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Original Article

Effect of hemoglobin powder substituted for fishmeal on growth performance, protein digestibility, and trypsin gene expression in *Litopenaeus vannamei*

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Abstract

Recent increases in the price and demand of fishmeal, the primary protein source in shrimp feeds, have caused a search for alternative protein sources. Hemoglobin powder (HE) is a by-product produced by separating hemoglobin from plasma of farm animal un-coagulant blood. HE contains high protein content but low lipid content, and thus has high potential for fishmeal substitution.

A six week feeding trial was carried out to investigate effects of HE substituted for fishmeal protein on growth performance, protein digestibility and trypsin gene expression in Pacific white shrimp (*Litopenaeus vannamei*). Six diets with 0%, 12.5%, 25%, 50%, 75% and 100% of HE replacing fishmeal protein were fed four times daily to six groups of shrimp with an average initial weight of 3.53 g/shrimp. Growth of shrimp decreased with increasing level of HE substitution. Although the 12.5% HE substitution caused significantly lower final weight, weight gain, SGR, feed intake, PER and PPV in comparison with the control diet, FCR of this diet was not statistically different ($p<0.05$).

The activity of trypsin of shrimp was similar among the groups fed diets with HE substitution not higher than 50% ($p>0.05$). *In-vitro* and *in-vivo* protein digestibility of 12.5% HE substitution were significantly lower than that of the control group in which the trypsin gene expression of shrimp fed 12.5% HE substituted diet was the highest.

Keywords: protein digestibility, trypsin gene expression, hemoglobin powder, fishmeal substitution, *Litopenaeus vanamei*

1. Introduction

The culture of white shrimp, *Litopenaeus vannamei*, is becoming economical important in Thailand because of the ability of this species to adapt to a wide range of salinities and temperatures and to cope with diseases under high intensity culture conditions. Increasing shrimp production worldwide has resulted in an increased feed demand, which directly raises demand for shrimp feed ingredients, particularly fishmeal. Fishmeal (FM) is the main protein source in many aquatic feeds due to its suitable amino acid profile and palatability. While fishmeal production is stable or even decreasing, the rising price and increasing demand for fishmeal in animal production systems is expected to cause a shortage of the meal in the future. Thus, the shrimp feed industry is searching for other suitable protein sources.

The protein sources, which are used to substitute for fishmeal in the shrimp feed, include plant products such as soybean meal and terrestrial processing by-products such as meat and bone meal, poultry by-products, blood meal, and hemoglobin meal (Lim and Dominy, 1990; Tacon and Akiyama, 1997; Bureau *et al*., 1999; Abery *et al*., 2002; Forster *et al*., 2003). The level of fishmeal substitution is variable depending on protein sources. Inclusion of different protein sources at the same protein level may not give the same muscle growth because of disparity in protein quality and

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digestibility. Commercially, the highest substitution level for fishmeal is preferred to lower the production cost. However, diets with high levels of fishmeal replacement may not support good growth due to the inferior amino acid profile and protein digestibility of the alternative protein sources used in the diets.

Hemoglobin powder (HE) has a potential as an alternative protein source due to its high digestible protein content with high lysine and leucine content (Hertrampf and Piedad-Pascual, 2000). Little is known about the nutritional quality of HE relative to fishmeal in diets for shrimp. This study was therefore carried out to investigate fishmeal substitution with HE on growth performance, protein digestibility, and trypsin gene expression.

2. Materials and Methods

2.1 Experimental diets and leaching tests

The fishmeal used in this study was a premium grade (65.36% protein) purchased from a fishmeal plant (Pattani Fishmeal Industry Co., LTD) in Pattani Province, Thailand. Hemoglobin powder (84.16% protein) was donated by a feed company (Inteqe Feed Co., LTD).

Six diets were formulated to contain protein and lipid at 45% crude protein and 8% lipid with hemoglobin substituted for fishmeal at 0%, 12.5%, 25%, 50%, 75%, and 100% (Table 1). The coarse ingredients were finely ground to pass through a 30 mesh screen. Dry ingredients were mixed using a Hobart mixer (A200 MIYERS model A200T ML 104568, Troy, Ohio, USA) for 10 min, then lecithin and oil were gently added and mixed for a further 5 min. Distilled water was gradually added at 35% of diet and mixed for another 10 min. The resulting mash was pelleted using a pelleted mill with a 2 mm diameter pore size die and cut into 2 mm length pellets. Pelleted diets were dried at 60°C for 24 hrs. The dried diets were sieved through a 2 mm diameter mesh screen and stored in polyethylene bags at -20°C in the dark until used. The proximate composition of ingredients and experimental diets was determined (AOAC, 1995). The amino acid profiles of

Table 1. Composition (g/100 g), proximate composition (% as fed basis) and leaching loss (dry matter) of experimental diets.

Ingredients	Experimental diets					
	Control	12.5% HE	25% HE	50% HE	75% HE	100% HE
Fishmeal (65.36% protein)	44	38.50	33	22	11	-
Hemoglobin powder (84.16% protein)	-	4.27	8.54	17.09	25.63	34.17
Squid meal	8	8	8	8	8	8
Wheat flour	20	20	20	20	20	20
Rice flour	15	15	15	15	15	15
Wheat gluten	6	6	6	6	6	6
Lecithin	2	2	2	2	2	2
Fish oil	-	0.50	1	2	3	4
Vitamin premix[1]	2	2	2	2	2	2
Vitamin C	0.10	0.10	0.10	0.10	0.10	0.10
Trace mineral premix[2]	0.50	0.50	0.50	0.50	0.50	0.50
Calcium phosphate	0.20	0.20	0.20	0.20	0.20	0.20
BHT	0.02	0.02	0.02	0.02	0.02	0.02
Cholesterol	0.50	0.50	0.50	0.50	0.50	0.50
CMC	1	1	1	1	1	1
Cellulose	0.68	1.41	2.14	3.59	5.05	6.51
Proximate composition (% as fed basis) and leaching loss (% dry matter)						
Protein	45.89	46.75	46.51	44.83	45.16	45.79
Lipid	7.89	7.53	7.48	7.67	8.38	7.56
Ash	7.64	6.76	6.19	4.96	3.61	2.27
Leaching loss (%)	12.07	12.45	11.23	8.43	8.70	6.86

[1] Vitamin premix (g/kg vitamin premix): thiamin HCl 0.5, riboflavin 3.0, pyridoxine HCl 1.0, DL Ca-pantothenate 5.0, nicotinic acid 5.0, biotin 0.05, folic acid 0.18, cyanocobalamine 0.002, choline chloride 100, inositol 5.0, menadione 2.0, retinol acetate (20,000 IU/g) 5.0, cholecalciferol (400,000 IU/g) 0.002, DL-alfa-tocopheryl acetate (250 IU/g), wheat flour 865.266.

[2] Mineral premix (g/100 g mineral premix): cobalt chloride 0.004, cupric sulfate pentahydrate 0.250, ferrous sulfate 4.0, magnesium heptahydrate 28.398, manganous sulfate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, wheat flour 53.428.

diets were determined by HPLC (AOAC, 1995).

Diet leaching test was performed using three replicates according to the method modified from Aquacop (1978) and Cruz-Suarez *et al*. (2001). Five grams of the pellets were put on fine mesh baskets and immersed in water for 1 hr with aeration, simulating the culture conditions in glass aquaria. The percent dry matter loss (%DML) was calculated as: %DML = 100x(DWd-DWwid)/DWd; where DWd and DWwid were the dry matter weights of the diet before and after immersion, respectively.

2.2 Growth trial

1) Shrimp, culture and feeding

L. vannamei juvenile shrimp were nursed at the Aquatic Science Research Station, Satun Province, Thailand until used. The shrimp were stocked into 24 glass aquaria (45x45x115 cm) containing 200 L of seawater (water flow 33.260 L/h; water temperature 26-30°C, salinity 29-33 ppt) and acclimatized to the experimental conditions for one week. Twenty shrimp (initial weight 3.53±0.06 g) were then selected and randomly distributed into 24 glass aquaria and fed with experimental diets. Feeding was done by hand to satiation 4 times daily at 8.00 a.m., 12.00 a.m., 5.00 p.m., and 10.00 p.m. for 6 weeks.

2) Sampling

At the end of the feeding period, ten shrimp from each aquarium were sampled. Six shrimp from each aquarium were used for proximate analysis. Two shrimp were decapitated and the hepatopancreas were fixed in TRIzol reagent and kept at -80°C until used for trypsin gene expression analysis, while the final two shrimp were decapitated and hepatopancreas were taken for enzyme extraction for *in-vitro* protein digestibility. The remaining shrimp were left in the aquaria for 30 days for *in-vivo* protein digestibility determination.

2.3 *In-vitro* protein digestibility

1) Enzyme extraction and activity determination

In-vitro protein digestibilities of experimental diets were determined using crude enzyme extract from the hepatopancreas from the shrimp in the experiments described above. The crude enzyme extracts were prepared and the *in-vitro* protein digestibility determined on pooled samples using the method modified from Bassompierre (1997). Crude enzymes were extracted from the hepatopancreas and homogenized (1:10 w/v) in 0.05 M Tris buffer pH 7.5 at 4°C. The homogenate was centrifuged twice at 12,000 x g for 30 min at 4°C. The crude enzyme was obtained and kept at -80°C for further analysis. Protein in extracted enzyme was measured by modified Lowry's Method using Bovine Serum Albumin (BSA) as a standard. Crude enzyme was diluted to 1 mg protein/ml before *in-vitro* digestion study. The trypsin activity of the crude enzyme extract was determined using BAPNA as a substrate by mixing 950 µl of 0.1 M BAPNA and 50 µl of crude enzyme, incubated at 37°C for 10 min, then the reaction was terminated by adding 100 ml of 30% trichloroacetic acid and measured for absorbance at 410 nm. The enzyme activity was calculated as follows (Rathore *et al*., 2005): Unit of Enzyme activity (mmole/ml/mg protein)=[Abs at 410 nm/ min) * 1000 ml * ml of reaction volume]/[Extinction of chromagen * mg protein in reaction mixture]. The molar extinction coefficient of p-nitroanilide is 8800.

2) Preparation of feed for enzyme digestibility assay

A sample of each diet was ground and weighed to the exact weight at 30 mg protein calculated using dietary protein content. Forty ml of 0.01 M phosphate buffer (pH 7.8) and 1 ml of 0.5% chloramphenicol (in 96% ethanol) were added and mixed thoroughly. The mixture was incubated at 30°C for 18 hrs in a shaking water bath.

3) Pre-digestion concentration

Prior to sample incubation, 0.5 ml of mixture from each treatment was sampled as a control, immediately heated at 100°C for 5 min to terminate the enzyme activity, rapidly frozen at -80°C for later determination of total reactive amino group using the trinitrobenzene sulfonic acid (TNBS) assay as described below.

4) Post-digestion concentration

Digestion concentration was determined by adding 0.5 ml of the crude enzyme extract (1 mg/ml of protein). The digestion process was performed in a shaking water bath for 18 hrs at 30°C. At the end of incubation time, 1 ml of each digested mixture was sampled, immediately heated at 100°C for 5 min and rapidly frozen at -80°C for the later determination of free reactive amino group of the peptides using the trinitrobenzene sulfonic acid (TNBS) assay as described below.

5) Determination of free reactive amino acid groups

Dilution of 0.2 ml of either the undigested control or the digested mixture with 2 ml of 0.05 M phosphate buffer pH 8.2 were mixed thoroughly with 1 ml of 0.1% TNBS in 0.01 M phosphate buffer and were incubated at 60°C for 1 h in the dark. The reaction was stopped by adding 1 ml of 1 N HCl and cooling to room temperature. The absorbance was measured at 420 nm and the concentration of free amino groups was calculated using DL-alanine as a standard. *In-vitro* digestibility was expressed as mole alanine equivalent liberated reactive amino group cleaved peptides per 200 µl sample: Alanine equivaqlent liberated (mole)=alanine conc. (g/L)*(1/89.10 g/mole)*(0.2 ml/L,000 ml).

2.4 *In-vivo* protein digestibility

The apparent digestibility coefficient (ADC) of dietary protein in diets was measured. Diets were prepared as described above with chromic oxide (Cr_2O_3) as a marker included at 0.5% of diet.

After growth trial termination, feeding was continued with diets containing chromic oxide for 30 days. Feces collection commenced 2 days after changing to chromic oxide diet by siphoning method twice daily at 2:00 p.m. and 8:00 p.m. Feces were separated from feed particles, kept at -20°C and oven dried at 105°C. Determination of chromic oxide was carried out according to the method of Lall (1991). The ADC was estimated using the following equation.

$$\text{ADC} = \%\ \text{of protein digestibility} = 100 - \left(100 * \frac{I_a}{I_h} * \frac{P_h}{P_a}\right)$$

where ADC = apparent digestibility coefficient, I_a = % Cr_2O_3 in feeds, I_h = % Cr_2O_3 in feces, P_a = % protein in food, and P_h = % protein in feces.

2.5 Trypsin gene expression

Trypsin gene expression was studied using a 2-step RT-PCR. First step cDNA synthesis, total RNA was extracted from the hepatopancreas of shrimp fed six different diets using TRIzol reagent. Intact total RNA was used for reverse transcription; cDNA were synthesized from each individual sample using the Superscript III™ first-strand synthesis system for RT-PCR. Reverse transcriptions were performed using 8 µl of (100 ng/µl) total RNA, 1 µl of (10 mM) dNTP mix and 1 µl of (50 ng/ìl) random hexamer. The reaction mixtures were incubated for 5 min at 65°C then further for 2 min at 4°C. After 2 min at 4°C of incubation, 10 µl of cDNA synthesis mixture containing 2 µl of (10X) RT buffer, 4 µl of (25 mM) $MgCl_2$, 2 µl of (0.1 M) DTT, 1 µl of RNase out, and 1 µl of (50 units) Superscript III™ RT, was added. The volume of combined two portion mixture was 20 µl. Then the reaction was extended by incubation for 10 min at 25°C, 50 min at 50°C and 5 min at 85°C, followed by addition of 1 µl of RNase inhibitor and incubation for 20 min at 37°C, after which the reaction was terminated and the mixture held at 4°C until used for the second step.

The second step RT-PCR, trypsin primers for PCR amplification were based on three trypsin genes reported for *L. vannamei* (Klein *et al*., 1996). Primer sequences were Forward trypsin- CTCAACAAGATCGTCGGAGGAACTGA- and Reward trypsin-GACACTCGTCGTCAGAACACGATG- that matched positions 81-106 and 545-567, respectively. PCR amplifications were performed in a 25 ìl final reagent mixture containing 12.5 µl of H_2O, 2.5 µl of (10X) PCR buffer, 1.5 µl of (25 mM) $MgCl_2$, 1 µl of (10 mM) dNTP mix, 1 µl of (6 µM) each primer, 5 µl of the obtained cDNA (100 ng/µl) of each sample and 0.5 µl of Tag DNA polymerase. A thermocycler was used with the following program: 5 min at 95°C, 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C (35 cycles); and over-extension step for 10 min at 72°C. The resulting PCR products for trypsin were analyzed in a single 1.5% agarose gel and stained with ethidium bromide (Sambrook and Russell, 2001). The intensity of the bands in gel images obtained was evaluated relative to that of EF-1 alpha (Wongpanya *et al*., 2007) using the Scion Image program (Phongdara *et al*., 2006).

2.6 Statistical Analysis

Data were analyzed using analysis of variance to determine if significant differences exist among treatment means. Tukey's HSD test was used to determine significant differences between treatments. Final weight was analyzed using linear regression procedures with fishmeal replacement level as the independent variable. A 5% error rate for significance was used for all analyses.

3. Results

3.1 Amino acid composition of experimental diets

The amino acid profile of the diets was related to the level of fishmeal substitution by hemoglobin meal (Table 2). Glutamic acid was highest in the control diet (22.95% of protein) and decreased with increasing HE levels particularly 100% HE diet had the lowest one (16.32% of protein). Moreover, arginine, glycine, methionine, proline, isoleucine, tyrosine, and lysine declined as FM was replaced by HE. In contrast, leucine, phenylalanine, aspartic acid, alanine, serine, histidine, valine, threonine, and tryptophan increased with HE increment. In addition, EAA/NEAA balance was changed and proportion of leucine and isoleucine dramatic changed with increasing HE.

3.2 Survival rate

The survival rate of shrimp was not affected ($p>0.05$) by the treatment (Table 3).

3.3 Growth and feed utilization

Shrimp fed the control diet had the highest final weight, weight gain, SGR, PER and PPV (Tables 3 and 4). Feed intake of the shrimp in the control treatment was also significantly ($p<0.05$) higher while FCR was the lowest with values of 9.33±0.42 g and 1.44±0.15, respectively. Final weight, weight gain, SGR, PER and PPV of shrimp fed diets decreased with increasing levels of FM replacement by HE. Regression between final weight and levels of hemoglobin powder was $R^2 = 0.8446$ (Figure 1). Feed intake of shrimp fed diets with hemoglobin replacement was lower than that of the control shrimp and was the lowest in 100% replacement. Replacement with hemoglobin powder caused higher FCR although the trend of increment was not correlated with levels of replacement, but 100% replacement diet gave the highest FCR. The

Table 2. Amino acid composition of experimental diets (% of protein).

Amino acid	Experimental diets					
	Control	12.5% HE	25%HE	50%HE	75%HE	100%HE
Arginine	5.46	5.33	5.20	4.42	3.90	3.38
Histidine	4.19	4.12	4.30	4.92	5.28	5.64
Isoleucine	1.35	1.53	1.40	0.94	0.74	0.53
Leucine	5.69	6.71	7.28	7.31	8.12	8.93
Lysine	6.05	6.11	6.19	5.88	5.79	5.71
Methionine	2.43	2.30	2.09	1.84	1.54	1.25
Phenylalanine	4.00	4.60	5.04	5.22	5.83	6.44
Threonine	3.30	3.82	3.84	3.53	3.64	3.76
Tryptophan	0.70	0.78	0.71	0.87	0.96	1.05
Valine	2.01	2.58	2.75	2.50	2.74	2.99
Alanine	7.58	7.49	7.60	8.49	8.94	9.39
Aspartic acid	9.45	10.32	10.46	10.54	11.08	11.62
Cystine	1.09	1.14	1.13	1.00	0.96	0.92
Glutamic acid	22.95	20.15	18.66	19.63	17.98	16.32
Glycine	7.67	7.28	7.42	7.00	6.66	6.33
Proline	7.01	6.51	6.70	6.45	6.16	5.88
Serine	5.04	5.55	5.43	5.82	6.20	6.59
Tyrosine	4.02	3.69	3.80	3.65	6.46	3.28
Essential amino acids	35.18	37.88	38.80	37.43	38.55	39.67
Non essential amino acids	64.82	62.12	61.20	62.57	61.45	60.33
EAA/NEAA	0.54	0.61	0.63	0.60	0.63	0.66

Table 3. Growth of *L. vannamei* fed diets with hemoglobin powder substituted for fishmeal over 6 week feeding trial.

Experimental diets	Final weight (g/shrimp)	Weight gain (g/shrimp)	SGR[2] (%/day)	Survival rate (%)
Control	10.38±0.45^{a1}	6.88±0.41^{a}	2.59±0.08^{a}	96.25±4.79
12.5% HE	8.76±0.29^{b}	5.13±0.34^{b}	2.13±0.12^{b}	95.00±4.08
25% HE	8.51±0.40^{b}	4.95±0.42^{b}	2.07±0.13^{bc}	83.75±10.31
50% HE	7.67±0.28^{c}	4.15±0.24^{bc}	1.85±0.06^{cd}	95.00±4.08
75% HE	7.19±0.23^{c}	3.65±0.19^{c}	1.68±0.06^{d}	91.25±7.50
100% HE	5.79±0.23^{d}	2.26±0.26^{d}	1.17±0.12^{e}	96.25±2.50

[1] Means with the same superscript are not statistically different ($p>0.05$, n=4).
[2] Specific growth rate = (ln W2-ln W1/T2-T1)*100, W1 = initial weight, W2 = final weight, T2-T1 = cultured period (days).

highest ash content was found in shrimp fed the 100% HE diet, while protein and lipid content were not significantly ($p>0.05$) different among treatments (Table 5).

3.4 Protein digestibility

The *in-vitro* and *in-vivo* apparent protein digestibility values of the 12.5% hemoglobin substituted diet were the lowest without significant difference ($p>0.05$) from other groups (Table 6). *In-vitro* protein digestibility of diets was related to the level of hemoglobin substitution and coincided with *in-vivo* apparent protein digestibility.

3.5 Gene expression

Trypsin gene expression of shrimp fed the 12.5% HE diet (Figure 2) was the highest, whereas the apparent protein digestibility of this treatment was the lowest. However, the expression of this gene was not related to the levels of FM substitution by HE. Regardless of 12.5% HE treatment, the

Table 4. Feed utilization of *L. vannamei* fed diets with hemoglobin powder substituted for fishmeal over 6 week feeding trial.

Experimental diets	Feed intake (g/shrimp)	FCR[2]	PER[3]	PPV[4] (%)
Control	8.20±0.37[a1]	1.19±0.09[d]	1.83±0.14[a]	34.57±2.79[a]
12.5% HE	7.32±0.18[b]	1.43±0.10[cd]	1.50±0.10[b]	27.27±0.03[b]
25% HE	7.55±0.30[b]	1.53±0.09[c]	1.44±0.08[b]	26.24±1.62[b]
50% HE	7.01±0.29[b]	1.70±0.16[c]	1.32±0.13[bc]	24.95±3.33[b]
75% HE	7.24±0.11[b]	1.99±0.11[b]	1.12±0.06[c]	20.67±1.70[bc]
100% HE	5.91±0.35[c]	2.63±0.18[a]	0.84±0.06[d]	14.34±1.71[c]

[1] Means with the same superscript are not statistically different ($p>0.05$, n=4).
[2] Feed conversion ratio = feed intake (g)/weight gain (g).
[3] Protein efficiency ratio = weight gain (g)/protein intake (g).
[4] Productive protein value = (protein gain (g)/protein intake (g)) * 100.

Table 5. Proximate composition (%) of *L. vannamei* fed diets containing various levels of hemoglobin powder for 6 weeks (dry matter basis).

Composition	Experimental diets					
	control	12.5% HE	25% HE	50% HE	75% HE	100% HE
Moisture	75.00±0.60[b1]	75.63±0.61[ab]	75.54±0.34[ab]	75.55±0.45[ab]	75.87±0.31[ab]	76.55±0.31[a]
Crude protein	72.22±0.93	73.62±1.11	73.40±1.65	73.04±1.39	71.96±0.12	71.57±1.16
Crude fat	5.73±0.71	4.20±0.17	6.83±1.12	3.56±1.23	3.83±0.85	4.27±0.95
Ash	11.94±0.37[ab]	11.74±0.15[b]	11.63±0.34[b]	11.65±0.33[b]	12.13±0.80[ab]	13.05±0.48[a]

[1] Means with the same superscript are not statistically different ($p>0.05$, n=4)and means without superscript are not statistically different among treatments ($p>0.05$).

Table 6. *In-vitro* and *in-vivo* protein digestibility of shrimps fed diets substituted fishmeal with hemoglobin powder at varying levels.

Experimental diets	AG liberated by experimental feed induced enzyme (10^{-7} mole ala/200 µl sample)	*In-vivo* digestibility (%)	Feces (g wet weight/shrimp/29 days)
Control	1.6122±0.1873[1]	86.03±1.09[1]	4.33±0.56[1]
12.5 % HE	1.1844±0.1951	83.25±0.34	4.62±0.43
25 % HE	1.4044±0.3120	85.70±0.44	4.59±1.46
50 % HE	1.5914±0.1647	84.77±1.61	4.96±1.19
75 % HE	1.5171±0.4175	85.36±1.61	3.70±0.53
100 % HE	1.8225±0.3468	83.54±0.30	3.70±0.58

[1] Means are not statistically different in all parameter ($p>0.05$, n=3)

other HE diets showed an inverse trend with inclusion levels on trypsin gene expression.

4. Discussion

The decreasing growth responses and feed utilization with increasing levels of HE had two main causes. The first was that the reduction in feed intake indicated an unpalatability of HE. Feed intake of shrimps fed 100% HE diet was only 5.91 g/shrimp whereas those of the control group was 8.20 g/shrimp. The increment of hemoglobin replacement caused the reduction of a non-essential amino acid, glutamic acid, which play an important role as a palatable agent in food and feed, particularly for shrimp and fish (D'Abramo,

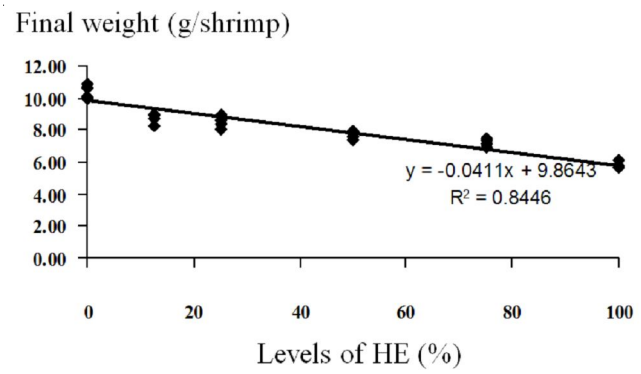

Figure 1. Regression between shrimp final weight and levels of HE substituted for fishmeal

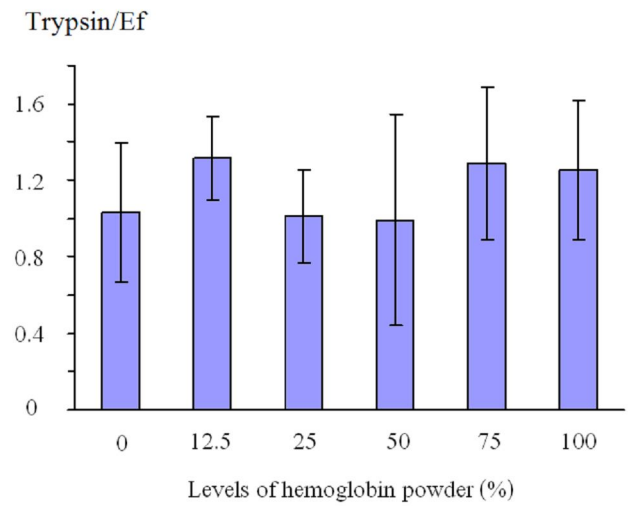

Figure 2. Trypsin gene expression of shrimp fed varying diets with hemoglobin powder replacing fishmeal at different levels (average gene expression from 3 replicates).

1997). Yousif *et al*. (1996) reported a reduced feed intake of tilapia when blood meal was substituted for fishmeal. FCR reflected feed utilization ability of ingested protein by test animals. The reduced consumption of diets with increasing levels of HE indicate that this ingredient acts as a feed suppressant (Lee and Myers, 1996).

The second cause of the reduced feed utilization by shrimp was an imbalanced amino acid profile of the HE diets. Increasing HE levels resulted in the reduction of dietary arginine, isoleucine, and methionine, while increasing leucine levels leading to an imbalance of branched-chain amino acid (BCAA), leucine, isoleucine, and valine. Greater FM substitution by HE resulted in a higher imbalance of BCAA, particularly those between leucine and isoleucine. Arginine and methionine contents in all HE substituted diet were lower than the requirement levels for *L. vannamei,* which are 5.8 and 2.4% of protein, respectively (Akiyama *et al*., 1991), whereas those of the 100% HE diet were only 3.38 and 1.25% of protein, respectively. The present study is the first report on hemoglobin substitution for fishmeal in shrimp diets. The ability of hemoglobin to substitute for fishmeal in *L. vannamei* in this study was lower than those reported for juvenile Japanese eel. Lee and Bai (1997a) reported that the dietary level of hemoglobin inclusion can be up to 50% without essential amino acid supplementation and up to 75% with essential amino acid supplementation without an adverse effect on growth performance and feed utilization. A similar result was reported in Nile tilapia where Lee and Bai (1997b) successfully replaced FM with HE up to 50%. Martinez-Llorens *et al*. (2008), in contrast, reported that juvenile and on-growing gilthead sea beam fed diets containing hemoglobin powder substituted for fishmeal up to 15% showed a reduction in growth, which is similar to the level of replacement in the current study. The difference among these species could be due to differences in essential amino acid requirements of animals. Substitution for fishmeal with hemoglobin powder in high quantity may elevate the iron level in the body, resulting in pathological effects. Despite no pathological evidence in this study, shrimp fed 100% HE diet had the highest ash content, which was similar to those observed in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) when fed diets with spray-dried blood meal replacing 17.5% fishmeal (Fowler and Banks, 1976). Blood meal is known to contain high levels of iron (2,769 mg/kg) and zinc (306 mg/kg) (NRC, 1993). In general, the responses of different species to blood meal incorporated diets have been varied.

Whole blood meal is closely related to hemoglobin powder although it contains blood plasma. Many researchers have studied replacement of blood meal for fishmeal either in shrimp and fish. Dominy and Ako (1988) reported that 15% blood meal products (ring-dry blood meal, sun-dried blood meal, sun-dried blood meal added crystalline methionine or sun-dried blood meal accompanied covalently linked methionine) could be substituted for marine protein in diets fed to *L. vannamei* without any effect on survival, growth performance and feed utilization as compared to the control groups. However, Abery *et al*. (2002) found that Murray cod *Maccullochella peelii peelii* (Mitchell) receiving a diet containing 8% blood meal had lower final weight and higher FCR compared to those fed the control diet. In addition, Martinez-Llorens *et al*. (2008) reported that blood meal can substitute for fishmeal up to 15% with no effect on growth performance while hemoglobin substituted at the same level caused a reduction in fish growth (Martinez-Llorens *et al*., 2008).

Both *in-vitro* and *in-vivo* digestibility were high with increasing HE levels. The results of this study demonstrate that hemoglobin powder used in the diets were easily digested by white shrimp, which was similar to those reported in Australian snapper *Pagrus auratus* (Booth *et al*., 2005). The ingested and digested diets nevertheless could not be utilized by shrimp because of the amino acid imbalance and deficiencies associated with the HE levels included in diets.

Growth response was not reflected by the trypsin gene expression in the present study. In 12.5% HE fed shrimp

with the growth response second to the control group gave the highest gene expression, while *in-vitro* and *in-vivo* digestibility values were the lowest. The results might be the responses to the better amino acid profile of the diet rather than to the HE inclusion level. Meanwhile, 25% and 50% HE diet showed trypsin gene expression equal to that of the control diet (0% HE) whereas 75% and 100% HE diet were second to the 12.5% diet. This could be due to the replacement of the HE at 25%, whereas 50% HE had an equal digestibility resulting in the similar gene expression. 75% and 100% HE diets had affected the palatability of that diet resulted in unsatisfying feed intake and finally affected physiological functions, which is the high gene expression that took place in this case due to the hunger. It may be deduced that the quality and unpalatability of diets, diet digestibility, and the physiological responses of shrimp resulted in the low growth response of shrimp fed high levels of hemoglobin powder. This is the first report of the relationship between diet quality and gene expression in this species. Muhlia-Almazan *et al*. (2003) studied trypsin gene expression in response to dietary protein levels in *L. vannamei* and found that 30% protein fed shrimp showed the highest gene expression as compared to 15% and 50% protein fed shrimp. In addition, Sanche-Paz *et al*. (2003) reported that trypsin-encoding RNA levels were strongly influenced by starvation by *L. vannamei*, resulting in an increase after 24 hrs of starvation.

The gelling property of HE can be observed during feed production of the present study. The 100 % HE diet had good water stability with leaching loss of only 6.86% while that of the control diet was 12.07%**.** Incorporation of hemoglobin powder in shrimp diets will be an advantage for the pellet binding capacity (Hertrampf and Piedad-Pascual, 2000) besides the alternative protein source.

From an economic point of view, HE could be considered as an alternative protein source for fishmeal due to its high protein content, good binding property and digestibility. However, the appropriate level of substitution in shrimp diets needs to be investigated and its use in combination with other protein sources maybe necessary to overcome nutritional deficiencies.

5. Conclusion

The results from this study clearly demonstrate the inability of *L. vannamei* to utilize HE, even at 12.5% replacement for fishmeal protein. Further studies on the appropriate level of HE in diet for *L. vannamei* need to be conducted.

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