コイ補体第1成分(C1)の血清からの単離

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Isolation of the First Component of Complement (C1) from Carp Serum*1

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The first component of complement (C1) was isolated from carp serum by a 6-step purification procedure: 1) affinity chromatography (I) on Blue Cellulofine, 2) QAE-Sephadex A-50 chromatography, 3) gel filtration on Sepharose CL-6B, 4) Blue Cellulofine chromatography (II), 5) DEAE-Toyopearl 650 M chromatography (I), and 6) DEAE-Toyopearl 650M chromatography (II). The final recovery of C1 hemolytic activity was 18%, representing a 788-fold purification. The purity of the isolated carp C1 was established by gel filtration on Sepharose CL-6B and by immunoelectrophoresis against anti-whole carp serum (rabbit). The molecular weight of carp C1 was estimated to be 1,020,000. The hemolytic activity of carp C1 was inhibited by EDTA treatment, but this inhibition was overcome by dialysis against a Ca^{2+}-containing buffer. The hemolytic activity of carp C1 was destroyed by heating (50°C, 10 min) or incubation with carrageenan, but was retained when incubated with ammonia, hydrazine or zymosan. This indicates that carp C1 resembles mammalian C1 in chemical properties.

During the past 20 years, it has become increasingly evident that the complement system of teleosts, like that of mammals, consists of multiple components which are activated sequentially *via* the classical pathway or the alternative pathway.[1-7] However, there is little information about the kinetics of immune hemolysis and it has not yet been clarified how many components take part in the lysis of target cells, though complement components homologous to mammalian C3 and C5 have been purified from rainbow trout plasma by Nonaka *et al.*[8,9]

In a previous paper,[10] we have shown that the hemolytic action of carp complement on sensitized sheep red blood cells (EA) takes place in two successive steps. The first of these necessitates Ca^{2+} specifically and involves the fixation of components C1 and C4, with formation of a complex EAC1,4. In the second step, which requires Mg^{2+}, the complex EAC1,4 reacts with the remaining components.

In this paper, we deal with the isolation of the component C1 from carp serum by use of Cn (carrageenan-treated carp serum) as a reagent for detecting C1.

Materials and Methods

Chemicals

Blue Cellulofine (cellulose gel with covalently coupled Cibacron Blue F3GA), anti-human C1q (goat) and anti-human IgM (goat) were obtained from Seikagaku Kogyo Co., Tokyo, Japan. QAE-Sephadex A-50 and Sepharose CL-6B were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-Toyopearl 650M was a product of Toyosoda Mfg. Co., Tokyo. EDTA, carrageenan, agarose and Tris were purchased from Wako Pure Chemical Industries Co., Osaka, Japan. Zymosan, PMSF and proteins used as molecular-weight markers were obtained from Sigma Chemical Co., St. Louis, USA. Anti-whole carp serum (rabbit) was obtained from Medical Biological Laboratory Co., Nagoya, Japan. Sheep blood in Alsever's solution was purchased from Japan Bio-Supply Center, Tokyo. All other chemicals were of the highest grade available.

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Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; QAE, quartenary aminoethyl; GGVB, veronal-buffered saline, pH 7.8, containing 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 2.5% glucose; EDTA·GVB, veronal-buffered saline, pH 7.4, containing 0.1% gelatin and 10 mM EDTA.

Blood Sampling

The blood was collected from carp *Cyprinus carpio* by peduncle amputation, allowed to clot at room temperature for 30 min and cooled at 0°C for 1 h. The serum was then separated by centrifugation at 3000 rpm for 5 min and stored in small aliquots at −80°C until use.

Preparation of SRBC Sensitized with Carp Antibody (EA)

Sheep red blood cells (SRBC) in Alsever's solution were washed with EDTA·GVB, standardized spectrophotometrically to a concentration of 1×10^9 cells/m*l*, and sensitized with an equal volume of an appropriate dilution of carp antiserum at 30°C for 20 min. The sensitized SRBC (EA) formed were washed with GGVB and brought to a final concentration of 3×10^8 cells/m*l*.

Preparation of Carp Serum Deficient in Component C1 (Cn)

To 1 m*l* of carp serum was added 0.1 m*l* of 0.2% carrageenan and the mixture was incubated at 30°C for 10 to 15 min.[10]

Hemolytic Assays of Carp C1

The carp C1 content of column eluates was measured qualitatively as follows. A dilution (10 μl) of sample (equivalent to 0.1–5 μl of eluates) was incubated with 0.1 m*l* of EA suspension (3×10^8 cells/m*l*) at 30°C for 10 min. This was followed by the addition of 1.4 m*l* of GGVB and 10 μl of Cn. After incubation at 30°C for 30 min, the unlysed cells were removed by centrifugation and the degree of lysis was measured by reading the optical density at 414 nm (OD_{414}) of the supernatant.

C1 hemolytic activity was quantitated as follows. Serial dilutions (100 μl) of C1 pool in each purification step were incubated with 0.1 m*l* of EA suspension (3×10^8 cells/m*l*) at 30°C for 10 min. This was followed by addition of 1.4 m*l* of GGVB and 10 μl of Cn, and each mixture was incubated at 30°C for 60 min. After centrifugation, the degree of hemolysis was calculated from the OD_{414} of the supernatant. The data were evaluated in terms of "effective molecules" according to Mayer.[11]

Purification Procedure of Carp C1

Carp C1 was purified by a 6-step procedure. All of the purification procedures were performed at 5°C. Fractions obtained from each step were assayed for absorbance at 280 nm with a Shimadzu spectrophotometer (Shimadzu Corp., Kyoto) and for ionic strength with an MS conductivity meter (M & S Instruments Trading Inc., Tokyo). The concentration of protein solutions was conducted by ultrafiltration with a Q-2000 membrane (Advantec-Toyo Co., Tokyo). The protein concentrations of the pooled and concentrated fractions were calculated assuming arbitrarily $E^{1\%}_{280\,mm}=10$ for carp serum protein.

Step 1: Affinity Chromatography (I) on Blue Cellulofine. Frozen carp serum (100 m*l*) was thawed, made 1 mM in PMSF, and centrifuged at 10,000 rpm for 15 min at 4°C to remove any sediment. The cleared supernatant was poured into 400 m*l* of 10 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 5 mM $CaCl_2$, and applied to a Blue Cellulofine column (5.4×22 cm) equilibrated with the same buffer. The column was washed with 1.7 *l* of the starting buffer and adsorbed proteins were eluted with 2.0 *l* of 10 mM Tris-HCl buffer (pH 8.0) containing 750 mM NaCl, 15% saccharose and 0.2 mM PMSF, and then with 2.5 *l* of the same buffer adjusted to contain 1.8 M NaCl and 20% saccharose. The flow rate was kept at 780 m*l*/h and 20-m*l* fractions were collected. The C1-containing fractions were pooled on the basis of hemolytic activity and concentrated to approximately 150 m*l*.

Step: 2: QAE-Sephadex A-50 Chromatography. The concentrated carp C1 pool from Step 1 was dialyzed overnight against three changes of 2 *l* of 20 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl, 5 mM $CaCl_2$ and 0.2 mM PMSF, and applied to a QAE-Sephadex A-50 column (3.5×15 cm) equilibrated with the same buffer as that used for dialysis. The column was washed with 100 m*l* of the starting buffer, and adsorbed proteins were eluted with 100 m*l* of 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl. Ten-m*l* fractions were collected at a flow rate of 50 m*l*/h.

The procedure from Step 1 to Step 2 was repeated twice more, using 100 m*l* of carp serum each time, and all the C1-containing fractions were pooled (total volume, ca. 800 m*l*) and concentrated to approximately 6 m*l*.

Step 3: Gel filtrations on Sepharose CL-6B. The concentrated carp C1 pool from Step 2 was centrifuged at 10,000 rpm for 20 min at 4°C to remove any sediment and the supernatant was subjected to gel filtration on a Sepharose CL-6B column (2.0×90 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl

and 5 mM $CaCl_2$. Two-ml fractions were collected at a flow rate of 5 ml/h, and the fractions containing most of the carp C1 activity were pooled.

Step 4: Affinity Chromatography (II) on Blue Cellulofine. The carp C1 pool from Step 3 was applied to a small Blue Cellulofine column (2.0×10 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 5 mM $CaCl_2$. The column was washed with 50 ml of the starting buffer and adsorbed proteins were eluted with 250 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 750 mM NaCl, 5 mM $CaCl_2$ and 15% saccharose, and then with 550 ml of 10 mM Tris-HCl buffer (pH 8.3) containing 3 M NaCl, 5 mM $CaCl_2$ and 20% saccharose. Ten-ml fractions were collected at a flow rate of 30 ml/h. The C1-containing fractions were pooled and concentrated to about 60 ml, and dialyzed overnight against 20 mM Tris-HCl buffer (pH 8.3) containing 60 mM NaCl and 5 mM $CaCl_2$.

Step 5: DEAE-Toyopearl 650M Chromatography (I). The conductivity of the dialyzate in Step 4 was adjusted to that of the starting buffer using 20 mM Tris-HCl buffer (pH 8.3) containing 5 mM $CaCl_2$, and the solution was then applied to a DEAE-Toyopearl 650M column (1.6×10 cm) equilibrated with the same buffer as that used for dialysis. The column was washed with the starting buffer and then a linear NaCl gradient (total volume of 200 ml) was developed to a limiting concentration of 160 mM NaCl. Four-ml fractions were collected at a flow rate of 15 ml/h. The C1-containing fractions were combined with the pool of the pass-through fractions; the protein in the path-through fractions was used as a carrier in order to minimize the loss of carp C1 in subsequent chromatography.

Step 6: DEAE-Toyopearl 650M Chromatography (II). The C1 pool in Step 5 was diluted to give the same conductivity as the starting buffer, and then applied to a DEAE-Toyopearl 650M column (1.2×10 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.3) containing 60 mM NaCl and 5 mM $CaCl_2$. The column was washed with 30 ml of the starting buffer and then connected to a linear NaCl gradient. The total volume of the gradient was 120 ml and the limiting NaCl concentration of the buffer was 140 mM. Three-ml fractions were collected at a flow rate of 12 ml/h and the C1-containing fractions were pooled.

Immunoelectrophoresis

Electrophoresis was run in 1% agarose gel set in veronal buffer (pH 8.6, μ=0.05) containing 2 mM calcium lactate with a constant voltage gradient of 21 V/cm at 4°C for 3 h.

Molecular Weight Determination

The determination of the molecular weight of carp C1 was carried out according to the method of Whitaker[12] and Andrews[13] on a Sepharose CL-6B column (2.0×90 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl and 5 mM $CaCl_2$. Two-ml fractions were collected at a flow rate of 5 ml/h. The proteins used as molecular weight markers were: carbonic anhydrase (29,000), bovine serum albumin (66,000), yeast alcohol dehydrogenase (150,000), sweet potato β-amylase (200,000), horse spleen apoferritin (443,000), and bovine thyroglobulin (669,000). Complement component C1 (740,000) and IgM (950,000) partially purified from human serum were also used as marker proteins. The detection of the C1 and IgM in eluates was carried out by immunodiffusion using anti-human C1q and anti-human IgM, respectively.

Results

Purification of Carp C1

Complement component C1 was isolated from carp serum by the 6 step purification procedure.

Step 1: Affinity Chromatography (I) on Blue Cellulofine. The carp C1 adsorbed on the column was eluted with 10 mM Tris-HCl buffer (pH 8.0) containing 1.8 M NaCl, 20% saccharose and 0.2 mM PMSF. The pool of C1-containing fractions was found to contain approximately 115% of the initial hemolytic activity, but only 4.1% of the initial serum protein, representing a 28-fold purification. Upon subsequent ultrafiltration on an Advantec Q-2000 membrane (M. W. cut-off limit, 200,000), the loss of carp C1 was less than 2%.

Step 2: QAE-Sephadex A-50 Chromatography. Approximately 98% of carp C1 activity was recovered in the pool of the pass-through fractions. The hemolytic activity of this C1 pool was stable for at least 3 weeks when stored at 4°C in a solution containing 0.05% NaN_3, although some precipitation occurred.

Step 3: Gel Filtration on Sepharose CL-6B. A large part of carp C1 was found in the first protein peak as shown in Fig. 1. The recovery of carp C1 activity from the column was nearly 100%, though a part was discarded to avoid contamination with inactive proteins, giving a final recovery

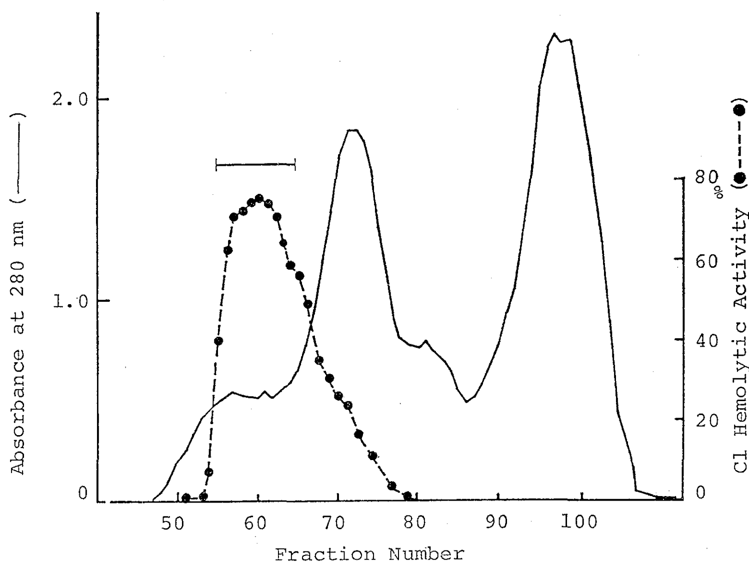

Fig. 1. Purification of carp C1 on a Sepharose CL-6B column (Step 3). The column (2.0×90 cm) was equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl and 5 mM $CaCl_2$. The sample applied to the column was a concentrated pool of the pass-through fractions obtained in Step 2. Two-m*l* fractions were collected at a flow rate of 5 m*l*/h. Fractions from 55 to 65 were pooled as indicated by the bar.

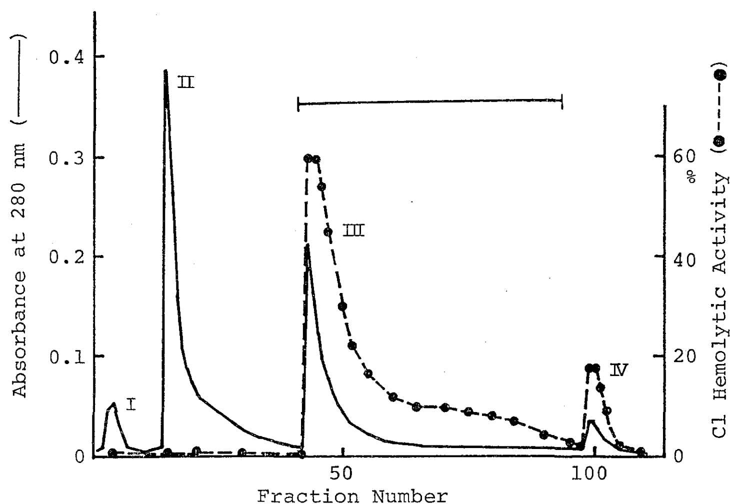

Fig. 2. Purification of carp C1 on a Blue Cellulofine column (Step 4). The column (2.0×10 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 5 mM $CaCl_2$. After sample loading and washing with the starting buffer, the adsorbed proteins were eluted with 10 mM Tris-HCl buffer (pH 8.0) containing 750 mM NaCl, 15% saccharose and 5 mM $CaCl_2$, and then with 10 mM Tris-HCl buffer (pH 8.3) adjusted to contain 3 M NaCl and 20% saccharose. Ten-m*l* fractions were collected at a flow rate of 30 m*l*/h. Fractions from 46 to 95 were pooled as indicated by the bar.

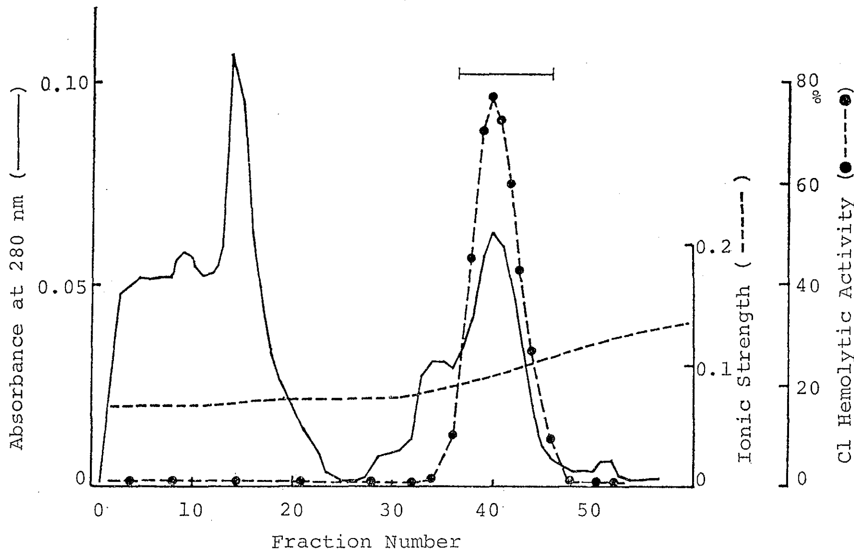

Fig. 3. Purification of carp C1 on a DEAE-Toyopearl 650M column (Step 5). The column (1.6×10 cm) was equilibrated with 20 mM Tris-HCl buffer (pH 8.3) containing 60 mM NaCl and 5 mM $CaCl_2$. After sample loading and washing with the starting buffer, a linear gradient of NaCl to 160 mM (total volume of 200 m*l*) was developed. Four-m*l* fractions were collected at a flow rate of 15 m*l*/h. Fractions from 35 to 45 were pooled as indicated by the bar.

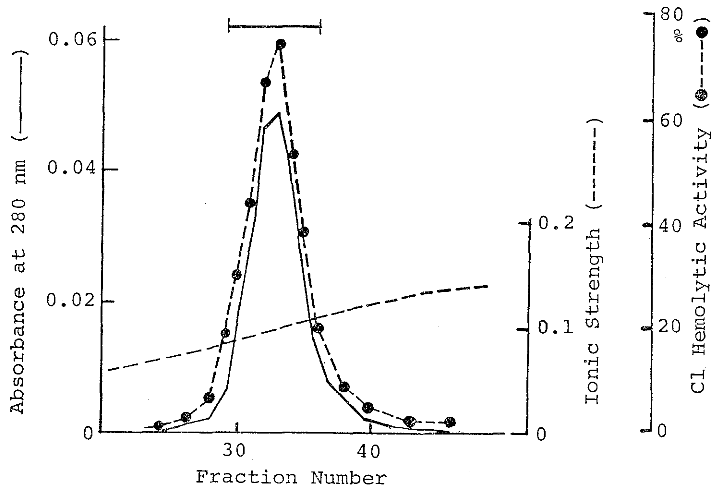

Fig. 4. Purification of carp C1 on a DEAE-Toyopearl 650M column (Step 6). The column (1.2×10 cm) was equilibrated with 20 mM Tris-HCl buffer (pH 8.3) containing 60 mM NaCl and 5 mM $CaCl_2$. After sample loading and washing with the starting buffer, a linear gradient of NaCl to 140 mM (total volume of 120 m*l*) was developed. Three-m*l* fractions were collected at a flow rate of 12 m*l*/h. Fractions from 29 to 36 were pooled as indicated by the bar.

of about 74%.

Step 4: Affinity Chromatography (II) on Blue Cellulofine. Carp C1 was eluted with the buffer containing 3 M NaCl and 20% saccharose (peak III), and proteins still adsorbed on Blue Cellulofine (peak IV) were eluted with 500 mM NaSCN containing 5 mM $CaCl_2$ (Fig. 2). Approximately 50% of the C1 hemolytic activity was recovered

Table 1. Summary of C1 purification

Step	Total protein* (mg)	Total volume (m*l*)	Total activity** (eff.mol.)	Recovery (%)	Specific activity (eff.mol./mg)	Purification (-fold)
Serum	12000	300	4.31×10^{13}	100	3.59×10^{9}	1
Blue Cellulofine (I)	492	6000	4.95×10^{13}	115	1.01×10^{11}	28
QAE-Sephadex	233	800	4.22×10^{13}	98	1.81×10^{11}	50
Sepharose CL-6B	38.6	22	3.20×10^{13}	74	8.29×10^{11}	231
Blue Cellulofine (II)	16.0	500	1.54×10^{13}	36	9.63×10^{11}	268
DEAE-Toyopearl (I)	4.0	40	8.27×10^{12}	19	2.07×10^{12}	577
DEAE-Toyopearl (II)	2.8	24	7.93×10^{12}	18	2.83×10^{12}	788

* Calculated assuming arbitrarily $E_{1\%}^{280\,\mathrm{mm}}=10$ for carp serum protein.
** Determined by hemolytic titration using EA cells and Cl-deficient carp serum.

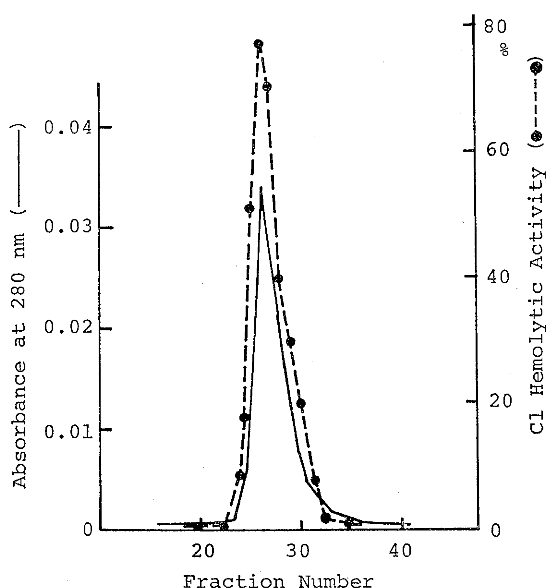

Fig. 5. Gel filtration of purified carp C1 on a Sepharose CL-6B column (1.5×50 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl and 5 mM $CaCl_2$. Two-m*l* fractions were collected at a flow rate of 5 m*l*/h.

in peak III, and about 3% appeared in peak IV.

Step 5: DEAE-Toyopearl 650 M Chromatography (I). Carp C1 activity was found in fractions having an ionic strength of between 0.09 and 0.11 (Fig. 3). The recovery of C1 hemolytic activity in this step was 50 to 60%.

Step 6: DEAE-Toyopearl 650M Chromatography (II). Carp C1 was detected as a single symmetrical peak (Fig. 4). The isolated carp C1 remained stable at least one month when stored at 0°C in 20 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl, 5 mM $CaCl_2$ and 0.05% NaN_3.

In this 6-step purification procedure, about 2.8 mg of carp C1 protein was obtained from 300 m*l* of carp serum, assuming arbitrarily $E_{280\,\mathrm{mm}}^{1\%}=10$ for carp C1 protein. This final preparation contained 18% of the initial C1 hemolytic activity and 0.023% of the initial serum protein, representing a 788-fold purification (Table 1).

Criteria of Purity

The purity of the final preparation of carp C1 was evaluated by gel filtration and immunoelectrophoresis. As shown in Figs. 5 and 6, the final preparation of carp C1 was eluted as a symmetrical peak upon gel filtration on Sepharose CL-6B and

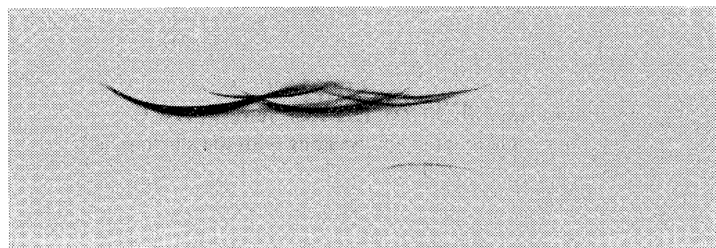

Fig. 6. Immunoelectrophoresis of carp C1. Whole carp serum (upper well) and purified carp C1 (lower well) were electrophoresed in 1% agarose gel set in veronal buffer (pH 8.6, μ=0.05) containing 2 mM calcium lactate with a constant voltage gradient of 21 V/cm at 4°C for 3 h. After electrophoresis, a precipitin reaction was developed against rabbit anti-whole carp serum (trough). The left side is anode.

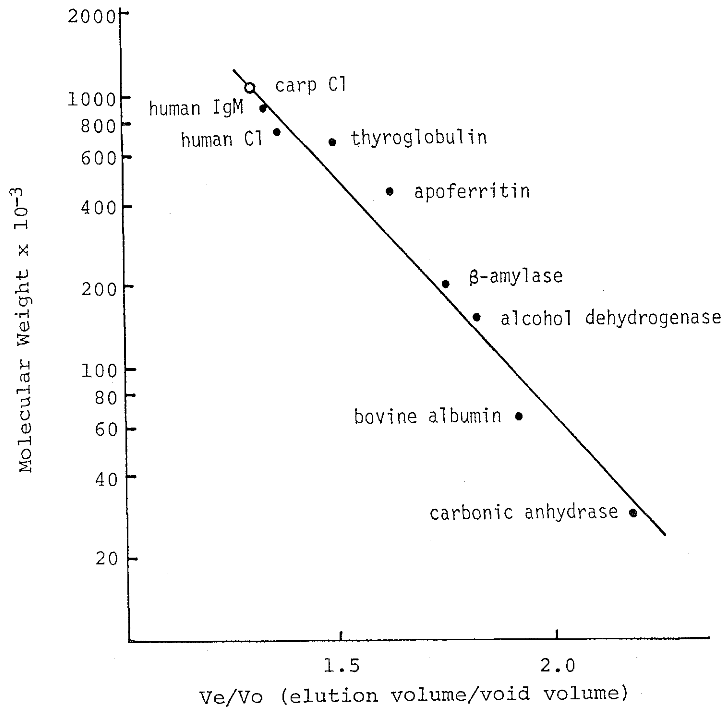

Fig. 7. Molecular weight determination of carp C1. The molecular weight of carp C1 was determined by gel filtration on a Sepharose CL-6B column (2.0×90 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl and 5 mM $CaCl_2$.

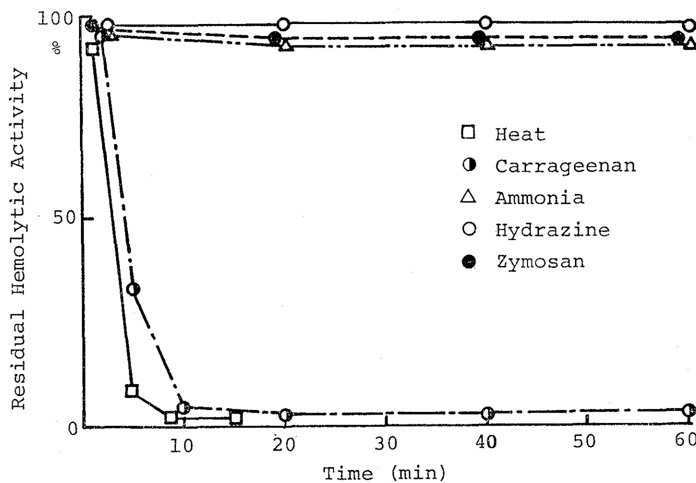

Fig. 8. Effect of heat, carrageenan, ammonia, hydrazine or zymosan treatment on carp C1 hemolytic activity. Residual hemolytic activity was expressed as a percentage of the initial one.

gave a single precipitin arc against anti-whole carp serum (rabbit) upon immunoelectrophoresis.

Molecular Weight of Carp C1

The molecular weight of carp C1 was estimated to be 1,020,000 on Sepharose CL-6B gel filtration (Fig. 7).

Properties of Carp C1

The chemical properties of carp C1 were examined as follows: A dilution (1 m*l*, GGVB) of carp C1 was heated at 50°C for 15 min or incubated with 0.1 m*l* of 1 N ammonia, 40 mM hydrazine, 0.1% carrageenan or 5% zymosan at 30°C for 1 h. At timed intervals, an aliquot (0.1 m*l*) was with-

drawn from each mixture and assayed for C1 hemolytic activity as described in Materials and Methods. As shown in Fig. 8, carp C1 was quickly destroyed upon heating at 50°C or by incubation with carrageenan, but retained its activity upon incubation with ammonia, hydrazine or zymosan. These results are in fair agreement with the data in the previous report.[10]

Next, carp C1 (1 m*l*) was dialyzed against two changes of 100 m*l* of veronal-buffered saline (pH 8.0) containing 0.1, 0.2, 0.5, 1.0 or 2.0 mM EDTA at 4°C for 3 h, and then against 100 m*l* of veronal-buffered saline (pH 8.0) containing 5 mM $CaCl_2$ for 1 h. The content of each bag was sampled at 60-min intervals and mixed with packed EA cells (3×10^7) which had been washed with GVB (gelatin veronal buffer, pH 8.0, free of divalent cations), and assayed for C1 hemolytic activity. The hemolytic activity of carp C1 was inhibited by EDTA at any concentrations tested, and was recovered by dialysis against a Ca^{2+}-containing buffer. This indicates that the reaction between EA and carp C1 needs Ca^{2+}.

Discussion

We have isolated the first component of complement (C1) from carp serum by a combination of affinity chromatography, ion-exchange chromatography and gel filtration.

It is known that mammalian serum proteins can be separated into a soluble and an insoluble fraction by dilution,[14,15] and that the first complement component C1 is recovered at a good yield from the insoluble euglobulin fraction. In a preliminary experiment, we attempted to separate carp serum proteins into a soluble and an insoluble fraction based on the procedure of Gigli *et al.*[16] for the purification of human C1, but all efforts were unsuccessful. Carp C1 quickly lost its activity at pH values lower than 5.5, while at values higher than 5.5, separation was incomplete. At pH 5.5, for example, 30 to 40% of the hemolytic activity of carp C1 remained in the supernatant.

Affinity chromatography with Blue Cellulofine (cellulose gel with covalently coupled Cibacron Blue F3GA[17]) proved valuable as an initial step in the purification of carp C1. Carp C1 was eluted from the Blue Cellulofine column with a buffer containing 1.8 M NaCl, 20% saccharose and 0.2 mM PMSF, while serum albumins were not eluted under these conditions. In this chromatographic procedure, addition of saccharose to the eluent was essential to increase the recovery of carp C1, and PMSF was essential to prevent spontaneous activation of serum serine proteases.

The molecular weight of purified carp C1 was estimated to be 1,020,000 by the gel filtration method. The molecular weight of carp C1 is therefore higher than that of human C1, which has been reported to be about 740,000 by Siegel *et al.*[18] and Lepow *et al.*[19]

Ross and Jensen purified the first component (C1n) of complement from nurse shark serum.[20,21] This nurse shark C1n could be measured hemolytically with EAn (SRBC sensitized with natural antibody of nurse shark) and guinea-pig serum devoid of C1. They found in addition 1) that C1n was incompatible with rabbit IgG or IgM, 2) that EAn and C1n formed an intermediate complex (EAnC1n) even in the absence of Ca^{2+}, and 3) that the inactivation of C1n by EDTA could not be reversed upon addition of divalent cations.

The properties of carp C1 obtained in the present experiment were different from those of nurse shark C1n in several points. 1) Carp C1 was incompatible not only with rabbit antibody, but also with guinea pig complement (data not shown), 2) EA (SRBC sensitized with carp anti-SRBC) and carp C1 formed an intermediate complex (EAC1) only in the presence of Ca^{2+}, indicating that Ca^{2+} is indispensable for the reaction between EA and carp C1. Recently, we have purified another complement component which reacts with EAC1 in the absence of Ca^{2+} and identified it as carp C4 (unpublished data). 3) Inactivation of carp C1 by EDTA treatment could be recovered upon addition of Ca^{2+}. These data suggest that the C1 protein of the carp (a teleost) is nearer to that of mammals than to that of the nurse shark (an elasmobranch).

Finally, it is known in mammals that the C1 molecule is a complex of three subcomponents (C1q, C1r and C1s),[22,23] and that these combine with each other in the presence of Ca^{2+}. We observed that carp C1 was fragmented into 2 to 3 pieces by EDTA treatment (data not shown). Presumably, this was a consequence of dissociation of carp C1 into its subcomponents. We are now making efforts to isolate each subcomponent of carp C1. Details will be reported in a subsequent paper.

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