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<https://doi.org/10.5109/23914>

出版情報：九州大学大学院農学研究院紀要. 33 (1/2), pp.29-36, 1988-10. Kyushu University
バージョン：
権利関係：

Optimum Conditions for the Assay of the Classical Pathway- Complement Titer of Tilapia (*Tilapia nilotica*) Serum

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(Received March 17, 1988)

The optimum conditions for the assay of the classical pathway-complement titer (CH50) of tilapia serum were investigated by use of sheep red blood cells (SRBC) sensitized with tilapia antibody. The antiserum was obtained from tilapia immunized with SRBC stroma for 35 days, and heat-treated at 46°C for 20min to inactivate the complement coexisting *in* the serum. The optimum conditions for the hemolytic complement reaction were found to be as follows : reaction temperature, 25°C ; pH, 7.5-8.0 ; reaction period, 120 min ; concentrations of Ca^{2+} and Mg^{2+} , 1.0 mM and 1.0 mM, respectively. Under these conditions, the CH50 of 15 tilapia specimens (240-960 g) which were obtained from a fish farm in Fukuoka City from September in 1987 to January in 1988 were assayed. The titers ranged from 153 to 392 CH50 units/ml, mean \pm SD being 250170 CH50 units/ml. These CH50 values of tilapia sera were extremely high as compared with those reported for other bony fishes.

INTRODUCTION

Our previous studies (Yano et al., 1984; Matsuyama et al., 1985) have shown that the classical pathway-complement titer (CH50) of carp *Cyprinus carpio* and porgy *Pagrus major* could be assayed by Mayer's method with some modifications. In this study, we investigated the optimum conditions for assaying the CH50 of tilapia *Tilapia nilotica*, a freshwater fish extensively cultured in Japan, by use of sheep red blood cells sensitized with tilapia antibody.

MATERIALS AND METHODS

Reagents

The sources of reagents used in the present work were as follows : EDTA (ethylenediamine tetraacetic acid disodium salt) and barbital sodium, Katayama 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 : GVB²⁺, veronal-buffered saline, pH 7.5, containing 0.1% gelatin, 0.15 mM CaCl_2 and 0.50 mM MgCl_2 ; GVB²⁺(T), veronal-buffered saline, pH 7.5, containing 0.1% gelatin, 1.0 mM CaCl_2 and 1.0 mM MgCl_2 ; EDTA . GVB, veronal-buffered saline containing 10 mM EDTA and 0.1% gelatin ; GGVB, veronal-buffered saline containing 2.5% glucose,

0.1% gelatin, 0.15 mM CaCl_2 and 0.50 mM MgCl_2 ; GVB(f), veronal-buffered saline free from divalent cation, pH 7.5, containing 0.1% gelatin. GVB(f) was prepared by passing 200 ml of veronal-buffered saline containing 0.1% gelatin through an Amberlite IRC-50 (5 ml) column and readjusting its pH to 7.5. Except where noted, GVB^{2+} was employed in this experiment.

Fish

Specimens of tilapia *Tilapia nilotica*, weighing 830 to 970 g were purchased in early September from a fish farm in Fukuoka City. Prior to use in the experiment, the fish was maintained in an artificial outdoor pond for more than a month at a water temperature of $24 \pm 2^\circ\text{C}$.

Collection of antiserum and normal serum

In order to immunize tilapia against sheep red blood cells (SRBC), SRBC stroma suspension (1 mgN/ml) was prepared as immunogen according to Yano *et al.* (1984). Three tilapia were anesthetized with MS-222 and injected intraperitoneally with the SRBC stroma suspension (0.4 ml/fish) at 5-day intervals. At the same time, 0.4 ml of blood was collected from the caudal vein to follow the time course of antibody production. The injection of the stroma suspension was repeated 7 times, whereas the blood-collecting was repeated 8 times.

Five days after the last injection, the fish whose serum showed the highest hemagglutination titer was anesthetized with MS-222 and blood was withdrawn from the caudal vein. The blood was allowed to stand at room temperature for 30 min, cooled at 0°C for 30 min, and centrifuged at 3,000 rpm for 5 min. Then anti-SRBC tilapia serum obtained as the supernatant was heated at 46°C for 20 min and stored at -80°C . The hemagglutination titer against SRBC was determined by microtiter method using EDTA•GVB as diluent.

Normal tilapia serum was collected as a source of complement in a similar manner from uninjected fish, and stored at -80°C .

Preparation of sensitized SRBC (EA)

SRBC were washed twice with EDTA•GVB and suspended in the same buffer to a concentration of 1×10^9 cells/ml. Anti-SRBC tilapia serum was optimally diluted (1 : 25) with EDTA•GVB. Equal volumes of the SRBC suspension and of the antiserum dilution were mixed and incubated at 20°C for 30 min with occasional shaking. The mixture was cooled and centrifuged at 1,600 rpm for 3 min. The precipitate (EA) was washed twice with EDTA•GVB and once with GGVB, and then suspended in GGVB to give a cell density of 5×10^8 /ml. The EA suspension was stored at 4°C after addition of 1/500 volume of 10% NaN_3 and used for experiment within one week. Just prior to use, EA cells were resuspended in fresh GVB^{2+} or GVB^{2+} (T).

Optimum conditions for assaying the classical pathway-complement titer (CH50)

1) reaction temperature

The effect of temperature on the hemolytic reaction was investigated by reducing the scale of Mayer's method (5×10^8 cells/7.5 ml) to one-fifth (1×10^8 cells/1.5 ml) : the

mixture of 0.2 ml of EA suspension (5×10^8 cells/ml), 0.8 ml of GVB²⁺ and 0.5 ml of tilapia serum diluted with GVB²⁺ to give 70 to 80% hemolysis (1 : 300 to 1 : 375) was incubated at different temperatures ranging from 0 to 37°C for 90 min. After addition of 1.5 ml of EDTA•GVB, the mixtures were centrifuged at 3,000 rpm for 5 min. Percent hemolysis at each temperature was calculated from the OD₅₄₁ of the supernatant.

2) pH

The pH of GVB²⁺ was readjusted to 6.5, 7.0, 7.5, 8.0 or 8.5, and the hemolytic reaction (total volume 1.5 ml) was carried out at 25°C for 90 min. After centrifugation, the percent hemolysis at each pH was calculated from the OD₅₄₁ of the supernatant.

3) incubation period

The reaction mixture consisting of 4 ml of EA suspension (5×10^8 cells/ml), 16 ml of GVB²⁺ and 10 ml of the diluted tilapia serum was incubated at 25°C for 140 min. Every 10 min during the incubation, an aliquot (0.75 ml) of the reaction mixture was taken out, mixed with 0.75 ml of EDTA•GVB and centrifuged. Percent hemolysis at each point was calculated from the OD₅₄₁ of the supernatant and plotted on a graph paper to follow up the time-course of hemolysis.

4) concentrations of Ca²⁺ and Mg²⁺

The optimum concentration of Ca²⁺ was determined in the presence of 0.50 mM MgCl₂, whereas that of Mg²⁺ in the presence of 0.15 mM CaCl₂.

In order to remove as much Ca²⁺ and Mg²⁺ as possible from tilapia serum, 0.2 ml of the serum (ca. 300 CH50 units/ml) was added to an Amberlite IRC-50 (2 ml) column, and the column was eluted with 2.5 ml of GVB(f). The first eluate (ca. 0.2 ml) was discarded and the next eluate (2.0 ml) was collected. This eluate was further diluted 1/25 with GVB(f).

Aliquot (0.5 ml) of the diluted eluate and appropriate volumes of 1 M CaCl₂ and 1 M MgCl₂ were added to EA suspension [5×10^8 cells/ml, GVB(f)], then the total volume was adjusted to 1.5 ml with GVB(f). The mixtures were incubated at 25°C for 120 min, and centrifuged to estimate the percent hemolysis.

Assay of the classical pathway-complement titer (CH50)

One-fifth ml of EA suspension (5×10^8 cells/ml) was added to 0.1, 0.2, 0.3, 0.4, or 0.5 ml of tilapia serum diluted 1/300 with GVB²⁺(T), then the total volume was adjusted to 1.5 ml with GVB²⁺(T). Each mixture was incubated at 25°C for 120 min and centrifuged at 3,000 rpm for 5 min. The percent hemolysis was estimated from the OD₅₄₁ of the supernatant and the classical pathway-complement titer (CH50 units/ml) was calculated according to the method of Mayer (1961).

RESULTS AND DISCUSSION

Anti-SRBC tilapia serum

Three tilapia were injected intraperitoneally with 0.4 ml of SRBC stroma suspension 7 times at 5-day intervals, and the antibody production of the fish was followed. As shown in Fig. 1, the titers were at low levels on the 10th day, thereafter they began to increase and reached to 2²-2¹² on the 35th day.

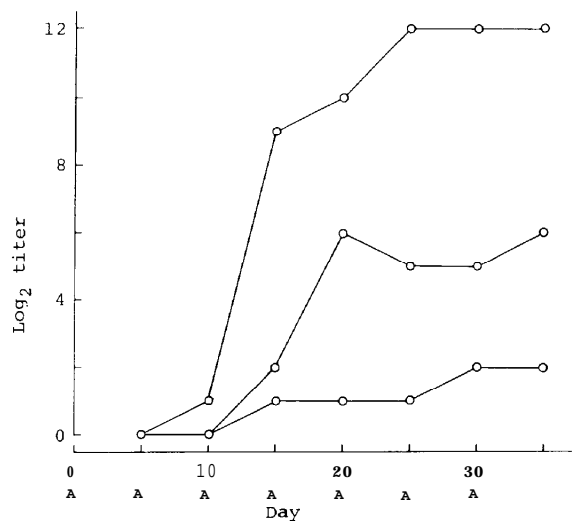


Fig. 1 Changes in the hemagglutination titers of 3 tilapia injected with SRBC stroma suspension (1 mgN/ml). Triangles indicate the days of injection (0.4 ml/fish)

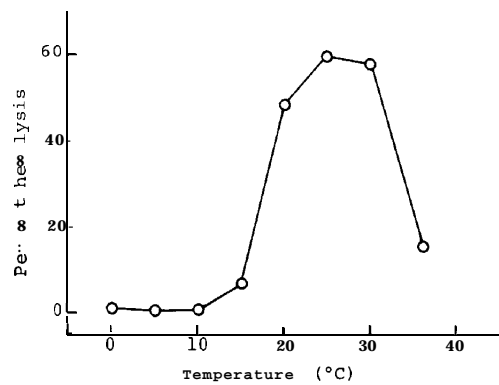


Fig. 2 Effect of incubation temperature on the hemolytic activity of tilapia complement. The reaction was carried out at pH 7.5 for 90 min.

Heat inactivation of the complement in tilapia antiserum

In order to determine the optimum condition for heat inactivation of the complement in anti-SRBC tilapia serum, normal tilapia serum was heated at various temperatures for 20 min, and the residual hemolytic activity was assayed by incubating the heat-treated serum with EA cells in GVB²⁺. Consequently, tilapia complement was completely inactivated by heating at 46°C for 20 min. This inactivation temperature is nearly equal to that for porgy (45°C) and lower than that for carp (50°C).

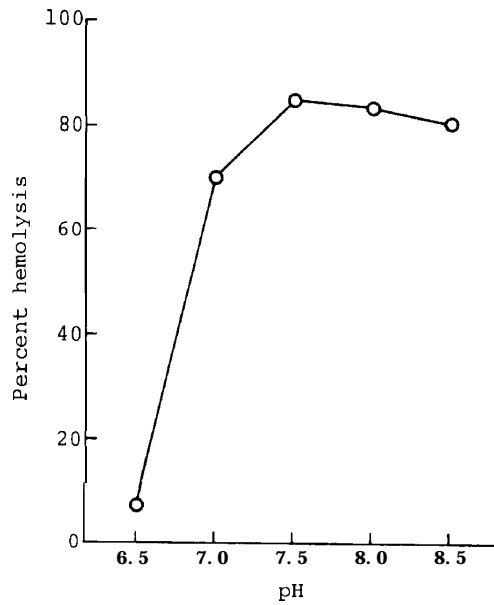


Fig. 3 Effect of pH on the hemolytic activity of tilapia complement. The reaction was carried out at 25°C for 90 min.

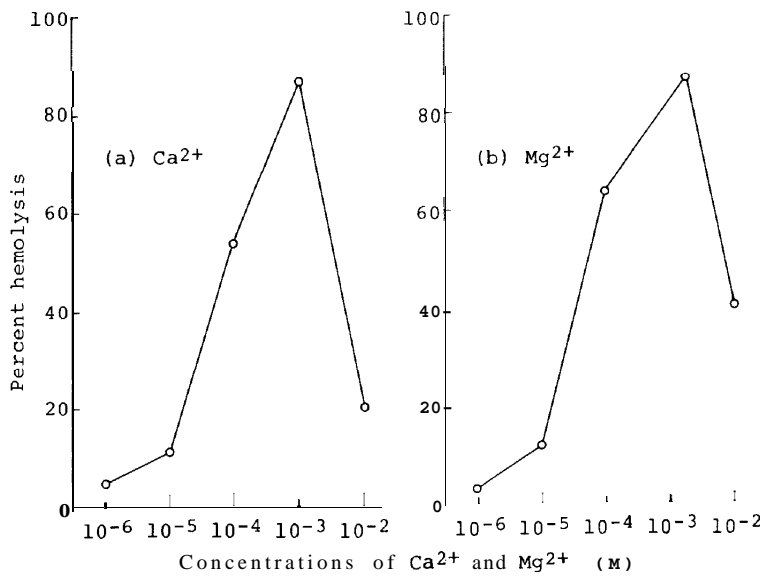


Fig. 4 Effect of concentration of Ca²⁺ or Mg²⁺ on the hemolytic activity of tilapia complement. The reaction was carried out at 25°C for 90 min.

(a) Concentration of Ca²⁺ was altered in the presence of 0.50 mM MgCl₂.

(b) Concentration of Mg²⁺ was altered in the presence of 0.15 mM CaCl₂.

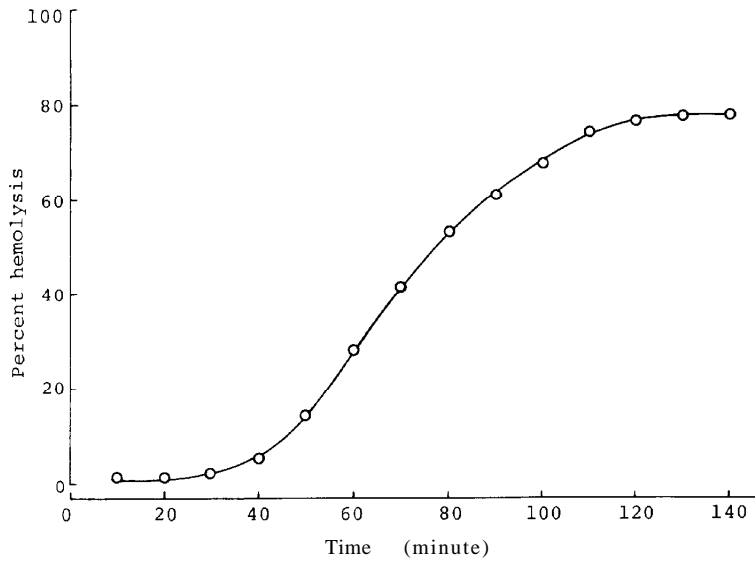


Fig. 5 Time-course of hemolysis in the reaction of tilapia complement with sensitized SRBC. The reaction was performed at 25°C at pH 7.5 in the presence of 1.0 mM MgCl_2 and 1.0 mM MgCl_2 .

Optimum conditions for the assay of the classical pathway-complement titer (CH50)

The total volume of the reaction mixture in Mayer's method was reduced to 1/5, and the incubation was carried out at various temperatures for 90 min. As shown in Fig. 2, the tilapia complement showed high hemolytic activity at temperatures between 20°C and 30°C, and the highest activity was observed at 25°C.

Fig. 3 shows the effect of pH on the hemolytic activity of tilapia complement. The optimum pH lay between pH 7.5 and 8.0, and at pH values below 7.5, the activity dropped rapidly.

Fig. 4 shows the effects of concentrations of Ca^{2+} and Mg^{2+} on the complement activity. The optimum concentrations of Ca^{2+} and Mg^{2+} were 1.0 mM and 1.0 mM, respectively.

Fig. 5 shows the time-course of hemolysis of EA by tilapia complement under the optimum conditions (25°C, pH 7.5, 1.0 mM Ca^{2+} and 1.0 mM Mg^{2+}). The hemolysis took place rather slowly and reached a plateau in about 120 min.

Consequently, the conditions for the hemolytic reaction in Mayer's method (37°C, 60 min, pH 7.4, 0.15 mM Ca^{2+} and 0.50 mM Mg^{2+}) should be modified to 25°C, 120 min, pH 7.5-8.0, 1.0 mM Ca^{2+} and 1.0 mM Mg^{2+} for the titration of CH50 of tilapia serum.

It is noteworthy that the optimum conditions for the titration of CH50 of carp, porgy and tilapia were quite similar except for reaction time which was different among these fishes from 60 to 120 min. Titration of these fishes can be performed under an uniform condition as follows : reaction temperature, 25°C ; pH, 7.5 ; concentrations of Ca^{2+} and Mg^{2+} , 1.0 mM and 1.0 mM, respectively.

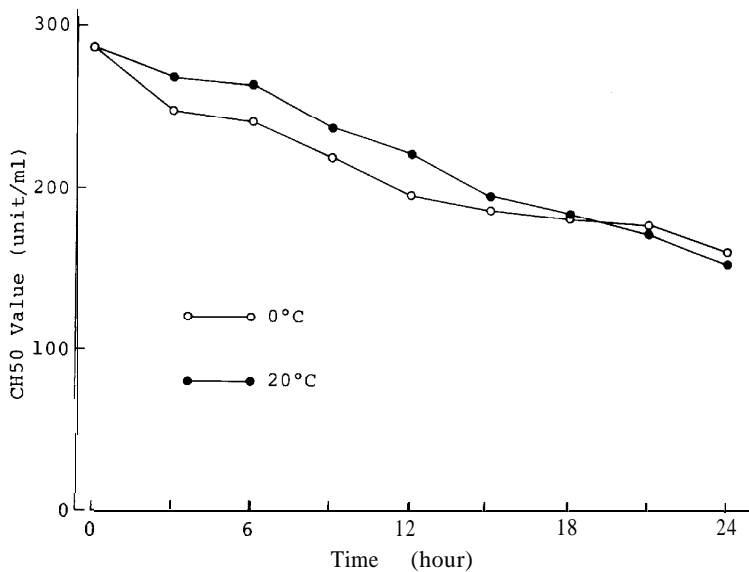


Fig. 6 Stability of tilapia complement. The tilapia serum stored at -80°C was thawed at room temperature, allowed to stand at 0°C or 20°C for 24 h, and the complement titer (CH50) was assayed every 3 hours after thawing.

Assay of classical pathway-complement titer (CH50) of tilapia

The CH50 of 15 tilapia (240 to 960 g), which were obtained from a fish farm in Fukuoka City from September in 1987 to January in 1988, were assayed under the conditions determined above. The titers ranged from 153 to 392 CH50 units/ml, the mean \pm SD being 250 ± 70 CH50 units/ml.

These CH50 values of tilapia were remarkably high as compared with those of other fishes. Until today, the CH50 values of channel catfish (*Ictalurus punctatus*), bigmouth buffalo (*Ictiobus cyprinellus*), longnose gar (*Lepisosteus osseus*), bowfin (*Amia calva*), paddlefish (*Polydon spathula*), lemon shark (*Negaparon brevirostris*), nurse shark (*Ginglymostoma cirratum*), goldfish (*Carrasius auratus*), rainbow trout (*Salmo gairdneri*), carp (*Cyprinus carpio*) and porgy (*Pagrus major*) have been assayed by use of sensitized sheep red blood cells (Legler and Evans, 1967 ; Legler *et al.*, 1967 ; Day *et al.*, 1970 ; Legler *et al.*, 1971; Nonaka *et al.*, 1981 ; Sakai, 1981 ; Yano *et al.*, 1984 ; Matsuyama *et al.*, 1985). The titers of these fishes were in the range of 9.8 to 57 units/ml except those of nurse shark and lemon shark (300-964 units/ml). Direct comparison of these titers is difficult since it is obscure whether these measurement were carried out under optimum conditions, but it is apparent that tilapia showed the highest hemolytic activity among bony fishes (Osteichthyes) ever examined.

Stability of tilapia complement

An experiment was performed to check the stability of tilapia complement. The tilapia serum stored at -80°C was thawed at room temperature and allowed to stand

at 0°C and 20°C for 24 h. The CH50 titer was measured every 3 h after thawing (Fig. 6). There was little difference between the titers of the sera kept at 0°C and 20°C. The titers gradually decreased with time and by the end of the experiment, both sera lost about 50% of their hemolytic activities.

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