New Antimicrobial Characteristics of Lysozyme–Dextran Conjugate

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Lysozyme–dextran conjugate prepared by Maillard reaction revealed significant antimicrobial activity for both Gram-negative and Gram-positive bacteria. Estimating from the binding weight ratio, about two molar dextrans were linked covalently with one molar lysozyme. Lytic activity of the conjugate was considerably reduced when measured by using *Micrococcus lysodeikticus* as a substrate, while it remained about 80% that of native lysozyme when measured by using glycol chitin. The heat stability of lysozyme activity was increased by linking with dextran. The emulsifying properties of the conjugate were much higher than those of native lysozyme. These results suggest that an antimicrobial activity of the lysozyme–dextran conjugate for Gram-negative bacteria comes from the remaining stable lysozyme activity and the excellent emulsifying properties by which the outer membrane is solubilized.

INTRODUCTION

Since most Gram-negative bacteria cause food poisoning, many attempts have been made to control these contaminating microorganisms in processed foods. Food preservatives have been used to destroy these food-poisoning bacteria. However, the problem with food applications of these preservatives is their toxicity, and hence much attention has been directed to safe materials such as hen egg white lysozyme. Lysozyme has been reported to be utilized as a preservative for processed food (Akashi and Ohno, 1972). Although many attempts have been made to use the enzyme as a food preservative, lysozyme was unsuitable to use as a general antimicrobial agent because the lytic spectrum was limited only to Gram-positive bacteria and lytic action is inert with food components such as carbohydrates (Hayashi et al., 1989).

Gram-negative bacteria, whose peptidoglycan is coated with an outer membrane mainly consisting of lipopolysaccharide (LPS), are known to be resistant to native lysozyme. Sodium deoxycholate (Nakamura and Mizushima, 1975), sodium dodecyl sulfate (Sekizawa and Fukui, 1973), and Triton X-100 (DePamphilis, 1971; Schnaitman, 1971) were used as media dissolving the outer membrane, since these detergents extensively dissociate lipopolysaccharide into its subunits. Therefore, if lysozyme is converted to amphiphilic proteins without loss of lytic activity, it is expected to extend the spectra of antimicrobial activity.

We found that a soluble protein–dextran conjugate prepared by Maillard reaction showed excellent emulsifying and heat-stable properties (Kato et al., 1988, 1990). Lysozyme–dextran conjugate may act on the outer membrane of Gram-negative bacteria, and the conjugate is expected to show a lytic action spectrum different from that of native lysozyme. This paper describes the characteristics of the conjugate and the possibility of application as a new food preservative.

MATERIALS AND METHODS

Materials. Dextran (average molecular weight of 60 000–90 000) was obtained from Wako Pure Chemicals. Lysozyme was crystallized from fresh egg white at pH 9.5 in the presence of 5% sodium chloride and recrystallized five times. Sephacryl S-300 was obtained from Pharmacia. *Micrococcus lysodeikticus* was obtained from Wako. Glycol chitin was provided by Dr. Daizo Koga, University of Yamaguchi, Japan. Bacto-tryptone and bacto-yeast extract were obtained from Difco Laboratories, and MacConkey medium was obtained from Nissui Seiyaku Co.

Preparation of Lysozyme–Dextran Conjugate. Lysozyme and dextran were mixed in water at the weight ratio of 1:5 and lyophilized. The mixture was incubated at 60 °C under a relative humidity of 78.9% in the container saturated with KBr solution for a given time (0–3 weeks). The lysozyme–dextran conjugate thus prepared was separated from the unreacted lysozyme by gel permeation chromatography using a Sephacryl S-300 column (85 × 1.6 cm). The column was equilibrated and eluted with 50 mM acetic acid–sodium acetate buffer, pH 5.0. The protein content in each fraction was detected by measuring the absorbance at 280 nm, and the carbohydrate content was determined by measuring the absorbance at 470 nm after color development with the phenol–sulfuric acid reaction. All fractions containing lysozyme–dextran conjugate were collected together, dialyzed against deionized water, and lyophilized.

Measurement of Amino Groups. The contents of free amino groups in the lysozyme–dextran conjugate were determined by the trinitrobenzenesulfonate method (Haynes et al., 1967).

Lysozyme Activity. Lysozyme activity was measured by the lysis and glycolysis assay using *M. lysodeikticus* cells and glycol chitin as substrates, respectively. Suspensions of *M. lysodeikticus* cells were prepared in 50 mM potassium phosphate buffer, pH 7.0. The absorbance at 660 nm was adjusted to 0.8, and then to 2.4 mL of the suspensions was added 0.1 mL of 0.003% lysozyme solution. Initial velocities were determined by measuring the decrease in the turbidity of the cells automatically monitored at 660 nm with a spectrophotometer (Hitachi Co. U-2000). Glycolysis was measured as follows: To 0.5 mL of lysozyme solution in 10 mM acetic acid–sodium acetate buffer (pH 4.5) was added 1.0 mL of 0.05% solution of glycol chitin. The mixture was incubated at 40 °C for 30 min. After the reaction, 2 mL of the color reagent (made by dissolving 0.5 g of potassium ferricyanide in 1 L of 0.5 M sodium carbonate) was added and the mixture was immediately boiled for 15 min to estimate the reducing power resulting from hydrolysis of glycol chitin (Imoto and Yagishita, 1971).

Measurement of Emulsifying Properties. The emulsifying properties were determined by the method of Pearce and Kinsella (1978). To form emulsion, 1.0 mL of corn oil and 3.0 mL of sample solution in 100 mM sodium phosphate buffer, pH 7.4, were shaken together and homogenized in an Ultra Turrax (Hansen Co.) at 12 000 rpm for 1 min at 20 °C. One hundred microliters of emulsion was taken from the bottom of the test tube after different times and diluted with 5.0 mL of 0.1% sodium dodecyl sulfate solution. The absorbance of diluted emulsion was then determined at 500 nm. The emulsifying activity was

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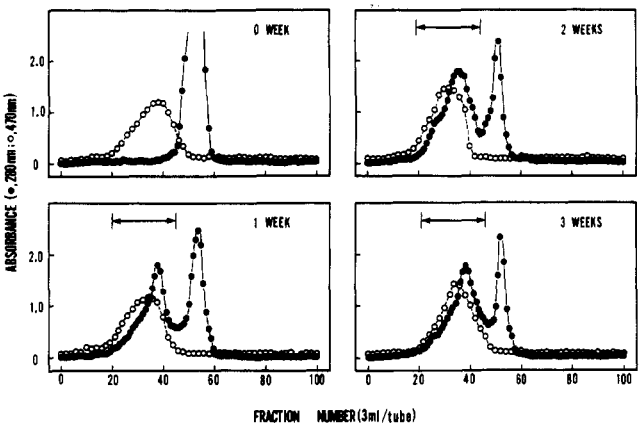

Figure 1. Elution profiles in 50 mM acetate buffer (pH 5.0) on a Sephacryl S-300 column on the lysozyme–dextran mixtures incubated at 60 °C for 0–3 weeks under 78.9% relative humidity condition. The fractions indicated by horizontal arrows were pooled, dialyzed, and used for further experiments. (●) Absorbance at 280 nm for protein; (○) absorbance at 470 nm to follow the color development by the phenol–sulfate method for polysaccharide.

determined from the absorbance measured immediately after emulsion formation. The emulsion stability was estimated by measuring the half-time of the turbidity of emulsion.

Bacterial Strains and Culture Condition. Five Gram-negative parental strains (*Vibrio parahaemolyticus* IFO 12970, *Escherichia coli* IFO 12713, *Aeromonas hydrophila* IFO 13286, *Proteus mirabilis* IFO 12668, and *Klebsiella pneumoniae* IFO 14438) and two Gram-positive parental strains (*Bacillus cereus* IFO 13690 and *Staphylococcus aureus* IFO 14462) were obtained from the Institute for Fermentation, Osaka. All strains except *V. parahaemolyticus* were grown in L-broth (10 g of bacto-tryptone, 5 g of bacto-yeast extract, 5 g of NaCl, 1 g of glucose, and water to 1 L). *V. parahaemolyticus* was grown in L-broth containing 2.5% NaCl at the final concentration. Each subcultured strain was inoculated into 100 mL of L-broth in a 500-mL round flask and incubated at 35 °C with shaking at 90 rpm on an Eyela shaker (Tokyo Rikakikai Co.) for overnight.

Detection of Antimicrobial Effects. Microorganisms were harvested from the culture medium and washed three times by centrifugation and resuspended in 50 mM potassium phosphate buffer (pH 7.0). Washed preparations of microorganisms were diluted with 50 mM potassium buffer (pH 7.0) to give a concentration of 10^5 cells/mL by using a hematometer. Lysozyme or lysozyme–dextran conjugate was added to the cell suspensions to give a final lysozyme concentration of 0.05%. Five milliliters of the conjugate–cell mixture suspensions was incubated at 50 °C in a water bath equipped with a Lab-thermo shaker (Advantec Co.) at 90 rpm to provide constant temperature and cell suspension. After a given heating time, the heated suspension was immediately added to a sterile test tube immersed in an ice bath. After cooling to room temperature, decimal dilutions were subsequently carried out in physical saline solution adjusted to pH 7.2. A 100-μL portion was spread over MacConkey agar plates for Gram-negative bacteria and over standard agar plates for Gram-positive bacteria. For *V. parahaemolyticus* (halophilic bacteria) the agar supplemented with NaCl at the final concentration of 2.5% was used to ensure complete growth. Colonies were counted after incubation for 24 h at 35 °C. Data were represented as means ■ standard deviations obtained from two separate experiments run in duplex.

RESULTS

Figure 1 shows the elution profiles of lysozyme–dextran conjugate separated by gel chromatography on a Sephacryl S-300 column. As lysozyme was incubated with dextran, significant shifts in the protein peak from a smaller to a higher molecular fraction were observed, suggesting that lysozyme was covalently attached to dextran to form the lysozyme–dextran conjugate. It is assumed from elution patterns that lysozyme was effectively linked with dextran by incubating for 2 weeks. The higher molecular weight fractions were collected and used as samples in the experiments.

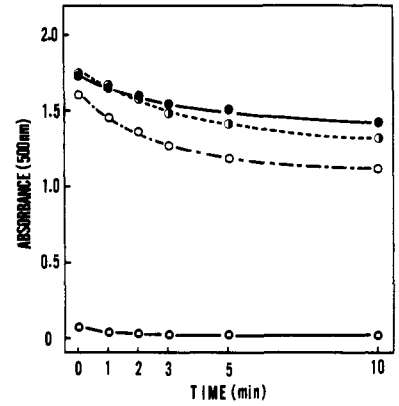

Figure 2. Emulsifying properties in 1/15 M phosphate buffer (pH 7.4) of lysozyme–dextran conjugate purified by gel filtration. (○) 1-week-incubated conjugate; (●) 2-week-incubate conjugate; (◑) 3-week-incubated conjugate; (◉) native lysozyme.

Table I. Binding Ratio of Dextran to Lysozyme and Free Amino Groups in Lysozyme–Dextran Conjugate

	native lysozyme	conjugate	
		lysozyme	dextran
binding ratio			
weight		1	9.4
molar		1	1.8
free amino groups (number per mole)	7	4.8	

Figure 2 shows the emulsifying properties of the lysozyme–dextran conjugates incubated at 60 °C for 1–3 weeks under 78.9% relative humidity. The turbidity of emulsion is plotted as ordinate and standing time after emulsion formation as abscissa. The emulsifying activity of the conjugates was about 30 times that of native lysozyme. The 2-week-incubated conjugate possessed an additional characteristic, more stable emulsion formation, compared with the 3-week-incubated conjugate. From these results, the 2-week-incubated conjugate was used for further experiments.

Table I shows the binding mode of lysozyme–dextran conjugate. The molar ratio was calculated by assuming that the molecular weight of dextran is 75 000. From the binding weight ratio about two molar dextrans appear to link to one molar lysozyme. To get further information on the binding mode, the measurement of free amino groups of the conjugate was carried out, as shown in Table I. Two free amino groups per molecule were decreased in the conjugate. This result is consistent with the binding ratio of lysozyme–dextran conjugate estimated from the binding weight ratio between lysozyme and dextran, as described above. It was reported in a previous paper (Kato et al., 1991) that the average molecular weight of the lysozyme–dextran conjugate was 150 000 in the presence of SDS. This suggests that lysozyme binds with dextran through only one reducing end group in the polysaccharide and amino groups in the protein without polymerized network structure by Maillard reaction.

The lysozyme activity of the conjugate was measured by both lytic activity of *M. lysodeikticus* and glycolysis of glycol chitin. Table II indicates that the conjugate still reveals about 80% of its enzymatic activity when glycol chitin was used as a substrate. On the other hand, the lytic activity of the conjugate decreased to 13.3% of the

Table II. Enzymatic Activity and Heat Stability of Native Lysozyme and Lysozyme–Dextran Conjugate

lysozyme	enzymatic activity, %, with substrate of glycol chitin	enzymatic activity, %, with substrate of *M. lysodeikticus*	heat stability[a] (half-life), min
native	100.0	100.0	15
conjugate	79.8	13.3	40

[a] Half-life was represented as the time when lytic activity lowered to 50% on heating at 80 °C.

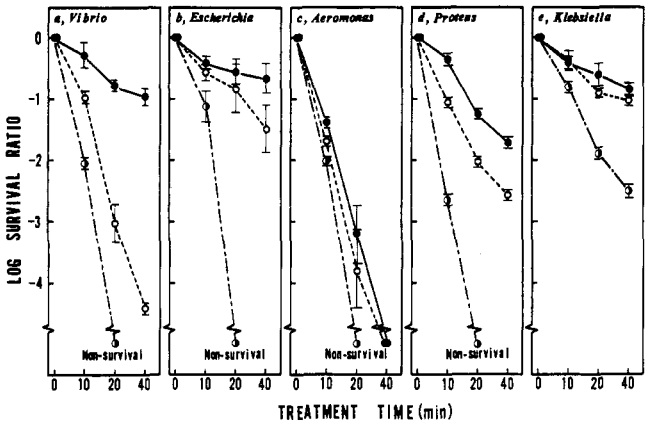

Figure 3. Antimicrobial activity of lysozyme–dextran conjugate for five laboratory cultured Gram-negative bacteria. (a) *V. parahaemolyticus* IFO 13286; (b) *E. coli* IFO 12713; (c) *A. hydrophila* IFO 13286; (d) *P. mirabilis* IFO 12668; (e) *K. pneumoniae* IFO 14438. (●) Control (medium without the addition of lysozyme or conjugate); (○) native lysozyme; (◑) lysozyme–dextran conjugate. Vertical lines represent standard deviations of the mean.

relative activity when *M. lysodeikticus* was used as substrate. The heat stability of lysozyme was enhanced by the conjugation with dextran. The half-life period when 50% of the lysozyme activity remained after heating at 80 °C was 40 min for lysozyme–dextran conjugate, while it was 15 min for native lysozyme.

Antimicrobial effects of the conjugate were investigated for five Gram-negative bacterial strains that were food-poisoning bacteria. Figure 3 shows the survival cell numbers in the presence of free lysozyme or lysozyme–dextran conjugate combined with heating at 50 °C. The log survival ratio means survival decimal fraction based on the log values. The possibility of heat injury for each bacteria has been tested in all experiments. Solid circles in Figure 3 indicate the proliferating cell numbers in control medium without lysozyme and lysozyme–dextran conjugate. Compared to control medium, the medium containing lysozyme–dextran conjugate reveals stronger antimicrobial effects. Four strains, all except *K. pneumoniae*, did not survive after 20 min of incubation at 50 °C in the presence of lysozyme–dextran conjugate. Although all tested strains were slightly affected by heating at 50 °C in a control medium without lysozyme or lysozyme–dextran conjugate, the lethal effects were induced in the presence of the conjugate.

Antimicrobial effects of the conjugate for Gram-positive bacteria were also investigated by using two typical strains (Figure 4). It is well-known that lysozyme has antimicrobial effects for Gram-positive bacteria. As expected, the antimicrobial effects of lysozyme–dextran conjugate were almost the same as those of lysozyme for *S. aureus* and were more lethal than those of lysozyme for *B. cereus*.

DISCUSSION

We have reported (Kato et al., 1990) that ovalbumin–dextran conjugate obtained by naturally occurring Maillard reaction reveals excellent emulsifying properties superior to those of commercial emulsifiers. Strikingly, lysozyme–dextran conjugate obtained by a similar method showed much higher emulsifying properties than ovalbumin–dextran conjugate. In addition, the conjugate has lytic activity, although its activity was considerably decreased by steric hindrance. Thus, we have succeeded in making a protein emulsifier having lytic activity.

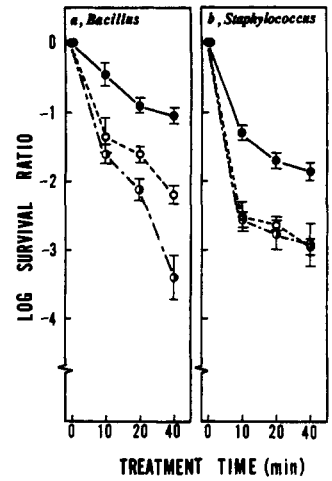

Figure 4. Antimicrobial activity of lysozyme–dextran conjugate for two Gram-positive bacteria. (a) *B. cereus* IFO 13690; (b) *S. aureus* IFO 14462. (●) Control (medium without lysozyme or conjugate); (○) native lysozyme; (◑) lysozyme–dextran conjugate. Vertical lines represent standard deviations of the mean.

The cell envelope of Gram-negative bacteria contains significant amounts of lipopolysaccharide and lipoprotein in association with the thin peptidoglycan layer. The layer of lipid material outside the peptidoglycan is called the outer membrane. The enzymatic breakdown of the peptidoglycan layer within the cell envelope is prevented by the outer membrane.

The antimicrobial effects of lysozyme–dextran conjugate for the Gram-negative bacteria may come from the excellent surfactant properties. It is well-known that the outer membrane was solubilized with sodium deoxycholate in the presence of NaCl and EDTA (Nakamura and Mizushima, 1975); 1% SDS solubilizes the purified outer membrane isolated from *E. coli* K-12 without any sediments (Sekizawa and Fukui, 1973). DePamphilis (1971) reported that dissociated outer membrane was prepared with Triton X-100 coexisting in EDTA. This suggests that the outer membranes of Gram-negative bacterial cells were very sensitive to surface-active agents.

The antimicrobial ability of the lysozyme–dextran conjugate was strengthening in association with heating at 50 °C. Hitchener and Egan (1977) reported that about 20% of the cellular LPS was released from the outer membrane into medium during 60 min at 48 °C. It has been also reported in endotoxic experiments that LPS was liberated into the suspending fluid from a watery suspension of Gram-negative organisms by heating (Roberts, 1966). Physical stress such as heating causes considerable damage to Gram-negative bacteria including structural change and loss of viability. Therefore, the thermal stresses seem to result in synergistic effects in destroying the outer membrane by lysozyme–dextran conjugate.

Although lysozyme is known to exert antimicrobial effects only for Gram-positive bacteria, as we have seen, the conjugation of lysozyme with dextran resulted in the extension of the antimicrobial spectrum to Gram-negative bacteria. Lysozyme–dextran conjugate prepared without

the use of chemical reagents can be potentially used in formulated food or drug systems possessing novel bifunctional properties, either emulsifier or antimicrobial reagent.

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