

Effects of Maillard reaction on allergenicity of buckwheat allergen Fag t 3 during thermal processing

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Abstract

BACKGROUND: Fag t 3 is a major allergenic protein in tartary buckwheat. The Maillard reaction commonly occurs in food processing, but few studies have been conducted on the influence of thermal processing on the allergenic potential of buckwheat allergen. The aim of the present study was to investigate the effects of autologous plant polysaccharides on the immunoreactivity of buckwheat Fag t 3 (11S globulin) following the Maillard reaction.

RESULTS: Fag t 3 and crude polysaccharides were prepared from tartary buckwheat (*Fagopyrum tataricum*) flour. After heating, the polysaccharides were covalently linked to Fag t 3 via a Maillard reaction, and the IgE/IgG-binding properties of Fag t 3 decreased dramatically, with significant changes also being observed in the electrophoretic mobility, secondary structure and solubility of the glycated Fag t 3. The great influence of glycation on IgE/IgG binding to Fag t 3 was correlated with a significant change in the structure and epitopes of the allergenic protein. These data indicated that conjugation of polysaccharides to Fag t 3 markedly reduced the allergen's immunoreactivity.

CONCLUSION: Glycation that occurs via the Maillard reaction during the processing of buckwheat food may be an efficient method to reduce Fag t 3 allergenicity.

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Keywords: buckwheat allergy; Fag t 3; allergenicity; Maillard reaction; food processing

INTRODUCTION

China is one of the countries of origin of buckwheat, with over 2000 years of history of buckwheat culture. Buckwheat belongs to the family Polygonaceae and includes the two species common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.). As an important traditional food, tartary buckwheat is used as a key ingredient in various products such as biscuits, noodles and cakes. A previous study by He *et al.*¹ demonstrated that buckwheat has a cholesterol-lowering effect. Nosratabadi *et al.*² found that tartary buckwheat contains specific bioactive substances such as rutin and other polyphenols that can attenuate endotoxin-induced airway contraction in isolated rat lungs. Nowadays, buckwheat is regarded as one of the main health foods, and there is great interest in studying its preventive effects on ailments such as diabetes, hypertension and arthritis. Foods that contain buckwheat have become more popular, especially tartary buckwheat tea. Compared with traditional tea, tartary buckwheat tea is processed through frying and roasting. The brown colour and special flavour of buckwheat tea after thermal treatment indicate that the Maillard reaction has occurred during processing. The Maillard reaction in food processing is a reaction between free amino groups of proteins and aldehyde or ketone groups of sugars, resulting in aromatic compounds that modify the sensory aspects of cooked foods.³

The effects of glycation (the Maillard reaction) on food allergens have been reported in fruit, seafood, milk, hazelnut and other foods.^{4–7} However, almost all carbohydrates used in those studies

were standard commercial materials. Few studies had been conducted on the influence of homologous plant polysaccharides on the allergenic potential of buckwheat proteins. The effects of thermal processing on the properties of peanut extract have been observed.^{8–10} The results suggest that roasting influences the extraction efficiency of peanut allergens.

A previous study indicated that the main tartary buckwheat allergen Fag t 3 (TBt), a seed storage protein, belongs to the cupin superfamily and has high similarity to the legume-like 11S storage protein.¹¹ The most widespread groups of plant proteins that contain allergens are the cupin and prolamin superfamilies and the protein families of plant defence systems. The cupin superfamily includes allergenic seed storage proteins of the vicilin and legumin types present in soybeans, peanuts and tree nuts.¹² In this study, to investigate the influence of thermal processing on the allergenicity of Fag t 3, polysaccharides (FTPS) were isolated from tartary buckwheat flour, and the effects of FTPS on the immunoreactivity of Fag t 3 were analysed by immunological methods. The major buckwheat allergen Fag t 3 may be covalently

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linked with FTPS through controlled dry heating, which likely occurs in the food processing of tartary buckwheat.

EXPERIMENTAL

Materials

Tartary buckwheat seeds were harvested from the Academy of Agricultural Science, Shanxi, China. Dextran (molecular weight 10 kDa) was obtained from Sigma-Aldrich (St Louis, MO, USA). Resource Q, Superdex 200 and Sephacryl S-200 were obtained from GE Healthcare (Uppsala, Sweden). Horseradish peroxidase (HRP)-conjugated mouse anti-human IgE and HRP-conjugated goat anti-rabbit IgG were purchased from Southern Biotech (Birmingham, AL, USA).

Serum and allergen preparation

Human serum samples were collected from five patients who had a history of respiratory, dermatological or gastrointestinal symptoms within 1 h after ingestion of buckwheat in the Blood Center of Taiyuan, Shanxi, China (Table 1). Serum samples from healthy volunteers not allergic to buckwheat were used as negative control.

The extraction of Fag t 3 was performed as described by Zhang *et al.*¹¹ Fag t 3, with an apparent molecular mass of 56 kDa, was purified using ion exchange chromatography followed by size exclusion chromatography of the extracted crude protein sample. All chromatographic procedures were performed in an AKTA Purifier system (GE Healthcare), and the proteins were monitored by absorbance at 280 nm.

Preparation of polysaccharides

The extraction of polysaccharides was carried out in a water bath at 80 °C for 4 h. To precipitate soluble proteins, the solution of polysaccharide extracts was treated with 30 g L⁻¹ trichloroacetic acid (TCA) at 4 °C overnight. After centrifugation at 13 000 × *g*, the solution was concentrated in a rotary evaporator and then mixed with four volumes of 950 mL L⁻¹ ethanol for isolation of the polysaccharides. Precipitates were obtained by centrifugation (8000 × *g*, 10 min), washed with acetone and then dried under reduced pressure at -50 °C to obtain crude polysaccharides.

Total carbohydrate was determined by the phenol/sulfuric acid colorimetric method at 490 nm with D-glucose as standard.¹³ Absorbance spectra of the samples were recorded with an ultraviolet (UV) spectrophotometer (U-2010, Hitachi, Tokyo, Japan) between 190 and 400 nm. Residual protein was determined by the Coomassie brilliant blue (CBB) method,¹⁴ starch by iodination, and reducing sugar by Fehling's test.¹⁵

Protein glycation and electrophoresis analysis

Fag t 3 and polysaccharides were individually dissolved in 20 mmol L⁻¹ phosphate buffer (pH 7.5) at a weight ratio of 1:1 and then freeze-dried. The resulting lyophilised mixture was placed in an incubator and heated for 3 days at 70 °C or for 15 min at 160 °C to generate a naturally occurring Maillard linkage. Fag t 3 treated under the same conditions but without polysaccharide was used as control. Fag t 3 conjugated with polysaccharide was separated from free proteins and free carbohydrates by size exclusion chromatography on a Superdex 200 column.

Samples were separated by 125 g L⁻¹ sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 1 g L⁻¹ SDS. The protein samples were prepared in Tris/glycine buffer

containing 10 g L⁻¹ SDS (without reducing agent). The gels were stained with CBB for proteins or with 10 g L⁻¹ periodic acid/fuchsin (PAS) for carbohydrates. Native-PAGE was performed similarly to SDS-PAGE, except that the samples were not heated and no SDS was added to the samples or the electrophoretic buffer.

Measurement of free amino groups

Changes in concentration of free amino groups in Fag t 3 and Fag t 3-polysaccharide conjugates were determined by spectrophotometric assay at 344 nm using trinitrobenzene sulfonic acid (TNBS) according to the method of Haynes *et al.*¹⁶

Immunisation of rabbits and measurement of antibody production

Antibodies were produced from three New Zealand rabbits by mixing purified Fag t 3 (0.5 mg kg⁻¹) with an equal volume of Freund's adjuvant. Complete Freund's adjuvant was used in the first dose and incomplete Freund's adjuvant in subsequent doses. Rabbits were immunised subcutaneously, three times, at 10 day intervals. Serum IgG antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) with different dilutions. Controls were performed with normal rabbit antibodies. Western blotting was used to determine the rabbit antiserum specificity. The Institute and Animal Care and Use Committee approved the animal-handling and experimental procedures.

ELISA and immuno-dot blotting

ELISA and dot-blotting analysis was performed as described previously¹⁷ to detect the IgE/IgG-binding activities of Fag t 3 proteins and conjugates. All protein samples were incubated at 100 µg mL⁻¹. The non-heated protein was used as control. Human serum samples and rabbit anti-Fag t 3 antibodies were added as primary antibodies. HRP-conjugated mouse anti-human IgE or HRP-conjugated goat anti-rabbit IgG was added as the secondary detection antibody, then o-phenylenediamine was added for colour development.

Circular dichroism analysis

Circular dichroism (CD) spectral measurements were performed using an MOS-450 spectropolarimeter (Bio-Logic, Grenoble, France) at a scan speed of 100 nm min⁻¹ with three accumulations. The secondary structure of Fag t 3 and conjugates in the far-UV range (195–250 nm) was recorded at a protein concentration of 0.3 mg mL⁻¹ in phosphate-buffered saline (PBS) at room temperature. Buffer scans were recorded under the same conditions and subtracted from the protein spectra before further analysis.

Temperature and pH stability of Fag t 3

Fag t 3 was incubated at various temperatures between 50 and 100 °C for 30 min. IgE-binding activities of these samples were detected by ELISA, and secondary structures were determined after the samples had cooled to room temperature. To analyse the effect of pH, Fag t 3 was incubated for 4 h in the following buffers at a final concentration of 20 mmol L⁻¹: glycine/HCl (pH 2–3), sodium acetate/acetic acid (pH 4–6), Tris/HCl (pH 7–9) and borax/NaOH (pH 10–12). All samples were analysed by SDS-PAGE and CD spectroscopy.

Table 1. Characterisation of sera from patients with positive IgE binding to buckwheat

Serumno.	Immediate hypersensitivity reaction	Sex	Age	Buckwheat specific IgE level (kU L ⁻¹)	Major diagnosis
1	Positive	F	22	18.5	Buckwheat allergy and severe atopic dermatitis
2	Positive	F	40	17.2	Buckwheat and elm pollen allergy
3	Positive	M	46	42.3	Food allergy and allergic rhinitis
4	Positive	F	38	16.4	Buckwheat and mite allergy
5	Positive	M	16	25.1	Food allergy and bronchial asthma

Statistical analysis

All experiments were conducted in triplicate and data expressed as mean $\pm$ standard deviation. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test at a significance level of $P < 0.05$ using SigmaStat 3.1 (Systat Software Inc., San Jose, CA, USA).

RESULTS AND DISCUSSION

Extraction and isolation of FTPS

A water-soluble crude polysaccharide was extracted from the defatted flour of tartary buckwheat. After precipitating the soluble proteins with TCA and isolating the polysaccharide with ethanol, almost all other soluble materials were removed, including proteins, nucleic acids, free sugars, amino acids and some phenols. The purity of FTPS was determined by UV spectrophotometry. The UV spectrum showed a characteristic absorption of polysaccharide at 190 nm, with no absorption at 260 nm (nucleic acid) or 280 nm (protein). The FTPS concentration was estimated by the phenol/sulfuric acid colorimetric method. The FTPS was obtained as a white powder with a final yield 62 g kg⁻¹ buckwheat flour.

Purification of Fag t 3 and Fag t 3–polysaccharide conjugates

Purified Fag t 3 was used to prepare a carbohydrate conjugate with polysaccharides via a non-enzymatic Maillard reaction. When a mixture of lyophilised Fag t 3 and polysaccharides (1:1 w/w) was incubated at 70 or 160 °C, major changes in the colour and solubility of the conjugated Fag t 3 were observed. Compared with the control sample, the conjugated Fag t 3 was much less soluble after heating in the presence of polysaccharides, and the powder turned brown in colour, with a notable flavour. The reaction likely reached a stage with the formation of insoluble brown products.

Most foods are subjected to thermal processing either at home or during their manufacture. In the manufacturing process of tartary buckwheat tea, buckwheat seeds are roasted at 150–180 °C. Such thermal processing may bring major changes in allergenicity by introducing covalent modifications to proteins. Perhaps the most important and well-defined covalent modification is the Maillard reaction, in which the attachment of reducing sugars to free β -amino groups of lysine occurs. These reactions take place rapidly at or above 100 °C.¹⁸ It was reported that IgE-reactive proteins in chestnut were greatly reduced by boiling, probably owing to protein denaturation.¹⁹ Lower heating temperatures and longer reaction times were adopted in several other studies.^{4–7} The minimum antigenicity of bovine α -lactalbumin was achieved at a 52.8 °C for 78 h.²⁰ In our experiment the Maillard reaction conditions were 70 °C for 3 days or 160 °C for 15 min. However, the higher temperature reduced the immunoreactivity of Fag t 3 by protein denaturation, so we focused on the reaction at 70 °C

for 3 days to ensure that the protein was still in its folded state. Therefore, in the following, only Fag t 3 conjugation obtained at 70 °C was analysed.

The Fag t 3–polysaccharide conjugates were first analysed by PAGE. The same gels were stained with different staining solutions. The glycosylated proteins were stained with both CBB and PAS, while the control protein was only stained with CBB. SDS-PAGE analysis (Fig. 1A) showed that the purity of Fag t 3 was greater than 95% (lane 1) and the lower-molecular-weight protein band (~34 kDa) was an N-terminal subunit degraded from Fag t 3.¹¹ The 11S globulins are typically hexameric, with 60 kDa subunits that are proteolytically cleaved to yield an acidic 30–40 kDa polypeptide linked by a disulfide bond to a basic polypeptide of approximately 20 kDa.¹² This was confirmed in Fig. 1C, where all samples were treated with dithiothreitol (DTT). At both 70 and 160 °C the conjugate bands were in a range of molecular weights higher than that of the native control protein, suggesting that the polysaccharides were covalently attached to Fag t 3, as confirmed by PAS staining (Fig. 1B).

Compared with the single band of the native control protein, the Fag t 3 conjugate samples had a broad protein band that was positive for both protein and carbohydrate stains. This broad band may be attributed to the fact that FTPS contains a variety of molecular sizes. This result was confirmed by the measurement of free amino groups in the conjugates. A naturally occurring Maillard linkage was generated between the ϵ -amino group of the protein and the reducing carbonyl group of the polysaccharides. The number of attached polysaccharides was calculated from the decrease in N-terminal and ϵ -amino groups of Fag t 3 (14 residues). Approximately 15 and 21% decreases in free amino groups were observed for Fag t 3 conjugated with dextran and FTPS respectively, suggesting that the conjugates are still useful as lysine-rich food sources.

The electrophoretic properties of Fag t 3 and its conjugates in the natural state were analysed by native-PAGE (Fig. 2). Compared with the native control protein (lane 1), the conjugate bands were at different positions, indicating a change in the charge of the conjugates.

In the far-UV CD spectrum (Fig. 3), Fag t 3 showed specific features of the cupin family proteins containing β -sheets, with a large negative minimum at about 222 nm and a positive peak between 190 and 210 nm, a typical β -sheet-dominant structure. However, the CD spectra of the glycosylated proteins were different, with more random coil structure being observed. This result showed there were changes in protein conformation following glycation during the heating process.

Part of Fag t 3 was insoluble after heating in the presence of polysaccharides. Reduced solubility was also reported for protein from peanut flour following 10 min of roasting.²¹ On the other hand, the solubility of Fag e 1 was improved considerably by

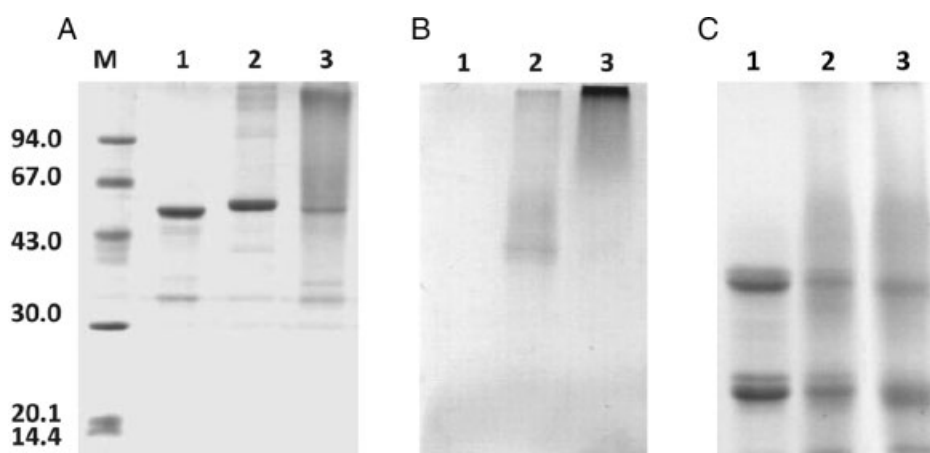


Figure 1. SDS-PAGE analysis of control and glycosylated Fag t 3 proteins: lane M, protein molecular weight marker (kDa); lane 1, Fag t 3; lane 2, Fag t 3–dextran conjugate; lane 3, Fag t 3–FTPS conjugate. Samples in A and B were heated at 70 °C, while those in C were roasted at 160 °C. After electrophoresis, gels were stained with CBB for proteins (A, C) or with PAS for carbohydrates (B).

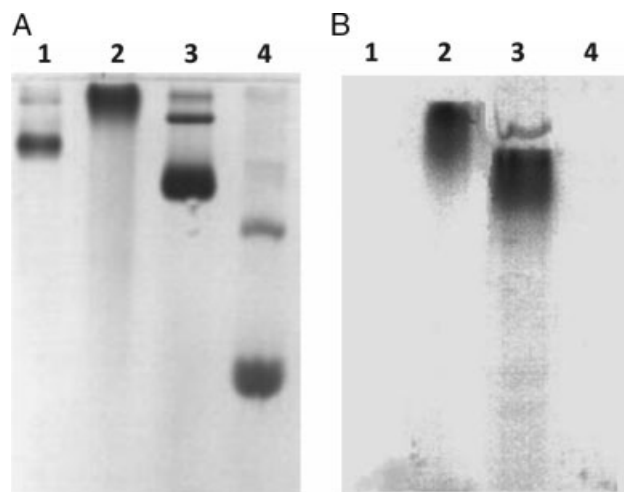


Figure 2. Native-PAGE analysis of control and glycosylated Fag t 3: lane 1, Fag t 3; lane 2, Fag t 3–dextran conjugate; lane 3, Fag t 3–FTPS conjugate; lane 4, BSA. After electrophoresis, gels were stained with CBB for proteins (A) or with PAS for carbohydrates (B).

the introduction of polysaccharide chains on its molecule.²² The different reaction products were influenced by the stage of reaction and the type of sugar. Significant improvement in solubility was observed owing to the newly attached hydrophilic molecules on the surface of the protein, while the loss of solubility was due to the formation of aggregates and insoluble brown products.

Effect of Maillard reaction on IgE/IgG binding of Fag t 3

Fag t 3 and its conjugates were compared for their IgE-binding activity by ELISA using human serum samples. A decrease of over 80% in the IgE reactivity of Fag t 3–polysaccharide conjugates was observed (Fig. 4A), suggesting that conjugation of polysaccharides can mask the epitope sites sterically.

The IgG-binding activities of the native control and glycosylated Fag t 3 were also determined by ELISA using polyclonal rabbit IgG antibodies against Fag t 3 (Fig. 4B). The decrease in IgG-binding activity was less than that in IgE reactivity, suggesting that the polyclonal IgG antibodies recognise different epitopes compared with IgE. Dot-blotting analysis, as a visualised allergenic assay, was

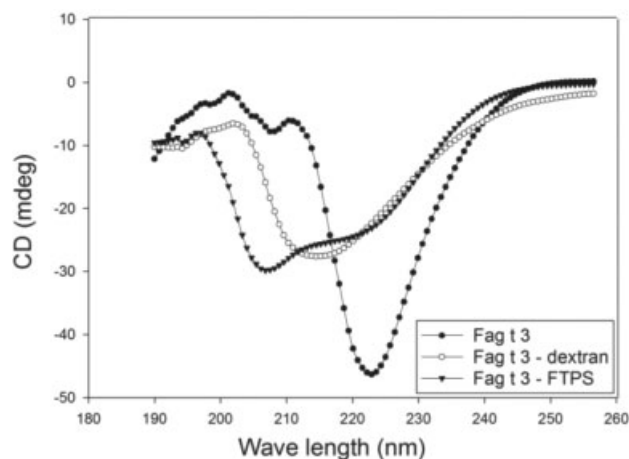


Figure 3. Far-UV CD spectra of control and glycosylated Fag t 3 proteins.

further performed (Fig. 5). Bovine serum albumin (BSA) was used as negative control. The dot-blotting results were consistent with the ELISA data.

The portion of a food protein that may cause an allergic reaction is related to linear and conformational epitopes, maybe a few amino acids along the primary structure or a unique three-dimensional motif of the protein structure respectively.²³ Different food-processing conditions may change the allergenicity by altering the immunoreactive epitopes of allergenic proteins.³ Previous studies have revealed that the Maillard reaction affects the allergenicity of allergens differently when allergenic proteins are thermally treated in the absence of carbohydrate. It was found that glycation of β -lactoglobulin during the Maillard reaction has a clear 'masking' effect on the recognition of epitopes by IgE of cow milk-allergic patients.⁶ However, peanut-specific IgE binds peanut allergens Ara h 1, 2 and 3 more strongly from roasted compared with boiled or fried peanuts.²⁴ This indicates that certain types of thermal processing can introduce additional IgE-binding sites. Therefore processing may destroy existing epitopes on a protein or expose new ones (neoallergen formation) as a result of the change in protein conformation.²³ Various saccharides have been evaluated for their effects on protein allergenic activity, including monosaccharides, oligosaccharides and polysaccharides.^{4,25} It

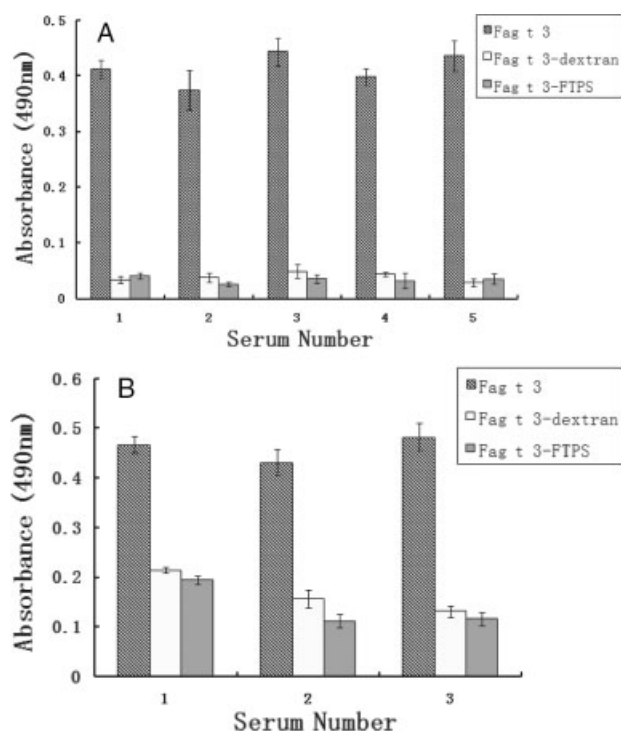


Figure 4. IgE or IgG binding to Fag t 3 and Fag t 3-polysaccharide conjugates: A, ELISA using human sera from patients for IgE-binding activity of control and glycosylated Fag t 3 proteins; B, ELISA using polyclonal rabbit anti-Fag t 3 IgG antibodies for IgG-binding activity of control and glycosylated Fag t 3 proteins. Results are mean $\pm$ standard deviation of three independent experiments.

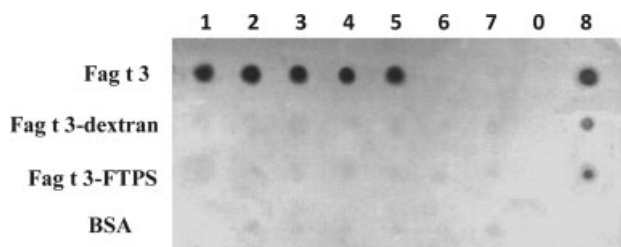


Figure 5. Immuno-dot assay of control and glycosylated Fag t 3 proteins. Nitrocellulose-dotted Fag t 3, Fag t 3-dextran, Fag t 3-FIPS and BSA were exposed to buffer (0), human sera from patients with allergy (1–5), healthy individuals without allergy (6, 7) or rabbit anti-Fag t 3 antibodies (8).

seems that larger molecular size of the attached saccharides plays an important role in masking epitope sites.

In the present study we focused on the effect of tartary buckwheat polysaccharide on the immunoreactivity of Fag t 3 after heating, since it is certain that glycation will take place in buckwheat food processing. Epitope mapping and identification were performed wherein the critical core amino acid residues were charged and exposed on the surface of the protein.¹⁷ The main effect of the Maillard reaction on the conformational structure of proteins involves changes in hydrophobic interactions.²⁶ Thus the binding of polysaccharides can mask the hydrophobic amino acid residues and those epitope sites located on the molecular surface.

Temperature and pH stability

Fag t 3 was very stable in basic buffer (pH 12) but was precipitated in acidic buffer (pH < 6). However, the acid-induced precipitation

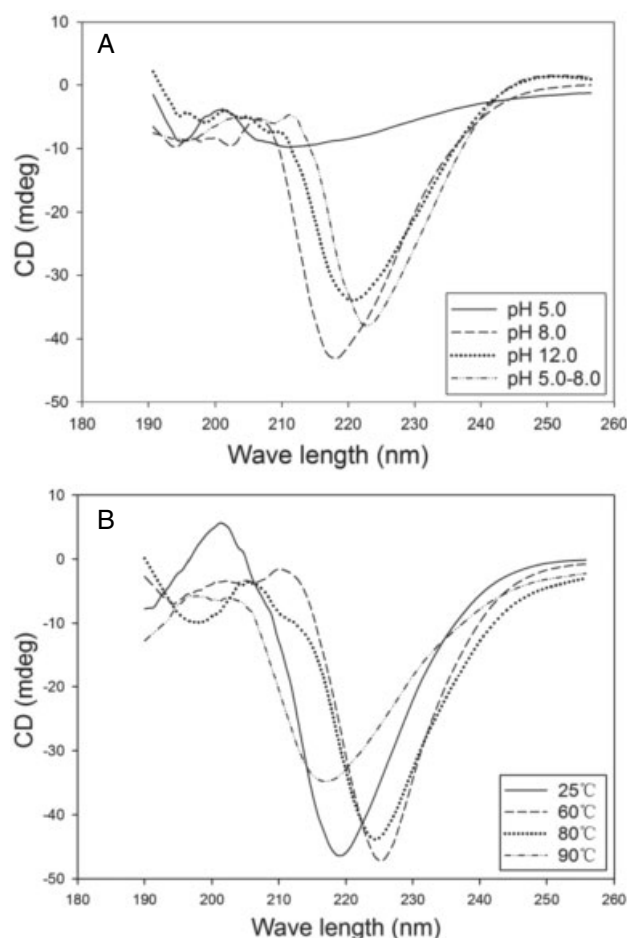


Figure 6. Effect of pH and temperature on secondary structure of Fag t 3: A, Fag t 3 was incubated at various pH values for 4 h before far-UV CD spectra were recorded; B, Fag t 3 was incubated at various temperatures for 30 min before far-UV CD spectra were recorded. Acid-induced conformational changes in Fag t 3 were reversed when incubated in basic buffer (pH 5–8).

was reversed upon incubation in pH 8 buffer. The far-UV CD spectrum showed structural changes under both acidic and basic conditions (Fig. 6A). Fag t 3 was stable even after incubation at 90 °C for 30 min, and the increase in temperature resulted in marginal changes in the far-UV CD spectrum (Fig. 6B). When the effect of high temperature on the IgE-binding activity of Fag t 3 was evaluated, there was no significant difference between samples heated below 90 °C and the non-heated sample. However, there was a 40–50% decrease in thermal stability when samples were heated above 95 °C.

The effects of the Maillard reaction on the stability of Fag t 3-polysaccharide conjugates were also evaluated. The thermal stability of the conjugates decreased slightly, with some precipitation being observed after 30 min at 90 °C. However, the Maillard reaction increased the acid resistance of Fag t 3-polysaccharide conjugates significantly. The far-UV CD spectra indicated that they were stable at pH 5–6 (data not shown).

Major food allergens share a number of common features such as water solubility, 10–70 kDa molecular weight and relative stability to heat, acid and proteases.²⁷ The 11S globulins unfold only at temperatures above 94 °C, and this remarkable stability property may play a role in predisposing members of the cupin superfamily to become allergens.²⁸ The cupin tertiary structure provides a stable scaffold that allows these proteins to survive and function

under a wide range of extreme conditions. The thermotolerance and resistance of Fag t 3 have been confirmed in this study, and its stability properties were not significantly altered by glycation.

CONCLUSIONS

The results of this study proved that conjugation with polysaccharides significantly reduced the antigenicity of Fag t 3. The structural changes of the allergen due to heating in the presence of polysaccharides led to significantly reduced IgE- and IgG-binding activities. In comparison, thermal treatment at 70 °C in the absence of any carbonyl compound did not influence the IgE-binding activity of the protein. In conclusion, we hypothesise that Fag t 3 and FTPS form a conjugate during the Maillard reaction and during the thermal processing of tartary buckwheat. The great influence of glycation on the IgE/IgG-binding activity of Fag t 3 is correlated with a significant change in its structure and conformational epitopes. Thus glycation may be a promising way to achieve buckwheat products with lower allergenicity.

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