

Application of Carp Glycophorin from Red Blood Cell (RBC) Membranes as an Antibiotic Reagent: Effect of Carp Glycophorin as an Antibiotic Reagent

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ABSTRACT

Carp red blood cell (RBC) membranes contain a glycophorin (GP) that is responsive to a carp pathogen. The efficacy of carp GP as an antibacterial reagent was evaluated for the control of disease in rainbow trout (*Oncorhynchus mykiss*). The results suggest that intravenous administration of GP significantly delays the time of death following challenge with *Vibrio anguillarum*. In addition, we showed that carp GP was effective at adsorbing *V. anguillarum* and *Micrococcus luteus* from suspension following GP-binding column chromatography. After conducting small column experiments, we performed an upscaling column attached to a 500 L water tub with *Escherichia coli*.

Keywords: Fish, Carp, Red Blood Cell Membranes, Glycophorin, Antibiotic Reagent.

INTRODUCTION

Glycophorins (GPs) are transmembrane glycoproteins that contain sialic acid. These proteins are found in the red blood cell (RBC) membranes of mammals [1] [2] [3] [4], birds [5] [6] and teleosts [7]. We demonstrated that the teleost GP, isolated from carp (*Cyprinus carpio* L.) inhibits bacterial activity by attachment to the flagellum of *Vibrio anguillarum* or the cell surface of *Micrococcus luteus*. Moreover, the carp GP appears to be responsive to a broad range of

bacteria. The adsorbent properties of GP are attributable to the oligosaccharide moiety of the GP fraction [8]. The carp glycophorin has also shown bacteriostatic action against a carp pathogen, *Pseudomonas fluorescens*, and the GP content in diseased carp RBC membranes clearly decreases compared with the content in healthy carp [9]. This physiological property also exists in the GPs of yellow tail (*Seriola quinqueradiata*) and red sea bream (*Pagrus major*). Thus, we concluded that this antimicrobial activity is not confined to these teleost species but can be found in all fish [10].

Our objective was to evaluate the effect of exogenous treatment with carp GP on the control of vibriosis in rainbow trout (*Oncorhynchus mykiss*). In addition, the ability of carp GP to exclude bacteria from suspension was evaluated using a GP-binding column, which reacted to bacteria within the column bed.

MATERIALS AND METHODS

Fish

Rainbow trout (*Oncorhynchus mykiss*) (mean body weight: 92.5 g) were obtained from Uomatsu Salmonids Culture Farm in Mie Prefecture, Japan. The fish were held in flow-through water (16 °C) in a 500 L tub and fed ad libitum with commercial trout pellets. The fish were acclimated to these conditions for at least 14 days prior to the experiment.

Chemicals

Ficoll-Paque PLUS was purchased from Amersham Biosciences (GE Healthcare, Little Chalfont, U.K.). Ethyl 3-aminobenzoate methanesulfonate (MS-222) was purchased from Sigma (St. Louis, MO, U.S.A.). The *V. anguillarum* PT-499 strain was a gift from Dr. T. Nakai, Hiroshima University. *M. luteus* ATCC 9341 and *Escherichia coli* IFO3301 were cultured in our laboratory. The micro-organic media were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Formyl-cellufine was obtained from Seikagaku Corp. (Tokyo, Japan), and glycine ethyl ester was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were analytical grade.

Preparation of the Red Blood Cell Membrane

Blood was collected from the dorsal aorta of anaesthetized (MS-222) carp using a heparinized syringe that was inserted orally. The blood was diluted 1:1 with fish Ringer (145 mM NaCl, 5 mM CaCl₂, 1 mM MgSO₄, 4 mM KCl, 10 mM Hepes, and 5 mM glucose; pH 7.9) [11]. The white blood cells and plasma were removed using Ficoll-Paque PLUS. The RBC membranes were prepared as described previously [8].

Preparation of the Carp Glycophorin

The carp GP was extracted as described previously [8] using lithium 3,5-diiodosalicylate (LIS)-phenol. After solubilization by LIS, followed by phenol extraction, aggregation of GP did not occur in the preparation solution. The GP preparation was stored at -20 °C until use.

Miscellaneous Assays

Protein analysis was conducted by the method of Lowry et al. [12] with bovine serum albumin as the standard. The number of colony forming units (CFU) was quantified by plating a 10-fold serial dilution on agar medium and counting the number of colonies after incubation at 25 °C (*V. anguillarum* and *E. coli*) or 37 °C (*M. luteus*). The bacterial suspension was diluted with

phosphate-buffered saline (PBS) to the desired concentration. The minimum inhibitory concentration (MIC) of *E. coli* and *M. luteus* was determined by the broth dilution method [13] [14].

Artificial Infection and GP Injection Test

The *V. anguillarum* strain was prepared by inoculation into liquid medium (2.5% heart infusion broth containing 1.5% NaCl) and incubation at 25°C for 48 h with shaking. The culture was centrifuged at $1,500 \times g$ for 15 min, and the resulting precipitate was resuspended in PBS to the desired concentration.

The fish were randomly divided into two groups, control (physiological saline only) and treatment (physiological saline plus glycophorin) ($n = 11/\text{group}$). The fish were anaesthetized with 150 ppm MS-222 and then injected intramuscularly (dorsal musculature) with the bacterial suspension (ca. 100 μl , 3.0×10^6 CFU/100 g fish body weight). Following recovery from anaesthesia, each group of fish was transferred to a 500 L tub. After 1, 3, and 5 h, we injected saline solution containing GP (approximately 100 μl , 55 μg protein/100 g fish body weight) into the caudal vein of the fish in the treatment group. The control group was injected with saline alone.

Statistics

The response of the two groups was compared using a two-tailed Student's test. A p value of ≤ 0.05 was considered statistically significant.

Preparation of the GP-Binding Column

A GP-binding column was prepared using the reducing agent trimethylamine borane (TMAB) [15]. The formyl-cellulose gel was first washed thoroughly with water. Excess water was removed by gentle blotting prior to use. The GP preparation (approx. 2 mg protein) was suspended in a 0.2 M phosphate buffer (pH 6.8) and then dialyzed against the same buffer for 24 h at 4 °C. All subsequent procedures were carried out at 4 °C. The dialyzed GP solution (approx. 40 ml) was added to the formyl-cellulose gel (4 g wet gel). The suspension was stirred for 2 h, after which TMAB (up to 7 mg/g wet gel) was added and the suspension was stirred for another 8 h. The coupling reaction mixture was filtered using a suction funnel. The gel was then washed with $20 \times$ gel volumes of water. Excess formyl groups were blocked by adding 1 M glycine ethyl ester to the gel. Following this, TMAB was added to the suspension (same concentration as above) and then stirred for 2 h to prevent a reverse reaction. The GP-binding gel was packed into a column (1.5 $\times$ 3.0 cm) and washed with ten bed volumes of PBS. The control column was prepared as described above with the omission of the GP preparation.

GP-Binding Column Chromatography

V. anguillarum and *M. luteus* were inoculated into their respective liquid media (*V. anguillarum*: 2.5% heart infusion broth containing 1.5% NaCl; *M. luteus*: 2.5% heart infusion broth) and incubated at 25 °C with shaking for 24 h. The culture was diluted with PBS to the desired concentration and 1 ml of each dilution of the bacterial suspension was applied to the column. Then, the column was washed with 10 ml PBS, and the eluates were pooled. We quantified the CFU in each eluate from the treatment and control columns and determined the rate of bacterial

exclusion (%) as follows: (CFU of applied suspension - CFU of eluent)/CFU of applied suspension $\times$ 100 (%).

Upscaling Column Experiment Using a 500 L Water Tub

We prepared an upscaling GP-binding column (300 cm³ gel bed volume/3.6 cm $\times$ 30 cm column) by the same method previously mentioned. The control column was made of Celite (Johns-Manville Corp., Denver, CO, USA) as a column gel. The column was connected to a variable liquid delivery pump (CERAMIC PUMP CPA-2, AS ONE Corp., Osaka, Japan) and attached to a 500 L polycarbonate water tub (Figure 1). The flow rate was 100 mL/min and the temperature of the tub water was 16 °C (control column) or 18 °C (GP-binding column) when the experiment was performed. *E. coli* was inoculated into 5 mL of liquid media (2.5% heart infusion broth) and incubated at 25 °C with shaking for 24 h. After the liquid medium was added into the tub, the tub water was mixed with a rod. After mixing, the tub water was circulated through the column. The aliquot was sampled from the tub after 1, 3, and 7 days. We quantified the CFU in each 50 μ L of tub water.

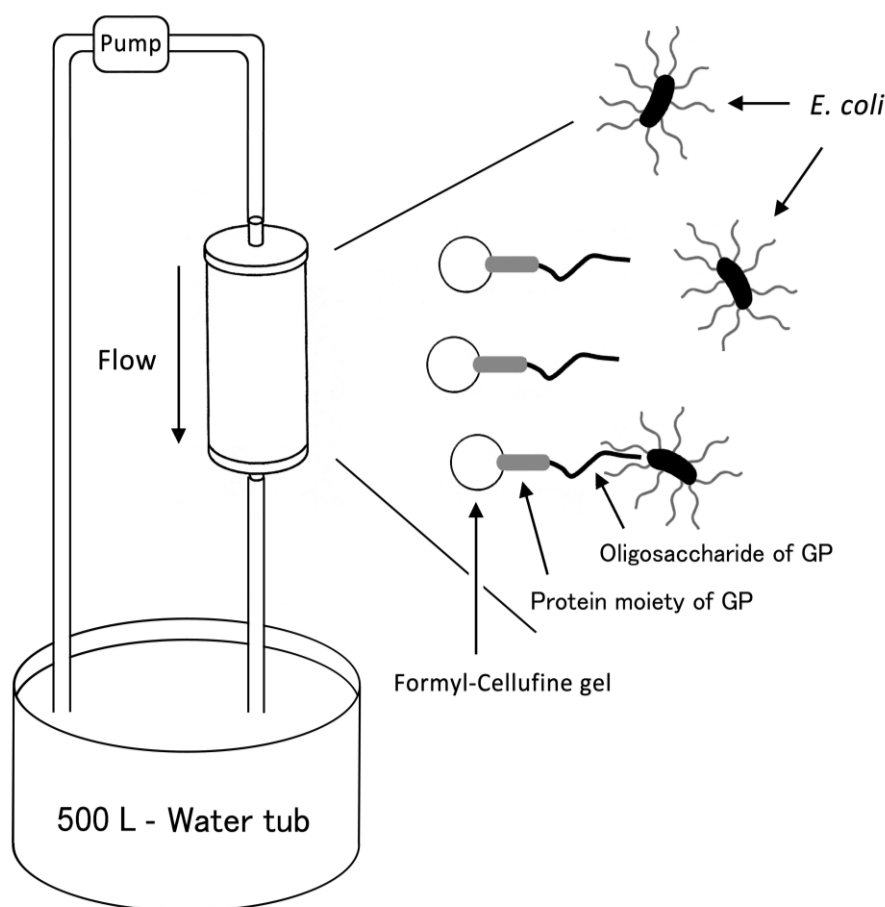


Figure 1: Schematic representation of the experiment with the GP-binding column attached to a 500 L water tub with *E. coli*.

RESULTS

MIC of *E. coli* and *M. luteus*

As shown in Table 1, the MIC value for carp glycoporphin against *E. coli* was 2.5 µg/ml, and that against *M. luteus* was also 2.5 µg/ml. This value against *E. coli* was higher than that of oxolinic acid (OA) and miloxacin (MLX), whereas, against *M. luteus*, it was lower than that of sulfamonomethoxine (SMM).

GP Injection Test

The fish in the control group exhibited symptoms of vibriosis, including a darkened body and enlarged anus, 12 h after the injection and died within 30 h. We observed a large number of *V. anguillarum* colonies in the samples of liver and kidney tissue from each infected fish.

The relationship between body weight and time to death is illustrated in Figure 2. The treatment group lived significantly longer than the control group ($p \leq 0.05$).

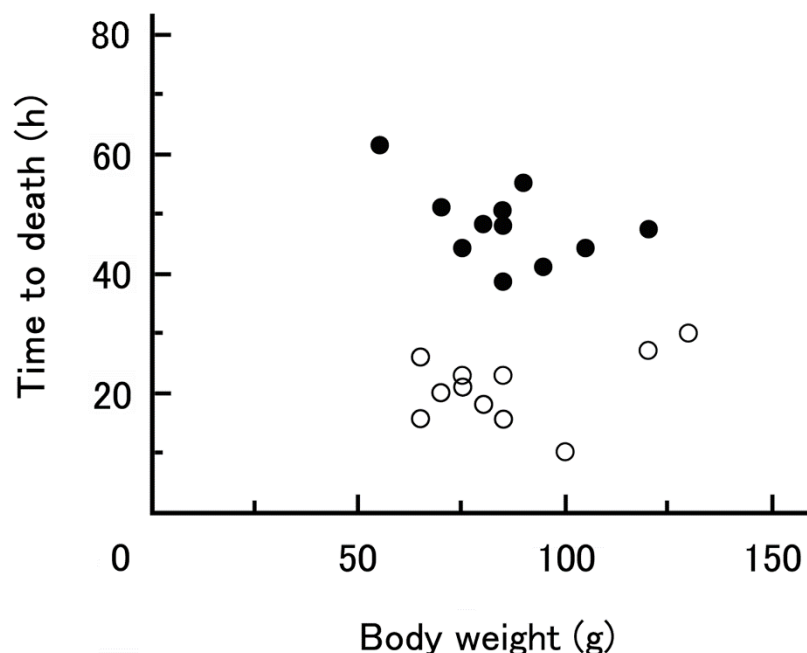


Figure 2: Correlation between time to death and body weight in rainbow trout treated with *V. anguillarum*. (●) Rainbow trout injected with glycoporphin; (○) control group.

GP-Binding Column Chromatography

The effect of carp GP was tested on the exclusion of *V. anguillarum* (4 concentrations) and *M. luteus* (5 concentrations) (Figure 3). For *V. anguillarum* the rate of exclusion was $75 \pm 8.2\%$ (initial concentration: 5.7×10^6 CFU), $71 \pm 1.4\%$ (4.8×10^5 CFU), $95 \pm 1.1\%$ (1.1×10^4 CFU), and 100% (2.5×10^3 CFU). For *M. luteus*, the rates of exclusion were $62 \pm 6.3\%$ (1.6×10^6 CFU), $53 \pm 4.6\%$ (6.1×10^5 CFU), $81 \pm 1.2\%$ (3.7×10^4 CFU), $90 \pm 1.4\%$ (3.1×10^3 CFU), and 100% (2.3×10^2 CFU). The effect of the control column was not observed upon the exclusion of each bacterium, *V. anguillarum* and *M. luteus*.

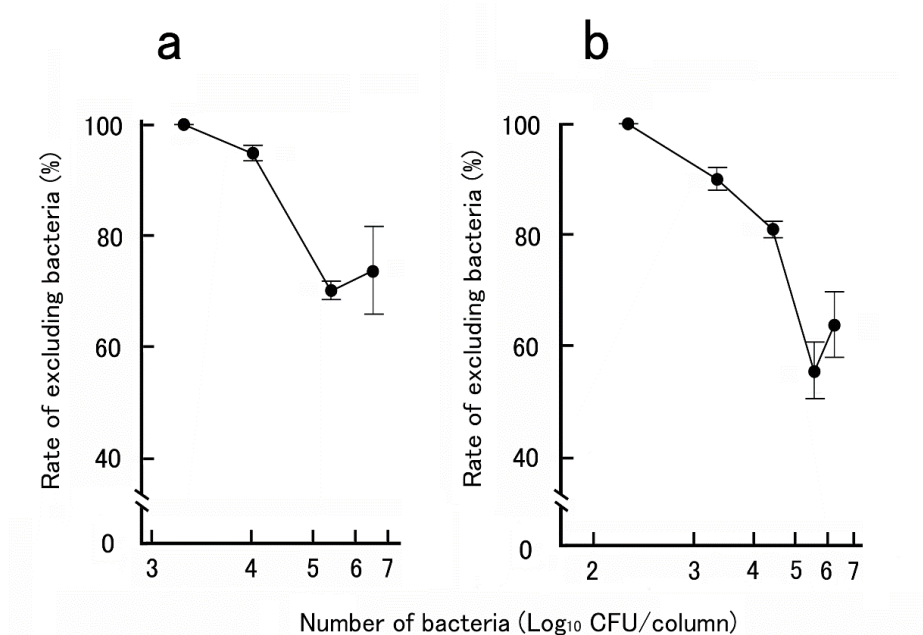


Figure 3: Rate of excluding bacteria by GP-binding column chromatography. (a) *V. anguillarum* as a test bacterium, (b) *M. luteus*.

Upscaling Column Experiment Using a 500 L Water Tub

The effect of an upscaling GP-binding column was tested. The CFU value of the tub water decrease after 24 running cycles (Figure 4).

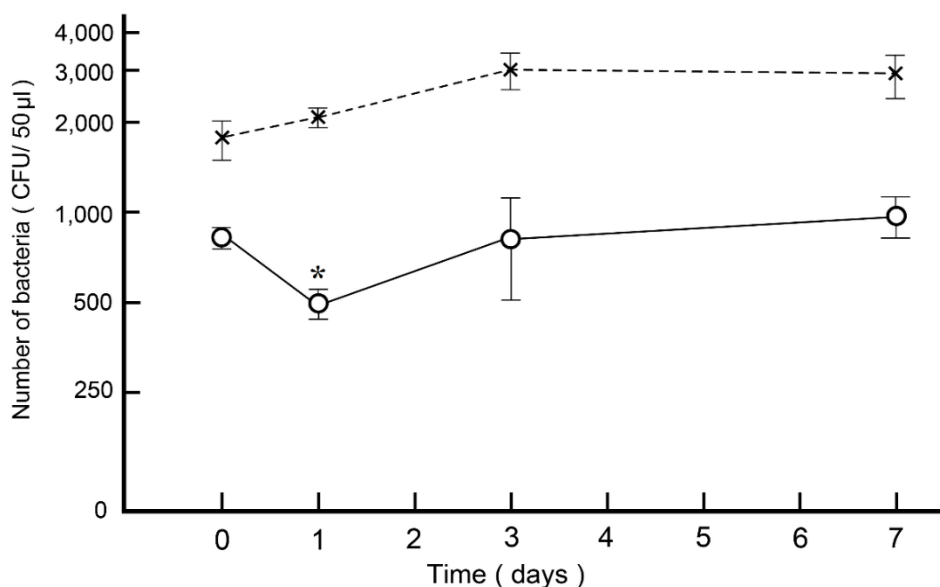


Figure 4. CFU of *E. coli* in the water of a 500 L polycarbonate tub. ○; Tub attached to a GP-binding column, ×; tub attached to a Celite column. * Column clogging.

However, it proved to have no effect on CFU after one day of operation because the column became clogged and blocked the water. Therefore, we tried a batch processing to remove the

GP-binding gel from the column and put it into the tub followed by mixing with a rod. The CFU values of the tub water before and after processing are shown in Figure 5. It also showed efficacy as a degerming agent.

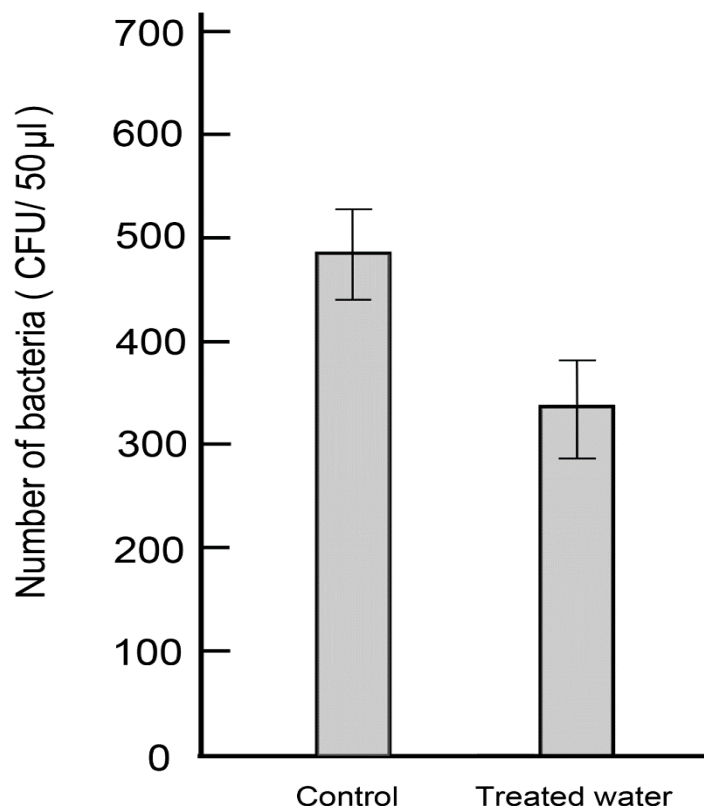


Figure 5: CFU of *E. coli* in 500 mL of the tub water by batch processing of the GP-binding gel.

DISCUSSION

Human GPs have receptor sites for viruses [16] [17] and malaria parasites [18]. However, the structural complexity of GPs does not allow easy identification of the functional site.

Previously, we developed a test for the effect of carp GP on the growth of a carp pathogen, *Pseudomonas fluorescens*, using a disc plate. Using this method, the carp GP fraction exhibited bacteriostatic activity against these fish pathogens, and the level of GP in the diseased carp RBC membranes clearly decreased compared with that in healthy carp [9].

In this study, we evaluated the utilization of the GP fraction as an antibiotic reagent. As shown in Table 1, the MIC value for carp glycophorin against *E. coli* (2.5 µg/ml) was similar to the value against *M. luteus* (2.5 µg/ml). This MIC value was higher than that of oxolinic acid (OA) (0.2 µg/ml) and miloxacin (MLX) (0.39 µg/ml), whereas the value against *M. luteus* (2.5 µg/ml) was lower than that of sulfamonomethoxine (SMM) (25 µg/ml) [19]. Thus, when compared with these antibiotics, the GP fraction was more effective against gram-positive than gram-negative bacteria. These similar efficacies of GP for the growth of both gram-negative and gram-positive

bacteria were also suggested by the almost same diameter of the inhibition zones around the paper disc on the test bacteria plates [8].

Table 1: MIC values for carp GP

	Carp GP	oxolinic acid (OA)	miloxacin (MLX)
MIC (μg/ml) against <i>E. coli</i>	2.5	*0.20	*0.39
	Carp GP	sulfamonomethoxine (SMM)	
MIC (μg/ml) against <i>M. luteus</i>	2.5	*25	

* [19]

GP administration significantly increased the time to death in rainbow trout. The fish receiving GP lived an average of 2 times longer (48 h/85.9 g body weight) than the controls (20.9 h/86.4 g body weight). Taken together, these results suggest that GP is as effective as synthetic antibiotics for the control of vibriosis.

Our previous study suggested that GP aggregates attach to the flagellum of *V. anguillarum* or to the cell surface of *M. luteus* [8]. These bacteriostatic activities of the GP are caused by the contained sialyl-oligosaccharide and are attributed to the property of the lectin receptor [8] [20]. Antibiotic activity was not detected on the protein moiety of the GP fraction. Therefore, we prepared the GP-binding column using the protein moiety as a ligand to affinity column gel. The adsorbent properties of the GP fraction were further evaluated using a GP-binding column. The results presented in this paper suggest that the adsorbing ability of the column was ca. 1.7×10^6 CFU/cm³ bed volume for *V. anguillarum* and ca. 3.8×10^5 CFU/cm³ for *M. luteus*. Given the density of carp GP, the column was 0.8 mg glycophorin/ cm³ bed volume, 2.5 g of glycophorin should over 1 t of bacterial suspension (10^3 CFU/cm³). Moreover, we performed an upscaling GP-binding column attached to a 500 L water tub with *E. coli*. The results suggested the efficacy of the GP-binding column as a degerming device, although the column was clogged to block water. It was necessary to examine the gel and a mixed rate of Celite to prevent column clogging.

In summary, we showed that the carp GP fraction is effective for the treatment of some fish diseases and may also be used to remove bacteria from infected water sources.

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