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Rapid Formation of Biologically Active Neoglycoprotein from Lysozyme and Xyloglucan Hydrolysates through Naturally Occurring Maillard Reaction

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Abstract

Hen egg-white lysozyme was conjugated with 7~9 mers xyloglucan hydrolysates (MW=1,400) at 60°C and 79% relative humidity for 3 days. SDS-PAGE showed that the conjugation between lysozyme and the oligosaccharide began from 1-day incubation, and three molecules of carbohydrate chains were attached to a protein molecule after 3-day incubation. The enzymatic activity of lysozyme was totally conserved in the neoglycoprotein, when measured by using glycol chitin as substrate. Besides, the emulsifying properties of lysozyme were vastly improved by the conjugation with the oligosaccharide, in which emulsifying activity of the neoglycoprotein was five times higher than that of native one.

Key words: neoglycoprotein, hen egg-white lysozyme, xyloglucan hydrolysate, Maillard reaction

INTRODUCTION

In order to expand the utilization of food proteins as human health applications, newly emerging techniques have been developed (1). Glycosylation is one of the most promising techniques, as it is anticipated that the attachment of carbohydrate chains to protein brings about dramatically novel functional properties. One of the principal advantages is that glycosylation will convert proteins to more stable form just like many glycoproteins in which the carbohydrate groups confer important physical properties such as conformational stability, protease resistance, charge effects and water-binding capacity (2-4).

Maillard-type protein-polysaccharide conjugate, namely neoglycoprotein, has been proposed to be useful as a safe functional biopolymer, since the conjugate can be prepared by binding of the free amino groups in the protein to the reducing-end carbonyl group in the polysaccharide through a controlled heating system without using any chemical reagents (5). However, polyglycosylation appears to be inappropriate to use for the production of biologically active proteins because of lowering the biological activity due to glycosylation with excessively long chains. It has been reported that the lytic activity of the polyglycosyl lysozyme with galactomannan was only 78% of that of the native counterpart when the insoluble cell wall of *Micrococcus lysodeikticus* was used as substrate (6). On the other hand, the conjugation with small carbohydrate molecules such as glucose or lactose resulted in insoluble aggregates having poor surface properties (5), which means glycosylation with monosaccharides disintegrates functional activity expected. Thus, it is assumed that a desirable neoglycoprotein with high biological activity is required a conjugation with oligosaccharide.

The objective of this study was to synthesize protein-carbohydrate conjugate with high biological activity using oligosaccharide as conjugation substrate. This paper also describes the effects of binding number of oligosaccharide chains on the functional properties of protein-oligosaccharide conjugate. Our attempts were made to improve the surface functional properties of hen egg-white lysozyme by the covalent attachment of xylogucan hydrolysates without any loss of the enzymatic activity.

MATERIALS AND METHODS

Materials

Xyloglucan, β-glucanase hydrolysate of tamarind seed was supplied from Dainihon Phermaceutical (Osaka). *Micrococcus lysodeikticus* cells and ethylene glycol chitin for the enzymatic assay for lysozyme were from Sigma-Aldrich Japan (Tokyo) and Nakarai Tesuque (Kyoto), respectively. Other chemicals were all of analytical grade.

Preparation of lysozyme conjugate with galactomannan

Lysozyme was crystallized from fresh egg white at pH

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9.5 in the presence of 5% sodium chloride, and it was recrystallized five times (7). Xyloglucan hydrolysates were purified using JASCO HPLC system (Tokyo) equipped with RI-1530 detector (JASCO). The hydrolysates were loaded on a TSK-GEL Amide-80 column (4.6×250 mm, Tosoh, Tokyo) equilibrated with 1% acetonitrile, and eluted with 50% acetonitrile at a flow rate of 0.6 ml/min for 30 min. As shown in Fig. 1, seven to nine mers of xyloglucan hydrolysates (XG) were collected together, dialyzed against deionized water using a Spectra/Por dialysis membrane (MWCO 1,000, Spectrum Lab., Inc.), and used as the oligosaccharide sample in this study. Lysozyme was once mixed with XG in a weight ratio of 1/10 and dissolved in water, and then lyophilized. The powdered lysozyme-XG mixture was stored at 60°C under the relative humidity of 79%, as previously described (6,8). The degree of the conjugation of protein with oligosaccharide was determined by SDS slab polyacrylamide gel electrophoresis.

Electrophoresis in SDS-slab polyacrylamide gel

SDS-slab polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli (9) using 15% acrylamide separating gel and 5% stacking gel containing 0.1% SDS. Samples (0.1%) were heated at 100°C for 3 min in Tris-glycine buffer (pH 8.8) containing 1% SDS. Electrophoresis was carried out at a constant current of 10 mA for 5 hr using an electrophoretic buffer of Tris-glycine containing 0.1% SDS. The gels were stained for proteins and carbohydrates with Coomassie blue G-250 and Fuchsin (10), respectively.

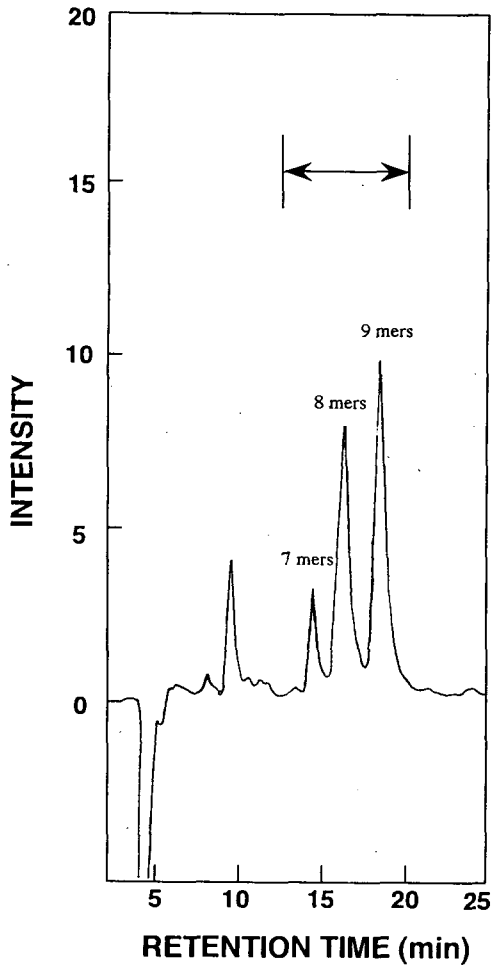

Fig. 1. Purification of xylogulan hydrolysates using HPLC. The fractions indicated by an arrow were collected together and used for further experiments after dialysis against deionized water.

Measurement of lysozyme activity

The lysozyme activity was measured by the lytic assay using *M. lysodeikticus* cells as substrate. Cell suspension was prepared in 50 mM potassium phosphate buffer (pH 7.0). The absorbance at 660 nm was adjusted to 0.8, and then 0.1 ml of 0.003% lysozyme solution were added to 2.4 ml of the suspensions. The initial velocities were determined by measuring the decrease in the turbidity of the cells automatically monitored at 660 nm with a spectrophotometer (JASCO V-530). The hydrolytic activity with glycolysis was also measured by following the reducing procedure using glycol chitin (11). To 0.5 ml of sample solution in 10 mM acetic acid-sodium acetate buffer (pH 4.5) were 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 potassium ferricyanide in 1 liter of 0.5 M sodium carbonate) were added, and the mixture was immediately boiled for 15 min. The reducing power resulting from hydrolysis of glycol chitin was estimated by absorbance at 420 nm (11). The amount, micromoles per minute of reducing groups produced by the reaction of lysozyme was defined as one unit.

Measurement of emulsifying properties

The emulsifying properties were determined according to the method of Pearce and Kinsella (12). An emulsion was prepared by homogenizing 1.0 ml of corn oil and 3.0 ml of a 0.1% sample solution, using a homogenizer (Polytron PT3100, KINEMATICA, 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% SDS solution. The absorbance of the diluted emulsion was then determined 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 time for emulsion decay during standing for 10 min.

RESULTS

Oligoglycosylation of lysozyme with xylogucan hydrolysate

Fig. 2 shows SDS-PAGE profiles of lysozyme-XG mixtures incubated at 60°C and 79% relative humidity for seven days. The patterns confirmed that lysozyme molecule was successfully linked with XG after one day of incubation. A new band was visualized at an apparent molecular mass of 17 kDa in addition to a band at 15 kDa which corresponds to the native

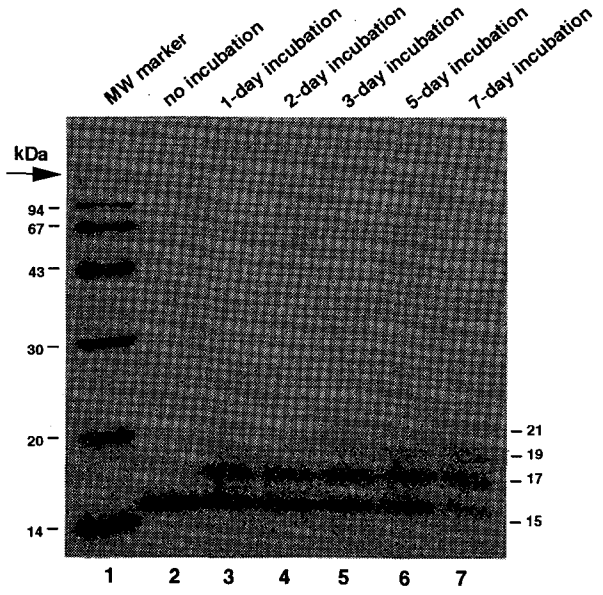

Fig. 2. Electrophoretic patterns of lysozyme-XG mixtures incubated at 60°C and 79% relative humidity for seven days, stained for protein. An arrow indicates the position of the boundary between stacking (upper) and separating (under) gels. Lane 1, molecular weight markers (94,000, phosphorylase *b*; 67,000, bovine serum albumin; 43,000, ovalbumin; 30,000, carbonic anhydrase; 20,000, trypsin inhibitor; 14,300, *α*-lactoalbumin); lane 2, lysozyme-XG mixture; lane 3, lysozyme-XG mixture incubated for 1 day; lane 4, lysozyme-XG mixture incubated for 2 days; lane 5, lysozyme-XG mixture incubated for 3 days; lane 6, lysozyme-XG mixture incubated for 5 days; lane 7, lysozyme-XG mixture incubated for 7 days.

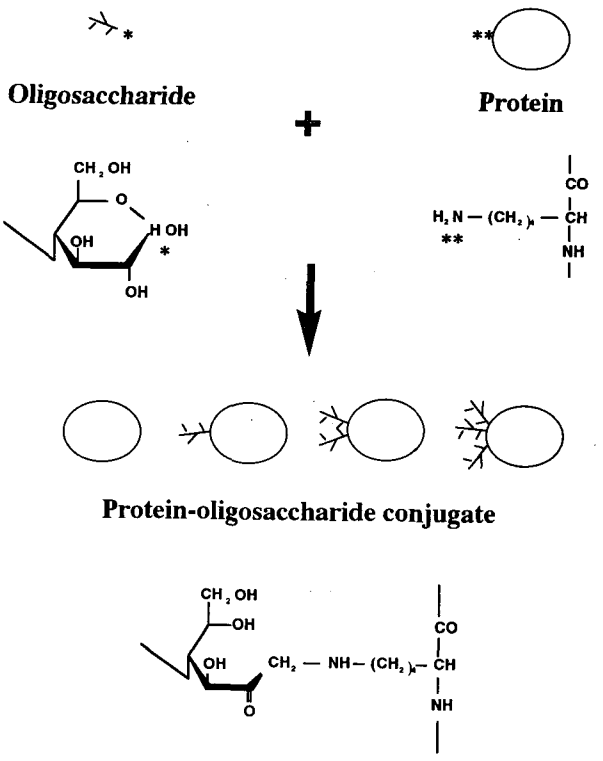

Fig. 3. Scheme of conjugation of protein (lysozyme) and oligosaccharide (XG) under the controlled dry state at 60°C and 79% relative humidity for 7 days. The symbol * and ** represent the only one reducing-end in the reducing-end-side residue in XG molecule and free amino groups in lysozyme molecule, respectively.

protein (Fig. 2, lane 3), whereas the no-incubation sample showed a single band at 15 kDa (Fig. 2, lane 2). Another band appeared at an apparent molecular mass of 19 kDa in the sample after the 3-day incubation. Finally, three types of neoglycosylated proteins were formed at 17, 19, and 21 kDa positions indicated in Fig. 2 (lane 7). These new bands were slightly stained with carbohydrate-specific reagent (periodic acid/Schiff reagent) (10) here (data not shown), resulting from low carbohyrate concentration on these bands and low sensitivity of the staining method. However, the 7-day incubated sample showed clear carbohydrate-stained bands here (data not shown). Thus, lysozyme was glycosylated with one to three molecules of the oligosaccharide by the Maillard reaction under the controlled heat process (Fig. 2, lane 7). A schematic figure demonstrating the production of the protein-oligosaccharide conjugate using the Maillard reaction is shown in Fig. 3.

Changes of lysozyme activity during lysozyme-XG conjugate formation

The effect of oligoglycosylation with XG on the lysozyme activity was determined by both lysis of *M. lysodeikticus* cells and glycolysis of glycol chitin. Table 1 shows the lysozyme activities of neoglycoproteins modified with XG. The lytic activities of 1- to 7-day incubated samples decreased dramatically with increase of incubation period up to 75% of native lysozyme when insoluble bacterial cells of *M. lysodeikticus* were used as substrate. As indicated in Table 1, changes of the lytic activity of lysozyme were closely related to the maximum binding number of XG. The lysozyme activity showed 91% in the 3-day incubated sample, where at most three carbohydrate chains were linked with the protein molecule. However, an excessive incubation significantly lowered the biological

Table 1. Change of enzymatic activity of lysozyme by the conjugation with XG

Incubation time (day)	Maximum binding number of XG to LZ molecule[1)]	Enzymatic activity (%)[2)]	
		Glycol chitin[3)]	*M. lysodeikticus*[4)]
0	0	100	100
1	1	100±1.4[5)]	95±1.6
2	2	100±1.5	92±1.5
3	3	100±1.5	91±1.3
5	3	95±1.1	80±2.1
7	3	95±1.0	75±1.5

[1)]Maximum binding numer was estimated from the SDS-PAGE (Fig. 1). [2)]Enzymatic activity was represented as the percentages of 0-day incubation. [3)]Glycolysis assay was done in 10 mM acetate buffer (pH 4.5) with glycol chitin. [4)]Lytic activity was measured in 50 mM potassium phosphate buffer (pH 7.0) with *M. lysodeikticus*. [5)]Each value is the mean±standard deviation of three replications. XG, xyloglucan hydrolysates; LZ, lysozyme.

activity of lysozyme.

On the other hand, the glycolysis activities were greatly conserved in the sample materials when glycol chitin was used as substrate. It seems likely that the steric hindrance with oligosaccharide chains occurs around the substrate binding site when the cell wall was employed for the determination of lysozyme activity. While the soluble material such as glycol chitin could be accessible to the substrate binding site in the neoglycosyl protein. According to the glycolysis assay, it can be concluded that the enzymatic activity of lysozyme was totally sustained for 3-day incubation.

Emulsifying properties of lysozyme-XG conjugates

Emulsifying properties were measured using corn oil under neutral pH condition (0.1 M phosphate buffer, pH 7.4). Table 2 shows changes of emulsifying properties of the lysozyme-XG mixture during incubation. Emulsifying properties of lysozyme were considerably improved by conjugation with XG. The improvement in the emulsifying properties reached a steady state when the conjugation of lysozyme with three molecules of the oligosaccharide was done for three days. The relative emulsifying activity of 0.1% 3-day incubated sample was 5 times that of the lysozyme-XG mixture (0-day incubated sample) at the same protein concentration. Furthermore, the emulsion stability of the incubated samples was much more stable than that of the mixture. The half-life of the decay of emulsion in 3-day incubated sample was 1.6 (min), while that of the lysozyme-XG mixture was less than 1.0 (min).

It is assumed that the hydrophobic residues of protein moiety partially denaturated during emulsion formation at the oil-water interface may be anchored to the surface of oil droplets in emulsion, while the hydrophilic residues of the extended branched oligosaccharide chains oriented to water may cover oil droplets, resulting in inhibiting the coalescence of the droplets. Thus, the lysozyme-XG conjugate showed the stable emulsion formation.

Table 2. Changes of relative emulsifying activity and emulsion stability of lysozyme by the conjugation with XG

Incubation time (day)	Relative emulsifying activity[1)]	Emulsion stability[2)]
0	0.072±0.002[3)]	<1.0
1	0.141±0.004	<1.0
2	0.282±0.005	1.2±0.1
3	0.360±0.011	1.6±0.2
5	0.381±0.018	1.6±0.3
7	0.390±0.005	1.6±0.2

[1)]An emulsion was prepared by homogenization of 1.0 ml of corn oil and 3 ml of a 0.1% sample solution at 12,000 rpm for 1 min at 20°C. A 100 µl portion of emulsion was taken from the bottom of the test tube and diluted with 5 ml of 0.1% SDS solution. The turbidity of diluted emulsion was determined at 500 nm, and the relative emulsifying activity was represented as the absorbance measured immediately after emulsion formation (0 min). [2)]The emulsion stability was estimated by measuring the half-life time of the decay of emulsion. [3)]Each value is the mean±standard deviation of three replications. XG, xyloglucan hydrolysates.

DISCUSSION

A Maillard-type conjugation was initiated from 1-day incubation in the lysozyme-XG mixture by a controlled heating at 60°C in the dry state of 79% relative humidity. The glycolysis activity of 3-days incubated mixture was 100% of 0-day incubated mixture when glycol chitin was used as substrate. Emulsifying properties of lysozyme were substantially improved after 3-day incubation. These data strongly indicate that, through the naturally occurring Maillard reaction, a biologically active neoglycoprotein was rapidly formed in the oligosaccharide (WM=1,400) mixture, whereas it took more than two weeks in the polysaccharide (galactomannan, MW= 15,000) mixture (6,8). Although tightly folded proteins have favorable surface activities due to their hydrophilicity, they are not generally suitable as a food additive in their native form because of its insufficient stability. They are generally unstable to heating for the pasteurization and homogenization that is necessary to prepare an emulsion, in which the proteins are easily coagulated during the emulsifying process. Therefore, if functional food proteins can be modified to a form being stable to the physical treatments, they will be further broadened for food applications as a new ingredient. Lysozyme with poor emulsifying properties in the native form has been converted to highly emulsifying proteins by conjugating with polysaccharides, dextran (13) and galactomannan (6). However, the enzymatic activity fell dramatically to 13.3% and 78.9% in the lysozyme-dextran conjugate (13) and lysozyme-galactomannan conjugate (6), respectively, when *M. lysodeikticus* was used as substrate. In this study, it has been revealed that the enzymatic activity was highly stored in the lysozyme-XG conjugates. On the whole, the chain length of the glycan added is the most important factor in the maintenance of the biological activity of lysozyme. The oligoglycosylation in the 3-day incubated mixture conjugate may be preferable for producing a stable ingredient with biologically activity and improved emulsifying properties.

In this paper, xyloglucan derived from tamarind seed was used as the model carbohydrate, because this is a well-characterized and favorable polysaccharide that is utilized as sticker binder and stabilizing agent in food applications. Xyloglucan is composed of a (1→4)-β-D-glucan backbone substituted with a side chain of α-D-xylose and β-D-galactosyl-(1→2)-α-D-xylose linked (1→6) to glucose residue. The conformation of xyloglucan is relatively similar to that of galactomannan (6). Although 7~9 mers xyloglucan hydrolysates were used to prepare lysozyme-oligosaccharide conjugate, physiological effects could be expected as an indigestible dietary fiber that serves as a soluble agent and lowers the blood sugar level in rat small intestine (14). Recently, pronounced antimutagenic effects were found for xyloglucan against 1-nitropyrene induced mutagenicity, by protecting the tested organisms from the mutagenic attack (15). Also, it has been reported that

wounds of rabbit corneal epithelium cells with n-heptanol significantly healed by the treatment with xyloglucan, a tamarind seed polysaccharide under the influence of the integrin recognition system (16). In addition to the good emulsifying properties, the beneficial effects as dietary fiber can be anticipated in the lysozyme-XG conjugate.

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