Studies on the Carotenoids in the Muscle of Salmons I. Intracellular Distribution of Carotenoids in the Muscle

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Studies on the Carotenoids in the Muscle of Salmons

I. Intracellular Distribution of Carotenoids in the Muscle

Hikaru HENMI, Tsutomu IWATA*, Masahiro HATA and Mitsuo HATA**

Laboratory of Fishery Chemistry, Department of Fishery Science, Faculty of Agriculture, Tohoku University, Sendai, 980, Japan

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Summary

Astaxanthin in the muscle of sockeye salmon existed in the water-insoluble fraction only.

Subcellular fractionation of the muscle revealed that astaxanthin and/or canthaxanthin existed in the myofibrils. The analysis of the actomyosin prepared from the myofibrils demonstrated that these carotenoids bind to actomyosin. The treatment of the muscle with detergents, salts and EDTA suggest that these carotenoids bind to actomyosin directly by weak hydrophobic bond.

It is well known that red color of the muscle in salmonids is due to the presence of red carotenoid pigments such as astaxanthin and canthaxanthin (1-3). These carotenoids are present in small crustaceans that form an important part of the diet of salmonids. Canthaxanthin is not metabolized to astaxanthin in salmonids. Therefore the existence ratio of these carotenoids in the muscle is due to the difference of the dietary carotenoids. In nature, astaxanthin is the main red carotenoid in salmons and canthaxanthin is only found in fresh water trouts (4-6).

In muscle, astaxanthin exists in free form. Crozier (7) reported that astaxanthin existed in the specific lipoprotein bound to mitochondria in muscle cells. Keith (8) reported that astaxanthin was stored in the sarcoplasmic membrane. Their results are very different and detailed results were not reported until now.

* Present address: Nakano Biochemical Research Institute, Nakano Vinegar Co., Handa, Aichi, 475, Japan
** Present address: Toyoko Women's College, Todoroki, Setagaya, Tokyo, 158, Japan

In the present study, subcellular fractionation of the muscle was carried out by the method of differential centrifugation to elucidate the intracellular distribution of carotenoids, and it was confirmed that astaxanthin and/or canthaxanthin exists in actomyosin.

Experiments

Materials

Chum salmon, *Oncorhynchus keta*, collected at Otsuchi river, coho salmon *Oncorhynchus kisutch*, fed canthaxanthin as a pigment source and sockeye salmon, *Oncorhynchus nerka*, from Alaska were used.

These specimens were stored at −80°C until use.

Subcellular fractionation of the muscle of sockeye salmon by differential centrifugation

Skeletal muscles (50 g) were cut into small pieces and homogenized for 3 min in 5 volumes (v/w) of cold Tris-HCl buffer, pH 7.0, containing 0.25 M sucrose and 100 mM ascorbic acid using a Nippon Seiki Universal homogenizer. The homogenate was centrifuged for 5 min at 150 xg at 4°C (PPT1, SUP1), and the supernatant (SUP1) was rehomogenized for 30 sec and centrifuged for 5 min at 2000 xg at 4°C (PPT2, SUP2). Cell organelles in each fraction are shown in Fig. 1.

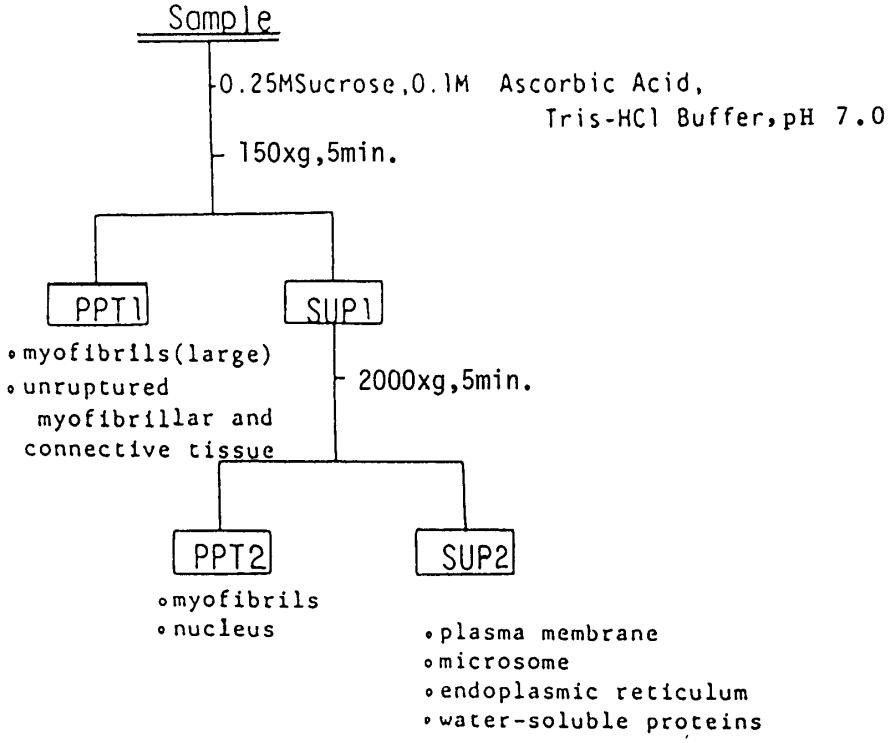


FIG. 1. Subcellular fractionation of sockeye salmon muscle.

Preparation of myofibrils and actomyosin

Myofibrils were prepared by the method of Perry (9).

Actomyosin was prepared by the method of Arai (10).

A microphotograph of the myofibrillar fraction was taken by the method of Takahashi et al. (11).

Treatment of muscle with detergents, salts and EDTA

The muscle was homogenized for 2 min in 5 vol. (v/w) of 0.1 M phosphate buffer, pH 6~9, containing various detergents or NaCl or EDTA, and centrifuged for 20 min 1000 rpm.

The astaxanthin in supernatant was extracted with acetone-petroleum ether and the content was determined.

The supernatant obtained by Triton X-100 treatment was applied to a Sepharose CL-6B column (2.5×41 cm) equilibrated with the 0.1 M phosphate buffer, pH 7.0, containing 0.5% Triton X-100 and eluted with the same buffer. The astaxanthin fractions were pooled and the excess Triton X-100 was removed with Bio-Beads SM-2 (Bio-Rad) and subjected to polyacrylamide slab gel electrophoresis. Electrophoresis was carried out at 30 mA for 4 hr. After electrophoresis, the gel was stained with Coommassie Brilliant Blue.

Analysis of carotenoids

Carotenoids were extracted with acetone and identified by the following methods; (i) absorption spectrum, (ii) behavior of chromatogram on thin layer chromatography (silica gel, Wakogel-B5), (iii) comparison with authentic standards on TLC.

Carotenoid content was determined using $E^{1\%}_{1cm}=2680$ (471 nm, petroleum ether).

Determination of protein content

Protein content was determined by the method of Lowry et al. (12), and bovine serum albumin was used as the standard.

Results

Distribution of astaxanthin in subcellular fractions from sockeye salmon muscle

Carotenoid was extracted from sockeye salmon muscle and identified as astaxanthin by the absorption spectrum and the behavior of the chromatogram on silica-gel TLC (Fig. 2, 3).

Astaxanthin contents in subcellular fractions are shown in Fig. 4. Astaxanthin contents in whole muscle (50 g), the Homogenate, SUP1, PPT1, SUP2, and PPT2 were 1036, 95, 910, 0, and 57 μg respectively. Purification folds in the

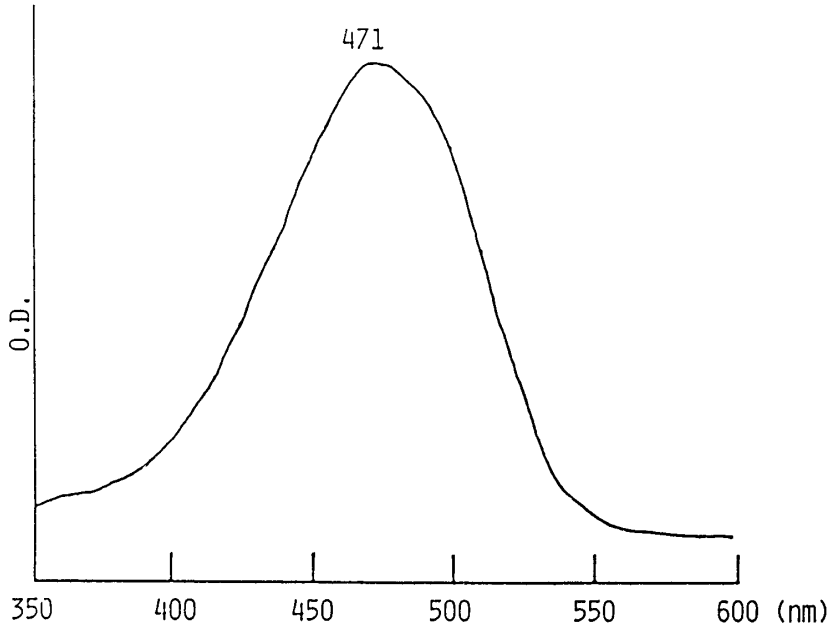


FIG. 2. Absorption spectrum of the carotenoid (astaxanthin) from sockeye salmon muscle in PE.

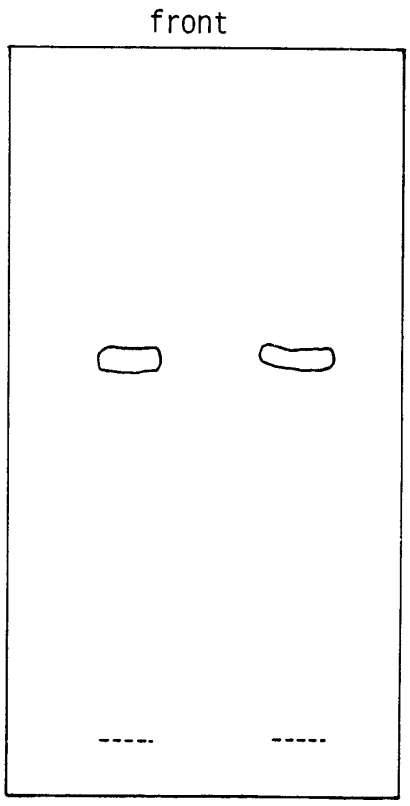


FIG. 3. Thin-layer chromatogram of the carotenoid from sockeye salmon muscle. Adsorbent: silica gel Solvent: PE-acetone (70 : 30)

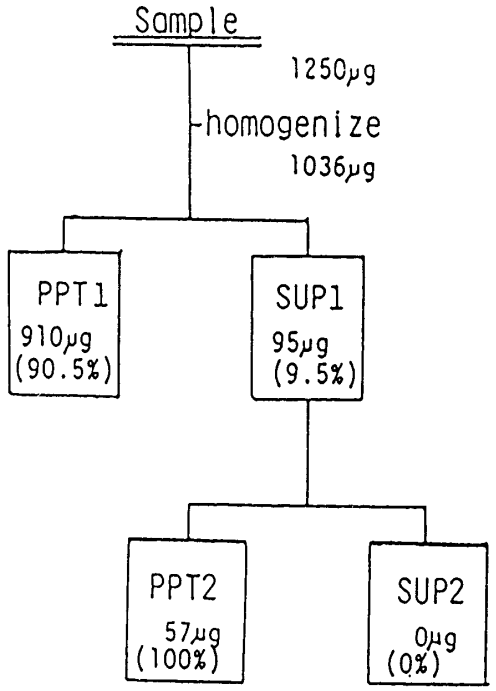


FIG. 4. Distribution of astaxanthin in subcellular fractions from sockeye salmon muscle.

TABLE 1. *Recovery of Astaxanthin and Proteins*

Fraction.	Astaxanthin (μg)	Protein (mg)	Astaxanthin (μg) / Protein (mg)	Purification fold
Homogenate	1036	3012	0.34	100
SUP 1	95	447	0.21	62
PPT 1	910	2142	0.42	124
PPT 2	57	98	0.58	171

Homogenate, SUP1, PPT1, and PPT2 were 100, 62, 124, and 171 respectively (Table 1). In PPT2, myofibrils and nucleus are present. However, colored myofibril was only observed by lightmicroscopy.

These results suggest that astaxanthin exists in myofibrils.

Existence of ketocarotenoids in myofibrils

As mentioned above, it was suggested that astaxanthin exists in myofibrils. To confirm this result, preparation of myofibrils, and identification of carotenoids were carried out. Coho salmon muscle myofibrils were prepared by the method of Perry (9).

After centrifugation of the myofibrillar fraction of coho salmon for 20 min at 600 xg, orange colored myofibrils were obtained (Fig. 5, 6). Carotenoids of the myofibrils were extracted with acetone and subjected to TLC. Canthaxanthin was found as a main carotenoid (Fig. 7, 8).

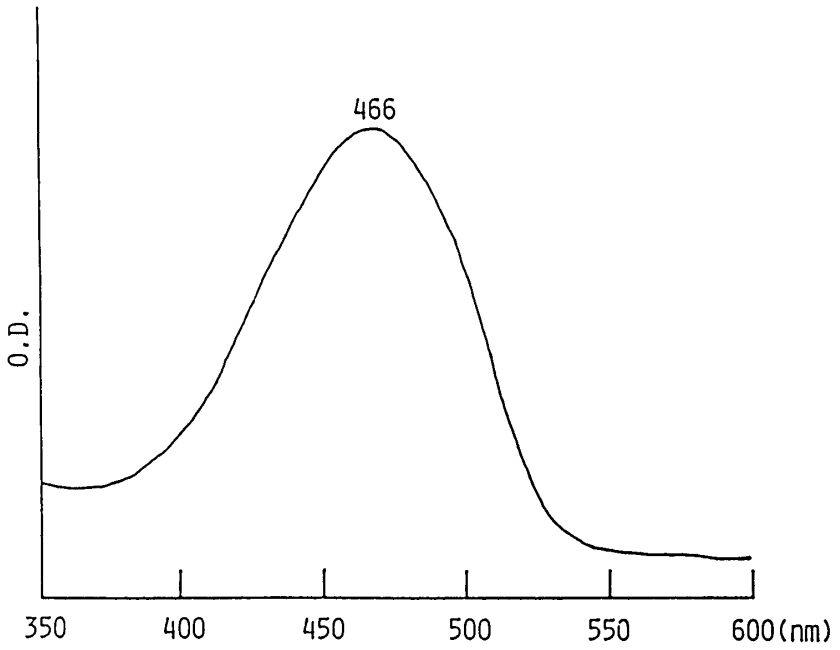


FIG. 7. Absorption spectrum of the carotenoid from coho salmon myofibrils in PE.

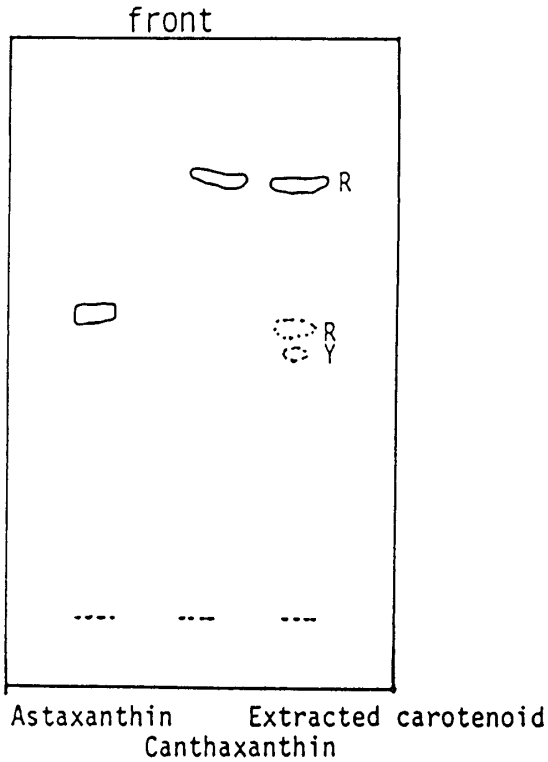


FIG. 8. Thin-layer chromatogram of the carotenoids from coho salmon myofibrils. Adsorbent: silica gel Solvent: PE-acetone (70 : 30) R: red, Y: yellow.

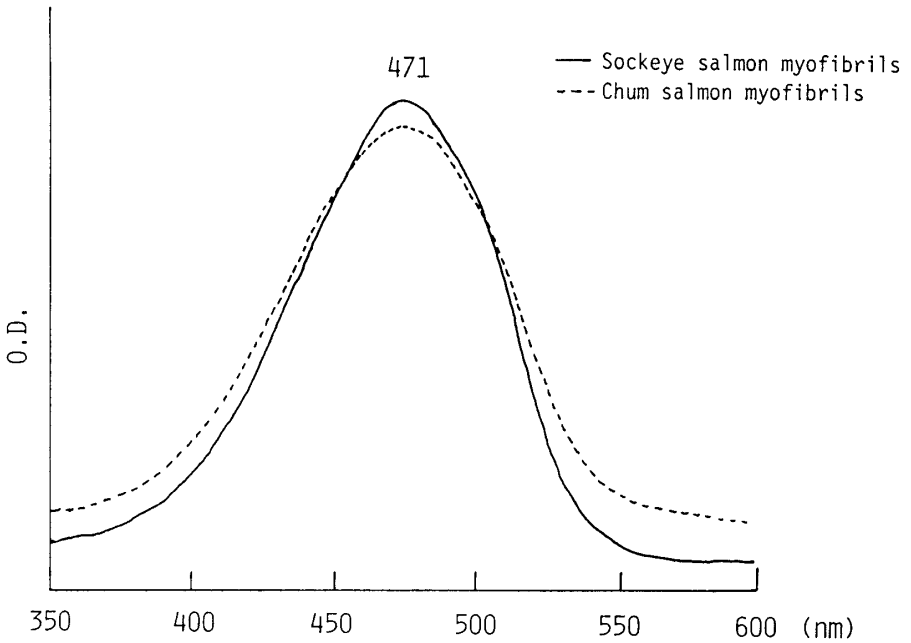


FIG. 9. Absorption spectra of the carotenoids from sockeye salmon and chum salmon myofibrils in PE.

The carotenoid in the myofibrils of sockeye salmon and chum salmon was astaxanthin (Fig. 9).

Existence of ketocarotenoids in actomyosin

Actomyosin was extracted from the muscle of sockeye salmon with 0.6M KCl. The purified actomyosin was dissolved in 0.6M KCl-phosphate buffer, pH 7.5, and trichloro acetic acid (TCA) was added to the solution to a concentration of 5% (w/v) and centrifuged for 10min at 600xg. Reddish colored actomyosin coagulate was observed (Fig. 10).

ATP was added to the actomyosin solution to a concentration of 1mM, a contractible precipitation (ATP-superprecipitation) occurred and the precipitate showed red color (Fig. 11, 12).

Carotenoid was extracted from the precipitate and identified as astaxanthin by the absorption spectrum and the behavior of the chromatogram on silicagel-TLC (Fig. 13, 14).

Canthaxanthin was identified as ketocarotenoid from the actomyosin of coho salmon.

These results indicate that astaxanthin and/or canthaxanthin bind to actomyosin.

The effect of detergents, salts and EDTA

Astaxanthin was solubilyzed with sodium dodecyl sulfate (SDS), Triton X-100 and sodium cholate. Salts and EDTA had no effect (Table 2). The

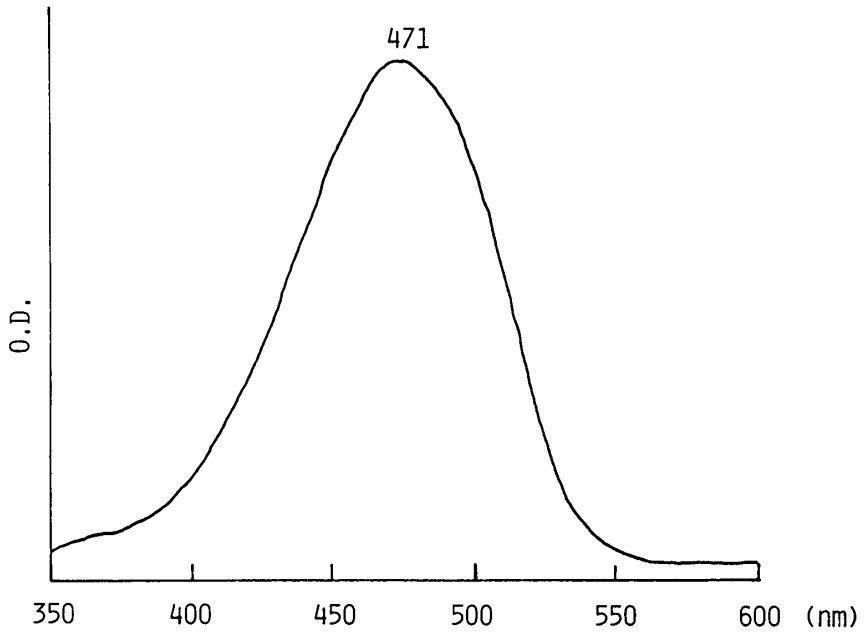


FIG. 13. Absorption spectrum of the carotenoid from sockeye salmon actomyosin in PE.

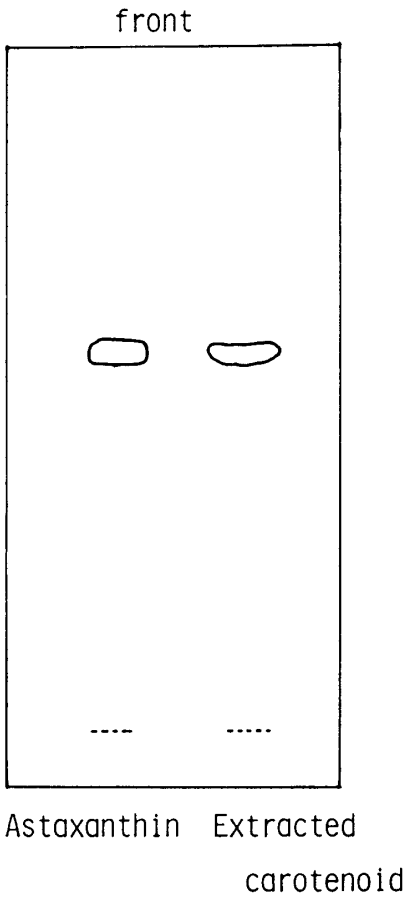
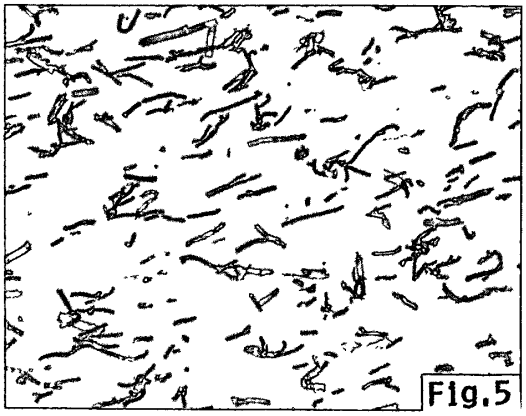


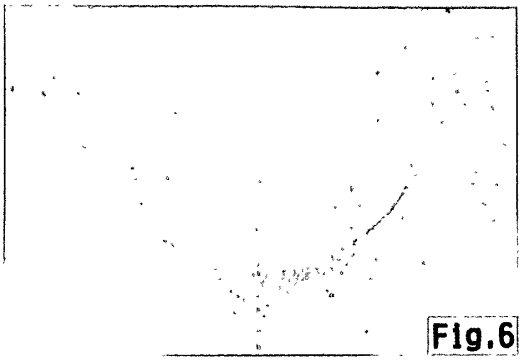
FIG. 14. Thin-layer chromatogram of the carotenoid from sockeye salmon actomyosin. Adsorbent: silica gel Solvent: PE-acetone (70 : 30)

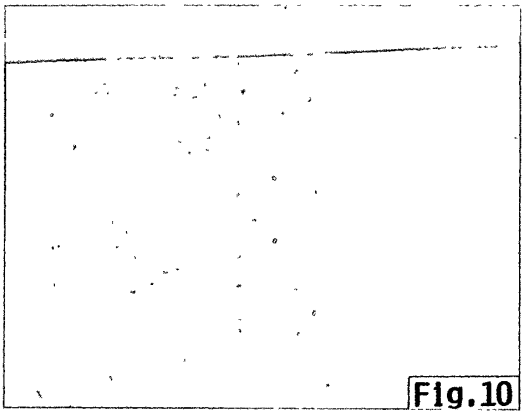
TABLE 2. *Solubilyzation of Astaxanthin from the Salmon Muscle*

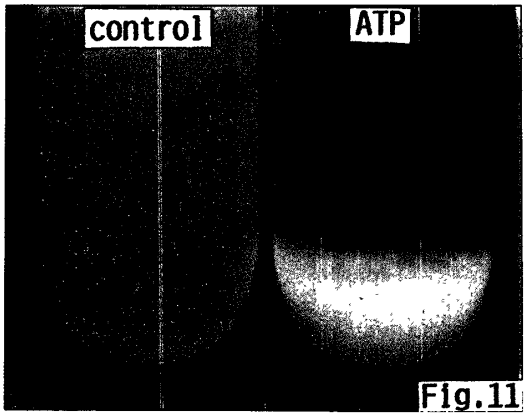
	pH		
	6.0	7.0	8.0
Control (0.1 M Phosphate buffer)	−	−	−
+0.5% SDS	−	−	−
+0.1% SDS	−	++	++
+2.0% SDS	−	++	++
+1.0% Triton X-100	++	++	++
+2.0% Triton X-100	++	++	++
+1.0% Tween 20	+	+	+
+2.0% Tween 20	++	++	++
+0.2% Sodium deoxycholate	−	−	−
+1.0% Sodium cholate	−	−	+
+0.1 M NaCl		−	
+0.5 M NaCl		−	
+0.4 M Ammonium sulfate		−	
+50 mM EDTA		−	

supernatant obtained by Triton X-100 treatment was applied to a Sepharose CL-6B column, and the partially purified astaxanthin fraction was subjected to polyacrylamide slab gel electrophoresis. The protein corresponding to astaxanthin band was not detected by Coommassie Brilliant Blue staining after electrophoresis, suggesting that astaxanthin-protein complex is not solubilyzed by detergents.









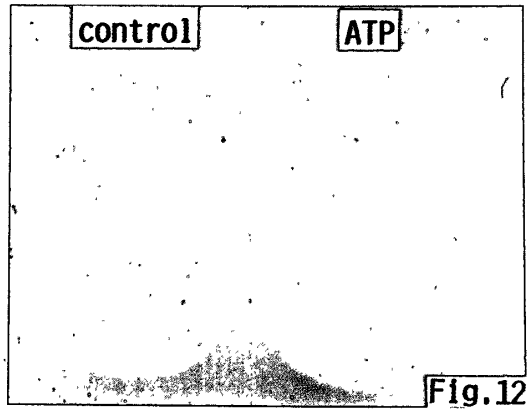


FIG. 5. Microphotograph of the myofibrils of coho salmon (×200).

FIG. 6. Myofibrils of coho salmon (2000×g, 10 min.).

FIG. 10. TCA-precipitation of sockeye salmon actomyosin.

FIG. 11. ATP-superprecipitation of sockeye salmon actomyosin.

FIG. 12. ATP-superprecipitation of sockeye salmon actomyosin.

Discussion

From the results of subcellular fractionation, it was confirmed that astaxanthin did not exist in plasma membrane fraction and mitochondria fraction, but in myofibrillar fraction only. These results were different from other reports (7, 8).

In the muscle of sockeye salmon, the carotenoid was astaxanthin only, but, in the muscle of coho salmon or chum salmon, a small amount of other carotenoids in addition to astaxanthin or canthaxanthin existed.

These results suggest that myofibrils and actomyosin of salmonid combine not only astaxanthin and canthaxanthin but also non-ketocarotenoids. The presence of a small amount of non-ketocarotenoids such as lutein, zeaxanthin, diatoxanthin and cynthiaxanthin in the muscle of salmonid have been reported by many investigators (4, 13–15). Astaxanthin and/or canthaxanthin-protein complex was not solubilyzed by detergents, salts and EDTA. However astaxanthin was solubilyzed by non-ionic detergents, especially by Triton X-100. These results indicate that astaxanthin and/or canthaxanthin bind to actomyosin directly by a weak hydrophobic bond.

Acknowledgement

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References

1) Kanemitsu, T., and Aoe, H., *Bull. Japan. Soc. Sci. Fish.*, **24**, 209 (1958) (in Japanese, with English summary)
2) Khare, A., Moss, G.P., Weedon, B.C.L., and Matthews, A.D., *Comp. Biochem. Physiol.*, **45B**, 971 (1973)
3) Kitahara, T., "*Carotenoids of Aquatic Animals*", ed. by Japan. Soc. Sci. Fish. Koseisha-Koseikaku, Tokyo, Japan, 1978, pp 78～89
4) Matsuno, T., Katsuyama, M., and Nagata, S., *Bull. Japan. Soc. Sci. Fish.*, **46**, 879 (1980) (in Japanese, with English summary)
5) Thommen, H., and Wackernagel, H., *Die Naturwissenschaften*, **51**, 87 (1964)
6) Matsuno, T., Nagata, S., Katsuyama, M., Matsutaka, H., Maoka, T., and Akita, T., *Bull. Japan. Soc. Sci. Fish.*, **46**, 473 (1980) (in Japanese, with English summary)
7) Crozier, G.F., in "*Chemical Zoology*" (M. Florkin and B.T. Scheer ed.), Vol. 3, Academic Press, New York, 1974, pp 509～521
8) Keith, M., *The International Symposium on Feeding and Nutrition in Fish*, **Abstract**, Aberdeen July, 10～13, 1984
9) Perry, S.V., and Grey, T.C., *Biochem. J.*, **64**, 184 (1956)
10) Arai, K., in "*Suisan Seibutsu Kagaku. Shokuhingaku Jikkensho*", Koseisha-

Koseikaku, Tokyo, Japan, 1974, pp. 179～188
11) Takahashi, K., Fukazawa, T., and Yasui, T., *J. Food. Sci.*, **32**, 409 (1967)
12) Lowry, O.H., Rowebrough, N., Farr, A., and Randall, R., *J. Biol. Chem.*, **193**, 265 (1951)
13) Hata, M., and Hata, M., *Tohoku J. Agr. Res.*, **26**, 35 (1975)
14) Kitahara, T., *Comp. Biochem. Physiol.*, **76B**, 97 (1983)
15) Kitahara, T., *Comp. Biochem. Physiol.*, **78B**, 859 (1984)