Protective effect of lysozyme-galactomannan or lysozyme-palmitic acid conjugates against *Edwardsiella tarda* infection in carp, *Cyprinus carpio* L.

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Received 19 January 1996; revised version received 28 February 1996

Abstract The protective effect of lysozyme-galactomannan or lysozyme-palmitic acid conjugates orally administered to carp, *Cyprinus carpio* L. was investigated using a virulent strain of Gram-negative *Edwardsiella tarda* isolated from an infected fish. Lysozyme-galactomannan conjugate was prepared through controlled Maillard reaction. Lysozyme-palmitic acid conjugate was prepared through base-catalyzed ester exchange using *N*-hydroxysuccinimide ester of palmitic acid. The conjugates provided substantial protection to carp infected with a Gram-negative bacteria fish pathogen *E. tarda* NG 8104. Lytic activities of lysozyme conjugates with galactomannan and palmitic acid were about 80 and 71% of native lysozyme using *Micrococcus lysodeikticus* as a substrate. Feeding with lysozyme conjugates, for 8 days, significantly enhanced fish protection against *E. tarda* infection. The survival rate was 30% for lysozyme-galactomannan conjugate treated fish and 20% for lysozyme-palmitic acid conjugate treated fish after 6 days cultivation while all control fish died within 3 days. On the other hand, a recovery rate of 40% after 6 days was observed in the fish group that were fed lysozyme-palmitic acid conjugate 3 and 2 h before and after *E. tarda* challenge, respectively, and for 6 consecutive days. The results of this work show the possibility of utilizing lysozyme conjugates with galactomannan or palmitic acid as a therapeutic for infection in fish.

Key words: Lysozyme-galactomannan conjugate; Lysozyme-palmitic acid conjugate; Bacterial infection; Fish therapeutic

1. Introduction

Antimicrobial activity of lysozyme is limited to Gram-positive bacteria. In mammalian and fish cells, lysozyme plays an important defense role in the earlier stage of bacterial infection by enhancing the phagocytosis of bacteria [1–3]. In an effort to expand the antibacterial activity of lysozyme to Gram-negative bacteria, lysozyme derivatives having bactericidal activity against Gram-positive as well as Gram-negative bacteria were prepared by covalent attachment of polysaccharides or fatty acids to lysozyme lysyl residues [4–7]. The antimicrobial effects of lysozyme conjugates against Gram-negative bacteria were associated with the degradation of the outer membrane in the bacteria and subsequent lysis of the peptidoglycan in the cells. The safety of lysozyme conjugates prepared through controlled Maillard reaction has been reported [8].

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Edwardsiella tarda (Edwardsiellosis) is a serious problem to the farming industry because the disease often occurs in fish grown in farming ponds. *E. tarda*, a Gram-negative bacteria belonging to the family of Enterobacteriaceae causes disease in fish. *E. tarda* NG 8104 was isolated, for the first time, from a flat-fish as a virulent pathogen in Japan. The use of antibiotics to control the bacterial infection in fish is a concern because it promotes the growth of drug-resistant bacterial strains [9]. Lysozyme is widely distributed in fish at their body, surface, skin, gill, intestinal tract, and serum as a protection factor against bacterial infection [2]. The physical antimicrobial action of lysozyme is suitable for use in aquaculture because it may provide an effective approach to prevent bacterial infection in fish without concern over residual antibiotics in the fish. The purpose of the present paper was to investigate the protective effect of orally administering lysozyme-galactomannan or lysozyme-palmitic acid conjugates to fish against a virulent strain of *E. tarda*.

2. Materials and methods

2.1. Materials

Mannase hydrolysate of guar gum (galactomannan, average molecular weight of 15000) was supplied by Taiyo Chemicals Co.(Mie, Japan). Palmitic acid was obtained from Sigma Chemical Co.(St. Louis, MO). *Micrococcus lysodeikticus* cells for lysozyme assay was from Wako Pure Chemicals, Tokyo, Japan. Bactotrypton and bactoyeast extract were from Difco Laboratories (Detroit, MI). Heart infusion medium and MacConkey medium were from Nissui Seiyaku Co., Tokyo, Japan. Commercial fish pellet was from Nippai Shiryo Co., Tokyo, Japan. All other chemicals were of analytical grade.

2.2. Preparation of lysozyme-galactomannan conjugate

Lysozyme was purified from fresh egg white at pH 9.5 in the presence of 5% sodium chloride and recrystallized five times. Galactomannan was dialyzed against deionized water for 2 days at 4°C. Lysozyme and galactomannan, in a weight ratio of 1:1, were dissolved in water and freeze-dried. Lyophilized mixture was spread over a petri dish and incubated at 60°C for 2 weeks at 79% relative humidity as previously described [4]. The resulting powder was stored at 4°C and used as lysozyme-galactomannan conjugate.

2.3. Preparation of lysozyme-palmitic acid conjugate

Palmitic acid was esterified with *N*-hydroxysuccinimide as previously reported [10]. The esterified fatty acid was covalently attached to a lysyl residue of lysozyme by base-catalyzed ester exchange according to the method of Haque et al. [10]. The degree of incorporation was controlled by adjusting the molar ratio of *N*-hydroxysuccinimide ester of fatty acid to lysozyme to obtain dipalmitoylated lysozyme [6]. 10 ml of tetrahydrofuran (THF) containing 1.4 mM *N*-hydroxysuccinimide ester of palmitic acid was gradually added to 40 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.7 mM lysozyme. The pH was then brought to 9.0 with 1 N NaOH at

SSDI S0014-5793(96)00260-8

25°C. The reaction mixture was incubated for 1 h at 25°C with gentle agitation. The pH was then adjusted back to 7.0 with 1 N HCl, and the mixture was subsequently dialyzed for 2 days at 4°C against potassium phosphate buffer containing 1 M NaCl. Following dialysis, the reaction mixture was washed five times with ice-cold diethyl ether, dialyzed against deionized water for 3 days at 4°C and lyophilized. The resulting powder was stored at 4°C and used as lysozyme-palmitic acid conjugate.

2.4. Measurement of lysozyme activity

Lysozyme activity was measured by the lytic assay using *M. lysodeikticus* cells as a substrate [11]. The lysis of cells in 50 mM potassium phosphate buffer (pH 7.0) was monitored at 660 nm. A 0.1 ml portion of the lysozyme conjugates solution (final lysozyme concentration, 0.003%) was added to 2.4 ml of *M. lysodeikticus* cell suspension (final concentration, 250 mg/ml; dry weight in the same buffer). This lysis mixture gave an initial OD of 0.8–0.85 at the onset of the experiment. The initial velocities were determined by measuring the decrease in turbidity of the cells automatically monitored at 660 nm (25°C) using a Hitachi U-2000 recording spectrophotometer (Tokyo, Japan).

2.5. Bacteria

E. tarda NG8104, a virulent strain for fish, was obtained from Dr. T. Kitao, Miyazaki University, Japan. The strain was cultured on heart infusion agar for 24 h at 37°C and orally administered three times to carp to maximize its virulence [12].

2.6. Antibacterial assays

Overnight cultured *E. tarda* cells were harvested from the heart infusion broth by centrifugation and were resuspended in 50 mM potassium phosphate buffer (pH 7.0) after washing twice with the same buffer. Washed cell preparations were diluted with the same buffer to give a concentration of 10^5 cells/ml using a hematometer. The cell suspension was added to the cell suspension to give a final lysozyme concentration of 50 mg/ml. 5 ml of the suspension was incubated at 37°C in a water bath equipped with a Lab-thermoshaker (Advantec Co., Tokyo, Japan) at 90 rpm to provide constant temperature and cell suspension. After a given incubation time, decimal dilution of the sample was subsequently carried out in physiological saline adjusted to pH 7.2. A 100 ml volume of cell suspension or dilution was spread onto two separate nutrient agar plates (MacConkey). All plates were incubated at 35°C for 24 h, and then the viable cell numbers were counted. Percentage of survivors was presented with respect to control mixture. The experiment was performed in triplicate.

2.7. Fish

Carp, *Cyprinus carpio* L., weighing 23.7 ± 2.8 g were obtained from a fish farm in Kumamoto, Japan, and were acclimatized to laboratory conditions with a commercial fish pellet in a 50 l aquarium at 22 ± 1°C for 2 weeks.

2.8. Challenge test

The fish were fed a pelleted food containing lysozyme conjugates at a daily ratio of 1.5% body weight at 10:00 h and 19:00 h for 8 days prior to challenge with *E. tarda*. Lysozyme conjugates were given to the fish at a dosage of 40 mg kg^{-1} day^{-1}. Challenge was performed on the 8th day by intraperitoneal injection of 0.2 ml of bacterial suspension in physiological saline (1.9×10^8 CFU100 g^{-1}). The fish were subsequently fed a commercial pellet to observe lysozyme conjugates-induced protection of carp against the bacterial infection. 10 fish per treatment were used in each experiment. Dead fish were immediately removed from the experimental aquarium and survivors were recorded for 6 days. In a separate set of experiments, lysozyme conjugates were orally administered to the acclimatized carps 3 h before and 2 h after intraperitoneal challenge with 2.1×10^8 CFU100 g^{-1} of *E. tarda*, respectively. The fish were subsequently fed with a pelleted food containing lysozyme conjugates at a dosage of 40 mg kg^{-1} day^{-1} and survivors were recorded for 6 days in a similar manner to the above. In this study, the administered dosage and the dose of bacteria used to challenge the fish were determined according to a preliminary experiment and previous studies on immunostimulation effect of polysaccharides [12,13].

3. Results and discussion

Maillard-type protein-polysaccharide conjugates are formed between the α- or ε-amino groups in protein and the reducing-end carbonyl group in polysaccharide through Schiff base. Covalent attachment of lysozyme to galactomannan was confirmed by SDS-slab polyacrylamide gel electrophoresis [5]. The binding molar ratio was calculated on the assumption that the molecular weight of lysozyme and galactomannan were 14000 and 15000, respectively. About 2 molecules of galactomannan appeared to link to one molecule of lysozyme on the basis of the binding weight ratio. The measurement of free amino groups in the conjugate were carried out to obtain further information on the binding. The results of the TNBS method demonstrated a decrease of 1.8 free amino groups per molecule in the conjugate. This result is consistent with the binding weight ratio described above. Diacylation of lysozyme with palmitic acid was adjusted by controlling the mole ratio of *N*-hydroxysuccinimide ester of the fatty acid to lysozyme molecules. The stoichiometry of acylation of the dipalmitoylated lysozyme was confirmed by measuring the TNBS-nonavailable free amino groups of the lysozyme conjugate which was 1.9 mol palmitic acid/mol of lysozyme. When the lytic activity of the conjugates was measured using *M. lysodeikticus*, lysozyme-galactomannan and lysozyme-palmitic acid conjugate exhibited about 80 and 71% enzymatic activity of control lysozyme, respectively. The observed decrease in residual activities of lysozyme conjugates may be attributed to the decrease in surface positive charge [14–16]. Modified lysozyme with approx. 2 molecules of polysaccharide or hydrocarbon chains per molecule of lysozyme maintained considerably high enzymatic activities.

In order to assess the protective effect of lysozyme conjugates against *E. tarda* NG8104, the mid-logarithmic phase culture was mixed with lysozyme conjugates at 37°C, and viable cell numbers were determined using MacConkey agar plates. Fig. 1 shows antimicrobial effects of lysozyme-galactomannan and lysozyme-palmitic acid conjugates against *E. tar-*

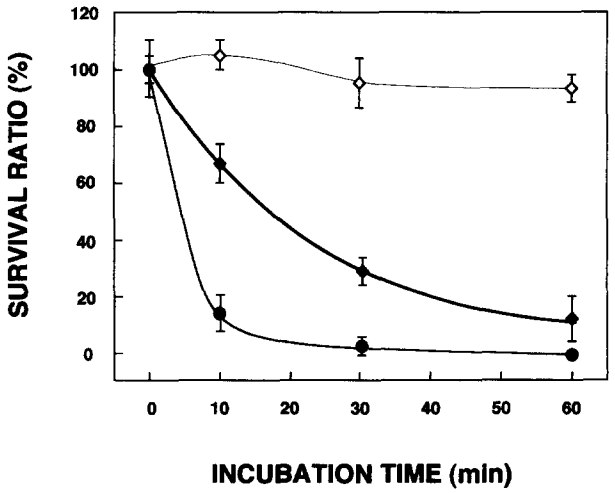

Fig. 1. Bactericidal activity of native lysozyme and lysozyme conjugates against *E. tarda* cells as a function of incubation time at 37°C. 50 mg/ml native lysozyme (◇), lysozyme-galactomannan conjugate (◆), or lysozyme-palmitic acid conjugate (●) were incubated with *E. tarda* cells (10^5 cells/ml) in 50 mM potassium phosphate buffer, pH 7.0. Values are means of three independent experiments, and the bars indicate the standard deviation.

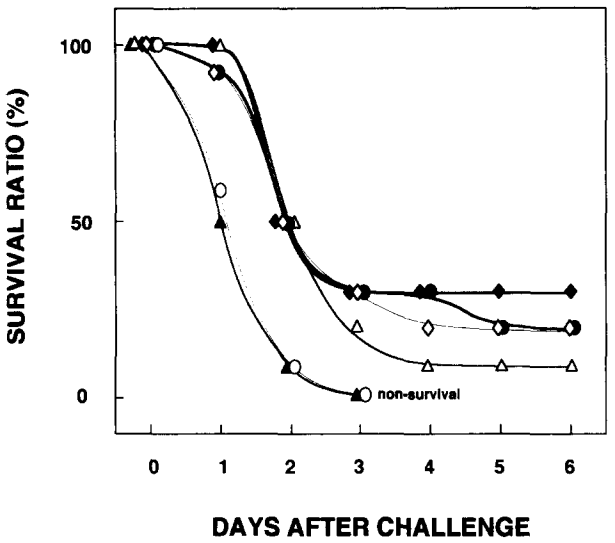

Fig. 2. Protective effect of lysozyme conjugates administered to carp challenged with *E. tarda* (1.9×10^8 CFU100 g^{-1}). Lysozyme conjugates were given at a dosage of 40 mg kg^{-1} day^{-1} for 8 days prior to challenge. $N=10$ per group. (△) Native lysozyme, (◆) lysozyme-galactomannan conjugate, (◇) galactomannan conjugate, (●) lysozyme-palmitic acid conjugate, (○) palmitic acid, (▲) control without tested material. Data are from a representative experiment repeated three times with similar results.

dt as a function of incubation time. A significant decrease in the viable cell numbers was observed in the incubation medium containing lysozyme-palmitic acid conjugate. The antimicrobial effect of the palmitoylated lysozyme on *E.tarda* was similar to the bactericidal action against *Escherichia coli* that was previously reported by Ibrahim et al. [6]. Lysozyme-galactomannan conjugate exhibited remarkable antimicrobial activity but was less effective than palmitoylated lysozyme (Fig. 1). It seems to be related to the evidence that polyglycosylated lysozyme could be lethal to Gram-negative bacteria including Enterobacteriaceae following incubation at 50°C, but could not be as lethal following incubation at 20°C [5]. In addition, to verify the specific role of lysozyme in the conjugates, we used α-lactalbumin as a conjugate protein control, which has a close structural similarity to hen egg-white lysozyme [17]. α-Lactalbumin conjugates did not exhibit any antimicrobial activities against *E. tarda* (data not shown). Thus, it could be demonstrated that lysozyme plays an important role to act as an antimicrobial agent in the conjugates.

Lysozyme conjugates were given at a dosage of 40 mg kg^{-1} day^{-1} for 8 days prior to challenge. On day 8, the fish were challenged with *E. tarda* and survivors was monitored for 6 days. As shown in Fig. 2, oral administration of lysozyme conjugates to carp enhanced protection against *E. tarda* strain. The survival rate was 30% for lysozyme-galactomannan conjugate treated fish and 20% for lysozyme-palmitic acid conjugate treated fish after 6 days cultivation, while all control fish died within 3 days. Recently, the administration of some polysaccharides was reported to enhance protection of fish against bacterial infection as immunostimulants [12,13]. In mammals, polysaccharides potentiate the non-specific immune responses, such as activation of macrophages, NK cells, T-lymphocytes and interferon products, and that their activities are associated with the activation of the alternative complement pathway [18–20]. Fish given galactomannan without lysozyme had a survival rate of 20% 6 days after the challenge, whereas there were no survivors in the group fed palmitic acid only. The protective effect of lysozyme-galactomannan on carp may indicate that polysaccharide moiety of the conjugate stimulated the non-specific immune system of the host fish in addition to the bactericidal activity of lysozyme moiety. The therapeutic effect of galactomannan has been also anticipated to be important in overcoming bacterial infection. Yamamoto et al. [21] reported that oral administration of galactomannan reduced total contents of lipids in rat livers.

The survival rate of carp infected with *E. tarda* (2.1×10^8 CFU100 g^{-1}) was also monitored to investigate the effects of the conjugates on fish recovery. Lysozyme conjugates were orally administered to the test carp 3 h before and 2 h after intraperitoneal challenge with *E. tarda*. The fish were subsequently fed a pelleted food containing lysozyme conjugates at a dosage of 40 mg kg^{-1} day^{-1}. As shown in Fig. 3, fish fed lysozyme-palmitic acid conjugate had a significantly greater survival rate than fish fed native or lysozyme-galactomannan conjugate. The survival rate of infected fish was 40% for lysozyme-palmitic acid conjugate group after 6 days while all control fish died within 3 days. Fish fed lysozyme-galactomannan conjugate or native lysozyme had a 20% survival rate. In the same experimental system, any survival effect of galactomannan was not observed. All fish fed galactomannan died within 3 days. The higher survival rate of palmitoylated lysozyme compared to polyglycosylated lysozyme was compatible with the differences in bactericidal activities between palmitic acid conjugate and galactomannan conjugate. It is well known that in vivo modification of proteins by fatty acids such as myristic, palmitic and stearic acids, is a widespread behavior of protein anchoring and fusion into membranes [22]. Attachment of fatty acids to lysozyme is important for its bactericidal effect against *E. tarda* by interaction with and subsequent insertion into the outer membrane of Gram-negative bacteria. Fatty acylation of lysozyme have been regarded

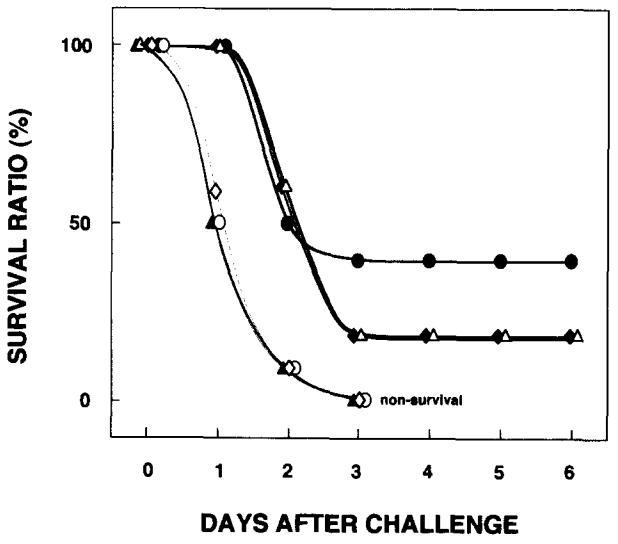

Fig. 3. Recovery of carp challenged with *E. tarda* (2.1×10^8 CFU100 g^{-1}). Lysozyme conjugates were given at a dosage of 40 mg kg^{-1} day^{-1} with commercial fish pellet for 6 days after challenge with *E. tarda*. $N=10$ per group. (△) Native lysozyme, (◆) lysozyme-galactomannan conjugate, (◇) galactomannan conjugate, (●) lysozyme-palmitic acid conjugate, (○) palmitic acid, (▲) control without tested material. Data are from a representative experiment repeated three times with similar results.

as directly mediating the interaction and penetration of lysozyme into the membrane to perform its action on the peptidoglycan of Gram-negative bacteria [7]. Although antimicrobial drugs have been employed for prophylactic and therapeutic purposes, recent occurrence of drug-resistant strains has made treatment with antibiotics more difficult. Lysozyme-palmitic acid conjugate may provide a safe alternative for the prevention of *E. tarda* infection in farmed fish. The development of lysozyme-galactomannan conjugate containing palmitic acid chain as a mean of improving the bactericidal activity of lysozyme-galactomannan conjugate is in progress.

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