J. Food Sci. Nutr.
Vol. 3, No. 4, p. 320~323 (1998)

Antibacterial Activity of Lysozyme-Galactomannan Conjugate against *Escherichia coli*

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Abstract

Lysozyme was covalently conjugated with galactomannan through a amino-carbonyl reaction between the lysine ε-amino groups of lysozyme and the reducing ends of galactomannan at a relative humidity of 79% and 60°C. The resulting lysozyme-galactomannan conjugate (LGC) was investigated for its antibacterial activity against *Escherichia coli*. Lysozyme alone did not exhibit antibacterial activity against *E. coli*. In contrast, significant bactericidal effect was observed for LGC, depending on the reaction temperature. The degree of conjugation between lysozyme and galactomannan was dependent on the incubation time, which affected the antibacterial efficiency against *E. coli*. This study demonstrated that the amino-carbonyl reaction between lysozyme and galactomannan could be a potential tool to modify lysozyme toward broadening its antibacterial spectrum to Gram-negative bacteria.

Key words: lysozyme, polysaccharide, conjugation, antibacterial activity

INTRODUCTION

Many attempts have been made to produce natural food preservatives, since a majority of chemical preservatives may possess some toxicities (1). Lysozyme acts as a natural antibacterial protein by effectively hydrolyzing the β-1,4 linkages between *N*-acetylglucosamine and *N*-acetylmuraminic acid in Gram-positive bacterial cell wall (2-4). However, antibacterial activity of lysozyme for Gram-negative bacteria is not as effective as that for Gram-positive bacteria, since the outer membrane of Gram-negative bacteria excludes lysozyme and prevents it from binding to its site of action on the peptidoglycan cell walls (5-7). Therefore, if lysozyme can be converted to amphiphilic protein without using any chemical reagents, lysozyme could be extensively employed as a natural food preservative.

It has been well documented that protein-polysaccharide conjugates (PPC) are versatile biomaterials providing desirable functional properties of proteins and polysaccharides (8-10). Lysozyme has been most frequently used as a protein source for PPC. In this research, lysozyme was conjugated with galactomannan which has been used widely as a thickening and stabilizing agents in food systems. Thus, the lysozyme-galactomannan conjugate (LGC) can provide combined features of lytic enzyme and functional polysaccharide. This research is aimed to investigate the antibacterial activity of LGC prepared by amino-carbonyl reaction against Gram-negative bacteria, using *Escherichia coli* as a target microorganism.

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MATERIALS AND METHODS

Materials

Egg white lysozyme (EC 3.2.1.17) purchased from Sigma Chemical Co. (USA) was used without further purification. Galactomannan, of approximately 12,000 of molecular weight, was provided by Taiyo Kagaku Co. (Japan). Galactomannan was dissolved in distilled water, in which isopropanol was added to remove small molecular fractions. Then, the precipitates were washed with isopropanol and acetone, successively, and dried at room temperature.

Sephadex G-50 (super fine) and S-Sepharose were obtained from Pharmacia (Sweden), and nutrient agar from Difco Laboratories (USA). *E. coli* (IFO 12713) and *E. coli* O157 (ATCC 43894) were used for this study. Dialysis membrane (MWCO: 1,000) was supplied by Spectrum (USA). All other reagents were of analytical grade.

Preparation of lysozyme-galactomannan conjugate (LGC)

Lysozyme was throughly mixed with galactomannan in distilled water at the weight ratio of 1:1. In order to induce a amino-carbonyl reaction between lysozyme and galactomannan, the mixtures were lyophilized, followed by incubation at 60°C under the relative humidity of 79% for 5~20 days (11). Relative humidity was controlled by saturated KBr solution. Binding ratio of galactomannan to lysozyme was measured by the TNBS (trinitrobenzenesulfonate) method (12,13).

Gel filtration chromatography

Unreacted lysozyme was separated from LGC by gel filtra-

tion chromatography using a Sephadex G-50 column (1.5×100 cm) at a flow rate of 0.3 ml/min using 50 mM of pH 5.0 sodium acetate buffer as an eluant. Lysozyme content in each fraction was measured by absorbance at 280 nm and galactomannan content at 470 nm after phenol-sulfuric acid reaction (14). Each fraction containing LGC was collected, dialyzed against deionized water and lyophilized for further antibacterial assay.

SDS-PAGE

SDS-PAGE was conducted according to the method of Laemmli (15). Purified LGC and lysozyme were incubated at 100°C for 3 minutes with 10% SDS, 1 M Tris-HCl (pH 6.8), 2-mercaptoethanol and 1% bromophenol blue. 50 to 100 μl of samples were loaded on slab gel consisting of 14% separating gel and 5% stacking gel. Electrophoresis was carried out at a constant current of 30 mA. The gel was stained with coomassie brilliant blue R-250.

Cation exchange chromatography

S-Sepharose column (2.5×20 cm) was used to separate free galactomannan from LGC. 20 mM of pH 7.0 sodium phosphate buffer was used as an equilibrating buffer and the same buffer containing 1 M NaCl was used as a gradient buffer. Flow rate was 0.3 ml/min.

Assay of antibacterial activity

Antibacterial activity was measured by the viable cell count method. *E. coli* was incubated at 37°C for 14 hours and then washed twice with 50 mM of pH 7.0 potassium phosphate buffer and diluted to give a final concentration of 10^5 cells/ml. The reaction mixtures, containing 4 ml of diluted cells and 1 ml of 0.05% antibacterial agent, were incubated at 30~50°C for a given time. Viable cells were counted by the pour plating method after incubation for 2 days at 37°C.

RESULTS AND DISCUSSION

Purification of LGC

Fig. 1 shows gel filtration chromatography profiles for lysozyme-galactomannan mixture (LGM) and conjugates (LGC) as a function of incubation time. When compared to LGM of Fig. 1 (a), newly shifted protein peaks in LGC appear as shown in Figs. 1 (b)-1 (d). These shifted protein peaks represent lysozymes conjugated with galactomannan, i.e., LGC. The amount of shifted protein peaks of LGC, originated from native lysozyme, progressively increased with incubation time. The LGC fractions, indicated by the horizontal arrow in Fig. 1, were collected for further antibacterial experiments.

SDS-PAGE was carried out to confirm that lysozyme was in fact conjugated with galactomannan. Fig. 2 shows the SDS-PAGE patterns of LGC, which was separated from gel filtration chromatography, in conjunction with native lysozyme. Lane C, obtained from the first protein peak of Fig. 1 (d), contains nearly no native lysozyme residues. This indicates that a majority of lysozyme in the first peak of gel filtration is conjugated with galactomannan. The high polydispersity of galactomannan and the various number of galactomannan bound

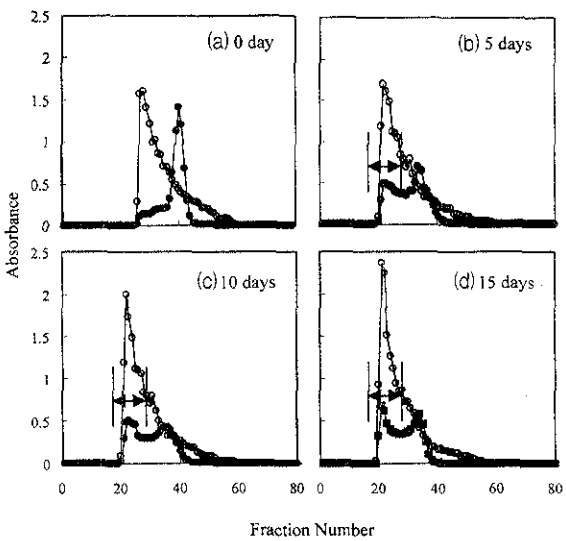

Fig. 1. Elution profiles of LGM and LGC on Sephadex G-50 column. ●, absorbance at 280nm for protein; ○, absorbance at 470nm after color development by phenol-sulfuric acid reactions for polysaccharide.

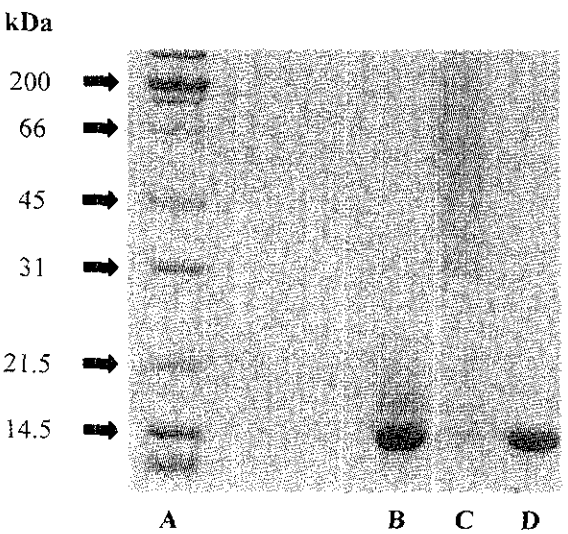

Fig. 2. SDS-PAGE patterns of LGC (15 days) and lysozyme. (a) molecular weight marker; (b) the second protein peak of LGC on Sephadex G-50 column; (c) the first protein peak of LGC on Sephadex G-50 column; (d) native lysozyme.

to lysozyme may cause the broad molecular weight distribution of LGC, leading to the long trailed band of lane C in Fig. 2. A similar result was also reported for α-lactalbumin-galactomannan conjugate by Nakamura et al. (16). In contrast, the major portion of the second protein peak shown in Fig. 1 (d) was lysozyme as demonstrated in lane B. Lane D in Fig. 2 represents the molecular weight of native lysozyme, 14,000.

LGC can be differentiated in terms of the binding ratio and molecular size of galactomannans conjugated with lysozyme. Cation exchange chromatography was performed to separate neutral galactomannans from LGC. Fig. 3 shows the elution profile of LGC, obtained from the first peak of Fig. 1 (d), on

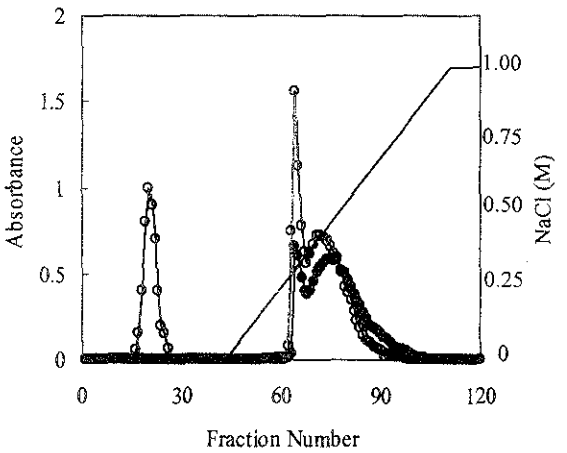

Fig. 3. Elution profile of the first protein peak fractions of LGC (15days) from Sephadex G-50 on S-Sepharose column.
●, absorbance at 280nm for protein; ○, absorbance at 470nm after color development by phenol-sulfuric acid reactions for polysaccharide.

S-Sepharose cation exchange chromatography. The first peak of conjugates were eluted at the NaCl gradient buffer concentration from 0.3 M to 0.4 M and for the second peak, from 0.4 M to 0.5 M. These two distinct peaks indicate that variation exists in the number of galactomannan conjugated with ε-lysine residues of lysozyme.

Antibacterial effect of LGC

Fig. 4 shows the antibacterial effect of lysozyme and LGC on *E. coli* (IFO 12713), depending on the reaction temperature. The living cell numbers are demonstrated in terms of log survival ratio, representing the survival decimal fraction based on log values. Differences in the antibacterial activity for *E. coli* were not evident between lysozyme and LGC at 30°C. In contrast, the antibacterial activity of LGC was significantly enhanced with increasing reaction temperature. There were no detectable cells after 30 min of incubation at 50°C in the presence of LGC. The results indicate that the antibacterial activity of LGC is greatly dependent on reaction temperature. This may be ascribed to the thermal damage of the cell, inducing blebbing and vesiculation of the outer membrane (17).

It is conceivable that the degree of conjugation between lysozyme and galactomannan can be affected by incubation time, which in turn may affect the antibacterial activity of LGC. Therefore, 4 different LGC samples were prepared, in which the incubation time for amino-carbonyl reaction was controlled up to 20 days at 5 day intervals. Fig. 5 demonstrates the antibacterial activity of LGC against *E. coli* (IFO 12713) and *E. coli* O157, depending on incubation time. LGC incubated for 5 days exhibited the similar antibacterial activity to lysozyme. In contrast, significant increase in antibacterial activity was observed with increasing incubation time, and LGC conferred much higher antibacterial activity than native lysozyme. These results strongly suggest that the antibacterial activity of LGC increased by incubation time or, correspondingly, the degree of conjugation. The same experiments were also carried out for *E. coil* O157, one of the pathogenic food microorganisms (Fig. 5(b)). *E. coli* O157 was more resistant to lysozyme and LGC, taking the total reaction time into consideration. The results also demonstrated that LGC provided the substantial antibacterial activity against *E. coil* O157 as compared to native lysozyme.

Lysozyme molecules contain 7 lysine ε-amino residues for amino-carbonyl reaction with galactomannan (11). Fig. 6 shows the binding ratio (mole%) of galactomannan to lysine ε-amino groups of lysozyme. The binding ratio consistently increased up to 10 days of incubation time, after which it re-

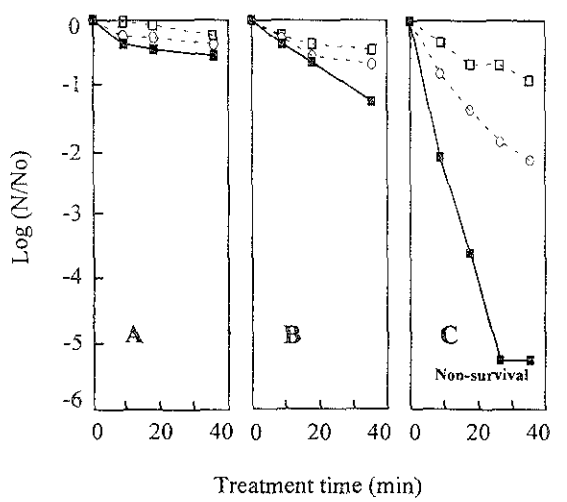

Fig. 4. Effect of reaction temperature on antibacterial activity of lysozyme and LGC against *E. coli*.
(a) reaction temperature of 30°C; (b) reaction temperature of 40°C; (c) reaction temperature of 50°C. □, buffer; ○, lysozyme; ■, LGC incubated for 15 days.

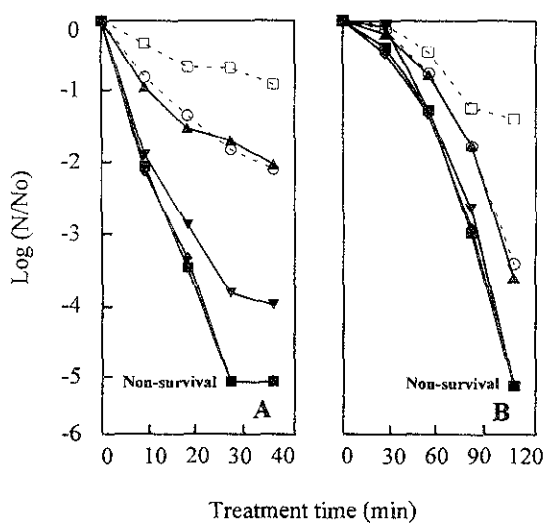

Fig. 5. Antibacterial activity of LGC against *E. coli* and *E. coli* O157 at the reaction temperature of 50°C.
(a) *E. coli*; (b) *E. coli* O157. □, buffer; ○, lysozyme; ▲, LGC incubated for 5 days; ▼, LGC incubated for 10 days; ■, LGC incubated for 15 days; ◆, LGC incubated for 20 days.

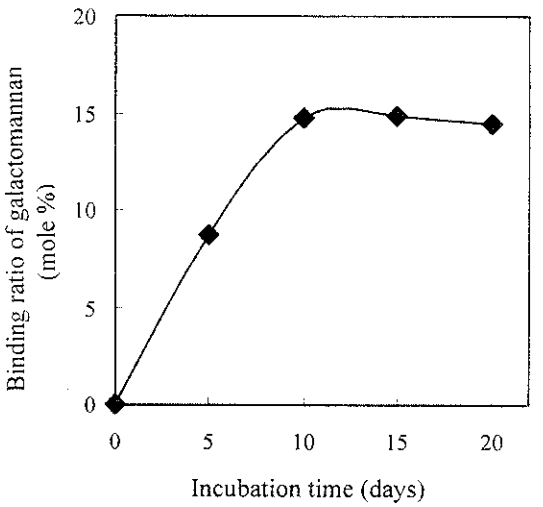

Fig. 6. Binding ratio of galactomannan to ε-amino groups of lysozyme.

mained nearly constant, regardless of incubation time. In the light of experimental results that the antibacterial activity of LGC significantly increased after 10 days of incubation (Fig. 5), the binding degree of lysine ε-amino groups may be closely related to antibacterial activity. In this research, however, the binding ratio approximated to 13.0 mole% per lysozyme molecule. This corresponds to about 1 mole of ε-amino group among the maximum 7 moles residues available. The amount of ε-amino group conjugated with galactomannan was less than that reported by Nakamura et al. (18). They showed that 2 moles of galactomannan seemed to link to one mole of lysozyme in 2 weeks-incubated LGC. Further studies are necessary to develop the potential tools to enhance the binding degree or, correspondingly, the bactericidal effect of LGC.

This research demonstrated that the conjugation of lysozyme with galactomannan rendered lysozyme to a higher and broader antibacterial activity. The conjugation by amino-carbonyl reaction to date has been confined to the neutral polysaccharides such as dextran or galactomannan. Presumably, the charge of the polysaccharide in combination with molecular size can be a significant contributor to the antibacterial activity of the conjugates. Therefore, more systematic research is necessary to investigate the effects of the molecular structure of the polysaccharides.

ACKNOWLEDGEMENTS

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Bioproducts Research Center at Yonsei University (97U4-0407-00-02-3).

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(Received November 12, 1998)