The Biosynthesis of Astaxanthin—III****. The Carotenoids in Sea Breams

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It has commonly considered that fish, like all other animals, do not possess any ability to synthesize carotenoids de novo, but they can alter alimentary carotenoids by oxidation and can store the resulted products.

It is often observed the bright red color of natural sea breams fades to almost white while they are in captivity at the fish farm. In the previous report,^{1,2)} it was clarified that the faded color of the cultured sea breams was caused by the extremely small amount of astaxanthin contained in them, and the contents of the stomach of natural sea breams were examined and the half digested *Squilla oratoria* and other *Crustacea* were found in them. It was therefore assumed that most of astaxanthin in natural sea breams was brought about their food.

Besides astaxanthin the existence of ϵ -carotene, 3,3'-dihydroxy- ϵ -carotene, lutein and α -doradecin was confirmed in the carotenoids extracted from natural sea breams. All of those carotenoids exist in the form of esters except ϵ -carotene and it is very interesting to note that the carotenoids of sea breams contain lutein ester and α -doradexanthin ester (3,3'-dihydroxy-4'-keto- α -carotene ester^{3,4)}).

Materials and Methods

Fresh wild sea breams (length, 20 cm) were bought at the local fish shop. The dorsal and ventral sections of the cuticle and all of the epidermis of cuticle were collected and extracted repeatedly with acetone until no further pigment could be obtained. The acetone solutions were combined, transferred to petroleum ether by addition of water, and washed repeatedly with water to remove acetone.

Petroleum ether solution of the pigments was concentrated under vacuum and dried over sodium sulfate. The absorption spectra of the extracted carotenoids in petroleum

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ether is shown in Fig. 1.

The pigments were separated chromatographically on columns of aluminum oxide (grade 3, WAKO PURE CHEMICALS CO. LTD.) and were developed with 3% acetone in petroleum ether. Three pigments bands were found (Fig. 2).

Fig. 1. The absorption spectrum of the crude carotenoids extracted from Sea breams in petroleum ether.

Fig. 2. Carotenoids from Sea breams as they appear on aluminum oxide when developed with 3% acetone in petroleum ether.

Pigment identification:

ϵ -Carotene and 3,3'-dihydroxy- ϵ -carotene. Band A pigments were saponified by dissolving it in 100 ml of absolute ethyl alcohol, adding 10 ml of 60% (w/v) aqueous potassium hydroxide solution and leaving it overnight under nitrogen at room temperature. The saponified pigments were transferred to petroleum ether with addition of water, and dried with anhydrous sodium sulfate.

Thus obtained, saponified pigments were rechromatographed on aluminum oxide (grade 2, WAKO PURE CHEMICALS, for chromatographic adsorption) and were developed with acetone in petroleum ether of different proportions (from 0.5% to 8% acetone in petroleum ether).

Two bands of pigments were obtained. Band A-I; Lower band, Band A-I was eluted with acetone and retransferred to petroleum ether by addition of water. It was epiphasic when partitioned between 90 to 95% methanol and petroleum ether.^{5,6)} The absorption spectra in petroleum ether (416, 439, 468 $m\mu$) and benzene (426, 451, 481 $m\mu$) were in full agreement with those of ϵ -carotene.⁴⁾ Band A-II; Upper band, Band A-II was eluted from aluminum oxide column with acetone and transferred to petroleum ether

by adding water. It was hypophasic to 90 and 95% methanol.^{5,6)} The pigment exhibited absorption maxima at 416, 440, 469 m μ in petroleum ether. Those were identical with that of known 3,3'-dihydroxy- ϵ -carotene⁶⁾ (Fig. 3 and 4).

Fig. 3. The absorption spectra of ϵ -carotene from Sea breams.

Fig. 4. The absorption spectra of 3,3'-dihydroxy- ϵ -carotene from Sea breams.

Lutein and α -doradecin (3-hydroxy-3'4'-diketo- α -carotene). The pigments of the Band B were rechromatographed on Microcel-C column using 2% acetone in petroleum ether as developing solvent. Two bands were obtained. The B-1 band, lower band was saponified by dissolving in 10 ml of absolute ethyl alcohol, adding 1 ml of 60% aqueous potassium hydroxide. The saponified pigments were separated chromatographically on magnesium oxide-hyflsuperpel (1:2) column, using 25% acetone in petroleum ether as developing solvent. The main zone of pigments was dissolved in petroleum ether. The pigment exhibited absorption maxima at 426, 448, 475 m μ in petroleum ether, and the behavior on column and absorption maxima were identical with that of lutein (Fig. 5).

The pigments of the upper zone, the B-2 band were redissolved in 50 ml of absolute ethyl alcohol and then 5 ml of 60% (w/v) aqueous potassium hydroxide were added to make final concentration about 6%. The alkaline mixture was left overnight in the dark at room temperature. After saponification the carotenoids were retransferred to petroleum ether by addition of water, the saponified pigments were rechromatographed on magnesium oxide-hyflsuperpel (1:2) in order to remove trace of lutein, using 30% acetone in petroleum ether as developing solvent. The pigments of the band were eluted with 10% glacial acetic acid in ethyl ether, and transferred to petroleum ether by addition of water.

Fig. 5. The absorption spectra of lutein from Sea breams.

Fig. 6. The absorption spectra of a new keto-carotenoid, α -doradecin which was reported in the previous paper³⁾, from Sea breams.

Thus purified pigments were rechromatographed on dried, powdered sugar, using 2% acetone in petroleum ether as developing solvent. The band of pigments was eluted with acetone, retransferred to petroleum ether and washed repeatedly with water to remove acetone. In petroleum ether this pigment exhibited an absorption maximum at 454 and 470 $m\mu$.

The pigment was reduced by the addition of sodium borohydride in 95% ethyl alcohol at 5°C. The absorption spectrum of the reduced pigment agreed with that of lutein. These results were identical with that of α -doradecin found in gold fish³⁾ (Fig. 6).

Astaxanthin and astacin. Before saponification the pigments of the Band C were purified chromatographically on Microcel-C, using 2% acetone in petroleum ether. It presented a single maximum at 468 $m\mu$ in petroleum ether and after reduction with sodium borohydride in ethanol, three maxima appeared at 425, 451, 478 $m\mu$, being characteristic for the β -carotene polygenic system (Fig. 7).

After saponification the pigment was transferred to ethyl ether, the solution was

Fig. 7. The absorption spectra of astaxanthin ester from Sea breams.

acidified by the addition of glacial acetic acid and washed repeatedly with water to remove the acetic acid. The pigments were rechromatographed on dried, powdered sugar, using 2% acetone in petroleum ether. The main zone was extracted with acetone and retransferred to petroleum ether. The absorption maximum was 472 m μ . The pigment was reduced by addition of sodium borohydride. The absorption maximum agreed with that of synthesized astacin.

Results and Discussion

The existence of ϵ -carotene, 3,3'-dihydroxy- ϵ -carotene, lutein, α -doradecin and astacin was revealed in the pigments extracted from sea breams by the chromatographic separation, and all of those pigments existed in the form of esters except ϵ -carotene.

The contents of astaxanthin in the cultured sea breams were extremely little, and the mixture of astaxanthin ester and α -doradexanthin ester in the natural sea breams seem to make them brightly red, so it was assumed that the faded color of the cultured sea breams was caused by the extremely small amount of astaxanthin ester and α -doradexanthin ester in them.

It is the subject of further work to clarify that α -doradexanthin ester is oxidized from lutein ester in sea breams de novo or comes from their food, and also 3,3'-dihydroxy-

Fig. 8. The metabolic pathway from ϵ -carotene to 3,3'-dihydroxy- ϵ -carotene ester.

Fig. 9. The metabolic pathway from lutein ester to α -doradexanthin ester.

ϵ -carotene is metabolized from ϵ -carotene in sea breams or comes from their food as follows (Fig. 8 and 9).

Summary

1. The existence of ϵ -carotene, 3,3'-dihydroxy- ϵ -carotene, lutein, α -doradecin and astacin was confirmed in the carotenoids of sea breams and those exist in the form of esters except ϵ -carotene.

2. It was assumed that the bright red color of natural sea breams would come from the mixture of astaxanthin ester (red color) and α -doradexanthin ester (golden color).

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