Pigmentation of Cultured Black Tiger Prawn by Feeding with a *Spirulina*-Supplemented Diet

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Black tiger prawn *Penaeus monodon* were fed with diets containing different carotenoid sources: β -carotene, *Spirulina*, *Phaffia*, and krill oil. A marked increase of carotenoid content in the carapace was observed when fed with *Spirulina*-supplemented diets. The best effect of *Spirulina* was obtained at 3% in diets. The analysis of carapace carotenoids in the *Spirulina*-fed prawns suggests that zeaxanthin, one of major carotenoids in *Spirulina*, has been rapidly converted to astaxanthin. In view of these findings, we propose that a practical strategy for pigmentation of cultured *P. monodon* is to feed it with a diet containing *Spirulina* at 3% for 1 month before harvest.

The black tiger prawn *Penaeus monodon* is cultivated widely in South East Asia, and production has been increasing year by year. The greater part of the product is consumed in Japan, but the external color of cultured prawns is generally pale. The color affects the price in the market, well-pigmented prawns being more acceptable because the desirable red coloration can be expected when cooked.

Carotenoids and carotenoproteins are responsible for the color of crustaceans.¹⁾ The distribution and metabolic pathways of carotenoids in Crustacea have been reviewed by Goodwin,²⁾ Davis,³⁾ and Matsuno and Hirao.⁴⁾ Tanaka *et al.*⁵⁾ investigated the metabolism of dietary carotenoids in the prawn *P. japonicus* and suggested the presence of the following two oxidative pathways:

β -carotene $\longrightarrow$ isocryptoxanthin $\longrightarrow$ echinenone
 $\longrightarrow$ canthaxanthin $\longrightarrow$ phoenicoxanthin $\longrightarrow$
astaxanthin: zeaxanthin $\longrightarrow$ 4-ketozeaxanthin
 $\longrightarrow$ astaxanthin.

They also reported that the prawn could directly deposit dietary astaxanthin in their tissues. Yamada *et al.*⁶⁾ and Chen and Jeng⁷⁾ carried out a pigmentation test on *P. japonicus* by various carotenoid sources and levels and feeding regimes.

Recently, astaxanthin was shown to be a predominant carotenoid associated with the body color of the black tiger prawn *P. monodon*.⁸⁾

However, no pigmentation attempt has been made so far for *P. monodon*.

The purpose of this study was to find a suitable pigmenter and to establish a practical strategy of pigmentation for cultured *P. monodon* through feeding experiments.

Materials and Methods

Composition and Preparation of Diets

Four kinds of carotenoid sources, *i.e.* β -carotene, *Spirulina*, *Phaffia*, and krill oil were used. β -Carotene was purchased from Sigma Chem. Co. *Spirulina* was a spray-dried product of mass-cultivated blue-green alga *S. maxima* from Japan Spirulina Co., Ltd. The content and composition of carotenoids were analyzed by the method of Miki *et al.*⁹⁾ with slight modifications, and were found to be 346 mg/100 g excluding carotenoid glycosides and 52% β -carotene, 10% echinenone, 6% β -cryptoxanthin, 5% 3'-hydroxyechinenone, 21% zeaxanthin including *cis*-zeaxanthins, and 7% decomposed and unidentified carotenoids. *Phaffia* and krill oil were supplied by Nippon Suisan Co. The former was a dried preparation of the yeast *Phaffia rhodozyma*, of which carotenoid content and composition were 84 mg/100 g and 4% astaxanthin diester, 33% astaxanthin monoester, 45% astaxanthin, and 18% unknown. The

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Table 1. Composition of test diets for feeding experiments

(g/100 g)

Ingredients	Diet no.						
	1	2	3	4	5	6	7
White fish meal	50	50	50	50	50	50	50
Wheat flour	10	10	10	10	10	10	10
Corn gluten	5	5	5	5	5	5	5
Beef liver meal	3	3	3	3	3	3	3
Mineral mixture* ¹	5	5	5	5	5	5	5
Oil* ²	2	2	2	2	2	2	2
Vitamin mixture* ³	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Vitamin E* ⁴	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Dextrin	22.4	22.39	19.4	10.4	11.4	21.4	17.4
β -Carotene	—	0.01	—	—	—	—	—
<i>Spirulina</i>	—	—	3	—	—	1	5
<i>Phaffia</i>	—	—	—	12	—	—	—
Krill oil	—	—	—	—	11	—	—

*¹ $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O} : \text{CaCO}_3 = 3 : 1$.*² Pollack liver oil: Soybean lecithin = 1 : 1.*³ A preparation of Nippon Roche K.K.*⁴ The DL- α -tocopheryl acetate content was 50%.

latter contained almost exclusively astaxanthin diester at a concentration of 93 mg/100 g in terms of astaxanthin.¹⁰⁾ The composition of test diets are presented in Table 1.

Two feeding experiments were carried out. In experiment I, the effects of the four pigments were examined. Diet 1 was the control diet (carotenoid-deficient). Diets 2 to 5 were supplemented with one of the four pigments, and total carotenoid level was adjusted to around 10 mg/100 g with dextrin. As will be described in the Results section, *Spirulina* was found to be most effective, so in experiment II the minimum dose of *Spirulina* required for desirable pigmentation was checked using diets 1, 3, 6, and 7. Diet 1 again was the control, and diets 6, 3, and 7 contained *Spirulina* at 1, 3, and 5%, respectively.

The ingredients were mixed with 30 to 35% (v/w) water and kneaded. The dough so obtained was extruded into pellets using a meat mincer with holes of 1.9 mm diameter, and was then dried in a freeze vacuum dryer at -40°C overnight until the moisture content was reduced to about 10%. They were stored in a freezer at -25°C until use.

Feeding Experiments

Live juvenile prawns *P. monodon* were obtained from Taiwan. The feeding experiments were carried out at the Fisheries Laboratory, Faculty of Agriculture, The University of Tokyo, Shizu-

oka, Japan. In experiment I, prawns were fed with the control diet for 10 days and then randomly divided into 5 groups, each with 45 individuals (average weight 1.1 g), and placed in outdoor concrete pools (2×1 m; depth of water, 42 cm). In experiment II, prawns were fed with short-neck clam meat and the control diet for 31 days before starting the experiment. The animals (average weight 0.61 g) were divided into 4 groups, each with 14 individuals, and reared in plastic containers (40×30 cm; depth of water 20 cm).

In both experiments sea water was supplied at a flow rate of 4 to 6 l/min. Salinity was maintained at 29 to 30‰ with freshwater and constant aeration. Water temperature varied between 24 and 30°C during the experiments. Diets were given twice a day at 8:00 and 17:30. The feeding levels were 7 to 8%. Faeces, sloughs, and uneaten diets were cleaned everyday. Feed intake was calculated by deducting the amount of diet uneaten from that given.

On the basis of preliminary tests, we set the feeding duration to about 1 month. The adequacy of this period was confirmed for *P. japonicus* by Chen and Jeng⁷⁾ who reported that there was no difference in pigmentation among prawns fed with the pigmented diets for 1, 2, or 3 months. Both experiments I and II were carried out for 28 days and the weight of each prawn was measured on day 14 and 28. The

Table 2. Results of the feeding trial in experiment I

Diet no.	Body weight (g)* ¹			Percent gain		Feed efficiency* ²	
	Initial	14 days	28 days	14 days	28 days	14 days	28 days
1	1.13±0.26	1.99±0.67	3.99±1.34	76	253	0.59	0.70
2	1.14±0.24	2.04±0.54	4.19±1.00	79	268	0.62	0.75
3	1.13±0.25	1.91±0.65	4.04±1.13	69	258	0.54	0.78
4	1.14±0.21	1.72±0.42	3.14±0.73	51	175	0.40	0.56
5	1.14±0.21	2.13±0.59	4.48±1.11	87	267	0.68	0.68

*¹ Mean ± standard deviation (initial number = 45).*² g gain/g feed.

prawns were harvested on day 28 and stored at -20°C until being analyzed.

Carotenoid Analysis

The carapace of individual prawns was removed, cut into small pieces, and ground in a mortar with anhydrous sodium sulfate. Carotenoids were extracted repeatedly with acetone until they could not be detected by color. The extracts were combined and concentrated under reduced pressure. The concentrate was transferred to a separating funnel and the carotenoids were taken up in diethyl ether. The ethereal layer was washed with water, dried on anhydrous sodium sulfate, and concentrated to dryness under reduced pressure. The residue was dissolved in benzene to make a volume. Total carotenoid was determined at 480 nm by the method of McBeth¹¹ with a Hitachi 330 spectrophotometer. The carotenoids were subjected to silica gel column chromatography. β -Carotene was eluted with *n*-hexane, followed by the remaining carotenoids with methanol. The carotenoid content of both fractions was determined by the same methods as for total carotenoid. The carotenoids, except β -carotene, were hydrolyzed with lipase according to the method of Matsuno *et al.*,¹² and separated by HPLC on a column of Superiorex-ODS (150×4.6 mm) with a mobile phase of methanol/ethylacetate/water (54:15:10) at a flow rate of 0.7 ml/min. Individual carotenoids were identified by spectroscopic analysis with a Shimadzu SPD-M6A photodiode array and by co-HPLC with authentic standards. The relative percentages were determined from peak areas on HPLC.

Measured Parameters and Data Treatment

Percent gain and feed efficiency were calculated according to Deshimaru.¹³ The carotenoid contents were analyzed by Student's "t" test at a significant level of 5%.

Table 3. Content of carotenoids in the carapace on day 28 in experiment I (mg/100 g)*¹

Diet no.	Pigmenter	Content
1	None	8.08±1.27
2	β -Carotene	9.15±0.89
3	<i>Spirulina</i>	12.02±1.95* ²
4	<i>Phaffia</i>	8.50±1.30
5	Krill oil	8.46±0.62

*¹ Mean ± standard deviation (n = 4).*² The value is significantly different from that of diet 1 at the 5% level.

Results

Table 2 shows the results of the feeding trial in experiment I. The prawns receiving *Phaffia* (diet 4) showed retarded growth, though the reason is not apparent. In the other diet groups the animals grew well. At the final stage of the feeding trial the body color of prawns in the control group remained light, whereas that of prawns receiving *Spirulina* (diet 3) was very dark. The animals receiving diets 2, 4, and 5 showed intermediate coloring between the control and *Spirulina* groups. The body color turned to red on boiling for 15 min at 100°C and the same trend in the deepness of body color was observed as before boiling. The content of carotenoids in the carapace was determined individually and is shown in Table 3. Evidently, the figures in Table 3 supported the above observation; carotenoids of the prawns receiving *Spirulina* increased conspicuously. Thus *Spirulina* proved to be most effective for pigmentation of cultured *P. monodon* among the pigmenters examined.

In experiment II, the effects of diets formulated at different levels of *Spirulina* on the pigmentation were examined. Table 4 shows the results of the feeding trial. The prawns fed with *Spirulina* showed less growth compared to that of the control group for 2 weeks, probably due to unfamiliarity with the *Spirulina*-containing diet.

Table 4. Results of the feeding trial in experiment II

Diet no.	Level of <i>Spirulina</i> supplement (%)	Body weight (g)* ¹			Percent gain		Feed efficiency** ²	
		Initial	14 days	28 days	14 days	28 days	14 days	28 days
1	0	0.61±0.29	1.04±0.53	1.43±0.6	70	134	0.49	0.36
6	1	0.61±0.29	0.96±0.45	1.62±0.3	57	166	0.41	0.41
3	3	0.61±0.27	0.94±0.43	1.50±0.4	54	146	0.38	0.39
7	5	0.60±0.24	0.89±0.41	1.32±0.6	48	120	0.36	0.32

*¹ Mean±standard deviation (initial number=14).*² g gain/g feed.Table 5. Content of carotenoids in the carapace on day 28 in experiment II (mg/100 g)*¹

Diet no.	Level of <i>Spirulina</i> supplement (%)	Content
1	0	3.62±0.65
6	1	4.30±0.78
3	3	8.01±0.64* ²
7	5	7.86±0.92* ²

*¹ Mean±standard deviation (n=3).*² The values are significantly different from that of diet 1 at the 5% level.Table 6. Composition of carotenoids in the carapace of prawns fed the 3% *Spirulina*-supplemented diet on day 28 (%)

Carotenoid	Composition
β-Carotene	12.4
Phoenicoxanthin	0.5
4-Ketozeaxanthin	0.4
Astaxanthin	68.7
Unknown	18.0

This tendency was also found in diet 3 of experiment I (Table 2). Thereafter, however, the prawns fed with diets containing 1 and 3% *Spirulina* grew well, body weight surpassing that of the control group on day 28. In contrast, the growth of prawns receiving 5% *Spirulina* remained retarded. This may be due to the high content of *Spirulina*, because the alga was found to have an adverse effect on the growth of some cultured fish when fed too much of it.¹⁴⁾

The body color of the prawns at the final stage of the experiment was deeper with the increase of the level of *Spirulina* from 1 to 3%. However, little difference was observed in the prawns fed with diets supplemented with 3 and 5% *Spirulina*. This observation was evidenced by the analysis of carotenoid content in the carapace as shown in Table 5. The contents gradually increased in the prawns fed with 1 and 3% *Spirulina*, when compared to the control group.

A slightly lower value was obtained in the prawns fed with 5% *Spirulina*, suggesting a 3% supplement in the diet to be the saturation level as far as carotenoid capacity in the carapace is concerned.

The composition of carotenoids deposited in the carapace of prawns fed with the 3% *Spirulina*-supplemented diet in experiment I was analyzed to follow the bioconversion of dietary carotenoids. Table 6 shows the results. Astaxanthin, unknown, and β-carotene accounted for 69, 18, and 12% of the whole carotenoids, respectively. Unknown carotenoids consisted of decomposed products and highly polar ones. Only phoenicoxanthin was present in the oxidative pathway from β-carotene to astaxanthin. On the other hand, no zeaxanthin and only a trace amount of 4-ketozeaxanthin were found, both of which are members in the other oxidative pathway to astaxanthin.

Discussion

We estimated astaxanthin to be most effective as a pigmenter, because it has been reported that *P. japonicus* can directly deposit dietary astaxanthin in the tissues.^{5,6)} However, feeding of *Phaffia* and krill oil, both of which contained astaxanthin esters as predominant carotenoid, to *P. monodon* proved less effective. The reason is not known, but there is a possibility that the incorporation of esterified carotenoids by crustaceans is more delayed than that of free carotenoids.

Bioconversion of β-carotene to astaxanthin requires many oxidative steps,⁵⁾ suggesting β-carotene to be less effective as a pigmenter. Actually, the low rate of incorporation of this carotenoid was found in the present feeding experiment and similar results were reported for *P. japonicus*.^{6,7)} *Spirulina* has β-carotene and zeaxanthin as major carotenoids. Thus, the effectiveness of *Spirulina* as a pigmenter of *P.*

monodon is attributable to zeaxanthin, that can be converted into astaxanthin via 4-ketozeaxanthin.⁵⁾ Analysis of carapace carotenoids of prawns which received the *Spirulina*-supplemented diet revealed that no zeaxanthin and only a small amount of 4-ketozeaxanthin were found (Table 6). This finding suggests that dietary zeaxanthin has rapidly been metabolized to astaxanthin in *P. monodon*. That may be the reason why *Spirulina* worked effectively in pigmentation.

In experiment II, we found that there was essentially no difference in the pigmentation of prawns by feeding with 3 or 5% *Spirulina*-supplemented diet, and that the growth in the latter group was somewhat retarded (Tables 4 and 5). In this context, Watanabe *et al.*¹⁴⁾ observed that over-supplementation of dietary *Spirulina* for cultured striped jack caused retardation of growth. Economically, the smaller the amount of *Spirulina*, the better.

In conclusion, a practical strategy for pigmentation of cultured *P. monodon* is to feed it with a diet containing *Spirulina* at 3% for 1 month before harvest.

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