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Effect of dietary protein level on its *in vitro* and *in vivo* digestibility in the tiger shrimp *Penaeus monodon* (Crustacea: Penaeidae)

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Protein being the most important and expensive nutrient in shrimp feed, determination of its appropriate level in relation to the digestive capacity of shrimp is essential in order to make the feed cost effective as well as to minimize the nitrogenous waste excretions. Six diets having different levels of crude protein (30-41 %) were investigated by *in vitro* digestibility method using the homogenate of hepatopancreas (digestive proteases) of tiger shrimp *Penaeus monodon*. Peak digestibility of dietary protein was observed at 3 hours incubation. Maximum digestibility of protein (69.19 %) was recorded with diet having 35.28 % protein. The *in vivo* results in tiger shrimp (2.0 g) also showed that the weight gain in the shrimp was also highest at this dietary protein level. The average apparent protein digestibility was highest (76.02 %) in animals fed with diet having 35 % crude protein. The results of the study suggested that dietary protein for *P.monodon* can be lowered to 35 %, considerably reducing the cost of the feed and making it more environmental friendly.

[**Key words**: Digestibility, enzyme studies, *Penaeus monodon*, shrimp nutrition]

Compounded feeds play a major role in penaeid shrimp culture, constituting up to 60 % of total costs[1]. Protein is the most critical ingredient in shrimp diets in terms of its cost[2] as well as growth of shrimp[3].The feeds must be nutritionally adequate and economical. The protein requirement for optimal growth of penaeid species has been reported to be in the range of 35 and 61 %[4-6]. The pronounced differences in reported protein requirements may be due to different protein sources, dietary energy levels in diets and rearing conditions used in these studies. One of the factors in the environmental management in aquaculture is the feed related nitrogenous wastes. Nitrogen is provided in high concentrations in shrimp feed but most (80 %) of it is added to the ponds and it is not retained as shrimp biomass[7]. The low retention of dietary N can be caused by several factors : sub optimal feed formulations or quality of ingredients, and poor water stability of feeds[8]. Therefore, determination of protein level vis-à-vis the digestive capability of the shrimp, will help not only in optimizing the protein levels in feed but also reducing the nitrogenous waste generation, and makes the feed more cost effective and environmental friendly.

The digestibility of protein in shrimp diets is principally determined by feeding trials using inert markers, which are often time consuming and expensive. The *in vitro* digestibility method, though may not replace the conventional digestibility method, but it can be used to assess the potential digestibility of diets and feed stuffs[9]. Digestive proteases from the test animal rather than those commonly used and commercially available (i.e. from mammals or micro- organisms), can better assess the digestibility of protein[10]. The present study was aimed at investigating the effect of dietary protein level on its *in vitro* and *in vivo* digestibilities in the tiger shrimp *Penaeus monodon* Fabricius (Crustacea: Decapoda: Penaeidae), based on which more appropriate level of protein in shrimp diets could be suggested.

Materials and Methods

Six practical diets having different levels of protein were prepared by replacing the protein base with wheat flour on w/w basis (Table 1). Ingredients like fish meal, mantis shrimp (*Oratosquilla nepa*) and squid (*Loligo* sp.) were ground in a micropulveriser and passed through a 300-µm mesh screen. All the dry ingredients including 1 % chromium oxide (as an inert marker) were mixed in an electrical blender and the lipid sources were added and thoroughly homogenized. Water was then added (30 ml/100 g feed) to the diet mix and kneaded into a dough. It was steamed for 5 minutes at atmospheric pressure and pelleted in a hand pelletizer with a 2-mm die. The pellets were dried at 60 °C for 12 hours and stored in desiccator until use. The diet samples were powdered in a cyclotec sample mill (Tecator) and sieved (No. 20) for

incubation for *in vitro* digestion study. The proximate composition of the test diets was analysed as per the standard AOAC[11] methods. All the analyses were done in duplicate.

Specimens of wild tiger shrimp, *P.monodon* were obtained from the wild and stored in a deep freezer (–20 °C). Frozen animals were cut open and the digestive gland (hepatopancreas) was dissected out and quickly weighed. The glands from ten animals were pooled and homogenized in 10 ml distilled water in a tissue homogenizer. The homogenate was centrifuged in a refrigerator centrifuge at 10,000 rpm for 15 min. The supernatant (enzyme solution) was decanted and stored at –70 °C.

Fat free casein (Sigma) solution (0.01 %) in 0.1 *M* Phosphate buffer (*p*H 7.6) was used as standard. About 1 g of dry feed powder was suspended in 49 ml of 0.1 *M* phosphate buffer (*p*H 7.6). The protein concentration in the buffer solutions was 0.75, 0.69, 0.63, 0.60, 0.57 and 0.54 % for diet 1 to diet 6, respectively. To this 1 ml of enzyme solution was added and incubated. Simultaneously casein solution (as control) was also incubated in the same way with the enzyme solution. After the incubation, at time intervals of 1,2,3,4 and 5 hour, 2 ml aliquots from each sample were taken out and the reaction was arrested by adding 3 ml of 5 % trichloroacetic acid (TCA). After centrifugation, the supernatant was collected and colour was developed by using the Folin Ciocalteu phenol reagent. From this the amount of tyrosine released was measured using spectrophotometric method against tyrosine standard[12] at 691 nm. One unit of protease activity was expressed[13] as milligrams of tyrosine liberated in 15 minutes. All the incubations were carried out in triplicate. The enzyme activity in the tissue solution prepared and used in the incubation was calculated[14] as:

$$\text{Protease activity} = \frac{C \times 1000}{T \times V} \text{ units/ml}$$

where C = µg/ ml of casein hydrolyzed, T = duration of incubation (minutes) and V = volume of enzyme solution (µl).

For *in vivo* digestibility determination of test diets, juveniles of *P. monodon* weighing 1.5 to 2.4 g were used. Ten shrimps per tank (three tanks per treatment) were randomly distributed in 100 liter oval Fibre Reinforced Plastic (FRP) indoor tanks equipped with a system supplying seawater and air through a porous stone. During the experiment, the water temperature

Table 1 — Ingredient and proximate composition of test diets

Ingredients/ proximate parameters	Diet no.					
	1	2	3	4	5	6
Protein base* (%)	75.00	65.00	55.00	45.00	35.00	25.00
Wheat flour (%)	16.50	26.50	36.50	46.50	56.50	66.50
Fish oil (%)	3.00	3.00	3.00	3.00	3.00	3.00
Lecithin (%)	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin Mix[1] (%)	0.50	0.50	0.50	0.50	0.50	0.50
Mineral Mix[2] (%)	1.00	1.00	1.00	1.00	1.00	1.00
Guar Gum (%)	1.00	1.00	1.00	1.00	1.00	1.00
Chromium Oxide (%)	1.00	1.00	1.00	1.00	1.00	1.00
Dry matter (%)	91.18	90.12	89.63	90.83	90.43	89.13
Crude protein (%)	41.16	38.42	35.28	33.12	31.63	30.18
Crude fibre (%)	3.92	4.06	4.52	4.68	5.16	5.30
Ether extract (%)	12.28	10.12	11.63	10.83	10.86	10.02
Ash (%)	23.38	20.81	18.17	16.66	15.81	13.18
NFE[3] (%)	19.26	26.59	30.40	34.71	36.54	41.31

*Protein base: Fish meal 40 parts, Squid meal 5 parts, Mantis shrimp (Squilla) meal 5 parts and Soya flour 25 parts.

[1]Vitamin mixture: (mg/100g) Vitamin A 2.0, Vitamin D 0.4, Vitamin E 12.0, Vitamin K 6.0, Choline Chloride 600.0, Thiamine 18.0, Riboflavin 24.0, Pyridoxine 18.0, Niacin 108.0, Pantothenic acid 72.0, Biotin 0.2, Folic acid 3.0, Vitamin B_{12} 0.015, Inositol 150.0, Vitamin C 900.0.

[2]Mineral mixture: (g/kg) $CaCO_3$ 28.0, $NaHPO_4$ 22.0, K_2SO_4 10.0, Mg SO_4 12.5, Cu SO_4 0.2, $FeCl_3$ 0.5, $MnSO_4$ 0.5, KI 0.01, $ZnSO_4$ 1.0, $CoSO_4$ 0.01, Cr_2 SO_4 0.05, Bread flour 7.14.

[3]NFE calculated by difference = 100 - (moisture % + Crude protein % + Crude fibre % + Ether extract % + Ash %)

was at 29 ± 1 °C, salinity 33 $^{0}/_{00,}$ and dissolved oxygen at 7 mg/l. Eighty percent of the water in the tanks was exchanged daily. Shrimp were fed on a ration equal to 7 % of body weight daily. The feeding was done twice daily, at 11.00 AM and 5.00 PM. Weight of the shrimp was recorded once in 10 days and the quantity of feed was adjusted accordingly. Uneaten food was removed from the tank every morning and oven dried at 100 °C for 24 hours for determination of dry feed intake. Duration of the experiment is 30 days. Faecal matter was collected after 4 h of feeding from 4th day of experiment. Faeces were carefully collected with a pipette on to a bolting silk cloth, gently washed with distilled water and freeze dried immediately. Chromium content in diet and faeces was analysed [15] and was used for calculating the apparent protein digestibility[16]:

$$\text{Apparent digestibility of protein (\%)} = 1 - \frac{(\%\text{ protein in faeces}/\%\ Cr_2O_3\text{ in faeces})}{(\%\text{ protein in diet}/\%\ Cr_2O_3\text{ in diet})} \times 100$$

The average percent weight gain, survival (%), feed conversion ratio (FCR) and protein efficiency ratio (PER) per tank were calculated[16], as:

Weight gain (%) = $[w_1 - w_0] \times 100/w_0$

FCR = Dry feed consumed/wet weight gain

PER = $[w_1 - w_0]/D_p$

where w_1 = final wet weight (g), w_0 = initial wet weight (g) and D_p = dry protein intake (g).

The *in vitro* digestibility experiment was conducted under factorial randomized block design and *in vivo* digestibility trial was conducted under completely randomized design (CRD)[17]. Statistical analysis was done using MSTAT-C statistical software package.

Results

The proximate composition of different diets is presented in Table1. The digestibility of crude protein (CP) in feed as determined by the *in vitro* method increased with time up to three hours and after that no improvement on digestibility was observed. The digestibility of dietary protein was significantly ($P < 0.05$) higher (62.72 %) at three hours after incubation when compared to 1st and 2nd hour and no significant difference in digestibility was observed between three and five hours (Table 2). The protein level had significantly ($P < 0.05$) influenced its digestibility. The diet with 35.28 % of CP showed highest digestibility of 69.19 % at the end of three hours.

The results of growth with digestibility trial conducted on the juveniles of *P. monodon* indicate that all groups had high survival (more than 85 %) and values were not significantly different ($P > 0.05$), indicating that differences in dietary protein did not influence survival (Table 3). The weight of shrimps increased with time in all the treatments (Fig. 1) during the feeding trial and it was measured once in every ten days. Significantly ($P < 0.05$) higher weight gain (100.13-102.35 %) was observed in shrimp fed with higher protein diets 1, 2 and 3 (41.16-35.28 %) when compared to those fed with low protein diet 6 (30 %). However, increasing the dietary protein levels higher than 35 % in the diet did not improve weight gain in shrimp. But, significantly ($P < 0.05$) lower FCR was observed in diet 1 (Table 3). However, no significant difference in FCR was observed in animals fed with 38.42 % and 35.28 % CP. PER did not differ significantly ($P > 0.05$) among treatments (Table 3).

The apparent protein digestibility of protein in diet 3 is significantly ($P < 0.05$) higher than that of other

Table 2 — *In vitro* digestibility of dietary protein at different time intervals

Diets [CP %]	Time (hours)					
	1	2	3	4	5	Mean ± S.D.
1 [41.16]	48.76	54.31	61.42	60.73	62.18	57.48 ± 5.79^{b}
2 [38.42]	41.38	58.63	64.26	60.13	60.02	56.88 ± 8.92^{ab}
3 [35.28]	54.33	65.11	69.19	68.21	67.83	64.94 ± 6.12^{d}
4 [33.12]	48.62	58.21	62.63	64.02	63.81	59.46 ± 6.5^{c}
5 [31.63]	50.02	51.38	58.11	60.12	59.23	55.77 ± 4.7^{a}
6 [30.18]	49.12	54.26	60.72	60.31	58.19	56.52 ± 4.87^{ab}
Mean± S.D.	48.71 ± 4.17^{x}	56.98 ± 4.82^{y}	62.72 ± 3.77^{z}	62.25 ± 3.27^{z}	61.88 ± 3.56^{z}	
Caesin [control]	54.31	69.23	82.36	80.41	78.17	

Mean values bearing different superscripts in a column differ significantly (P<0.05).

Mean values bearing different superscripts in a row differ significantly (P<0.05).

CD at 5 % level of significance for Diet x Time interaction is 3.39.

Table 3 — Results of feeding trial with test diets fed to *P.monodon* for 30 days

Diets	Initial weight (g)	Final weight (g)	Weight gain (%)	Survival (%)	FCR	PER
1	1.98±0.18	3.98±0.14	101.20^{c}	96.7	2.22^{a}	1.09
2	2.06±0.09	4.12±0.08	100.13^{c}	100.0	2.38^{bc}	1.09
3	1.89±0.12	3.82±0.11	102.35^{c}	96.7	2.36^{b}	1.20
4	2.21±0.11	3.55±0.20	60.90^{b}	93.0	2.51^{c}	1.20
5	2.11±0.16	3.37±0.13	59.84^{b}	90.0	3.18^{d}	0.99
6	1.75±0.08	2.70±0.09	54.52^{a}	86.7	3.24^{d}	1.02

Mean values bearing different superscripts in a column differ significantly (P<0.05).

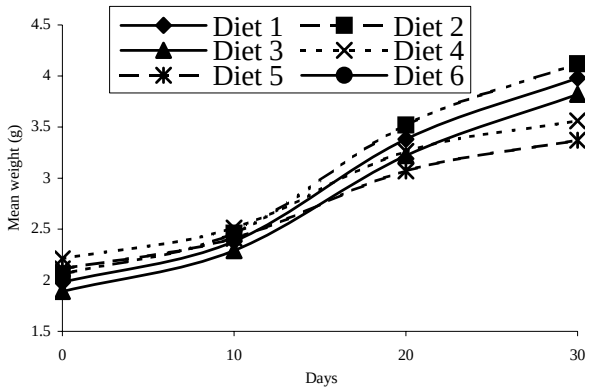

Fig. 1 — Growth of *P. monodon* with time when fed with test diets

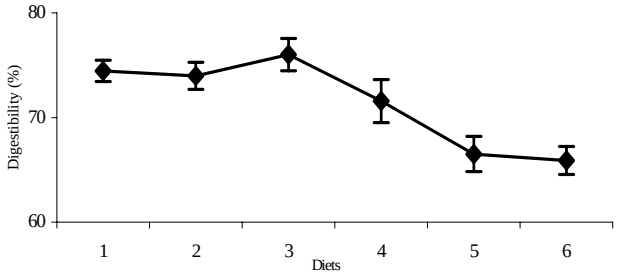

Fig. 2 — Apparent digestibility (*in vivo*) of dietary protein in *P. monodon*

feeds (Fig. 2). The apparent protein digestibility is significantly lower in diets with protein levels < 35 % and this has reflected in poor growth rate in animals fed with low protein diets 4, 5 and 6 (Table 3).

Discussion

The digestibility of dietary protein determined by *in vitro* method in the present study ranged from 41.38 to 69.19 %. These values are comparable to that of compounded diets having casein and albumin as protein sources in *P. indicus* [18,19] . Peak digestibility values were reported after three hours of incubation. This observation is in line with the findings that evacuation of food in the digestive tract of shrimps takes 5–6 hours after feeding[20] with maximum digestibility up to three hours[21].

Dietary protein levels had significant effect on *in vitro* digestibility. Significantly (P < 0.05) higher protein digestibility (69.19 %) was recorded with diet having 35.28 % crude protein. These results suggest that under the experimental conditions the tissue homogenate of the hepatopancreas can handle specific levels of protein in the range of 35 % for effective digestion. The apparent protein digestibility (Fig. 2) as determined by the inert marker also has shown highest (76.02 %) in the shrimp fed with 35.28 % CP. Decreasing trend of apparent protein digestibility (Fig. 2) was observed in animals fed with diets 4, 5 and 6, this may be due to the increase of crude fibre content from 4.52 to 5.30 % (Table 1) in these diets. In a similar study in kuruma prawns, the apparent protein digestibility was decreased as the protein level decreased in the diets[16].

The results in the growth with digestibility trial have shown that shrimp fed with 35 % protein diet had better growth (Table 3), supporting the findings of the *in vitro* digestibility methods[16]. The present study revealed that 35 % CP could sustain good growth and survival of *P.monodon*. The reduction of dietary protein from 41.16 to 35.28 % and consequent increase in non-protein nutrients, mainly carbohydrate (Table 1), did not reduce weight gain significantly in the shrimp, indicating that energy from non-protein sources spared protein utilization. Discrepancies in optimum dietary protein reported[1] by different studies may be due to use of different dietary protein sources, and/or different digestible energy contents. The amino acid profile of dietary protein is more important for better anabolic utilization[16]. The growth of *P.merguiensis* did not change when diets in which the dietary protein content was reduced from 51 to 34 %, while increasing the non-protein nutrients[22]. The growth differences were not due to differences in digestible energy content among diets but due to

differences in dietary protein content[16]. These observations are supportive of the observation made on dietary protein for *P.monodon* in the present study.

The protein levels in practical feeds for most of the penaeid shrimp species is in the range of 40-50 %. It is also observed that considerable quantities of feed related nitrogenous waste is excreted into the system during shrimp aquaculture[23]. This may be due to the feeds having protein levels in excess the digestive capacity by the shrimp. Consequently, the excess undigested protein in feed may be just passed out. In an elaborate study it has been reported[24] that the digestive enzymes set a physiological limit on growth rate and food conversion efficiency in fish. Based on the results obtained in the present study, it is worthwhile to optimize protein levels in shrimp feeds, based on the digestive capacity of the *P.monodon.* This would facilitate in minimizing the nitrogenous waste excretion through faeces making the feed environmental friendly. The reduction in protein level in practical feeds also makes the feed more cost effective.

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