種々の環境で飼育したコイ(Cyprinus carpio)の殺菌活性

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Serum Bactericidal Activity of Carp (*Cyprinus carpio*) under Supposed Stressful Rearing Conditions

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ABSTRACT. The serum bactericidal activity of cultured carp was examined under different supposed stressful rearing conditions. A cell suspension of *Escherichia coli* was incubated with untreated, EGTA- and EDTA-treated sera at 25°C for 3hr, and serum bactericidal activity was determined as the log killing of bacteria. Similar bactericidal activities of EGTA-chelated sera and untreated sera indicated the predominance of the alternative complement pathway, while the incomplete inactivation by EDTA chelation suggested the activity of lysozyme and/or other bactericidal substances. Generally, all the stressful conditions, such as starvation, low dissolved oxygen and high salinity, induced a decrease in the complement and lysozyme activity. When carp were subjected to transportation stress, the lysozyme activity increased. These results suggest that the effect of the short period stress on the self-defense system of carp significantly differs from that of the long period stress.

Key words: Complement, Live transportation, Lysozyme, Serum bactericidal activity, Stressful conditions

Since the evaluation of the fish defense system is not yet determined, we can not predict sudden mass mortalities of fish (fish kill) and thus, we are helpless to prevent it. But if we are familiar with the general state of health of fish, for example an increase of stressed fish, we can take the necessary steps to prevent further possible deterioration. Therefore it is important to know the effect of stressful conditions on fish, in order to understand their health condition. When fish are invaded by pathogens or foreign particles, they are able, as well as mammals, to resist the invasion first by non-specific means and then by specific immune responses [1]. They are also known to be more dependent upon temperature and other external environmental factors [2, 3]. The non-specific response of the fish includes the complement alternative pathway, lysozyme, mucus, chitinase and interferons [1, 2]. Different methods have been used to measure the activity of each agent in the self-defense mechanism of fish. Fish health was assessed by using the non-specific haemolytic activity, SH_{50} and ACH_{50} [4–6]. The activity of lysozyme was reported in several fishes [7–10]. In this study, using a bacterial cell *Escherichia coli*, the bactericidal activity of carp serum was examined through the alternative complement pathway and other bactericidal substances when fish were subjected to supposed stressful conditions. These conditions were: feeding rate, dissolved oxygen (DO) and salinity. The effect of transportation stress on carp, was also examined.

Materials and Methods

Carp, weighing from 680 to 1664 g in body weight, were purchased from a commercial supplier and maintained in 500 l tanks equipped with recirculating water systems for more than one month. The stocking density was 6 fish per tank, and fish were fed a pelleted feed (Nihon Haigo Shiryo, Tokyo) at about 0.25% body weight per day.

In the feeding experiment, four different amounts of the feed ranging from 0 to 1% body weight per day were given in 4 times a day during the treatment. In the DO experiment, three different concentrations of DO, were set; 2.8, 4.4, and 6.2 ppm while in the salinity experiment,

three different concentrations of Herbt's artificial seawater ranging from 0 to 25% of seawater were adjusted [11]. The salinity experiment was conducted twice. After anesthetization with MS-222, a blood sample was taken by caudal puncture before and after the treatment. The experimental period lasted two weeks for all the treatments.

E. coli IAM 1264 (=ATCC 10798) was used in this study. A stock culture was maintained at −85°C and 4°C, on the slant of Trypticase soy agar [TSA] (BBL). The strain was incubated on Trypticase soy agar plates for 48 hr at 25°C. Then it was suspended in phosphate buffer saline (PBS), pH 7.5, at a concentration of 1×10^6 cells /ml. To examine the roles of complement and other substances in the serum bactericidal activity against *E. coli*, the following sera treatments were carried out: (i) untreated sera; (ii) sera chelated with 8 mM ethylene glycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) in PBS containing 2 mM magnesium chloride to inactivate the classical complement pathway while preserving the alternate pathway function (EGTA sera); and (iii) sera chelated with 7 mM EDTA in PBS to inactivate both the classical and the alternate complement pathways (EDTA sera) [14].

In the experimental incubations, 0.1 ml of the range of sera and 0.1 ml of bacterial suspension was added to 0.8 ml of PBS, while in the control incubations, 0.1 ml of PBS was added to the medium, instead of serum. Incubations were performed for 3 hr at 25°C. After a serial 10-fold dilution in PBS of the samples and a duplication on TSA plates (at 37°C for 2 days), surviving bacteria were counted. The bactericidal activity was calculated as the $\log_{10}$ CFU in the control minus the $\log_{10}$ CFU with serum [14].

In addition, some carp weighing from 680 to 1530 g were supplied from an aquaculture farm in Annaka, Gumma. These carp were placed in plastic bags into which oxygen was pumped, and were transported by car for 4 hr to Setagaya, Tokyo (about 120 km). Some fish were bled before and after the transportation.

Results and Discussion

In the feeding experiment (Table 1) when the fish were starved for 2 weeks, even though there was a great variation between individual fish (S.D. 0.68 and 0.31), the bactericidal activity of the untreated sera decreased from 3.47 to 2.90. In contrast, when the fish were fed, the bactericidal activity of the untreated sera slightly increased, irrespective of the feeding rate. The EGTA-chelated sera followed the same trend as the untreated sera, i.e. a slight decrease after starvation. The EDTA-sera showed a low activity with a trend to decrease except for the 0.25% feeding rate.

Table 1 Bactericidal activity of carp sera against *E. coli* in the feeding experiment

Feeding rate (%)	Before the experiment			After the experiment		
	Untreated sera	EGTA sera	EDTA sera	Untreated sera	EGTA sera	EDTA sera
0	3.47±0.68[a]	2.90±0.39	3.39±0.73	2.90±0.31	2.64±0.22	0.17±0.26
0.25	2.91±0.95	2.35±0.49	0.86±0.49	3.26±0.68	2.38±1.69	1.02±0.92
0.50	3.03±0.93	2.42±0.90	1.08±0.33	3.14±0.75	2.28±0.83	4.41±0.15[b]
0	3.37±0.87	3.87±1.05	1.06±0.38	3.50±0.87	3.03±0.87	0.75±0.58

[a] Mean bactericidal activity±standard deviation (n=6).
[b] Significant difference (t test at $p<0.01$) between the values before and after the experiment.

Table 2 Bactericidal activity of carp sera against *E. coli* in the DO treatment

DO (ppm)	Before the experiment			After the experiment		
	Untreated sera	EGTA sera	EDTA sera	Untreated sera	EGTA sera	EDTA sera
2.8	2.54±0.66[a]	1.51±0.95	0.30±0.40	2.18±0.77	2.18±0.75	0.00±0.05
4.4	2.58±0.64	2.17±0.66	0.71±0.59	2.22±0.27	1.95±0.65	0.54±0.42
6.2	2.95±0.76	2.62±0.49	1.07±0.48	3.61±0.55	2.74±0.44	0.38±0.30[b]

[a] Mean bactericidal activity±standard deviation (n=6).
[b] Significant difference (t test at $p<0.10$) between the values before and after the experiment.

At low levels of DO (2.8 and 4.4 ppm), the bactericidal activity of the untreated sera (Table 2) slightly decreased while at a higher DO level (6.2 ppm), it showed a small increase. In all DO levels, the EGTA-chelated sera and the untreated sera exhibited similar values while the low activities of EDTA sera showed a trend to decrease.

In the salinity experiment (Table 3), the bactericidal activity of the untreated sera decreased slightly at the 25% seawater concentration while in the other concentrations, it generally increased slightly. As in the feeding and DO experiments, the EGTA-chelated sera followed the same trend as the untreated sera. Since the EGTA sera inactivates only the classical complement pathway, the indications are that the bactericidal activity of carp is mainly attributable to the alternative complement pathway, as reported in previous reports [4, 6, 12, 13]. In all treatments, the EDTA-treated sera showed a low activity with a general trend to decrease. Since the EDTA chelation generally inactivates both the classical and alternate complement pathways, this activity may be due to complement-independent factors such as lysozyme and/or other bactericidal substances present in the serum. Lysozyme activity have been reported in the serum, mucus and different organs of several fishes as well as its slight inhibition by EDTA [8–10]. These results show that under supposed stressful conditions such as starvation, low DO and 25% seawater concentration, the alternate complement pathway and the complement-independent substances including lysozyme, showed a general trend to decrease, suggesting a deterioration of the carp defense system, although a great individual-to-individual variation was observed.

In the transportation experiment (Table 4), the highest bactericidal activity of the untreated sera was observed in the specimens sampled in summer, before transportation, (August: 3.01), while in the other seasons, the bactericidal activity was stable of about 2.49. In summer and winter, the bactericidal activity of the untreated sera decreased significantly after transportation, while in spring it stayed about the same and in autumn it increased. However, the activity of EDTA sera increased significantly after transportation, in all seasons. The inconsistent variation of untreated sera may be related to the physiological condition

Table 3 Bactericidal activity of carp sera against *E. coli* in the salinity experiment

Seawater (‰)	Before the experiment			After the experiment		
	Untreated sera	EGTA sera	EDTA sera	Untreated sera	EGTA sera	EDTA sera
0	3.45±1.09[a]	2.95±0.72	0.54±0.58	3.61±0.68	3.70±0.88	0.54±0.45
10	3.23±1.12	2.86±1.58	1.15±1.24	3.46±0.83	2.87±1.48	1.01±1.15
25	4.70±0.43	3.92±0.87	1.79±0.96	4031±0.49	3.94±0.65	1.53±0.88
0	3.62±0.95	2.79±0.80	0.39±0.34	3.23±0.85	2.28±0.43	0.26±0.11
10	2.95±0.68	2.51±0.48	0.70±0.26	3.27±0.80	2.61±1.04	0.33±0.15[b]
25	3.33±0.72	2.92±0.72	0.60±0.43	2.97±0.62	2.27±0.60	0.25±0.26

[a] Mean bactericidal activity±standard deviation (n=6).
[b] Significant difference (t test at $p<0.01$) between the values before and after the experiment.

Table 4 Bactericidal activity of carp against *E. coli* in the transportation experiment

Month	Water temp. (°C)	Before the experiment			After the experiment		
		Untreated sera	EGTA sera	EDTA sera	Untreated sera	EGTA sera	EDTA sera
May	21.5	2.47±0.63[a]	2.46±0.10	0.08±0.12	2.46±0.33	2.24±0.23	0.51±0.32[b]
Aug.	20.5	3.61±0.12	1.99±0.20	0.16±0.10	2.19±0.51[c]	1.96±0.73	0.58±0.37[b]
Nov.	10.8	2.55±9.17	1.64±0.53	0.05±0.04	3.10±0.12[d]	2.72±0.56[c]	0.57±0.32[c]
Feb.	4.5	2.44±0.30	2.04±0.16	0.04±0.08	2.01±0.28[d]	2.20±0.32	0.24±0.11[c]

[a] Mean bactericidal activity±standard deviation (n=5).
[b] Significant difference (t test at $p<0.10$) between the values before and after the experiment.
[c] Significant difference (t test at $p<0.05$) between the values before and after the experiment.
[d] Significant difference (t test at $p<0.01$) between the values before and after the experiment.

of the fish. Seasonal and sexual variation in the lysozyme level of plaice serum was reported [14] as well as a seasonal variation of the serum bactericidal activity in rainbow trout [15]. In this case also, the EGTA values followed the same trend as the untreated sera.

The establishment of an infection in a fish can be caused not only by the introduction of pathogens, which are frequently present in the environment, but also by the state of the fish's health, which may be closely related to its rearing condition [1]. In the present study, under supposed stressful conditions, the bactericidal activity of the untreated sera generally decreased even though there was a large standard deviation. However, when the stress was over a short period of time, the activities of lysozyme and other bactericidal substances were stimulated, as opposed to longer periods of time when they were inhibited. These results suggest that the effect of the short period stress, such as transportation, in the self-defense system of carp significantly differs from that of the long period stress. The great individual variation of serum bactericidal activity observed within the carp used in the former three experiments, may be attributed to the stressful conditions in the tanks because the carp in the culture pond, had bactericidal activities which were distributed closely to each other. Further studies along these lines are in progress.

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種々の環境で飼育したコイ (*Cyprinus carpio*) の殺菌活性: Nouama HAJJI・杉田治男・石井　秀・出口吉昭

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コイを種々の環境で2週間飼育し，血清の殺菌活性の変化を調べた。未処理血清と EGTA 処理血清との殺菌活性の類似はコイの殺菌活性の大部分が補体代替経路によることを示唆した。また EDTA 処理血清において若干の活性が認められ，これはリゾチームまたは類似の殺菌物質によるものであると考えられた。無給餌，低溶存酸素または高塩分濃度条件でコイを飼育すると，一般に補体代替経路とリゾチームの活性が減少した。輸送によるストレスではリゾチーム活性が増加した。これらの結果から，短期間と長期間のストレスではコイに及ぼす影響が著しく異なることが示唆された。