スピルリナ投与による養成アユの体色改善

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Pigmentation of Cultured Sweet Smelt Fed Diets Supplemented with a Blue-Green Alga *Spirulina maxima*[*1]

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Pigmentation of cultured sweet smelt *Plecoglossus altivelis* was attempted by using diets supplemented with a commercial preparation of *Spirulina maxima*, whose main carotenoid is zeaxanthin. Addition of 3 to 6% *Spirulina* to diets and feeding them for 10 weeks improved successfully the color of test fish. Supplementation of 4 to 8 mg lutein A per 100 g of diet was also tried for comparison and was found to be effective. Carotenoids in the diets were accumulated not only in the integuments but in the subcutaneous tissues. The contents of the integuments and subcutaneous tissues in the test groups were 4.2 to 5.7 mg/100 g and 19.5 to 44.0 mg/100 g, whereas those in the control group were 2.9 mg/100 g and 12.7 mg/100 g, respectively. In the integuments major carotenoids were zeaxanthin, β-cryptoxanthin, lutein B, and diatoxanthin together with minor ones such as 3′-hydroxyechinenone, cynthiaxanthin, and β-carotene triol. No lutein A was detected in the *Spirulina*-fed groups. In the subcutaneous tissues appreciable amounts of β-carotene and canthaxanthin were found in addition to the carotenoids mentioned above, but no lutein B was detected. Presumed metabolic pathways of carotenoids from diets to the integuments via the subcutaneous tissues were discussed.

Wild sweet smelt *Plecoglossus altivelis* is said to be one of the most palatable freshwater fishes in Japan, and recent progress in fish culture has succeeded in its mass production. However, the cultured fish are considerably different from wild ones in appearance; the formers are bluish and have a less sharp-pointed forehead, whereas the latters are tinged with yellowish orange with a characteristic light yellow spot near the pectoral fin. Furthermore, wild ones are generally slender and exhibit an orange band below the lateral line when matured. Because of these distinct differences in appearance, cultured ones are disposed of at a low valuation in the market, so that the improvement of their appearance, especially of the integumentary color is desired.

The color of sweet smelt is evoked by integumentary pigments, mainly carotenoids of which zeaxanthin constitutes the major part.[1] However, the orange hue of the band manifested frequently by matured fishes is attributed not to the integumentary pigments but to carotenoids present in the subcutaneous tissues.[*6] Therefore, we have anticipated that the supplementation of a blue-green alga *Spirulina maxima* to diets as a pigmenter is effective for improvement of the color of cultured sweet smelt because this alga contains quantities of carotenoids of which the main component is zeaxanthin[2] and because the alga is actually used for pigmentation of goldfish, fancy red carp, and rainbow trout.[3,4]

In the present study, we carried out feeding experiments of cultured sweet smelt with diets supplemented with *S. maxima* and analyzed carotenoid composition of the integuments and subcutaneous tissues. We also employed lutein

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A-dipalmitate as a pigmenter for comparison, since we have found that lutein B is one of main carotenoids in the integuments and subcutaneous tissues of sweet smelt.*[6] We have confirmed that not only *S. maxima* but also lutein A-dipalmitate is effective for improvement of the color of cultured sweet smelt. This paper deals with the results.

Materials and Methods

Pigmenters

S. maxima cultured and spray-dried by Japan Spirulina Co., Ltd. and lutein A-dipalmitate oil (lutein A content 5%) produced by F. Hoffman-La Roche & Co., Ltd. were used. The content and composition of carotenoids in the *S. maxima* powder (henceforth referred to as *Spirulina*) were analyzed by the method reported previously,[2] and found to be 336 mg/100 g and β-carotene 15%, β-carotene-5,6-epoxide 3%, echinenone 10%, β-cryptoxanthin 12%, 3'-hydroxyechinenone 7–8%, zeaxanthin 18%, diatoxanthin 7%, cynthiaxanthin 6%, carotenoid-glycosides 6%, and decomposed carotenoids 11–15%.

Test Diets

The compositions of test diets are presented in Table 1. Diet No. 1 was a commercial diet without carotenoids as the control and the other test diets contained carotenoids. Diets No. 2 and 3 contained *Spirulina* at concentrations of 3 and 6% and diets No. 4 and 5 lutein A-dipalmitate oil at concentrations of 0.08 and 0.16%, respectively. That is, the fish fed diets No. 2 and 3 received 1.8 and 3.6 mg zeaxanthin/100 g diet, respectively, together with other carotenoids, and those fed diets No. 4 and 5 ingested only lutein A as the carotenoid source as shown in Table 1.

Feeding Experiment

Sweet smelt *P. altivelis* weighing 11.5 g on average were divided into 5 groups and reared for 10 weeks with each test diet. Ten fish were sampled from each group at the end of the feeding experiment and frozen immediately. They were stored below −20°C until analysis. Their average weight and length were 56 g and 15 cm, respectively.

Analytical Methods

The integuments of each fish were removed and then the subcutaneous tissues were scraped off the muscle with a scalpel while the fish was still in a frozen state. The integuments were cut into small pieces and ground with anhydrous sodium sulfate. The subcutaneous tissues were also ground with the dehydrating agent. Carotenoids were extracted repeatedly with acetone until the extract became colorless. The extracts were combined, concentrated under reduced pressure, and transferred into diethyl ether by adding water. The aqueous phase was extracted repeatedly with diethyl ether to effect complete pigment transfer. The combined ethereal layer was washed with water, dehydrated on anhydrous sodium sulfate, and dried up under reduced pressure. Oily materials obtained were dissolved in benzene and made up to volume. The total carotenoid content was determined by the method of McBeth[5] with a Hitachi 330 spectrophotometer. After the determination, carotenoids were saponified as usual.[6] The relative percentage was measured with a Gelman ACD-18 densitometer after subjecting the carotenoids to thin layer chromatography (TLC) on silica gel $60F_{254}$ in ethyl acetate/dichloromethane (1:4). Each carotenoid was isolated and purified by column chromatography on dry silica gel and MgO: Celite 545 (1:1) and by high performance liquid chromatography (HPLC) on ERC-CN. Each carotenoid was identified by means of co-TLC and co-HPLC with authentic specimens, spectroscopic analyses, and chemical treatments as described previously.[2,7–9]

Table 1. Composition of test diets

Ingredient (%)	Diet No.				
	1*	2	3	4	5
Fish meal		44	42	55	47
Wheat flour		20	20	20	20
α-Starch		10	10	10	10
Mineral mixture		2	2	2	2
Vitamin mixture		2	2	2	2
Feed oil		6	6	0	5
Cellulose		13	12	11	14
Spirulina		3	6	0	0
Lutein A-dipalmitate oil		0	0	0.08	0.16
Carotenoid (mg/100 g diet)					
Total carotenoids	0	10	20	4	8
Zeaxanthin	0	1.8	3.6	0	0
Lutein A	0	0	0	4	8

* A commercial Ayu diet containing no carotenoids.

Results

The fish fed diets containing *Spirulina* or lutein A showed a tendency to be pigmented in almost

Table 2. Carotenoid contents of the tissues (mg/100 g)

	Diet No.				
	1	2	3	4	5
Integuments	2.9	4.5	4.2	4.3	5.7
Subcutaneous tissues	12.7	31.3	19.5	44.0	24.0

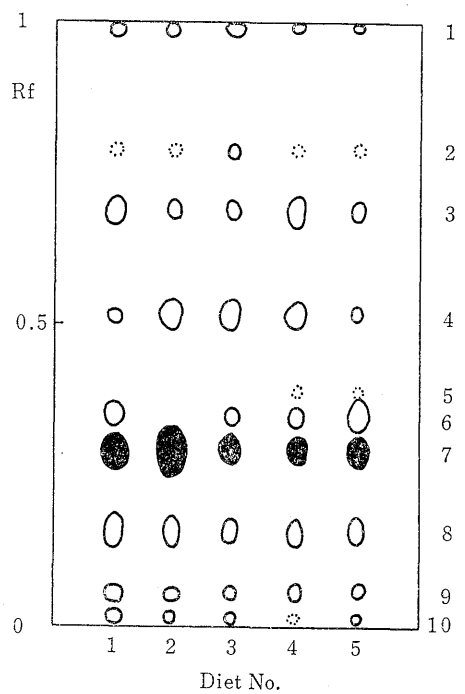

Fig. 1. Thin layer chromatogram of whole carotenoids in the subcutaneous tissues after saponification.
Stationary phase: silica gel $60F_{254}$
Mobile phase: ethyl acetate/dichloromethane (1:4)

the same degree as wild ones, though considerable differences were noticed from individual to individual. In contrast, all the fish in the control group were poorly pigmented. Two fish in each group were sampled for analysis of carotenoids. In the carotenoid-fed groups two individuals pigmented satisfactorily were selected because of little macroscopic differences between them. Average carotenoid content of the two fish is shown in Table 2. The contents of the integumentary carotenoids in the carotenoid-fed groups were more than 4.2 mg/100 g while that of the control group was 2.9 mg/100 g, which indicates that carotenoids in the diets were obviously accumulated in the integuments. This is also true of the

Table 3. Relative composition of caroteoids in the integuments (%)

	Diet No.				
	1	2	3	4	5
β-Carotene	—	—	—	—	+
Canthaxanthin	—	—	—	+	1
β-Cryptoxanthin	10–16	22	21	21	14
3′-Hydroxy-echinenone	7–9	7–8	2	7	3
Lutein B	11–12	4	15	16	20–22
Lutein A	2–3	—	—	12	8–9
Zeaxanthin	31–32	28–29	31–33	25	23–25
Diatoxanthin	17	19	16–19	14	12–23
Cynthiaxanthin	7–8	12	9	2	9
β-Carotene triol	9–11	7–8	4	4	3–4

+, trace; —, not detected.

Table 4. Relative composition of carotenoids in the subcutaneous tissues (%)

	Diet No.				
	1	2	3	4	5
β-Carotene	4	3–4	9–10	5	3
Canthaxanthin	2	2	4	2–3	2
β-Cryptoxanthin	9–10	5–7	6–7	11–14	8
3′-Hydroxy-echinenone	5–6	14–17	18–20	13–15	3–4
Lutein B	—	—	—	1	1–2
Lutein A	10–11	—	7	10–11	24–27
Zeaxanthin	34	42–43	26	27–28	34–37
Diatoxanthin	16–17	17–20	15	17–18	14–17
Cynthiaxanthin	9–10	7–8	9	7–8	7–8
β-Carotene triol	10	4	4–5	1–2	4

—, not detected.

subcutaneous tissues; the contents in the carotenoid-fed groups were 19.5–44.0 mg/100 g, whereas that in the control group was 12.7 mg/100 g.

TLC of the whole carotenoids in the subcutaneous tissues is depicted in Fig. 1, in which the separated carotenoids are referred to spots 1–10 in order of increase of polarity. The same patterns were obtained for those in the integuments, though their relative percentages differed considerably. Spots 1–10 were isolated and identified as β-carotene, canthaxanthin, β-cryptoxanthin, 3′-hydroxyechinenone, lutein B, lutein A, zeaxanthin, diatoxanthin, cynthiaxanthin, and β-carotene triol, respectively.

The relative compositions of carotenoids in both tissues are shown in Tables 3 and 4. In the integuments zeaxanthin was found to be the main component, and β-carotene and canthaxanthin were trace ones or not detected at all in all the

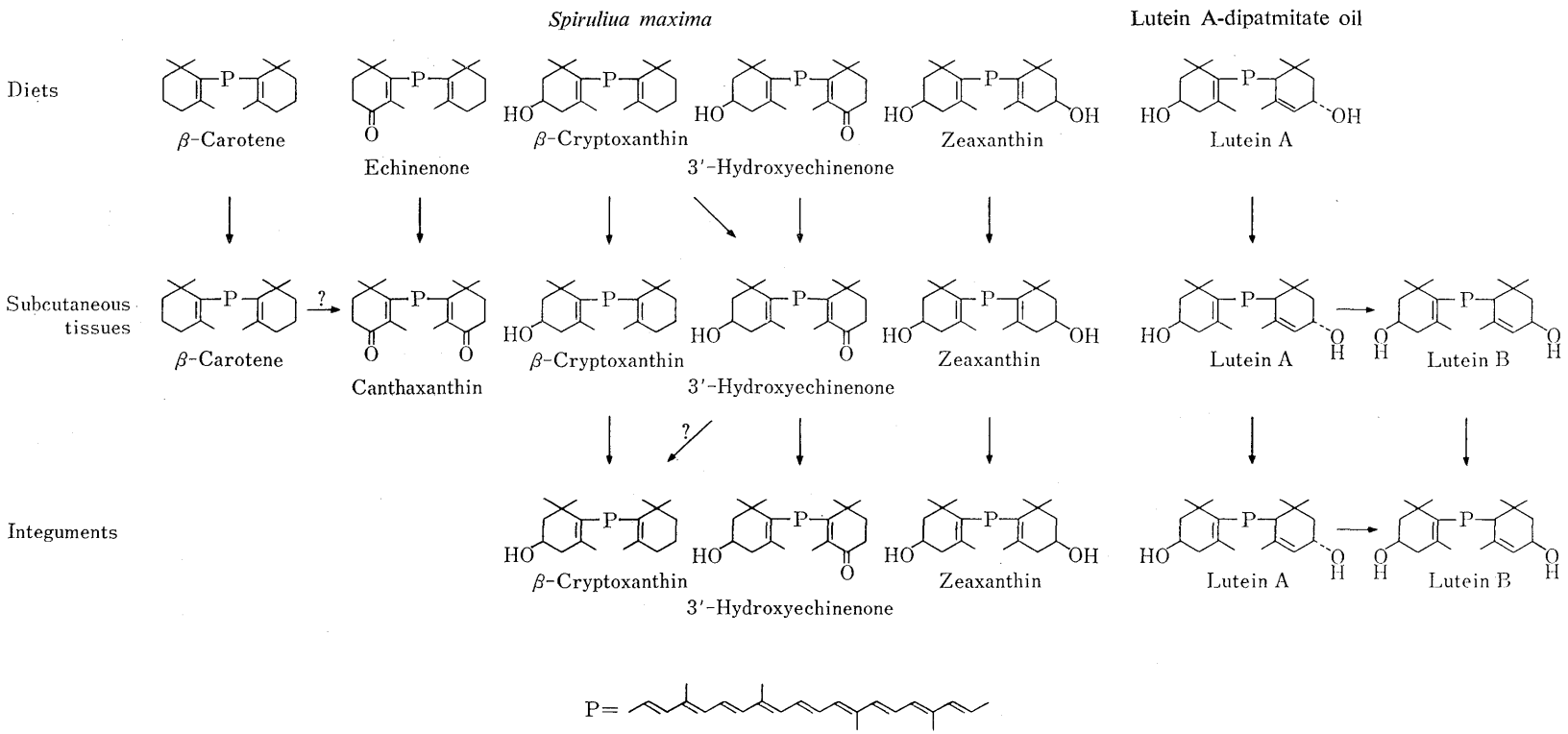

Fig. 2. Presumed metabolic pathway of carotenoids in sweet smelt.

groups. 3′-Hydroxyechinenone, diatoxanthin, cynthiaxanthin, and β-carotene triol in the carotenoid-fed groups did not show a tendency to increase when compared with those in the control group. This suggests that the supplemented carotenoids to the diets had little influence on those components. In contrast, taking the increased amounts shown in Table 2 into consideration, apparent increases of β-cryptoxanthin in the carotenoid-fed groups, of zeaxanthin in the *Spirulina*-fed groups, and of lutein B and lutein A in the lutein A-fed groups could be indicated.

In the subcutaneous tissues, zeaxanthin was also the major component, but appreciable amounts of β-carotene and canthaxanthin were detected in all the groups. Considering the changes in the contents of carotenoids, we could conclude that β-cryptoxanthin and 3′-hydroxyechinenone tended to increase more or less, whereas β-carotene triol to decrease in the carotenoid-fed groups. The amount of zeaxanthin varied from group to group and higher amounts of lutein A were found in the lutein A-fed groups. It should be noted that there was a distinct difference in the amount of lutein B between the integuments and subcutaneous tissues.

Discussion

Carotenoids in the diets were considered to have been accumulated in both tissues because the carotenoid-fed groups contained more carotenoids than the control group. Their contents in the subcutaneous tissues were markedly higher than those in the integuments.

In the subcutaneous tissues of the lutein A-fed groups, lutein A was found but almost no lutein B. On the other hand, in the integuments of these groups the contents of lutein B were obviously greater than those of lutein A. These results suggests that carotenoids in diets should have been initially stored and metabolized in the subcutaneous tissues and that some carotenoids have been subsequently further metabolized, for example, isomerization of lutein A to lutein B, when they were transferred to the integuments. With respect to β-carotene and canthaxanthin they were present in the subcutaneous tissues but scarcely in the integuments. Echinenone which was contained in an appreciable amount in *Spirulina* was detected neither in the integuments nor in the subcutaneous tissues. This carotenoid seems to have been metabolized to canthaxanthin. These findings are further evidences to support above supposition.

There is a possibility that β-cryptoxanthin and 3′-hydroxyechinenone are convertible each other in the tissues of sweet smelt through oxidation and reduction at 4-position of ionone ring because the former was present more abundantly in the integuments and the latter in the subcutaneous tissues. Since no oxidation at 3,3′-positions of ionone ring in fish has been reported so far,[10] β-cryptoxanthin could not be metabolized to zeaxanthin, so that the increase of zeaxanthin in the *Spirulina*-fed groups may be due to direct transfer from the diets. However, the reason why zeaxanthin in the subcutaneous tissues of the diet No. 5 group was increased is not clear. Insignificant variations observed in diatoxanthin, cynthiaxanthin, and β-carotene triol suggest that no distinct metabolism as to these carotenoids should take place in the tissues of sweet smelt. The presumed metabolic pathway of carotenoids found in the present study is shown in Fig. 2.

In the previous feeding experiment of sweet smelt fed diets supplemented with krill oil or *Spirulina*, we found that both astaxanthin and zeaxanthin in the diets were metabolized to 4-ketozeaxanthin by reduction and oxidation at 4,4′-positions, respectively.*[6] In the present study, however, not a trace of 4-ketozeaxanthin was detected. The reason of this discrepancy is not known at this time but probably due to the fact sweet smelt changes the food habit drastically with growth, which might induce large alternations in the metabolism of carotenoids. Further work is clearly needed to elucidate this problem.

From the practical point of view we have established that the pigmentation of cultured sweet smelt can be successfully attained by supplementing *Spirulina* to a diet up to 3%, that is approximately 10 mg/100 g in terms of total carotenoids. Further addition does not necessarily bring satisfactory results as evidenced in the present study. Since the content of carotenoids in commercial preparations of Spirulina powder varies considerably,[2] care should be taken in the actual use of this alga.

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