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Improving the Surface Functionality of Curdlan by Conjugation with Unfolding Protein through Naturally Occurring Maillard Reaction

Soichiro Nakamura†, Masahiro Ogawa*, Hiroki Saeki, Masayoshi Saito***, Satoko Miyasaka, Junya Hata, Naoko Adachi and Jae-Kwan Hwang*****

Department of Living Science, Shimane University, Matsue 690-8504, Japan
**Food, Nutrition and Health, University of British Columbia, Vancouver, BC V6T 1Z4, Canada*
***Graduate School of Fisheries Science, Hokkaido University, Hakodate 041-8611, Japan*
****National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba 505-8642, Japan*
*****Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea*

Abstract

Protein conjugation of curdlan belonging to β-1,3-glucan was carried out to improve its surface functionalities. The glucan was mixed with phosvitin, α_s-casein, lysozyme or ovalbumin, respectively. The mixture was freeze-dried, and the resulting powder was incubated at 60°C and 79% relative humidity for 12 days in order to generate a controlled Maillard reaction between curdlan and proteins. Conjugation with unfolding proteins, *i.e.*, phosvitin and α_s-casein, drastically increased the solubility of the glucan, whereas lysozyme and ovalbumin did not. The solubility in water of curdlan was 3.44% for the phosvitin conjugate and 1.09% for the α_s-casein conjugate. SDS-slab polyacrylamide gel electrophoresis showed that curdlan was solubilized due to covalent binding with phosvitin. Emulsifying properties of curdlan were substantially improved by the conjugation with phosvitin and α_s-casein. Emulsion stability of the curdlan-phosvitin conjugate was about 2.9 times greater than that of the curdlan-phosvitin mixture.

Key words: curdlan, solubility, emulsifying properties, unfolding protein, Maillard-type conjugation

INTRODUCTION

Although many researchers have reported that selected polysaccharides potentiate the non-specific immune responses which may be involved in anti-cancer activity, the insolubility of the polysaccharides sets limits their utilization in application systems. It is well-known that β-1,3-glucans show strong immunomodulation effects such as activation of macrophages, NK cells and T-lymphocytes, and production of interferon (1-3). The glucans are ubiquitous in bacteria, fungi, and higher plants as structural constituents of cell walls or as reserve polysaccharides (1). Among them, curdlan is the first known bacterial β-1,3-glucan produced by a mutant strain of *Alcaligenes feacalis* var. *myxogenes* (4), which is composed of a linear polysaccharide chain consisting mainly of β-(1→3)-linked D-glucose residues (5). This glucan has been approved for use by the FDA in December 1996 as a formulation aid, processing aid, stabilizer and thicker or texturizer use in food (6). Therefore, curdlan is a typical β-1,3-glucan which is desirable to be utilized in food systems for health promotion.

In order to improve the solubility of β-1,3-glucans, many studies were done on the conformational behavior of the glucans in aqueous solution. Ogawa et al. (7) suggested a mild alkaline treatment with trisodium phosphate (0.05~0.4 M Na_3PO_4) increases the solubility of curdlan. Ohno et al. (8) successfully developed a protocol to obtain a soluble β-1,3-glucan by sodium hypochlorite (NaClO) oxidation and subsequent dimethyl sulfoxide (Me_2SO) extraction. Seljelid (9) reported that amination of β-1,3-glucan was effective for the improvement of the solubility. However, the solubilities of glucans increased by the methods described above is not enough to apply to either food ingredients or pharmaceuticals, because considerable amounts of polysaccharides remain as insoluble substance. In 1990, an effective method has been proposed for improving the surface functional properties of proteins by covalent linking with carbohydrate chains through a naturally occurring Maillard reaction (10). The novel approach is the most promising way to solubilize β-1,3-glucans for food applications, because the conjugate can be prepared by joining the free amino groups in the protein with the reducing-end carbonyl group in the polysaccharide through a controlled heating system without using any chemical reagents. Kato et al. (11) recently reported that an improvement of the solubility and functional properties of insoluble wheat gluten were achieved by the conjugation with dextran. Thus, the conjugation with protein would be important and effective for achieving surface functional changes of the β-1,3-glucans.

In this study, the Maillard-type conjugation with food proteins was employed to develop a neoglycoconjugate of curdlan. Our objective of this study was to improve surface functional

†Corresponding author. E-mail: soichiro@edu.shimane-u.ac.jp
Phone: 81-852-32-6351, Fax: 81-852-32-6351

properties of the water-insoluble β-1,3-glucan through the controlled Maillard reaction with food proteins. This approach may be promising in the further use of insoluble polysaccharides and the improvement of their functional properties such as immunostimulizing activities.

MATERIALS AND METHODS

Materials

Curdlan was the courtesty from the Takeda Chemical Industry Ltd. (Osaka, Japan). Phosvitin was preparted from fresh egg-yolk by the method of Mecham and Olcott (12). α_s-casein was prepared from fresh milk by the method of Zittle and Custer (13). Lysozyme and ovalbumin were prepared from fresh egg-white by the crystallization as described by Alderton and Fevolt (14), and Kekwick and Cannan (15), respectively. Chemical reagents used here were of special grade commercially available.

Conjugation with food proteins

Curdlan was conjugated with soluble proteins using naturally occurring Maillard reaction as previously reported (16). The glucan was mixed with phosvitin, α_s-casein, lysozyme or ovalbumin in distilled water at the weight ratio of 1:1, respectively, and stirred for overnight (14 hr) at 4°C, and then completely lyophilized. The resulting lyophilized powder was incubated at 60°C under a relative humidity of 79%. After a given time, a portion of the heated powder was removed to a sealed bottle and stored at 4°C until further experiments.

Measurement of solubility

Curdlan and its conjugate with proteins were suspended in distilled water at 20°C, and vigorously shaken for 1 min using a vortex machine (VORTEX-2 GENIE, Scientific Industries Inc, Bohemia, N.Y.). The suspension was centrifuged at 15,000 rpm for 15 min at 20°C, and then supernatant was immediately separated from insoluble materials. Water solubility of the polysaccharide was evaluated by determining the total carbohydrate content of the soluble fraction. Carbohydrate analysis was carried out according to the phenol-sulfuric acid reaction (17) employing glucose as standard sugar.

Electrophoresis in SDS-slab polyacrylamide gel

SDS-slab polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (18) using 15% acrylamide separating gel and 5% stacking gel containing 0.1% SDS. Samples (20 μL, 0.1%) were heated at 100°C for 5 min in Tris-glycine buffer (pH 8.8) containing 1% SDS. Electrophoresis was carried out at a constant current of 15 mA for 3 hr using an electrophoretic buffer of Tris-glycine containing 0.1% SDS. The gels were stained with Coomassie Brilliant Blue R-250.

Measurement of emulsifying properties

The emulsifying properties were determined according to the method of Pearce and Kinsella (19). An emulsion was prepared by homogenizing 1.0 mL of corn oil and 3.0 mL of a 0.1% sample solution in a test tube, 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, 5, 10, 20 and 30 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 30 min of standing.

RESULTS

Effects of incubation time on development of curdlan-phosvitin conjugate

Figure 1 shows the effect of Maillard-type conjugation with phosvitin on the solubility of curdlan. After 2-day incubation, phosvitin initiated to solubilize the glucan. An equilibrated state in soluble capacity of the glucan was reached after 12-day incubation. SDS-slab polyacrylamide gel electrophoresis (PAGE) was thus conduced in order to determine the development of curdlan-phosvitin conjugate. The SDS-PAGE pattern of the high molecular weight fraction in phosvitin conjugate showed a dense broad band for protein stain near the boundary between stacking and separating gels with another higher molecular weight band which was unable to enter into the staking gel, that implied the formation of conjugate (Fig. 2). Thus, it was shown that curdlan was solubilized due to covalent binding of phosvitin with periods of incubation time by dry-heating. The highest degree of the conjugation was at 12-day incubation. Therefore, 12 days incubation was taken to make proteins conjugate with the glucan for further experiments.

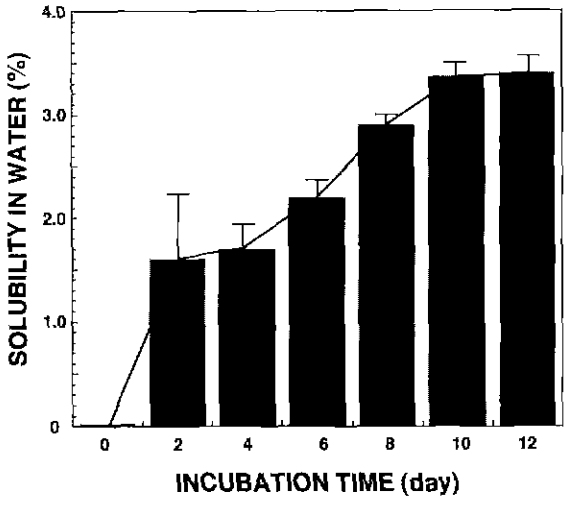

Fig. 1. Effect of Maillard-type conjugation with phosvitin on the solubility of curdlan. Vertical bars show standard deviation (n=5).

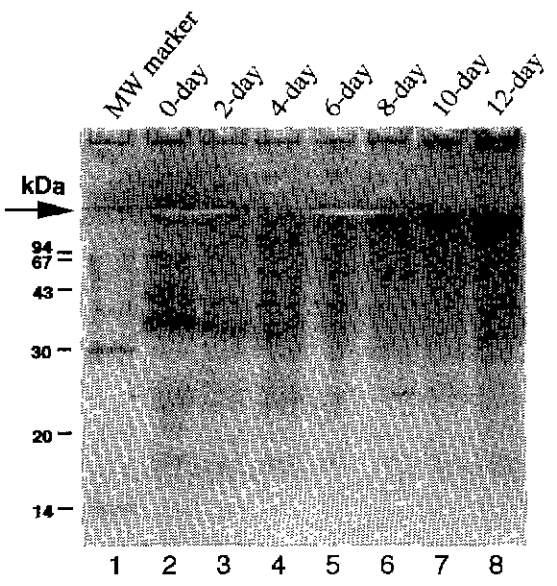

Fig. 2. SDS-slab polyacrylamide gel electrophoretic patterns of curdlan-phosvitin conjugate. Lane 1 represents molecular weight marker. Lane 2, 3, 4, 5, 6, 7 and 8 represent 0-day, 2-day, 4-day, 6-day, 8-day, 10-day and 12-day incubation samples of the curdlan-phosvitin mixture, respectively. Arrows indicate the position of the boundary between the upper staking gel (5%) and the lower separating gel (15%).

Changes in solubility of curdlan by Maillard-type conjugation with proteins

In order to make curdlan soluble in water by protein conjugation, phosvitin, α_s-casein, lysozyme or ovalbumin was mixed with the glucan, and then freeze-dried, respectively. The resulting powder mixture was incubated at 60°C and 79% relative humidity for 12 days in order to generate a Maillard reaction between the glucan and the food proteins. Table 1 shows changes of the solubility of the glucan in water at 20°C by conjugation with food proteins. Soluble capacity in water (at 20°C without protein) of curdlan was less than 0.01%. The solubility was slightly improved by mixing with the unfolding proteins, *i.e.*, phosvitin and α_s-casein. In contrast, the incubation of the protein mixture by dry-heating for 12 days brought about high solubility of the glucan. The conjugation with phosvitin or α_s-casein drastically improved the solubility of curdlan to 3.44% and 1.09%, respectively, whereas the conjugation with lysozyme and ovalbumin improved the solubilities only to an extent of 0.18% and 0.22%, respectively.

Emulsifying properties of curdlan-protein conjugates

Changes in emulsifying properties of curdlan by the conjugation with proteins were investigated using corn oil. Both emulsifying activity and emulsion stability of curdlan-protein conjugates were much higher than those of the mixtures of 0-day incubation. Among the substances, it has been revealed that phosvitin and α_s-casein were effective since they belong to certain types of charged polymer with unfolded structure, whereas lysozyme and ovalbumin with rigid and folded structure were not (data not shown). Especially, phosvitin conjugation caused a remarkable improvement of the emulsifying properties of the glucan. Relative emulsifying activity of the curdlan-phosvitin conjugate was 1.31, while that of the mixture was 1.18. The emulsifying activity increased about 1.1 times by the Maillard-type conjugation. Besides, it was revealed that the emulsion stability of the conjugate was 2.9 times greater than that of the mixture. As shown in Fig. 3, the phosvitin conjugation was effective for the modification of curdlan.

Table 1. Changes of solubility of curdlan in water by the conjugation with food proteins[1)]

Protein	Solubility (%)[2)]	
	Mixture[3)]	Conjugate[4)]
Phosvitin	0.03±0.002[5)]	3.44±0.005
α_s-casein	0.03±0.001	1.09±0.003
Lysozyme	ND[6)]	0.18±0.002
Ovalbumin	ND	0.22±0.002

[1)]Curdlan and protein were mixed, stirred for overnight at 4°C, and lyophilized. The lyophilized powder was incubated at 60°C for 12 days under 79% relative humidity.
[2)]The measure of solubility was carried at 20°C as described in the text.
[3)]Mixture means "the 0-day incubation sample".
[4)]Conjugate means "the 12-day incubation sample".
[5)]Data represent the average±standard deviation of five independent experiments.
[6)]ND means "not detected (less than 0.01%)".

DISCUSSION

Curdlan was conjugated with phosvitin, α_s-casein, lysozyme or ovalbumin through a naturally occurring Maillard reaction in order to improve the surface functional properties of the β-1,3-glucan. The covalent bond formation with the food proteins was demonstrated by SDS-slab polyacrylamide gel elec-

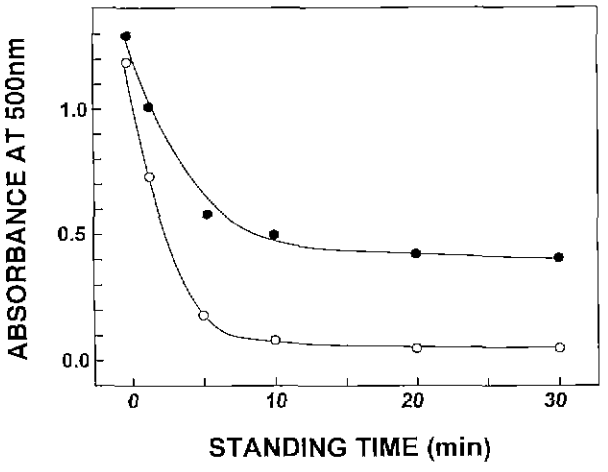

Fig. 3. Emulsifying properties of curdlan-phosvitin conjugate (●) and curdlan-phosvitin mixture (○). Data are from a representative experiment repeated five times.

trophoresis. Conjugation with unfolding proteins, *i.e.*, phosvitin and α_s-casein, dramatically increased the solubility of the glucan. The solubility of curdlan was improved more than 100 times by the conjugation with phosvitin. The glucan-phosvitin conjugate also showed the excellent emulsifying properties. It has been reported that both polysaccharide and protein have a role in the stabilization of oil-water emulsion (20). Polysaccharides confer colloid stability through their thickening and gelation behavior in the aqueous, while proteins adsorb an oil droplet at the oil-water interface during emulsification to form a covalent viscoelastic layer (20). Therefore, the protein conjugation with glucan has been expected to show good emulsifying properties. Recently, we have stated that the covalent attachment of polysaccharide by chemical modification brings about excellent surface functional properties of phosvitin (21). In the present study, among protein conjugations with curdlan, phosvitin was a distinguishing attribute of the modification. Since curdlan is mainly composed of a linear β-(1→3)-D-glucose chain (5) and forms a tight conformation as triple-helix aggregates (7), the partially solubilized polysaccharide due to the covalent binding of the unfolding protein was more favorably oriented in an oil-in-water interface, thus resulting in an excellent improvement of emulsifying properties in the curdlan-phosvitin conjugate. Hence, novel surface functional properties of neoglycoconjugates from the glucan, in addition to the increased solubility, suggest a new direction of the design of new functional glucans by the artificial modification without any chemicals, the so-called controlled Maillard reaction under dry-heating.

In the last three decades, numerous β-1,3-glucans have been isolated from mushrooms and used as a source of therapeutic agents. The most promising bio-pharmacological activities of these biopolymers are their immunomodulation and anti-cancer effects (1). Although the mechanism of their antitumor action is still not completely clear, these polysaccharides are suggested to enhance cell-mediated immune responses both *in vivo* and *in vitro* and act as biological response modifiers (22). Potentiation of the host defense system may result in the activation of many kinds of immune cells that are vitally important for the maintenance of homeostasis (23). Some interesting studies focus on investigation of the relationship between their structure and antitumor activity, and explain that improvement of their various biological activities could be caused from their solubility (24,25). The improvement of soluble capacities of curdlan may increase its immunostimulizing activities.

In addition to curdlan, we have successfully solubilized ilulin belonging to β-2,1-fructan by the conjugation with phosvitin (data not shown). Since the water-soluble inulin-phosvitin conjugate exhibited excellent surface functional properties similar to the curdlan-phosvitin conjugate, the Maillard-type conjugation will contribute to the development of novel applications for water-insoluble polysaccharides. The results presented in this paper will be beneficial to produce a new type of immunostimulating pharmaceuticals and food additives in the near future. The research is in progress to assess biological and physiological activites of the curdlan-phosvitin conjugate.

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