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Reduction of *in vitro* allergenicity of buckwheat Fag e 1 through the Maillard-type glycosylation with polysaccharides

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Abstract

A major allergenic protein of buckwheat, Fag e 1 prepared from common buckwheat (*Fagopyrum esculentum*), was covalently linked with food-grade polysaccharides, arabinogalactan or xyloglucan through the controlled dry-heating at 60 °C under 65% relative humidity. The introduction of polysaccharide chain onto the molecular surface of Fag e 1 reduced the allergenicity of Fag e 1. The results revealed that the Maillard-type glycosylation of Fag e 1 with polysaccharides brought about a drastic reduction of the reactivity against human sera of buckwheat-allergy subjects, using immuno dot-blotting, QCM analysis and ELISA. In addition, the glycosylation of Fag e 1 yielded a great improvement of its surface functionality. Solubility of Fag e 1 at the neutral pH was substantially increased up to 13.5 times and 9.6 times by the conjugation with arabinogalactan and xyloglucan, respectively. Emulsifying properties of Fag e 1 were also improved by the glycosylation, of which both emulsifying activity and emulsion stability were more than 6 times higher than those of the native protein.
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Keywords: Fag e 1; Buckwheat; Allergenicity; Arabinogalactan; Xyloglucan; Maillard reaction; Solubility; Emulsifying properties

1. Introduction

Buckwheat has become popular as a healthy food, since it was reported that its seeds contain many biologically active compounds, such as flavonoids (Jiang et al., 2007; Quettier-Deleu et al., 2000) and dietary fibre (Li & Zhang, 2001), as well as rich proteins with well-balanced amino acids (Pomeranz & Robbins, 1970). Except its rich-bioactive compound properties, the allergy caused by buckwheat proteins has been recognized as a common food allergy in the world because of increasing consumption of buckwheat (Park et al., 2000; Wieslander, 1996; Wieslander & Norback, 2001; Wieslander et al., 2000). Buckwheat proteins are known to be a potent allergen causing serious symptoms with anaphylaxis in patients with hypersensitivity (Wieslander, 1996). Among buckwheat proteins recognized by the sera immunoglobulin E (IgE) from buckwheat-allergic patients, the 22 kDa protein was identified as the major allergen of buckwheat, with the molecular mass belonging to the globulin protein legumin-like β subunit (Park et al., 2000; Wang et al., 2004). A clinical study has shown that the globulin protein has a broad binding activity with almost all sera from different patients (Urisu et al., 1994), and thus it was considered to be a major allergen and was termed as Fag e 1 (Nair & Adachi, 1999). The respective cDNA of Fag e 1 was cloned, expressed in *Escherichia coli*, and characterized as allergenic protein (Nair & Adachi, 1999; Yoshioka, Ohmoto, Urisu, Mine, & Adachi, 2004). Thus, we have focused on Fag e 1 as a typical model allergenic protein from buckwheat in order to develop a new method that could

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decrease its allergenicity. We have reported that naturally occurring Maillard reaction could be useful to prepare neoglycoprotein from protein and polysaccharide, in which multiple functional improvements could also be expected due to modification of molecular surface of the target protein (Jing & Nakamura, 2005; Nakamura & Kato, 2000).

In protein molecules, 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. Therefore, most food proteins have potent surface functional properties arising from their amphiphilic properties (Nakamura, 2007). Fag e 1 in native form has less solubility and poor amphiphilicity. Introduction of polysaccharide chains onto the protein molecular surface will bring about not only reduction of the allergenicity but also improvement of the solubility and emulsifying properties of Fag e 1. Complex carbohydrates, i.e., polysaccharides from plant sources are widely used for medical, cosmetic, agricultural and food-related purposes. Current evidence indicates an immunostimulating role for polysaccharides. Accordingly, in this study, we made use of arabinogalactan as the template for glycosylation. Arabinogalactan has been registered as food ingredients in Japan and USA. Natural arabinogalactan from larch showed specific *in vivo* effects on natural killer (NK) cells, as well as a variety of other hemopoietic cells in both bone marrow and spleen of healthy young adult mice (Currier, Lejtenyi, & Miller, 2003). Serum lipids were decreased after 2-months administration of 8.4 g/day larch arabinogalactan in healthy human subjects (Marett & Slavin, 2004). Natural xyloglucan extracted from tamarind seed exhibited conjunctival cell adhesion ability and corneal epithelium wound healing effects (Burgalassi et al., 2000).The present study describes changes of allergenicity and surface functionalities of Fag e 1 after conjugation with the polysaccharide.

2. Materials and methods

2.1. Materials

Common buckwheat (*Fagopyrum esculentum* Moench) flour was obtained from Dr. S. Kasuga of the AFC research center in Shinshu University. Arabinogalactan (AG) and xyloglucan (XG) were from MRC Polysaccharide Inc. (Toyama, Japan) and Dainippon Sumitomo Seiyaku Inc. (Osaka, Japan), respectively. Sunsoft SE-16 (sucrose-fatty acid ester) was supplied from Taiyo Kagaku Co. (Tokyo, Japan). Rabbit anti-Fag e 1 antibody was entrusted with Kitayama Labes Co. Ltd (Nagano, Japan). Human sera were obtained from six food-allergy subjects, consisting of two males and four females ranging in age from 12 to 45 years old (Table 1). Superdex 200 and Q Sepharose FF were purchased from GE Healthcare in Japan (Tokyo, Japan). Goat anti-rabbit IgG labeled with HRP and goat anti-human IgE labeled with HRP were purchased from Stressgen Bioreagents Co. (Victoria, BC, Canada) and Morphosys UK Ltd. (Oxford, UK), respectively. All other reagents were of biochemical levels.

Table 1
Clinical characteristics of human sera used here

No.	Age (years)	Sex	IHR (symptoms)	Diagnosis
1	45	Male	APX	FA, AD, BA, AR
2	28	Female	APX	FA, AD, BA, AR
3	12	Female	UR	FA
4	32	Male	APX	FA, AD, BA, AR
5	18	Female	UR	FA
6	13	Female	UR	FA

APX, anaphylaxis including generalized skin, abdominal and respiratory symptoms; UR, generalized urticaria or angioedema only; FA, food allergy; AD, atopic dermatitis; BA, bronchial asthma; AR, allergic rhinitis.

2.2. Protein isolation and purification of Fag e 1

The extraction procedure of globulin protein was that of Wang et al. (2004) with some modifications. Common buckwheat flour was defatted with acetone and air-dried for one hour to remove acetone completely, and then the resulting powder was stirred with 10 times volume of an extraction solution containing 1.1% (w/v) NaCl and 0.28% (w/v) $NaHCO_3$ overnight at 4 °C. The extract was centrifuged at 10,000*g* for 30 min at 4 °C to remove lees, after filtration using four layers of gauze. The supernatant was once filtered through No. 5A filter paper (Advantec Co. Ltd., Tokyo, Japan), and proteins were salted out with 80% saturation level of ammonium sulfate. Resulting precipitate was collected by the centrifugation at 10,000*g* for 30 min at 4 °C, and dissolved into the extraction solution. The solution was dialyzed against distilled water, lyophilized and used as crude buckwheat protein powder for the next purification step. Fag e 1 with an apparent molecular mass of 22 kDa was purified using size exclusion chromatography followed by ion exchange chromatography from the crude protein sample. A Superdex 200 column 10/300 (GE Healthcare, Tokyo, Japan) was equilibrated and eluted with 50 mM sodium phosphate buffer (pH 7.0) containing 8 M urea and 1% 2-ME. Q Sepharose FF column (GE Healthcare, Tokyo, Japan) was also equilibrated with the same buffer and eluted with a linear salt gradient of 0–1.0 M NaCl in the buffer. The fractions of Fag e 1 were gathered and dialyzed against distilled water for 24 h, and finally the protein solution was concentrated and freeze-dried.

2.3. Preparation of Fag e 1–polysaccharide conjugate

The protein was conjugated with AG or XG using the method of Kato, Sasaki, Furuta, and Kobayashi (1990) Nakamura, Kato, and Kobayashi (1990). AG is a polysaccharide derived from Japanese larch with 5–20% concentration, consisting of arabinose and galactose. The molecular

weight of AG is polydispersed between 10 and 120 kDa, with average molecular weight of 20 kDa. On the other hand, XG is a relatively small size polysaccharide with average molecular weight of 1.4 kDa, which was prepared from tamarind seed by the β-glucanase hydrolyzation. XG is consisted of glucose and xylose. Fag e 1 and polysaccharides were individually dissolved in 50 mM phosphate buffer (pH 7.0) with a given weight ratio of 1:1 (weight base), and freeze-dried. Resulting lyophilized mixture was put into an incubator and stored at 60 °C for one week under the relative humidity of 65% to generate a naturally occurring Maillard-linkage. Fag e 1 conjugate with polysaccharide was separated from free protein and carbohydrate by the size exclusion chromatography using Superdex 200 column.

2.4. SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted according to the method of Laemmli (1970) using 15% (w/w) acrylamide separating gel with 5% (w/w) stacking gel containing 1% (w/v) sodium dodecyl sulphate (SDS). Samples were heated at 100 °C for 5 min in Tris–glycine buffer (pH 8.8) containing 1% SDS and 1% (v/v) 2-mercaptoethanol. Electrophoresis was carried out at a constant current of 15 mA for 3 h using an electrophoretic buffer of Tris–glycine containing 0.1% (w/v) SDS. After electrophoresis, the gels were stained for protein and carbohydrate with 0.025% (w/v) Coomassie Brilliant Blue R-250 solution and 0.5% (w/v) periodic acid–Fuchsin solution (Zacharius, Zell, Morrison, & Woodlock, 1969), respectively.

2.5. Determination of free amino groups

The content of free amino groups in the Fag e 1–polysaccharide conjugates was determined according to the method of Haynes, Osuga, and Feeney (1967) using trinitrobenzene sulphonate in determining free amino groups.

2.6. Circular dichroism (CD) spectrum analysis

The far-ultraviolet (190–260 nm) CD spectra of Fag e 1 and Fag e 1 conjugates were recorded at a protein concentration of 300 μg-protein ml^{-1} in 50 mM acetate buffer (pH 3.0) using a JASCO spectropolarimeter J-600 (Japan Spectroscopic Co. Ltd., Tokyo, Japan) using a 1.0-cm cuvette. The temperature of the cuvette was maintained at 25 °C with a circulating water bath. Accumulation of the CD spectra was initiated within 5 min of recording the absorbance spectra of the samples. The resulting averaged spectra were normalized to molar ellipticities (molar ellipticity $[\theta]$ in deg cm^2 $dmol^{-1}$).

2.7. Immuno dot-blotting

A sheet of nitrocellulose membrane was soaked into distilled water overnight, and then once dried before spotting the protein samples. A 5 μl sample solution of Fag e 1 and Fag e 1 conjugates was spotted to the membrane. Human sera were added to the membrane and incubated for overnight at 37 °C, after blocking the membrane with 0.3% skim milk. Subsequently, the antibody was removed, and the secondary antibody, goat anti-human IgE labeled with HRP, was added then incubated for 4 h at 37 °C. Finally, Ez-West-Blue solution (Atto Co. Ltd., Tokyo, Japan) containing 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 were added to the membrane to develop it as colouring form. The membrane was washed well by PBS with Tween-20 during whole processes.

2.8. Quartz crystal microbalance analysis

Antigen–antibody interaction was also determined according to the quartz crystal microbalance (QCM) analysis (Okahata, Niikura, Sugiura, Sawada, & Morii, 1998; Yoshida et al., 2003). A 5 μl of a human serum mixture from six subjects was immobilized on a gold electrode surface of a 27-MHz QCM with hydrophobic interactions, and the frequency of the electrode was detected and recorded with a frequency counter-equipped computer (Affinix Q System, Initium Inc, Tokyo, Japan) in the mixing chamber containing 50 mM phosphate buffer (pH 7.0) at 37 °C. Then 0.1% bovine serum albumin (BSA) was added to block the electrode. After stabilization, Fag e 1 or Fag e 1 conjugate was added to the chamber at the final concentration of 0.04, 0.2, 1.0, 5.0, 25.0 μg/ml with an interval of 10 min, and the changes in the 27-MHz frequency at the electrode were recorded.

2.9. Enzyme-linked immunosorbent assay (ELISA) using human sera

A 50 μl of protein samples was applied in a well of the microtiter plate (Iwaki Co. Ltd., Tokyo, Japan) and incubated for overnight at 4 °C. After blocking with a 100 μl of 1% BSA for 90 min at 37 °C, a 50 μl of human serum was added and incubated for 4 h at 37 °C. Subsequently, a 50 μl of anti-human IgE conjugate with HRP was added and incubated 90 min at 37 °C. And then, a 50 μl of substrate containing 0.4 mg/ml *o*-phenylenediamine and 5 μl H_2O_2 was added, and finally added with 50 μl of 2 N H_2SO_4 to stop the enzyme reaction. The absorbance 490 nm was measured by the microplate reader Model 680 (Bio-Rad Co. Ltd., Tokyo, Japan).

2.10. ELISA using rabbit anti-Fag e 1 antibody

A 50 μl protein samples was incubated in a well of the microplate overnight at 4 °C, and a 100 μl of 1% BSA was added for blocking for 90 min at 37 °C. Then, a 50 μl of the anti-Fag e 1 antibody was added as the primary antibody, and incubated for 90 min at room temperature. Subsequently, a 50 μl of the HRP labeled Goat Anti-Rabbit IgG was added as the secondary antibody, and also

incubated under the same condition. Colour development and measurement of the absorbance procedures were the same as those mentioned in Section 2.9. In addition to the antigen-immobilizing procedure, the sandwiching type ELISA was done using the microplate detection kit for buckwheat provided from Morinaga Bioscience Laboratory Inc. (Tokyo, Japan). A 100 μl/well of sample was added into the microplate previously immobilized with the anti-buckwheat antibody, and incubated for 60 min at room temperature. Then, enzyme-labeled anti-buckwheat antibody was added and incubated for 30 min at room temperature. After that, substrate containing TMB was added for the colour development. Absorbance 450 nm was measured, using a microplate reader after stopping the enzyme-substrate reaction with 50 μl of 1 N H_2SO_4 according to the instruction of the detection kit.

2.11. Determination of surface functionality

Solubility of Fag e 1 in the given buffer was measured based on the absorbance at 280 nm of the sample supernatant after centrifugation at 15,000*g* for 20 min at 4 °C. Emulsifying properties of Fag e 1 and Fag e 1 conjugates were determined according to the method of Pearce and Kinsella (1978). Samples were dissolved in 50 mM phosphate buffer, pH 7.0, at the concentration of 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. A 100 μl of emulsion was taken from the bottom of the test tube after standing for 0, 1, 2, 5, 10 and 20 min, and diluted with 5.0 ml of 0.1% SDS solution. The absorbance of the diluted emulsion was then determined at 490 nm. The relative emulsifying activity was presented as the absorbance at 490 nm measured immediately after emulsion formation. The emulsion stability was estimated by measuring the half-life time for emulsion decay during standing for 20 min. The turbidity of emulsion is plotted as ordinate and standing time after emulsion formation as abscissa.

2.12. Statistical analysis

All experiments were conducted in triplicate. Data were analyzed using the Student's *t* test (Microsoft Excel ver. 5.0) for comparing differences between treatment means.

3. Results

3.1. Purification of Fag e 1 and preparation of Fag e 1-arabinogalactan conjugate

Crude globulin protein was extracted from the defatted flour of common buckwheat with a yield of 5%, and Fag e 1 with 22 kDa molecular mass was purified using size exclusion chromatography with Superdex 200 column (SEC) followed by ion exchange chromatography with Q Sepharose FF column (IEC). SDS–PAGE verified that the combination of SEC and IEC is acceptable procedure to obtain pure Fag e 1 (data not shown). Resulting protein was used to prepare a carbohydrate conjugate with arabinogalactan via naturally occurring Maillard reaction. When lyophilized Fag e 1 and polysaccharide mixture was incubated at 60 °C under 65% relative humidity for 1 week, a naturally occurring Maillard-linkage was generated between the ε-amino groups of the protein and the reducing-end carbonyl group of the polysaccharides. As shown in Fig. 1, the conjugates had a broad band for both protein and carbohydrate stains near the boundary between stacking and separating gels, whereas the native protein had a single band. This suggested that Fag e 1 was covalently attached to the polysaccharides. By using Alpha Imager™ (Alpha Innotech Co., Tokyo, Japan), the molecular mass distributions of protein (CBB) and glycosylated protein (PAS) were analyzed. As a result, it is revealed that XG is more reactive than AG, in which 83% Fag e 1 was conjugated with XG, while that with AG was 32%.

The Fag e 1–polysaccharide conjugate was separated using SEC with Superdex 200 column and used for further experiments. The binding molar ratio was calculated on the assumption that the molecular weights of Fag e 1, AG and XG were 22, 20 and 1.4 kDa, respectively. On the basis of the binding weight ratio, about two and four molecules of AG and XG appear to link to one molecule of the protein, respectively. The results were confirmed by the measurement of free amino groups in the conjugate, which suggested that Fag e 1 is linked with polysaccharides by only

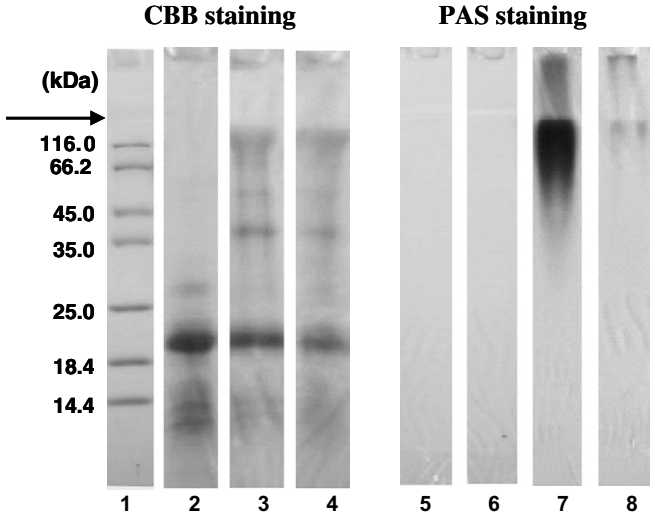

Fig. 1. Electrophoretic patterns of Fag e 1 and Fag e 1–polysaccharide conjugates. After electrophoresis, the gel sheets were stained for protein and carbohydrate with CBB and periodic acid–Fuchsin (PAS), respectively. *Lane 1*, molecular mass standard proteins with β-galactosidase (116,000), bovine serum albumin (66,200), ovalbumin (45,000), lactate dehydrogenase (35,000), REase Bsp98I (25,000), β-lactogloblin (18,400), and lysozyme (14,400); *lane 2*, Fag e 1; *lane 3*, conjugated sample between Fag e 1 and arabinogalactan; *lane 4*, conjugated sample between Fag e 1 and xyloglucan.

one reducing-end group in the polysaccharide and free amino groups in the protein through the controlled Maillard reaction, and no formation of polymerized network structure. It is also noteworthy that all 11 free amino groups including lysine residues were not occupied with the carbohydrate moiety, indicating that the conjugates are still useful as a lysine rich food source. In the far-UV region of CD spectra, Fag e 1 and Fag e 1–polysacharide conjugates showed specific features of the globule protein containing α-helices, having a negative minimum peak at 208 nm and a broad shoulder around 215–222 nm. The results indicated that the secondary structures of the proteins were completely destroyed in the conjugates (data not shown) with conformational changes, and the polysaccharides were covalently linked with Fag e 1 under the controlled heating process.

3.2. Reduced immunogenicity of Fag e 1 by the conjugation with polysaccharides

Table 1 listed sera types obtained from six subjects that were used in this study. Based on medical diagnosis, the sera from six subjects were all confirmed to have buckwheat-specific IgE antibody.

One of the widely-used allergenic assays, the immuno dot-blotting procedure, was performed. As a result, it was demonstrated that human sera were undoubtedly reacted with Fag e 1 and the immunogenicity of Fag e 1 was strongly reduced by the conjugation with polysaccharides. All patients' sera were not reacted with Fag e 1–polysaccharide conjugates, as shown in Fig. 2. Especially the introduction of XG seems to be more effective than that of AG for the reduction of the reactivity of the anti-Fag e 1 antibody. The result was confirmed by the QCM analysis. As shown in Fig. 3, the 27-MHz frequency at the electrode for native Fag e 1 significantly decreased at the final concentration of 5.0 μg/ml (40 min) and 25 μg/ml (50 min) that is an evidence for the interaction between antigen and antibody in the serum sample. Whereas the 27-MHz frequency was not changed by adding Fag e 1–XG conjugate at the final concentration of 25.0 μg/ml, while that of Fag e 1–AG conjugate was slightly changed. Thus, we investigated in more detail about this phenomenon using the enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 4, it is demonstrated that there were IgE antibodies recognizing Fag e 1 in all sera. The reactivates of human IgE's from six patients against Fag e 1–AG conjugate were reduced with reacting rates of 3.6–10.9% compared with the native form. More effective reductions of the reactivity of human IgE's were observed for Fag e 1–XG conjugate with 1.9–10.7% reacting rates. These results indicate that the epitope sites recognized by human IgE antibodies are different among patients.

The above result was supported by ELISA using rabbit polyclonal anti-Fag e 1 antibody. Two types of ELISA were further employed to determine the reduction of antigen-antibody interaction of Fag e 1 in the neoglycoproteins. One was the antigen immobilizing type and the other was the sandwich type. As a result of antigen immobilized type assessment, it was shown that the antibody binding capacity of Fag e 1 was drastically reduced, compared to that of native form. Considerable reductions (96.2% and 98.1%) were observed with the Fag e 1–AG conjugate and Fag e 1–XG conjugate, respectively. On the other hand, the sandwich type assessment showed a strong effect for Fag e 1–XG conjugate, but less effect for Fag e 1–AG conjugate. The reactivity of Fag e 1 with antibody was decreased to 98.8% by the conjugation with XG, whereas that of AG was 87.0%. It was probably caused by the difference of polymerization degree and length of the polysaccharides. As indicated above, XG is easier conjugated with Fag e 1 than AG. The carbohydrate chains

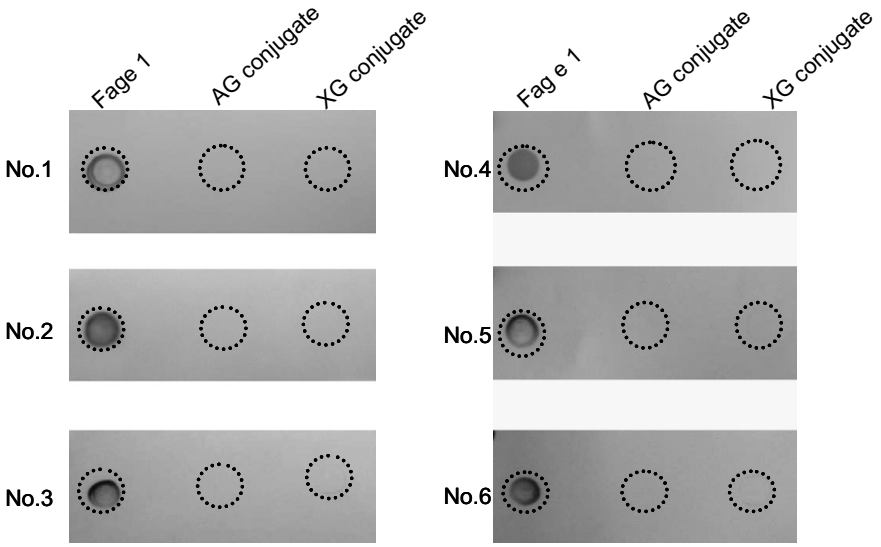

Fig. 2. Immuno dot-blotting profiles of Fag e 1 and Fag e 1–polysaccharide conjugates for buckwheat allergy patients.

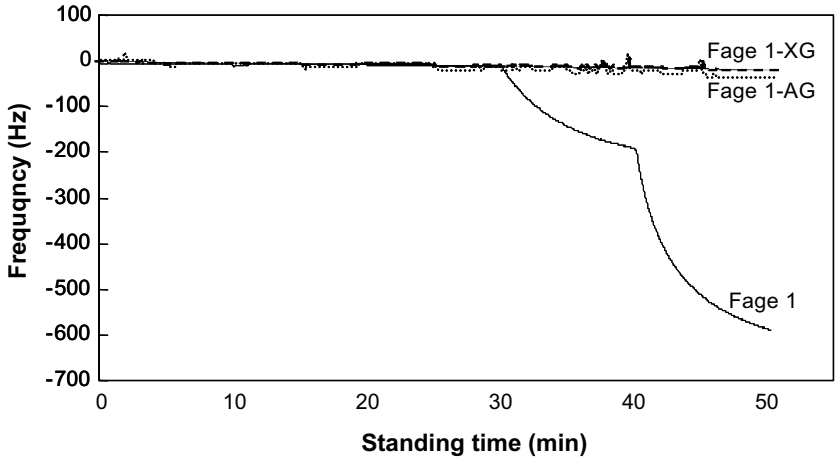

Fig. 3. QCM analysis of Fag e 1 and Fag e 1–polysaccharide conjugates. A human serum (subject no.1) was immobilized on a gold electrode surface of a 27-MHz QCM, and Fag e 1 or Fag e 1 conjugate was added to the chamber at the final concentration of 0.04, 0.2, 1.0, 5.0, 25.0 μg/ml with an interval of 10 min, and the changes of the frequency were monitored. The data are from a representative experiment repeated three times with similar results.

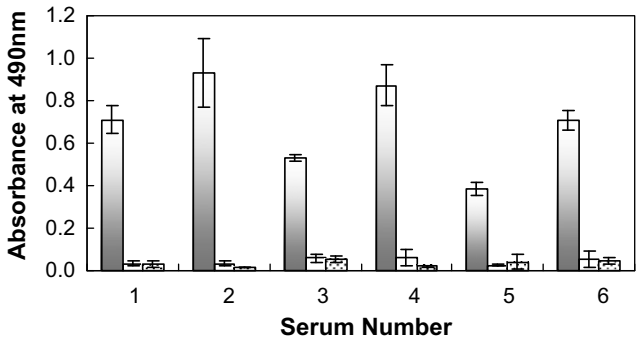

Fig. 4. ELISA using patients' sera for Fag e 1 and Fag e 1–polysaccharide conjugates. Using anti-human IgE conjugate with HRP, changes of antigen–antibody interaction was monitored using the Microplate reader Model 680. The results are mean ± SD of three independent experiments. ▩, Fag e 1; □, Fag e 1–AG conjugate; ⬚, Fag e 1–XG conjugate.

introduced on the protein molecule was 4.1 for Fag e 1–XG conjugate and 1.9 for Fag e 1–AG conjugate. The smaller polysaccharide may easily cover with the protein molecular surface.

3.3. Changes of surface functionalities of Fag e 1 by the conjugation with polysaccharides

The effects of the Maillard-type glycosylation on the surface functionalities of Fag e 1 were investigated. The solubility of Fag e 1 in 50 mM phosphate buffer (pH 7.0) was improved considerably by the introduction of polysaccharide chains on its molecule. The solubility of Fag e 1 increased 13.5 times and 9.6 times for AG conjugation and XG conjugation, respectively. To determine the structural changes of Fag e 1 during dry-heating process, the far-ultraviolet (190–260 nm) CD spectra of native Fag e 1 and neoglycoproteins were recorded using a spectropolarimeter (JASCO J-600, Tokyo, Japan) using a 1.0-cm cuvette. Accumulation of the CD spectra was initiated within 5 min of recording the absorbance spectra of the samples. The resulting spectra ($n = 10$) were normalized to molar ellipticities (molar ellipticity $[\theta]$ in deg cm^2 dmol^{-1}). As the result, it is confirmed that there are visible differences for the secondary structures of the Fag e 1 moiety in the neoglycoproteins. Significant improvement of the molecular surface functionalities of Fag e 1 was observed due to the newly attached hydrophilic molecules on the surface of the protein. Emulsifying properties of Fag e 1 were remarkably improved by the conjugation with AG and XG. As shown in Fig. 5, both neoglycoproteins revealed better emulsifying properties than native Fag e 1, although the properties were interior to those of commercial emulsi-

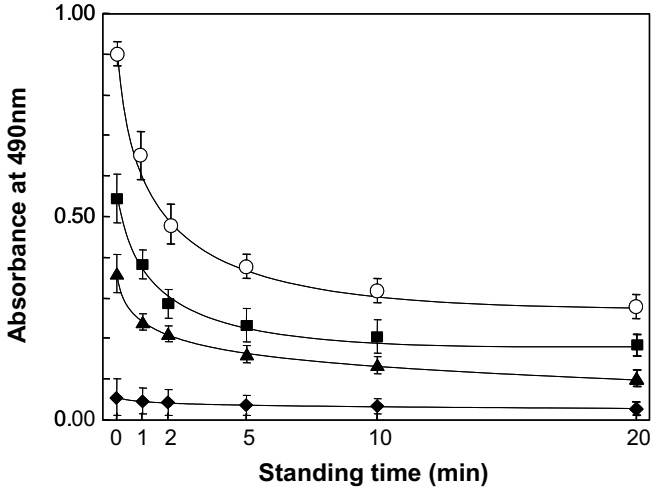

Fig. 5. Changes of the emulsifying properties of Fag e 1 by the conjugation. Sample solution was homogenized with corn oil, and resulting emulsion was taken from the bottom of the test tube and stored in 0.1% SDS solution. The absorbance of the diluted emulsion was then determined at 490 nm after standing for 0, 1, 2, 5, 10 and 20 min. ◆, Native Fag e 1; ■, Fag e 1–AG conjugate; ▲, Fag e 1–XG conjugate; ○, commercial emulsifier.

fier. The emulsifying activities of Fag e l-AG conjugate and Fag e l-XG conjugate were 9.9 times and 6.4 times, respectively, much higher than that of Fag e 1. The emulsion stability of Fag e l-AG conjugate and Fag e l-XG conjugate were improved 6.2 times and 6.0 times, respectively, compared with that of the native protein.

4. Discussion

Buckwheat protein is close to animal proteins because of its nutritive value of high lysine and arginine content (Pomeranz & Robbins, 1970), and it has been proposed to be an excellent supplement for cereal grains (Sure, 1953). Buckwheat has been abundantly consumed in Asian countries and has become increasingly popular in the United States, Canada, and Europe (Stember, 2006). With increasing consumption, food allergy caused by buckwheat allergy or allergic symptom becomes a serious health concern. Consequently, the utilization of buckwheat protein would be limited as general food gradients. The 22 kDa protein (BK24kDa) in buckwheat proteins displayed the high IgE-binding frequency with almost all sera from the patients with buckwheat allergy, and recently the major allergen named Fag e 1 was cloned and characterized (Nair & Adachi, 1999; Yoshioka et al., 2004). In this study, we focused on function of Fag e 1 and applied the naturally occurring Maillard-type glycosylation to decrease the allergenic activity of the protein. Arabinogalactan (AG) and xyloglucan (XG) were used as macromolecular modifiers for this purpose. As the result, the neoglycoproteins were obtained in this study with not only surface functional change but also drastically reduced allergenicity. The mechanisms responsible for the low allergenicity in the conjugates could be attributed to the shielding of the epitopes by the covalently attached polysaccharide chains, and the partial denaturation of Fag e 1 during the dry-heating at 60 °C for the Maillard-type glycosylation, which could cause protein conformational change and destruction of the epitopes. As the evidence, the amphiphilicity of Fag e 1 was greatly enhanced by the conjugation with polysaccharide. In addition to the improved solubility, the Maillard-type glycosylation was brought about the excellent emulsifying properties to the protein. Both emulsifying activity and emulsion stability were more than 6 times higher than those of the native form. The combination between protein and polysaccharide play an important role in the stabilization of O/W emulsions. Partially denaturated protein moieties may adsorb at the oil–water interface during emulsification to form a coherent layer, where polysaccharide chains confer colloid stability through their thickening behavior in the aqueous phase. Therefore, the protein–polysaccharide conjugate is expected to exhibit the good emulsifying properties (Kato et al., 1990; Nakamura, 2007; Nakamura & Kato, 2000; Nakamura et al., 1990).

Gene manipulation techniques have been employed for rice and soybean to modify, decrease and/or remove allergens (Herman, Helm, Jung, & Kinney, 2003; Tada et al., 1996). However, it may cause new allergenic potentials of genetically modified organism (GMO) food, because most traits introduced into genetically engineered crops could result in the expression of new proteins (Yum, Lee, Lee, Sohn, & Kim, 2005). Previous studies revealed that enzymatic modification and microbial formation were effective to reduce the allergenicity of wheat and soybean without transgenic techniques (Kobayashi, Hashimoto, Taniuchi, & Tanabe, 2004; Ogawa, Samoto, & Takahashi, 2000; Watanabe, Watanabe, Sonoyama, & Tanabe, 2000; Yamanishi et al., 1996), but limited studies have reported for a practical application of hypoallergenic buckwheat (Handoyo, Maeda, Urisu, Adachi, & Morita, 2006; Morita et al., 2006). In this study, we have succeeded in obtaining a new functional neoglycoprotein from Fag e 1 with hypoallergenicity and improved solubility and emulsifying properties. Although the storage proteins of plants have potentially functionalities, they are insoluble in pure water or ethanol, but soluble in dilute salt solution and insoluble at high salt solution. One limitation to Fag e 1 usage is poor water solubility. A great improvement in solubility was observed in the neoglycoproteins derived from Fag e 1. Thus, we have also succeeded in creating new functional properties from the water/ethanol insoluble protein by the conjugation with polysaccharides. The method for modification of Fag e 1 allergenicity could be used for other applications. For example, it might be useful for developing a desensitization procedure for cereal allergies except buckwheat allergy, because their allergens share a similar amino acid sequences.

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