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Novel surface functional properties of polymannosyl lysozyme constructed by genetic modification

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The surface functional properties of glycosylated lysozyme were investigated by using polymannosyl and oligomannosyl enzymes at the position 49 by genetic modification [Nakamura, S. et al. (1993) J. Biol. Chem. 268, in press]. The polymannosyl lysozyme exhibited excellent emulsifying properties superior to those of commercial emulsifiers in addition to heat stability, while the oligomannosyl lysozyme did not. The surface tension of the polymannosyl lysozyme was greatly decreased correspondingly to the enhanced emulsifying properties, although that of oligomannosyl protein was not. The emulsifying activity and the emulsion stability of the polymannosyl lysozyme were stable in acidic pH or high salt conditions; in addition, they were greatly enhanced also by preheating the polymannosyl lysozyme. Thus novel surface functional properties of polymannosyl lysozyme in addition to heat stability suggest the direction of the design of new functional proteins by genetic modification.

Polymannosyl lysozyme; Genetic modification; Emulsifying properties; Surface tension

1. INTRODUCTION

Glycosylation of proteins by polysaccharide is one of the most promising methods to overcome their instability to heating [1] and proteolytic attack [1], and to further improve their functional properties [2–6]. Although the glycosylation of proteins with monosaccharides or oligosaccharides were attempted to improve their functional properties [7–10], the effects seemed to be poor compared to the glycosylation with polysaccharides. Both proteins and polysaccharides have a role in the stabilization of oil-in-water emulsions. Proteins adsorb at the oil–water interface during emulsification to form a coherent viscoelastic layer [11]. In contrast, polysaccharides confer colloid stability through their thickening and gelation behavior in the aqueous phase [12]. Therefore, the protein–polysaccharide conjugate is expected to exhibit the good emulsifying properties. Thus, we have reported that the covalent attachment of polysaccharide to lysozyme in a controlled dry-heating state [4] brings about the heat stability, excellent emulsifying property and favorable bactericidal effect [5,6]. However, it is difficult to control the chain length and number of attached polysaccharides in a case of chemical modification. On the other hand, if lysozyme is genetically modified with polysaccharide, it is easy to construct more effective glycosylated enzyme and to evaluate the effect of glycosylation on the functional properties of protein on the molecular basis. Thus, we developed the genetic engineering using yeast-secretion system to attach a polysaccharide and a oligosaccharide to lysozyme and succeeded in constructing two types of glycosylated lysozymes, a large polymannose chain ($Man_{310}GlcNAc_2$)-linked form and a small ologimannose chain ($Man_{18}GlcNAc_2$)-linked form [13]. As predicted, the polymannosyl lysozyme showed the striking heat stability in that no coagulation was observed under conditions in which the wild type lysozyme coagulated [13]. In the present paper, the emulsifying properties of the glycosylated lysozymes were investigated to evaluate the effects of the length of saccharide chains on the functionality and were compared with commercial emulsifiers for industrial applications. The polymannosyl lysozyme revealed the excellent emulsifying properties superior to commercial emulsifiers.

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2. MATERIALS AND METHODS

2.1. *Materials*

The wild-type hen egg white lysozyme was obtained from yeast harboring the lysozyme expression plasmid as described [14]. Polymannosylated lysozyme with molecular mass of 71 kDa was obtained from yeast harboring the recombinant lysozyme expression plasmid constructed to carry the signal sequence for *N*-linked glycosylation at position 49 by genetic modification as described [13]. Oligomannosyl lysozyme with molecular mass of 18 kDa was also obtained from yeast harboring the same recombinant plasmid as described [13]. *Saccharomyces cerevisiae* AH22 (*MATa, Leu2, His4, Cir*$^+$) was used for a host yeast cell. The commercial emulsifiers, Sunsoft SE11 (sucrose-fatty acid ester, HLB11) and Sunsoft Q-18S (decaglyceryl monoesterate, HLB12) were supplied from Taiyo Kagaku Co. (Japan). All other chemicals were of analytical grade.

2.2. *Emulsifying properties*

Emulsifying activity and emulsion stability were estimated according to the modified method of Pearce and Kinsella [15]. 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 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 turbidity detected immediately after the emulsion had formed

2.3. *Surface tension*

Surface tension was measured by an interfacial tensiometer (Kyowa Kagaku Co., Tokyo), a du Nouy tensiometer, which is essentially a torsion balance with platinum ring (2 cm diameter) suspended from the beam. The platinum ring was put into 2 ml of 0.05% protein solution, and then the force required to release the ring from the surface is measured using the apparatus. Since the exhibitions of the stable surface tension required 5 min after preparation of the interface, the measurements were carried out five times at 5 min after preparation of the interface.

3. RESULTS AND DISCUSSION

The emulsifying properties of the polymannosyl lysozyme constructed by genetic modification were measured under various solution systems. Fig. 1 shows emulsifying properties of the polymannosyl and oligomannosyl lysozymes, wild-type lysozyme and commercial emulsifiers in neutral pH solution system (1/15 M phosphate buffer, pH 7.4). The turbidity of emulsion is plotted as the ordinate and standing time after emulsion formation as the abscissa. The value of the ordinate at zero time is the relative emulsifying activity, and the half-life of initial turbidity reflects the stability of the emulsion. Excellent emulsifying properties were observed in the emulsion of the polymannosyl lysozyme. The emulsifying activity was about 15 times that of wild-type lysozyme, and the emulsion stability was too high to measure in the emulsion. The emulsifying property was almost the same as that of the lysozyme–dextran conjugate prepared by chemical modification [6]. In addition, the polymannosyl lysozyme revealed much better emulsifying activity and emulsion stability than

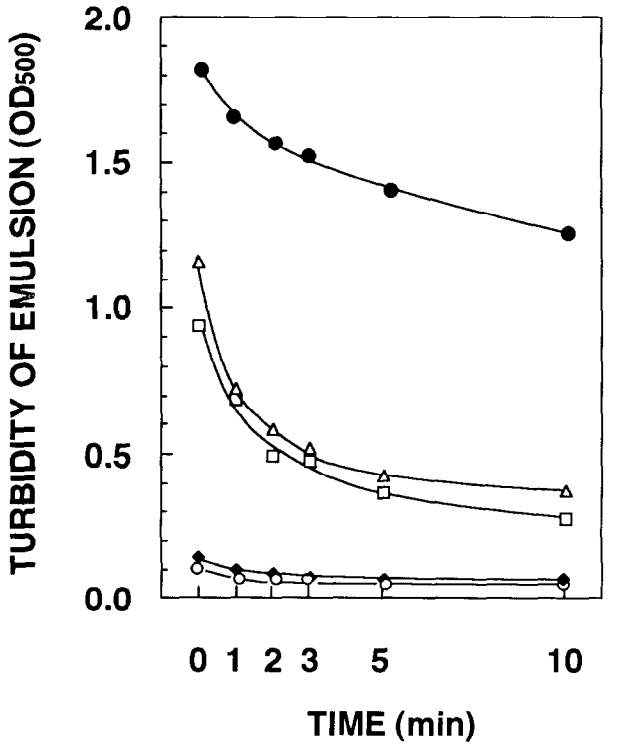

Fig. 1 Emulsifying properties of polymannosyl lysozyme, oligomannosyl lysozyme, wild-type lysozyme and commercial emulsifiers in neutral pH system (1/15 M sodium phosphate buffer, pH 7.4). (●) Polymannosyl lysozyme; (◆) oligomannosyl lysozyme; (○) wild-type lysozyme; (△) commercial emulsifier, Sunsoft Q-18S, decaglyceryl monoesterate; (□) commercial emulsifier, Sunsoft SE11, sucrose fatty ester. Data are from a representative experiment repeated two times with similar results

Table I

Surface tension of polymannosyl lysozyme, oligomannosyl lysozyme, wild-type lysozyme and commercial emulsifiers in water system

	Surface tension (dynes/cm)[a]
Polymannosyl lysozyme	59.8 ± 0.3
Oligomannosyl lysozyme	70.1 ± 0.4
Wild-type lysozyme	71.0 ± 0.6
Water[b]	75.6 ± 0.1

[a]Values are means ± S.D. of independent experiments performed in quintuplicate.
[b]Distilled water without any proteins, for control.

those of commercial emulsifiers, Sunsoft SE-11 and Q-18S that are a sucrose–fatty acid ester and a polyglycerin–fatty acid ester, respectively. This observation suggests that the polysaccharide chain in the polymannosyl lysozyme is an important role to form the stable emulsion, because the oligomannosyl lysozyme revealed poor emulsifying properties similar to wild-type lysozyme. Thus, it is confirmed by the genetic modification that the length of attached carbohydrate chain is critical for the formation of excellent emulsifying properties. 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 polymannosyl chains oriented to water may cover oil droplets to inhibit the coalescence of oil droplets, resulting in the stable emulsion formation. On the other hand, the oligomannosyl chain is not enough to suppress the coalescence of oil droplets, failing to form stable emulsion. In addition, the measurement of surface tension may supply a good information to elucidate the mechanisms of the excellent surface functional property of the polymannosyl lysozyme. Table I shows the surface tension

of the polymannosyl, oligomannosyl, and wild-type lysozymes at 0.05% protein concentration in water system. The surface tension of lysozyme was extremely reduced by the polymannosylation, while that of the oligomannosyl lysozyme remained almost the same value as wild-type lysozyme. The reduction of surface tension value induces excellent surfactant activity [16], strongly suggesting that the polymannosylation only allows lysozyme to convert to a surface-active protein.

The emulsifying properties of the polymannosyl lysozyme were similarly investigated in acidic pH and high-salt conditions. As shown in Fig. 2, the emulsifying properties of the polymannosyl lysozyme were still excellent in an acidic solution system (1/15 M acetate buffer, pH 3.0), whereas those of the commercial emulsifiers were greatly reduced, especially Sunsoft SE-11. In addition, the excellent emulsifying properties of the polyglycosyl lysozyme were slightly reduced even in a high-salt solution system (1/15 M phosphate buffer, pH 7.4, containing 0.2 M NaCl), as shown in Fig. 3. Because acidic pH and high-salt concentration are commonly used in industrial applications, the polymannosyl lysozyme constructed by genetic modification is a suitable ingredient for food stuffs.

Further, the effect of heat-treatment on the emulsifying properties of the polymannosyl lysozyme were compared with those of the commercial emulsifiers in a water emulsion system. As shown in Fig. 4, the polyglycosyl lysozyme revealed excellent emulsifying properties superior to those of commercial emulsifiers in water solution system where each sample tested exhibited better emulsifying properties than in any salt solution systems (1/15 M buffer with or without 0.2 M NaCl). The emulsifying activity of the polyglycosyl lysozyme was increased 1.1 times that of the native form by preheating the polymannosyl lysozyme to 95°C from 30°C at a rate of 3°C/min in the water system, whereas the emulsifying properties of the commercial emulsifiers were not quite altered by the preheating treatment (data not shown). We have already reported that the polymannosyl lysozyme was heat-stable and any coagulate was not observed during heat treatment at 95°C [13]. This observation suggests that the resulting unfolded form of the polymannosyl lysozyme was kept by the attached polymannosyl outer chains without coagulation of protein portion and amphiphilic balance was formed to have better emulsifying properties.

It is well known that amphiphilic proteins such as casein, serum albumin and β-lactoglobulin show good

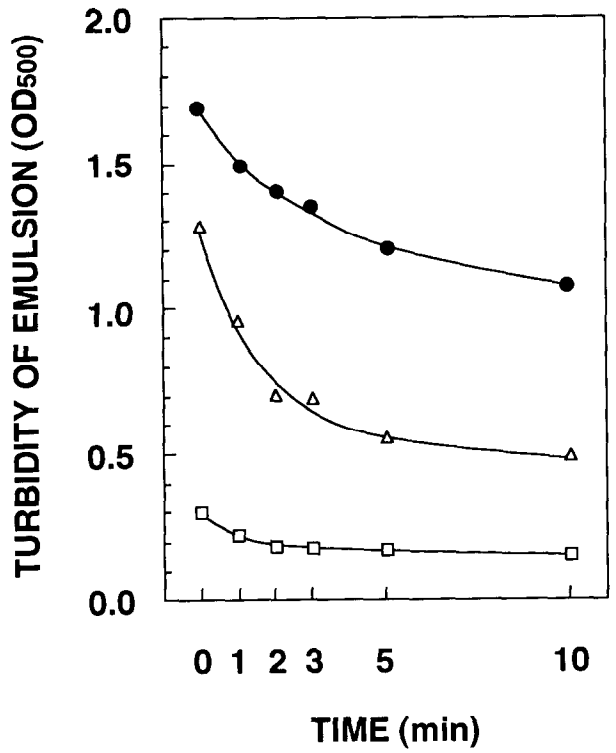

Fig. 2. Comparison of emulsifying properties between polymannosyl lysozyme and commercial emulsifiers in an acidic solution system (1/15 M sodium citrate buffer, pH 3.0). (●) Polymannosyl lysozyme; (△) commercial emulsifier, Sunsoft Q-18S, decaglyceryl monoesterate; (□) commercial emulsifier, Sunsoft SE11, sucrose fatty ester. Data shown are from a representative experiment repeated twice with similar results.

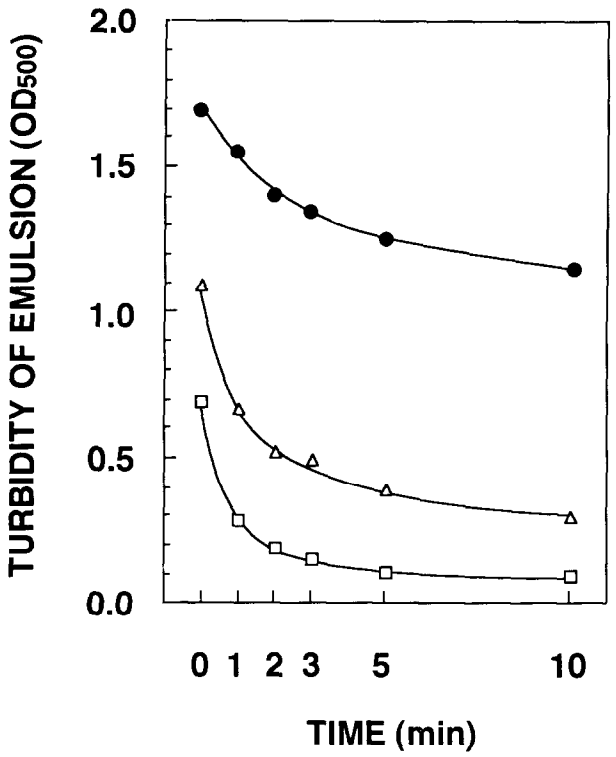

Fig. 3. Comparison of emulsifying properties between polymannosyl lysozyme and commercial emulsifiers in a high-salt solution system (1/15 M sodium phosphate buffer, pH 7.4, containing 0.2 M NaCl). (●) Polymannosyl lysozyme; (△) commercial emulsifier, Sunsoft Q-18S, decaglyceryl monoesterate; (□) commercial emulsifier, Sunsoft SE11, sucrose fatty ester. Data are from a representative experiment repeated two times with similar results.

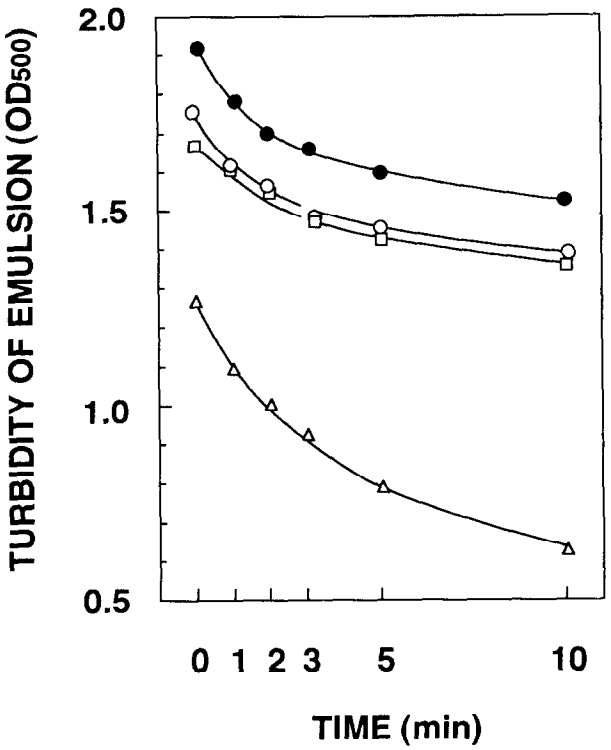

Fig. 4. Effect of heat treatment of polymannosyl lysosome on the emulsifying properties. (○) Untreated polymannosyl lysozyme, (●) heat-treated polymannosyl lysozyme; (△) untreated commercial emulsifier, Sunsoft Q-18S, decaglycеryl monoesterate; (□) untreated commercial emulsifier, Sunsoft SE11, sucrose fatty ester Samples were heated to 95°C from 30°C at a rate of 3°C/min in distilled water and immediately cooled to 20°C, and then the emulsifying properties were measured. Data shown are from a representative experiment repeated twice with similar results.

emulsifying properties [17]. However, the polymannosyl lysozyme revealed much better emulsifying properties, especially emulsion stability, than those of the native-type amphiphilic proteins, suggesting the special role of polysaccharide chain in emulsion stability. In addition, the polymannosyl lysozyme exhibited better emulsifying properties than commercial emulsifiers. The novel emulsifying properties of polymannosyl lysozyme in addition to heat-stability suggest the direction of the design of new functional proteins by genetic modification.

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