Biochemical Studies on Carp Plasma Protein—I Isolation and Nature of an Albumin

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The presence and nature of an albumin in carp plasma were investigated. It was estimated by cellulose acetate electrophoresis that the albumin was 34.6 per cent of the total plasma protein. The albumin obtained from carp plasma was a kind of lipoprotein with a molecular weight of approximately 15×10^4 , having a lipid content of 22.3 per cent of which 12.2 per cent consisted of free fatty acids. More than half of plasma lipid is conjugated with the albumin. The albumin evidently differs from the human type in its ability to bind BCG and BPB, molecular size, electrophoretic mobility, and nature of the lipid moiety.

Fish serum or plasma proteins of extensive species have been investigated by means of the biochemical and electrophoretic techniques. However, very little is known in detail on the profound nature of specific fish serum.

Since the cyprinid fish possesses several advantages for serological observation, its serum proteins were examined by many workers¹⁻⁸⁾. They show evidences that variations in fish serum proteins occur as a result of physiological, genetic, and environmental factors. It was reported by LYSAK and WOJCIK¹⁾ that the proportion of albumin fraction changed under the influence of food in carp breeding. SAITO⁹⁾ found that the concentrations of serum protein and albumin of carp are lower in winter than the other seasons. LIEBMAN *et al.*²⁾, RIEDMÜLLER⁵⁾, and OLLENSCHLÄGER⁶⁾ described the importance of the albumin in the study of fish pathology.

In the present paper the authors report the identification of plasma albumin in order to gain the basic information on the nature of fish plasma protein.

Materials and Methods

Materials: Cultured red carp, *Cyprinus carpio*, obtained from the Umeda's Carp Breeding Station, Hiroshima Prefecture were maintained in a tank of the university for several days. The fish used were between 100 and 300 g in weight. Blood was collected from the heart of carp with a syringe treated with Anglot^{*2} (Nippon Shoji Co.) to prevent blood coagulation. The blood was centrifuged at 3,000 rpm for 10 min. Plasma sample was stored in a freezer at -20°C until analyses. For comparison, human serum, Consera (Nissui Seiyaku Co.), was used.

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^{*2} contains 1.5% stabilized heparin in water.

Chemical analyses of plasma: Protein and albumin concentrations were determined by the biuret method and Bromocresol green (BCG) method¹⁰⁾, respectively. Lipid concentration was determined by sulfo-phosphate-vaniline (SPV) method¹¹⁾.

Cellulose acetate electrophoresis: The plasma sample (2 μ l) was spotted on cellulose acetate film (Sartorius-Membrane filter) of 7 cm in length and 1.5 cm in width, and the electrophoresis was carried out in a field strength of 0.5 mA per cm width, in veronal buffer of pH 8.6 and ionic strength of 0.06. The cellulose acetate film was stained with Ponceau 3R for protein detection. Glycoprotein was stained by the periodic acid-Schiff's (PAS) method. Lipoprotein was detected by staining with Schiff's reagent after preliminary ozonization according to the method of KOHN¹²⁾. A quantitative analysis of the electrophoretic pattern was conducted by using an Ozumor Densitometer 82 (Asuka Kogyo Co.) after making the film transparent with decahydronaphthalene.

Disc electrophoresis: Polyacrylamide gel disc electrophoresis was carried out by using 7.5% acrylamide gel. Five μ l of plasma in buffer-containing 2 M sucrose were applied on the gel. A current intensity of 2 mA/gel was charged for 90–120 min. For staining of protein 0.3% Coomassie Blue was used. Glycoprotein was detected with the periodic acid-Schiff's reagent by the method of FELGENHAUSER¹³⁾. For lipoprotein detection, 10 μ l of plasma was used, and the gel was stained with Sudan black B according to the method of PRAT *et al.*¹⁴⁾

Gel filtration: Plasma protein dialyzed against elution buffer was placed on a column of Sephadex G-200. The elution was performed by using a 0.45 M KCl-containing 0.05 M phosphate buffer (pH 7.2) at 10°C. The fraction volume was 5 ml. The optical density of effluent was measured at 280 nm with a spectrophotometer.

Starch-grain electrophoresis: Potato starch (Katayama Chemical Co.) was packed in a migration cell (6 $\times$ 1.5 $\times$ 2.6 cm). The sample was loaded in the groove of starch and applied to electrophoresis. Buffer of veronal at pH 8.6 of ionic strength 0.06 was used. Current intensity of 1.9 mA/cm² was charged for 25 hr. Each of the migrated proteins was extracted with the veronal buffer, and the protein concentration was determined by the Folin method.

Bromphenol Blue (BPB) binding test: BPB binding test of plasma protein was carried out by the method of NISHIYAMA *et al.*¹⁵⁾ (1) Three volumes of plasma sample were mixed with one volume of 0.05% BPB solution. After incubation, the mixture was applied to the cellulose acetate electrophoresis. (2) Plasma-BPB mixture was passed through a Sephadex G-200 gel filtration column, and optical density of the effluent was measured at 595 nm. Elution was carried out by 0.005 M phosphate buffer at pH 7.2.

Molecular weight estimation: Disc electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was used to estimate the molecular weight of the isolated protein. The standard proteins which were purchased as a kit of the molecular weight markers

(Mann Res. Lab.) were bovine albumin, ovalbumin, chymotrypsinogen A, myoglobin, and cytochrome C.

Results

Some chemical properties of the pooled carp plasma are shown in Table 1. The cellulose acetate electrophoretic diagrams of human serum and carp plasma are illustrated in Fig. 1. There appeared 6 components on cellulose acetate film in carp plasma. The percentage composition of the various components estimated by a densitometer is shown in Table 1.

Schematic disc electrophoretic diagrams of carp plasma are shown in Fig. 2. At least 22 protein components have been found in the carp plasma⁶⁾. Twenty four bands

Table 1. Some chemical properties of the pooled carp plasma

Hematocrit value	39.2
Total protein	2.62 g/100 ml
Albumin* ¹	1.17 g/100 ml
Total lipid	588 mg/100 ml
Protein fraction* ²	
I	11.7%
II	21.0%
III	12.7%
IV	15.4%
V	34.6%
VI	4.6%
Albumin-globulin ratio	
BCG method	0.81
Cellulose acetate electrophoresis	0.64

*¹ Calculated by BCG method.

*² Estimated from cellulose acetate electrophoretic pattern.

Fig. 1. Cellulose acetate electrophoretic diagrams of carp plasma and human serum.

Fig. 2. Disc electrophoresis of carp plasma.

- A: Whole plasma (protein staining).
- B: Whole plasma (lipid staining).
- C: Whole plasma (carbohydrate staining).
- D: Soluble protein in a half saturated $(\text{NH}_4)_2\text{SO}_4$.
- E: Insoluble protein in a half saturated $(\text{NH}_4)_2\text{SO}_4$.

Table 2. Percentage composition of carp plasma lipoproteins

Disc electrophoresis	Fr. No.	1	2	3	4	5	10	12, 14, 15	20
	%	9.1	8.1	18.2	4.5	1.0	3.0	2.0	54.0
Cellulose acetate electrophoresis	Fr. No.	IV							V
	%	30.9							69.1

were detected by the staining of Coomassie blue. They were numbered from the original spot toward the positive side. Fraction 20 was dominant protein component. Ten lipoprotein components were observed in disc electrophoresis as shown in Fig. 2-B. Their percentages estimated by densitometry are shown in Table 2. Identity of the fraction 20 of Fig. 2 to the fraction V of Fig. 1 was confirmed by two dimensional electrophoresis. The dominance of fraction 20 was observed in both protein and lipid stainings.

Carp plasma was applied for the starch grain electrophoresis. Then the migrated proteins were extracted from starch block. When the extracts were measured by BCG reaction, BCG reaction could be found distinctly only in human serum albumin, but nonspecifically in carp plasma.

Human serum and carp plasma were fractionated by gel filtration with Sephadex G-200, resulting in the appearance of three peaks, as shown in Fig. 3. In human serum BCG reaction was positive only in albumin-containing peak, whereas nonspecific in carp plasma. Lipid was found in the first and middle peaks of human serum and carp plasma by SPV method.

Fig. 3. Gel filtration of human serum and carp plasma on Sephadex G-200. Column of length 40 cm and diameter 2.5 cm was used.

Fig. 4. Gel filtration of carp plasma proteins fractionated by salting-out with 50% saturated ammonium sulfate solution. Column of length 60 cm and diameter 2.6 cm was used.

Carp plasma protein was fractionated into crude albumins and globulins by ammonium sulfate precipitation. The soluble fraction in a half saturation was designated as crude albumins. Parallel electrophoresis of the crude albumins and globulins indicated that the former consisted of fraction 20 as main component and the latter relatively low mobility components (Fig. 2).

Fig. 4 shows the elution patterns of both crude globulins and albumins on gel filtration. The former revealed two peaks, while the latter revealed three peaks. The middle peak of the crude albumins mainly consisted of a high mobility protein corresponding to the fraction 20 of Fig. 2.

Cellulose acetate electrophoresis of plasma sample incubated with BPB was carried out. The schematic electrophoresis diagrams are shown in Fig. 5. In human serum, albumin band was rigidly pigmented with BPB. Only fraction V of carp plasma was ascertained to bind with BPB. No difference of electrophoretic mobility of plasma protein was observed before and after BPB binding. The carp and human serum albumins complexed with BPB held their forms on the cellulose acetate film during migration, while on the disc electrophoresis the former showed somewhat feeble binding affinity with BPB. Moreover, each sample incubated with BPB was passed through the Sephadex gel column. The elution pattern showed that BPB binding protein was found in the last

Fig. 5. BPB binding test of human serum and carp plasma on cellulose acetate electrophoresis. Buffer: 0.06 M veronal buffer, pH 8.6. Current: 0.5 mA/cm, 90 min. Staining: Ponceau 3R. The solid bands represent BPB-bound fractions.

A: BPB. B: Human serum. C: Human serum+BPB. D: Carp plasma. E: Carp plasma+BPB.

peak of human serum, while in the middle of carp plasma, as shown in Fig. 6.

The carp plasma albumin was purified by the following method. Plasma was mixed with the same volume of saturated ammonium sulfate solution. The protein in soluble fraction was salted-out on further addition of solid ammonium sulfate until 70% saturation. Then the protein was passed through a column of Sephadex G-200. The effluents corresponding to the middle peak shown in Fig. 4 were collected. After dialyzing against veronal buffer the protein was applied for preparative starch-grain electrophoresis.

The high mobility zone in starch block was taken and extracted with water. Thus obtained protein was homogeneous in disc electrophoretic analysis. The molecular weight of the albumin was of the order of approximately 15×10^4 by SDS-disc electrophoresis. The absorption spectrum of the albumin was found only in ultraviolet region, but not in visible region. The lipid content of the purified albumin estimated by SPV method was

Fig. 6. BPB binding test of carp plasma protein on Sephadex G-200 gel filtration. Column size: 2.5×40 cm. Elution buffer 0.005 M phosphate buffer, pH 7.2.

22.3 per cent.

The lipid of the albumin was extracted with methanol-chloroform mixture at room temperature, then applied to thin-layer chromatography. For comparison, the effluent from the first peak, as shown in Fig. 4, was collected into 5 ml fractions and the lipid composition was also analyzed. Thus obtained both lipids were fractionated into several lipid classes by developing solvent of petroleum ether-ether-acetic acid (85: 15: 1, v/v/v). For the determination of phospholipid composition, solvent system of methanol-chloroform-water (65: 25: 4, v/v/v) was used. The spots were detected by spraying saturated potassium perchromate-containing 70% sulfuric acid, and charring. Their color intensities were measured by densitometry. The compositions are listed in Table 3. Free fatty acids were apparently higher in the lipid of the albumin. The comparatively lower proportion of neutral lipid and cholesterol ester was characteristic to the albumin. Phosphatidyl choline was higher in the albumin, while other lipoproteins were somewhat lower.

Table 3. Lipid class and phospholipid compositions of the albumin and other lipoproteins of carp plasma

	Albumin	Other lipoproteins
Lipid class		
Phospholipids	14.4%	9.7%
Partial glycerides	5.0	4.0
Sterols	21.6	13.1
Fatty alcohols	4.3	2.6
Fatty acids	12.2	3.7
Triglycerides	18.0	40.2
Wax esters	6.5	2.3
Sterol esters	18.0	24.5
Phospholipids		
Sphingomyelin	1.4	1.6
Phosphatidyl choline	85.2	71.9
Phosphatidyl ethanolamine	6.7	18.8
Lysophosphatidyl choline	3.8	7.8
Unknown	2.9	0.0

Discussion

PETERS¹⁶⁾ described an exacting definition of serum albumin in human blood. These properties are (a) solubility, (b) high negative charge, (c) molecular size, and (d) absence of carbohydrate by customary tests.

Fish serum albumin has been investigated by many workers^{4,7,8,17-25)}, who classified it by color reaction such as BPB, solubility test, and electrophoretic mobility. In any case the fish serum albumin occurred in lesser concentration than human.

The albumin fraction separated by using salting-out procedure mainly consisting of fractions 21-24 were probably prealbumins, as presented by BALAKHNIN⁷⁾. Fraction 20

would be the serum albumin styled by many workers. It is known that one of the lipid staining fractions is localized in the serum albumin component of cyprinid fish^{8,9)}. Fraction V in cellulose acetate electrophoresis was consistent with fraction 20 in disc electrophoresis in the carp plasma protein. In general fish serum albumin migrated electrophoretically somewhat behind human serum albumin.

The carp plasma protein fraction 20 was soluble in water and a half saturated ammonium sulfate solution. The protein showed higher mobility in electrophoretic behavior, and about 15×10^4 in molecular weight. It showed higher lipid and carbohydrate contents than human serum albumin. It is well known that free fatty acids preferentially fixed on and are practically transported only by serum albumin in mammal. The result that the fraction 20 containing more fatty acids than the other lipoproteins in carp plasma seems to play a transport function of free fatty acids, as has already been indicated^{8b)}.

Many workers, however, pointed out the inadequacy of the application of the human nomenclature to fish serum proteins^{17,27-29)}. The nomenclature to human serum albumin seemed to be inapplicable to that of carp. The evolutionary difference of plasma protein distribution of some fish was presented by GUNTER *et al.*²⁰⁾ Therefore, fraction 20 was thought to be the albumin corresponding to that of human.

Polymorphism of serum albumin was demonstrated in various species of fish by electrophoresis^{4,17,22,24,25)}. SUMMERFELT⁴⁾ demonstrated the presence of two components of albumin in Cyprinidae. Polymorphism of prealbumin of carp plasma was reported by BALAKHNIN *et al.*⁷⁾ In the carp plasma, disc electrophoresis also revealed four prealbumins.

Albumin-globulin ratio was responsible for various biological and physiological differences. In the pooled carp plasma it was found to be 0.64 by cellulose acetate electrophoresis, and 0.81 by BCG method. In cellulose acetate electrophoresis fraction V and VI were regarded as albumin. In carp plasma the albumin concentration as determined by BCG was higher than that by electrophoretic analysis. It could be due to that BCG associate preferentially with human albumin, reacts nonspecifically with carp plasma proteins.

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