamei* has expanded dramatically, leading to a sharp increase in demand for fish meal as a high-quality protein source and a corresponding rise in price [1]. Considering both production costs and industry development requirements, exploring new dietary protein sources has become a research hotspot [2-4]. Animal absorption of various amino acids from feed is not limited by single restrictive amino acids alone [5]. In the 1960s, Newey et al. [6-8] demonstrated that glycine dipeptides could be absorbed intact, providing strong evidence for peptide absorption by the digestive tract. Xu et al. [9] found that adding small peptide products to the diet of *Penaeus vannamei* significantly increased protease activity and improved protein utilization efficiency.

Numerous studies have shown that enzymatic degradation technology can be used to prepare feather meal peptides rich in peptide content. Yao et al. [10] successfully produced palatable feather meal peptides using different techniques. Poultry by-products such as feather meal and blood meal are excellent protein sources, but their utilization is limited by extremely low digestibility of keratin and scleroprotein. Using enzymatically hydrolyzed soy protein as a carrier, biological enzyme engineering technology can decompose poultry feather meal and blood meal into polypeptides, small peptides, and individual amino acids to produce functional protein peptides—peptides hydrolyzed from poultry by-products (PHFP)—which can improve digestibility [11]. However, amino acid balance is a critical consideration for fish meal alternatives. Although PHFP is rich in essential amino acids such as cysteine, threonine, and isoleucine, its methionine content is far lower than that of fish meal, which is a limiting amino acid for *L. vannamei*. Studies have shown that combining feather meal with amino acids can replace some fish meal in aquatic animal diets to reduce feed costs [12]. Therefore, this study used PHFP supplemented with coated methionine as a raw material to investigate its effects on growth performance and non-specific immune indices of *L. vannamei* when used to partially replace fish meal in the diet.

1.1 Feed Preparation

PHFP was produced by Qinhuangdao Yier Biotechnology Co., Ltd., with small peptide content 12%, crude protein content 60%, and acid-soluble protein content 30%. Its main nutritional components are shown in Table 1 and molecular weight distribution in Table 2 . Since the methionine content in PHFP was lower than that in domestic fish meal, coated methionine was added to the PHFP to match the methionine content of domestic fish meal (1.7%), producing premixed PHFP. The premixed PHFP replaced 0% (control), 2%, 4%, 6%, and 8% of fish meal in the basal diet to formulate five experimental diets, designated as T0, T1, T2, T3, and T4. Feed ingredients were ground through an 80-mesh sieve, mixed according to the formulation (Table 3), extruded into 1.2 mm diameter pellets using a twin-screw extruder, cooked at 60°C for 40 min, dried at 30°C

for 3 h, cooled, sealed in ziplock bags, and stored at -20°C.

1.2 Experimental Management

Postlarvae were purchased from Huanghua Xinhai Biotechnology Co., Ltd., as imported *L. vannamei* inbred line F1 generation. After a 2-week acclimation period, 900 healthy and active postlarvae [average body weight (0.31±0.10) g] were randomly stocked into 15 aquaria (60 cm×40 cm×50 cm) at 60 shrimps per aquarium. Three aquaria were randomly selected as one group, forming five groups, each fed one experimental diet for an 8-week trial. During the experiment, shrimps were fed quantitatively at 7–10% of body weight three times daily (08:00, 14:00, 20:00). Waste was siphoned twice daily, and one-third to one-half of the water was exchanged. Feed intake and mortality were recorded, and water salinity, pH, dissolved oxygen, and ammonia nitrogen were measured every two weeks. Water parameters were maintained at salinity (30.5±0.5)‰, temperature (27±1)°C, dissolved oxygen (7.6±0.7) mg/L, ammonia nitrogen (0.48±0.13) mg/L, and pH 8.0–8.6. After six weeks of feeding, feces were collected 2 h after feeding using a siphon method, intact fecal pellets were selected, dried at 70°C, and stored at -20°C for determination of nutrient apparent digestibility.

1.3 Sample Collection

At the end of the experiment, shrimps were fasted for 24 h, counted, and weighed. Six shrimps per aquarium were randomly selected, surface moisture was removed, and they were weighed for whole-body proximate composition analysis. Another ten shrimps per aquarium were randomly selected, and 0.5 mL hemolymph was drawn from the pericardial cavity behind the carapace using a 2.5 mL syringe, mixed with 1.5 mL anticoagulant, and immediately centrifuged at 100×g for 10 min at 4°C. The supernatant (plasma) was collected and stored at 4°C. After hemolymph collection, shrimps were dissected on an ice tray, hepatopancreas was excised, weighed, rapidly frozen in liquid nitrogen, and stored at -80°C.

1.4.1 Preparation of Hepatopancreas Homogenate

Hepatopancreas tissue was rinsed in ice-cold physiological saline to remove blood, blotted dry with filter paper, and 0.5 g was placed in a 10 mL centrifuge tube. Homogenization medium (solution containing 0.01 mol/L Tris-HCl, 0.0001 mol/L EDTA-2Na, 0.01 mol/L sucrose, and 0.137 mol/L NaCl, pH=7.4) was added, and tissue was homogenized five times for 10 s each using an internal tissue homogenizer (in ice water bath) to prepare 10% hepatopancreas homogenate for digestive and immune index determination.

1.4.2 Measurement Methods

Chromium oxide (Cr O) content in feed and feces was determined by acid digestion colorimetry according to SCT 1089-2006 “Method for Determination of

Digestibility in Fish" . Moisture content was determined by drying at 105°C to constant weight, crude protein by Kjeldahl method, crude fat by Soxhlet extraction, and ash content by muffle furnace incineration at 550°C. Plasma glucose (GLU) and triglyceride (TG) contents were measured by Qinhuangdao Harbor Hospital Clinical Laboratory. Activities of amylase (iodine-starch colorimetry), lipase (turbidimetry), pepsin (Folin reagent colorimetry), trypsin [N-benzoyl-L-arginine ethyl ester (BAEE) method], lysozyme (LZM, self-control method), peroxidase (POD, phenol colorimetry), and total antioxidant capacity (T-AOC, FRAP method) were determined using assay kits from Nanjing Jiancheng Bioengineering Institute.

Growth performance and nutrient apparent digestibility were calculated as follows:

Survival rate (SR, %) = 100 × Nt/N
Weight gain rate (WGR, %) = 100 × (Wt-W)/W
Specific growth rate (SGR, %/d) = 100 × (lnWt-lnW)/t
Protein efficiency ratio (PER, %) = 100 × (Wt-W)/(F×P)
Feed conversion ratio (FCR) = F/(Wt-W)
Condition factor (CF, %) = 100 × Wt/L^3
Apparent digestibility of crude protein (ADCP, %) = 100 × (1-A' /A×C/C')
Apparent digestibility of crude fat (ADCF, %) = 100 × (1-B' /B×C/C')

Where: N = initial shrimp number; Nt = final shrimp number; W = initial body weight (g); Wt = final body weight (g); t = experimental days; P = crude protein content in feed (%); F = feed intake (g); L = body length (cm); A = crude protein content in feed (%); A' = crude protein content in feces (%); B = crude fat content in feed (%); B' = crude fat content in feces (%); C = Cr O content in feed (%); C' = Cr O content in feces (%).

1.5 Data Analysis

Results are expressed as mean±SD. Data were analyzed by one-way ANOVA using SPSS 17.0 software, and Duncan' s multiple range test was used for intergroup comparisons. Significance level was set at $P<0.05$.

Results

2.1 Effects of PHFP on Growth Performance of *L. vannamei*

As shown in Table 4 , final body weight, weight gain rate, specific growth rate, and protein efficiency ratio of *L. vannamei* showed a gradual decreasing trend with increasing PHFP replacement levels. However, T1, T2, and T3 groups did not differ significantly from T0 ($P>0.05$), while T4 was significantly lower than T0 and T1 ($P<0.05$). Feed conversion ratio showed an increasing trend, but T1, T2, and T3 groups were not significantly different from T0 ($P>0.05$), whereas T4 was significantly higher than T0 ($P<0.05$). No significant differences were observed in survival rate or condition factor among groups ($P>0.05$). Condition

factor increased initially and then decreased with increasing replacement levels, reaching its maximum in T2.

2.2 Effects of PHFP on Body Composition of *L. vannamei*

As shown in Table 5 , whole-body crude fat content decreased gradually with increasing PHFP replacement levels, showing significant differences from T0 when replacement exceeded 2% (T2, T3, T4) ($P<0.05$). Whole-body ash content increased gradually, also differing significantly from T0 when replacement exceeded 2% ($P<0.05$). No significant differences were observed in moisture or crude protein content among groups ($P>0.05$).

2.3 Effects of PHFP on Plasma GLU and TG Contents of *L. vannamei*

As shown in Table 6 , all replacement groups (T1, T2, T3, T4) had significantly lower plasma GLU content than T0 ($P<0.05$). Plasma TG content was significantly lower than T0 in all replacement groups except T3 ($P>0.05$ for T3).

2.4.1 Effects of PHFP on Nutrient Apparent Digestibility of *L. vannamei*

As shown in Table 7 , apparent digestibility of crude protein decreased gradually with increasing PHFP replacement levels, while apparent digestibility of crude fat increased initially and then decreased. However, no significant differences were observed among groups ($P>0.05$).

2.4.2 Effects of PHFP on Digestive Enzyme Activities in Hepatopancreas of *L. vannamei*

As shown in Table 8 , hepatopancreatic pepsin and trypsin activities decreased gradually with increasing PHFP replacement levels. Pepsin activity in T1, T2, and T3 did not differ significantly from T0 ($P>0.05$), but T4 was significantly lower than T0 ($P<0.05$). Trypsin activity in T2 and T4 was significantly lower than T0 ($P<0.05$), while other replacement groups showed no significant differences ($P>0.05$). Hepatopancreatic lipase activity was higher in all replacement groups than in T0, with T1 showing the highest activity, though no significant differences were observed among groups ($P>0.05$). No significant differences were found in hepatopancreatic amylase activity among groups ($P>0.05$).

2.5 Effects of PHFP on LZM Activity in Plasma and Hepatopancreas of *L. vannamei*

As shown in Table 9 , plasma LZM activity increased initially and then decreased with increasing PHFP replacement levels. T1, T2, and T3 groups showed significantly higher plasma LZM activity than T0 ($P<0.05$), with T3 having the

highest activity, while T4 did not differ significantly from T0 (P>0.05). Hepatopancreatic LZM activity also increased initially and then decreased, being 19.3%, 42.5%, and 5.0% higher in T1, T2, and T3 than in T0, respectively, though these differences were not significant (P>0.05). T4 showed lower activity than T0, but the difference was not significant (P>0.05).

2.6 Effects of PHFP on POD Activity in Plasma and Hepatopancreas of *L. vannamei*

As shown in Table 10 , plasma POD activity in T1 did not differ significantly from T0 (P>0.05), but T2, T3, and T4 were significantly lower than T0 (P<0.05). Hepatopancreatic POD activity decreased gradually with increasing PHFP replacement levels, though no significant differences were observed among groups (P>0.05).

2.7 Effects of PHFP on T-AOC in Plasma and Hepatopancreas of *L. vannamei*

As shown in Table 11 , no significant differences were observed in T-AOC of plasma or hepatopancreas among replacement groups and T0 (P>0.05). Except for T1, which showed slightly lower plasma T-AOC than the control, all other replacement groups had higher plasma T-AOC than T0. Hepatopancreatic T-AOC increased initially and then decreased with increasing PHFP replacement levels, reaching its maximum in T2.

Discussion

3.1 Effects of PHFP on Growth, Feed Intake, and Body Composition of *L. vannamei*

Teshima et al. [13], Zambonino et al. [14], and Erba et al. [15] all suggested that small peptide products significantly promote growth in shrimp larvae. Jiang [16] found that appropriate supplementation of small peptides could improve muscle quality and protein utilization in juvenile starry flounder. In this study, premixed PHFP replaced fish meal at different levels, but no significant growth-promoting effects were observed at 2%, 4%, or 6% replacement levels, as weight gain rate and specific growth rate did not change significantly. However, whole-body crude protein content was slightly higher in all replacement groups than in the control, while whole-body crude fat content was significantly lower and ash content was significantly higher when replacement exceeded 2%. This increased ratio of crude protein to crude fat in whole body is desirable for *L. vannamei* as a high-protein food product.

The lack of significant growth promotion in this study may be related to the unique feeding and digestive characteristics of *L. vannamei* and the supplementation of free amino acids. Newey et al. [7] confirmed that small peptides containing 2–3 amino acids can be directly absorbed by intestinal cells through carriers,

with the intestine being the primary absorption site for most animals. However, studies by Mai et al. [17] and Liu [18] demonstrated that digestion, enzymatic hydrolysis, and absorption of protein ingredients in *L. vannamei* occur primarily in the midgut gland (hepatopancreas), where most free amino acids from feed are absorbed before entering the intestine. This suggests that absorption of small peptides and free amino acids may occur in different locations, which could be one reason why PHFP did not produce significant growth-promoting effects in this study. Given the unique feeding and digestive characteristics of shrimp, further research on the absorption and transport mechanisms of small peptides in the hepatopancreas and intestine is warranted.

Additionally, research has suggested that crystalline amino acids can be directly absorbed by the body but not synchronously with protein-bound amino acids from feed [19]. Therefore, coated methionine rather than crystalline methionine was added to the premixed PHFP to address asynchronous absorption. The lack of significant growth promotion may be attributed not only to the type of coating material affecting amino acid absorption but also to spatial and temporal differences in absorption of small peptides and different forms of free amino acids in shrimp. Based on the molecular weight distribution of PHFP, small peptides with molecular weight <1,000 u accounted for approximately 76% of the product, and such small peptides are highly active and diverse, with most being directly absorbable. Therefore, we speculate that asynchronous absorption with crystalline methionine may not be an issue in this study, and the coated methionine may have actually caused absorption asynchrony. Adding crystalline methionine might produce more significant growth-promoting effects, which will be tested in future studies.

The significant reduction in weight gain rate at 8% replacement may also be due to diminished effects of certain growth-promoting factors in fish meal as replacement level increased. The specific reasons require further investigation. Overall, when PHFP replaced less than 6% of FM, no significant differences were observed in growth indices such as weight gain rate and specific growth rate. Considering resource conservation and cost-effectiveness, partial replacement of fish meal with PHFP in *L. vannamei* diets is feasible.

3.2 Effects of PHFP on Hepatopancreatic Digestive Enzyme Activities and Nutrient Apparent Digestibility

Nutrient digestion and absorption in aquatic animals primarily depends on enzymes in digestive organs, and digestive capacity, growth performance, and immunity are all related to digestive enzyme activities [20]. The hepatopancreas is the main digestive enzyme-secreting organ in shrimp, making its enzyme activities important indicators of digestive capacity [21]. Theoretically, increased digestive enzyme activity should lead to increased digestibility of corresponding nutrients such as protein and fat [22]. This study found that hepatopancreatic lipase activity was higher in all replacement groups than in the control, with the highest activity at 2% replacement. Hepatopancreatic pepsin and trypsin ac-

tivities decreased with increasing PHFP replacement levels, being significantly lower than the control at 8% replacement. No significant differences were observed in hepatopancreatic amylase activity among groups. The trends in apparent digestibility of protein and fat generally matched the trends in corresponding enzyme activities, supporting theoretical expectations. This study did not confirm the conclusion of Hu et al. [23] that small peptides can increase certain digestive enzyme activities in fish, promoting earlier transition from cytoplasmic to membrane digestion and enhancing nutrient utilization, likely due to the unique digestive and absorptive mechanisms of crustaceans.

3.3 Effects of PHFP on Plasma Biochemical Indices of *L. vannamei*

Blood glucose content reflects carbohydrate metabolism and can indicate whether dietary nutrient levels are appropriate and whether liver function is normal [24]. Within a certain threshold, higher plasma GLU content indicates better shrimp condition, but exceeding this threshold causes nutritional physiological stress and damages health [25]. Plasma TG content reflects lipid metabolism, and elevated levels indicate excessive fat accumulation in the liver, which can lead to fatty liver and hepatomegaly [26]. This study showed that replacing 2–8% of FM with PHFP reduced plasma GLU and TG contents, indicating that PHFP replacement affects carbohydrate and lipid metabolism in *L. vannamei*. Plasma GLU and TG contents vary significantly with dietary composition. The reduction in plasma GLU may be because small peptides in PHFP promote glycolysis more than gluconeogenesis, accelerating glucose utilization. Alternatively, small peptides may enhance the efficiency of crustacean hyperglycemic hormone (CHH), a multifunctional neuroendocrine hormone unique to crustaceans that regulates plasma GLU. The decreased plasma TG content in replacement groups may be due to certain small peptides in PHFP promoting plasma lipid metabolism, accelerating TG hydrolysis and clearance, or due to effects of enhanced or reduced antioxidant capacity on lipid metabolism, which requires further investigation.

3.4 Effects of PHFP on Non-Specific Immune Indices in Plasma and Hepatopancreas of *L. vannamei*

Lysozyme (LZM) is a stable alkaline protease and an important antimicrobial protein in shrimp [27]. Teng et al. [28] found that 5% fish protein hydrolysate replacement significantly increased hepatopancreatic LZM activity in Chinese shrimp, while Liu et al. [29] reported that 2% yeast protein hydrolysate significantly increased serum LZM activity in juvenile turbot. This study showed that hepatopancreatic LZM activity increased with PHFP replacement level but did not differ significantly among groups. Plasma LZM activity increased initially and then decreased, with 2%, 4%, and 6% replacement groups being significantly higher than the control, suggesting that PHFP enhances antibacterial and antiviral capacity by increasing plasma LZM activity.

Peroxidase (POD) catalyzes the oxidation of hydrogen peroxide, phenols, and

amines, eliminating their toxicity. Located in peroxisomes, POD is an antioxidant enzyme that protects cells. This study found that plasma POD activity did not differ significantly from the control at 2% replacement but was significantly lower at 4%, 6%, and 8% replacement. No significant differences were observed in hepatopancreatic POD activity among groups.

Total antioxidant capacity (T-AOC) reflects the overall antioxidant level of the body' s defense system [30]. Shi et al. [31] found that antimicrobial peptides significantly increased serum T-AOC in shrimp. In this study, plasma and hepatopancreatic T-AOC in replacement groups were higher than in the control (except plasma T-AOC at 2% replacement), suggesting that PHFP may enhance defense capacity by improving antioxidant ability. Antioxidant capacity also plays an important role in lipid metabolism, and its enhancement can help alleviate stress and increase lipoprotein lipase activity, thereby reducing plasma TG content [32-33]. Therefore, enhanced T-AOC may provide a possible explanation for the reduced plasma TG content observed in this study.

Conclusion

Under the conditions of this experiment:

1. Replacement of 2-6% fish meal with PHFP did not significantly affect survival rate, weight gain rate, specific growth rate, or feed utilization efficiency of *L. vannamei*.
2. Replacement of more than 2% fish meal with PHFP significantly reduced whole-body crude fat content and significantly increased whole-body ash content, without significantly affecting other body composition indices.
3. At 2% replacement level, PHFP produced the highest hepatopancreatic lipase activity, significantly reduced plasma GLU and TG contents, and significantly increased plasma LZM activity.
4. Based on growth performance and considering digestive and non-specific immune indices comprehensively, the optimal replacement level of fish meal with PHFP in *L. vannamei* diets is 2%.

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Note: Figure translations are in progress. See original paper for figures.