Clotting Time of Common Carp Blood

Hiroshi Kawatsu*

(Accepted August 20, 1985)

Recalcification time, prothrombin time (PT), partial thromboplastin time (PTT) and activated partial thromboplastin time (APTT) were determined for common carp plasma. Clotting times were 37.6 ± 8.7 s (mean $\pm$ SD) recalcification time, 23.5 ± 3.4 s PT, 17.9 ± 3.2 s PTT, and 5.1 ± 0.7 s APTT, respectively. The difference between APTT and PTT suggests that the intrinsic hemostatic system is developed in carp blood. Citrated plasma lost its clotting activity within 12 h during incubation at 37°C , whereas clotting activity was maintained for long hours at 25°C . The shortest clotting time was obtained for the mixture of BaSO_4 adsorbed plasma and serum. This indicates that coagulation factors of carp blood can be classified into the serum factors and barium sulphate adsorbed factors.

The blood coagulation mechanism in fishes has been investigated from two standpoints: comparative physiology and fish pathology. Teleost fish blood clots very rapidly, while elasmobranch blood clots very slowly.¹⁻³⁾ The blood of cyclostome lacks intrinsic clotting activity.¹⁾ Clotting time is dependent on the concentration of sodium chloride, of which the optimum concentration is different between marine and freshwater fish.⁴⁾ The effect of temperature on the clotting time is different between warm and cold-water fish. The latter blood clots rapidly at low temperature.⁵⁾

Retardation of the clotting time has been observed in sick fish,⁶⁾ spent fish,⁷⁾ and in fish being exposed to chromium,⁸⁾ dehydroabietic acid,⁹⁾ and molinate.¹⁰⁾ On the other hand, shortening of the clotting time has been reported in fishes exposed to cobalt,¹¹⁾ malathion,¹²⁾ and chlor-dane.¹³⁾ It has been demonstrated that the clotting time declines and thrombocyte count increases after stress.^{14,15)} It seems that many factors may be involved in these alterations of the clotting time. The present paper aims to establish a standard procedure for the determination of clotting time in order to detect the factors associated with disorders in the hemostatic system in common carp blood.

Materials and Methods

Test Animal

Common carp *Cyprinus carpio* was used for

the experiments. Fish were obtained from a private fish farm and kept in the ponds until used.

Preparation of Citrated Plasma, Serum and Adsorbed Plasma

Citrated plasma: Blood was taken from the caudal vessel using plastic syringes. Nine volumes of the blood were withdrawn into a syringe already containing 1 volume of 3.13% trisodium citrate solution. Plasma was obtained by centrifugation at 3,000 rpm for 5 min. Equal volumes of the plasma samples collected from three or four individuals were pooled except that the clotting time was determined individually.

Serum: Clotted blood was left at room temperature for 1 h and was centrifuged at 3,000 rpm for 5 min. Three or four serum samples were pooled and stored at 4°C for 2 or 3 days until used.

Adsorbed plasma with BaSO_4 : Barium sulphate was added to the pooled citrated plasma (200 mg/ml plasma) and stirred with a glass rod for 15 min at 25°C . After centrifugation at 3,000 rpm for 5 min, the supernatant was stored at 4°C .

Adsorbed plasma with bentonite: Bentonite (Kanto Kagaku Co. Ltd., Tokyo) was added to the citrated plasma (50 mg/ml plasma) and stirred with a glass rod for 10 min at 25°C . After centrifugation at 3,000 rpm for 15 min, the supernatant was stored at 4°C .

Stored citrated plasma: Citrated plasma was incubated at 25°C and 37°C for 24 h according to the purpose of the experiment.

* Department of Fisheries, Faculty of Agriculture, Miyazaki University, Kumano, Miyazaki 889-21, Japan (川津浩嗣: 宮崎大学農学部水産増殖学科).

Procedures for Determination of the Clotting Time

Recalcification time: One-tenth ml of citrated plasma was pipetted into a 10×90 mm test tube and placed in a 25°C or 37°C water bath for 3 min. Then, 0.1 ml of M/40 calcium chloride solution being kept at the test temperature was added and a stop-watch was started. After adding calcium chloride solution, the lower end of the test tube was moved every second so that the liquid content of the tube flowed up and down the length of the tube for a distance of 1–2 cm. When the tube could be tilted to a horizontal position without the gel moving, the watch was stopped and the time was noted. Duplicate readings were obtained from each sample and their mean clotting time was recorded.

Prothrombin time (PT): A commercially available complete thromboplastin, "Activated thromboplastin" (International Reagent Corp., Tokyo) was used. A complete thromboplastin-calcium chloride mixture was prepared by mixing equal volumes of "Activated thromboplastin" and M/40 calcium chloride solution. This mixture was kept in a water bath at 37°C prior to use. In this study PT was determined using 4-fold diluted plasma (one part of the citrated plasma was diluted with 3 parts of saline). One-tenth ml of the diluted plasma was pipetted into a 10×90 mm test tube and placed in a 37°C water bath for 3 min. Then, 0.2 ml of complete thromboplastin-calcium chloride mixture was added and a stop-watch was started. The test tube was moved in the same manner as described in the determination of the recalcification time. The first appearance of a fibrin web was taken as the clotting time. Duplicate readings were obtained from each plasma sample and their mean clotting time was recorded.

Partial thromboplastin time (PTT): A commercially available partial thromboplastin reagent, "Platelin" (Warner-Lambert Co., New Jersey, USA) was used. Reconstituted "Platein" solution was kept at 37°C prior to use. One-tenth ml of the citrated plasma was pipetted into a 10×90 mm test tube and placed in a 37°C water bath for 1 min. Then, 0.1 ml of "Platelin" solution was added and the mixture allowed to stand for 2 min. Finally, 0.1 ml of M/40 calcium chloride solution was added and a stop-watch was started. The test tube was moved in the same manner as described in the determination of the recalcification time. The clotting time was taken as the time when the fibrin clot formed. Duplicate readings were obtained from each plasma sample and their mean

clotting time was recorded.

Activated partial thromboplastin time (APTT): A commercially available activated partial thromboplastin reagent, "Actin" (Dade diagnostic, Inc., Aguada, Puerto Rico) was used. This reagent is cephalin extracted from dehydrated rabbit brain in 1.0×10^{-4} M ellagic acid with added buffer, stabilizers and preservative. One-tenth ml of "Actin" solution was pipetted into a 10×90 mm test tube and kept in a 37°C water bath for 1 min. Then, 0.1 ml of the citrated plasma was added and kept for 2 min. Finally, 0.1 ml of M/40 calcium chloride solution was added and a stop-watch was started. The test tube was moved in the same manner as described in the determination of the recalcification time. The clotting time was taken as the time when a fibrin clot formed. Duplicate readings were obtained from each sample and their mean clotting time was recorded.

Results

Recalcification Time

The recalcification times were determined at 25°C and 37°C for each of 10 plasma samples. The mean value was 52.7 s at 25°C and 37.6 s at 37°C, respectively (Table 1). The clotting time obtained at 25°C was prolonged about 15 s longer than that at 37°C.

Changes of Recalcification Time during Incubation at 4°C, 25°C and 37°C

Pooled citrated plasma samples sub-divided into 3 tubes were incubated at 4°C, 25°C and 37°C, and recalcification time was determined for each sample at intervals of 1–12 h (Table 2). The

Table 1. Recalcification time determined at 25°C and 37°C

Sample No.	Body weight (g)	Recalcification time (s)		A-B (s)
		25°C (A)	37°C (B)	
1	210	37.9	27.1	10.8
2	220	43.1	32.8	10.3
3	235	47.1	37.9	9.2
4	195	47.5	28.5	19.0
5	200	48.9	32.8	16.1
6	225	52.3	32.8	19.5
7	210	53.8	38.6	15.2
8	295	57.4	42.7	14.7
9	390	69.0	44.8	24.2
10	265	69.7	58.1	11.6
mean±SD		52.7±9.8	37.6±8.7	15.1±4.6

Table 2. Changes of recalcification time during incubations at 4°C, 25°C and 37°C

Incubation time (h)	37°C	25°C	4°C
0	45.4 s	45.4	45.4
1	57.2	44.4	—
3	86.4	48.0	—
5	144.9	—	—
6	—	50.3	50.9
7	incomplete	—	—
12	—	58.8	62.7
24	—	67.8	63.7

Table 3. Prothrombin time (PT), partial thromboplastin time (PTT) and activated partial thromboplastin time (APTT) of carp plasma

Sample No.	Body weight (g)	PT (s)	PTT (s)	APTT (s)
1	256	17.3	13.4	4.2
2	255	20.7	15.8	4.7
3	263	21.3	16.7	4.7
4	228	21.7	15.6	5.3
5	262	22.7	19.2	4.8
6	272	23.0	16.7	5.4
7	259	25.0	17.9	4.7
8	245	25.7	17.6	5.3
9	304	27.7	21.6	5.3
10	257	29.7	25.3	6.9
mean		23.5	17.9	5.1
±SD		±3.4	±3.2	±0.7

clotting time of the original pooled plasma was 45.4 s. Under incubation at 37°C, the clotting time rapidly increased and exceeded 2 min after 5 h. Fibrin clot formation was incomplete after 7 h and consequently the end point was indistinct. Under incubation at 25°C and 4°C, the clotting time gradually increased and reached 67.8 s and 63.7 s after 24 h, respectively. No significant difference was observed between the clotting times of the plasma incubated at 25°C and 4°C.

Prothrombin Time (PT)

The PT, PTT and APTT were determined for 10 plasma samples (Table 3). Mean, standard deviation, and range of the PT were 23.5 s, 3.4 s and 17.3–29.7 s, respectively.

PT of Graded Dilutions of the Plasma in Saline

A serially diluted plasma was prepared by diluting 1:2, 1:4, 1:8, 1:16, and 1:32, in saline. Each dilution should have 50, 25, 12.5, 6.25 and

Fig. 1. Prothrombin time (PT) of graded dilutions of plasma in saline. **Fig. 2.** Changes of prothrombin time (PT) during incubations at 4°C, 25°C and 37°C.

3.13% activities, respectively. For each dilution, PT was determined. The PT of graded dilutions of the plasma in saline were plotted against the plasma concentration (Fig. 1). No difference was found in PT between the original plasma and 4-fold dilution (25% concentration), but remarkable increase was recorded at more than 8-fold

Table 4. Prothrombin time (PT) of plasma mixtures

	PT (seconds) before mixing	PT (seconds) after mixing with		
		BaSO ₄ adsorbed plasma	Stored serum	Bentonite adsorbed plasma
Citrated plasma incubated at 37°C for 24 h	63*	70	37	63
BaSO ₄ adsorbed plasma	> 360	—	37	143
Stored serum	> 360	37	—	> 360
Bentonite adsorbed plasma	> 360	143	> 360	—

* Prothrombin time of citrated plasma was 28 s before incubation.

dilutions. This fact indicates that decrease of coagulation factor to 25% has no effect on PT prolongation. Although fibrin web diminished in quantity in high dilutions, it was clearly distinguishable up to 32-fold dilution.

Changes of PT During Incubations at 4°C, 25°C and 37°C

Pooled citrated plasma samples sub-divided into 3 tubes were incubated at 4°C, 25°C and 37°C, and PT was determined for each sample at intervals of 1–12 h (Fig. 2). The PT of the original pooled plasma was 24.3 s. Prolongations of PT during 24 h incubation were 12 s at 4°C, 25 s at 25°C, and 253 s at 37°C, respectively. Two phases were distinguished in the prolongations of PT. Under incubation at 37°C, the first rapid increase was observed during the first 3 hours, and the second after 6 h. A similar trend was found in the prolongation of PT during incubation at 25°C. Under incubation at 4°C, only a slow increase was observed. These trends suggest the existence of two or more heat labile coagulation factors in carp plasma.

PT of the Plasma Mixtures

In order to differentiate the coagulation factors, measurements of PT were performed on the mixtures of the following plasma and serum samples: citrated plasma incubated at 37°C for 24 h, adsorbed plasma with BaSO₄, adsorbed plasma with bentonite, and stored serum. To prepare the plasma mixtures, equal volumes of each plasma or serum sample were mixed. Measurements of PT for the original plasma and serum samples were performed using 4-fold dilution with saline. To avoid the effects of dilution of the coagulation factors, 2-fold dilution with saline was used for the determination of PT of the plasma mixtures.

As shown in Table 4, PT of the citrated plasma

incubated at 37°C was shortened by mixing with stored serum, whereas no noticeable change was observed after mixing with each of two adsorbed plasmas. This fact suggests that the heat labile coagulation factors were supplemented only from stored serum. The PT of the adsorbed plasma with BaSO₄ was remarkably shortened by mixing with stored serum. On the other hand, PT of adsorbed plasma with bentonite was not shortened by mixing with serum. From these results it may be considered that the deficient coagulation factors in the adsorbed plasma with BaSO₄ and the stored serum completely supplement each other by mixing. The PT of the adsorbed plasma with BaSO₄ was shortened to a certain extent by mixing with the adsorbed plasma with bentonite.

Partial Thromboplastin Time (PTT)

As shown in Table 3, mean, standard deviation and range of PTT were 17.9 s, 3.2 s and 13.4–25.3 s, respectively. The correlation coefficient between PT and PTT was 0.90.

Changes of PTT during Incubation at 4°C, 25°C and 37°C

Pooled citrated plasma samples sub-divided into 3 tubes were incubated at 4°C, 25°C and 37°C, and PTT was determined for each sample at intervals of 1–12 h (Table 5). The PTT of the original plasma was 14.8 s. Prolongations of PTT during 24 h incubation were 1.6 s at 4°C and 2.4 s at 25°C. Under incubation at 37°C, fibrin clot formation was incomplete after 6 h and no clot was formed after 12 h.

Activated Partial Thromboplastin Time (APTT)

As shown in Table 3, mean, standard deviation and range of APTT were 5.1 s, 0.7 s, and 4.2–6.9 s, respectively. The correlation coefficient was 0.80 between PT and APTT, and was 0.81 between PTT and APTT. Since clot formation was very

Table 5. Changes of PTT during incubations at 4°C, 25°C and 37°C

Incubation time (h)	37°C	25°C	4°C
0	14.8 s	14.8	14.8
1	17.1	14.8	—
2	18.0	14.3	—
3	19.5	14.4	—
6	26.8	15.7	—
12	not clot	16.0	14.8
24		17.2	16.4

Table 6. Changes of APTT during incubations at 4°C, 25°C and 37°C

Incubation time (h)	37°C	25°C	4°C
0	4.8 s	4.8	4.8
1	5.1	4.8	—
2	5.4	5.2	—
3	5.4	5.3	—
6	5.8	5.2	—
12	not clot	5.3	5.0
24		5.1	5.1

fast, skill seems to have a large influence upon errors in APTT measurement.

Changes of APTT during Incubation at 4°C, 25°C and 37°C

Pooled citrated plasma samples sub-divided into 3 tubes were incubated at 4°C, 25°C and 37°C, and APTT was determined for each sample at intervals of 1–12 h (Table 6). The APTT of the original plasma was 4.8 s. Prolongations of APTT during 24 h incubation were 0.4 s at both 4°C and 25°C. Under incubation at 37°C, fibrin clot formation was incomplete after 12 h.

Discussions

The citrated plasma incubated at 37°C lost its clotting activity within 12 h. On the other hand, plasma incubated at 4°C and 25°C maintained their clotting activity for more than 24 h, and no remarkable differences were found between their clotting times. This indicates that the heat labile factors are quickly destroyed at 37°C while they can remain for many hours at 25°C. In this study, 37°C was adopted as the test temperature, because it was necessary to use commercially available reagents prepared from rabbit brain in order to obtain reproducible results. Fujikata and Ikeda¹⁰ reported that the shortest clotting time was obtained at 34°C.

It is noteworthy that APTT was very short

compared with PTT. The reagent for APTT determination contains ellagic acid, which activates factor XII at a concentration of 10^{-8} M in mammalian plasma.¹⁷⁾ The present result suggests that the XII-like factor exists and the intrinsic coagulation mechanism is highly developed in carp blood.

The deficient coagulation factors in adsorbed plasma with BaSO₄ and stored serum completely supplemented each other by mixing. Although the properties and characteristics of the coagulant factors have not been fully investigated in fishes, coagulant factors in carp blood can be classified into two categories: the serum factors and the barium sulphate adsorbed factors.

This work was supported by Grant-in-Aid for Scientific Research (57560191) from the Ministry of Education.

References

- 1) R. F. Doolittle and D. M. Surgenor: *Am. J. Physiol.*, **203**, 964–970 (1962).
- 2) R. F. Doolittle: *Brit. J. Haemat.*, **9**, 464–470 (1963).
- 3) J. H. Lewis: *Comp. Biochem. Physiol. A.*, **42**, 233–240 (1972).
- 4) R. Holst: *J. Physiol. (Lond.)*, **247**, 32–33 (1975).
- 5) R. E. Feeney, J. Vandenhede, and D. T. Osuga: *Naturwissensch.*, **59**, 22–29 (1972).
- 6) C. Hougie: *Adv. Exp. Med. Biol.*, **22**, 89–102 (1971).
- 7) M. Katz and M. Southward: *Copeia*, **1950**, 150 (1950).
- 8) A. K. Srivastava, S. J. Agrawal, and H. S. Chaudhry: *Ecotoxicol. Environ. Safety*, **3**, 321–324 (1979).
- 9) G. K. Iwama, G. K. Greer, and P. A. Larkin: *J. Fish. Res. Bd. Canada*, **33**, 285–289 (1976).
- 10) T. Ochiai and S. Kubota: *Bull. Fac. Fish. Mie Univ.*, **5**, 129–153 (1978).
- 11) A. K. Srivastava and S. J. Agrawal: *Acta Pharmacol. Toxicol. (Copenh.)*, **44**, 197–199 (1979).
- 12) J. Mishra and A. K. Srivastava: *Environ. Res.*, **30**, 393–398 (1983).
- 13) S. Verma, S. Bansal, A. Gupta, and R. Daiela: *Bull. Environ. Contam. Toxicol.*, **22**, 467–474 (1979).
- 14) E. Casillas and L. S. Smith: *J. Fish Biol.*, **10**, 481–491 (1977).
- 15) A. Fujikata and Y. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, **51**, 1093–1096 (1985).
- 16) A. Fujikata and Y. Ikeda: *Bull. Japan. Soc. Sci. Fish.*, **51**, 933–939 (1985).
- 17) O. D. Ratnoff: in "Progress in hematology" (ed. by C. V. Moore and E. Brown), Vol. 5, Grune & Stratton, New York and London, 1966, pp. 204–244.