Isolation of apolipoproteins from carotenoid-carrying lipoprotein in the serum of chum salmon, *Oncorhynchus keta*

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Abstract Carotenoid-carrying lipoprotein (CCL) was rapidly isolated from the high density lipoprotein (HDL) fraction of the upstream migrating male chum salmon (*Oncorhynchus keta*) by a single-step density gradient ultracentrifugation. The two apolipoproteins (M_r = 24,000 and 12,000; designated apo-I and apo-II, respectively) were readily dissociated and separated in 0.1% SDS by gel filtration chromatography. Prominent features of the amino acid composition in the CCL included the relative high levels of glutamic acid, alanine, leucine, and lysine, and the low cysteine content. Apo-I, as well as the CCL, was rich in glutamic acid, alanine, leucine, and lysine. Compared to the amino acid composition of apo-I, apo-II included relatively high levels of glycine and tyrosine, and low threonine, serine, and arginine contents. When the intact CCL particle was treated with trypsin, apo-I was rapidly proteolyzed, while apo-II was resistant. However, both apo-I and apo-II isolated from the CCL particle were readily digested with trypsin. This suggested that a different structural arrangement rather than the amino acid compositions of the apolipoproteins was associated with the limited trypsin digestion of the CCL particle. Apo-II may be sheltered from the aqueous environment and lie partly within the CCL particle. The properties of both the HDL fraction and apolipoproteins from pink salmon (*Oncorhynchus gorbuscha*) were similar to those of the CCL from chum salmon. —**Ando, S., and M. Hatano.** Isolation of apolipoproteins from carotenoid-carrying lipoprotein in the serum of chum salmon, *Oncorhynchus keta*. *J. Lipid Res.* 1988. **29:** 1264–1271.

Supplementary key words apolipoproteins • density gradient ultracentrifuge • gel filtration • high density lipoprotein

Pacific salmon (*Oncorhynchus* spp.) are typically anadromous fish, developing in the ocean and breeding in fresh water. This biological feature governs the pattern of changes in their chemical composition (1). For example, after migrating chum salmon enter a river, the muscle discolors and the integument becomes dark yellow or red. These changes in muscle or integument color may be closely associated with the physiological state of the fish which is probably controlled by sex hormones (2–7).

Kitahara (8) has reported that carotenoid pigments (mainly astaxanthin) absorbed through the food (zooplankton) are carried from the muscle to the integument and gonads via the serum during spawning migration of chum salmon. Little has been reported concerning carrier proteins of carotenoids in the serum of salmon, although several studies on the distribution and characterization of serum lipoproteins in salmon have been made (9–12). Nakamura, Hata, and Hata (13) have recently found that astaxanthin in the serum of upstream migrating chum salmon was exclusively transported by the high density lipoprotein (HDL) fraction. We have previously demonstrated that three types of carotenoid-carrying lipoproteins (CCLs) such as low density lipoprotein (LDL), HDL, and very high density lipoprotein (VHDL) fractions were present in the serum of chum salmon. The CCL from the VHDL fraction was derived from vitellogenin, a female-specific serum protein (14–17). The CCL from the HDL fraction became a main component during spawning migration (14, 18–20). The CCL from the HDL fraction in both males and females was associated with carotenoid transport from the muscle to the integument, while the carotenoids were transported into the ovaries via vitellogenin. We have recently isolated the CCL from the HDL fraction in the serum of upstream migrating chum salmon by a sequential ultracentrifugal technique, and DEAE-cellulose and gel filtration column chromatography (18). The CCL gave rise to two apolipoproteins whose molecular weights were 24,000 (apo-I) and 12,000 (apo-II), and no disulfide bond was detected between the two apolipoproteins. The molar ratio of apo-I to apo-II was close to 1:1. The CCL from the HDL frac-

Abbreviations: HDL, high density lipoprotein; CCL, carotenoid-carrying lipoprotein; LDL, low density lipoprotein; VHDL, very high density lipoprotein; Tris, tris(hydroxymethyl)amino methane; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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tion was a unique lipoprotein with high carotenoid levels (20, 21). Thus, the interaction between apolipoproteins and carotenoids in the CCL is of interest.

In order to broaden our basic understanding of structure and function in the CCL, we have isolated apolipoproteins from the CCL of the upstream migrating male chum salmon, *Oncorhynchus keta*, and we report their amino acid compositions. A difference in exposure of the two apolipoproteins in the aqueous environment is also suggested from the results of amino acid composition and limited trypsinization of the CCL from the HDL fraction.

MATERIALS AND METHODS

Animals

Male adult chum salmon, *Oncorhynchus keta*, 3 and 4 years old, were caught at the Yurappu River of Hokkaido. The gonadosomatic index (gonad weight × 100/body weight) and hepatosomatic index (liver weight × 100/body weight) were 3.6–4.3 and 1.7–1.9, respectively.

Preparation of lipoproteins

Blood was collected from the caudal vasculature of live salmon and left at room temperature for several hours. The clotted blood was centrifuged at 3,500 rpm for 15 min at 10°C to obtain the serum. The serum was analyzed by density gradient ultracentrifugation (22). An equal amount of 0.75% NaCl was gently layered over 4.74 ml of serum containing 1.9 g of KBr, and centrifuged at 218,000 *g* for 5 hr at 10°C. The carotenoid-carrying lipoprotein (CCL), which forms a sharp band at density 1.16 g/ml, was removed by injecting a needle directly into the centrifuge tube beneath the CCL band and withdrawing the solution containing the bright orange-colored portion. The KBr was then removed by dialysis overnight against 0.75% NaCl–20 mM Tris-HCl buffer (pH 7.5).

Density, lipid:protein ratio, and lipid and carotenoid analyses

The density of CCL was measured from the refractive index of KBr solution on an Abbe refractometer (Atago Co., Ltd.) following density gradient ultracentrifugation. Protein level in the CCL was measured by the biuret method (23), using bovine serum albumin as the standard. Lipid extraction from the CCL was done by the method of Bligh and Dyer (24). The extracted lipid was analyzed quantitatively by thin-layer chromatography (TLC). The TLC plates (Kieselgel 60, ready-made plate from Merck) were developed using *n*-hexane–diethyl-ether–acetic acid 85:15:1 (by volume) for neutral lipids, and chloroform–methanol–acetic acid–water 25:15:4:2 (by volume) for phospholipids. The TLC plate was sprayed with 3% copper acetate–8% phosphoric acid, heated on a hot plate, and quantitated using a Cosmo F-808 densitometer. The amount of phospholipid was calculated from lipid-phosphorus assayed by the method of Fiske and Subbarow (25). Carotenoid content was calculated assuming the $E^{1\%}_{1\,cm}$ value in acetone at 477 nm to be 2,200. High performance liquid chromatographic (HPLC) analysis of carotenoids was carried out on a Shimadzu LC-6A instrument with a Shimadzu SPD-2A VIS spectrophotometer set at 470 nm. The column used was a 250 × 4 mm i.d. stainless steel column packed with 5 μm Sumipax OA-2000 (Sumitomo Chemical Co., Ltd.). Separation was achieved with a mobile phase of *n*-hexane–dichloromethane–ethanol 50:20:0.5 (by volume) at a flow-rate of 0.8 ml/min. Identification of each carotenoid was accomplished by co-TLC and co-HPLC with authentic specimens. The authentic carotenoids used were as follows: astaxanthin diester, astaxanthin monoester, and astaxanthin were extracted and purified from the Antarctic krill *Euphausia superba* (26, 27); zeaxanthin was extracted and isolated from *Spirulina maxima* (28).

Separation of apolipoproteins

Apolipoproteins of the CCL were dissociated in 1% SDS–1% 2-mercaptoethanol at 25°C overnight, and isolated by gel filtration chromatography on a 3.2 × 89 cm Sephadex G-100 column equilibrated in 0.1% SDS–20 mM Tris-HCl buffer (pH 7.5) containing 0.02% NaN_3. Column eluent was monitored at 278 nm and protein peaks were combined and dialyzed against distilled water.

Amino acid analysis

The amino acid compositions of the delipidated CCL and the apolipoproteins were determined on a Hitachi model 835 automatic amino acid analyzer after hydrolysis with 4 N methanesulfonic acid at 115°C for 24 hr.

Gel electrophoresis

Electrophoresis in 5% polyacrylamide gel was carried out at pH 8.3, 2 mA per tube, as described by Davis (29). SDS-slab-15% polyacrylamide gel electrophoresis (PAGE) was performed in the presence of 0.1% SDS, by the method of Laemmli (30). After electrophoresis, gels were stained with Coomassie Brilliant Blue.

Limited trypsinization

The native CCL and the apolipoproteins from the CCL were subjected to limited trypsinization. One mg of the CCL and the apolipoproteins in 20 mM Tris-HCl buffer (pH 7.5) was incubated for various times with trypsin (Sigma Chemical Co., Ltd.) at a concentration of 1:100 (trypsin:sample) for times ranging from 0 to 60 min at 25°C. The reaction was initiated by the addition of trypsin and stopped by addition of SDS-PAGE sample treatment buffer (pH 6.8) containing 20 mM Tris-HCl, 2% SDS, 2% 2-mercaptoethanol, and 40% glycerol, and boiling at

100°C for 2 min. Samples were subsequently analyzed by SDS-slab-15% PAGE.

RESULTS

The major lipoprotein in the serum of upstream migrating male chum salmon was isolated from the high density lipoprotein (HDL) fraction by a single-step density gradient ultracentrifugal separation. A bright orange band was present in the middle portion, corresponding to the HDL fraction (d 1.16 g/ml) of the tube. The homogeneity of the HDL fraction, which corresponded to carotenoid-carrying lipoprotein (CCL), was checked by PAGE. Under native conditions the CCL migrated as a single homogeneous band. The CCL gave two apolipoproteins whose molecular weights were 24,000 (apo-I) and 12,000 (apo-II) as judged by SDS-slab-PAGE (**Fig. 1**).

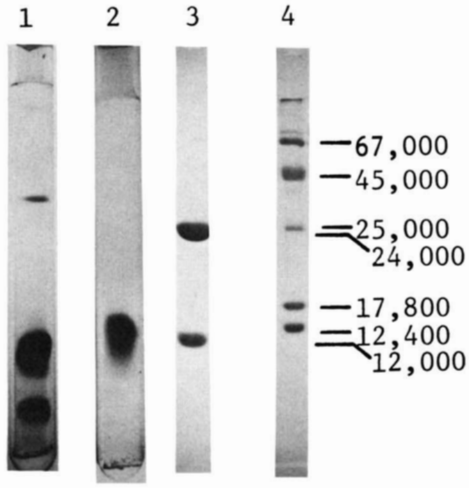

Fig. 1. Native PAGE (5% gel) of 1) whole serum protein (50 μg) and 2) isolated CCL (50 μg), and SDS-slab-PAGE (15% gel) of 3) isolated CCL (50 μg) and 4) molecular weight standards (bovine serum albumin, 67,000; egg albumin, 45,000; chymotrypsinogen, 25,000; myoglobin, 17,800; cytochrome, 12,400).

Lipid and carotenoid analyses

Table 1 shows the properties of CCL, along with those of the HDL fraction from pink salmon (9). Compared to the HDL fraction from pink salmon, the CCL contained more protein and less lipid, which explains the differences in density. In each of them, cholesteryl ester, cholesterol, and triglyceride accounted for most of the neutral lipid. The major components of phospholipid were phosphatidylcholine and sphingomyelin. Also, the CCL was bright orange, due to the presence of carotenoids, mainly astaxanthin.

Separation of apolipoproteins

Separation of apolipoproteins from the CCL was accomplished by gel filtration chromatography in the presence of 0.1% SDS. The purity of separated apolipoproteins was assessed by SDS-slab-PAGE (**Fig. 2**). The first and second peaks contained apo-I (molecular weight 24,000) and apo-II (molecular weight 12,000), respectively. The third peak contained no proteins, indicating the presence of 2-mercaptoethanol. Apo-I and apo-II separated from the CCL were soluble after removal of SDS by dialysis. A slight difference of the ultraviolet absorption spectra was found in between apo-I and apo-II (**Fig. 3**). The ratios of absorbances 278 nm/260 nm for apo-I and apo-II were 1.21 and 1.78, respectively.

Amino acid composition

Table 2 shows the amino acid composition of the CCL and apo-I and apo-II of chum salmon, and HDL and apo-I and apo-II of pink salmon (9). Prominent features of the compositions included the relative high levels of glutamic acid, alanine, leucine, and lysine, and the low cysteine content in both the CCL and the HDL fraction from pink salmon. Apo-I and CCL were rich in glutamic acid, alanine, leucine, and lysine. Compared to the amino acid composition of apo-I, apo-II included relatively high levels of glycine and tyrosine, and low threonine, serine, and arginine contents. No tryptophan was detected in the CCL, apo-I and apo-II. The amino acid analyses revealed similarities in the compositions of chum salmon and pink salmon.

Trypsin treatment

Limited trypsinization of the CCL and the apolipoproteins from the CCL was performed for various times with a 1:100 concentration of trypsin to protein. SDS-slab-PAGE of the trypsinized CCL showed that apo-I was more susceptible to proteolytic cleavage than apo-II (**Fig. 4**). The apo-I band was completely degraded by limited trypsin digestion, while apo-II remained resistant to trypsin cleavage for 60 min. The decrease in color intensity of apo-I with Coomassie Brilliant Blue corresponded with the appearance of several lower molecular weight bands.

Fig. 5 and **Fig. 6** show SDS-slab-PAGE of the trypsinized apo-I and apo-II, respectively. Apo-II as well as apo-I could be seen to be clearly degraded by limited trypsin digestion. Apo-II isolated from the CCL was readily digested with trypsin (Fig. 6), although apo-II in the CCL particle was resistant to trypsin cleavage (Fig. 4). The trypsin digestion pattern of apo-I was distinct from that of the CCL particle (Figs. 4 and 5).

No detectable band derived from trypsin was found in SDS-slab-PAGE because of its low concentration, although the molecular weight of trypsin was similar to that of apo-I (data not shown).

TABLE 1. Properties of carotenoid-carrying lipoprotein from chum salmon

Component	CCL from Chum Salmon	HDL Fraction from Pink Salmon[a]
	weight %	
Lipid		
Cholesteryl ester	14.2	18.3
Triglyceride	8.1	6.8
Free fatty acid	3.3	1.7
Cholesterol	5.0	3.2
Phospholipid	23.1	30.3
Phosphatidylcholine	(85.5)[b]	(83.0)
Phosphatidylethanolamine	(2.8)	(2.3)
Lysophosphatidylcholine	(4.3)	(3.1)
Sphingomyelin	(7.5)	(6.7)
Protein	46.0	39.9
Density (g/ml)	1.16	1.103
Carotenoids (μg/ml)	6.5	ND[c]
Astaxanthin	(80.2)	ND
Zeaxanthin	(5.2)	ND
4-Keto-zeaxanthin	(6.1)	ND
Others	(8.5)	ND

[a]Nelson and Shore (9).
[b]Individual phospholipids and carotenoids are expressed as percentages of total phospholipids and carotenoids, respectively, in parentheses.
[c]Not determined.

DISCUSSION

A number of studies on the structure and metabolism of serum lipoproteins have been made on mammalian species (31). Few detailed reports, however, have been made on the lipoproteins of nonmammalian species. Several studies on the distribution and characterization of serum lipoproteins in salmonid fish have shown that the lipoproteins consist of density classes that broadly resemble those of mammalian lipoproteins (31). Babin (32) has recently reported studies on the apoA-I-like, apoA-II-like, apoB-like, and apoC-like apolipoproteins of trout plasma lipoproteins.

There are few reports on the carotenoid-carrying lipoproteins in the serum. Chino (33) has demonstrated the presence of lipophorin in insects, which transports diacylglycerol, cholesterol, hydrocarbon, and carotenoids. Lipophorin is an HDL and the color is pale or deep yellow due to the presence of carotenoids. Ashes et al. (34) have reported the presence of an HDL component as a carrier of β-carotene in bovine plasma. We have recently isolated the CCL from the HDL fraction in the serum of upstream

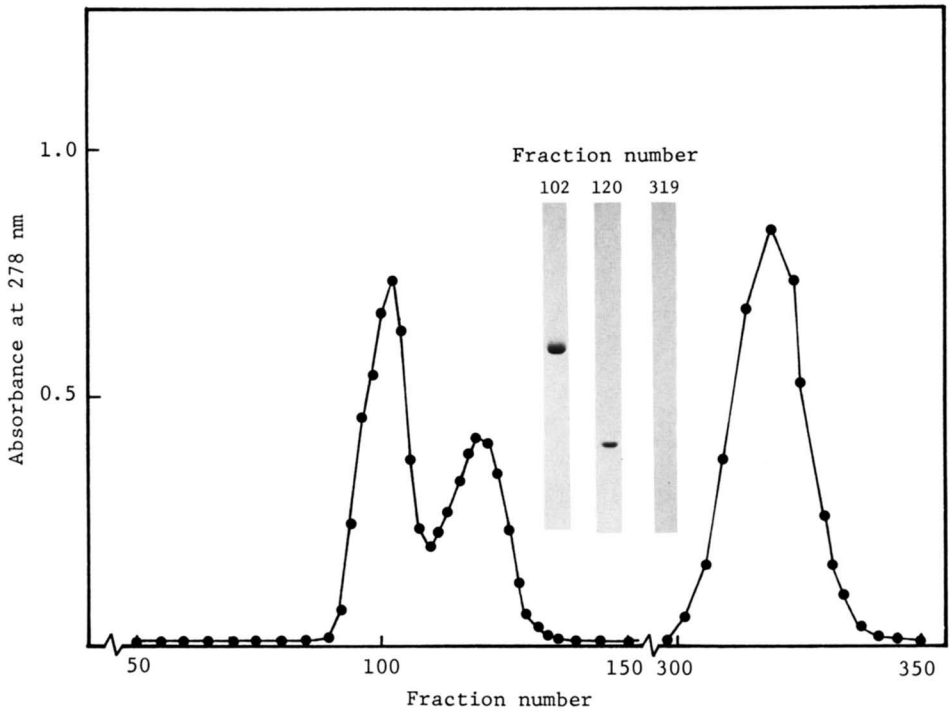

Fig. 2. Gel filtration chromatography of apolipoproteins in SDS. The CCL was dissociated in 1% SDS–1% 2-mercaptoethanol at 25°C overnight and isolated by gel filtration chromatography on a 3.2 × 89 cm Sephadex G-100 column equilibrated in 0.1% SDS–20 mM Tris-HCl buffer (pH 7.5) containing 0.02% NaN_3 at a flow-rate of 25 ml/hr. Two ml of sample was applied to the column, and 1.4-ml fractions were collected. Inset shows SDS-slab-PAGE (15% gel) of selected fractions.

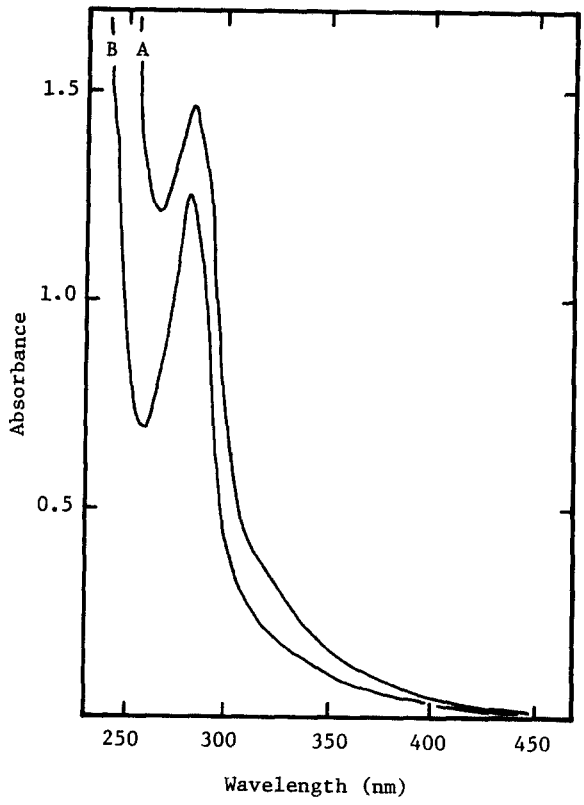

Fig. 3. Absorption spectra of apo-I (A) and apo-II (B) isolated from the CCL. Protein concentrations of apo-I and apo-II were 2.2 mg/ml and 1.9 mg/ml, respectively. Absorption spectrum was measured with a Hitachi 556 double wavelength spectrophotometer.

migrating chum salmon (18). The density of the CCL isolated from the HDL fraction was 1.16 g/ml. The CCL had a pI 5.1, and was rich in glutamic acid, alanine, leucine, and lysine. The CCL showed a high content of lipid, which consisted mainly of cholesteryl ester, cholesterol, triglyceride, phosphatidylcholine, and sphingomyelin (Table 1). The CCL gave rise to two apolipoproteins whose molecular weights were 24,000 (apo-I) and 12,000 (apo-II), and no disulfide bond was detected between the two subunits. The molar ratio of apo-I to apo-II was close to 1:1 (Fig. 1). The CCL from the HDL fraction has multiple functions such as carotenoid transport and bilirubin binding (20, 21).

In order to understand how the CCL functions during carotenoid transport, we need to know more about the apolipoprotein components of the CCL from the HDL fraction. We have developed a gentle and rapid density gradient procedure for isolating the CCL from the HDL fraction in the serum of upstream migrating male chum salmon and an efficient gel filtration chromatography procedure for separating the apolipoproteins from the CCL. Two apolipoproteins could be completely separated by gel filtration chromatography through use of SDS (Fig. 2). We performed complete amino acid analysis on the isolated apolipoproteins and found marked differences in levels of glycine, tyrosine, threonine, serine, and arginine between apo-I and apo-II (Table 2). Arginine residues are known to be cleaved by trypsin digestion. A marked difference in arginine content between apo-I and

TABLE 2. Amino acid compositions of carotenoid-carrying lipoprotein and its apolipoproteins

Amino Acids[a]	Carotenoid-Carrying Lipoprotein of Chum Salmon	HDL of Pink Salmon[b]	Apo-I		Apo-II	
			Chum Salmon	Pink Salmon[b]	Chum Salmon	Pink Salmon[b]
Asp	62.6	68.4	60.8	56.4	74.1	81.5
Thr	40.1	47.3	51.4	53.9	21.4	23.0
Ser	33.5	44.3	41.8	48.5	26.7	31.2
Glu	182.6	180.5	197.7	201.8	155.0	133.4
Pro	45.7	43.2	44.3	38.2	54.0	58.1
Gly	50.2	46.3	28.3	21.4	108.5	94.1
Ala	118.4	113.9	120.8	117.2	110.6	110.0
Cys/2	Tr	Tr	1.7	0.0	1.7	0.0
Val	76.4	74.7	70.1	72.6	85.9	91.8
Met	34.3	33.5	30.7	33.0	28.1	34.2
Ile	36.4	35.9	37.3	34.8	31.6	30.5
Leu	104.2	103.4	103.3	105.7	95.7	95.1
Tyr	48.0	45.4	37.4	38.8	63.4	66.9
Phe	16.5	17.1	18.0	13.6	17.8	16.8
His	13.6	16.6	13.0	15.7	12.0	23.8
Lys	93.0	90.6	88.4	98.1	87.2	86.4
Arg	44.3	39.0	55.1	50.4	26.4	23.4
Trp	0.0	0.0	0.0	0.0	0.0	0.0
Total	999.8	1000.1	1000.1	1000.1	1000.1	1000.2

[a]Residues per 1,000 amino acid residues; tr, trace.
[b]Nelson and Shore (9).

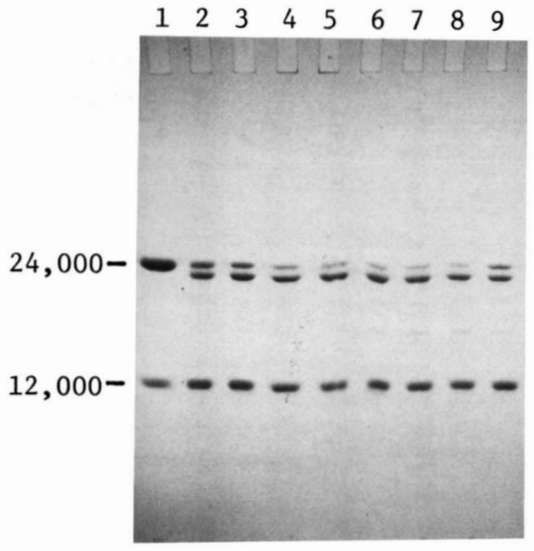

Fig. 4. SDS-slab-PAGE analysis of CCL particles after incubation with trypsin for 0 (1), 2.5 (2), 5 (3), 10 (4), 15 (5), 20 (6), 25 (7), 30 (8), and 60 (9) min. One mg of the CCL in 20 mM Tris-HCl buffer (pH 7.5) was incubated with trypsin at a concentration of 1:100 (trypsin:sample) for times ranging from 0 to 60 min at 25°C. The reaction was initiated by the addition of trypsin and stopped by addition of SDS-PAGE sample treatment buffer (pH 6.8) containing 20 mM Tris-HCl, 2% SDS, 2% 2-mercaptoethanol, and 40% glycerol, and boiling at 100°C for 2 min. Sixty μl (30 μg) of sample was subjected to SDS-slab-15% PAGE.

apo-II may affect the limited trypsin digestion of the CCL particle. SDS-slab-PAGE of the trypsinized CCL particle showed that apo-I was much more susceptible to proteolysis than apo-II (Fig. 4). Apo-I and apo-II isolated from

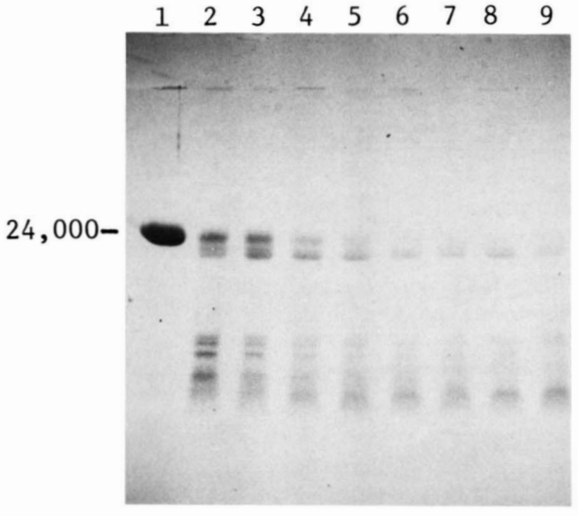

Fig. 5. SDS-slab-PAGE of apo-I isolated from the CCL following incubation with trypsin for 0 (1), 2.5 (2), 5 (3), 10 (4), 15 (5), 20 (6), 25 (7), 30 (8), and 60 (9) min. Apo-I was isolated from the CCL by Sephadex G-100 column (3.2 × 89 cm) equilibrated with 0.1% SDS–20 mM Tris-HCl buffer (pH 7.5) containing 0.02% NaN_3 and dialyzed against distilled water. One mg of apo-I in 20 mM Tris-HCl buffer (pH 7.5) was incubated with trypsin at a concentration of 1:100 (trypsin:sample) in the same manner as the CCL. Sixty μl (30 μg) of sample was subjected to SDS-slab-15% PAGE.

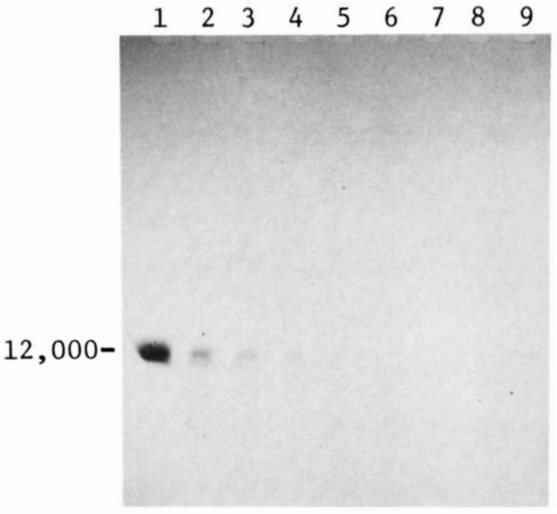

Fig. 6. SDS-slab-PAGE of apo-II isolated from the CCL following incubation with trypsin for 0 (1), 2.5 (2), 5 (3), 10 (4), 15 (5), 20 (6), 25 (7), 30 (8), and 60 (9) min. Apo-II was isolated from the CCL in the same manner as apo-I. One mg of apo-II in 20 mM Tris-HCl buffer (pH 7.5) was incubated with trypsin at a concentration of 1:100 (trypsin:sample) in the same manner as the CCL. Sixty μl (30 μg) of sample was subjected to SDS-slab-15% PAGE.

the CCL, however, were readily digested with trypsin (Figs. 5 and 6). This suggests that the different structural arrangement rather than the amino acid compositions of the apolipoproteins is associated with the limited trypsin digestion of the CCL particle. Apo-II may be sheltered from the aqueous environment and lie partly within the CCL particle.

The accessibility of the protein component of human HDL to trypsin has been compared with apoHDL (35). Human apoHDL was easily attacked by trypsin and degraded to small-size peptides, while human HDL was resistant to trypsin. This agreed with the results of the present study, in which apo-I and apo-II isolated from the CCL were readily digested with trypsin (Figs. 5 and 6). However, Camejo (35) did not mention which component of human apoHDL was more digested by trypsin. Taking into account the similarity of amino acid composition between human apoA-I and salmon apo-I (31), apoA-I as well as apo-I may be more exposed to the aqueous environment.

Lipophorin related to the lipid transport in insects has two apolipoproteins, apolipophorin-I (apoLp-I, mol wt 250,000–270,000) and apoLp-II (mol wt 85,000–88,000) (33). Pattnaik et al. (36), Shapiro, Keim, and Law (37), and Robbs et al. (38) have carried out structural studies on lipophorins using immunological probes and limited proteolysis. ApoLp-I was more susceptible than apoLp-II to reactions with antibodies and with trypsin digestion. The results of these studies indicated that apoLp-I was more exposed to aqueous medium than apoLp-II. A similar behavior of apolipoprotein arrangement was found

with CCL, although the molecular weights of the apolipoproteins were different from each other. The properties of both the HDL fraction and apolipoproteins from pink salmon were similar to those of the CCL (Tables 1 and 2), suggesting that the CCL was associated with carotenoid transport in salmonids including chum salmon and pink salmon. Apo-II may play an important role in interaction with carotenoids, because carotenoids are water-insoluble pigments and may be sheltered from the aqueous environment. Immunological approach is required to further reveal the homogeneity of the CCL in the HDL fraction of salmonids.

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