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Supplemental Effects of *Euglena gracilis* in a Casein Diet for *Penaeus japonicus*

M. HAYASHI and K. TODA

Tsukuba Research Laboratory
Harima Chemicals, Inc.
5-9-3 Tokodai, Tsukuba
Ibaraki 300-26, Japan

A. KANAZAWA and H. YAMATOYA

Faculty of Fisheries
Kagoshima University
4-50-20 Shimoarata
Kagoshima 890, Japan

S. KITAOKA

Department of Agricultural Chemistry
University of Osaka Prefecture
1-1 Gakuenmachi, Sakai
Osaka, Japan

Abstract

Feeding experiments with larvae and juveniles of prawn, *Penaeus japonicus*, were conducted to evaluate the supplemental effects of *Euglena gracilis* cells in a casein diet. All diets containing *Euglena* cells had a comparable dietary value to that of the casein diet. Supplementation with 13.25% *Euglena* cells in a casein diet improved growth of larvae, and growth and feed conversion efficiency of juveniles. Enriching the fatty acid composition in *Euglena* with eicosapentaenoic or docosahexaenoic acid gave no further improvement on growth or survival of the prawn. The experiments suggest that *Euglena* cells contain a growth-promoting factor for the prawn in addition to essential amino acids.

Introduction

Euglena cells do not have cell walls consisting of polysaccharide as do plant cells and green algae. The outer membrane complex of *Euglena* cells, called pellicle, contains about 60% protein and is readily digested by ordinary digestive enzymes (Hosotani and Kitaoka 1977; Nakano et al. 1987). While sulfur-containing amino acids in protein are the limiting factor in the nutritional value of *Chlorella* and *Spirulina*, *Euglena* protein is rich in sulfur-containing amino acids and other essential amino acids, and shows the amino acid scores of 78-80 (Kitaoka and Hosotani 1977). It is known that the dietary value of

Euglena protein is comparable to that of casein for rats and larval rainbow trout (Hosotani and Kitaoka 1977; Satoh et al. 1984a). In addition, *Euglena* incorporates useful fatty acids from growth media and accumulates them in the cells (Hayashi et al. 1993a). Recently, *Euglena* highly enriched with eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) has been used successfully as feed for enriching essential fatty acid in rotifer and *Artemia* (Hayashi et al. 1993b). In the present paper, the dietary value of adding *Euglena* to a casein diet for *Penaeus japonicus* is evaluated.

Materials and Methods

Cultivation of Euglena

Euglena gracilis strain Z was grown in a medium reported in the previous paper using a 50-l jar-fermentor in the dark (Hayashi et al. 1993a). After 72-h cultivation, cells were harvested with a centrifuge and spray-dried. Table 1 shows the chemical composition of *Euglena* cells used in Experiments 1 and 2. In Experiment 1, *Euglena* was not enriched with n-3 highly unsaturated fatty acid (HUFA). The spray-dried cells contained 56.6% protein and 5.3% moisture. In Experiment 2, EPA- or DHA-enriched *Euglena* were used in addition to non-enriched *Euglena* (protein 57.0%, moisture 5.1% in spray-dried cells). The EPA-enriched *Euglena* (protein 51.0%, moisture 4.8% in spray-dried cells; EPA 44.3% in the total fatty acid) was cultivated with supplementation of 0.5% EPA (purity 50.1%). DHA-enriched *Euglena* (protein 51.0%, moisture 3.8% in spray-dried cells; DHA 45.6% in the total fatty acid) was cultivated with supplementation of 0.5% DHA (purity 58.9%).

Table 1. Chemical composition of *Euglena* cells used in the experiments (on dry-matter basis).

	Moisture[1]	Protein[2]	Paramylon[3]	Lipid	EPA[4]	DHA[4]
Exp. 1 *Euglena* (non-enriched)	5.3	56.6	21.0	9.3	3.6	1.1
Exp. 2 *Euglena* (non-enriched)	5.1	57.0	20.8	9.0	4.2	1.3
Euglena (EPA-enriched)	4.8	51.0	20.1	17.1	44.3	2.5
Euglena (DHA-enriched)	3.8	51.0	23.7	13.9	7.9	45.6

[1] %, in spray-dried cells
[2] N x 6.25
[3] β–1, 3-glucan accumulated in *Euglena* cells
[4] %, in the total fatty acid

Diet Preparation and Feeding Scheme

The compositions of microbound diets (MBD) used in Experiment 1 are shown in Table 2; 5% *k*-carrageenan was used as a binder. The sole protein source of the control diet (diet 1) in Experiment 1 was vitamin-free casein. The sources of carbohydrates and lipids were dextrin and pollack liver oil, respec-

tively, in all diets. Acetone-insoluble material of commercial soybean lecithin was used as a source of phospholipids. Compositions of minerals and vitamins were reported in a previous paper (Kanazawa et al. 1977). In Experiment 1, 13.25 or 26.5% of spray-dried *Euglena* cells were added to the diets. In order to adjust the protein content in the diets to approximately 45%, the contents of vitamin-free casein and α-cellulose were reduced in the diets containing *Euglena* cells (Table 2). The essential amino acid composition of the diets were made similar to that of larval *P. japonicus* by supplementation with L-tryptophan and L-arginine-HCl. The amino acid composition was analyzed by high-performance liquid chromatography (HPLC) using *o*-phthaldialdehyde / 2-mercaptoethanol reagent (Lee and Drescher 1979; Teshima et al. 1986). The ingredients, except pollack liver oil, soybean lecithin, cholesterol and fat-soluble vitamins, were thoroughly mixed in 150 ml hot water per 100 g of dry diet. After the fat-soluble materials and 100 ml hot water were added, the mixture was agitated vigorously in a water bath at 70-80 °C until a pudding-like consistency was obtained. The diets were then cooled to room temperature, freeze-dried and sieved into adequate particle sizes (53 μm for zoea; 125 μm for mysis; 250 μm for postlarvae). All test diets were kept at -20 °C prior to feeding.

The daily feeding rates of the diet were 0.16 mg·larvae^{-1} for zoea, 0.20 mg·larvae^{-1} for mysis, and 0.24 mg·larvae^{-1} for postlarvae. Only the artificial diet was distributed in two equal feedings per day.

Table 2. Composition of the experimental test diets (MBD) (g·100 g^{-1} dry diet).

Ingredient	Diet 1	Diet 2	Diet 3
Casein (vitamin-free)	47.78	39.63	31.44
L-tryptophan	0.49	0.49	0.50
L-arginine-HCl	2.58	2.13	1.90
Euglena cells	-	13.25	26.50
Dextrin	4.01	4.01	4.01
α-cellulose	22.24	17.59	12.75
Glucosamine-HCl	0.80	0.80	0.80
Na-citrate	0.30	0.30	0.30
Na-succinate	0.30	0.30	0.30
Pollack liver oil	5.00	5.00	5.00
Soybean lecithin (PL)[1]	2.00	2.00	2.00
Cholesterol	0.50	0.50	0.50
Minerals[3]	6.00	6.00	6.00
Vitamins[3]	3.00	3.00	3.00
k-carrageenan	5.00	5.00	5.00
Total	100.00	100.00	100.00
Crude protein (%)[2]	45.84	45.59	45.51

[1] Polar lipid fraction of soybean lecithin
[2] On dry-matter basis (N x 6.25)
[3] Kanazawa et al. 1977

The composition of the moist pellets used in Experiment 2 are shown in Table 3, 5% agar being used as the binder. The sole protein source of the basal diet (diet 1) in Experiment 2 was vitamin-free casein. The sources of carbohy-

drates were glucose, sucrose and α-starch. The sources of lipids, minerals and vitamins were the same as in Experiment 1. In Experiment 2, 13.25% of either non-enriched, EPA-enriched or DHA-enriched *Euglena* cells was added to the test diet. In order to adjust the protein content in the diets to approximately 47%, the contents of vitamin-free casein and α-cellulose were reduced in the diets containing *Euglena* cells (Table 3).

Table 3. Composition of the experimental moist pellet (g·100 g^{-1} dry diet).

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
Casein (vitamin-free)	49.91	41.59	41.59	41.59
Euglena cells	-	13.25	-	-
Euglena (EPA-enriched) cells	-	-	13.25	-
Euglena (DHA-enriched) cells	-	-	-	13.25
α-cellulose	9.86	4.93	4.93	4.93
Arginine-HCl	2.60	2.60	2.60	2.60
Pollack liver oil	5.00	5.00	5.00	5.00
Soybean lecithin (PL) [1]	3.00	3.00	3.00	3.00
Cholesterol	1.00	1.00	1.00	1.00
Glucose	3.00	3.00	3.00	3.00
Sucrose	5.45	5.45	5.45	5.45
α-starch	2.18	2.18	2.18	2.18
Minerals[3]	8.60	8.60	8.60	8.60
Vitamins[3]	3.00	3.00	3.00	3.00
Glucosamine-HCl	0.80	0.80	0.80	0.80
Na-citrate	0.30	0.30	0.30	0.30
Na-succinate	0.30	0.30	0.30	0.30
Agar	5.00	5.00	5.00	5.00
Total	100.00	100.00	100.00	100.00
Water (ml)	170	170	170	170
Crude protein (%)[2]	47.92	46.81	46.22	47.39

[1]Polar lipid fraction of soybean lecithin
[2]On dry-matter basis (N x 6.25)
[3]Kanazawa et al. 1977

The ingredients, except agar, were thoroughly mixed with 20 ml water per 100 g of dry diet. The agar was separately solubilized at 100 °C in 150 ml water and mixed with the rest of the ingredients. The mixture was then heated to 100 °C and agitated until a pudding-like consistency was obtained. The diets thus prepared were kept at 5 °C prior to cutting before feeding.

The feeding rate of the diets was 6-9% body weight of the juveniles. The artificial diets alone were distributed in one feeding per day.

Cultures of Larvae and Juveniles

P. japonicus larvae, hatched from broodstock purchased from a commercial supplier in Japan, were used throughout the experiments.

For Experiment 1, 100 zoea 1-stage larvae were transferred from the hatching tank and randomly allocated to each of six 1-l beakers. Larvae were reared from the zoea 1 stage for 12 d using the four diets shown in Table 2. Body

length and number of survived larvae were measured once a day. The developmental stage of the larvae was determined by using Fujinaga's figures (Fujinaga 1942). The growth index was calculated by the following formula:

$$\text{Growth index} = (1xa)+(2xb)+(3xc)+(4xd)+(5xe)+(6xf)+(7xg)+(8xh)+(9xi)/N,$$

where, a = number of Z1 stage larva
b = number of Z2 stage larva,
c = number of Z3 stage larva,
d = number of M1 stage larva
e = number of M2 stage larva,
f = number of M3 stage larva,
g = number of P1 stage larva,
h = number of P2 stage larva,
i = number of P3 stage larva,
N = number of survived larva.

For Experiment 2, 15 juveniles were randomly allocated to each of eight 54-l tanks after the larvae had been reared on a commercial diet for 67 d. The juveniles were reared for a further 40 d using the four diets shown in Table 3. Body weight and number of survived juveniles were measured once every 10 d.

Water Management

For Experiment 1, water temperature was maintained at 28.0 ± 2.0 °C with the aid of a water bath. Water salinity was 33-35‰ and pH 8.2-8.6. Daily water renewal was 100%.

For Experiment 2, water temperature was maintained at 22.0 ± 3.0 °C with the aid of a ceramic heater. Water salinity was 33-35‰, and pH 8.1-8.3. Daily water renewal was 200% with fresh, filtered seawater.

Results

Experiment 1

The amino acid composition of the test diets and zoea 1-stage larvae are shown in Table 4. The essential amino acid compositions of the test diets were similar to that of the larvae.

The body length of larvae at 13 d rearing, survival rate and growth index are shown in Table 5. Body length was 4.3-4.7 mm. The survival rates of larvae except those fed diet 3 were around 55%. The growth index for all diets was about 7. All experimental groups had no significant differences ($P>0.05$) in survival rates. Diet 2 containing 13.25% *Euglena* cells showed a greater dietary value than the control diet (diet 1). The body length of larvae fed diet 2 containing 13.25% *Euglena* was 4.7 mm, and was significantly larger ($P<0.05$) than the others (4.3 mm).

Table 4. Amino acid composition of test diets and *Penaeus japonicus* (zoea I-stage larvae) (%).

Essential amino acids	Diet 1	Diet 2	Diet 3	Zoea I-stage larvae[1]
Methionine	2.60	2.54	2.48	2.91
Threonine	3.51	3.55	3.58	4.28
Valine	5.85	5.81	5.78	5.42
Isoleucine	4.73	4.63	4.48	4.78
Leucine	8.12	8.09	8.02	7.23
Phenylalanine	5.85	5.57	5.27	4.70
Histidine	2.23	2.22	2.18	2.38
Lysine	8.86	8.25	7.58	7.19
Tryptophan	5.34	4.63	3.91	1.26
Arginine	8.42	7.81	7.56	8.96
Non EAA[2]	44.50	46.90	49.15	50.89
Total	100.00	100.00	100.00	100.00

[1]All nitrogen compounds including proteins, peptides, free amino acids, etc.
[2]Essential amino acids

Table 5. Results of feeding experiment 1[1].

Experimental group		Body length (mm)[2]	Survival rate (%)	Growth index
Diet 1	47.78% casein	4.3±0.2[b]	57	7.0
Diet 2	39.63% casein+13.25% *Euglena*	4.7±0.3[a]	54	7.1
Diet 3	31.44% casein+26.50% *Euglena*	4.3±0.5[b]	38	6.9

[1]Average value from duplicate experiments
[2]Mean±SD, values with the same letters are not statistically significant (P>0.05) on Fisher PLSD test.

Table 6. Results of feeding experiment 2.

Experimental group		Body weight (g)[1]		Survival rate (%)	Feed conversion efficiency (%)[2]
		Initial	Final		
Diet 1	49.19% casein	0.93±0.03	1.35±0.13[b]	100	20
Diet 2	41.59% casein +13.25% *Euglena* (EPA)	0.93±0.03	1.58±0.24[a]	100	27
Diet 3	41.59% casein +13.25% *Euglena* (EPA)	0.93±0.03	1.44±0.11[ab]	100	23
Diet 4	41.59 casein +13.25% *Euglena* (DHA)	0.93±0.03	1.57±0.14[a]	100	27

[1] Mean±SD, average value from duplicate experiments.
Values with the same letters are not statistically significant (P>0.05) on Fisher PLSD test.
[2] {[final body weight (g)]-[initial body weight (g)]} X 100/[total amount of dry feed intake ($g \cdot prawn^{-1}$)].

Experiment 2

Body weight, survival rate and feed conversion efficiency of juveniles at 40 d rearing are shown in Table 6. Juveniles fed test diets containing *Euglena* had improved body weights. Although the juveniles had a slightly lower mean body weight on diet 3 (1.44 g) than those on diets 2 and 4 (1.57-1.58 g), all juveniles fed diets containing *Euglena* showed higher mean body weight than those fed the control diet (1.35 g). The body weight of juveniles fed diet 2 or 4 was significantly different ($P<0.05$) from those on diet 1. The survival rate of juveniles was 100% in all experimental groups. Feed conversion efficiency of juveniles fed diet 1 was 20%, while those fed diets 2-4 was 23-27%. The efficiency on diets 2 and 4 was 27%. These results show that the diets containing *Euglena* cells have greater dietary value than the casein diet for prawn juveniles, and that enrichment of *Euglena* cells with EPA or DHA did not enhance its dietary value to the prawns.

Discussion

Euglena protein is as easily digested by pepsin or trypsin as casein, and has a dietary value for rats equal to that of casein (Hosotani and Kitaoka 1977). Also it was shown that the diet with *Euglena* as the sole protein source resulted in better growth and survival of rainbow trout juveniles than the casein diet (Satoh et al. 1984a). The results of Experiment 1 show that the dietary value of the diets containing *Euglena* for *P. japonicus* larvae was equal to or greater than that of the control diet. Dietary supplementation with only 13.25% *Euglena* cells (diet 2) greatly improved growth of larvae.

It was shown in the previous paper that the amino acid composition of the diet, which simulated the essential amino acid composition of whole body protein of prawn larvae improved growth and survival of larvae (Teshima et al. 1986). In Experiment 1, however, analysis of the amino acid composition of the diets showed that the essential amino acid compositions of all diets used in Experiment 1 were similar to that of larval prawn (Table 4); no significant difference in the essential amino acid composition was found among the diets. Therefore, it is suggested that *Euglena* cells contain a growth-promotiong factor for larval prawn in addition to essential amino acids. More studies, however, are required to confirm this.

The diets containing *Euglena* as a sole protein source were reported to have higher feed conversion efficiency and protein efficiency with rainbow trout juveniles (Satoh et al. 1984a). In Experiment 2 of the present work, diets containing *Euglena* showed improved growth and feed conversion efficiency in prawn. Results of Experiment 2 show that the dietary value of diets for prawn containing 13.25% *Euglena* is apparently greater than that of the casein diet. This is consistent with results of Experiment 1.

Since *Euglena* is reported to contain a large amount of vitamins (Satoh et al. 1984b; Kitaoka 1989), it was reported that diets containing *Euglena* as the sole protein source with no supplementation of vitamins gave similar growth and survival rates in rainbow trout juveniles as did a diet supplemented with

vitamins (Satoh et al. 1984a). In the present study, although the vitamins in *Euglena* cells effectively improved growth and feed conversion efficiency in prawn, more studies are required concerning the effect of vitamins.

It is known that EPA and DHA are effective essential fatty acids for growth of larval prawn (Kanazawa, et al. 1978; Jones et al. 1979; Kanazawa et al. 1979). It has also been shown that DHA is more effective than EPA as an essential fatty acid for prawn juveniles (Kanazawa et al. 1993). However, larvae fed the diet containing *Euglena* enriched with either EPA or DHA showed similar growth and survival to those fed the diet containing *Euglena* without fatty acid enrichment (data not shown). Since all the diets used in Experiment 1 contained 5% pollack liver oil as a source of lipids and essential fatty acids, the effect of fatty acid composition of enriched *Euglena* appeared not to be manifest in the results of Experiment 1. In Experiment 2, the influence of the fatty acid composition of enriched *Euglena* was also not apparent. It is suggested that a sufficient amount of essential fatty acids was supplied by the pollack liver oil in the diets. However, when high-protein diets (55% CP) were used, the juveniles fed the diet containing DHA-enriched *Euglena* showed a higher growth of 1.10 g after 40 d rearing from 67 d after hatching than those fed the diet containing EPA-enriched *Euglena* (0.82 g) or the casein diet (0.80 g). Feed conversion efficiency was also improved with the high CP diets; the efficiencies of juveniles fed diets containing DHA-enriched and EPA-enriched *Euglena* and casein were 46%, 33% and 33%, respectively (unpubl. data). However, in order to evaluate the effect of enriching fatty acid composition in *Euglena* lipids, more studies are required.

Since test diets in the present study did not contain feeding stimulants, growth and feed conversion efficiency might have been poor. However, results indicate that the diet containing *Euglena* cells has a higher dietary value than the casein diet for prawn, as reported in rainbow trout and other animals. Although the essential amino acid compositions of the diets did not differ, all diets contained more than minimum amounts of vitamins, minerals and essential fatty acids. This suggests the possible presence of an unknown growth factor in *Euglena* cells. Clearly, further work is warranted to confirm this finding and to elucidate the factor within *Euglena* which exhibits this growth-promoting effect.

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