Double-Glycosylated Lysozyme at Positions 19 and 49 Constructed by Genetic Modification and Its Surface Functional Properties

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Complementary DNA encoding hen egg white lysozyme (HEWL) was subjected to site-directed mutagenesis to introduce two N-linked glycosylation sites (Asn^{19}-Try^{20}-Thr^{21} and Asn^{49}-Ser^{50}-Thr^{51}) into both positions 19 and 49 by substituting Arg-21 with Thr and Gly-49 with Asn, respectively. The double-glycosylated lysozyme R21T/G49N was expressed in *Saccharomyces cerevisiae* carrying the yeast expression plasmid inserted the double-mutant HEWL cDNA. The mutant lysozyme mainly secreted a polymannosyl form with a small amount of two oligomannosyl forms. The polymannosyl lysozyme R21T/G49N was glycosylated at two positions, 19 and 49, with a polymannosyl and an oligomannosyl chain. The lengths of the polymannosyl and oligomannosyl chains attached to R21T/G49N were approximately 272 and 18 mannose residues, respectively. The R21T/G49N showed better emulsifying properties than two types of single-polymannosyl lysozymes R21T and G49N. With regard to single-polymannosyl lysozyme, G49N showed somewhat better emulsifying properties than R21T. In addition, the cleavage of polymannosyl chain from lysoyme with endo-β-*N*-acetylglucosaminidase resulted in a dramatic decrease in the emulsifying properties of polymannosyl lysozymes.

Keywords: *Lysozyme; yeast expression system; genetic modification; polymannosylation; double glycosylation; emulsifying properties*

INTRODUCTION

We have reported that protein−polysaccharide conjugates can be prepared through a naturally occurring Maillard reaction in a dry state without the use of any chemical reagents (Kato et al., 1990, 1992a). Maillard-type lysozyme−polysaccharide conjugates have shown excellent emulsifying properties superior to those of commercial emulsifiers (Nakamura et al., 1992; Kato et al., 1993). To elucidate the molecular mechanism of the dramatic improvement in the functional properties of protein by glycosylation, we were successful in constructing polymannosyl lysozymes by genetic engineering. The single polymannosyl lysozymes R21T and G49N, which were subjected to site-directed mutagenesis to have the N-linked glycosylation site Asn-X-Ser/Thr at positions 19 and 49 by substituting Arg-21 and Gly-49 with Thr and Asn, respectively, were secreted in a yeast expression system (Kato et al., 1994; Nakamura et al., 1993a). The most striking aspect was the attachment of a polymannosyl chain consisting of ≈300 mannose residues. As expected, the polymannosyl lysozymes showed excellent emulsifying properties superior to those of commercial emulsifiers (Nakamura et al., 1993b). The effect of the length of polysaccharide chains on the emulsifying properties of lysozyme−polysaccharide conjugates was investigated using Maillard-type lysozyme−polysaccharide and lysozyme−oligosaccharide conjugates (Shu et al., 1996). The results suggested that the emulsifying properties of lysozyme−polysaccharide conjugates increased in proportion to the length of polysaccharide chains. Thus, it has been demonstrated that the length of the polysaccharide chain is critical for improving the emulsifying properties of proteins. However, the effect of the number of glycosylation sites in protein on the emulsifying properties has not been elucidated yet, because it is difficult to control the binding sites and number in chemical reactions such as the Maillard reaction. This is possible only through genetic modification of protein. In the present paper, to investigate the effects of binding sites and number of polysaccharide chains on the emulsifying properties of lysozyme−polysaccharide conjugates, the double-glycosylated mutant lysozyme R21T/G49N was constructed to create the N-linked glycosylation sites at positions 19 and 49. The emulsifying properties of the double-glycosylated lysozyme R21T/G49N were compared with those of single-polymannosyl lysozymes R21T and G49N. In addition, to assess the effects of polymannosyl chains on the emulsifying properties, polymannosyl chains were removed from the double-glycosylated lysozyme through treatment with endo-β-*N*-acetylglucosaminidase (Endo-H), and its emulsifying properties were compared with those of intact polymannosyl lysozymes.

MATERIALS AND METHODS

Materials. Lysozyme was crystallized from fresh hen egg white at pH 10.0 in the presence of 5% sodium chloride and recrystallized five times. Sephadex G-75 gel was purchased from Pharmacia Biotech Co. (Uppsala, Sweden), and CM-Toyopearl 650M resin was from Tosoh (Tokyo). Endo-H (15 units/mL) was obtained from Seikagaku Kyoto Co. (Japan),

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and hydrazine anhydrous was from Nacalai Tesque Kyoto Inc. (Japan). The yeast expression plasmid pYG-100 was supplied from Dr. K. Matsubara, Osaka University. The recombinant plasmid pKK-1, which contains a full-length hen egg white lysozyme cDNA (Kumagai and Miura, 1989), was provided by Dr. I. Kumagai, University of Tokyo. T_4 DNA ligase, alkaline phosphatase, and restriction enzymes were purchased from Takara Shuzo Co. (Japan). The oligonucleotide-directed in vitro mutagenesis system (version 2) for site-directed mutagenesis was from Amersham Co. (Japan). All other chemicals were of analytical grade for biochemical use.

Construction of Double-Glycosylated Lysozyme cDNA. The *Eco*RI/*Hin*dIII fragment of lysozyme cDNA from pKK-1 plasmid was subcloned into the *Eco*RI/*Hin*dIII sites of the bacteriophage vector M13mp19. The mutant lysozyme cDNA was constructed in M13mp19 vector by the Amersham oligonucleotide-directed mutagenesis system. Two mutagenetic oligonucleotide primers, 5′-GGACTTGATAACTATACGGGATACAGCCTG-3′ (R21T) and 5′-AACACCGATAACAGTACCGA-3′ (G49N) were synthesized according to the phosphoamidate method using a Pharmacia DNA synthesizer. They were used to convert Arg-21 (CGG) and Gly-49 (GGG) codons to Thr (ACG) and Asn (AAC), respectively, to introduce two N-linked glycosylation sites (Asn-X-Ser/Thr) onto the lysozyme molecular surface. The presence of the mutations was confirmed by dideoxy DNA sequencing analysis (Sanger et al., 1977). The double-mutant lysozyme cDNA was inserted into the *Sal*I site of yeast expression plasmid pYG-100 to construct the expression plasmid pYGKK-1 of the double-mutant lysozyme R21T/G49N, as described previously (Kumagai and Miura, 1989; Kato et al., 1992b).

Expression of the Mutant HEWLs. The expression plasmid pYGKK-1 was transformated into *S. cerevisiae* AH22 (*MAT*a, *Leu*2, *His*4, *Cir*$^{\pm}$) according to the lithium acetate procedure (Itoh et al., 1983). Leu^{+} transformants were screened by subculturing in the modified Burkholder minimum medium (Toh-e and Wickner, 1981) plates supplemented with histidine (20 μg/mL) at 30 °C. After cultivation, the colonies that appeared were replica-cultivated in the yeast medium on a small scale (5 mL) and the overexpression subcolonies with the highest levels of lysozyme activity were screened. The overexpression colonies were directly subcultured on a large scale in the yeast minimum medium at 30 °C for 5 days.

Purification of Polymannosyl Lysozymes. Six liters of growth medium of host cells were centrifuged at 6000*g* at 4 °C for 15 min. The supernatant was directly applied to a CM-Toyopearl 650M cation ion exchange opened column (1.8 × 5 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and then the column was washed with the same buffer until the washing solution was free from protein. The adsorbed polymannosyl lysozyme was eluted with a linear gradient elution from 0 to 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 7.5). All fractions containing glycoprotein were collected and dialyzed against deionized water at 4 °C for 2 days to remove salt. The fractions were subsequently purified by gel filtration on a column of Sephadex G-75 (1.2 × 60 cm) equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.5). Purified polymannosyl lysozyme was lyophilized and used for the next experiments.

Enzymatic Assay. The hydrolytic activity of lysozyme was measured according to the method of Imoto et al. (1971) using ethylene glycol chitin as substrate. After its absorbance at 280 nm had been adjusted to 0.05, 0.5 mL of assay lysozyme solution in 10 mM acetic acid/sodium acetate buffer (pH 6.0) was added to 1.0 mL of 0.05% ethylene glycol chitin solution. The mixture was incubated at 40 °C for 40 min, and 2 mL of the color reagent (made by dissolving 0.5 g of potassium ferricyanide in 1 L of 0.5 M sodium cabonate) was added into the reaction solution. The mixture was immediately boiled for 15 min to estimate the reducing power resulting from hydrolysis of ethylene glycol chitin at 420 nm with a Hitachi U-2001 spectrophotometer. The amount (micromoles per minute) of reducing groups produced by the action of lysozyme was defined as 1 unit.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was conducted according to the method of Laemmli (1970) using a 15% acrylamide running gel and 5% stacking gel containing 1% SDS. After electrophoresis, gels were stained for protein and carbohydrate with 0.025% Coomassie Brilliant Blue R-250 and 0.5% periodic acid/Fuchsin solutions, respectively.

Endo-H Treatment. The double-glycosylated lysozyme (2.0 mg/mL) was boiled for 5 min in 50 mM sodium citrate buffer (pH 6.0) containing 0.1% SDS and 200 μg/mL phenylmethanesulfonyl fluoride. After cooling, an equal volume of Endo-H (0.1 unit/mL) in 50 mM sodium citrate buffer (pH 6.0) was added and sequentially incubated at 37 °C for 20 h. After incubation, samples were analyzed by SDS−PAGE.

Chemical Analysis. Total hexose content of the double-glycosylated lysozyme (R21T/G49N) was estimated using the phenol−sulfuric acid reaction (Dubois et al., 1956) employing mannose as a standard. The hexoses liberated from glycosylated lysozyme were identified by HPLC after hydrolysis with 2 N HCl at 100 °C for 3 h in a sealed glass ampule. The hydrolysates were dried, dissolved in water, and chromatographed on an Asahipak NH2P-50 column (Asahi Chemical, Tokyo) in 75% acetonitrile using the Hitachi HPLC system equipped with a refractive index (RI) detector. For the analysis of hexosamine, the samples issued from hydrolysates with 3 N HCl at 100 °C for 4 h were dried and analyzed using an amino acid analyzer (Tosoh, HLC 805).

Isolation of Polymannosyl Chains. The method of Takasaki et al. (1982) was employed to isolate polymannosyl chains from R21T/G49N after slight modification. The polymannosyl lysozyme powder was put into a tube and dried at 40 °C for 2 h, and 2 mL of pure hydrazine anhydrous was added into the tube. The tube was then sealed and kept at 100 °C for 10 h to digest the protein moiety of the polymannosyl lysozyme. After cooling, the treated solution containing polymannosyl chains was dialyzed against deionized water at 4 °C for 3 days using dialysis tubes with a molecular weight cutoff of 3500 to remove the amino acids digested from lysozyme. The dialyzed solution was collected, lyophilized, and used for the measurement of the molecular weight of the polymannosyl chain.

Cleavage of Polymannosyl Chain from Lysozyme with Endo-H. The method of Yamamoto et al. (1987) was used to release the polymannosyl chains from lysozyme with Endo-H, which cleaves between *N*-acetylglucosamine residues without SDS treatment. One milligram of polymannosyl lysozyme was incubated at 37 °C for 20 h with 1.5 units of the Endo-H in a 0.1 mL reaction mixture containing 100 mM acetate buffer (pH 6.0). After analysis by SDS−PAGE, the reaction solution was then diluted to 3 mL with 1/15 M phosphate buffer (pH 7.4) and directly used for measuring the emulsifying properties.

Determination of the Molecular Weight Using Low-Angle Laser Scattering Technique. The molecular weight of the sample was determined according to the low-angle laser scattering method (Kato et al., 1992c). The solution of sample (0.1%) in 200 mM sodium phosphate buffer (pH 6.9) was applied to a high-performance gel chromatography system connected with a TSK gel G3000SW column (Tosoh, 0.75 × 60 cm). The column was equilibrated and eluted with the same buffer at a flow rate of 0.3 mL/min. The elution from the column was monitored using a low-angle laser light scattering photometer (Tosoh, LS-8) and a precision differential refractometer (Tosoh, RI-8). The molecular weight of samples (M_r) was estimated from the ratio of the height of the peak from the low-angle laser light scattering photometer (LS) to that of the refractometer (RI), according to the equation

$$M_r = M_{std}(dn/dc)_{std}(LS/RI)_{sam}/(dn/dc)_{sam}(LS/RI)_{std} \quad (1)$$

where M_{std} is the molecular weight of standard protein, $(dn/dc)_{std}$ is the refractive index increment of standard protein, $(dn/dc)_{sam}$ is the refractive index increment of samples, $(LS/RI)_{std}$ is the ratio of the top peak of the low-angle laser scattering to that of the refractometer of standard protein, and $(LS/RI)_{sam}$ is that of samples. Ovalbumin (45 kDa) was used as a standard.

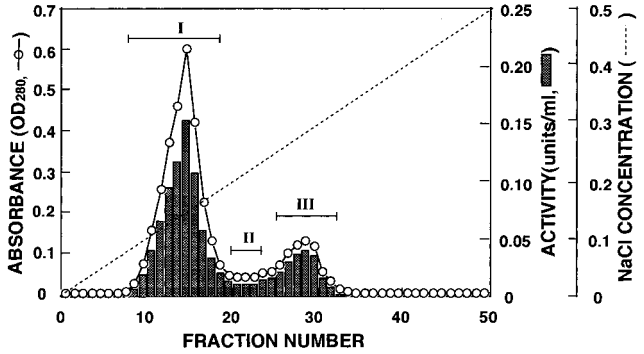

Figure 1. Isolation of mutant lysozyme secreted from *S. cerevisiae* carrying R21T/G49N lysozyme cDNA with CM-Toyopearl column. The lysozyme secreated in the yeast medium was adsorbed to a CM-Toyopearl column. The adsorbed lysozyme was eluted with a linear gradient elution from 0 to 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 7.5). Fractions indicated by horizontal lines were pooled and used for the next experiments.

Measurement of Emulsifying Properties. Emulsifying properties were determined according to the modified method of Pearce and Kinsella (1978). An emulsion was formed by homogenizing a mixture of 1.0 mL of corn oil and 3.0 mL of sample solution in 1/15 M sodium phosphate buffer (pH 7.5) using a Polytron homogenizer PT 10-35 (Kinematica Co., Switzerland) at 12 000 rpm at 20 °C for 1 min; 100 μL of the emulsion was taken from the bottom of the test tube at various times (0, 1, 2, 3, 5, and 10 min) and diluted with 5 mL of a 0.1% SDS solution. The absorbance of the diluted emulsion was then measured at 500 nm. The relative emulsifying activity was determined from the turbidity measured immediately after the emulsion was formed (0 min). The emulsion stability was also estimated by measuring the half-time of the turbidity detected immediately after the emulsion had formed.

RESULTS AND DISCUSSION

Expression and Purification of Double-Glycosylated Lysozyme R21T/G49N. The mutant lysozyme R21T/G49N secreted into the culture medium of *S. cerevisiae* was purified on a column of CM-Toyopearl cation ion exchange chromatography (Figure 1). The first main fractions (peak I) were eluted between 0.12 and 0.2 M NaCl solution, and the second and third fractions were further separated into two peaks (II and III) that have higher lytic activity. Although peak I did not show lytic activity for *M. lysodeikticus*, it did show hydrolysis activity for glycol chitin. This may be due to the steric hindrance of polysaccharide attached to lysozyme. Figure 2 is the SDS−PAGE analysis of peaks I−III. Peak I appeared as a high molecular weight band (near the boundary) between stacking and running gels. This suggests that peak I is a polymannosyl form of R21T/G49N as shown in polymannosyl lysozymes G49N and R21T (Nakamura et al., 1993a; Kato et al., 1994). The presence of the two bands (17 and 20 kDa) in peak II suggests that two types of oligomannosyl lysozymes were also secreted in small amount. Peak III seems to be a nonglycosylated form of lysozyme. To confirm the type of glycosylation, the polymannosyl form of R21T/G49N (peak I) was treated with Endo-H. As shown in Figure 3A, the molecular band of the polymannosyl lysozyme was completely diminished by the digestion with Endo-H. In addition, the SDS−PAGE patterns stained with the carbohydrate-specific reagent, periodic acid−Schiff solution, showed only one broad band near the boundary between the stacking and running gels

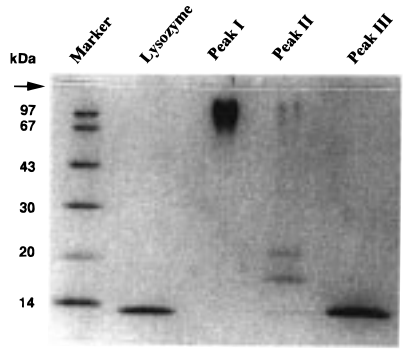

Figure 2. SDS−PAGE patterns of each peak of mutant lysozyme R21T/G49N. The gel was stained for protein with Coomassie Brilliant Blue. The arrow indicates the boundary between the stacking (upper) and separating (lower) gel. Markers: phosphorylase *b* (94 000); bovine serum albumin (67 000); ovalbumin (43 000); carbonic anhydrase (30 000); trypsin inhibitor (20 100); α-lactalbumin (14 300) as molecular weight markers; lysozyme, native lysozyme (from fresh hen egg white, MW 14 300). Peaks I−III were collected by CM-Toyopearl column chromatography in Figure 1.

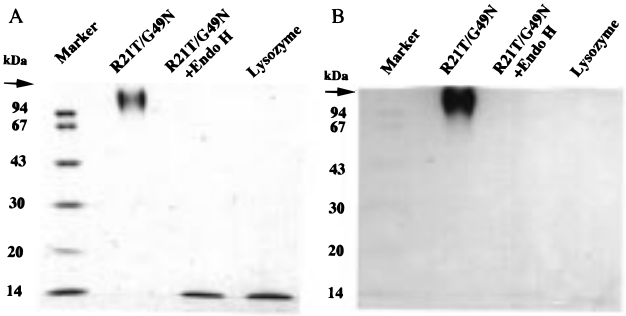

Figure 3. SDS−PAGE patterns of the mutant lysozyme R21T/G49N treated with Endo-H. The gel was stained for proteins and carbohydrates with Coomassie Brilliant Blue (A) and periodic acid−Fuchsin (B), respectively. The arrow indicates the boundary between the stacking (upper) and separating (lower) gel. Markers: phosphorylase *b* (94 000); bovine serum albumin (67 000); ovalbumin (43 000); carbonic anhydrase (30 000); trypsin inhibitor (20 100); α-lactalbumin (14 300) as molecular weight markers; R21T/G49N, R21T/G49N alone; R21T/G49N + Endo H, R21T/G49N treated with Endo-H; lysozyme, native lysozyme (from fresh hen egg white, MW 14 300).

before treatment with Endo-H (Figure 3B). When two bands of 17 and 20 kDa are also treated with Endo-H, the molecular weight of the bands changed to the same band as lysozyme (data not shown). The band of 20 kDa may be a double-oligomannosyl lysozyme glycosylated at both positions 19 and 49, and the 17 kDa band may be a single-oligomannosyl lysozyme. These results indicated that the double-mutant lysozyme was mainly expressed in a polymannosyl lysozyme with a small amount of two types of oligomannosyl lysozyme glycosylated in the N-linked form.

Molecular Mass Distribution of Polymannosyl Lysozyme R21T/G49N. The molecular mass distribution of the polymannosyl form of R21T/G49N was analyzed by the low-angle laser light scattering technique, which is a promising procedure for the estimation of the molecular mass of heterogeneous biopolymers such as protein−polysaccharide conjugates (Kato et al., 1992c). Figure 4 shows the elution pattern of the polymannosyl lysozyme R21T/G49N obtained with a low-angle laser light scattering photometer (LS) and a differential refractometer (RI). The molecular mass at each elution position plotted in Figure 4 was calculated

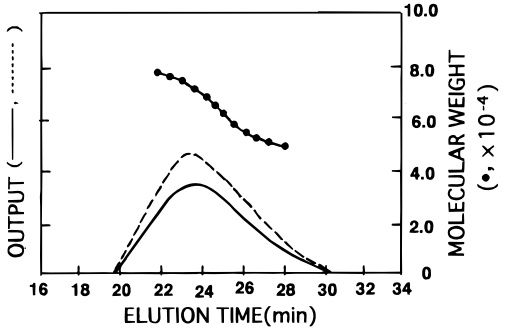

Figure 4. Molecular mass distribution of polymannosylated lysozyme R21T/G49N using the low-angle laser light scattering technique combined with HPLC. The polymannosyl lysozyme (0.1%) in 200 mM sodium phosphate buffer (pH 6.9) was applied to a TSK-gel G3000SW column (0.75 × 60 cm, Tosoh) at a flow rate of 0.3 mL/min. Elutions from the column were monitored with a low-angle laser light scattering detector (- - -) and a precision differential refractometer (—). The molecular mass distribution was determined from the ratio of the height of the LS peak to that of the RI peak by the equation described under Materials and Methods.

Table 1. Carbohydrate Composition of Polymannosyl Lysozyme

	content (mol/mol of lysozyme)	
polymannosyl lysozyme	*N*-acetylglucosamine[a]	mannose[b]
R21T/G49N	4	290 ± 3.1
G49N[c]	2	300 ± 4.6
R21T[d]	2	338 ± 20

[a] Determined with an amino acid analyzer using the hydrolysates with 3 N HCl at 100 °C for 4 h. [b] Mannose was identified by HPLC analysis using an NH2P column, and the content was determined by the phenol−sulfuric acid method. Values are means of triplicate without any significant variations. [c] Nakamura et al. (1993). [d] Kato et al. (1994).

from the ratio of LS to RI, according to the equation under Materials and Methods. The obtained molecular mass was distributed in the range of 50−79 kDa, indicating the degree of polymerization of polymannosyl lysozyme R21T/G49N is about ∼200−360 residues.

Chemical Composition Analysis. Table 1 shows the carbohydrate composition of the polymannosyl lysozyme R21T/G49N compared with that of other polymannosyl lysozymes R21T (Kato et al., 1994) and G49N (Nakamura et al., 1993a). On the basis of the chemical composition, 290 mol of mannose appeared to bind to one molecular lysozyme for the polymannosyl lysozyme R21T/G49N. In addition, 4 mol of glucosamine residues attached to one molecular R21T/G49N. This suggests that the polymannosyl lysozyme R21T/G49N was glycosylated at both positions. Despite the presence of two glycosylated sites in R21T/G49N, the carbohydrate content is almost the same as that of the single-polymannosyl lysozymes (G49N, R21T). This may be possible if the polymannosylation site of R21T/G49N is limited to either position 19 or 49, whereas another site is oligomannosylated.

Molecular Weight of the Polymannosyl Chain Removed from R21T/G49N with Hydrazine Anhydrous. The polymannosyl chains were removed from the polymannosyl lysozyme R21T/G49N with hydrazine anhydrous. This treatment results in the release of polysaccharide chains without degradation of cabohydrate chains (Takasaki et al., 1982). The molecular mass of polymannosyl chains was determined by the low-angle laser light scattering technique. As shown

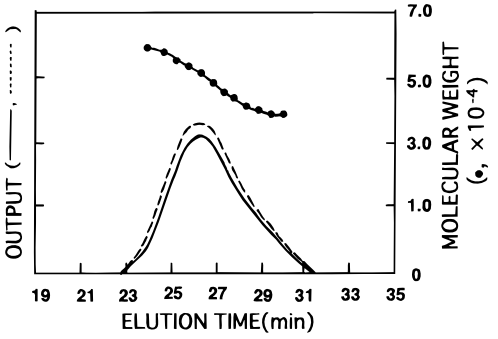

Figure 5. Molecular mass distribution of polymannosyl chain removed from R21T/G49N with hydrazine anhydrous using the low-angle laser light scattering technique. Conditions of measurement are the same as in Figure 4.

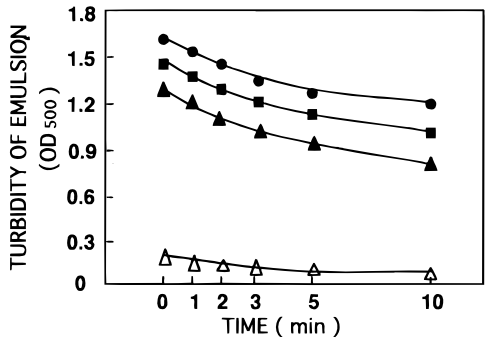

Figure 6. Emulsifying properties of various polymannosyl lysozymes R21T/G49N (●), G49N (■), R21T (▲), and native lysozyme (△) measured in 1/15 M sodium phosphate buffer (pH 7.4). The concentration of each sample was 0.025%. Values are means of triplicate measurements without any significant variations.

Table 2. Emulsifying Activity and Emulsion Stability of Polymannosyl Lysozymes[a]

polymannosyl lysozyme	emulsifying activity (OD_{500})	emulsion stability (min)
R21T/G49N	1.635	27.25
G49N	1.472	19.93
R21T	1.218	14.18
native lysozyme	0.216	<1

[a] The emulsifying activity and emulsion stability were determined from the curves in Figure 6.

in Figure 5, the molecular mass of polymannosyl chains removed from R21T/G49N was distributed in the range 40−59 kDa, and the average of molecular mass is ≈49 kDa. This indicates the possibility that R21T/G49N was glycosylated at one site with a polymannosyl chain consisting of 272 mannose residues and may be oligoglycosylated at another site with an oligomannosyl chain of 18 mannose residues. The double-polymannosylation could not occur in Golgi apparatus after N-oligomannosylation in endoplasmic reticulum of yeast. This finding may be useful to further elucidate the mechanism of polymannosylation of glycoproteins in yeast.

Emulsifying Properties of Various Polymannosyl Lysozymes. Emulsifying properties of polymannosyl lysozymes R21T/G49N, G49N, and R21T were compared to elucidate the effects of binding site and number of polymannosyl chains on the functional properties of polymannosyl lysozymes. Figure 6 and Table 2 show the emulsifying properties of various polymannosyl lysozymes at 0.025% concentration in a neutral pH solution system (1/15 M phosphate buffer,

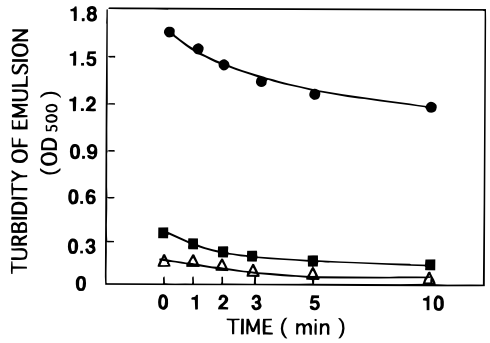

Figure 7. Emulsifying properties of the double-glycosylated lysozyme R21T/G49N (●), R21T/G49N deglycosylated with Endo-H (■), and native lysozyme (△) measured in 1/15 M sodium phosphate buffer (pH 7.4). The concentration of each sample was 0.033%. Values are means of triplicate measurements without any significant variations.

pH 7.4). Although a polymannosyl and an oligomannosyl chain attached to lysozyme, the double-glycosylated lysozyme G49N/R21T exhibited much higher and more stable emulsifying properties than the single lysozymes G49N and R21T. These suggest that the number of binding sites of the glycosyl chain closely relates to the emulsifying properties of polymannosyl lysozymes. It is possible that the formation of a thick bound water layer adsorbed around the emulsion of R21T/G49N is further enhanced and that coalescence of oil droplets was more effectively inhibited by increasing the oligomannosyl chain. A similar result was observed in Maillard-type lysozyme−polysaccharide conjugates (Shu et al., 1996). On the other hand, single-polymannosyl lysozyme G49N showed a significant improvement in emulsifying properties than another single-polymannosyl lysozyme R21T. This result demonstrated that the binding site of polymannosyl chain also relates to the emulsifying properties of polymannosyl lysozymes. This may be because the differences in binding site affect the protein structure relating emulsifying property although the structure change is not addressed.

Effect of the Cleavage of Polymannosyl Chain on the Emulsifying Properties of Polymannosyl Lysozymes. As described above, polymannosyl lysozymes constructed by genetic modification exhibit a dramatic improvement in emulsifying properties compared with native lysozyme. It may be possible that the polymannosyl chain plays independently a role for enhancing the emulsifying properties. To elucidate the role of the polymannosyl chain attachment in the excellent emulsifying properties, the polymannosyl chains were completely cleaved from lysozyme with Endo-H where the deglycosylation was confirmed by SDS−PAGE (data not shown). As shown in Figure 7, the emulsifying property of deglycosylated R21T/G49N was greatly decreased to almost the same as that of native lysozyme. Similarly, the deglycosylation with Endo-H of single-polymannosyl lysozymes R21T and G49N also resulted in a dramatic decrease in the emulsifying properties (data not shown). These results suggest the mixture of polymannosyl chains and lysozyme could not display good emulsifying properties without their covalent attachment and indicated that the dramatic improvement in the emulsifying properties of polymannosyl lysozymes is attributed to the attachment of the polymannosyl chains to lysozyme. The hydrophobic residues of the protein moiety exposed by the denaturation during the emulsion formation may be anchored to the surface of oil droplets in the emulsion, while the hydrophilic residues of polysaccharide chains oriented toward water may cover oil droplets to inhibit the oil coalescence, resulting in the stability of the emulsion. The effect of the glycosylation on the conformation of lysozyme was confirmed by CD analysis. No conformation change was observed with the attachment of the polymannosyl chain to the protein (data not shown). Therefore, the polysaccharide chain attachment to proteins is essential for the excellent emulsifying properties of polymannosyl lysozymes.

In conclusion, the polymannosyl lysozyme R21T/G49N was found to be glycosylated at both sites 19 and 49 with a polymannosyl (272 mannose residues) and an oligomannosyl chain (18 mannose residues) by genetic engineering. R21T/G49N showed better emulsifying properties than two types of single-polymannosyl lysozymes (R21T and G49N). G49N demonstrated a considerable improvement in emulsifying properties over R21T. These observations indicate that the binding site and number of polysaccharide chains relate to the improvement in the emulsifying properties of lysozyme−polysaccharide conjugates. Polymannosyl lysozymes did not exhibit excellent emulsifying properties when the polysaccharide chains were released from the protein moiety with Endo-H. These findings suggest that the dramatic improvement in the excellent emulsifying properties of lysozyme−polysaccharide conjugates is due to the attachment of a polysaccharide chain to the protein.

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