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Food Safety of Functional Neoglycoproteins Prepared by Covalent Attachment of Galactomannan to Food Proteins

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Abstract

Hen egg-white lysozyme, ovalbumin, egg-yolk phosvitin, acid-precipitated soy protein and α_{s1} milk casein were covalently linked with galactomannan through a controlled dry-heating at 60°C under 79% relative humidity without any chemical reagent. Neoglycosylation by the covalent binding of polysaccharide chains brought a significant improvement into the surface functionalities of food proteins. Excellent emulsifying properties and foaming properties were observed in all protein-galactomannan conjugates. Bacterial mutagenesis tests and animal dose test were done to evaluate the food safety of the protein-galactomannan conjugates. The neoglycoproteins were negative for *Ames* test using *Salmonella typhimurium* TA100 (*hisG46*) and TA98 (*hisD3052*) strains, and *rec*-assay using *Bacillus subtilis* H17 (*rec*$^-$) and M45 (*rec*$^+$) strains. All substances were also nontoxic for oral administration to rats. LD_{50}'s of these substances were all more than 7.5 g/kg body-weight of rat. No effect was also observed in the weight increases and the concentrations of total cholesterol, triglyceride and phospholipids in blood serum of the administrated rats with 7.5 g/kg conjugates. Thus, Maillard-type protein-polysaccharide conjugates prepared by covalent attachment of galactomannan to food proteins were proposed to be useful as a safe functional biopolymer in this study.

Key words: food protein, galactomannan, neoglycoprotein, Maillard-type conjugate, food safety

INTRODUCTION

In most proteins, all hydrophobic residues are not completely buried in the interior because of steric constraints imposed by the polypeptides chain, while almost all the hydrophilic and charged residues are located on the surface (1). Therefore, most food proteins have potent surface functional properties arising from their amphiphilic properties. However, applications of food proteins are limited, since they are generally unstable against physical and chemical stress such as heating, organic solvents and proteolytic attack in their natural form due to their large molecular size. Hence, newly emerging techniques have been developed to overcome these disadvantages and to expand the utilization of food proteins as human health applications. Glycosylation is the most promising technique for this purpose. Protein molecules will be converted to a stable form just as many natural glycoproteins in which carbohydrate groups confer important physical properties such as conformational stability, protease resistance and water-binding capacity (2). Thus, we have succeeded in preparing novel functional neoglycoproteins from food compounds by the attachment of biopolymer templates, polysaccharides, to food proteins (3).

By the increasing consumer's awareness on food safety, changes to foods and food processing have always produced public concerns. This was the case for margarine, pasteurization milk, microwave cooking, and is the case of functional foods as well as biotechnology-derived foods. Traditional foods are viewed by the government offices as safe based on a long history of use. Consumers also regard traditional foods as safe. However, many of them contain naturally occurring toxins that can present hazards to consumers under some circumstances of exposure. The newly developed foods may contain some unique components that are not individually or collectively assessed for safety. Recently, a particular concern has been directed towards the food safety of functional foods for consumers.

Although lysozyme, ovalbumin and phosvitin from hen egg, acid-precipitated protein from soy bean, α_{s1}-casein from bovine milk, and galactomannan from guar bean are all natural food compounds, the food safety of the Maillard-type conjugates between the food proteins and the polysaccharide has not been still confirmed for toxicological evaluation. Thus, in addition to bacterial mutagenesis tests, acute and semi-acute tests using rats were

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carried out to determine the food safety of food protein-galactomannan conjugates in this study. Bacterial mutagenicity was investigated by *Ames* test using *Salmonella typhimurium* and *rec*-assay using *Bacillus subtilis*.

MATERIALS AND METHODS

Materials

Lysozyme and ovalbumin were prepared from fresh egg-white by the crystallization as described by Alderton and Fevolt (4), and Kekwick and Cannan (5), respectively. Phosvitin was prepared from fresh egg-yolk according to Mecham and Olcott (6). Acid-precipitated soy protein was prepared from defatted soy flakes as isoelectric soy isolate at pH 4.5 (7). α_{s1}-Casein was prepared from fresh milk by the method of Zittle and Custer (8). Galactomannan was prepared from mannase hydrolysate of guar gum (Taiyo Chemicals Co., Japan) by dialyzing against deionized water for 2 days at 4°C (9). Sunsoft SE11 (sucrose-fatty acid ester, HLB1) and Sunsoft Q-18S (decaglyceryl monoesterate, HLB12) were supplied from Taiyo Kagaku Co., Japan. The S9 fraction from rat liver was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). 2-(2-Furyl)-3-(5-nitro-furyl) acrylamide, 2-aminoanthracene and mitomycin C were from Sigma-Aldrich Japan. All chemicals used here were special grades commercially available.

Preparation of food protein conjugates with galactomannan

Food proteins were conjugated with a soluble polysaccharide, galactomannan through naturally occurring Maillard reaction as previously reported (10). Galactomannan was mixed with lysozyme, ovalbumin, phosvitin, acid-precipitated soy protein or α_{s1}-casein, in distilled water at the weight ratio of 1:1, respectively, and then completely lyophilized. The resulting lyophilized powder was incubated at 60°C under the relative humidity of 79%. After a given time, the dry-heated powder was removed to a sealed bottle and stored at 4°C until further experiments. The degree of the conjugation of food proteins with galactomannan was determined by SDS-slab polyacrylamide gel electrophoresis (11) after purification by gel filtration with a Sephacryl S-300 HR column (2×60 cm) and ion-exchange chromatography with CM-Toyopearl column (1.8×5 cm) or DEAE-Toyopearl column (1.8×5 cm).

Measurement of emulsifying properties

Emulsifying properties of food protein-galactomannan conjugates were determined according to the method of Pearce and Kinsella (12). Samples were dissolved in 20 mM phosphate buffer, pH 7.0, to be 0.1%, and 3 mL of the sample solution was homogenized with 1.0 mL of corn oil using a homogenizer (Polytron PT3100, KINEMATICA, Switzerland) at 12,000 rpm for 1 min at 20°C to prepare an O/W type emulsion. 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. The emulsion stability was estimated by measuring the half-life time for emulsion decay during standing for 10 min.

Measurement of foaming properties

Foaming properties were determined by measuring the volume of foams produced by an inactive gas. One mL of a 0.1% sample solution in 20 mM phosphate buffer (pH 7.0) was put into a glass column (1.1×20 cm) with a G4 glass filter (VIDREX, Japan), and nitrogen gas was then introduced for 10 seconds at a constant flow rate of 0.5 L/min. The resulting foams were scaled after standing for 0, 5, 10, 15, 20, 30 and 60 sec. The foaming activity was represented the volume (height) of foams immediately after foaming formation. The foam stability was estimated by measuring the half-life time (sec) of the initial height of the foam. This experiment was performed under room temperature (20°C).

Bacterial mutagenesis test

Ames test: The mutagenicity of food protein-galactomannan conjugates was assessed in two *Salmonella typhimurium* strains, a base-pair substitution strain TA 100 (*hisG46*) and a frame shift strain TA98 (*hisD3052*) obtained from Dr. Y. Noguchi (Ube scientific analysis Lab., Inc., Japan), by preincubation method as described (13). The test strains were incubated with 10 mg/mL sample at 37°C for 20 min with or without the S9 mix from rat liver, and then poured onto a minimum agar plate with top agar (1 mg of L-histidine, 1.2 mg of D-biotin 1.2 mg, 0.5 g of NaCl, 0.6 g of agar, and water to 100 mL). The number of colonies formed after incubation at 37°C for 48h was measured to calculate the revertant ratio. 2-(2-furyl)-3-(5-nitro-furyl) acrylamide (AF-2) and aminoanthracene (2-AA) were used as a positive control in this study.

Rec-assay: The repair test with *Bacillus subtilis* was carried out to determine the genotoxicity of food protein-galactomannan conjugates. The wild-type strain *B. subtilis* H17 (*rec*$^{+}$) and a recombinationless strain *B. subtilis* M45 (*rec*$^{-}$) were used as a set in this study. The *Bacillus* strains were the courtesy of Dr. S. Matsui, Kyoto University. Over-night cultivated *Bacillus* strains were streaked to be crossed at the center of standard agar plates (Nissui Co., Ltd., Japan), and a paper disk with 10.0 mm diameter

containing the sample solution is placed on the center of the streaks. The diameters of the growth inhibition were measured after incubation at 37°C for 24h to evaluate the repair ratio (14). Mitomycin C was used as a positive control in this study.

Experiments using animal

Male closed colony of SPF/VAF rats (Crj:CD (SD) IGS) of 4 weeks old, weighing 120 ~ 130 g was obtained from Charles River Japan Inc. (Yokohama). The rats were fed with a standard pelleted food (Charles River CRF-1) for 1 week prior to oral administration experiments. Food protein-galactomannan conjugates were orally given to the animals using a sonde at a maximum dosage of 7.5 g kg^{-1} body-weight. The executed animals were subsequently fed with the commercial pellet for 2 more weeks in order to observe semi-acute toxicity of the conjugates.

The 7 weeks old rats were submitted to dissection, and the weight of each internal organ was measured after directly drawing blood from heart. Total cholesterol, triglyceride and phospholipids in serum were determined by using TC kit-K, TG kit-GN and PL kit-K (AZWELL Inc., Japan), respectively.

Six rats per treatment were used in each experiment. Handling and feeding conditions of the rats were as followed: Each of animals was individually brought up in an isolated cage under free with food and water. The temperature of animal room was kept at 20°C with filtrated fresh air under the condition of 60% relative humidity. Lighting was turned on for 12 h by the illumination from 6 am to 6 pm.

Statistical analysis

All experiments were conducted in multiple. Data were analyzed using Student *t* test (15) for comparing differences between treatment means (SAS Institute Inc., 1988).

RESULTS AND DISCUSSION

Improved functional properties of food proteins by the conjugation with galactomannan

Freeze-dried food protein-galactomannan mixtures were incubated at 60°C under 79% relative humidity (RH) for 3 days except for hen-egg white lysozyme-galactomannan mixture. Conjugation of lysozyme and galactomannan was required for 2 weeks. The resulting protein-polysaccharide conjugates were separated from the unreacted protein and polysaccharide molecules by size-exclusion chromatography and ion-exchange chromatography. Formation of covalent linkage between food proteins and galactomannan was confirmed by SDS-slab polyacrylamide gel electrophoresis as previously reported (2).

Emulsifying properties of protein-galactomannan conjugates were measured and compared with those of protein-galactomannan mixtures (without incubation at 60°C). As shown in Table 1, the introduction of the polysaccharide moiety drastically improved emulsifying properties of all proteins. Emulsifying activities of food proteins were improved 1.6 ~ 10.2 times by the conjugation with galactomannan. In addition, emulsifying properties of all protein-galactomannan conjugates were superior to those of commercial emulsifiers, sucrose-fatty acid ester HLB11 and decaglyceryl monoesterate HLB12. Emulsifying activities of HLB11 and HLB12 were 0.950 and 1.195, respectively. Especially, emulsion stabilities of HLB11 and HLB12 were poor of 4.1 and 2.5, respectively, while all food protein-galactomannan conjugates showed high scores. The observation suggests that the affinity of macromolecule protein to oil was remarkably improved by covalent linking with polysaccharide chains.

Foaming properties of the food protein-galactomannan conjugates were also investigated. Table 2 summarizes the foaming properties of food protein-galactomannan conjugates. Foaming activities of galactomannan conjugates with lysozyme, ovalbumin, phosvitin, soy protein and α_{s1}-casein were 12.3, 8.4, 4.7, 4.4 and 3.8 times better than those of the control galactomannan mixtures with food proteins (0 h-incubation). Furthermore, foam stabilities of all food proteins were drastically improved. In particular, phosvitin-galactomannan conjugate and α_{s1}-casein-galactomannan conjugate showed excellent foam stabilities. Thus, we have succeeded in developing macromolecular emulsifiers having excellent foaming properties that can be used in practical food processing.

Table 1. Changes of emulsifying properties of food proteins by the conjugation with galactomannan

	Mixture	Conjugate
Relative emulsifying activity[1)]		
Lysozyme-galactomannan	0.190 ± 0.05	1.880 ± 0.05
Ovalbumin-galactomannan	0.280 ± 0.03	1.310 ± 0.07
Phosvitin-galactomannan	1.190 ± 0.04	1.920 ± 0.06
Soy protein-galactomannan	0.350 ± 0.03	1.360 ± 0.04
α_{s1}-casein-galactomannan	1.170 ± 0.05	1.890 ± 0.06
Emulsion stability[2)]		
Lysozyme-galactomannan	0.50 ± 0.1	>10
Ovalbumin-galactomannan	0.40 ± 0.1	7.50 ± 0.3
Phosvitin-galactomannan	6.20 ± 0.2	>10
Soy protein-galactomannan	5.50 ± 0.3	>10
α_{s1}-casein-galactomannan	2.80 ± 0.3	>10

[1)] The relative emulsifying activity was represented as the absorbance at 500 nm that was measured immediately after emulsion formation.
[2)] The emulsion stability was estimated by measuring the half-life time (min) of the decay of emulsion. Each value is the mean ± standard deviation of three replications.

Table 2. Changes of foaming properties of food proteins by the conjugation with galactomannan

	Mixture	Conjugate
Relative foaming activity[1]		
Lysozyme-galactomannan	1.10±0.2	13.50±0.5
Ovalbumin-galactomannan	1.70±0.2	14.20±0.6
Phosvitin-galactomannan	2.10±0.3	9.80±0.5
Soy protein-galactomannan	1.60±0.3	7.10±0.5
α_{s1}-casein-galactomannan	2.60±0.2	9.80±0.6
Foam stability[2]		
Lysozyme-galactomannan	ND[3]	10.51±0.2
Ovalbumin-galactomannan	ND	20.32±0.5
Phosvitin-galactomannan	ND	>60
Soy protein-galactomannan	ND	17.12±0.2
α_{s1}-casein-galactomannan	3.50±0.5	>60

[1]The foaming activity was represented the volume (height, cm) of foams immediately after foaming formation.
[2]The foam stability was estimated by measuring the half-life time (sec) of the initial height of the foam. Each value is the mean±standard deviation of three replications.
[3]Not detected.

Bacterial mutagenicities of food protein-galactomannan conjugates

Food safety of the food protein-polysaccharide conjugates was evaluated by the general bacterial mutagenesis test (*Ames* test) and another bacterial mutagenesis test based on DNA-recombination (*rec*-assay).

Ames test: Spontaneous revertants of *Salmonella typhimurium* TA 100 were 145 and 131 CFU/plate with and without S9 mix, respectively. 0.5 μg/plate of aminoanthracene (2-AA) with S9 mix and 0.01 μg/plate of AF-2 (2-(2-furyl)-3-(5-nitro-furyl) acrylamide) without S9 mix caused 362 and 663 CFU/plate revertants, respectively. On the other hand, the spontaneous colonies of *S. typhimurium* TA 98 were 28 and 18 CFU/plate with and without S9 mix, respectively, while 0.5 mg/plate of 2-AA with S9 mix and 0.01 μg/plate of AF-2 without S9 mix produced 135 and 210 CFU/plate revertants, respectively. As shown in Table 3, *Salmonella* revertants were detected 135~181 and 130~183 CFU/plate revertants with and without S9 mix, respectively, in the coexisting systems of food protein-galactomannan conjugates. All conjugates were evaluated as negative, because samples expressing mutagenicity twice higher than spontaneous revertants with a dose-dependent relationship were considered as positive (16).

Rec-assay: Table 4 shows genotoxic effects of food protein-galactomannan conjugates. No growth inhibitory of the recombinationless strain *B. subtilis* M45 (rec^-) was observed in the *rec*-assay system for galactomannan conjugates with ovalbumin, acid-precipitated soy protein and

Table 3. Mutagenicity of food protein-galactomannan conjugates based on *Ames* test

	Amount (mg/plate)	CFU numbers of revertants/plate			
		TA 100		TA 98	
		with S9 mix	without	with S9 mix	without
Protein-galactomannan conjugates					
Lysozyme conjugate	0.5	143±15[1]	148±26	31±0.2	18±2
	1.0	148±29	138±25	29±0.2	18±3
Ovalbumin conjugate	0.5	175±18	157±19	30±0.2	20±3
	1.0	181±24	163±18	33±0.2	25±3
Phosvitin conjugate	0.5	146±20	130±26	28±0.2	18±2
	1.0	135±21	139±19	27±0.2	17±3
Soy protein conjugate	0.5	153±19	166±11	31±0.2	22±4
	1.0	169± 9	178±19	35±0.2	24±2
α_{s1}-casein conjugate	0.5	145±12	163±11	34±0.2	23±3
	1.0	167±24	183±25	37±0.2	25±3
Positive controls					
AF-2[2]	0 (negative control)	ND[4]	131± 8	ND	18± 3
	0.01	ND	663±12	ND	210±28
	0.1	ND	1985±22	ND	645±31
2-AA[3]	0 (negative control)	145± 7	ND	28± 2	ND
	0.5	362±11	ND	135± 5	ND
	1.0	963±25	ND	513±12	ND

[1]Each data is the mean±standard deviation of three replications.
[2]AF-2: 2-(2-furyl)-3-(5-nitro-furyl) acrylamide.
[3]2-AA: 2-aminoanthracene.
[4]ND: Not determined.

Table 4. Genotoxic effects of food protein-galactomannan conjugates

	Amount (mg/disc)	Diameter of growth inhibitory (mm)		M45 - H17 (mm)
		H17 (rec^-)	M45 (rec^+)	
Protein-galactomannan conjugates				
Lysozyme conjugate	0.5	11.3±0.5[1)]	12.5±0.6	1.2
	1.0	15.2±0.4	15.9±0.4	0.7
Ovalbumin conjugate	0.5	<10.0[2)]	<10.0	0
	1.0	<10.0	<10.0	0
Phosvitin conjugate	0.5	11.5±0.5	11.9±0.4	0.4
	1.0	12.2±0.3	12.6±0.5	0.4
Soy protein conjugate	0.5	<10.0	<10.0	0
	1.0	<10.0	<10.0	0
α_{s1}-casein conjugate	0.5	<10.0	<10.0	0
	1.0	<10.0	<10.0	0
Positive controls				
Mitomycin C	0 (negative control)	<10.0	<10.0	0
	0.0001	<10.0	19.3±0.5	9.3

1)Each data is the mean±standard deviation of three replications.
2)<10.0 represents that no growth inhibitory was observed.

α_{s1}-casein, whereas lysozyme and phosvitin conjugates showed some bacteriostatic effects against the test strain. However, the same inhibition of growth was observed in the assay system using wild-type strain *B. subtilis* H17 (*rec*$^+$) for the both conjugates, as shown in Table 4. In this study, the DNA-damaging activity was determined as positive if the difference exhibits more than 3.0 mm between the diameters of growth inhibition zones that were produced in *B. subtilis* H17 and M45 strains. The difference was 9.3 mm against mitomycin C, a positive control. Thus, it was concluded that the genotoxicity was negative for a range of all tested protein-galactomannan conjugates.

Acute and semi-acute toxicities of food protein-galactomannan conjugates

Further food safety of the food protein-polysaccharide conjugates was investigated according to animal dose test. Lethality of each conjugate was determined by oral administration to rats and the value represents an amount of compound that cause 50% death of tested rats (LD_{50}, g/kg). All conjugates were negative for the acute toxicity test in which LD_{50}'s of all conjugates showed more than 7.5 g/kg body-weight of rats. Then, the animals orally administrated with the maximum dosage of 7.5 g/kg were subsequently fed with standard pelleted food for 2 weeks, and monitored by changes of body weight. As the result, it was demonstrated that growth rates of the prescribed animals were not significantly ($p<0.05$) different from that of the control animal (Fig. 1). The body weights of 7 weeks old rats given the galactomannan conjugates with lysozyme, ovalbumin, phosvitin, soy protein and α_{s1}-casein were 311±17 g (n=6), 317±19 g (n=6), 311±20 g (n=6), 315 ±15 g (n=6) and 318±21 g (n=6), respectively, where that of control group was 313±15 (n=6).

The weight of intestines and blood characteristics were investigated in the 7 weeks old animals in addition to the measurement of the weight of intestines. The weights of liver, spleen and kidney were respectively 9.1±0.8 g (n=6), 0.7±0.1 g (n=6) and 2.5±0.2 g (n=6) in the control rats. The weight ranges of liver, spleen and kidney were 9.0~9.8 g, 0.6~0.7 g and 2.3~2.7 g, respectively, in the orally administrated rats with food protein conjugates (n=60). Any significant difference was not observed in the intestine weights between the administrated groups and the control group ($p<0.05$). On the other hand, the ranges of the concentration of total cholesterol, triglyceride and phospholipids were 38.6~42.9 mg/100 mL (n=60), 43.9~47.1 mg/100 mL (n=60) and 31.1~35.6 mg/100 mL (n=60), respectively, in serum from the orally administrated rats with food protein conjugates. Since the contents of total cholesterol, triglyceride and phospholipids were 41.5±2.5 mg/100 mL (n=6), 48.6±5.8 mg/100 mL (n=6) and 32.3±4.3 mg/100 mL (n=6), respectively, in serum from the control rats, no significant difference ($p<0.05$) was observed between the administrated animals and the control group.

If galactomannan conjugates were constantly administrated to the tested rats for 2 weeks, some reducing effects might be observed, because Yamamoto et al. (17) reported that oral administration of galactomannan decreased total content of lipids in the liver of rats. Recently, the administration of some polysaccharides was reported to enhance protection of animals against bacterial infection as immunostimulants (18,19). In mammals, polysaccharides potentiate

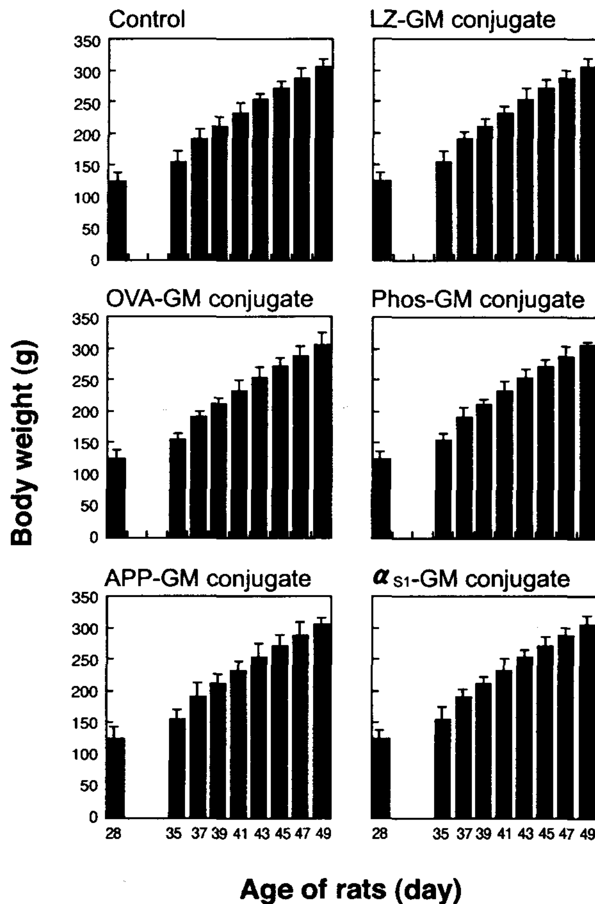

Fig. 1. Growth curves of rats. Four weeks old (28 days) rats were preliminarily fed with a standard pelleted food for 1 week, and then 7.5 g/kg body-weight of food protein-galactomannan conjugates was orally administrated to the 5 weeks old (35 days) animals, and the animals were subsequently fed with the commercial pellet until 7 weeks old (49 days). LZ, OVA, Phos, APP, α_{s1} and GM represent hen egg-white lysozyme, ovalbumin, egg-yolk phosvitin, acid-precipitated soy protein, α_{s1} milk casein and galactomannan, respectively. T-shaped line indicates standard deviation (n=6).

the nonspecific immune responses, such as activation of macrophages, NK cells, T-lymphocytes and interferon products (20-22). These healthy effects of polysaccharides have been also anticipated to be important in using galactomannan conjugates as food ingredient. In this study, five galactomannan conjugates were prepared by covalent liking with hen egg-white lysozyme and ovalbumin, egg-yolk phosvitin, acid-precipitated soy protein, and α_{s1} milk casein in the dry-heating at 60°C with 79% relative himidity. It was confirmed that all conjugates showed excellent emulsifying properties and foaming properties. Food safety of the conjugates was evaluated by bacterial mutagenesis tests and animal dose tests, and confirmed that all conjugates were found to be negative for *Ames*-test and *rec*-assay and were nontoxic in oral administration tests. Thus, we succeeded to develop natural macromolecular reagents with safety having good surface functional properties.

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REFERENCES

1. Damodaran S. 1994. Structure-function relationship of food proteins. In *Protein Functionality in Food Systems*. Hettiarachchy NS, Ziegler GR, eds. Marcel Dekker Inc, New York. p 1-37.
2. Nakamura S, Kato A. 2000. Multifunctional biopolymer prepared by covalent attachment of galactomannan to egg-white proteins through naturally occurring Maillard reaction. *Nahrung/Food* 44: 201-206.
3. Kato A, Nakamura S, Takasaki H, Maki S. 1996. Novel functional properties of glycosylated lysozymes constructed by chemical and genetic modifications. In *Macromolecular Interactions in Food Technology*. Parris N, Kato A, Creamer LK, Pearce J, eds. American Chemical Society, Washington DC. p 243-256.
4. Alderton G, Fevolt HL. 1946. Direct crystallization of lysozyme from egg white and some crystalline salts of lysozyme. *J Biol Chem* 164: 1-5.
5. Kekwick RA, Cannan RK. 1936. The hydrogen ion dissociation curve of the crystalline albumin of the hen eggs. *Biochem J* 30: 277-283.
6. Mecham DK, Olcott HS. 1946. Phosvitin, the principal phosphoprotein of egg yolk. *J Am Chem Soc* 71: 3670-3679.
7. Rhee KC. 1994. Functionality of soy proteins. In *Protein Functionality in Food Systems*. Hettiarachchy NS, Ziegler GR, eds. Marcel Dekker Inc, New York. p 311-324.
8. Zittle CA, Custer JH. 1963. Purification and some of the properties of α_s-casein and α-casein. *J Dairy Sci* 46: 1183-1187.
9. Nakamura S, Kato A, Kobayashi K. 1992. Bifunctional lysozyme-galactomannan conjugate having excellent emulsifying properties and bactericidal effect. *J Agric Food Chem* 40: 735-739.
10. Nakamura S, Ogawa M, Saeki H, Saito M, Miyasaka S, Hata J, Adachi N, Hwang J-K. 2000. Improving the surface functionality of curdlan by conjugation with unfolding protein through naturally occurring Maillard reaction. *J Food Sci Nutr* 5: 200-204.
11. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
12. Pearce KM, Kinsella JE. 1978. Emulsifying properties of proteins: Evaluation of a turbidimeric technique. *J Agric Food Chem* 26: 716-723.
13. Maron DM, Ames BN. 1983. Revised methods for *Salmonella* mutagenicity test. *Mutat Res* 113: 173-215.
14. Kada T, Tutikawa T, Sadaie Y. 1972. In vitro and host-mediated *rec*-assay procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected. *Mutat Res* 16: 165-174.
15. SAS Institute Inc. 1988. SAS/STAT User's Guide. release

6.03 ed. Statistical Analysis System Institute, Cary, NC. p 987-994.
16. De Serres FJ, Shelby MD. 1979. Recommendations on data production and analysis using *Salmonella* microsome mutagenicity assay. *Mutat Res* 64: 159-165.
17. Yamamoto T, Yamamoto S, Miyahara I, Matsumura Y, Hirata A, Kin M. 1990. Isolation of a β-mannan hydrolyzing enzyme and hydrolysis of guar gum by the enzyme isolated. *Denpun Kagaku* 37: 99-105.
18. Yano T, Matsuyama H, Mangindaan REP. 1991. Polysaccharide induced protection of carp, *Cyprinus carpio* L., against bacterial infection. *J Fish Disease* 14: 577-582.
19. Matsuyama H, Mangindaan REP, Yano T. 1992. Protective effect of schizophyllan and scleroglucan against *Streptococcus* sp. infection in yellowtail (*Seriola quinqueradiata*). *Aquaculture* 101: 197-203.
20. Okuda T, Yoshioka Y, Ikekawa T, Chihara G, Nishioka K. 1972. Anti-complementary activity of anti-tumour polysaccharides. *Nature: New Biology* 238 (12): 59-60.
21. Hamuro J, Hadding U, Bitter-Suermann D. 1978. Solid phase activation of alternative pathway of complement by β-1,3 glucans and its possible role for tumour regressing activity. *Immunology* 34: 695-670.
22. Matsuyama H, Yano T, Yamakawa T, Nakano M. 1992. Opsonic effect of the third complement component (C3) of carp (*Cyprinus carpio*) on phagocytosis by neutrophils. *Fish & Shellfish Immunology* 2: 69-78.

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