

Phosphorylation and Enzymatic Hydrolysis with Alcalase and Papain Effectively Reduce Allergic Reactions to Gliadins in Normal Mice

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Supporting Information

ABSTRACT: Gliadins are major allergens responsible for wheat allergies. Food processing is an effective strategy to reduce the allergenicity of gluten. In the present study, we determined the secondary and tertiary structures of gluten and gliadins treated by chemical, physical, and enzymatic means through FTIR, surface hydrophobicity, intrinsic fluorescence spectra, and UV absorption spectra. The results showed that the three treatments of phosphorylation and alcalase and papain hydrolyses significantly changed the conformational structures of gliadins, especially the secondary structure. Then, the potential allergenicity of the phosphorylated and alcalase and papain hydrolyzed gliadins were further characterized, and we observed a significant decrease in the allergenicity through the results of the index of spleen, serum total IgE, gliadin-specific IgE, histamine, and serum cytokine concentrations. An elevation of Th17 cells, the absence of Treg cells, and an imbalance in Treg/Th17 are associated with allergy. On the basis of the expression levels of related cytokines and key transcription factors, we also confirmed that phosphorylation and alcalase and papain hydrolysis could effectively reduce the allergenicity of gliadins by improving the imbalance of both Th1/Th2 and Treg/Th17 in the spleens of sensitized mice. This study suggested that the changes in conformational structure contribute to gliadin hyposensitization and that phosphorylation and alcalase and papain hydrolysis may be promising strategies for the production of wheat products with low allergenicity.

KEYWORDS: gliadins, allergenicity, protein structure

INTRODUCTION

As one of the three most prevalent harvest cereals, wheat is a most important crop in the daily diet of human beings throughout the world.¹ Although it plays an important role in the diet, it exhibits wheat-related allergy in some individuals due to the presence of antigenic proteins capable of inducing allergic reactions.² Therefore, this means that many people cannot eat wheat and wheat-related foods. Similar to other common food allergens, the incidence of allergies to wheat has increased over the past decade.³ The wheat sensitization rate is close to 1% of the global population: about 0.4–1.3% in children and 0.2–0.9% in adults.⁴ However, the only effective way to avoid these allergies is to completely eliminate the wheat-based food.⁵ In addition to removing all gluten-containing products from the diet, effective processing methods could be used to modify immunoreactive components in wheat.⁶

On the basis of solubility, wheat proteins are divided into water-soluble albumins, salt-soluble globulins, and insoluble proteins.⁷ The insoluble fraction is called prolamins and consists of gliadins and glutenins, which together form gluten.⁸ Prolamins are divided into high-molecular-weight prolamin (HMW), cysteine-deficient (S-poor) prolamins, and cysteine-rich (S-rich) prolamins. According to biochemical and genetic

characteristics, the gliadin content up to 40% of wheat proteins can be divided into α -, β -, ω -, and γ -type subgroups with molecular weights ranging from 28 to 55 kDa.⁹ The α - and γ -gliadins belong to S-rich prolamine, and ω -gliadin is the main S-poor prolamine. α -Gliadin accounts for 15–30% of wheat proteins and is the most abundant of the gliadins.¹⁰

There are many allergens that have been found in wheat flour. Different types of wheat proteins cause different allergy symptoms, such as asthma and rhinitis, known as immunoglobulin E mediated inhalation of flour allergy.¹¹ Among these allergies, gliadins are the major types of proteins responsible for wheat allergies in gluten.¹² Gliadin allergy is an IgE-mediated immune reaction.¹³ IgE is present on the surface of mast cells or basophils that binds to allergen epitopes and then triggers the release of vasoactive inflammatory mediators such as histamine and β -hexosaminidase.¹⁴

The gliadin structure comprises a repeating amino acid sequence called a central domain consisting of a peptide motif enriched in Pro and Gln.¹⁵ An epitope refers to a portion of a

Received: January 24, 2019

Revised: May 4, 2019

Accepted: May 9, 2019

Published: May 9, 2019

macromolecule that is recognized by the immune system. IgE antibodies in sensitive patients react with wheat epitope peptides to induce human immune responses and antiallergen effects. The pentapeptide repeat Gln-Gln-Gln-Pro-Pro of the low-molecular-weight glutenin subunit was found to be an IgE binding epitope. The secondary and tertiary structures of the protein are critical for the immune system to recognize epitopes.⁹ Structural changes that occur during processing can alter protein allergenic epitopes, which affect the likelihood that cells recognize gliadin epitopes.¹⁶ Lupi et al. reported that thermal treatment reduced the recognition of gliadins by IgE by aggregating gliadins.⁴ According to Li et al., enzymatic hydrolysis cleaves peptide bonds and converts proteins into peptides with fewer epitopes, thus leading to impaired IgE recognition.⁶

In this study, we chose nine different treatments, including physical, chemical, and enzymatic methods. Among them, physical methods include ultrasound, hot water bath, and microwave treatment with powers of 210 and 70 W, chemical methods include phosphorylation, acetylation, and deamidization, and alcalase and papain were used for enzymatic hydrolysis, which are commonly used for enzymatic processing of gluten meal. By measuring the structures of proteins, we found that phosphorylation and enzymatic hydrolysis had significant effects on the secondary and tertiary structures of gliadins. In order to investigate the details of the structural changes of phosphorylated and alcalase and papain hydrolyzed gliadins, SDS-PAGE was performed and the content of free SH was determined. The digestibility of the three treated gliadins was studied by *in vitro* simulated gastrointestinal digestion. Furthermore, BALB/c male mice were sensitized with untreated and treated gliadins to study *in vivo* potential allergenicity. Taken together, this study provides a new strategy for reducing the allergenicity of wheat protein and promoting the development of wheat protein related food.

METHODS AND MATERIALS

Materials and Reagents. Commercial wheat gluten was obtained from Hebei Huasheng Noodle Industry Co. Ltd. 1-Anilinonaphthalene-8-sulfonate is abbreviated ANS. The enzymes used in this study, including alcalase from the bacterium *Bacillus licheniformis* (>200 U/mg) and papain from papaya latex (90–150 U/mL), were purchased from Sigma-Aldrich. The enzymatic activities presented were determined by a universal protease activity assay using casein as substrate. Aluminum hydroxide was purchased from Thermo (77161, 40 mg/mL). Mouse IgE ELISA Kit was purchased from Multi Sciences (70-EK275). Mouse Histamine ELISA Kit was purchased from Nanjing Jiancheng Bioengineering Institute. All chemicals used were of analytical grade unless otherwise specified.

Treatments of Gluten. *Ultrasonic Processing.* An 80 g portion of gluten was added to 1000 mL of an aqueous solution with pH 4.58 and sonicated at 60 °C, 400 W for 20 min. The pH was then adjusted to 7 after cooling the reaction system to room temperature, and the sample was lyophilized for further analysis.

Hot Water Bath. An 80 g portion of gluten was added to 1000 mL of pH 3 liquor, and the reaction system was continuously stirred in a hot water bath at 75 °C for 1 h. It was then cooled to room temperature, and the pH of the reaction system was set to 7. The sample was lyophilized for further analysis.

Microwave. Two microwave processing methods with powers of 210 and 70 W were used. Gluten and solution were microwaved at a ratio of 2:25 (w:w) for 90 s. The pH of the reaction system was then adjusted to 7 and lyophilized for further analysis.

Phosphorylation. An 80 g portion of gluten was weighed and dissolved in 800 mL of PBS (pH 9.5), 16 g of sodium tripolyphosphate was added, and the reaction was carried out at 25

°C for 1 h; the pH was adjusted to 9.5 with 1 M NaOH throughout the reaction. The pH was then adjusted to 7.0 and the sample lyophilized for further analysis. The degree of phosphorylation was $40.98 \pm 0.64\%$. The method for determining the degree of phosphorylation was according to Shih.¹⁷

Acetylation. An 80 g portion of gluten was dissolved in pH 8.0–8.5 phosphate buffer solution (the reaction temperature was 37 °C), 12 mL of acetic anhydride was added in three to five batches with constant stirring, and NaOH (2 M) was added to keep the pH in the 8.0–8.5 range. After 2 h of reaction, the pH was set to 7 and the sample lyophilized for further analysis. The degree of acetylation was $29.56 \pm 0.96\%$, and the method of determining the extent of acetylation was according to Liu.¹⁸

Deamination. An 80 g portion of gluten meal was weighed and dissolved in 0.25 M HCl solution; the mass fraction of gluten was 10%.¹⁹ The reaction system was continuously stirred at 70 °C for 3 h, and after completion, the pH was adjusted to 7.0 to stop any further reaction. The samples were then lyophilized for further analysis. The degree of deamination was $31.95 \pm 0.78\%$, and the method of determining the extent of deamination was according to Adler-Nissen.²⁰

Enzymatic Hydrolysis. An 80 g portion of gluten was weighed and added to 1000 mL of PBS (pH 9.0); the ratio of alcalase to gluten powder was 200 U/g. The enzymatic reaction was carried out in a 65 °C water bath with stirring for 1 h. The pH of the sample solution was adjusted to 9.0 throughout the process. The reaction system was kept in a water bath at 85 °C for 15 min to terminate the reaction and then cooled to room temperature. The samples were then lyophilized for further analysis. In addition to the pH of the reaction of 7.0, the method of hydrolyzing gluten powder by papain was the same as that of alcalase hydrolysis of gluten. The enzymatic hydrolysis DH values of gliadins treated by alcalase and papain were $27.13 \pm 0.67\%$ and $19.81 \pm 0.78\%$, respectively, and the method of determining the DH was according to Cui.²¹

Extraction of Wheat Gliadins. The gliadins of untreated and treated gluten were extracted according to the method of Wang et al.²² The untreated and treated gluten samples were degreased twice with anhydrous ether in advance and dried overnight. A 20 g portion of defatted gluten was extracted with 200 mL of 65% ethanol at room temperature for 1 h. The extract was centrifuged at 5000g for 15 min, the supernatant was pooled, and the remaining ethanol was removed by a rotary evaporator at 40 °C. The supernatant was freeze-dried.

Animals. Six-week-old male BALB/c mice were obtained from Model Animal Research Center of Nanjing University and adapted to the laboratory environment for 2 weeks prior to the experiment. All mice were housed in ventilated shelves under pathogen-free conditions with air conditioning and a 12 h light/dark cycle. The temperature and humidity were kept at 24 ± 2 °C and 50%, respectively. The test was performed according to the Guide for the Care and Use of Laboratory Animals and approved by the Laboratory Animal Ethics Committee of Jiangnan University (Wuxi, People's Republic of China). Ethical approval number: JN. No. 20181015b0301201[221]. A total of 30 mice were randomly distributed to 5 groups: that is, control, mice sensitized with no treated gliadins (NT), mice sensitized with phosphorylated gliadins (Phos-T), mice sensitized with gliadins hydrolyzed with Alcalase (Alcalase-T), and mice sensitized with gliadins hydrolyzed with papain (Papain-T).

The sample concentration was adjusted to 1 mg/mL with phosphate-buffered saline PBS (0.01 mol/L pH 7.4). The gliadins were emulsified with 1:1 aluminum hydroxide prior to sensitization. BALB/c mice were immunized on days 0, 7, and 14 by intraperitoneal injection of 50 µg of gliadins emulsified in aluminum hydroxide, and the mice of the control group were injected with the same volume of PBS. Blood samples and spleens were collected 7 days after immune stimulation. All of the mice were euthanized using CO₂.

Structure Analysis. *Fourier Transform Infrared Spectroscopy (FTIR).* The secondary structures of the untreated and treated gluten were determined by Fourier transform infrared spectroscopy (Nicolet US IS10). The samples were measured from 400 to 4000 cm⁻¹ with

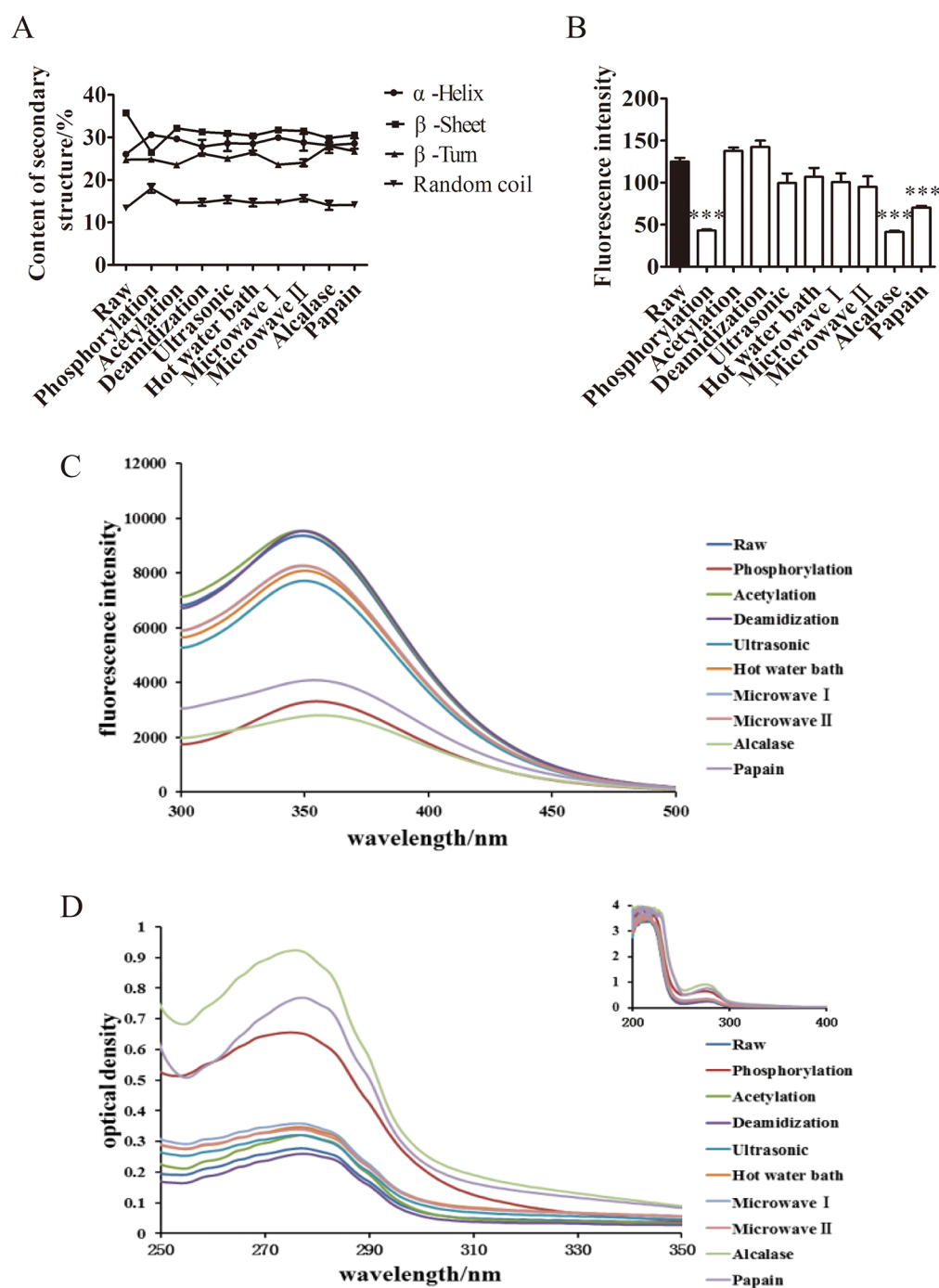


Figure 1. Conformational structures analysis. (A) Content of each secondary structure of raw and treated gluten. The results of significant difference analysis are noted in Table S1. The secondary structure of gluten was measured by Fourier transform infrared spectroscopy (FTIR). FTIR measurements were conducted for the powdery form of gliadins: microwave I, $P = 210$ W; microwave II, $P = 70$ W (the same is true below). (B) The hydrophobicity of raw and treated gliadins was measured with the hydrophobic fluorescence probe ANS, the excitation wavelength was 390 nm, and the emission wavelength was 470 nm. All of the samples were tested in triplicate, and data are expressed as mean $\pm$ SEM of three independent experiments for all panels. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the raw sample (Student's t test). What is not marked was that there was no significant difference. (C) The intrinsic fluorescence spectra of untreated and treated gliadins. The excitation wavelength was 280 nm, and the emission wavelength was from 300 to 500 nm with all sample concentration being homogeneous at 0.5 mg/mL. (D) The entire UV absorption spectra were scanned from 200 to 400 nm and is shown in the top right corner, while the major absorption peak spectra were from 250 to 300 nm.

32 scans and 4 cm^{-1} resolution. The samples were mixed with potassium bromide and then pressed into crystal sheets and recorded under the same conditions as for the background, and each sample was tested at least three times. The contents of different secondary structures were analyzed with Peakfit.

Surface Hydrophobicity Determination. The surface hydrophobicity (H_0) of untreated and treated gliadins was determined using 1-anilinonaphthalene-8-sulfonic acid (ANS).²³ The protein dispersion was diluted in phosphate buffer (pH 7.0) at a protein concentration ranging from 0.05 to 0.5 mg/mL. A 20 μL portion of 8.0 mM ANS solution was added per 4.0 mL of sample solution. The

fluorescence intensity was measured with a Hitachi F7000 fluorescence spectrometer (Tokyo, Japan). The excitation wavelength was 390 nm, and the emission wavelength was 470 nm. The initial slope of the fluorescence intensity versus protein concentration curve was used as an indicator of surface hydrophobicity. Each sample test was performed in triplicate.

Intrinsic Fluorescence Emission Spectra. The change in the tertiary structure of gliadins was determined through endogenous fluorescence spectroscopy.²⁴ Each sample had a uniform concentration of 0.5 mg/mL, an excitation wavelength of 280 nm, and an emission wavelength of 300–500 nm. The scan was performed at 1200 nm/min with a response time of 0.5 s, a PMT voltage of 500 V, and excitation and emission slits of 5 nm. Each experiment was performed at least three times to ensure accurate results.

UV Absorption Spectrum Analysis. UV absorption spectra were obtained and analyzed with a UV spectrophotometer (UV-1800). All samples were uniformly mixed at 1 mg/mL, and the UV absorption spectrum was scanned from 200 to 400 nm with an optical path of 1 cm and a response time of 0.1 s. Each sample was analyzed in triplicate.

SDS-PAGE Analysis. The SDS-PAGE patterns were used to show protein changes due to different treatments. Each protein sample (15 μ g/ μ L) was added to loading buffer (4:1) and heated at 100 °C for 5 min. The composition of the loading buffer was 50 mM Tris-HCl, 0.1% bromophenol blue, 10% glycerol (v/v), and 2% SDS (w/v) at pH 6.8. After the sample was cooled to room temperature, 50 μ g of protein was loaded onto the gel and the test run at 120 V for 60 min. The gel was stained with Coomassie Brilliant Blue, and then the gel was destained overnight at 37 °C with a mixture of 10% acetic acid and 5.5% ethanol.

Determination of Free Sulfhydryl (SH) of Gliadins. Determination of the free SH content of gliadins was by the method of Beveridge²⁵ with slight modification. A 3 mg gliadin sample was incubated in 5 mL of Tris-glycine-EDTA buffer (10.4 g of Tris, 6.9 g of glycine, and 1.2 g of EDTA per liter, pH 8.0) for 30 min and shocked every 10 min, 40 μ L of Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in Tris-glycine-EDTA buffer) was added, and the sample was covered with aluminum foil and then incubated at 25 °C for 30 min. The absorption of the mixture was measured at 412 nm against the blank (without the gliadins). The SH contents were calculated by the use of a molar extinction coefficient of 13600 M⁻¹ cm⁻¹ and expressed as μ mol/g of protein.

In Vitro Simulated Gastrointestinal Digestion. The method of simulating the digestion process was according to Minekus.²⁶ An 8 mg portion of each protein sample was mixed with 3.86 mL of simulated gastric fluid (SGF), and then added 1 μ L of 0.3 M CaCl₂ and 139 μ L of water were added. Each tube was then preheated to 37 °C, 0.16 mL of pig porcine pepsin (Sigma 500 U/mg) was added and mixed gently, and the tubes were kept in a 37 °C water bath for 2 h. Samples of 100 μ L were removed at 10 and 120 min, and the reactions were immediately terminated by 30 μ L of NaHCO₃. The 2 mL gastric digestion samples were mixed with 1.1 mL of simulated intestinal fluid (SIF), 0.25 mL of bile salts, 4 μ L of CaCl₂, 0.015 mL of NaOH, and 0.131 mL of water, the tubes were preheated to 37 °C prior to the addition of 0.5 mL of pancreatin, the digestion of samples was performed in a 37 °C water bath for 4 h, and the reaction was terminated in a 95 °C water bath for 10 min. The gastric digestive samples at 10 and 120 min and the intestinal digestive sample at 10 min were boiled at 100 °C for 10 min and then subjected to SDS-PAGE.

Allergenicity Analysis. IgE, Histamine, and Cytokine Levels Determined by ELISA. Blood samples of different groups were centrifuged at 1500 rpm for 20 min, and the serum total IgE and histamine concentration were estimated respectively by a Mouse IgE ELISA Kit (Multi Sciences), a Mouse Histamine (HIS) ELISA Kit, a Mouse IFN- γ ELISA Kit, and a Mouse IL-4 ELISA Kit.

ELISA for Specific-IgE Antibodies to Gliadin. For the determination of the levels of gliadin-specific serum IgE, we used ELISA as described previously.²⁷ A microtiter plate was coated at 37 °C overnight with 100 μ L of gliadin (Shanghai Yuanye Biological Co,

Ltd., S24514) at a concentration of 1 μ g/mL. Wells were washed five times with the washing buffer (phosphate-buffered saline buffer containing 0.05% Tween-20). The remaining binding sites were blocked with 3% bovine serum albumin–phosphate buffered saline Tween-20 for 1 h at room temperature. Then wells were incubated at room temperature with 50 μ L of serum. After 3 h, the plate was washed five times with washing buffer, and 100 μ L of goat antimouse IgE HRP conjugated antibody (Beyotime, A0216) was added to each well and incubated for 1 h at room temperature. After washing five times with washing buffer, colorimetric conversion was developed with 100 μ L of TMB substrate solution (Beyotime, P0209) for 10 min at room temperature. The HRP reaction was stopped by the addition of 50 μ L of 2 N sulfuric acid, and the absorbance was read at 450 nm by a 96-well microplate reader.

Index of Spleen. Seven days after the sensitization, the mice were sacrificed and the spleens were taken and weighed immediately to calculate the percentage by weight (mg/g).

Quantitative Real Time PCR Analysis. Total RNA was extracted from spleens using Trizol reagent (Invitrogen), and the RNA was quantified by Nanodrop (Thermo Scientific). RNA was transcribed into cDNA by the Prime Script RT system (Takara). For real-time PCR, primers were designed and the sequences are shown in Table S2. The expression of 18S was used as an internal control. Th1-biased and Th2-biased cytokines as well as critical transcription factors IFN- γ , T-bet, IL-4, IL-5, and GATA-3 expression levels were determined. Furthermore, the mRNA expression levels of Th17/Treg related cytokine (TGF- β) and critical transcription factors (Foxp3 and ROR γ t) were determined by quantitative real-time RT-PCR.

Statistical Analysis. In the study, data were expressed as mean $\pm$ standard errors of the mean (SEM). All samples were determined in triplicate. Student's *t* test was used to detect the differences between groups. All data were analyzed by GraphPad Prism 5.0 (GraphPad Software).

RESULTS

Effects of Nine Different Treatments on the Secondary and Tertiary Structure of Gliadins. Treatment-induced conformational structure alterations of gluten were analyzed through FTIR, fluorescence spectra, and UV absorption spectra. In comparison to the raw gluten, the amount of β -sheet decreased significantly in all treated gluten samples, especially phosphorylated gluten. The amount of α -helix and random coil increased significantly in the phosphorylated sample, but the changes in other samples were not significant (Figure 1A and Table S1). Alcalase- and papain-hydrolyzed gluten samples had a higher β -turn amount and were statistically significantly different from the raw sample. In all, phosphorylation significantly converted the β -sheet into α -helix and random coil, and alcalase and papain hydrolysis resulted in significant loss of the β -sheet and β -turn of gliadins, while the rest of the treatments had no significant effects on the secondary structure. We employed the alterations of H_0 as the probe to evaluate the change in the tertiary structure of gliadins. Except for acetylation and deamination, which increased the surface hydrophobicity of gliadins, other treatments showed a downward trend (Figure 1B). In these treatments, the surface hydrophobicities of phosphorylation- and enzyme-treated gliadins was significantly reduced by 65.5%, 67.6%, and 44.4% in comparison to raw gliadins, but the other treatments for protein surface hydrophobicity had no significant effect. The tertiary structure of gliadins can also be reflected in the intrinsic fluorescence spectrum. The fluorescence intensities of phosphorylated and two enzymatically hydrolyzed gliadins were significantly lower than that of the raw gliadins, but there was no significant difference between the other treated gliadins and raw gliadins

(Figure 1C). We observed an emission spectrum with a peak position of 349 nm in the raw sample upon excitation at 280 nm. In addition, phosphorylation and alcalase and papain hydrolysis not only altered the maximum fluorescence intensity but also induced a slight red shift in the position of the fluorescence peak (4–6 nm). This indicated a decrease in the surface hydrophobicity of the gliadins after phosphorylation and enzymatic hydrolysis, which were consistent with the results of H_0 determined by ANS. In addition, UV absorption spectra are also some of the structural characteristics that are generally used to assess conformational changes in proteins. Phosphorylation and enzymatic treatments significantly increased the maximum absorbance of gliadins (Figure 1D). The maximum UV absorbance peak position of gliadins was found to be at 277 nm, and a slight blue shift in the UV absorption peak was observed in the phosphorylated gliadins. The maximum absorption peak of enzymatic hydrolysis and the other treatments was almost identical with that of the untreated gliadins. These results indicated that phosphorylation and alcalase and papain hydrolysis significantly altered the gliadin secondary and tertiary structures, resulting in a more disordered protein structure.

Effects of Phosphorylation and Enzymatic Hydrolysis on Molecular Weight and Free SH Content of Gliadins.

According to the results of secondary and tertiary structures of nine different processed gliadins, phosphorylation and alcalase and papain enzymatic treatments have a significant influence on the protein structure, SDS-PAGE was performed, and the content of free SH was determined to learn more details about structural changes. The electrophoresis band of untreated gliadins was in the range of 28–55 kDa (Figure 2A, lane 1).

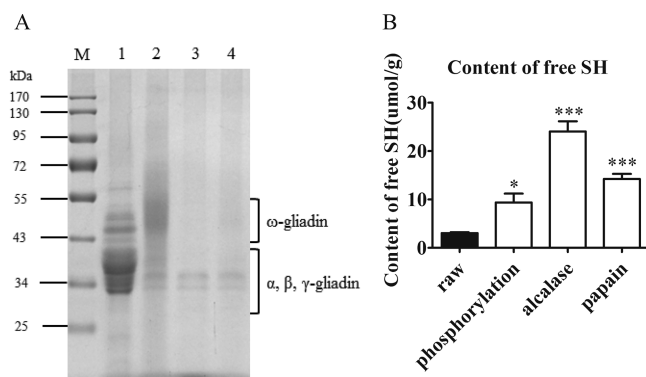


Figure 2. Effects of phosphorylation and enzymatic hydrolysis on molecular weight and free SH content of gliadins. (A) SDS-PAGE patterns of gliadins extracted from gluten exposed to different treatments: (lane M) molecular marker; (lane 1) raw gliadins; (lane 2) phosphorylated gliadins; (lane 3) gliadins hydrolyzed by alcalase; (lane 4) gliadins hydrolyzed by papain. An equal amount of gliadin (15 μ g) was loaded in each lane. (B) Content of free SH in the raw and phosphorylated gliadins and enzymatic hydrolyzed gliadins with alcalase and papain. Data are expressed as mean $\pm$ SEM of three independent experiments for all panels. * p < 0.05, ** p < 0.01, and *** p < 0.001 versus the raw (Student's t test); ns, not significant.

The electrophoresis band of phosphorylated gliadins was significantly different from that of the raw gliadins, with a heavier aggregation band ranging from 45 to 72 kDa (Figure 2A, lane 2), indicating that the phosphorylated gliadin molecules fold or aggregate to some extent. In contrast, the bands of gliadins became weak after hydrolysis with alcalase

and papain (Figure 2A, lanes 3 and 4), indicating that a part of the gliadins was hydrolyzed into small peptides.²⁸ The amount of disulfide bonds (SS) was found to be another structural factor of gluten that is closely related to its allergenicity. The result showed that phosphorylation and alcalase and papain hydrolysis increased the SH content significantly in comparison to the raw gliadins (Figure 2B). The results suggested that the disruption of SS bonds in the protein molecule increased the content of free SH groups.

Effects of Phosphorylation and Enzymatic Hydrolysis on the Digestibility of Gliadins.

Allergic reactions occur through interactions between epitopes and immune cells in the gastrointestinal digestion.²⁹ To assess the digestibility of gliadins treated with different methods, we performed in vitro simulated gastric and intestinal digestion and learned the changes in the digestibility of gliadins by SDS-PAGE patterns (Figure 3). The results showed that the band intensities of alcalase and papain hydrolyzed samples were significantly weakened after 10 min of in vitro gastric digestion, while the gliadin band around 34 kDa in the digest of raw gliadins was still evident (Figure 3A). The clear and distinct band at 43–55 kDa present throughout the gastric digestion process was unique to porcine pepsin. After 2 h of in vitro gastric digestion, most of the alcalase and papain hydrolyzed gliadins were digested into small fragments, but the raw and phosphorylated gliadins had not been completely digested (Figure 3B). After 10 min of intestinal digestion, the band intensity of gliadins in the digestive mixture of the raw sample significantly weakened (Figure 3C), and the protein bands all disappeared after the intestinal digestion stage. Together, phosphorylation and alcalase and papain hydrolysis could significantly improve the digestibility of gliadins.

Effects of Phosphorylation and Enzymatic Hydrolysis on the Characteristics of the Allergic Response.

In order to evaluate the effect of processing on gliadin allergenicity, we sensitized BALB/c mice with raw and treated gliadins and explored the potential allergenicity in vivo (Figure 4A). The mice sensitized with raw gliadins (NT) had many obvious red spots on the tails after the second sensitization during the course of the experiment, while the other three groups did not. This indicated that phosphorylation and enzymatic hydrolysis with alcalase and papain may alleviate the allergic appearance of mice. The spleen is the largest immune tissue in the human body. Antigen stimulation can cause immune responses and phagocytosis of exogenous substances in spleen macrophages and lymphocytes, consequently causing the swelling of the spleen.³⁰ In comparison with the control group, mice sensitized with gliadins had a higher spleen index (Figure 4B). The spleen index of the Phos-T and Papain-T mice was significantly lower than that of the NT group, but phosphorylated and papain hydrolyzed gliadins could still induce a more moderate increase in spleen size. There was no significant difference in the index of spleen between the mice in the Alcalase-T group and the control group. Allergic reactions induced by most food are mediated by allergen-specific IgE.³⁰ Serum total IgE and gliadin-specific IgE levels were significantly increased in the NT group in comparison to the Control group. The levels of serum total IgE and gliadin-specific IgE were significantly decreased in the Phos-T, Alcalase-T, and Papain-T groups, and especially the IgE level of Alcalase-T group achieved to the same level as that of the Control group (Figure 4C,D). On the mast cell surface, the cross-linking of specific IgE triggers the release of histamine

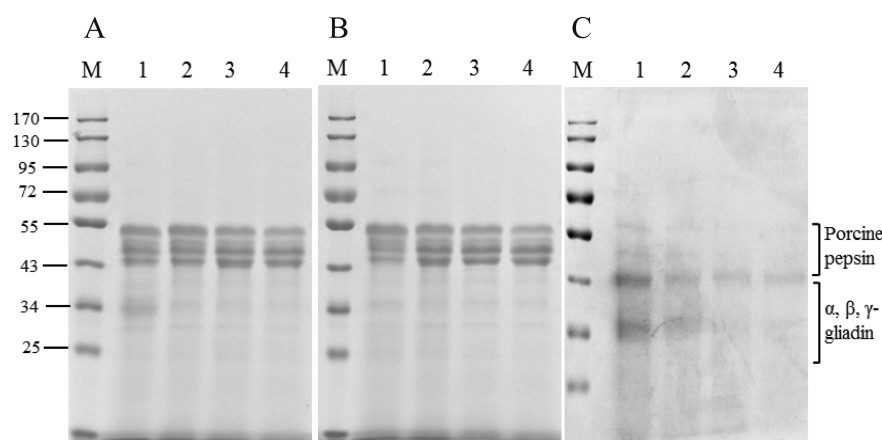


Figure 3. Effects of phosphorylation and alcalase and papain hydrolysis on the digestibility of gliadins. In vitro simulated gastrointestinal digestion was performed, followed by SDS-PAGE. The whole digestion process included 2 h of gastric digestion and 4 h of intestinal digestion. Three time points were selected to demonstrate the results of gastric and intestinal digestion patterns. (A) Gliadins digested in SGF (porcine pepsin, 2000 U/mL) for 10 min. (B) Gliadins digested in SGF (porcine pepsin, 2000 U/mL) for 2 h. (C) Gliadins digested in SIF (porcine pancreatin) for 10 min. All gels in the study were loaded in the same order with 12 μ g of gastric digestion mix and 100 μ g of intestinal digestive mix: (lane M) molecular marker; (lane 1) raw gliadins; (lane 2) phosphorylated gliadins; (lane 3) gliadins hydrolyzed by alcalase; (lane 4) gliadins hydrolyzed by papain.

while binding with the food allergen.³¹ Therefore, serum histamine concentration is a good indicator of the degree of hypersensitivity in sensitized mice. The NT group showed a serum histamine concentration significantly higher than that of the Control group, while the histamine productions in serum of the Phos-T, Alcalase-T, and Papain-T groups were decreased significantly in comparison with the NT group (Figure 4E). In particular, the histamine concentrations of Phos-T and Alcalase-T were not significantly different from those of the control group, which was consistent with the results of serum total IgE concentrations. The serum concentrations of the Th1 response cytokine (IFN- γ) and Th2 response cytokine (IL-4) were measured. The levels of IFN- γ and IL-4 were significantly decreased in NT group in comparison to Control group. The levels of serum IFN- γ were significantly decreased in the Phos-T, Alcalase-T, and Papain-T groups, and especially the IFN- γ level of the Alcalase-T group achieved the same level as that of the Control group (Figure 4F). In addition, cytokine IL-4 production in serum of Phos-T Alcalase-T and Papain-T groups were decreased significantly compared with NT group (Figure 4G). The IL-4 concentration of Alcalase-T was not significantly different from that of the control group, which was consistent with the results of the index of spleen and serum total IgE concentrations. These indicated that the mice were successfully sensitized with gliadins and that phosphorylation and enzymatic hydrolysis could reduce the hypersensitivity of gliadins.

Phosphorylation and Enzymatic Hydrolysis Modulate Allergic Factors. In order to better understand the effects of processing methods on the potential allergenicity of gliadins, we determined the expression of Th1-biased and Th2-biased cytokines (IL-4, IL-5) and transcription factor T-bet (Th1) and GATA-3 (Th2) in spleen by qPCR. The expression levels of Th1-biased cytokine IFN- γ were decreased significantly in the Phos-T, Alcalase-T, and Papain-T groups (Figure 5A). For Th2-biased cytokines IL-4 and IL-5, these were also significantly decreased in all experimental groups in comparison with the NT group (Figure 5B,C). Furthermore, the results showed that T-bet mRNA expression levels were significantly increased and GATA-3 mRNA expression levels were significantly decreased in the Phos-T, Alcalase-T, and

Papain-T groups (Figure 5D,E). The ratio of T-bet to GATA-3 expression levels in the four groups was the same as T-bet, as opposed to GATA-3 (Figure 5F). In addition to that, to investigate changes in the balance of Th17/Treg responses, we measured the expressions of TGF- β and key transcription factors FOXP3 and ROR γ t. Both TGF- β and FOXP3 had higher expression levels in the Phos-T, Alcalase-T, and Papain-T groups and had statistically significant differences in comparison to the control group (Figure 5G,H). In contrast, the expression levels of ROR γ t in the Phos-T, Alcalase-T, and Papain-T mice were significantly lower than in the control group (Figure 5I). Moreover, in comparison with the control group, the sensitized mice of the Phos-T, Alcalase-T, and Papain-T groups had significantly increased ratio of FOXP3 to ROR γ t expression levels (Figure 5J). The above results indicated that the treatment of phosphorylation and alcalase and papain hydrolysis reduced the allergenicity of gliadins by improving the imbalance between Th17 and Treg cells in mice.

DISCUSSION

Since the effects of some other treatments on the secondary and tertiary structure of the gluten were not significant, we only analyzed the structure, digestive properties, and potential allergenicity of phosphorylated and alcalase and papain hydrolyzed gliadins. It had been reported that processing methods could change the allergenicity of allergenic foods,^{10,32} and different treatments induced varying degrees of gliadin structural changes, thereby reducing or increasing allergenicity.¹⁶ In our study, the changes in secondary and tertiary structures of raw and treated gluten were determined through FTIR and fluorescence spectra. From the results, we observed four secondary structures sensitive to phosphorylation and enzymatic hydrolysis of alcalase and papain. The β -sheet structures of the three treated samples were decreased, while the α -helix content of the phosphorylated gluten and the β -turn content of the enzymatic hydrolyzed gluten increased significantly. This further indicated that β -sheets were partially converted to the other three secondary structures and that the protein structures tend to be more disordered. On the other hand, the intrinsic fluorescence spectra of proteins are mainly expressed as typical fluorescence emission spectra of

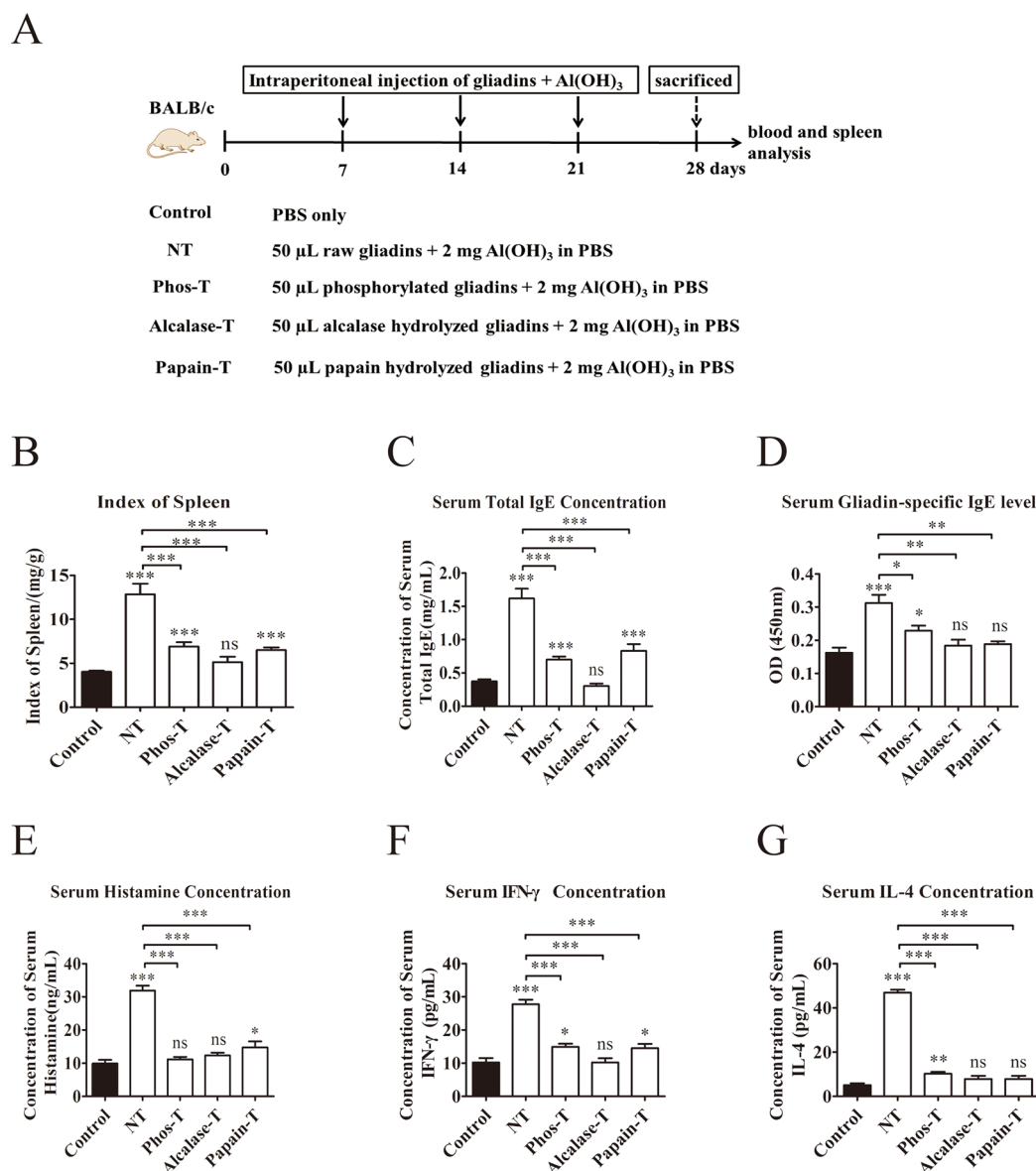


Figure 4. Effects of phosphorylation and enzymatic hydrolysis on the characteristics of the allergic response. (A) Experimental design. Mice were sensitized with 50 µg of raw gliadins, phosphorylated gliadins, and enzymatic hydrolyzed gliadins for 3 weeks, once a week. Blood and spleen samples were collected in the fourth week. (B) Index of spleen. (C) Serum total IgE concentration. (D) Serum gliadin-specific IgE level. (E) Serum histamine concentration. (F) Serum IFN- γ concentration. (G) Serum IL-4 concentration. BALB/c mice were stimulated by intraperitoneal injection with no treated gliadins (NT) and phosphorylated (Phos-T) and enzymatic hydrolyzed by alcalase (Alcalase-T) and papain (Papain-T) gliadins ($n = 5$). The index of the spleen is equal to the weight of the spleen divided by the body weight (mg/g), IgE and histamine level were determined by a Mouse ELISA Kit. Data are expressed as mean $\pm$ SEM of three independent experiments for all panels. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the Control (Student's t test); ns, not significant.

tryptophan (Trp) and tyrosine (Tyr) residues. A decrease in the fluorescence intensity of phosphorylated and alcalase and papain hydrolyzed gliadins indicated that fewer hydrophobic regions were exposed on the protein surface, and the decrease in the surface hydrophobicity of gliadins was also reflected in the change of UV absorption spectra. Furthermore, a slight red shift in the location of the fluorescence peak indicated that the microenvironment of Trp and Tyr side-chain groups had changed.³³

We applied SDS-PAGE to investigate the change in molecular weight and found that the molecular weights of phosphorylated gliadins were higher than that of the raw sample, while the bands of gliadins disappeared in the samples hydrolyzed by alcalase and papain. Therefore, alcalase and

papain hydrolysis resulted in breakdown of the primary structure, not only the secondary structure of proteins. The reasons for the increased molecular weight of phosphorylated gliadins included the binding of PO₄ groups to protein molecules and the folding of protein. The surface hydrophobicity of the protein would increase on binding to PO₄ groups. According to the results of tertiary structure and surface hydrophobicity, we could not rule out that the gliadins bind to certain PO₄ groups, and the surface hydrophobicity of phosphorylated gliadins was reduced significantly, indicating that the phosphorylation caused the protein molecules to fold and lead to an increase in molecular weight. Taken together, these results suggest that phosphorylation may fold the gliadins protein molecules together,³² while alcalase and papain

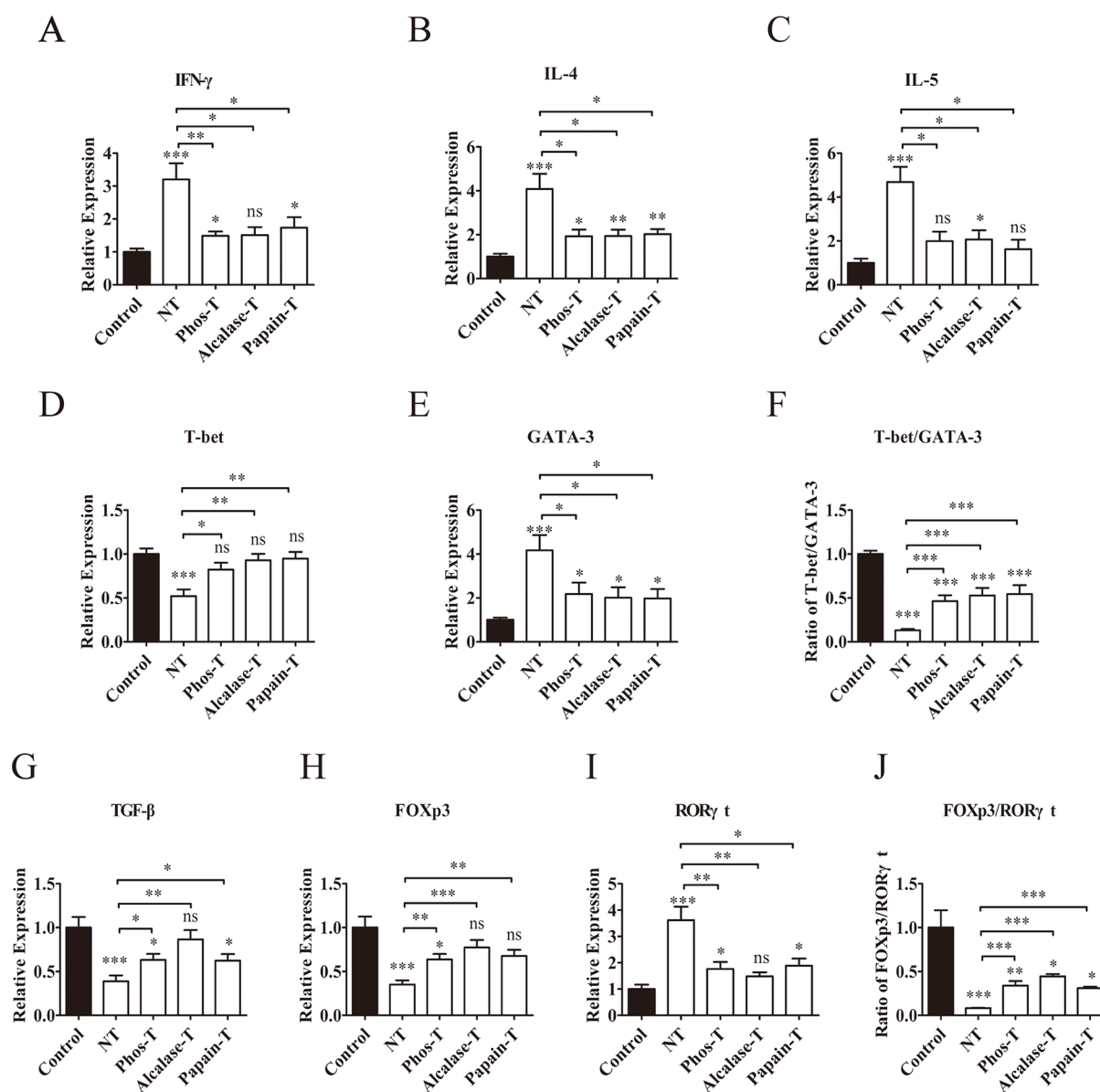


Figure 5. Phosphorylation and enzymatic hydrolysis modulate allergic factors: (A) IFN- γ (Th1-biased cytokine); (B) IL-4 (Th2-biased cytokine); (C) IL-5 (Th2-biased cytokine); (D) T-bet (Th1-biased transcription factor); (E) GATA-3 (Th1-biased transcription factor); (F) T-bet/GATA-3; (G) TGF- β (Treg-biased cytokine); (H) FOXP3 (Treg-biased transcription factor); (I) ROR γ t (Th17-biased transcription factor); (J) FOXP3/ROR γ t. BALB/c mice were simulated by intraperitoneal injection with no treated gliadins (NT) and phosphorylated (Phos-T) and enzymatic hydrolyzed by alcalase (Alcalase-T) and papain (Papain-T) gliadins ($n = 5$). The mRNA expression level was determined by RT-qPCR analysis. Data are expressed as mean $\pm$ SEM of three independent experiments for all panels. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the Control (Student's t test); ns, not significant.

hydrolyzed the gliadins into small peptides. The probable reason for the reduced surface hydrophobicity of the phosphorylated gliadins was that the aggregation of protein molecules would bury a portion of the hydrophobic regions. The gliadins were hydrolyzed into small peptide fragments by alcalase and papain, and these peptides would exist at the lowest surface hydrophobicity under hydrophobic force, which is the reason the surface hydrophobicity of gliadins decreased after phosphorylation and enzymatic hydrolysis. On the other hand, the content of free SH also reflects the change in the conformational structure of gliadins.⁸ It has been reported that reduction of the SS bonds to free SH groups reduced the affinity for specific antibodies,⁶ and the disulfide bonds played an important role in the integrity of the IgE-binding epitopes.³⁴

In accordance with this, we further determined the free SH content of gliadins. A significant increase in free SH in the three treated gliadins indicated the breaking of disulfide bonds in or between gliadin protein molecules. However, the result of SDS-PAGE analysis showed that proteins were clustered together after phosphorylation; therefore, we speculated that the disulfide bond within or between the protein molecules was destroyed, causing the protein molecules to unfold, thereby exposing a large number of hydrophobic amino acid groups. Under hydrophobic interaction, a large number of exposed hydrophobic groups cause protein molecules to aggregate, reduce surface hydrophobicity, and improve the stability of the whole system. This point was also illustrated by

the significant decrease in H_0 values measured by the methods using ANS as a fluorescent probe.

Another factor associated with the potential allergenicity of gliadins is its digestibility in the gastrointestinal tract, and changes in protein structure directly affect the digestibility of gliadins.²⁹ It is well-known that in many cases allergic foods quickly develop allergic symptoms after ingestion.³⁵ Some allergenic proteins, including gliadins, have lower digestibility than common proteins and exhibit hydrolysis resistance to the gastrointestinal and brush-border membrane enzymes.^{4,36} The results showed that the raw gliadins partially resisted the hydrolysis of gastric juice, while the phosphorylated gliadins and enzymatically hydrolyzed gliadins were more easily digested. Gliadins were hydrolyzed into small peptides by alcalase or papain and perhaps exposed more cleavage sites of the digestive enzymes.³⁷ Although the phosphorylated gliadins molecules aggregated, the destruction of SS and the production of a large amount of free SH indicated that the intermolecular or intramolecular structures of the gliadins were destroyed, and thus some cleavage sites of the digestive enzymes were also available. Sensitivity to proteolysis is a determinant of allergenicity of gliadins, as it partially controls the sensitization of the immune system after transport across the intestinal barrier. The results of allergenicity analysis validated that there were still some smaller peptides left after gastrointestinal digestion and that these peptides contain IgE-binding epitopes which relate to their allergenicity. Thus, phosphorylation and alcalase and papain hydrolysis enhanced the digestibility of gliadins, resulting in reduced allergenicity. However, the information obtained from in vitro simulated digestion was not sufficient to understand the potential allergenicity of treated gliadins. Therefore, we designed animal experiments to explore the in vivo allergic reactions of treated gliadins.

Because of the different protein structures, the potential allergenicities of the three treated gliadins were also different. Therefore, we further discuss the effects of phosphorylation and enzymatic treatments on allergenicity in the case of completely different changes in protein structure. The severity of food allergies is associated with elevated levels of IgE, an important clinical diagnosis and food allergy prevention indicator.¹⁴ In comparison with the Control group, mice in the Phos-T, Alcalase-T, and Papain-T groups significantly reduced total IgE and gliadin specific IgE production. On the other hand, mast cell degranulation plays an important role in the immediate allergic food induced anaphylactic reaction which is mediated by IgE, and histamine as a key anaphylactic mediator released by mast cells showed the same trend as IgE and gliadin-specific IgE. Modification of the gliadin structure contributes to the destruction of certain epitopes and thereby to reduction of serum IgE and histamine concentrations. The index of spleen of Phos-T, Papain-T, and Papain-T groups was significantly lower than that of the Control group, which was consistent with changes in serum IgE and histamine concentrations.

To further elucidate the hypersensitivity of treated gliadins clearly, we analyzed the cytokine and transcription factor expression levels of spleens collected from sensitized BALB/c mice. IgE production is reported to be due to the development and activation of Th2 cells and B cells.³⁸ An important function of IL-4 is to induce the class switching of IgE isoforms and their production.³⁹ The excess IL-4 production in Th2 cells was consistent with the increase in IgE and histamine concentrations in our results. On the other hand, IFN- γ is

produced by Th1 cells, and as a major cytokine secreted by Th1 cells, IFN- γ inhibits the production of IgE. Therefore, the balance of Th1 and Th2 could be an effective reflection of allergic diseases, and allergies are usually accompanied by excessive Th2-type immune responses.⁴⁰ In comparison to the NT group, significant declines in the expression levels of Th2 type cytokines (IL-4 and IL-5) were consistent with our expectations and previous conclusions, whereas the decrease in the expression level of IFN- γ appeared to be incompatible with the results obtained above. These results suggested that the mechanism of reducing allergen-specific IgE antibody mediated allergic responses was more complex than simply enhancing the Th1 immune response and reducing the Th2 immune response. Another possible reason is that different types of gliadins cause different allergic symptoms,⁴¹ and the degree of reduction of several allergens varies with the way of treatment. Moreover, the results of serum IFN- γ and IL-4 concentrations were consistent with changes in their mRNA relative expressions. On the other hand, it is well-known that both Treg and Th17 cells are derived from the CD4+ T-cell precursors,⁴² and both cells require TGF- β for differentiation, but distinct transcription factors determine the outcomes: ROR γ t for Th17 cells and FOXP3 for Treg cells.⁴³ The increased expressions of TGF- β and FOXP3 and the decreased expression of ROR γ t indicated that Th17 cells were less overdifferentiated. On the basis of the above results, we concluded that the potential allergenicity of gliadins is closely related to the conformational structure. Phosphorylation and enzymatic hydrolysis were effective ways to improve the allergenicity of proteins due to the epitopes being made less available to antibody receptors. In phosphorylated gliadins, molecular aggregation hid allergen epitopes that cause allergen epitopes to be hardly recognized by the immune system, while in enzymatically hydrolyzed gliadins, epitopes were destroyed after enzymatic hydrolysis. Castan et al.⁴⁴ observed that industrial processes influence food allergenicity. The difference is that they found deamidation induced a stronger allergic reaction and hydrolyzed gliadins displayed a minor effect, while our results showed that the deamidation had no significant effect on the structure of gliadins. The reason for the two completely different results was that there was a great difference in the conditions of the deamidation treatment. In addition, our results demonstrated that the allergenicity of phosphorylated and enzymatic hydrolyzed gliadins reduced rather than aggravated the potential allergenicity. We thought that the possible reason was that different processing conditions formed two kinds of proteins with widely different structures, although they were all deamidated gliadins.

In conclusion, we characterized the treated gliadins by both the structural characteristics and potential allergenicity and illustrated the relationship between structural changes and hypoallergenicity of phosphorylated and alcalase and papain hydrolyzed gliadins. Conformational changes in gliadins affected allergenicity mainly by altering the binding epitopes. Phosphorylation changed the tertiary and quaternary structures of gliadins, and the aggregation of protein molecules destroyed the conformational epitopes and buried linear epitopes. Enzymatic treatments with alcalase and papain destroyed allergenic epitopes by hydrolyzing gliadins into small peptides. Although the potential allergenicity of gliadins had not been completely eliminated, our results indicated that we can minimize the allergenicity of gliadins from the perspective of changing protein structure, such as destroying conformational

epitopes, hiding linear epitopes, or eliminating the allergenicity by hydrolyzing allergen proteins into small peptides that do not contain allergen epitopes. We further confirmed that reasonable processing methods could effectively reduce the allergenicity of wheat protein, which has good prospects for the production of wheat-related foods.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b00569.

Additional data as described in the text; genes and sense and antisense sequences (PDF)

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Funding

This work was supported by grants from the Fundamental Research Funds for the Central Universities (No. JUSR-PS1708A and No. JUSRP11842), the National Natural Science Foundation of China (31671890), Jiangsu Key Laboratory of Advanced Food Manufacturing Equipment & Technology (FMZ201807), National first-class discipline program of Food Science and Technology (JUFSTR20180103), Ministry of Science and Technology of China (Grants 2016YFA0500102 and 2016YFC1304905), and Major Science and Technology Projects in Anhui Province (18030701176).

Notes

The authors declare no competing financial interest.

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Supporting information

Table. S1

Items	α -Helix	β -Sheet	β -Turn	Random coil
Raw	26.05 $\pm$ 0.17 ^b	35.81 $\pm$ 0.03 ^a	24.76 $\pm$ 0.51 ^b	13.38 $\pm$ 0.37 ^b
Phosphorylation	30.63 $\pm$ 0.46 ^a	26.53 $\pm$ 0.69 ^c	24.84 $\pm$ 0.12 ^b	18.00 $\pm$ 1.03 ^a
Acetylation	29.66 $\pm$ 0.08 ^a	32.16 $\pm$ 0.09 ^{ab}	23.55 $\pm$ 0.11 ^b	14.63 $\pm$ 0.10 ^b
Deamidization	27.80 $\pm$ 1.62 ^b	31.28 $\pm$ 0.11 ^b	26.20 $\pm$ 0.70 ^{ab}	14.71 $\pm$ 0.82 ^b
Ultraphonic	28.02 $\pm$ 1.84 ^b	30.95 $\pm$ 0.48 ^b	25.06 $\pm$ 0.52 ^b	15.37 $\pm$ 0.90 ^b
Hot water bath	28.51 $\pm$ 1.64 ^b	30.38 $\pm$ 0.54 ^b	26.45 $\pm$ 0.21 ^{ab}	14.66 $\pm$ 0.90 ^b
Microwave I	29.94 $\pm$ 0.01 ^a	31.76 $\pm$ 0.37 ^b	23.59 $\pm$ 0.53 ^b	14.71 $\pm$ 0.17 ^b
Microwave II	28.80 $\pm$ 1.97 ^{ab}	31.47 $\pm$ 0.37 ^b	24.04 $\pm$ 0.81 ^b	15.69 $\pm$ 0.80 ^b
Alcalase	28.11 $\pm$ 1.80 ^b	29.85 $\pm$ 0.26 ^{bc}	28.00 $\pm$ 0.81 ^a	14.04 $\pm$ 1.09 ^b
Papain	28.58 $\pm$ 1.22 ^b	30.54 $\pm$ 0.54 ^b	26.74 $\pm$ 0.40 ^a	14.14 $\pm$ 0.29 ^b

Table. S2

Gene	Sense sequence	Antisense sequence
IFN- γ	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG
T-bet	CCTCTTCTATCCAACCAGTATC	CTCCGCTTCATAACTGTGT
IL-4	CTCATGGAGCTGCAGAGACTCTT	CATTCATGGTGCAGCTTATCGA
IL-5	TGACCGCCAAAAAGAGAAGTG	GAACTCTTGCAGGTAATCCAGGAA
GATA-3	CAGAACCGGCCCTTATCA	ACAGTTCGCGCAGGATGTC
TGF- β	CACCGGAGAGCCCTGGATA	TGTACAGCTGCCGCACACA
FOXp3	CCCATCCCCAGGAGTCTT	ACCATGACTAGGGGCACTGTA
ROR γ t	ACAGCCACTGCATTCCCAGTTT	TCTCGGAAGGACTTGCAGACAT