Note

Enhancement of Serum Lysozyme Activity by Injecting a Mixture of Chitosan Oligosaccharides Intravenously in Rabbits

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Chitin [(1→4)-linked 2-acetamido-2-deoxy-β-D-glucan] and chitosan (*N*-deacetylated chitin) are the main components of the cell walls of infectious pathogenes. These polysaccharides are hydrolyzed by lysozyme (EC 3.2.1.17), chitinase (EC 3.2.1.14), and chitosanase. Lysozyme is present in animal bloods[1] and tears,[2] and chitinase in plants, insects, and microorganisms.[3,4] Chitinase is induced in plants by their contact with chitosan and with the cell walls of phytopathogens.[4,5] However, little is known about the induction of lysozyme in animals with chitin, chitosan, and their oligosaccharides, although these compounds enhance macrophages in rats,[5,6] and lysozyme is secreted by animal macrophages.[7,8] Chitin oligosaccharides have an anti-tumor activity against Sarcoma 180[9] and Meth-A[10] solid tumors in BALB/*c* mice, and an inhibitory activity against infection by *Listeria monocytogenes* in mice.[11] We now report an enhancement of serum lysozyme activity by injecting a mixture of chitosan oligosaccharides intravenously in rabbits.

Male rabbits were individually raised in cages. These rabbits were fed a basal ration (Labo-R-stock, Nihon Nohsan Kogyo, Inc., Tokyo). A mixture of chitosan oligosaccharides [degree of polymerization (d.p.) 2–8 in a ratio of 4:12:21:20:19:14:10%] was furnished by Katakura Chikkarin, Inc., Tokyo,[12] a mixture of chitin oligosaccharides (d.p. 2–6 in a ratio of 20:36:24:14:6%) by Yaizu Suisan Inc., Shizuoka, and a mixture of (1→4)-α-D-galactosaminan oligosaccharides (d.p. 2–7) by Higeta Shoyu Inc., Chiba. A sample of low-molecular-weight (LMW) chitosan was prepared by an oxidative depolymerization of chitosan with chlorine gas.[13] Each of these compounds was dissolved in saline at a concentration of 10 mg/ml. An appropriate amount was intravenously injected daily through a 0.2 μm filter (Steradisc 25, Kurabo Inc., Osaka) into the vein of rabbit conchae for 5 days.

The fate of these compounds in rabbit blood was examined by injecting 4.5 mg/kg of body weight into rabbits weighing 2.0–2.6 kg (Table I). A portion of the blood was drawn on the 2nd, 5th, 7th, and 13th day after the last injection, and hydrolyzed at 100°C for 15 hr in a sealed glass-tube. The hexosamine value in the hydrolyzate was analyzed by the Blix method,[14] and calculated as μmol of D-glucosamine.

The enhancement of serum lysozyme activity was examined by injecting 7.1–8.6 mg of a compound/kg of body weight per day into rabbits weighing 3.5–4.2 kg (Table II). In this experiment, the total dosage of the compound was 150 mg per rabbit. For analysis of the serum lysozyme activity, 2 ml of the blood was drawn from the vein of rabbit conchae on the 1st, 3rd, 5th, 7th, and 60th day after the last injection. After standing at room temperature (25°C) for 30 min in a glass-test tube, the blood was centrifuged at 1,500 × *g* at 0°C for 20 min to give a serum fraction. The serum (0.2 ml) was mixed with 2.8 ml of a suspended mixture (0.25 mg/ml) of *Micrococcus lysodeikticus* (M-3770, Sigma) as a substrate in 0.1 M phosphate buffer (pH 7.4). The mixture was incubated at 37°C with mechanical shaking, and the decrease in the turbidity was monitored at 600 nm by a Shimadzu UV-2200 spectrophotometer. The turbidity decreased proportionally to the reaction time for up to 60 min. One unit (U) for the enzyme activity is defined as a decrease in the absorption of 0.001 at 600 nm per min.

No abnormal physiological symptom was observed in the rabbits during and after the intravenous injections of LMW chitosan, chitosan oligosaccharides, or galactosaminan oligosaccharides at a dosage of 4.5 mg/kg of body weight per day for 11 days.The serum hexosamine value increased to 11.6 μmol/ml of serum on the 2nd day after the last day of injection, and it decreased to 5.5–7.7 μmol/ml of serum on the 13rd day (Table I). The Rabbits' appetites decreased slightly when a mixture of chitosan oligosaccha-

Table I. THE FATE OF CHITOSAN OLIGOSACCHARIDES, LMW CHITOSAN, AND GALACTOSAMINAN OLIGOSACCHARIDES IN SERUM AFTER THE LAST INTRAVENOUS INJECTION IN RABBITS[a]

Substance injected	Hexosamine (μmol/ml of serum)[b] Day after the last injection		
	2nd	5th	13th
Control		5.4–7.7	
Chitosan oligosaccharides	10.2±0.4	n.d.	5.5±0.2
low-molecular-weight	11.6±0.2	9.6±0.9	7.7±0.3
Galactosaminan oligosaccharides	7.9±1.9	5.4±0.7	6.0±0.4

[a] Injected at a dosage of 4.5 mg/kg of body weight for 5 days into rabbits weighing 2.0–2.6 kg.
[b] The average value of three experiments.

Table II. THE ENHANCEMENT OF SERUM LYSOZYME ACTIVITY BY INTRAVENOUS INJECTION OF CHITOSAN OLIGOSACCHARIDES, CHITIN OLIGOSACCHARIDES, OR GALACTOSAMINAN OLIGOSACCHARIDES IN RABBITS

Substance injected[a]	Blood lysozyme activity (U/ml of serum)				
	Day after the last injection				
	1st	3rd	5th	7th	60th
Saline (control)[b]	4.4±1.2	4.4±2.0	n.d.[f]	n.d.	4.3±1.2
Chitosan oligosaccharides[c]	9.2±2.2	7.7±2.2	6.9±2.4	3.7±1.0	4.7±2.0
Chitin oligosaccharides[d]	4.4±1.5	3.7±1.6	n.d.	n.d.	n.d.
Galactosaminan oligosaccharides[e]	4.5±0.9	4.4±0.8	n.d.	n.d.	n.d.

[a] Injected at a dosage of 7.1–8.6 mg/kg of body weight per day for 5 days into rabbits weighing 3.5–4.2 kg per rabbit.
[b] Tested with 4 rabbits.
[c] Tested with 6 rabbits.
[d] Tested with 4 rabbits.
[e] Tested with 3 rabbits.
[f] Not determined.

rides was injected at a dosage of 7.1–8.6 mg/kg of body weight per day. However, the rabbits' appetites were unchanged when chitin oligosaccharides and galactosaminan oligosaccharides were injected at this dosage. The chitosan oligosaccharides were not hydrolyzed by lysozyme *in vitro*, but polymeric chitosan orally administered was digested in the rabbit digestive organs.[15] The detailed fate of these oligosaccharides in rabbit blood was not examined.

The normal value of lysozyme activity (U/ml of serum) in rabbit serum was 4.4±2.0. As shown in Table II, the activity was enhanced up to 9.2 after five injections of the chitosan oligosaccharides at a dosage of 7.1–8.6 mg/kg of body weight per day, and the high activity lasted for about 5 days after the last injection. This enhancement appeared after two or more injections. However, the lysozyme activity was neighter enhanced by injecting chitin oligosaccharides, galactosaminan oligosaccharides and saline, nor enhanced by adding chitosan oligosaccharides into a medium for an *in vitro* culture of the whole rabbit blood.

These data indicate that lysozyme is induced into blood by chitosan oligosaccharides from specific cells of blood vessels or organs. This phenomenon is essentially similar to the induction of chitinase in plants,[4] and to the induction of lysozyme in insects.[16] The enhancement of lysozyme and chitinase activities is probably a biological process for the body-defensive function, followed by activation of the macrophage system in animals,[5,6] and by production of phytoalexins in plants[17] and antibacterial proteins in insects.[18]

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