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https://doi.org/10.5109/23820

出版情報：九州大学大学院農学研究院紀要. 30 (2/3), pp.149-158, 1985-12. Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., 30 (2 · 3), 149-158 (1985)

Optimum Conditions for the Assay of Hemolytic Complement Titer of Porgy (*Pagrus major*) Serum

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(Received September 10, 1985)

The optimum conditions for the titration of complement activity of porgy serum were examined by the use of sheep red blood cells (SRBC) sensitized with porgy antiserum. The antiserum was obtained from porgy immunized with SRBC stromata for 45 days, and heat-treated at 45°C for 20 min to inactivate the complement coexisting within the serum. The optimum conditions for the hemolytic complement reaction were found to be as follows: reaction temperature, 20°C; reaction period, 2 h; pH, 7.0 to 8.0; concentrations of Ca^{2+} and Mg^{2+}, 0.5 to 1.0 mM and 1.0 mM, respectively. The complement titer of porgy specimens which were obtained from a fish farm near Fukuoka City in August 1985 were assayed under these conditions. The titers ranged widely from 19.5 to 54.0 CH_{50} units/ml, the mean value being 29.2 CH,, units/ml. It was found that porgy complement is quite labile compared with mammalian and carp complements. Porgy complement lost its hemolytic activity within 24 h.

INTRODUCTION

Hemolytic complement activity of mammals is usually assayed by Mayer's method (1961) : the complement is incubated with sensitized sheep red blood cells (5×10^8 cells) in 7.5 ml of isotonic buffer (pH 7.4), containing 0.15 mM Ca^{2+} and 0.50 mM Mg^{2+}, at 37°C for 60 min; one unit of CH_{50} is defined as the amount of complement which hemolyzes 50 % of the total cells.

Recently, the amount of literature concerned with fish complement has increased as more attention has become focussed on the defense mechanisms of lower vertebrates, and most classes of fish have been shown to possess a complement system which resembles that of mammals. Included in this literature, have been reports describing the measurement of fish complement activity by Mayer's method with modifications of the temperature and/or period of incubation. For example, Legler and Evans (1967) incubated sensitized SRBC with fish! complement at 28°C for 2 h; Legler *et* al. (1971) assayed the complement activity of paddlefish at 25°C for 4 h; Sakai (1981) titrated the complement activity of salmonid fishes at 30°C for 60 min; Nonaka *et al.* (1981) carried out an assay of the complement activity of rainbow trout at *25°C* for 60 min. However, as far as we are aware, the effects of pH and concentrations of Ca^{2+} and Mg^{2+} on the hemolytic activity of fish have not been fully investigated.

In our previous report (Yano et *al.*,1984), we examined the optimum conditions for the assay of the complement titer of carp. In this paper, we deal with the titration of the complement activity of the porgy, a marine fish, which is now extensively cultivated in Japan.

MATERIALS AND METHODS

1. Reagents

The sources of reagents used in the present work were as follows: EDTA and barbital sodium, Wako Pure Chemical Industries Co., Osaka; MS-222 (tricain methanesulfonate), Sankyo Co., Tokyo; Amberlite IRC-50, Organo Co., Tokyo; sheep blood in Alsever's solution, Japan Bio-Supply Center, Tokyo.

The buffers used in the experiment and their abbreviations were: GVB^{2+}, veronal-buffered saline, pH 7.4, containing 0.1% gelatin, 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$; $GVB^{2+}(P)$, veronal-buffered saline, pH 7.4, containing 0.1 % gelation, 1.0 mM $CaCl_2$ and 1.0 mM $MgCl_2$; EDTA . GVB, veronal-buffered saline containing 0.1% gelatin and 0.01 M EDTA; GGVB, veronal-buffered saline containing 2.5 % glucose and 0.1% gelatin; GVB(f), veronal-buffered saline containing 0.1 % gelation, free from divalent cations. GVB(f) was made by passing 200 ml of veronal-buffered saline containing 0.1% gelatin through Amberlite IRC-50 (5 ml) and readjusting its pH to 7.4. Unless otherwise noted, GVB^{2+} was used throughout this experiment.

2. Fish

Specimens of porgy, *Pagrus* ***major,*** weighing 450 to 500 g, were purchased from a fish farm near Fukuoka City, and maintained in aquaria for about one month at a water temperature of 18°±1°C under a photoregime of 12 L/12 D.

3. Immunization of fish

A sheep red blood cell (SRBC) stroma suspension was prepared by the method described in the previous paper (Yano ***et al.,*** 1984). Every 5 to 7 days, 4 porgys were intraperitoneally injected with this suspension (0.2 ml/fish), and at the same time, 0.5 ml of blood was collected from the dorsal aorta of each fish to measure the antibody titer (hemolysin titer). The blood was coagulated at 20°C for 30 min, cooled in ice-water for 1 h, and centrifuged at 3000 rpm for 5 min. The serum obtained was stored at -30°C until use.

Injection of the stroma suspension was repeated 7 times, while the blood collection was repeated 8 times (until 45 days after the first injection).

4. Collection of antiserum and normal serum

On the last day of the above experiment (the 45 th day of immunization), porgys were anesthetized with MS-222, and ca. 5 ml of blood was collected from the dorsal aorta of each fish using tuberculin syringes. The blood was allowed to clot at 20°C for 30 min, and cooled at 0°C for 1 h. After centrifuging at 3000 rpm for 5 min, the supernatant, anti-SRBC porgy serum, was stored at -30°C until use.

Normal porgy serum was collected as a source of complement in a similar manner to that from uninjected fishes, and stored at -30°C. Both porgy antiserum and normal serum were stable for at least one month at -30°C.

5. Heat inactivation of complement

Anti-SRBC porgy serum was diluted 1/40 with GVB^{2+}, and 0.5-ml aliquots of the dilution were heated at 25°, 30°, 43°, 45" and 50°C each for 20 min in a small test tube. To each tube, 0.1 ml of SRBC suspension (1×10^9 cells/ml) and 0.9 ml of GVB^{2+} were added, and the mixture was further incubated at 20°C for 90 min. After centrifugation, the hemolysis rate was calculated from the OD_{541} of the supernatant fluid.

6. **Preparation of sensitized sheep red blood cells (EA)**

SRBC were washed twice with GVB(f) and suspended at a concentration of $1\ x10^9$ cells/ml in the same buffer. Anti-SRBC porgy serum, which had been heat-treated at 45°C for 20 min, was diluted to between 1/50 and 1/100 with EDTA . GVB (the dilution for the optimum sensitization was determined in the usual way). Equal volumes of SRBC suspension and of the diluted antiserum were mixed and incubated at 20°C for 30 min with occasional shaking. Then the mixture was centrifuged at 2000 rpm for 3 min and the precipitate (EA) was washed well with GVB(f). Finally, EA cells were suspended in GGVB to give a cell density of 5×10^8/ml and stored at 0°C. Just before use, the EA cells were washed and resuspended in GVB^{2+} or GVB(f).

7. **Determination of the optimum conditions for the hemolytic reaction**

(**a**) reaction temperature

Hemolytic reaction was carried out by reducing the total volume used in Mayer's method (7.5 ml) to one fifth (1.5 ml). The reaction mixture, containing 0.2 ml of EA suspension (5×10^8/ml), 0.5 ml of diluted porgy serum and 0.8 ml of GVB^{2+}, was incubated at 10°, 20°, 25°, 30" and 37°C for 90 min, followed by cooling at 0°C and centrifuged at 3000 rpm for 5 min. Hemolysis rate at each temperature was calculated from the OD_{541} of the supernatant.

(b) pH

The pH of GVB^{2+} was altered to 6.0, 6.5, 7.0, 7. 5, 8.0, 8.5, 9.0 and 9.5 by adding 1 N HCI or 1 N NaOH, and hemolytic reaction was carried out at each pH. The reaction mixtures were incubated at 20°C for 90 min and centrifuged, and the hemolysis rate at each pH was calculated from the OD_{541} of the supernatant.

(c) incubation period

The hemolytic reaction mixtures were incubated at 20°C for 150 min in small test tubes. Every 10 to 20 min during incubation, a test tube was taken out and centrifuged. Hemolysis rate at each time point was calculated from the OD_{541} of the supernatant and plotted on graph paper to follow up the time course of hemolysis.

(d) concentrations of Ca^{2+} and Mg^{2+}

In order to remove as much Ca^{2+} and Mg^{2+} as possible, 0.5 ml of porgy

serum (ca. 20 CH_{50} units/ml) was passed through an Amberlite IRC-50 (3 ml), then 2.8 ml of GVB(f) was added to the column; the first eluate (0.3 ml) was discarded and the next eluate (2.5 ml) was collected. This eluate was further diluted 1/4 with GVB(f).

A 0.5-ml aliquot of the diluted eluate and 10 μl of $CaCl_2$ and 10 μl of $MgCl_2$ were added to 0.2 ml of EA suspension in GVB(f), and the total volume was made up to 1.5 ml with GVB(f). The optimum concentration of Ca^{2+} was examined in the presence of 0.50 mM Mg^{2+}, whereas that of Mg^{2+} was investigated in the presence of 0.15 mM Ca^{2+}. The mixtures were incubated at 20°C for 90 min, and centrifuged to estimate the hemolysis rates.

8. Assay of complement titer (CH_{50} unit/ml)

For assaying the hemolytic complement titer of porgy, GVB^{2+}(P) was used instead of GVB^{2+}. To 0.2 ml of EA suspension (5×10^8/ml) was added 0.3, 0.5 or 0.7 ml of diluted porgy serum (1/25 to 1/35), then the total volume was adjusted to 1.5 ml with GVB^{2+}(P). The mixture was incubated at 20°C for 120 min and centrifuged. The hemolysis rate was estimated from the OD_{541} of the supernatant and the complement titer (CH_{50} unit/ml) was calculated according to the method of Mayer (1961).

9. Assay of hemolysin titer

Anti-SRBC porgy serum was heat-treated at 45°C for 20 min and diluted with GVB^{2+}(P) in a two-fold manner (1/50, 1/100, 1/200...) at a volume of 0.5 ml in small test tubes, then 0.1 ml of SRBC suspension (1×10^9/ml) was added to each tube. After incubation at 20°C for 10 min, 0.5 ml of optimally diluted normal porgy serum (ca. 1/50) and 0.4 ml of GVB^{2+}(P) were added, and the reaction mixture was further incubated at 20°C for 120 min. After centrifugation at 3000 rpm for 5 min, the hemolysis rate (y) was plotted on semi-logarithmic graph paper (the logarithmic X-axis representing the dilution of hemolysin, the Y-axis representing y). Hemolysin titer, the dilution giving 50 % hemolysis (y=0.5), was read off from the graph.

RESULTS

1. Anti-SRBC porgy serum

Four porgys were injected intraperitoneally with 0.2 ml of SRBC stroma suspension 7 times at intervals of 5 to 7 days, and the change of hemolysin titer occurred during immunization was observed (Fig. 1). The hemolysin titer of the fish began to rise on the 25 th day of immunization and reached a high value (1300 to 15000) on the 45 th day.

2. Heat treatment of porgy antiserum

In order to determine the optimum condition for the heat inactivation of the complement coexisting within antiserum, anti-SRBC porgy serum was heated at various temperatures for 20 min, and the remaining hemolytic activity was assayed by incubating the heat-treated serum with SRBC.

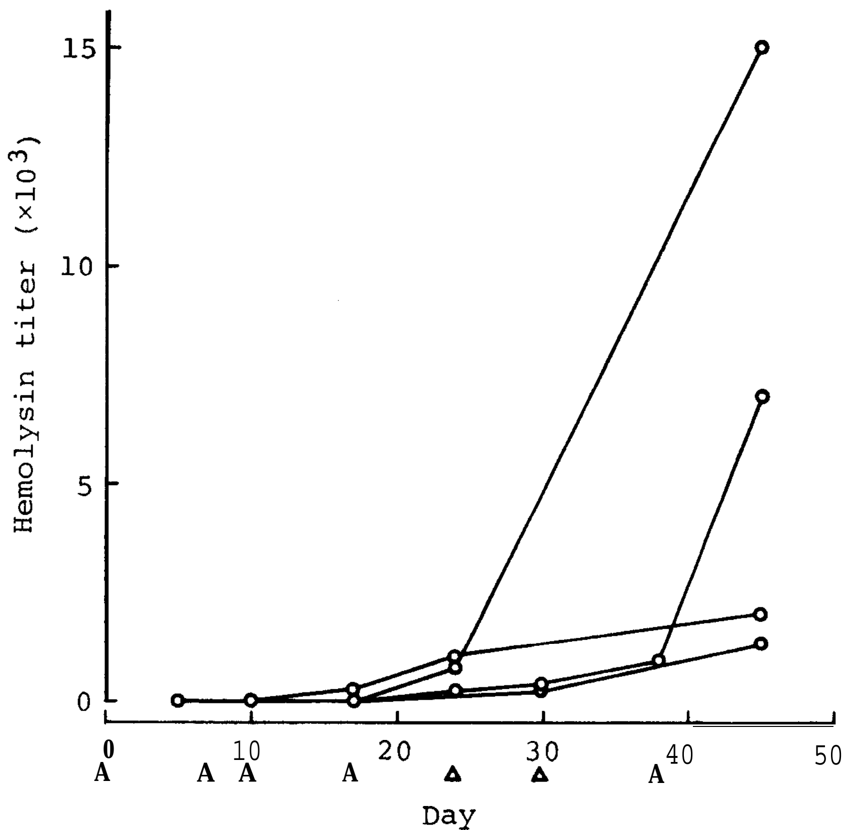

Fig. 1. Changes in the hemolysin titers of 4 porgys injected with SRBC stroma suspension (1 mgN/ml).
Triangles indicate the days of injection (0.2 ml/fish). On the 45 th day of immunization, all fish were bled and antisera were stored at -30°C.

As shown in Fig. 2, porgy complement was found to be completely inactivated by heating at 45°C for 20 min.

3. **Optimum conditions for the assay of complement titer**

The total volume of the reaction mixture used in Mayer's method was reduced to 1/5, and incubation was carried out at various temperatures for 90 min. As shown in Fig. 3, the porgy complement maintained high activity over the range of 20" to 30°C, and the highest activity was obtained at 20°C.

Fig. 4 shows the effect of pH on the activity of porgy complement. The optimum pH lay between pH 7.0 and 8.0, and at lower or higher pH values, the activity of porgy complement diminished.

Fig. 5 shows the time course of hemolysis by porgy complement. It was found that hemolysis takes place rather slowly, reaching an end-point in about 120 min.

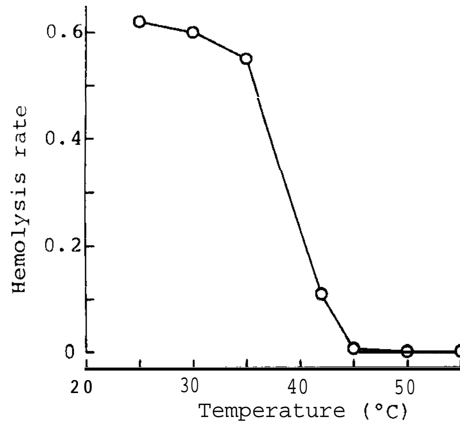

Fig. 2. Heat inactivation of porgy complement.
Anti-SRBC porgy serum was heated at 30" to 50°C for 20 min, and the remaining hemolytic activity was assayed by incubating with SRBC at 20°C for 90 min at pH 7.4.

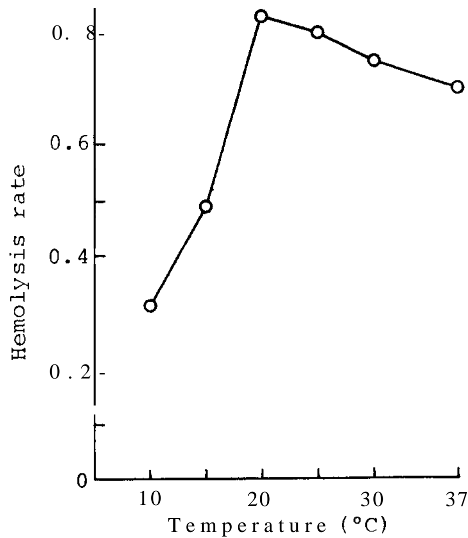

Fig. 3. Effect of incubation temperature on the hemolytic activity of porgy complement.
The reaction was carried out at 10' to 37°C for 90 min at pH 7.4.

The effect of concentrations of divalent cations on the complement activity was then investigated. As shown in Fig. 6, the optimum concentration of Ca^{2+} was 0.5 to 1.0 mM and that of Mg^{2+} was 1.0 mM.

In conclusion, the titration of porgy complement necessitates some modifications of Mayer's method, that is, incubation should be carried out at **20°C**

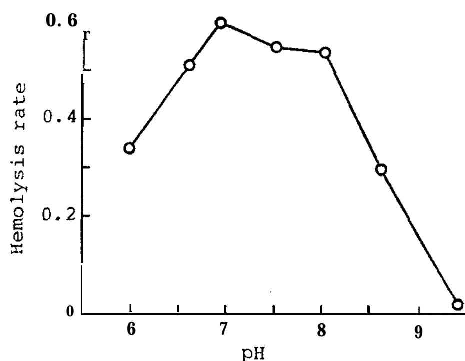

Fig. 4. Effect of pH on the hemolytic activity of porgy complement.
The reaction was carried out at 20°C for 90 min.

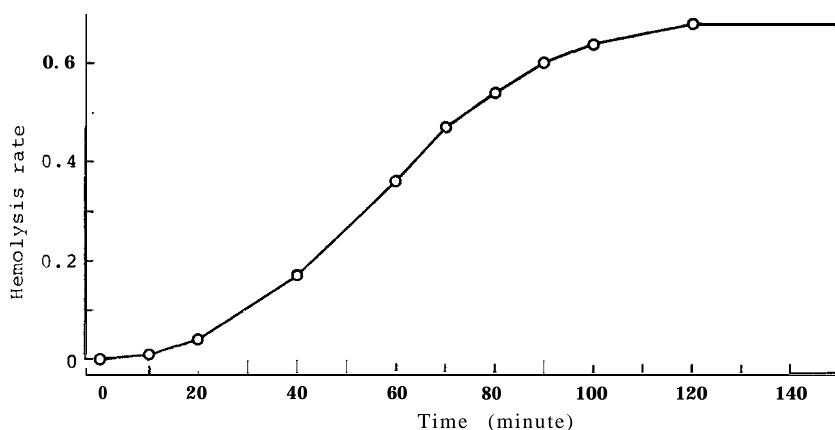

Fig. 5. Time course of hemolysis in the reaction of porgy complement with sensitized SRBC.
The reaction was performed at 20°C for 150 min at pH 7. 4.

for 120 min at pH 7.4 in the presence of 1.0 mM Ca^{2+} and 1.0 mM Mg^{2+}.

Under these conditions, the complement titer of 8 porgy specimens which were obtained from a fish farm near Fukuoka City in August 1985 were assayed. The titers ranged widely from 19.5 to 54.0 CH_{50} units/ml, the mean value being 29.2 CH,, units/ml.

4. **Stability of porgy complement**

An experiment was performed to check the stability of porgy complement. The porgy sera stored at -30°C were thawed at room temperature and then allowed to stand at 0°C or 20°C for 24 h. Hemolytic activity was measured at 3, 6, 12 and 24 h after thawing (Fig. 7). Within 3 h, the decreases in hemolytic activity were less than 5 % at both temperatures. However, this was followed

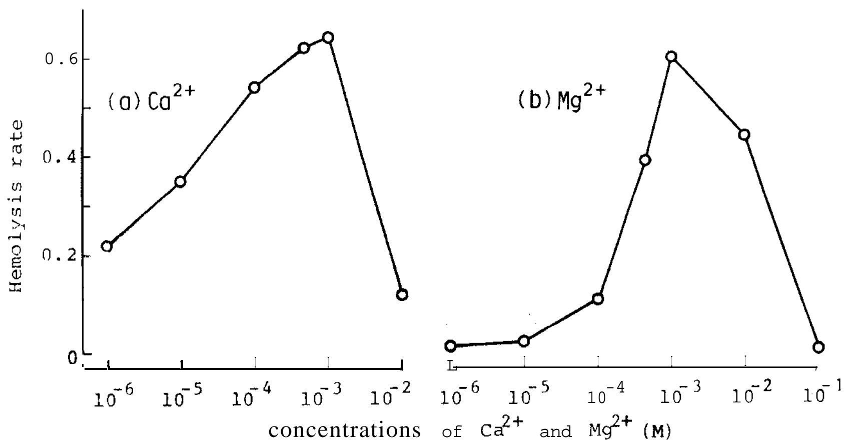

Fig. 6. Effect of concentration of Ca^{2+} or Mg^{2+} on the hemolytic activity of porgy complement.
The reaction was performed at 20°C for 90 min at pH 7.4.
(a) Concentration of Ca^{2+} was altered in the presence of 0.5 mM $MgCl_2$.
(b) Concentration of Mg^{2+} was altered in the presence of 0.15 mM $CaCl_2$.

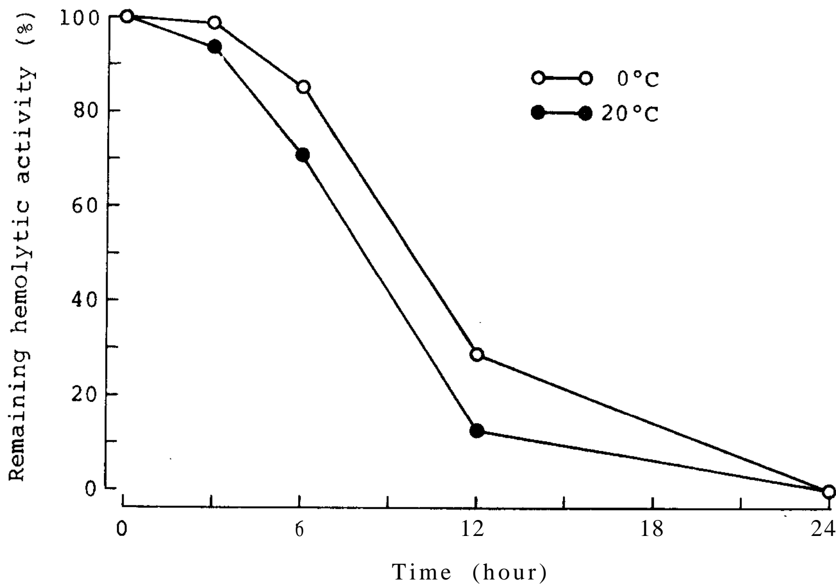

Fig. 7. Stability of porgy complement.
Porgy serum which had been stored at -30°C was thawed at room temperature and allowed to stand at 0°C or 20°C for 24 h. Residual hemolytic activity was expressed as a percentage of the initial one.

by a drop in the activities, i. e., after 12 h the sample kept at 0°C showed about a 70 % decrease in activity, while that kept at 20°C showed an 86 % de-

crease. By the end of the experiment, the samples completely lost their activities.

DISCUSSION

In preliminary experiments, we first attempted to use SRBC sensitized with carp antibody for the titration of porgy complement, as we had prepared carp antibody in the previous study (Yano et al., 1984), but carp antibody was found to be incompatible with porgy complement. Therefore, we prepared anti-SRBC porgy serum by immunizing porgy against SRBC.

The increase in the hemolysin titer of porgy was considerably slower than that of carp. As shown in Fig. 1, the hemolysin titer of porgy began to increase on the 25 th day of immunization and was still increasing at the end of the experiment, while that of carp began to increase on the 15 th day and reached its peak on the 25 th day (Yano *et al.*, 1984). This delay in antibody production of porgy is probably attributable to the difference in water temperature of the aquaria (18°C for porgy, 24°C for carp), because antibody production of fish is said to be temperature-dependent (Avtalion *et al.*, 1973; Tait, 1969 ; Harris, 1973 ; Rijkers *et al.*, 1980).

We have reported in the previous paper that the complement activity of carp can be assayed by Mayer's method if its incubation temperature is modified from 37°C to 30°C. However, the present work revealed that the titration of porgy complement necessitates some other modifications. (1) The incubation temperature should be lowered from 37°C to 20°C. (2) The incubation period should be extended from 60 min to 120 min, because the hemolytic reaction by porgy complement takes place slowly even at the optimum temperature (20°C). (3) The concentration of Ca^{2+} should be changed from 0.15 mM to 1.0 mM, and that of Mg^{2+} should be altered from 0.5 mM to 1.0 mM. These high requirements of Ca^{2+} and Mg^{2+} of porgy complement may reflect the high levels of concentration of these cations in porgy serum: porgy serum contained 2.90 mM Ca^{2+} and 1.63 mM Mg^{2+}, whereas carp serum contained 2.30 mM Ca^{2+} and 1.28 mM Mg^{2+} (Yano *et al.*, unpublished).

Finally, it must be noted that porgy complement is quite labile. When porgy serum was allowed to stand at 0°C, it showed a 15 % decrease in complement activity in 6 h, whereas the activity of carp complement decreased by less than 5 % within 6 h. We therefore paid careful attention to use the porgy serum as soon as possible after thawing it.

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