Occurrence of Multiple Albumins in Carp Blood Plasma^{*1}

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Five albumin components designated S₂D₁, S₂D₂, S₃E₁, S₃E₂, and S₃E₃, were isolated from carp plasma by a combination of Sephadex G-200 gel filtration, DEAE-cellulose column chromatography, and preparative electrophoresis. Their molecular weights, in the above order, were estimated to be 145,000, 145,000, 58,000, 71,000, and 71,000, respectively. It was demonstrated that each of components S₂D₁ and S₂D₂ is composed of several subunit chains, while each of the remaining components constitutes a single chain. Results of the dye binding tests showed that only components S₂D₁ and S₂D₂ bind with BPB, though all five components bind with HABA and BCG. Component S₃E₂ showed the strongest BCG-binding ability. All the components except S₃E₁ exhibited methemalbumin forming ability. Components S₃E₁ and S₃E₂ showed Sevin esterase activity. It was noted that component S₃E₂ is highest in these activities.

The blood plasma proteins of fish have so far been characterized less satisfactorily than those of the mammalian origin, especially of human¹⁻³). Recently, attempts to classify the plasma proteins of several fishes have been made mainly from the viewpoint of comparative biochemistry^{4,5}). However, even with carp, the fish whose plasma proteins have been examined most intensively⁶⁻⁹), much remain to be elucidated.

The present study was undertaken to characterize further the plasma albumin of carp. The results showed that unlike the mammalian albumin, carp albumin is composed of a number of components, each one sharing with other ones various physiological functions.

Materials and Methods

Materials The blood was individually collected by heart puncture from the cultured specimens (av. body weight: 800 g) of the carp *Cyprinus carpio*. Heparin was used as the anticoagulant. The blood samples were centrifuged to separate the plasma from the red cells. Non-hemolyzed plasmas were combined and immediately used for the following experiments. A portion of the red cell cake was repeatedly washed with the physiological saline and kept frozen below -20°C after being mixed with an approximately equal volume of the glycerin-citrate mixture¹⁰). The hemolyzate was prepared as usual from the red cells and used for examining the methemalbumin forming ability of plasma albumin as described below.

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The blood plasma and red cell hemolyzate of rabbit were similarly provided and used as the control.

Fractionation of plasma proteins Portions of carp plasma were 1/3-, 1/2-, and 2/3-saturated with ammonium sulfate at pH 7.0. The protein concentration in the mixture was fixed at 10–12 mg/ml. The mixtures were gently stirred overnight at 2–4°C, followed by centrifugation at $1,000\times g$ for 30 min or by filtration. The supernatants were then desalted by dialysis, concentrated by ultrafiltration with Amicon XM-50 up to 30 mg/ml. The fractions thus provided were analyzed electrophoretically. The plasma protein fractions of rabbit were similarly prepared and analyzed.

The carp plasma proteins were also salted out with sodium sulfate at 13.5, 17.4, and 21.5%⁽¹¹⁾ at pH 7.0 in almost the same procedures as above. The supernatants obtained were concentrated after dialysis and analyzed electrophoretically.

Isolation of albumin components After various preliminary experiments, carp albumin components were isolated by the method which consisted of Sephadex G-200 gel filtration, DEAE-cellulose column chromatography, and starch block electrophoresis. All the operations were carried out in a cold room at 2–4°C, unless otherwise stated.

Sephadex G-200 gel filtration Twenty ml of carp plasma was loaded onto a Sephadex G-200 column (5 $\times$ 100 cm) which was preequilibrated with 0.1 M Tris-HCl pH 8.0 containing 0.5 M NaCl and 0.1% 2-mercaptoethanol⁽¹²⁾. The same mixture was used as the solvent at a flow rate of 30 ml/hr. The upward system was adopted. Ten-ml fractions were collected and monitored at 280 nm.

DEAE-cellulose column chromatography Fraction S₂, as separated by Sephadex gel filtration (Fig. 2), was dialyzed against 5 mM phosphate buffer pH 7.2, and chromatographed on a DEAE-cellulose column (2 $\times$ 30 cm) by a linear gradient, using 200 ml each of the above phosphate buffer and 50 mM phosphate buffer pH 7.2, and then using 200 ml each of 50 mM phosphate buffer pH 6.0 and the same buffer containing 0.5 M NaCl⁽¹³⁾. The flow rate was maintained at 30 ml/hr, and 10 ml-fractions were collected and monitored at 280 nm.

Starch block electrophoresis Fraction S₃, as separated by Sephadex gel filtration (Fig. 2), was dialyzed against 0.06 M veronal buffer pH 8.6 containing 0.1% 2-mercaptoethanol, and electrophoresed for 48 hr in a starch block column (2 $\times$ 5 $\times$ 50 cm) under a constant current of 20 mA. After the run, filter strip was pressed onto the starch block, dried, and stained with amido black 10B, in order to locate the protein zones. The starch cakes corresponding to respective zones were scraped and extracted with water.

The albumin components thus isolated were finally concentrated by ultrafiltration, dialyzed against 0.033 M Tris-borate buffer pH 8.5, and subjected to the following analyses.

Methods of analysis Starch gel electrophoresis was performed after YAMANAKA *et al.*⁽¹⁴⁾. Molecular weight was estimated by thin layer chromatography (TLC) in Se-

phadex G-100¹⁵⁾. SDS-Polyacrylamide gel electrophoresis was carried out in 7.5 or 10% gels using bromophenol blue as the marker¹⁶⁾. Bovine serum albumin, ovalbumin, chymotrypsin, myoglobin, and cytochrome c (Mann Res. Lab.) were used as standard proteins. The free sulfhydryl group was determined by the DTNB^{**4} method¹⁷⁾. Protein concentration was measured by the biuret method. The concentration of isolated albumin component was calculated by use of its specific extinction, which was determined by dry weight and the absorbance at 280 nm.

The binding tests with bromophenol blue (BPB), hydroxyazobenzene carboxylic acid (HABA), and bromocresol green (BCG) were performed, referring to the method of SAITO¹⁸⁾, RUSTEIN *et al.*¹⁹⁾, and MIYADA *et al.*²⁰⁾, respectively. In the binding tests with BPB, 0.1 ml of 5% albumin, 2.0 ml of 0.1 M phosphate buffer pH 7.2, and 0.05 ml of 0.05% BPB (Tokyo Kasei Co.) were mixed, and the mixture was examined for the formation of albumin-BPB complex, spectrophotometrically and starch gel electrophoretically. With HABA, 0.2 ml of 5% albumin, 1.6 ml of 0.14 M phosphate buffer pH 6.2, and 0.2 ml of 10-fold dilute of the HABA (Daiichi Pure Chemicals Co.) stock solution were mixed, and the amount of the bound dye was calculated from the increment of O. D. at 510 nm after RUSTEIN *et al.*¹⁹⁾. With BCG, 0.05 ml of 5% albumin and 2.5 ml of 5-fold dilute of the BCG (Tokyo Kasei Co.) stock solution²⁰⁾ were mixed, and the amount of the bound dye was calculated from the increment of O. D. at 630 nm. In the present paper, HABA- and BCG-binding capacities of each albumin were expressed for convenience by $E_{510\text{ nm}} \times 1,000 / \text{protein concentration (mg/ml)}$, and $E_{630\text{ nm}} \times 1,000 / \text{protein concentration (mg/ml)}$, respectively. They will be referred to hereafter as HABA- and BCG-binding index.

The methemalbumin (MHA) forming ability of each albumin was examined essentially according to Bunn *et al.*²¹⁾. Briefly, 1% methemoglobin (methHb) solution was prepared as usual from the hemolyzate of carp or rabbit red cells, and mixed with an equal volume of 10–15% albumin. The mixture was incubated at 37°C for 2 hr, and subjected to starch gel electrophoresis. After the run, the gel was sliced horizontally into two halves, one being stained with amido black 10B and the other with benzidine. MHA bands were identified as usual by comparing both electropherograms.

Sevin^{**5} esterase (EC 3. 1. 1. 2) activity of albumin was analyzed essentially after CASIDA *et al.*²²⁾. Briefly, 0.25 ml of 4% albumin, 0.5 ml of 0.5 M phosphate buffer pH 8.0, and 0.25 ml of 4 mM Sevin (Union Carbide Co.) were mixed and incubated at 37°C for 20–60 min. After the incubation, 0.2 ml of 0.2% 4-aminoantipyrine and 0.2 ml of 0.8% ferricyanide were successively added to the mixture and gently shaken. After 5 min-standing, the mixture was made up to 3 ml with water. The dye formed was transferred into chloro-

^{**4} 5, 5'-Dithiobis-(2-nitrobenzoic acid).

^{**5} 1-Naphthyl *N*-methylcarbamate.

form, treated with anhydrous sodium sulfate, and analyzed spectrophotometrically at 460 nm.

Results and Discussion

Electrophoresis of plasma protein fractions In Fig. 1 are schematically shown the starch gel electropherograms of plasma protein fractions of carp and of rabbit for reference. In the case of rabbit, two components (bands 1 and 2) remained soluble at 1/2 saturation with ammonium sulfate, whereas a single one (band 1) regarded as albumin, remained at 2/3 saturation. In the case of carp, five (bands 1–5) and four (bands 1–4) components

Fig. 1. Starch gel electropherograms of some plasma protein fractions from carp and rabbit.

remained soluble at 1/2 and 2/3 ammonium sulfate saturation, respectively. In addition, a rather diffuse band seemed to be present over a wide range between bands 2 and 4. Essentially the same results were obtained when carp plasma was fractionated with sodium sulfate instead. From these results, it was concluded that carp plasma albumin is composed of at least four components, in a strong contrast to rabbit or other mam-

Fig. 2. Sephadex G-200 gel filtration of carp plasma protein.

malian albumins which usually appear as a single component.

Isolation of albumin components A gel filtration profile of carp plasma protein is presented in Fig. 2. As seen in the figure, three peaks designated fractions (Fr.) S_1 , S_2 , and S_3 , appeared. Fr. S_1 was discarded, since it was found to be non-albumin by electrophoresis described below. Ten-ml fractions for each of Fr. S_2 and S_3 , as indicated by both arrows, were combined and purified further: Fr. S_2 was subjected to DEAE-cellulose column chromatography, resulting in separation into two components, S_2D_1 and S_2D_2 , as shown in Fig. 3. The fractions for each component, as indicated by both arrows, were combined, concentrated by ultrafiltration, and dialyzed against 0.033 M Tris-borate buffer pH 8.5. On the other hand, Fr. S_3 was separated by starch block electrophoresis into three components designated S_3E_1 , S_3E_2 , and S_3E_3 in the order of decreasing mobility, as schematically given in Fig. 4. The three components were extracted from the respective starch blocks, concentrated, and dialyzed against the above buffer. Yields of components S_2D_1 , S_2D_2 , S_3E_1 , S_3E_2 , and S_3E_3 , were roughly 0.5, 0.5, 0.3, 0.3, and 0.3 g respectively per 100 ml of carp plasma. In Fig. 5 are shown the electropherograms of the five components together with those of the plasma and Fr. S_1 for reference. By comparison of this figure with Fig. 1, components S_3E_1 , S_3E_2 , and S_3E_3 were recognized to correspond to albumin bands 1, 2, and 3 in Fig. 1, while Fr. S_1 non-albumin. In this connection, components S_2D_1 and S_2D_2 were supposed to correspond to the diffuse band ranging from band 2 to 4, and

Fig. 3. DEAE-cellulose column chromatography of Fr. S_2 , by a linear gradient using sodium phosphate buffers.

Fig. 4. Starch block electrophoresis of Fr. S_3 .

Fig. 5. Starch gel electropherograms of isolated albumin components of carp and rabbit.

were electrophoretically indistinguishable from each other under the conditions applied. As seen in Fig. 5, any of the five components was still contaminated with trace of one or two other components or foreign proteins. Due to scarcity of the samples available, however, they were analyzed without further purification. Rabbit albumin was obtained as a component roughly corresponding to S_3E_1 of carp albumin, as far as the method of isolation is concerned. The yield was about 3 g/100 ml of the plasma.

Properties of carp albumin components The results obtained are collectively shown in Table 1.

Table 1. Properties of carp albumin components

	Carp albumin component					Rabbit albumin
	S_2D_1	S_2D_2	S_3E_1	S_3E_2	S_3E_3	
Molecular weight	145,000	145,000	58,000	71,000	71,000	69,000
Subunit mol. wt.	23,000	23,000	—	—	—	—
$E_{280m}^{1\%}$	6.29	6.55	6.01	11.0	12.5	6.76
Color of aq. soln.	yellow	yellow	—	—	red	—
Free sulfhydryl groups (mol/mol)	0.5	0.5	0.3	2	1	0.7
BPB-binding ability	+	+	—	—	—	—
HABA-binding index*	1.72	1.98	0.82	1.94	2.27	2.11
BCG-binding index*	12.8	15.2	18.9	27.1	21.6	25.3
MHA-forming ability	+	+	—	++	±	++
Sevin esterase activity	—	—	+	++	—	++

* Refer to the text for those definitions.

Molecular weight and subunit constitution By Sephadex-TLC and SDS-disc electrophoresis, it was demonstrated that components S_2D_1 and S_2D_2 have the same molecular weight of about 145,000 and are both composed of several subunit chains of a molecular

weight of 23,000. On the other hand, the molecular weights of S_3E_1 , S_3E_2 , and S_3E_3 were estimated to be 50,000, 67,000, and 67,000, respectively, by Sephadex-TLC. In SDS-disc electrophoresis, the three components presented the molecular weights of 58,000, 71,000, and 71,000, in the above order, indicating that each of those components is a single polypeptide chain. These values are given as their molecular weights in Table 1. Rabbit albumin was confirmed to have a molecular weight of about 69,000 and to be composed of a single polypeptide, in agreement with some mammalian albumins¹.

Absorption spectrum Each of the five albumin components exhibited an absorption maximum just at or around 280 nm. Their specific extinction ($E_{280\text{nm}}^{1\%}$) markedly differed from each other. Rabbit albumin resembled carp S_2D_1 or S_2D_2 in this respect. The aq. solution of S_2D_1 , S_2D_2 , and S_3E_3 were colored: Yellow with the former two and red with the last. Their visible spectra are shown in Fig. 6. As judged from their spectra, S_2D_1 and S_2D_2 may be complexing with some bile pigments and/or carotenoids, whereas S_3E_3 may be a transferrin²³. No further examination has not yet been carried out with these three components.

Free sulfhydryl group The free sulfhydryl groups of components S_3E_2 and S_3E_3 were 2 and 1 mol/mol, respectively, and those of the other components less than 0.5 mol/mol. The value of rabbit albumin was 0.7 mol/mol. FANTL²⁴ reported a value of 0.5–0.8 mol/mol for some mammalian albumins, suggesting their microheterogeneity.

Fig. 6

Fig. 6. Absorption spectra of components S_2D_1 , S_2D_2 , and S_3E_3 .

Fig. 7

Fig. 7. Shift of absorption maximum of BPB as caused by addition of component S_2D_1 (or S_2D_2) and rabbit albumin.

Binding with dyes 1) BPB As shown in Fig. 7, rabbit albumin shifted the absorption maximum of BPB from 590 to 605 nm, indicating the formation of a complex. The same degree of shift was observed when component S_2D_1 or S_2D_2 was added instead, but not at all when the remaining three components were added. When S_2D_1 - or S_2D_2 -BPB mixture was electrophoresed, a blue band of BPB-albumin complex appeared. It was

noted that the blue band tended to fade away during the migration, resulting in a complete disappearance usually after 20 min. This was not the case with rabbit albumin: Even after 90-min electrophoresis, the blue band was easily recognized, the fact that the binding of rabbit albumin with BPB is much stronger. No blue band was observed with the mixture with any other component.

2) *HABA and BCG* As seen in Table 1, HABA-binding indexes of carp albumins were 0.82–2.27, most of which were well comparable to that of rabbit albumin, 2.11.

On the other hand, BCG-binding indexes of carp albumins were ranging from 12.8 to 27.1, and that of rabbit albumin 25.3.

Both these dyes have been reported to bind specifically with albumin among plasma proteins^{19,25)}. To make it sure, the following two fractions of rabbit plasma protein were prepared and examined for the binding indexes with both dyes: Fr. A corresponding to band 2 and Fr. B corresponding to bands 3–6 in Fig. 1. According to the results, HABA-binding indexes of Fr. A and B were 1.36 and 2.20, respectively, and BCG-binding indexes of Fr. A and B, 4.2 and 17.5, respectively. These data clearly show that the binding with HABA or BCG is not so specific to albumin in the case of rabbit as well. Essentially the same results were obtained also with bovine plasma proteins.

Methemalbumin forming ability As shown in Table 1, all the carp albumin components except S_3E_1 exhibited a moderate to strong MHA forming ability under the conditions applied here. The results did not depend on the source of methHb. The MHA forming ability of S_3E_2 was found to match well that of rabbit albumin.

Sevin esterase activity Only components S_3E_1 and S_3E_2 showed this enzyme activity, as seen in Table 1. The activity of the latter was qualitatively comparable to that of rabbit albumin for reference. AUGUSTINSSON²⁶⁾ reported the presence of aromatic ester esterase in the piscine plasma, indicating that the enzyme activity is generally lower than that of mammalian plasma. On the other hand, NYMAN²⁷⁾ found by starch gel electrophoresis that the esterase activity of crucian carp plasma is located in 2–3 components which are close to our S_3E_1 and S_3E_2 in mobility.

As shown above, it was found that carp plasma albumin is composed of at least five components which differ from each other both in physico-chemical properties and in physiological functions such as dye-binding and Sevin esterase activity. It seems to deserve further research whether this holds true with other fishes as well. The carp seems to be the fish whose plasma proteins have so far been studied most intensively. CREYSSEL *et al.*⁶⁾ reported that the fastest migrating protein in paper electrophoresis could be of comparatively high molecular weight, as judged from its gel filtration behavior. Recently, HARA²⁸⁾ has reported a similar observation. The protein component in question seems to correspond to our Fr. S_2 . Incidentally, NAKAGAWA *et al.*⁹⁾ isolated a carp albumin by a similar procedure to that for our Fr. S_2 , and reported that it is a lipoprotein of a mole-

cular weight of 150,000, and is able to bind with BPB and BCG. They described that their albumin is composed of a single polypeptide chain, unlike our Fr. S₂. The cause of this difference remains to be elucidated.

SILBERZAHN *et al.*⁷⁾ found the presence of three hemopexic proteins in carp serum. It is not clear at present in what manners their proteins correspond to our albumin components possessing a MHA forming ability. They also isolated a transferrin, to which our component S₃E₃ may be related. On the other hand, NAGANO *et al.*⁸⁾ isolated in a highly pure state a carp serum protein which has the second fastest mobility in cellulose acetate electrophoresis. This protein was shown to have a molecular weight of 59,000, and to possess free sulfhydryl group at less than one mol/mol. We recognized in an experiment that our component S₃E₁ showing the fastest mobility in starch gel electrophoresis exhibits the second fastest mobility in cellulose acetate electrophoresis. From these facts, the protein purified by NAGANO *et al.*⁸⁾ seems to account for a portion of our S₃E₁.

As indicated by PETERS²⁹⁾, the definition that plasma (or serum) albumin is a single major component of the fastest electrophoretic mobility, is not applicable to the lower vertebrates. The present results seem to support this view clearly. In this connection, WALLACE *et al.*³⁰⁾ also disclosed, by comparing immunologically plasma albumins of various frogs that the difference between two given amphibians is often even wider than the average difference between the reptile and the bird. Making an attempt to characterize collectively the plasma protein of fish, HARA²⁵⁾ found that the interspecific differences of plasma protein are unexpectedly large. The knowledge of piscine plasma albumin is still lacking or insufficient in various aspects. A study is now in progress on the MHA forming ability of some carp albumin components, and the results will be reported in the succeeding paper.

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