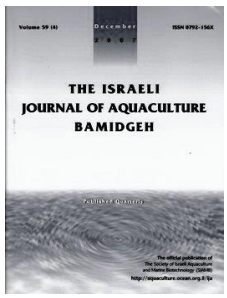

Replacement of Soybean Meal with *Rhizoclonium riparium* Protein Concentrate in the Diet of Pacific White Shrimp *Litopenaeus vannamei* Postlarvae

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Keywords: chlorophyte; seaweed growth promoting effect; body composition effects of seaweed

Abstract

The biological value of incorporating *Rhizoclonium riparium var. implexum* protein concentrate (RPC) meal in the diet of Pacific white shrimp *Litopenaeus vannamei,* postlarvae was evaluated for 60 days. Four diets were prepared: no seaweed inclusion (0%, control diet), 5.25% seaweed inclusion, 10.5% seaweed inclusion, and 15.75% seaweed inclusion with equivalent replacement rates of soybean meal at 0, 15%, 30% and 45%. Final average body weight (FABW) and weight gain (WG) of the shrimp were not significantly affected by the RPC supplementation ($P>0.05$) except those of shrimp fed the highest level of 15.75% RPC, which showed lower values but were statistically similar to those of the 5.25% group. Specific growth rate (SGR) and protein gained (PG) values of shrimp were not affected by the dietary RPC except those of the 15.75% group, which showed significantly lower values. Neither feed conversion efficiency (FCE) nor protein efficiency ratio (PER) showed a clear trend for graded concentration of RPC. Survival of shrimp was relatively good and statistically similar ranging from 76.0%-84.0%. In conclusion, *Rhizoclonium* protein concentrate can replace 30% soybean meal in the diet of *Penaeus vannamei* postlarvae or an equivalent of 10.5% inclusion rate without negative effects on the survival, growth, and feed conversion efficiency of white shrimp.

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Introduction

Protein sources in the animal feed industry contain more than 20% crude protein. Green seaweeds contain less than this amount: 9.9% in *Enteromorpha (Ulva) intestinalis* (Serrano and Aquino, 2014), 13.4% in *Ulva lactuca* (Santizo et al 2014), 13.93% and 15.6% d.w. in *Rhizoclonium riparium var implexum* (Cabanero et al 2015 and Sedanza and Serrano 2016, respectively). However, protein content can be improved by acid precipitation to 38.4% in *Ulva lactuca* (Santizo et al 2014), 31.6% in *Enteromorpha (Ulva) intestinalis* (Serrano and Aquino 2014). The concentration of crude protein in *Rhizoclonium riparium var implexum* was increased from 15.55% d.w. to 23.9% d.w. by solid state fermentation (Sedanza 2016). The chemical score (CS) and essential amino acid index (EAAI) of *Rhizoclonium riparium* protein concentrate (RPC) as a feed ingredient for penaeid shrimp is estimated to be 64 and 105% of shrimp EAA requirement, respectively (Serrano 2016). The EAAI indicates well-balanced essential amino acids with 100% of EAA requirement as a perfect score. In contrast, the CS reveals that when used as the sole source of protein, RPC can only support growth at 64% of its theoretical maximal growth (Serrano 2016); its most limiting amino acid was lysine. Soybean meal itself is deficient in methionine, threonine, and lysine in this order (Berry et al 1962). Thus, soybean itself can replace the deficiency of RPC together only with other protein sources in the diet of penaeid shrimp.

RPC has been evaluated in Nile tilapia *Oreocromis niloticus* (Cabanero et al., 2015a, b) but has not yet been studied in shrimp. The objective of this study was to evaluate crinklegrass *Rhizoclonium riparium var implexum* protein concentrate as an ingredient to replace soybean meal the diet of Pacific white shrimp *Litopenaeus vannamei.*

Materials and Methods

Collection and preparation of Rhizoclonium riparium. Rhizoclonium riparium var. implexum (Dillwyn) Kützing (1843) was previously identified by Dr. Anicia Q. Hurtado – Ponce, an expert in macroalgae identification in the Philippines (Bunda et al., 2015a). The seaweed was collected from brackish water ponds of the Brackishwater Aquaculture Center (BAC) of the Institute of Aquaculture, College of Fisheries and Ocean Sciences (CFOS-IA), University of the Philippines Visayas (UPV) in Brgy. Nabitasan, Leganes, Iloilo and transported to the Multi-Species Hatchery of CFOS-IA, UPV in Miagao, Iloilo for processing.

Protein concentrate of the seaweed was prepared following a modified method of Virabalin et al (1993) which included an acidification stage. Dried *Rhizoclonium* seaweed was homogenized with distilled water using a mechanical juicer. The slurry was acidified by adding HCL to pH 3.0, heated to 90°C for 20 min and the coagulated protein filtered through a muslin cloth. The thick protein concentrate slurry was oven-dried to about 10% moisture, ground into fine powder using a coffee grinder (KRUPS F203) and was kept at -20°C until further use.

A control and 3 test diets with different levels of inclusion of RM for Pacific white shrimp were formulated following Bunda (2015) (see Table 1) at the Nutrition Laboratory of the Institute of Aquaculture, University of the Philippines Visayas (UPV). Before mixing, all ingredients were passed through a 150µm sieve. All ingredients were mechanically mixed, pelleted and oven-dried for 8–12 h at 60°C. The pellets were then sieved to a size of 4mm and stored in polyethylene bags at -20°C until use.

Table 1. Ingredient and proximate composition of the *Rhizoclonium riparium var implexum* protein concentrate experimental diets fed to the Pacific white shrimp (*Penaeus vannamei*) postlarvae.

	0% RPC	*5.25% RPC*	*10.50% RPC*	*15.75% RPC*
Danish fish meal	250.00	250.00	250.00	250.00
Squid meal	29.00	29.00	29.00	29.00
Soybean meal	350.00	297.50	245.00	192.50
Bread flour	117.00	117.00	117.00	117.00
Cod liver oil	63.00	63.00	63.00	63.00
Lecithin	5.00	5.00	5.00	5.00
Carboxy Methyl Cellulose	130.50	130.50	130.50	130.50
Lignobond	15.00	15.00	15.00	15.00
*Vitamin mix**	10.00	10.00	10.00	10.00
Mineral mix	10.00	10.00	10.00	10.00
Dicalphosphate	20.00	20.00	20.00	20.00
BHT	0.50	0.50	0.50	0.50
Rhizoclonium Protein Conc.	0.00	52.50	105.00	157.50
Total	1000.0	1000.0	1000.0	1000.0
Dry matter	10.12	11.85	10.61	9.18
Crude protein	35.96	34.28	33.48	32.36
Crude lipid	9.20	8.37	9.08	8.21
Crude fiber	2.56	2.64	3.01	2.69
Ash	12.09	14.63	17.37	21.07
NFE	32.63	30.87	29.46	29.18
Total	100.0	100.0	100.0	100.0

*Vitamin mix: Vitamin A, 1 200 000 IU/kg; Vitamin D_3, 200 000 IU/kg; Vitamin E, 20 000 IU/kg; Vitamin B_1, 8 000 mg/kg; Vitamin B_2, 8000 mg/kg; Vitamin B_6, 5000 mg/kg; Vitamin B_{12} 1%, 2000 mcg/kg; Niacin, 40000 mg/kg; Calcium Pantothenate, 20000 mg/kg; Biotin, 40 mg/kg; Folic Acid, 1800 mg/kg; Ethoxyquin, 500 mg/kg

Growth trial. Two thousand *P. vannamei* post larvae shrimp from a commercial shrimp hatchery in Guimbal, Iloilo were nursed in a 1-ton capacity tank at the University of the Philippines Visayas Multi-Species Hatchery. The shrimp were acclimated and fed a commercial diet for 2 weeks. Before the experiment, the shrimp were randomly selected and screened, in the Fish Disease Laboratory of SEAFDEC, by one-step PCR for WSSV detection and were found to be virus free.

The growth trial was conducted using a recirculating system annexed to a sedimentation tank filtered by layers of cotton fiber, a biological filter containing oyster empty shells and mechanical filter tanks i.e., containing sand and gravel. The water was recirculated from the series of filter tanks to the 50 L capacity aquaria at a flow rate of about 600 ml/min/tank. Salinity ranged between 25-27ppt, temperature between 25–27°C, pH between 8.5-9.0 and dissolved oxygen was maintained at >5 ppm. These were monitored weekly. Nitrite (0-0.015 ppm) and total ammonia-nitrogen (TAN) that ranged between 0-0.02 were measured with commercially available kits (CP Aqua Test kits) and maintained at a low level by 100% water replacement in the recirculating system every 5-7 days. Each aquarium was provided with adequate aeration and cleaned daily by siphoning uneaten feed and feces before feeding.

Five hundred post larvae shrimps (0.06 ± 0.02 g) were distributed randomly into 20 substrate-free 50L culture tanks while the rest (1,500 post larvae) were sacrificed for the initial body composition. The shrimp were further acclimated to the experimental condition and to the control diet for 5 days. Four replicates of the experimental diets containing varying levels of the RPC, namely, 0% (control), 5.25%, 10.50% and 15.75% were fed to the shrimp in a completely randomized design. Feed was administered three times daily (08:00, 12:00 and 16:00) for 60 days and at a ration starting at 20% of average wet body weight and decreased to 6% towards the end of the experimental period. Sampling was conducted at the beginning of the experiment and every 15 days thereafter and shrimp from each tank were counted and bulk-weighed.

Growth measurement. The parameters measured were weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), percent survival, feed conversion efficiency (FCE) and protein gained (PG). These were calculated using the following formulas:

$$WG\ (g) = FBW - IBW$$
$$SGR\ (\%/day) = 100*(LnFBW - LnIBW)/D$$
$$\%\ FCE = 100*(FBW - IBW)/FI$$
$$PG\ (g) = (CP_f \times FBW) - (CP_i \times IBW) \times 100$$
$$PER = (FBW-IBW)/(FI \times FP)$$
$$Survival,\ \% = 100*\text{Final count of shrimps/Initial count of shrimps}$$

Where: FBW = final body weight (g) of individual fish; D = days of culture; IBW = initial body weight (g) of individual fish; FP = Feed Protein (in decimal); CP_i= Initial carcass protein (in decimal); CP_f= Final carcass protein (in decimal); FI = total feed intake of individual fish for the whole duration of the experiment

Analytical Methods and Calculations. Samples of the diets were submitted to Oversea Feeds Laboratory in San Fernando, Cebu, Philippines for proximate analysis (AOAC, 1990). Shrimp body composition analysis for dry matter, crude protein, and crude fat analysis (AOAC, 1990) were conducted in the laboratories of the Institute of Aquaculture and the Institute of Fish Processing Technology (CFOS-IFPT), University of the Philippines Visayas (UPV). All analyses were carried out in triplicate. Dry matter was determined by placing the sample in a constant weight crucible and dried in an oven at 100°C. Determination of crude fat was done using Foss®Soxtec 2055. Ash content was determined after incineration in a muffle furnace at 550° C for 12 h. Crude protein was measured after block digestion and steam distillation using Kjeltec digestion system set at a temperature of 400° C and Foss KjeltecTM 8200 auto-distillation unit.

Statistical Analyses. Data are presented as mean ± standard error of the mean (SEM) for each dietary treatment. They were analyzed using Levene's test for homogeneity of variances and Shapiro-Wilk test for normal distribution before using one-way analysis of variance (ANOVA). Tuckey test for mean separation was used to evaluate significant differences ($P< 0.05$) among treatment means.

Results

Growth and food conversion efficiency. Growth indices such as FABW, WG and SGR, food conversion efficiency (FCE, PER and PG) and survival rate of post larvae shrimp fed experimental diets are shown in Table 2. FABW and WG of shrimps were not significantly affected by the RPC supplementation ($P>0.05$) except those of shrimps fed the highest level of 15.75% RPC which showed lower values which were statistically similar with those of the 5.25% group. Similarly, SGR and PG values of shrimps were not affected by the dietary RPC except those of the 15.75% group. FCE values, in contrast, revealed that the groups fed diets containing 0% and 10.5% were statistically similar but significantly higher than those in 5.25% and 15.75% groups. PER values were statistically similar in the control, 5.25% and 15.75% but lower than the values exhibited by the 10.5% RPC group. Both FCE and PER did not show a clear trend *viz a viz* the graded concentration of RPC. Survival of shrimp was relatively good and statistically similar with a mean range of 76.0%-84.0%.

Table 2. Growth performance of *Penaeus vannamei* postlarvae fed experimental diets containing increasing levels *of Rhizoclonium* Protein Concentrate meal

	Parameters						
	FABW	*WG*	*SGR*	*FCE*	*PER*	*PG*	*%SURV*
0% RPC	1.30 ± 0.17^{b}	1.2 ± 0.17^{b}	5.1 ± 0.227^{b}	0.59 ± 0.02^{b}	1.6 ± 0.06^{ab}	0.82 ± 0.12^{b}	78.0 ± 6.2^{a}
5.25% RPC	0.97 ± 0.02^{ab}	0.91 ± 0.02^{ab}	4.7 ± 0.03^{b}	0.51 ± 0.02^{a}	1.5 ± 0.06^{a}	0.63 ± 0.01^{b}	79.0 ± 2.8^{a}
10.50% RPC	1.10 ± 0.03^{b}	1.1 ± 0.03^{b}	5.0 ± 0.05^{b}	0.58 ± 0.00^{b}	1.7 ± 0.01^{b}	0.63 ± 0.02^{b}	76.0 ± 1.6^{a}
15.75% RPC	0.69 ± 0.06^{a}	0.63 ± 0.06^{a}	4.1 ± 0.14^{a}	0.49 ± 0.01^{a}	1.5 ± 0.02^{a}	0.44 ± 0.04^{a}	84.0 ± 3.7^{a}

FABW = Final Ave. Body Weight; WG= Weight Gain; FCE= Feed Efficiency Conversion;
SGR= Specific Growth Rate; PG= Protein Gained (g); PER= Protein Efficiency Ratio; Surv= Survival. Values are expressed as mean ± SEM. Mean values in columns with different superscript letters are significantly different ($P< 0.05$).

Body composition. Whole body composition of shrimp fed the RPC supplemented and control diets are shown in Table 3. All the experimental groups of shrimp were statistically similar in body moisture, crude protein, and ash with the exception of body crude lipid which was significantly higher in shrimps fed the diet containing 15.75% RPC.

Table 3. Whole body composition of post larvae *Penaeus vannamei* fed graded levels of *Rhizoclonium riparium var implexum* Protein Concentrate (RPC) meal.

	Moisture	*Crude Protein*	*Crude Lipid*	*Ash*
Initial carcass	17.3 ± 0.30	56.6 ± 2.2	4.14 ± 0.0	3.9 ± 0.04
		Final carcass		
Control	23.3 ± 0.4 [a]	66.6 ± 2.5[a]	4.0 ± 2.4[a]	3.6 ± 0.2[a]
5.25% RPC	22.8 ± 0.7[a]	68.1 ± 1.6[a]	3.1 ± 0.1[a]	3.6 ± 0.2[a]
10.50% RPC	22.8 ± 0.1[a]	58.9 ± 4.7[a]	4.8 ± 1.1[a]	3.5 ± 0.2[a]
15.75% RPC	20.9 ± 0.4[a]	68.6 ± 1.7[a]	11.8 ± 0.0[b]	3.8 ± 0.1[a]

Values are expressed as mean ± SEM. Mean values in columns with different superscript letters are significantly different ($P < 0.05$). Data presented as percent dry basis.

Discussion

It has been previously reported that RPC contains crude protein (23.94%) and good essential amino acid index (1.1) which makes it a potential feed ingredient for aquafeeds (Sedanza & Serrano 2016; Serrano 2016). The present study evaluated the biological value of protein concentrate of crinkle grass *Rhizoclonium sp.* Incorporating RPC at low levels (5.25%-10.50%) improved or did not negatively affect the growth performance and feed efficiency of *Litopenaeus vannamei.* However, significant negative effects on SGR, FCE and PG were observed when 15.75% RPC (i.e. 45% replacement of soybean meal) was incorporated in the shrimp diet. These results suggest the inclusion level favorable for shrimp growth is between 5.25%-10.50%; however it was lower than that of the raw meal of *Rhizoclonium* which is as high as 15.75% not only for replacing soybean but also simultaneously replacing 100% of the mineral mix (Sedanza and Serrano 2016). This trend may be attributed to the process of concentrating proteins in the seaweed (Santizo et al., 2014; Kandasamy et al., 2012). Oxidized phenolic compounds may have reacted with the amino acids and proteins in the present study, inhibiting the activity of proteolytic enzymes and in turn lowering the nutritional value. Phenolic compounds lead to formation of insoluble/indigestible complexes with protein that interfere with its utilization (Wong and Cheung 2001). The findings of Santizo et al., (2014) showed similar results. The authors attribute these results to the small amounts of anti-nutrients found in the *Ulva* protein concentrate at higher inclusion level of 15.75% (i.e. 45% soybean meal replacement by weight in the diet); it could have led to the inferior growth performance of *P. monodon.* Another factor may be that due to the disproportionate amounts of amino acids found in the RPC diet. Although the EAAI of RPC was 110% of the shrimp's EAA requirement based on our recent findings, the chemical score was only 64% with lysine as the first limiting EAA (Serrano 2016). This means that RPC can only supply 64% of lysine required by the Pacific white shrimp and that the difference needs to be supplemented in the diet. The process of concentrating protein from *R. riparium* in the present study using both pH shift and heat treatment might have destroyed some of the lysine which is known to reduce the oxidation of other amino by improving the use of other EAAs (Kerr and Kerster, 1985). The remaining lysine in the present study resulted in lower than expected growth rate.

Although the results of this study showed that reduced growth and feed efficiency occurred at the highest inclusion rate, no other adverse effects were observed. Survival rate and the carcass composition, except for crude lipid content, did not vary significantly among all treatments. A high carcass lipid increase was observed at the highest inclusion level. The composition of dietary lipid affects that of the carcass lipid (Molina-Poveda, 2016). Pearson correlation analysis of the lipid content of the diet and that of carcass showed a similar trend in which an increased amount of dietary lipid linearly increased that of carcass. As amino acids cannot be stored in the body for later use, any amino acid not required for immediate biosynthesis is deaminated, and the carbon skeleton is

used as metabolic fuel or converted into fatty acids (FAs) via acetyl CoA (Molina- Poveda, 2016). The high amount of RPC inclusion might have exacerbated the already reduced lysine (Serrano 2016), which is a precursor of carnitine, a very important component in the transport of long-chain fatty acyl groups into the mitochondria for beta oxidation (Tanphaichitr et al., 1971).

In conclusion, RPC could be a substitute for soybean meal in the diet of *Penaeus vannamei* postlarvae up to 30% which is equivalent to 10.5% inclusion level, without compromising the survival, growth and feed utilization efficiency of the shrimp.

Acknowledgements

The authors wish to express their gratitude to the Philippine Department of Science and Technology (DOST)-Philippine Council for Agriculture, Aquatic and Natural Resources Development (PCAARRD) and DOST- Accelerated Science and Technology Human Resource Development Program (ASTHRDP). They also wish to thank the UPV Office of the Research and Extension for the partial research funding and publication support.

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