

Enhanced bactericidal action and masking of allergen structure of soy protein by attachment of chitosan through Maillard-type protein-polysaccharide conjugation

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The soy protein-chitosan conjugate was formed by the Maillard reaction in dry state (relative humidity 65%) at 60 °C for 2 weeks to improve the functional properties. The antimicrobial activity of the Maillard-type soy protein-chitosan conjugates enhanced 2–3 times that of soy protein-chitosan mixture. The soy protein-chitosan conjugate showed excellent emulsifying property with the progress of Maillard-type conjugation. The allergenicity of soy protein was greatly decreased by the

attachment of chitosan through Maillard reaction. The immunoblotting analysis with patient's sera revealed that soy protein-chitosan conjugate was more effective to mask the allergen structure of soy protein causing from 34 kDa-protein (Gly m Bd 30K) than soy protein-galactomannan conjugate. The Western blotting showed that allergen (34 kDa-protein) was completely masked by soy protein-chitosan conjugation, while it was not completely masked by soy protein-galactomannan conjugation.

1 Introduction

Soybeans are one of the important protein sources in food processing because of their nutritional and functional benefits. However, soybeans contain allergenic proteins. Therefore, human allergies to soybeans are becoming of a concern as soy products become more common in processed foods. Ogawa *et al.* [1] purified and characterized a 34 kDa-protein (Gly m Bd 30K) in soybean as a major allergen. It has been reported that about 65% of patients atopic to soybeans suffer from the 34 kDa-protein. We have developed a method to mask the allergenic structure of the responsible soy protein [2]. Soy protein was attached with galactomannan preparation (a group of polysaccharide of average molecular weight of 15 000), man-nase hydrolysate of guar gum, through spontaneous Maillard reaction between the ϵ -amino groups in protein and the reducing end of carbonyl group in polysaccharide. The soy protein-galactomannan conjugate was more effective to reduce the allergenicity than transglutaminase and protease treatments. Although we reported [2] the masking of the epitope in 34 kDa-protein by Maillard-type galactomannan conjugation, further effective reduction of allergenicity of soy protein should be investigated using other polysaccharide partner of the protein-polysaccharide conjugate. In addition, the endowment of further useful functional property such as antimicrobial action is desirable for the conjugate. The promising functional polysaccharide as a partner is chitosan that is a partially deacetylated polymer of *N*-acetyl glucosamine prepared from chitin in a wide range of natural source such as crustaceans,

fungi, and insects. Chitosan is a natural biopolymer, nontoxic, and biodegradable. The antimicrobial activity of chitosan is well observed in a wide variety of microorganisms including fungi, algae, and some bacteria. Therefore, the attachment of chitosan with soy protein is expected to form a functional soy protein-polysaccharide conjugate having antimicrobial activity. This paper describes the effect of chitosan attachment on the reduction of the allergenicity of soy protein to develop further functional protein-polysaccharide conjugate.

2 Materials and methods

2.1 Materials

Defatted soy protein extract was provided from Nissin Oil Mills Ltd (Japan). Chitosan (MW 3–30 kDa) was purchased from Wako Co. (Japan). A galactomannan preparation (average molecular mass, 15 kDa) was obtained by dialyzing a mannase hydrolysate of guar gum from Taiyo Chemical Co. (Japan). Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) and HRP-conjugated anti-human IgE were obtained from KPL (Gaithersburg, MD, USA). Coomassie Brilliant Blue R-350 and the ECL Western blotting kit were purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden). Unless otherwise stated, all reagents were of reagent grade.

2.2 Preparation of acid-precipitated soy protein (APP)

Acid-precipitated soy protein (APP) was prepared by the method of Iwabuchi *et al.* [3]. A sample of defatted soy protein extract (100 g) was extracted once with 2 L of 0.03 M Tris-HCl buffer (pH 8) containing 10 mM 2-mercaptoethanol (2-ME) at 20 °C. After centrifugation, the supernatant was acidified to pH 4.8 with 2 N HCl and then centrifuged. The precipitated protein was dissolved in water at 4 °C and the pH adjusted to 8. After centrifugation (8000 rpm), the clear supernatant was dialyzed against distilled water for 24 h at 4 °C and then freeze-dried.

2.3 Soy protein-polysaccharide conjugation

The soy protein-polysaccharide conjugate was prepared by the method of Kato *et al.* [4]. The APP-polysaccharide powder mixture in the weight ratio of 1:1 was dissolved in water at

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Abbreviations: APP, acid-precipitated soy protein; HRP, horseradish peroxidase; IgG, immunoglobulin G; RH, relative humidity

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10% w/v and freeze-dried. Powdered APP-polysaccharide mixtures were dry-heated at 60°C under 65% relative humidity (RH) in a desiccator containing saturated KI solution in the bottom for a given time (0, 7, 14 days). The APP-polysaccharide conjugates were separated from unbound protein and polysaccharides by anion-exchange chromatography on a column of DEAE-Toyoperl.

2.4 Antimicrobial activity of soy protein-chitosan conjugates

The antimicrobial activity was measured using *Escherichia coli* K-12. *E. coli* was cultured for 10 h (mid-exponential phase) in LB medium, and diluted 1000 times with 20 mM phosphate buffer (pH 7.0). A 4.5 mL of the cell suspension was mixed with 0.5 mL of sample solution (50 µg/mL) and incubated at 37°C for 30 min. Then 100 µL of cell suspension was surface-plated onto an agar plate. Colonies were counted after incubation at 37°C overnight. Sample-free solution was used as a control.

2.5 Measurement of emulsifying properties

The emulsifying properties of freeze-dried sample solutions were determined by the method of Pearce and Kinsella [5]. To prepare emulsions, 1.0 mL of corn oil and 3.0 mL of protein solutions (0.2%) in 0.1 M phosphate buffer (pH 7.0) were shaken together and homogenized in an Ultra Turrax (Hansen & Co., West Germany) at 12000 rpm for 1 min at 20°C. A 50 µL sample of emulsion was taken from the bottom of the container at different times and diluted with 5 mL of 0.1% SDS solution. The absorbance of the diluted emulsion was then determined at 500 nm. The relative emulsifying activity was determined from the absorbance immediately measured after the emulsion formation. The emulsion stability was expressed as the half time of the initial turbidity of the emulsion. Values obtained are means of triplicate samples.

2.6 SDS-PAGE and immunoblotting

SDS-PAGE was carried out on a 15% polyacrylamide gels according to the method of Laemmli [6]. Samples (20 µL, 0.2%) were prepared in a Tris-glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was done at a current of 10 mA for 1 h and thereafter at 20 mA for 2 h in electrophoretic Tris-glycine buffer containing 0.1% SDS. After electrophoresis, the gel sheets were stained for proteins and carbohydrate with 0.2% Coomassie Brilliant Blue R-250, then destained with 10% acetic acid containing 20% methanol. For immunoblotting analyses, the proteins separated by SDS-PAGE were electrotransferred onto nitrocellulose membranes and incubated with 10 mM sodium phosphate-buffered saline (PBS, pH 7.5) containing 0.1% Tween 20 and 2% skim milk for blocking. The membrane was incubated overnight with patient's sera diluted 1:6 in PBS. Bound IgE antibodies were detected using HRP-conjugated goat anti-human IgE and an ECL-Western blotting kit. Protein on the gel was stained by Coomassie Brilliant Blue R-350. In the case of the blotting with mouse monoclonal antibody F5 raised against Gly m Bd 30K [7], the bound F5 IgG antibody was detected using HRP-conjugated rabbit anti-mouse IgG and an ECL-Western blotting kit.

2.7 Human sera

Human sera were prepared from the patients allergic to soybean (RAST > 2), who were asked to donate blood sample for testing at Takamatsu Heiwa Hospital [8]. The pooled serum sample obtained from five nonallergic healthy individuals served as a negative control.

3 Results and discussion

3.1 Formation of Maillard-type soy protein-chitosan conjugate

The formation of covalently bound soy protein-chitosan conjugate through Maillard reaction was confirmed by SDS-PAGE, as shown in Fig. 1. The SDS-PAGE patterns display the broad high-molecular-weight bands for APP-galactomannan and APP-chitosan conjugates, indicating the formation of covalently attached protein-polysaccharide conjugates between ε-amino group in protein and the reducing end carbonyl group in polysaccharide through Maillard reaction during storage for two weeks at 60°C in dry state (RH 65%). The broad high-molecular weight peak of protein-polysaccharide conjugates appeared after storage for one week. In order to complete the formation of the conjugate, Maillard reaction was prolonged for another one week. As shown in Fig. 1, most soy protein components were reacted with polysaccharides after storage for two weeks. It has been reported in a case of other protein-polysaccharide conjugates that it took about two weeks for the maximum attachment of polysaccharides to rigid globular proteins [9, 10]. Therefore, the soy protein-polysaccharide conjugates were prepared by storage in dry state at 60°C for 2 weeks for the maximum attachment of polysaccharide to soy protein.

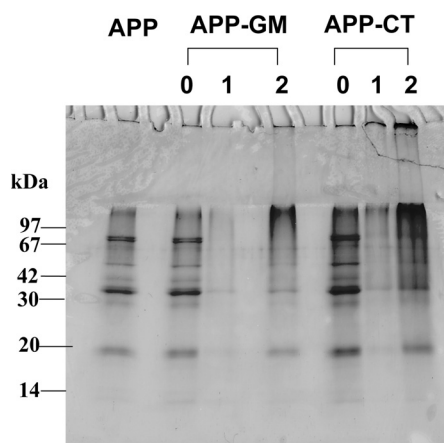


Figure 1. SDS-PAGE patterns of soy protein-polysaccharide conjugates. APP, soy protein; APP-GM, APP-galactomannan conjugate; APP-CT, APP-chitosan conjugate. 0, Untreated protein-polysaccharide mixture; 1, soy protein-chitosan conjugate obtained after 1 week storage in dry state at 60°C; 2, soy protein-chitosan conjugate obtained after 2 week storage in dry state at 60°C.

3.2 Improvement of the antimicrobial activity of soy protein-chitosan conjugate

The antimicrobial activity of chitosan is well observed in a wide range of microorganism. Therefore, the bactericidal action of soy protein-chitosan conjugate is expected to exhibit in a similar manner. Figure 2 shows the antimicrobial activity

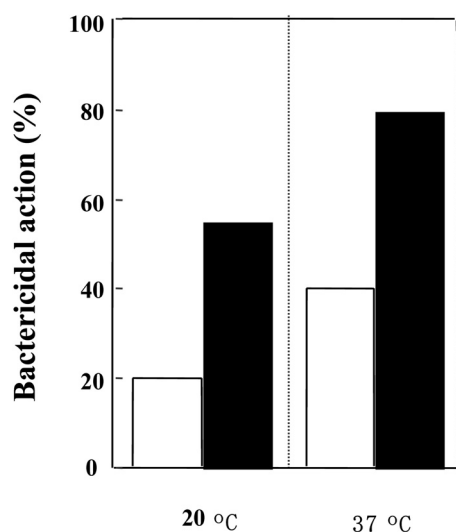


Figure 2. Bactericidal action of soy protein-chitosan conjugate against *E. coli*. (□) Soy protein-chitosan mixture; (■) soy protein-chitosan conjugate prepared after 2 week's storage in dry state at 60 °C. Bactericidal action was measured at 20 and 37 °C. Bactericidal action was indicated as a mean of triplicate measurements.

of Maillard-type soy protein-chitosan conjugate. Interestingly, the bactericidal action against *E. coli* of soy protein-chitosan conjugate was enhanced about 2–3 times that of soy protein-chitosan mixture. Since the protein-polysaccharide conjugate is known to become surface-active [9], the soy protein-chitosan conjugate may destabilize the cell membrane of *E. coli* to strengthen the antimicrobial activity in combination with chitosan. On the other hand, the soy protein-galactomannan conjugate did not show any antimicrobial effect at 20 and 37 °C (data not shown).

3.3 Improvement of the emulsifying properties of soy protein-chitosan conjugate

We reported that the emulsifying properties of soy protein greatly improved by Maillard-type protein-polysaccharide conjugation [2]. As shown in Fig. 3, the emulsifying property of soy protein-chitosan conjugate was also enhanced with the pro-

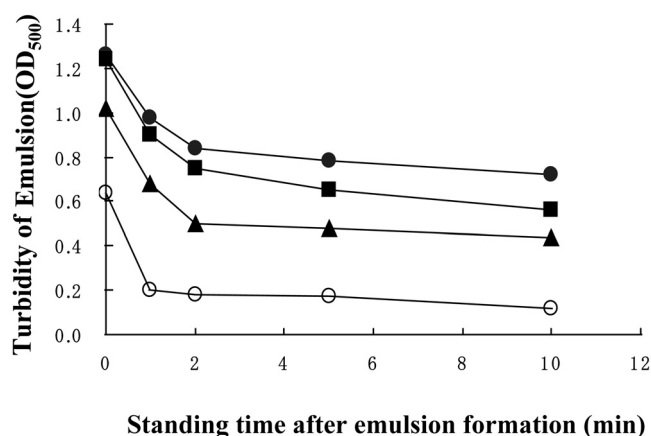


Figure 3. Changes in the turbidity of emulsion of soy protein-chitosan conjugates prepared by Maillard reaction for various periods. (○) Soy protein-chitosan mixture; (▲), (■), (●) soy protein-chitosan conjugates prepared by storage in dry state at 60 °C for 3, 7, and 14 days, respectively.

Table 1. Changes in the emulsifying properties of soy protein-chitosan conjugate during storage in dry state

Storage time (days)	Relative emulsifying activity (turbidity at 500 nm)	Emulsion stability (half times, min)
0	0.64	0.7
3	1.02	2
7	1.24	8
14	1.26	16

RH 65% at 60 °C

gress of the conjugation of soy protein-chitosan through Maillard reaction. The emulsifying property of native soy protein was low, but that of soy protein-chitosan conjugate was greatly improved with the dry-heating time. The relative emulsifying activity and emulsion stability of soy protein-chitosan conjugate during the progress of the conjugation was calculated from Fig. 2. As shown in Table 1, the emulsifying activity was increased about two times and the emulsion stability was increase eight times after 2-week storage. The similar improvement of emulsifying properties was observed for soy protein-galactomannan conjugate [10].

3.4 Reduction of allergenicity (IgE-binding ability) of soy allergen through the attachment of polysaccharide

The Western blotting analysis was carried out to investigate the masking of major allergen 34 kDa-protein (Gly m Bd 30K). As shown in Fig. 4, the band of soy allergen (34 kDa-protein) immunoblotted with F5 monoclonal antibody completely disappeared in soy protein-chitosan conjugate, although it considerably remained in soy protein-galactomannan conjugate. This suggests that the conjugation with chitosan is more effective to reduce the allergenicity of soy proteins than that

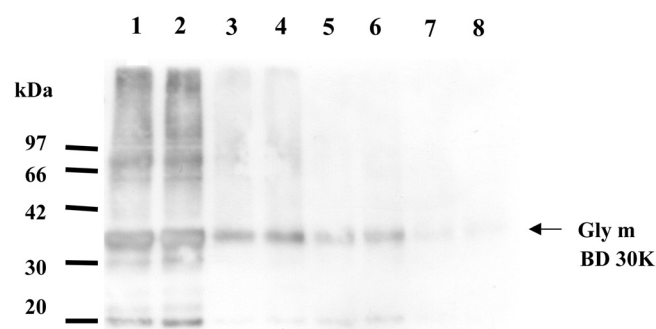


Figure 4. Western blotting patterns of soy protein-polysaccharide conjugates. 1, 2: APP; 3, 4: APP-GM conjugate (1 week); 5, 6: APP-GM conjugate (2 weeks); 7, 8: APP-CT conjugate. Gly m BD 30 K, soy allergen 34 kDa-protein; APP, soy protein; GM, galactomannan; CT, chitosan.

with galactomannan. This was further confirmed using patient's sera. The dot blotting with IgE antibody in patient's sera also indicated that the conjugation with chitosan is more effective to reduce the allergenicity of soy proteins than that with galactomannan (Fig. 5). The cross-reactivity of soy protein-galactomannan conjugate was slightly observed, while that of soy protein-chitosan conjugate was not observed. This was further supported from the dot-blotting with F5 monoclo-

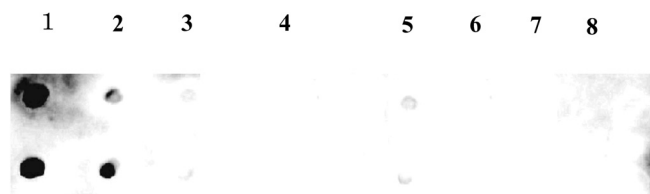


Figure 5. Dot immunoblotting of APP-polysaccharide conjugates against patient's sera. Lanes 1, 2, 3, and 4 are the dotting for APP, APP-GM (1 week), APP-GM (2 weeks), and APP-CT (2 weeks), respectively, when the antigen protein concentration is 0.3 mg/dot. Lanes 5, 6, 7, and 8 are the dotting for APP, APP-GM (1 week), APP-GM (2 weeks), and APP-CT (2 weeks), respectively, when the antigen protein concentration is 0.03 mg/dot.

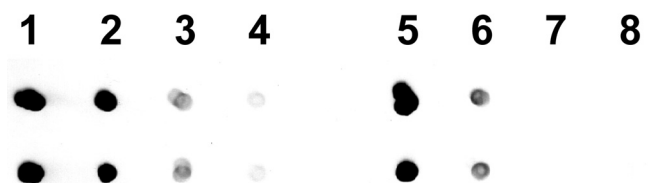


Figure 6. Dot immunoblotting of APP-polysaccharide conjugates against F5 monoclonal antibody. Lanes 1, 2, 3, and 4 are the dotting for APP, APP-GM (1 week), APP-GM (2 weeks), and APP-CT (2 weeks), respectively, when the antigen protein concentration is 0.3 µg/dot. Lanes 5, 6, 7, and 8 are the dotting for APP, APP-GM (1 week), APP-GM (2 weeks), and APP-CT (2 weeks), respectively, when the antigen protein concentration is 0.03 µg/dot.

nal antibody, as shown in Fig. 6. The cross-reactivity of soy protein-chitosan conjugate with F5 monoclonal antibody greatly decreased compared to that of soy protein-galactomannan conjugate. Thus, it was confirmed that the attachment of chitosan to soy protein is very effective to reduce the allergenicity of soy allergen. The reason why chitosan is effective polysaccharide to mask the allergen structure of 34 kDa protein (Gly m Bd 30K) remains to be elucidated. It is probable that

the reactivity of Maillard reaction between chitosan and protein may be higher than that between galactomannan and protein or chitosan may ease to mask the allergenic structure of 34 kDa-protein.

In conclusion, the allergenicity of soy allergen was greatly reduced by attachment of chitosan to soy protein extract through Maillard-type protein-polysaccharide conjugation. In addition to the reduction of allergenicity, soy protein-chitosan conjugate exhibited enhanced antimicrobial activity and high emulsifying properties. Therefore, the soy protein-chitosan conjugate can be used as a new functional soy protein having antimicrobial activity and nonallergenicity for food applications.

4 References

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