Role of Positive Charge of Lysozyme in the Excellent Emulsifying Properties of Maillard-Type Lysozyme–Polysaccharide Conjugate

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Maillard-type lysozyme–polysaccharide conjugates revealed the best emulsifying properties among various protein–polysaccharide conjugates. To elucidate the mechanism of the excellent emulsifying properties of Maillard-type lysozyme–polysaccharide conjugate, the emulsifying properties of the galactomannan conjugate with α-lactalbumin having extensive structural homology with lysozyme were compared to those of lysozyme–galactomannan conjugate. The lysozyme and α-lactalbumin were attached two and three galactomannans per mole of protein, respectively, through Maillard reaction in a dry state at 60 °C under 79% relative humidity for 2 weeks. The emulsion stability of α-lactalbumin was not so remarkably improved by conjugating with galactomannan as that of lysozyme, although the emulsifying activities of both protein–galactomannan conjugates were almost the same. The emulsion stability of lysozyme–galactomannan conjugates was greatly decreased with the extent of the acetylation of lysyl residues. These results suggest that the electrostatic repulsion of positive charge in the lysozyme molecule is attributed to the excellent emulsifying properties of lysozyme–galactomannan conjugate.

Keywords: *Lysozyme; α-lactalbumin; Maillard-type lysozyme–polysaccharide conjugate*

INTRODUCTION

We reported that protein–polysaccharide conjugates prepared by spontaneous Maillard reaction in a controlled dry state revealed dramatic improvements of the functional properties of proteins, such as solubility, heat stability, and emulsifying properties (Kato et al., 1990, 1991, 1992, 1993). The branched polysaccharides such as dextran and galactomannan efficiently improved the functional properties of proteins by the formation of protein–polysaccharide conjugates through a naturally occurring Maillard reaction between the ϵ-amino groups in the protein and the reducing-end carbonyl residue in the polysaccharide under a controlled condition at 60 °C and 65% or 79% relative humidity, while the straight-chain polysaccharides did not improve the functional properties of proteins. In addition to the types of branched or straight chains, the length of polysaccharides also affects the functional properties of protein-saccharide conjugates. The importance of the length of polysaccharide chains on the improvement of functional properties of protein–polysaccharide conjugates was evidenced by using oligomannosyl and polymannosyl lysozymes constructed by genetic modification (Nakamura et al., 1993). Polymannosyl lysozyme showed much better functional properties than oligomannosyl lysozyme. Thus, a considerable amount of informations on the polysaccharide have been obtained to prepare functional protein–polysaccharide conjugates. However, there is little information on the kinds of proteins to exhibit better functional properties of protein–polysaccharide conjugates. Among various Maillard-type protein–polysaccharide conjugates, lysozyme–polysaccharide conjugate revealed the best emulsifying properties (Nakamura et al., 1991, 1992). Thus, to elucidate why lysozyme exhibits the best emulsifying properties by conjugation with polysaccharide, we tried to compare the emulsifying properties of α-lactalbumin– and lysozyme–polysaccharide conjugate, because these two enzymes are homologous protein with closely similar three-dimensional structure (Browne et al., 1969) except their isoionic points. In addition, the effect of net charge in lysozyme was examined to clarify the mechanism of excellent emulsifying properties of lysozyme–galactomannan conjugate.

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MATERIALS AND METHODS

Materials. Lysozyme was purified by crystallizing from fresh egg white at pH 9.5 in the presence of 5% sodium chloride and recrystallized five times. Bovine α-lactalbumin was supplied from Sigma Chemical Co. These proteins were desalted by dialysis prior to use and then freeze-dried. A galactomannan preparation was obtained by dialyzing a mannase hydrolysate of guar gum (average molecular weight of 15 000) from Taiyo Chemicals Co. Sephadex G-50 was purchased from Pharmacia LKB. All other chemicals were of analytical grade.

Preparation of Protein Conjugates with Galactomannan. Protein and galactomannan were mixed in water at the weight ratio of 1:1 and then lyophilized. The powder mixture spread over Petri dish and was incubated at 60 °C for 2 weeks under the relative humidity of 79%, as previously described (Nakamura et al., 1991). The resulting protein–polysaccharide conjugates were separated from the unreacted protein by gel filtration using a Sephadex G-50 gel filtration column (1.1 × 45 cm) equilibrated and eluted with 50 mM acetic acid–sodium acetate buffer (pH 5.0). The lysozyme–galactomannan conjugate was purified by a cation-exchange chromatography on a CM-Toyopearl column (2 × 10 cm). The adsorbed conjugates on a CM-Toyopearl equilibrated with 0.02 M phosphate buffer (pH 7.0) were eluted with the same buffer containing 0.3 M Nacl. The α-lactoalbumin–galactomannan conjugate was purified by an anion-exchange chromatography on a DEAE-Toyopearl column (2 × 10 cm). All fractions containing protein-galactomannan conjugate were collected together, dialyzed against deionized water, and then lyophilized. The conjugates thus obtained were stored at 4 °C.

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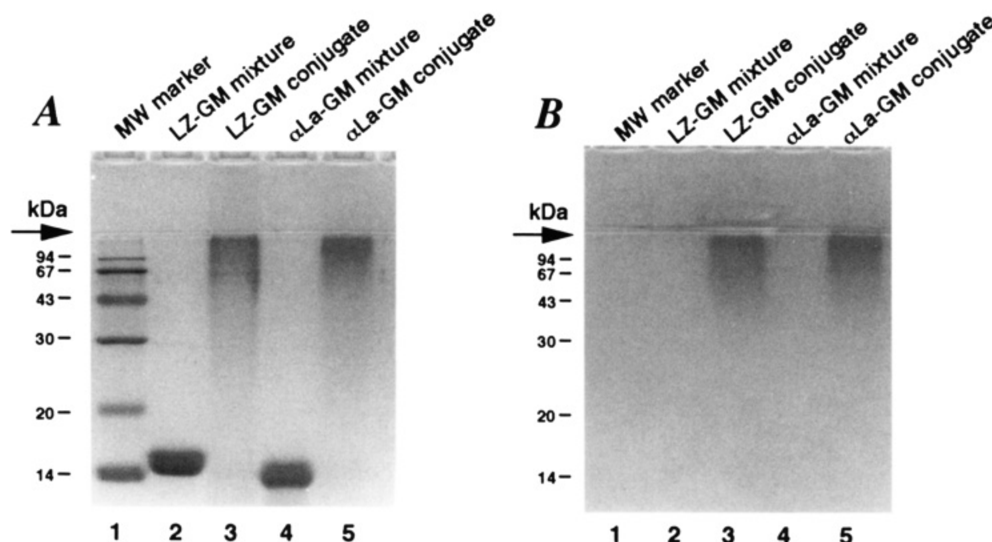

Figure 1. SDS–slab polyacrylamide gel electrophoretic patterns of the purified lysozyme– and α-lactalbumin–galactomannan conjugates. The gel sheets were stained for proteins an carbohydrates with Coomassie Brilliant Blue (*panel A*) and periodic acid–Fuchsin (*panel B*), respectively. Arrows indicate the position of the boundary between the stacking (*upper*) and separating (*lower*) gels. *Lane 1*, molecular weight markers; *lane 2*, powder mixture of lysozyme and galactomannan; *lane 3*, lysozyme–galactomannan conjugate; *lane 4*, powder mixture of α-lactalbumin and galactomanan; *lane 5*, α-lactalbumin–galactomannan conjugate.

Table 1. Binding Modes of Lysozyme– and α-Lactalbumin–Galactomannan Conjugates

	binding ratio[a]		
	wt ratio	molar ratio	free amino groups[a]
native lysozyme (LZ)			7
LZ–galactomannan conjugate			5.3
LZ:galactomannan	1:1.8	1:1.7	
native α-lactalbumin (αLa)			13
αLa–galactomannan conjugate			10.2
αLa:galactomannan	1:2.9	1:2.7	

[a] The data represent the means of four determinations.

Binding Ratio of Galactomannan to Proteins. The binding ratio of galactomannan to protein was determined from the content of protein calculated from the absorption spectrum at 280 nm and galactomannan calculated from the absorbance at 490 nm after color development with the phenol–sulfuric acid reaction.

Electrophoresis in SDS–Slab Polyacrylamide Gel. SDS–slab polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) using 15% acrylamide separating gel and 5% stacking gel containing 0.1% SDS. Samples were heated at 100 °C for 5 min in Tris–glycine buffer (pH 8.8) containing1% SDS and 1% β-mercaptoethanol. Electrophoresis was carried out at a constant current of 10 mA for 5 h using an electrophoretic buffer of Tris–glycine containing 0.1% SDS. After electrophoresis, the gels were stained for proteins and carbohydrates with 0.025% Coomassie Brilliant Blue R solution and 0.5% periodic acid–Fuchsin solution, respectively.

Peptide Mapping of Lysozyme–Galactomannan Conjugate. To determine the site of binding of the polysaccharide chain into lysozyme, peptide mapping of the lysozyme–galactomannan conjugate was performed. The trypsin digestion was carried out by using the suspension of reduced S-2-aminoethylated native lysozyme and the lysozyme–galactomannan conjugate, at 1% concentration in distilled water as described by Okazaki et al. (1985). The enzymatic digestion was allowed to proceed for 120 min. The analysis of the tryptic hydrolysates was carried out by the reversed-phase high-performance chromatography. A TSKgel ODS-120T column (4 × 200mm, Tosoh, Japan) was attached to a Hitachi 655-A-11 liquid chromatography equipped with a Hitachi 655-A LC detector. The column was equilibrated with 1% acetonitrile containing 0.1% concentrated HCl. Twenty microliters of tryptic hydrolysates containing 50 mg of peptides was injected, and the column was eluted with a gradient of 40 mL of 1% acetonitrile containing 0.1% concentrated HCl and 40 mL of 40% acetonitrile containing 0.1% concentrated HCl at a flow rate of 0.4 mL/min for 200 min. Peptide elution was monitored by absorbance at 210 nm. Peptide peaks were collected and used for the measurement of carbohydrate.

Measurement of Emulsifying Properties. Emulsifying activity and emulsion stability were estimated according to the modified method of Pearce and Kinsella (1978). An emulsion was prepared by homogenizing the mixture of 1.0 mL of corn oil and 3.0 mL of a 0.1% sample solution in a Polytron homogenizer PT 10-35 (Kinematica Co., Switzerland) 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 standing for 0, 1, 2, 3, 5, and 10 min and diluted with 5.0 mL of 0.1% sodium dodecyl sulfate solution. The absorbance of the diluted emulsion was then measured at 500 nm. The relative emulsifying activity was represented as the absorbance at 500 nm measured immediately after emulsion formation (0 min). The emulsion stability was estimated by measuring the half-life of the decay of emulsion, calculating from turbidity curves of emulsion during a standing time of 40 min.

Preparation of Acetylated Lysozyme–Galactomannan Conjugate. Five percent (w/v) concentration of lysozyme–galactomannan conjugate in half-saturated sodium acetate was acetylated by titrating with anhydrous acetic acid of 4-, 7-, and 40-fold volume (molar ratio). The solutions were kept on ice for 1 h at pH 7.5 with 1 N NaOH during the reaction by the addition of the reagent. The resulting products were dialyzed against deionized water and then lyophilized.

Determination of Free Amino Groups. The content of free amino groups in the acetylated lysozyme–galactomannan conjugates was determined according to the method of Haynes et al. (1967) using a specific reagent for amino groups, trinitrobenzenesulfonate.

RESULTS AND DISCUSSION

Figure 1 shows SDS–slab polyacrylamide gel electrophoretic patterns of lysozyme– and α-lactalbumin–galactomannan conjugates. The electrophoretic patterns indicated that galactomannan was covalently linked with α-lactalbumin in a similar manner as lysozyme through Maillard reaction at 60 °C under 79% relative humidity for 2 weeks. The galactomannan conjugates with lysozyme (lane 3) and α-lactalbumin (lane 5) showed the broader and higher molecular size bands in both protein and carbohydrate stains near the boundary between stacking and separating gels, indi-

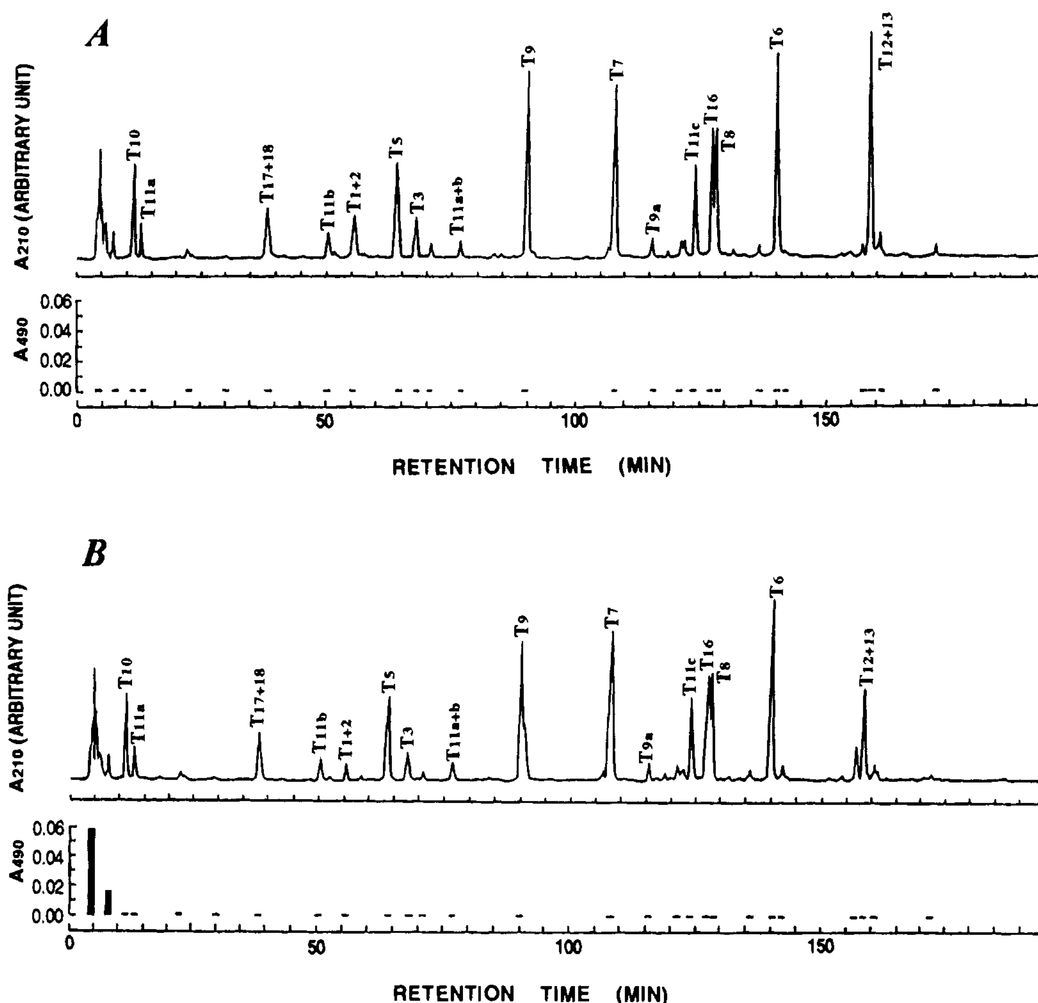

Figure 2. Reversed-phase HPLC elution patterns of tryptic digests of native lysozyme (*panel A*) and lysozyme–galactomannan conjugate (*panel B*). The bottom of panel B represents the absorbance at 490 nm to monitor the color development by the phenol–sulfate method for carbohydrate in each peak.

cating a polydispersed high molecular weight distribution. The broad molecular weight distribution of protein–polysaccharide conjugates are attributed to the polydispersed property of the galactomannan used in the experiment and the heterogeneity of the numbers of the binding of polysaccharide to proteins. Binding ratios of the polysaccharide to proteins were calculated on the assumption that the molecular weights of lysozyme, α-lactalbumin, and galactomannan were 14 300, 14 000 and 15 000, respectively. On the basis of the binding weight ratio, about 2 mol of galactomannan appear to link to one molecule of lysozyme, while about 3 mol of galactomannan to α-lactalbumin as shown in Table 1. These results were further confirmed by the measurement of free amino groups. About 2 and 3 mol of α- or ϵ-amino groups were decreased by the formation of lysozyme– and α-lactalbumin–galactomannan conjugates, respectively (Table 1). Maillard-type protein–polysaccharide conjugates are formed between the ϵ-lysyl amino groups in protein and the reducing-end carbonyl group in polysaccharide through Schiff base. The subsequent Maillard reaction is suppressed by the storage in low temperature after the formation of the conjugates. In addition, the steric hindrance occurs around the ϵ-lysyl amino groups in the molecular surface of proteins. Therefore, a limited number of polysaccharide seems to be attached to protein molecule.

The binding sites of galactomannan to lysozyme were determined by using peptide mapping with tryptic hydrolysate of reduced and S-2-aminoethylated lysozyme–galactomannan conjugate. Figure 2 shows the reversed-phase high-performance liquid chromatography elution patterns of tryptic peptides from native lysozyme (panel A) and lysozyme–galactomannan conjugate (panel B). The elution pattern of tryptic peptides of lysozyme–galactomannan conjugate was almost similar to that of native lysozyme. As shown in the bottom of the figure (panel B), the tryptic hydrolysate of lysozyme–galactomannan conjugate contained significant amounts of carbohydrate in void volume fractions and significantly decreased the peaks (T1+2) and (T12+13) corresponding to the peptide fragments 1–5 and 97–112, respectively. These results indicate that the amino terminal Lys-1 and Lys-97 in lysozyme are most susceptible nucleophiles for the polyglycosylation through the naturally occurring Maillard reaction. Although the binding sites of polysaccharide with α-lactalbumin was not identified, the same sites (N-terminal and Lys-97) may be involved in their binding because of the homology of tertiary structure and the similarity of the sequence around the position 97 lysyl residue. Another binding site is presumed to be Lys-13 in α-lactalbumin, because this site is not masked by C-terminal residue unlike lysozyme. Further studies are carrying out to identify the binding site of polysaccharide in α-lactalbumin.

Figure 3 shows the emulsifying properties of lysozyme- and α-lactalbumin–galactomannan conjugates. The turbidity of emulsion is plotted as ordinate and standing

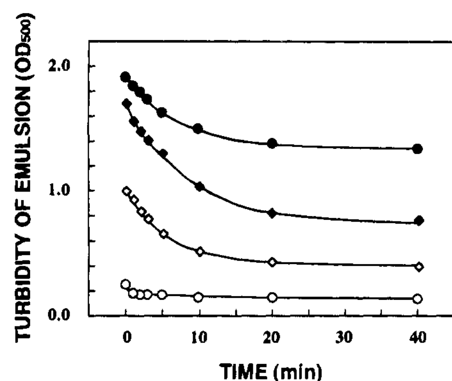

Figure 3. Comparison of emulsifying properties between lysozyme–galactomannan conjugate and α-lactalbumin–galactomannan conjugate in neutral pH system (1/15 M sodium phosphate buffer, pH 7.4). (○) Native lysozyme; (●) lysozyme–galactomannan conjugate; (◇) native α-lactalbumin; (◆) α-lactalbumin–galactomannan conjugate.

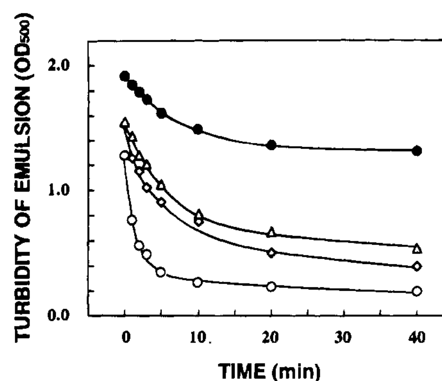

Figure 4. Effects of the acetylation of lysozyme in the conjugate on th emulsifying properties of lysozyme–galactomannan conjugate in neutral pH system (1/15 M sodium phosphate buffer, pH 7.4). (●) Lysozyme–galactomannan conjugate; (△) 68% acetylated conjugate; (◇) 85% acetylated conjugate; (○) 99% acetylated conjugate.

time after emulsion formation as abscissa. The value of the ordinate at zero time reflects the relative emulsifying activity, and the half-life of initial turbidity indicates the stability of the emulsion. By the Maillard-type conjugation with galactomannan, remarkable improvements in the emulsifying properties were observed for both lysozyme and α-lactalbumin. The emulsion stability of lysozyme–galactomannan conjugate was remarkably enhanced as well as high emulsifying activity. By contrast, the relative emulsifying activity of α-lactalbumin-galactomannan conjugate was almost the same as that of lysozyme–galactomannan conjugate, while the conjugate was not so remarkably improved as that of lysozyme–polysaccharide conjugate.

Although α-lactalbumin has a close structural similarity to hen egg white lysozyme, there is an apparent difference in the overall charge balance of these molecules between α-lactalbumin and lysozyme. The isoionic point of α-lactalbumin is about 4.5, while that of lysozyme is about 11.0. The difference in emulsifying properties of these conjugates may be due to the overall charge balance of protein molecules. It is presumed that the highly positive charge of lysozyme plays an important role in the excellent emulsion stability by lysozyme–galactomannan conjugate. To obtain further information on the excellent emulsion stabilizing properties of lysozyme–galactomannan conjugate, the effect of acetylation of free amino groups in the conjugate was investigated on the emulsifying properties. As shown in Figure 4, the acetylation of lysyl residues in the conjugate caused dramatically the lower of emulsifying properties of lysozyme–galactomannan conjugate in proportion to the extent of acetylation. This result indicates that positive charge of lysozyme molecule is contributed to keep the stable emulsion produced by lysozyme–galactomannan conjugate.

It is assumed that the hydrophobic residues of protein moiety partially denatured during emulsion formation at the oil–water interface may be anchored to the surface of oil droplets in emulsion, whereas the hydrophilic residues of the extended branched polysaccharide chains oriented to water may cover oil droplets to inhibit the coalescence of oil droplets, resulting in the stable emulsion. In addition to the explanation for the mechanism for enhanced emulsion stability of protein–polysaccharide conjugates, it is also revealed that the electrostatic repulsion of positive charge in the lysozyme is attributed to the excellent emulsion stability by the lysozyme–galactomannan conjugate. Thus, the most excellent emulsifying properties of lysozyme–galactomannan conjugate among various protein–polysaccharide conjugates seems to be due to the cationic electrostatic repulsion in lysozyme.

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