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Analytical Studies on the Lipids in Carotenoprotein purified form *Salmo Salar* Eggs

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연어알에서 분리 정제한 carotenoprotein 지질 성분에 관한 연구

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요 약

이온교환 크로마토그라피와 젤 여과법으로 연어알에서 carotenoprotein을 충분히 정제하였다. $CHCl_3$/MeOH (2/1)용매로 지질 성분을 추출한 후, Sephadex G-25로 정제하고 silicic acid 컬럼으로 분획하여 중성지질, 당지질, 인지질 그리고 콜레스테롤의 량을 각각 정량하였다. T.L.C.와 HPLC로 개별 성분을 분리, 정량한 결과 중성지질은 tripalmitate와 cholesteryl oleate로 구성되었고, 당지질은 esterified sterylglycoside와 소량의 cerebroside, 인지질은 p.c, ce, lp.c 및 p.e로 구성 되었음을 밝혔다. 결합당 성분으로서는 galactose만이 검출되었다.

지방산은 palmitate와 oleate가 대부분이었으며, 6종류의 sterol성분을 분리 확인하였다. 지질과 결합된 단백질의 아미노산도 분석하여 이들로부터 carotenoprotein의 본성을 추론하였다.

INTRODUCTION

Carotenoproteins are believed to be lipo(glyco) proteins[1] in which carotenoids are bound stoichimetrically to simple proteins, glycoproteins and/or lipoproteins. However, in spite of the large number of studies on the lipid compositions, the nature of the binding and its functions within tissues are unknown. In a *Salmo salar* eggs, biochemical characteristics of both enzymes and chromoproteins have been studied by us[2,3] for a long time. Our intentions are related to the facts how water-soluble protein

complexes are associated with the components of carotenoid-transfering low and/or high density lipoproteins, and stabilize the hydrophobic structures during the embryos in the fish-eggs. Therefore, carotenoprotein was highly purified from the *Salmo salar* eggs, and then its lipids, fatty acids, sterols, carbohydrate and amono acids of carotenoprotein were seriously analysed. On the basis of the analytical results the nature of bindings in the carotenoprotein was discussed in this paper.

MATERIALS AND METHODS

Extraction and purification

Carotenoprotein was purified from the *Salmo salar* eggs as previously described.[2,3] It was homogenated with physiological salt solution. To the homogenate, 5% TCA was added to remove a trace of acid soluble and metabolitic intermediates, and then centrifuged at 600 x g for 10 min. The residue was freeze dehydrated, lipids were extracted three times with binary solvent chloroform/methanol (2/1) for 48 hrs on a shaker. All extracts were pooled and concentrated to dryness under N_2 gas, below 20℃.

Nonlipid contaminants were freed by Sephadex G-25 column according to the method of Rouser *et al*[4]. A silicic acid column was also used for separation of the lipids into neutral, glycolipid and phospholipid fractions. The lipids eluted from Sephadex G-25 with water-saturated chloroform/methanol(19/1) were then subjected on the silicic acid column (Bio-gel 325 mesh, 0.5x10㎝), without additional treatment, equilibrated with chloroform. Fractionation was started with chloroform to separate neutral lipids at a flow rate of 1 ml/min, then continued with acetone for elution of glycolipids, and finally phospholipids were eluted with methanol.

Lipid assay

For preliminary lipid analysis, TLC method were performed. The fractionated lipids from silicic acid column were concentrated to dryness, thereafter 0.5μℓ samples were applied to a origin of the activated TLC plate had coated with silicagel G.

The plates were developed with chloroform/methanol/water (65 : 25 : 4)[4] system for separation of the polar lipids, and petroleum ether/diethylether/acetic acid (80 : 20 : 1)[5] for neutral lipids, respectively. The spots were visualized with iodine vapor and/or 40% sulfuric acid char spray and heating. The chromogenic reagents used for functional groups of lipids were : Lieberman-Burchard's reagent to identify neutral lipid, Molisch and anthrone reagents to glycolipid, Dragendorff's reagent to choline containing phospholipids, ninhydrin to amino group containing phospholipid, and molybdenum reagents to detect general phospholipids, respectively. HPLC and uv-spectroscopic method were performed for separation and assay of the individual lipid contents. The total lipid contents were determinated by the method of Joo *et al*[6] using phosphovanillin reagent(Vanillin, 0.6g;ethanol, 10mℓ;H_2O, 100dℓ;H_3PO_4, 400mℓ) at 525 nm after treatment with conc sulfuric acid, and heating in a water bath for 10 min. Glycolipids were assayed with anthrone. Phosphorus and phospholipids were estimated by the modified metnod of Fiske *et al*[7], after digestion with 50% perchloric acid and heating in a water bath for 10 min to hydrolyse phosphate. Neutral lipids were determined by the method of Briggs *et al*[8].

Cabohydrate assay

Glycolipid sample was hydrolysed in sealed ampoule at 100℃ for 4 hr with 1N－HCl. The ampule contents were filtered and passed through a Dowex cation exchange resin column, then washed with water. The filtrates were pooled, the sugars were then determined by HPLC.

Free fatty acids

The fractionated neutral, glyco-and phospholipids were concentrated, then hydrolysated with 2N alcoholic NaOH for 24hr at room temperature in the dark. The residue was filtered off, unsaponificable components were extracted with diethylether to assay of sterols, and subsquently made alkali free by washing with distilled water. The alkline solution layer was acidified with 2N－HCl, extracted with diethylether, dehydrated with sodium sulfate, and then concentrated with N_2. The free fatty acids were methylated, and assayed with the gas chromatograph with the general operating parameters.

Sterol analysis

Sterols were purified and cochromatographed with a standard cholesterol solvent. The sterol band (R_f =0.3) separated on TLC was scraped off. The total sterols were assayed with Liebermann-Burchard's reaction. The individual sterols were analysed by GC on the 10% OV-275 column.

RESULTS AND DISCUSSION

Purification and lipid assay

Lipid extracts were purified through a Sephadex G-25 column with water-saturated chloroform/methanol(19/1). After removal of the solvents, the total lipid contents by spectroscopic assay were found to be 21.5% on dry-weight basis. Elution of the lipids of a silicic acid column was started with pure chloroform, then with acetone and methanol respectively. Neutral lipid contents were 39.8%, then phospholipids were 57.3%, which were very rich. The analytical results were summarized in Table 1. Glycolipid contents were relatively low(2.9%), cerebride was also detected as minor.

Table 1. Analytical Results of the Carotenoprotein

In	carotenoprotein	
	total lipid	21.5 %
In	extracted lipid	
	netral lipid	39.8 %
	glycolipid	2.9 %
	phospholipid	57.3 %
	phosphorus	2.72%
	total cholesterol	23.6 %
	cerebroside	0.37%

With results of TLC and HPLC analysis, the carotenoprotein from *Salmo salar* eggs contained high cholesterol esters as major constituents. Total cholesterol contents by Liebermann-Burchard's reaction were 23.6%. The spots corresopnding in position to phospotidyl choline, lysophosphotidyl choline, and phosphotidyl ethanolamine were identified on thin-layer chromatography of the phospholipids.

All the lipids stained with iodine vapor, indicating unsaturated fatty acids. HPLC chromatograms of the fractioned lipids were given in Fig. 1 and Table 2. The neutral lipid contained high contents of cholesteryl oleate, in contrast to low tripalmitate. In the glycolipids the high steryl glyconside contents and relatively low cerebroside were confirmed in the TLC and HPLC, respectively.

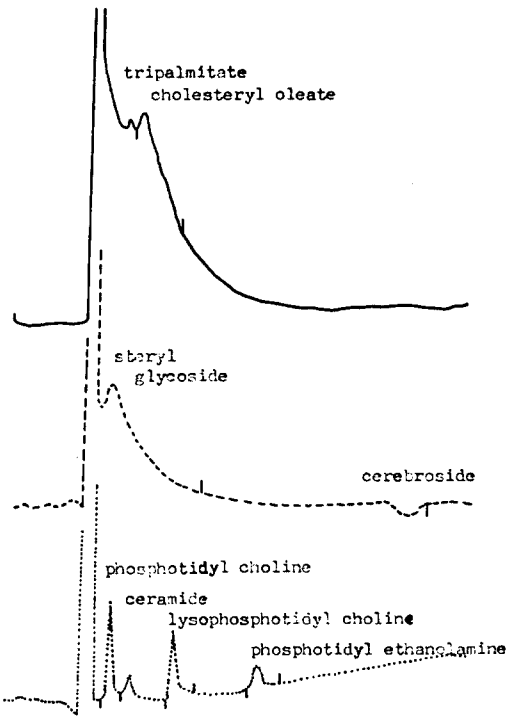

Fig. 1. HPLC Chromatograms of the lipids
——;neutral lipid
--- ;glycolipid
····· ;phospholipid

Phosphotidyl choline and lysophosphotidyl choline apperated to be the most abundant phospholipids in the carotenoprotein. They were thought to intract with cholesteryl moiety of cholesterol esters like LDL and/or HDL does. The glycolipid contained small amount of carbohydrate, a galactose was the only sugar component idetified with HPLC (Fig. 2), which was very simple in composition.

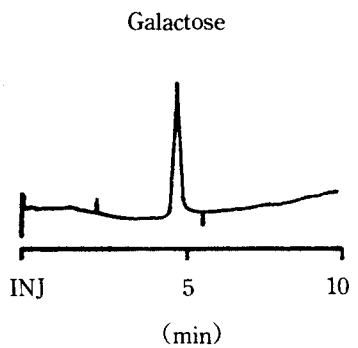

Fig. 2. HPLC chromatogram of the sugar in glycolipids

Table 2. Composition of the Lipids

Neutral lipids	Glycolipids	Phospholipids
tripalmitate(18.7%) cholesteryl oleate(81.3%)	esterified steryl glycoside(99.6%) cerebroside(0.37%)	phosphotidyl choline(42.5%) ceramide(8.7%) lysophosphotidyl choline(28.1%) phosphotidyl ethanolamine(20.7%)
-Bondapak C_{18} RI detector CH_3CN-Acetone(42/58	-Bondapak C_{18} uv 200 nm CH_3CN-iprOH(1/1)	ultrasil-NH_2 uv 206 nm Hexane-iprOH(5.5/8)

Fatty acids and sterols

The percentage composition of fatty acids on GC analysis were recorded in Fig. 3 and Table 3. The main fatty acids were oleate and palmitate. It was very similar to that of the pelyads lipids, fresh water-fish. The neutral and glycolipid were higher in oleate than in the other component, while linolenate and behenate were never contained. Saturated and unsaturated fatty acids were indiscriminately distributed in the phospholipids. Of them, the saturated fatty acids ($C_{18:0}$, $C_{16:0}$, $C_{14:0}$ and $C_{22:0}$) were particularly rich. The amino acids in the carotenoprotein were determined by HPLC. Hydrophobic groups were 41%, apolar residues, 24% and polarity index, 40.4%, respectively. This facts would be that

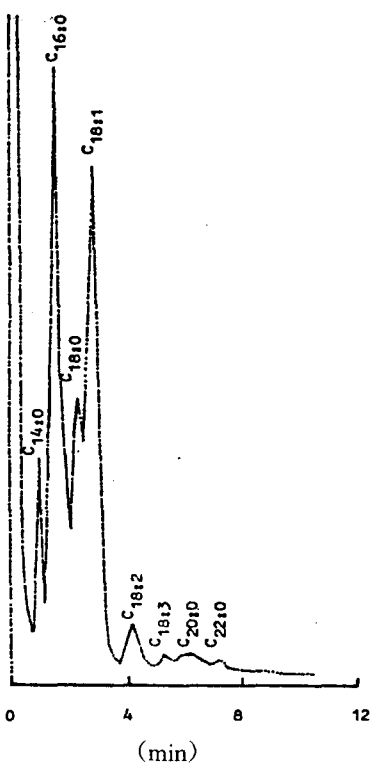

Fig. 3. Gas chromatogram of total lipid in carotenoprotein

Table 3. Fatty acid Composition of Lipids

Fatty acids	Total lipid	Neutral lipids	Glycolipids	Phospholipids
$C_{14:0}$	5.98	1.12	0.93	18.39
$C_{16:0}$	36.10	13.62	11.91	21.35
$C_{18:0}$	13.03	7.03	7.02	20.21
$C_{18:1}$	38.85	59.56	55.92	7.98
$C_{18:2}$	3.33	17.35	21.39	1.80
$C_{18:3}$	0.77	–	–	12.27
$C_{20:0}$	1.63	1.31	2.81	7.01
$C_{22:0}$	0.31	–	–	11.08

the hydrophobic groups of the phospholipids may intract with flexible branched-amino acids residues near the carotenoprotein surfaces, and/or may faciliate anchoring between the hydrophobic groups and the neighboring hydrogen bond angles. Thus, the main fatty acid chains of phospholipids were thought to associate hydrophobically with carotenoproteins in contrast to the HDL-protein structures had no intrinsic affinity for chelesterol esters, as it well known. However, their geometric trans-isomers were never detected in any of the unsaturated fatty acids. The sterol portions, after liberation by alkaline hydrolysis, were chromatographed on TLC, then analysed by GC as its TMS derivative. There were C_{26}-sterol, C_{22}-dehydrocholesterol, cholesterol, brassica sterol (and desmosterol), $\triangle^{5,7}$-sterol, and C_{24}-methylene cholesterol. Comparing the sterol contents, cholesterol was also present in particularly high amounts as shown in Fig. 4 and Table 4. Experimental results were close to that of cholesterols in pacific coast oysters studied by Cordon *et al*[9]. Djerassi *et al*[10] reported the isolation and structure elucidation of seventy-four sterols from the sponge.

Table 4. Percentage of Major Sterols in Carotenoprotein

Common-trivial name	Systematic nomenclature	Empirical formula	Sterol % Neutral Lipid	Glycolipid	Phospholipid
C_{26}-sterol	22-trans-24-norcholesta-5, 22-dien-3β-ol	$C_{26}H_{42}O$	17.27	18.03	4.76
C_{22}-dehydrocholesterol	22-trans-cholesta-5, 22-diene-3β-ol	$C_{27}H_{44}O$	16.42	10.24	8.16
Cholesterol	cholesta-5-en-3β-ol	$C_{27}H_{46}O$	39.14	52.15	4.90
Brassica sterol (and Desmosterol)	24-methylcholesta-5, 22-diene-3β-ol	$C_{28}H_{46}O$	10.80	17.32	1.49
$\triangle_{5,7}$-sterol	Cholesta-5,7-diene-3βol	$C_{27}H_{44}O$	6.81	0.61	1.33
C_{24}methylenecholesterol	24-methylcholesta-5en-3β-ol	$C_{27}H_{46}O$	3.93	0.55	0.72
Other			5.59	1.07	78.64

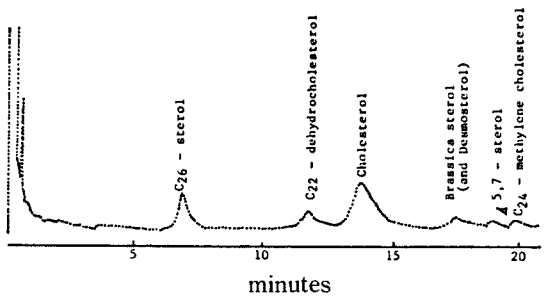

Fig. 4. GC chromatogram of the sterols.

Because of $\triangle^{5,7}$- sterol in bivalves or sponges, they were evaluated as the most diverse source of provitamin D sterols. However, apparently their sterol levels in marine products are thought to change with season, degree of starvation, and tissues. Though many other sterols were well known, there are no experimental studies on the functions acting as bigenergetic mass transfer in membranes, as some type of lipid solubilizing, and a role as biosynthetic intermediates for other sterols during embryos in the fish-eggs. So sterols' biosynthesis and their functions in the carotenoprotein will be discussed in future.

ABSTRACT

Carotenoprotein was highly purified by ion exchange chromatography and gel filtration from *Salmo salar* eggs. Its lipids were extracted with $CHCl_3$/MeOH(2/1), nonlipid contaminants were freed by Sephadex G-25, then a silicic acid column was used for separation of the lipids into neutral, glycolipid and phospholipid fractions. With the results of TLC and HPLC analysis, they are found to contain tripalmitate and cholesteryl oleate in the neutral lipid, esterified steryl glycoside and low cerebroside in glcolipid, and the phospholipid contained p.c, ce, lp.c and p.e, respectively.

A galactose was the only bound sugar component. Palmitate and oleate were particulary rich in total

lipids, six mjor sterols were analysed, and amino acids in the carotenoprotein also were determined. On the basis of analytical results the natures of carotenoprotein were discussed.

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