コイの体表粘液及び血清中の凝集素の物理化学的性状

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Physicochemical Properties of Agglutinin in Mucus and Serum of Carp

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ABSTRACT. Physicochemical properties of the naturally ocurring agglutinin in skin mucus and serum of reared carp were examined against bacterial cells of *Escherichia coli* IAM 1264 and *Pseudomonas* sp. 12-7. The agglutinin activity of mucus was stable between 30-55°C. From 60 to 90°C, the activity decreased and at 100°C, it was completely inactivated. The agglutinin activity of mucus was stable at a pH range of 5-12. Outside this range, at low pH of 2-4 and high pH of 13, the activity disappeared. In the case of serum, the agglutinin activity was stable between 30-45°C. From 50-65°C, it gradually decreased until it was completely inactivated at 70°C. The agglutinin activity of serum was stable at a pH range of 7-10. Outside this range, at pH 5-6 and 11, it decreased while it was inactivated at low pH of 2-4 and high pH of 12. Many monosaccharides, oligosaccharides and their derivatives were tested as inhibiting agents of agglutination in mucus and serum using the same bacteria. In skin mucus, agglutinin activity was partially inhibited by D-glucosamine. In serum, there was no significant difference in the agglutinin activity for most sugars, except for N-acetyl-D-glucosamine which partially inhibited it, although an individual variation among carp existed. Bactericidal activity in the mucus, tested for thirty bacterial strains was generally undetectable.

Key words: Carp, Lectin, Natural agglutinin, Serum, Skin mucus.

Due to their possible role in immune responses, a great deal has been written on natural antibodies [1,2]. The surface of fish which is always exposed directly to various pathogens in the water, is expected to have a self-defense mechanism. Isolation, purification and characterization of glycoproteins from mucus and serum of several species of fish have been determined [3-7]. Lectins or agglutinins are proteins or glycoproteins which agglutinate erythrocytes or other types of cells by combining with the carbohydrate moeity of cell membrane. Lectins were first discovered in plant seed but now their presence has been discovered in very diverse source including bacteria, fungi, lichens, invertebrates, fish and even mammals [8]. Nevertheless, lectins from fish have not been studied as extensively as have those from plants or invertebrates. Additionally, as we are aware, the activity range of temperature and pH of naturally ocurring agglutinin in skin mucus and serum of fish has not been established yet. Therefore, to detect the presence of lectins in serum and mucus of carp (*Cyprinus carpio*), an inhibition assay by sugars was attemped using the bacterial agglutination. The physicochemical properties of natural agglutinin were also examined.

Materials and Methods

1. Fish sera and mucus

Carp, weighing between 437 and 1855g, were reared in 500*l* tanks equipped with recirculating water systems for several months. After anesthetization with MS-222, carp were rinsed with distilled water. Then mucus was scraped off the body flanks of the fish, pooled and stored at − 85°C until use. For the mucus bactericidal test, mucus was additionally freeze-dried and finely ground to a powder. Then, mucus was brought to its original water content by sterile water and treated by a sonicator (Ohtake Works) at 50W for 15 mn. For the preparation

of a bacterium-free mucus, it was mixed with a same quantity of PBS, centrifuged at 3000rpm for 10 mn and filtered through a 0.22 μm membrane filter (Millipore).

Animals were also aseptically bled by caudal puncture and serum was obtained from each fish specimen, by allowing the blood to clot, first for a few minutes at room temperature, and then for 1 hr at 4°C, followed by centrifugation at 3000rpm for 10 min. The supernatant serum thus prepared was also stored at −85°C.

2. Bacterial cells

Escherichia coli IAM 1264 (= ATCC 10798) and *Pseudomonas* sp. I2-7 which was isolated from the intestine of carp [9], were used in the bacterial agglutination tests. Stock cultures were maintained at both −85°C and 4°C on the slants of Trypticase soy agar [TSA] (BBL). Each strain was incubated in Trypticase soy broth (BBL) for 48 hr at 25°C. Formalin was added to make a 0.5% concentration in the broth, and allowed to stand, first for 6 hr at 37°C, and then overnight at 4°C. After being harvested, the cells were washed three times with PBS (pH 7.5), containing 0.12% Na_2HPO_4, 0.07% KH_2PO_4 and 0.68% NaCl. Bacterial cells were refrigerated and used within 2 weeks after the preparation.

E. coli and thirty bacterial strains including *Pseudomonas* sp., which were isolated from a natural environment [9], were tested for bactericidal activity of the carp mucus. Stock cultures were maintained similarly as described earlier and each strain was incubated on TSA plates for 48 hr. Three different concentrations of bacterial cells ranging from 10^3-10^5 cells/ml were prepared in PBS.

3. Bacterial agglutination and bactericidal activity test

Serum and mucus were analysed for agglutination against *E. coli* or *Pseudomonas* sp. by using microtiter "V" plates. Serial twofold dilutions of either serum or mucus were made in physiological saline (0.85% NaCl). Bacterial cell suspensions, prepared at concentrations of 2×10^9 cells/ml for *E. coli* and 5×10^8 for *Pseudomonas* sp., were added to serum or mucus dilutions (0.025ml of serum or mucus plus 0.025ml of bacterial suspension). The microplate was gently agitated, covered, and incubated for 2 hr at 25°C, and then overnight at 4°C. Results were recorded using the highest dilution, which showed evidence of agglutination. Each agglutination test was run in triplicate and all agglutination experiments were done twice or more to confirm the result.

For the mucus bactericidal test, 0.9ml of the treated mucus was mixed to 0.1ml of bacterial suspension while the control was prepared with PBS instead of mucus. After incubation at 25°C for 3 hr, serial 10-fold dilutions of the experimental incubations were made with PBS and duplicated onto TSA plates. The plates were incubated aerobically for 2 days at 37°C and colony forming unit (CFU) per ml was determined. Bactericidal activity was calculated as the $\log_{10}$ CFU in the control minus the $\log_{10}$ CFU with mucus [10].

4. Effect of temperature and pH on agglutination

To determine the effect of temperature on the agglutinin activity, mucus and sera were heated for 30 min in water baths at temperatures ranging from 30 to 100°C. For the determination of pH stability, mucus and sera were adjusted to pH values ranging from 2 to 13 with HCl and NaOH. After each treatment, samples were rapidly cooled in an ice bath and the determination of the agglutination titer started within 2 hr. Only *Pseudomonas* sp. cells were used in these experiments. For the temperature and pH experiment, individual sera were pooled, as for mucus, and clots formed after the treatments were removed by centrifugation at 3000rmp for 10mn.

5. Saccharide-inhibition test

The following sugars were tested: D-mannose, D-glucose, D-glucosamine, N-acetyl-D-glucosamine, D-galactose, D-xylose, L-arabinose, sucrose, D-cellobiose, D-trehalose, D-sorbitol, L-sorbose, maltose, lactose, melibiose, D-ribose, D-fucose, D-fructose and L-rhamnose. D-glucose and melibiose were purchased from Merck, L-arabinose, sucrose, maltose, lactose, L-rhamnose and sorbose from Wako and the other sugars from Sigma. Each sugar, at a concentration of 10% (w/v), was mixed in two different ways. First, it was mixed with physiological saline, and then with the bacterial suspension to check a possible agglutination of sugar molecules to the bacterial cells. Besides the addition of sugar, the bacterial agglutination test was conducted as described above (3). Both *Pseudomonas* sp. and *E. coli* cells were used in this experiment. Pooled mucus and individual serum were used.

Results and Discussion

1. Effect of temperature and pH on the mucus and serum agglutinin activity

Fig. 1 shows the effect of heating for 30 min on the

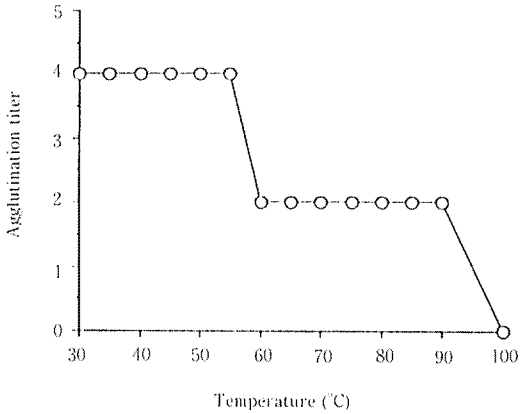

Fig 1 Stability of carp mucus agglutinin to temperature when heated for 30 min against *Pseudomonas* sp.12-7.

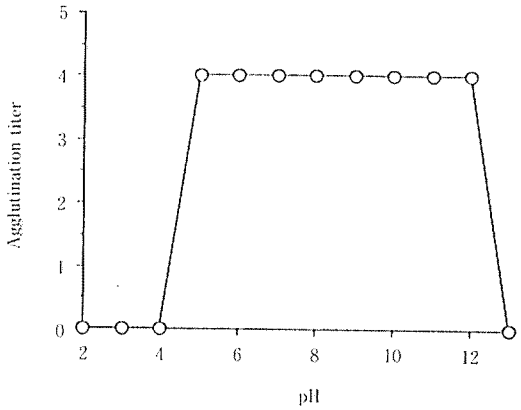

Fig 2 Stability of carp mucus agglutinin to pH against *Pseudomonas* sp. 12-7.

agglutinin activity of mucus. The activity was stable at temperatures ranging from 30 to 55°C. Beyond 55°C, the activity decreased and at 100°C, the activity was completely inactivated. The agglutinin activity of mucus was found to be stable over a wide pH range of 5-12 (Fig.2). The activity disappeared at low pH of 2-4 and at high pH of 13. The physiological pH of mucus was found to be slightly acidic, between 6.8 and 7.0. The low agglutination titer for mucus is not surprising since usually natural antibodies are found in extremely small quantities [1].

The agglutinin activity of serum was stable at a smaller temperature range, from 30 to 45°C, than in the mucus (Fig. 3). Then, it gradually decreased until 65°C, to become completely inactivated at 70°C. The agglutinin activity of serum was found to be stable over a smaller pH range of 7-10, than in the mucus (Fig. 4). The activity decreased at pH of 5-6 and 11, while at low pH of 2-4 and high pH of 12, it was completely inactivated. The physiological pH of carp serum was generally between 7.4 and 7.6.

These results suggest that the agglutinin of serum is less stable than that of the mucus, regarding temperature and pH, however the titer in serum was much higher than in the mucus.

2. Inhibition of mucus agglutinin by saccharides

All the sugars used did not inactivate the agglutinin activity of mucus except for D-glucosamine. The inhibition was complete when the sugar was mixed with the

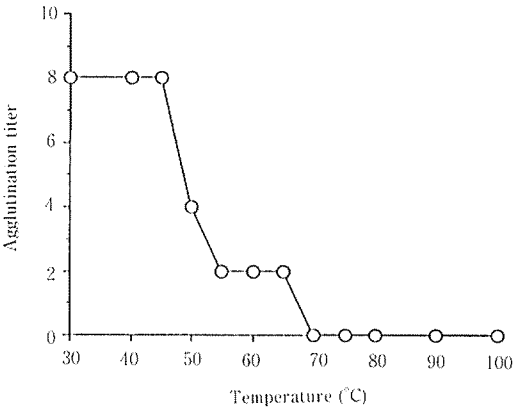

Fig 3 Stability of carp serum agglutinin to temperature when heated for 30 min against *Pseudomonas* sp. 12-7.

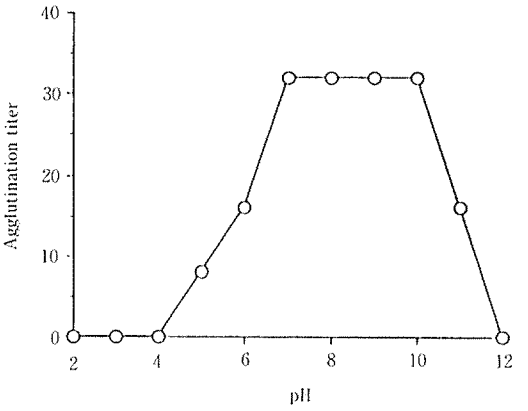

Fig 4 Stability of carp serum agglutinin to pH against *Pseudomonas* sp. 12-7.

Table 1 Effect of various sugars on the mucus agglutinin activity against *Pseudomonas* sp. 12-7.

Sugars	Untreated mucus	PS[a]	BS[b]
D-Mannose	4[c]	4	4
D-Glucose	4	4	4
D-glucosamine	4	2	<2
N-Acetyl-D-gluc.	4	4	4
D-Galactose	4	4	4
D-Xylose	4	4	4
L-Arabinose	8	8	4
Sucrose	4	4	4
D-Cellobiose	4	4	4
D-Trehalose	4	4	4
D-Sorbitol	4	4	4
L-Sorbose	4	8	4
Maltose	4	4	4
Lactose	4	4	4
Melibiose	2	4	4
D-Ribose	8	4	4
D-Fucose	4	4	4
D-Fructose	4	4	4
L-Rhamnose	4	4	4

[a] Sugar was added to the physiological saline.
[b] Sugar was added to the bacterial suspension.
[c] Average agglutinin titer of pooled mucus.

bacterial cells of *Pseudomonas* sp., and incomplete when it was mixed with physiological saline (Table 1).

The bactericidal activites of most strains tested, were undetectable at the three different bacterial concentrations assayed, while few strains showed weak activities, lower than 0.4.

3. Inhibition of serum agglutinin by saccharides

Of the sugars tested, none inhibited serum agglutinin activity completely (Table 2, 3). However, N-acetyl-D-glucosamine lowered the agglutinin titer against both *Pseudomonas* sp. 12-7 and *E. coli* IAM 1264, in most fish tested. Generally, *E. coli* and *Pseudomonas* sp. gave the same agglutination titer, however, autoagglutination was observed in the case of D-glucosamine with *E. coli* cells. Nine sugars, including D-mannose, D-glucosamine, D-xylose, L-arabinose, D-cellobiose, L-sorbose, D-ribose, maltose, and D-galactose, lowered the agglutinin titer only in some indivisuals, for both bacterial strains at the exception of L-arabinose. Such a difference among individuals may be the reflection of an individual variation in lectin composition and concentration [3]. Generally, serum showed a higher agglutinin titer than skin mucus: the highest titer being 64. Sometimes, the agglutination

Table 2 Effect of various sugars on the serum agglutinin activity against *Pseudomonas* sp. 12-7.

Sugars	Untreated sera	PS[a]	BS[b]	n[d]
D-Mannose	26.7± 9.2[c]	21.3± 9.2	21.3± 9.2	3
D-Glucose	18.7± 6.5	18.7± 6.5	17.3± 7.9	6
D-glucosamine	28.4±15.6	25.8±16.9	25.3±18.8	9
N-Acetyl-D-gluc.	24.0± 9.2	12.0± 4.6	12.0± 4.6	4
D-Xylose	26.7± 8.0	24.9± 8.4	26.7± 8.0	9
L-Arabinose	6.7± 2.3	5.3± 2.3	5.3± 2.3	3
Sucrose	16.0± 0.0	16.0± 0.0	16.0± 0.0	2
D-Cellobiose	25.6± 8.8	22.4± 8.8	25.6± 8.8	5
D-Trehalose	21.3± 8.3	21.3± 8.3	21.3± 8.3	6
D-Sorbitol	25.6± 8.8	25.6± 8.8	22.4± 8.8	5
L-Sorbose	22.4± 8.8	22.4± 8.8	22.4± 8.8	5
Maltose	26.7± 9.2	21.3± 9.2	21.3± 9.2	3
Lactose	22.9± 8.6	22.9± 8.6	22.9± 8.6	7
Melibiose	24.0± 9.2	24.0± 9.2	20.0± 8.0	4
D-Ribose	6.7± 2.3	4.0± 0.0	4.0± 0.0	3
D-Fucose	5.3± 2.3	5.3± 2.3	5.3± 2.3	3
D-Fructose	16.0± 0.0	16.0± 0.0	16.0± 0.0	2
L-Rhamnose	22.9± 8.6	22.9± 8.6	20.6± 7.8	7

[a] Sugar was added to the physiological saline.
[b] Sugar was added to the bacterial suspension.
[c] Mean agglutination titer ± standard deviation.
[d] Number of fish examined

Table 3 Effect of various sugars on the serum agglutinin activity against *E. coli*.

Sugars	Untreated sera	PS[a]	BS[b]	n[d]
D-Mannose	48.0±22.6[c]	32.0± 0.0	32.0± 0.0	2
D-glucosamine	36.0±20.1	−[e]	−	4
N-Acetyl-D-gluc.	36.0±20.1	20.0± 8.0	14.0±12.0	4
D-Galactose	36.0±20.1	28.0± 8.0	36.0±20.1	4
D-Xylose	48.0±22.6	32.0± 0.0	32.0± 0.0	2
L-Arabinose	12.0± 5.7	12.0± 5.7	12.0± 5.7	2
L-Sorbose	48.0±22.6	32.0± 0.0	48.0±22.6	2
Lactose	32.0	32.0	32.0	1
D-Ribose	12.0± 5.7	8.0± 0.0	12.0± 5.7	2

[a] Sugar was added to the physiological saline.
[b] Sugar was added to the bacterial suspension.
[c] Mean agglutination titer±standard deviation.
[d] Number of fish examined.
[e] not detected because of autoagglutination.

titer varied among individuals, between 16-64, like in the case of D-glucosamine which resulted in a large standard deviation (Table 2). These results strongly suggest that carp sera contain small amounts of lectins or lectin-like substances besides antibodies.

Since the bactericidal activity of mucus was undetected and the agglutination titer low, it is possible that the bacteria that live attached to the body surface of carp are not killed but only trapped by the agglutinin to be washed away in a first line of defense mechanism in carp.

Oda *et al.* [4] also reported an inhibition of lectin by N-acetyl-D-glucosamine in the external mucus of *Genypterus blacoides*. Even though the agglutinins in serum and mucus of carp were inhibited by derivatives of glucose and their stability about temperature and pH different, it is not possible at this stage to conclude about their inter-relationship or origin. Further studies involving a larger spectrum of sugars used at different concentrations, complemented with an isolation and characterization of lectins are necessary.

Lectins from the dragonet, eel and Arabian Gulf catfish were found to be inhibited by D-galactose related sugars [5-7]. A fructosan-specific protein in the serum of shark was also reported [3].

Although there are speculations on possible functions of lectins such as differential recognition and adhesion, their natural function in vertebrates is still unknown. Lectins find an application in serology and serve as reagents for the detection, isolation and characterization of carbohydrate-containing macromolecules. Further studies on lectins of fish are needed for the understanding of the relationships among these proteins, their origin and their function.

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コイの体表粘液及び血清中の凝集素の物理化学的性状

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Escherichia coli IAM 1264及び*Pseudomonas* sp. I2-7に対するコイ体表粘液及び血清中の凝集素の性状について検討した。体表粘液の凝集活性は30～55℃で30分間の加熱及び5～12のpHに対し安定であり、D-グルコサミンで阻害された。また、血清の凝集活性は30～45℃での加熱及び7～10のpHに対して安定であり、N-アセチル-D-グルコサミンで一部阻害された。以上の結果から，コイの体表粘液及び血清中には異なるレクチン様物質が存在することが示唆された。