Biochemical Studies on Carp Plasma Protein—III

Characterization of Lipoproteins of Globulin Fraction

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Five lipoproteins were fractionated from a globulin fraction of carp serum protein by gel filtration and DEAE-cellulose chromatography. The presence of a small quantity of the lipoprotein with a large molecular weight and zero mobility in electrophoresis was confirmed. The other 4 lipoproteins were purified and characterized. Their lipid contents were 23 to 64%, and their isoelectric points 4.2 to 5.2. In amino acid composition, these lipoproteins were very similar to each other, and resembled human serum chylomicron, very low density lipoprotein, and low density lipoprotein rather than human serum albumin and high density lipoprotein. The protein moiety of each lipoprotein was resolved into 5 major and several minor components by SDS-disc electrophoresis. Each lipoprotein consisted of various sets of apoprotein components.

Current studies on the serum lipoprotein have been carried out to understand evolutionary advancement and lipid transport system¹⁻⁷⁾. In fish serum, considerable amounts of lipid are associated with the albumin which migrates fastest in electrophoresis^{5,8,9)}. The author and co-workers extended this finding to characterize the lipid-containing albumin of fish plasma¹⁰⁻¹⁴⁾. A previous study¹⁰⁾ showed the presence of plural lipoproteins which behaved as globulin in a solubility test. These lipoproteins as well as the albumin are expected to be an instrument of lipid transport.

The present paper deals with the preparation and characterization of carp serum lipoproteins.

Materials and Methods

Blood Sampling

Two-year old carp, *Cyprinus carpio*, (100-300 g B.W.) cultured with fish pellets were kept in an out-door pond of Kumano Freshwater Biological Station of Hiroshima University. Blood sample withdrawn by cardiac puncture was allowed to clot, and the serum was isolated by centrifugation. Anticoagulant was not used to avoid any significant effect on lipoprotein.

Isolation of Lipoproteins

The globulin fraction salted-out with 50% saturated ammonium sulfate was passed through a column of Sephadex G-200 (2.6×33 cm). The elution was performed by using a 0.05 M phosphate

buffer pH 7.2 at 10°C. The first fraction was successively applied to a Sepharose 2B column (2.5×65 cm). The second fraction from the Sepharose 2B column was then submitted to a DEAE-cellulose column (1.5×30 cm) equilibrated with a 0.05M phosphate buffer pH 7.2. Elution of lipoproteins was carried out by stepwise elution, increasing the concentration of KCl in 0.05 M phosphate buffer pH 6.4 at 10°C.

Electrophoresis

The electrophoretic behavior of lipoprotein was examined on a cellulose acetate film (Sartorius-Membranfilter) and agarose gel plate (Corning Aci). Cellulose acetate electrophoresis was carried out in a veronal buffer (pH 8.6, 0.06 M) and with a constant current of 0.5 mA per cm width for 60 min. Agarose gel electrophoresis was done in 1% gel and 0.065 M veronal buffer pH 8.6 with a constant voltage of 9 V per cm for 60 min. Lipoprotein bands on the cellulose acetate film and agarose plate were visualized by ozonization-Schiff's and Fat red 7B stainings, respectively.

Isoelectrofocusing

For the determination of isoelectric point, the lipoprotein sample was subjected to an electrofocusing column of 80 ml capacity with a carrier ampholite of pH range 4-6. The protein was electrofocused for 48 h with 600 V at 10°C. The fractions were measured for optical density at 280 nm and pH value.

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Amino Acid Analysis

The lipoprotein was defatted by the procedure of HILLYARD *et al.*¹⁵ The protein moiety was washed with distilled water and hydrolyzed with 3 ml of 6 N HCl at $110 \pm 1^\circ\text{C}$ for 27 h, as described previously¹³. Amino acid composition was determined by a Hitachi 034 type liquid chromatograph. Tryptophan was not determined. The relatedness (*SAQ* value) among amino acid compositions of lipoproteins was estimated by the method of MARCHALONIS and WELTMAN¹⁶.

Analysis of Apoprotein

For the comparison of protein moiety of the lipoproteins, apoprotein was prepared by delipidation from the lipoproteins with an ether-ethanol mixture at 5°C according to the method of NELSON and SHORE⁶. The protein moiety was solubilized with 0.1% sodium dodecyl-sulfate (SDS)-containing 0.01 M phosphate buffer (pH 7.4). The apoprotein composition was determined by SDS-disc electrophoresis which was performed in 0.1% SDS-containing 10% polyacrylamide gel at 5 mA/gel. The molecular weight of apoprotein was estimated by the method of WEBER and OSBORN¹⁵. As a standard protein for molecular weight estimation, a Combithek (Boehringer Mannheim) was used.

Chemical Determination

Quantitative analyses of protein and lipid were determined by Folin-Ciocalteu's phenol method and sulfo-phospho-vanilline (SPV) methods, respectively. Molecular weight of the lipoprotein was estimated by gel filtration. Concentrations of protein and lipid in eluates from columns were measured by optical density at 280 nm and SPV method.

Results

In general, carp serum lipoprotein could be incompletely separated into 2-4 components by electrophoresis. However, disc electrophoresis using 7.5% polyacrylamide gel revealed a fair number of lipid stainable components¹⁰. The solubility test demonstrated that one major lipoprotein behaved as albumin, and the other minor lipoproteins as globulin. Fig. 1 shows typical serum lipoprotein patterns of human and carp. In agarose gel and cellulose acetate electrophoreses, a lipoprotein was located behind a major lipid-containing albumin. Its mobility was between those of α - and β -lipoproteins of human serum. In addition, a lipid-

Fig. 1. Electrophoretic patterns of human and carp serum lipoproteins. A cellulose acetate film was stained by ozonization-Schiff's method, and agarose gel with Fat red 7B. A component having zero mobility appeared in some cases.

Fig. 2. Fraction of a large molecular lipoprotein from carp globulin fraction by gel filtration with a Sepharose 2B column.

staining occasionally appeared on a sample well without migration.

The globulin fraction included at least 5 lipoproteins. When submitted to a Sepharose 2B column, the first fraction from a Sephadex G-200 column was separated into 2 lipid-containing fractions designated G_1 and G_2 , as shown in Fig. 2. Isoelectrofocusing of Fraction G_1 revealed about 9 protein components at pH 4-6, as shown in Fig. 3. The lipoprotein in the Fraction G_1 was conveniently designated Lp-I. However, detailed analysis of Lp-I could not be performed by the reason of minor sample.

Fig. 3. Isoelectrofocusing of Fraction G_1 from a Sepharose 2B column.

The lipoproteins in the Fraction G_2 were further purified by DEAE-cellulose column chromatography. The elution profile and designation of lipoproteins are presented in Fig. 4. The purity of 4 lipoproteins isolated was confirmed in electrophoreses and isoelectrofocusing. Approximately 70% of serum lipid was conjugated with the albumin. The remainder (30%) of the lipid distributed into lipoproteins of globulin fraction, in which Lp-II was the dominant component.

The 5 lipoproteins thus confirmed were subjected to cellulose acetate and agarose gel electrophoreses in order to compare their mobility (Fig. 5). Lp-I remained at the origin on the cellulose acetate and agarose gel. In agarose gel electrophoresis, the mobilities of a few lipoproteins were increased after purification. Their molecular weights, lipid contents, and isoelectric points are

Fig. 4. Typical elution profile of carp serum lipoproteins on DEAE-cellulose column chromatography. Elution was carried out by increasing the concentration of KCl in 0.05 M phosphate buffer pH 6.4.

Fig. 5. Electrophoretic patterns of isolated lipoproteins from carp serum. Detection method was the same as in Fig. 1.

Table 1. Some properties of carp serum lipoproteins

	CPA	Lp-I	Lp-II	Lp-III	Lp-IV	Lp-V
Lipid content (%)	22.3*	>15.9	63.3	29.9	63.5	39.1
Molecular weight ($\times 10^3$)	150*	>4,000			ca. 400	
Isoelectric point	5.2	4.2-4.5	4.6	5.2	4.5	4.6

* Cited from the previous data¹⁰⁾.Table 2. Amino acid composition of carp serum lipoproteins*¹

Amino acid	Lipoprotein			
	Lp-II	Lp-III	Lp-IV	Lp-V
Aspartic acid	12.3%	10.2%	12.1%	12.3%
Threonine	5.5	3.9	5.6	5.0
Serine	4.7	4.3	4.2	3.7
Glutamic acid	15.7	15.8	14.5	14.8
Proline	3.5	4.6	2.3	4.4
Glycine	4.8	6.0	4.8	4.9
Alanine	6.9	7.3	6.4	7.7
Half-cystine* ²	0.9	0.4	1.0	0.7
Valine	6.7	6.4	6.3	6.7
Methionine	0.7	1.2	0.7	0.8
Isoleucine	5.5	5.0	5.6	5.2
Leucine	9.4	8.8	9.5	9.3
Tyrosine	trace	trace	trace	trace
Phenylalanine	4.3	3.5	4.6	4.7
Lysine	7.5	6.7	7.1	6.9
Histidine	2.3	2.2	2.4	2.1
Arginine	4.6	6.2	6.2	5.8
Amide NH ₂	4.7	7.6	6.7	5.0
Total	100.0	100.1	100.0	100.0

*¹ Per cent of g amino acid residue.*² Estimated from the value of cysteic acid. Tryptophan was not analyzed.

listed in Table 1. The values of CPA were also estimated. The molecular weight of Lp-I was estimated to be more than 4000×10^3 , on the basis of the elution position from a Sepharose 2B column. The elution positions of the other lipoproteins were nearly equal to that of a standard apoferritin (mol. wt. 400×10^3). The values of isoelectric point were between 4.2-5.2. The lipid contents were significantly high with Lp-II and Lp-IV.

The amino acid composition of the lipoproteins is listed in Table 2. The high proportions of glutamic and aspartic acids were commonly recognized in the all lipoproteins. The relatedness of amino acid composition is shown in Table 3. The values (*S_dQ*) were estimated from the data of CPA¹⁸⁾, HSA¹⁸⁾, chylomicron¹⁸⁾, VLDL¹⁸⁾, LDL¹⁸⁾, and HDL⁸⁸⁾ from human serum. As to amino acid composition, the 4 lipoproteins of carp serum could not be significantly differentiated from each other. As far as the relatedness is concerned, the carp serum lipoproteins seemed to resemble human serum lipoprotein classes except for HDL. SDS-disc electrophoresis of the protein moiety from carp serum lipoproteins principally resolved into 5 major components including some minor com-

Table 3. Estimation of relatedness (*S_dQ*) of serum lipoproteins*¹

	CPA* ²	Lp-II	Lp-III	Lp-IV	Lp-V	HSA* ²	Chylo* ³	VLDL* ³	LDL* ³
Lp-II	178	0	—	—	—	—	—	—	—
Lp-III	199	23	0	—	—	—	—	—	—
Lp-IV	218	10	19	0	—	—	—	—	—
Lp-V	199	6	17	11	0	—	—	—	—
HSA* ²	60	151	184	180	162	0	—	—	—
Chylo* ³	109	70	77	75	74	48	0	—	—
VLDL* ³	178	69	89	66	82	116	28	0	—
LDL* ³	184	70	98	70	83	125	33	3	0
HDL* ³	54	115	115	128	129	22	28	83	91

Abbreviations: CPA, carp plasma albumin; Lp-II, III, IV, V, carp serum lipoprotein; HSA, human serum albumin; Chylo, chylomicron of human serum; VLDL, very low density lipoprotein of human serum; LDL, low density lipoprotein of human serum; HDL, high density lipoprotein of human serum.

*¹ The values were calculated according to the method of MARCHALLOIS and WELTMAN¹⁶⁾. The value given for a pair of proteins was the difference in g% values for each amino acid squared and summed. The values of tryptophan, cystine, and amide N were often neglected.

*² Calculated from the previous data¹⁸⁾.

*³ Calculated from the data presented by LEVY *et al.*¹⁸⁾.

Abbreviations: CPA, carp plasma albumin; HSA, human serum albumin; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

Fig. 6. Schematic disc electrophoretic diagrams of apoproteins prepared from carp serum lipoproteins in the presence of 0.1% SDS. Run in 0.05 M phosphate buffer, pH 7.4, for 1.5 h. Current: 5 mA/gel.

ponents, as expected (Fig. 6). The slow mobility bands were always very faint. The molecular weights estimated from relative mobility are also shown. The dominant bands among the 4 lipoproteins were as follows: Lp-II, band 8; Lp-III, band 9; Lp-IV, band 10; Lp-V, band 6.

Discussion

In most species of fish, a few lipid-containing components migrate in the region of α -globulin and albumin of human serum. Agarose gel electrophoresis of carp serum revealed distinct 3 or 4 lipoproteins of which the mobilities did not intimately correspond to that of human serum.

A previous study¹⁰⁾ showed that carp serum included plural lipoproteins behaved as globulin in addition to albumin. Several fish have a lipoprotein with zero mobility^{2,5,19,20)}. The deposition of a certain amount of lipid-containing protein at the origin of gel suggests the presence of a large molecular lipoprotein in fish serum. As to the electrophoretic mobility and the molecular weight, Lp-I was seemingly similar to chylomicron of mammal.

LEE and PUPPONE²¹⁾ found with Pacific sardine serum that the higher mobility lipoproteins were present in the high density class and those with the lower mobility were present in the low density class, in addition to VLDL remaining at the origin

in electrophoresis. Amino acid analysis indicated that the 4 lipoproteins isolated were similar to human serum chylomicron, VLDL, and LDL rather than HSA and HDL. In amino acid composition, moreover, CPA resembled HSA and HDL. Based on the results, lipid-containing albumins in fish sera probably correspond to the HDL designated by many workers in fish sera.

MILLS and TAYLOR¹⁾, and ALEXANDER and DAY⁵⁾ determined the distribution and composition of lipoproteins in several animal species, and found differences among mammals and fish. On the other hand, results of detailed analyses showed that general physical properties of fish serum lipoproteins were similar to those of mammals^{6,7,21)}. Sera of pink salmon and cod contain a lipoprotein very similar to human HDL in several properties^{6,21)}. Despite the presence of unusual lipids in the lipoprotein, the apoprotein composition of VLDL and LDL from shark serum are not unlike those from the corresponding lipoprotein of other animals.⁷⁾ The diversity in apoprotein composition, and the similarity in amino acid composition and molecular weight among the carp serum lipoproteins might show that these were metabolically related. It may be inferred from the above results that the physical diversities of these lipoproteins are dependent on partial replacement of apoproteins are dependent on partial replacement of apoprotein components relating to the binding feature of lipid to protein moiety.

Fish serum lipoprotein is unstable. In gel electrophoresis, the mobility of the purified lipoproteins no longer coincided with that of the intact ones. Mobility change is also caused by lipid moiety change¹¹⁾. Nevertheless, there was recognized a high reproducibility in the chromatographic and the cellulose acetate electrophoretic behaviors of lipoproteins.

LEE and PUPPONE²⁾ suggested the difference in mechanism of lipid transport between mammal and fish. On the other hand, the marked resemblance of lipid transport system between them was described²¹⁾. In spite of the seeming similarity in protein moiety of lipoprotein between fish and mammals, the heterogeneity in lipid moiety might indicate the complexity of lipid metabolism in fish.

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