

Mechanism of Reduction in Allergenicity and Altered Human Intestinal Microbiota of Digested β -Lactoglobulin Modified by Ultrasonic Pretreatment Combined with Glycation

Yan-hong Shao, Yao Zhang, Lu Zhang, Jun Liu,* and Zong-cai Tu*

Cite This: *J. Agric. Food Chem.* 2021, 69, 14004–14012

Read Online

ACCESS |



Metrics & More



Article Recommendations



Supporting Information

ABSTRACT: The effects of ultrasound combined with glycation (UCG) on the allergenicity and human microbial community of β -Lg during in vitro digestion were studied by ELISA, cell experiments, and 16S rRNA high-throughput sequencing. UCG modification and subsequent digestion significantly reduced allergenicity. The decrease in the allergenicity of β -Lg depended not only on the low digestibility of glycosylated β -Lg, which led to the decrease of some peptides with complete immunogenicity, but also the masking effect of glycation on allergen epitopes of β -Lg. Meanwhile, UCG modification and subsequent digestion could alter the structures of intestinal microbiota and the community abundance at phylum, family, and genus levels, such as Bacteroidota, Fusobacteriota, Enterobacteriaceae, Bacteroidaceae, Ruminococcaceae, *Bacteroides*, and *Faecalibacterium*. These results show that simulated in vitro digestion of modified β -Lg reduces allergenicity and alters human intestinal microbiota, which could provide a theoretical basis for studying the relationship between intestinal dysbiosis and cow's milk allergy.

KEYWORDS: Ultrasonic combined with glycation (UCG), β -lactoglobulin, in vitro digestibility, allergenicity, human intestinal microbiota

INTRODUCTION

β -lactoglobulin (β -Lg) is the main whey protein in milk and plays an extremely important role in food manufacturing due to its incredibly high content of essential amino acids and versatile functional properties.¹ However, β -Lg has been recognized as an allergenic component of bovine milk. According to previous research, ninety percent of people who are allergic to cow's milk has an allergy to β -Lg.²

Eliminating or reducing the antigenicity of β -Lg through various processing methods (heat treatment, glycation, enzymatic hydrolysis, fermentation, etc.) has been a research hotspot in recent years.^{3–6} Among them, glycation as a promising method not only changes the food function of proteins but also effectively reduces the allergenic potential of β -Lg.⁷ A great amount of attention has been paid to glycation, which produces powerful food-grade protein–carbohydrate conjugates. However, glycation alone is no longer enough to decrease allergenic activity to a satisfactory level. Thus, a number of researchers have pointed out that physical treatment combined with glycation has a better inhibitory effect on the allergenic potential of β -Lg as compared to glycation alone.^{2,8,9} Our previous research has also shown that ultrasonic treatment combined with glycation (UCG) modification can meaningfully reduce the allergenicity of β -Lg.¹⁰ Despite the number of works on UCG modification, information about the allergenic potential of β -Lg treated by UCG modification during in vitro digestion is still fragmented.

Some studies have shown that glycosylated proteins after gastrointestinal (GI) digestion can affect intestinal microbiota and thus alter their allergenicity.^{11,12} Corzo-Martínez et al.¹³ have studied the effect of glycosylated casein and β -Lg on human intestinal microbiota, and the results indicate that glycosylated

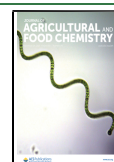
proteins can induce a significant increase in Bifidobacteria, whereas *Lactobacillus*–*Enterococcus*, *Clostridium histolyticum*, *Atopobium*, and *Bacteroides* have no effect. Caseinomacropeptide was treated in Maillard reaction used for in vitro intestinal microbial fermentation,¹⁴ and it was found that glycation showed bifidogenic activity. Glycoconjugates seemed to be more difficult to be digested and excreted, leading to microbiota fermentation in the intestine, which affected the growth of intestinal microbiota. Dietary carbohydrates, such as undigested polysaccharides and partially digested starch, can be consumed by intestinal microbiota, converting them into digestible compounds such as short-chain fatty acids (including acetic acid, propionic acid, and butyric acid) and changing the structure or diversity of the intestinal microbiota.¹⁵ Monosaccharides or disaccharides can be completely absorbed in the small intestine, and they have little effect on the intestinal microbiota.¹⁶ Studies have also shown that monosaccharides can escape the absorption from the small intestine, reach the colon, and be metabolized by the intestinal microbiota, affecting the commensal *Bacteroides thetaiotaomicron*, and high-monosaccharide diets can reduce the abundance of commensal *Bacteroides thetaiotaomicron*.¹⁷ Therefore, conducting research about the influence of dietary glycoproteins on the intestinal microbiota is necessary. However, the effect of β -Lg

Received: June 10, 2021

Revised: October 2, 2021

Accepted: October 4, 2021

Published: November 11, 2021



treated by UCG modification on human intestinal microbiota remains unknown. Abdel-Gadir et al.¹⁸ showed that different compositions of intestinal microbiota could influence food allergies. Certain specific bacteria in the intestine can protect humans from food allergies. Ling et al.¹⁹ have shown that the intestinal microbiota of infants allergic to milk is different from that of healthy infants, which is primarily manifested in the decrease of the microbiota diversity in their bodies. Therefore, exploring the effect of milk protein processed by UCG modification on human gut microbes after entering the body is of great importance. In this work, the influences of glycation combined with ultrasonic pretreatment on the structure, allergenicity, and human intestinal microbiota of β -Lg during in vitro digestion were investigated.

MATERIALS AND METHODS

Materials. β -Lg from bovine milk (L3908, $\geq 90\%$) was obtained from Sigma-Aldrich. *o*-Phthalaldehyde (OPA), acrylamide, sodium dodecyl sulfate, and other reagents were purchased from Solarbio. Polyclonal anti-bovine β -Lg antibody was produced in rabbit and was obtained from Bios (Beijing, China).

We purchased 10 serum samples from patients with milk allergy from Plasma Lab International (Everett, WA, U.S.A.). The specific IgE level of milk in the serum ranges from 7.12 to 117.43 kUA/L. A serum pool made by mixing equal amounts of sera of these 10 patients was used to study the IgE binding ability of β -Lg.

Sample Preparation. UCG modification of β -Lg using the method of Shao et al.¹⁰ Native β -Lg and glycated β -Lg ultrasonicated at 0 and 120 W/cm² were named β -Lg, β -Lg-G, and β -Lg-120-G, respectively. The degree of glycation and molecular weight (MW) of the glycated proteins were determined.

Degree of Graft. Free amino groups were determined by the OPA method using the Hitachi U-2910 spectrophotometer (Hitachi, Ltd, Tokyo, Japan) to measure the absorbance of all samples at 340 nm, as previously reported (Wang et al. 2016).²⁰

HPLC-ESI-QTOF-MS. Ultra-performance liquid chromatography (LC 30DA), equipped with a column provided by Waters (XBridge Protein BEH 125 Å SEC, 3.5 μ m, 7.8 $\times$ 300 mm), was used for separation to determine the MW of β -Lg, β -Lg-G, and β -Lg-120-G. Solvents A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. A flow rate of 0.3 mL/min was used to elute the sample, and the gradient conditions were set as follows: 0–1 min, 2% B; 1–7 min, 2%–90% B; 7–10.5 min, 90% B; 10.5–10.6 min, 90%–2% B; and 10.6–12 min, 2% B (isocratic elution). Triple TOF 4600 was used for mass spectrometry analysis, and ESI-QTOF-MS analysis was performed using a time-of-flight (TOF) mass detector under the following conditions: analysis time, 12 min; detection method, positive ion mode; precursor ion scan range, and 105–2500 *m/z*. PeakView 2.2 (ABI) software was used to process the MS data.

In Vitro Gastric and Duodenal Digestion. According to the method of Vassilopoulou et al.,²¹ the digestion of β -Lg, β -Lg-G, and β -Lg-120-G was performed. After 60 min of gastric digestion (GD) and 120 min of duodenal digestion (DD), samples were taken out and named as β -Lg-DD, β -Lg-G-DD, and β -Lg-120-G-DD.

Tricine-SDS-PAGE. Tricine-SDS-PAGE analysis was performed according to a previous description.²²

HPLC Determination of MW Distribution. The MW of hydrolysate was analyzed with an Agilent 1260 HPLC infinity system (Agilent, Palo Alto, CA).²³ Five standards (Cytochrome C, aprotinin, bacitracin, oxidized glutathione, and L-hydroxyproline) with different MWs were prepared, and the MW calibration curve on the basis of the average elution time was obtained.

Intrinsic Fluorescence Emission Spectroscopy. According to the previous report,²⁴ the Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Ltd, Tokyo, Japan) can be used to determine the intrinsic emission fluorescence intensity of the sample at a concentration of 1 mg/mL.

IgG/IgE Binding Ability. The IgG/IgE-binding ability was evaluated by the binding ability of samples to the human serum pool and polyclonal anti-bovine β -Lg antibody, which was determined by ELISA described by Liu et al. (2018).²⁵

Basophil Histamine Release Test. Based on the method by Appel et al. (2018),²⁶ the KU812 cells were seeded (5×10^5 cells/well) in 24-well plates using the RPMI-1640 medium, with 1×10^5 U/L penicillin/streptomycin and 20% fetal bovine serum, and incubated for 24 h at 37 °C in a humidified 5% CO₂ incubator. Subsequently, 10 μ L of human serum pool was added to passively activate the cells for 24 h and then stimulated with samples (50 μ g/well) for 4 h. The cells treated with PBS served as the negative control. The method used by Kuehn et al. (2010)²⁷ was used to determine the release of β -hex, whereas the release of histamine and IL-6 was evaluated using an ELISA kit.²⁸

In Vitro Fermentation of the Digested Juice in Human Feces. Fecal samples from three healthy people (one male and two female healthy donors without any known metabolic or GI disorders; 2–3 years old) were selected as research materials. The three children had not used probiotics, prebiotics, and antibiotics within 3 months. Small-scale in vitro fermentation systems were used for the incubation of 10 mg of β -Lg-DD, β -Lg-G-DD, and β -Lg-120-G-DD at 37 °C. All operations were completed in an anaerobic incubator.¹⁴

Statistical Analysis. All experimental data were analyzed by one-way ANOVA using SPSS for Windows version 22 (SPSS Inc., Chicago, IL). Values were expressed as mean $\pm$ SD.

RESULTS AND DISCUSSION

Degree of Glycation and MW of Glycated Proteins.

The results of OPA determination of free amino groups in samples are shown in Table S1 (Supporting Information file 1). Compared with β -Lg and β -Lg-G, the free amino groups of β -Lg-120-G showed a significant decrease, which indicated that the amino group of β -Lg reacted with the carbonyl group of galactose (Gal). Ultrasonic pretreatment promoted the reduction of free amino groups of β -Lg, and the degree of glycation of β -Lg and Gal increased.

The MWs of β -Lg, β -Lg-G, and β -Lg-120-G were determined using QTOF-MS. As shown in Figure S1 (Supporting Information file 1), the MWs of β -Lg-G and β -Lg-120-G were 19661.3 and 19985.2 Da, respectively, which were higher than the MW of β -Lg (18364.0 Da). The results showed that glycation reaction occurred between β -Lg and Gal, resulting in a large-molecular-weight glycoprotein complex, and ultrasonic pretreatment promoted more Gal molecules to combine with β -Lg, thereby producing a product with a large MW. Ultrasonic pretreatment changed the structure of β -Lg, thereby promoting the degree of glycation with Gal, which may change its functional properties to a certain extent. This result was consistent with the results of the degree of glycation (Table S1).

Tricine-SDS-PAGE. Tricine-SDS-PAGE was used to obtain the electrophoretic profiles of each sample, and the results are shown in Figure 1. After protein staining, high-molecular-mass bands were observed near the boundary between the stacking and separating gels, indicating that β -Lg had dimers, and dimers occurred after glycation reaction. β -Lg-G and β -Lg-120-G were above 18.4 kDa, and they migrated slower than β -Lg. The presence of these bands indicated the formation of covalent linkages between β -Lg and Gal. In addition, no evident band was observed in β -Lg-DD. Trypsin hydrolyzed β -Lg into small peptides, and ultralow-molecular-weight peptides (approximately 3 kDa) were easily leached from the gel, causing the peptide bands to disappear. These peptide molecules did not exhibit peptide aggregation, indicating that

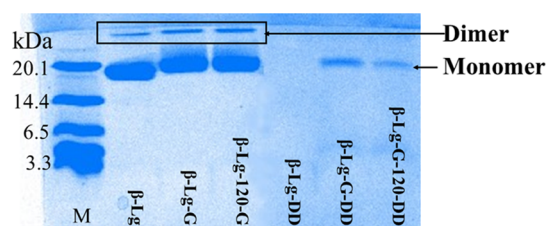


Figure 1. Effect of digested UCG-modified β -Lg on the molecular weight distribution. The molecular weight of the marker (represented by M) ranges from 3.3 to 20.1 kDa.

trypsin may prevent the aggregation of peptide molecules after β -Lg digestion to a certain extent. However, in the electrophoretic profiles of β -Lg-G-DD and β -Lg-120-G-DD, a broad

high-molecular-mass band was observed near the boundary between the stacking and separating gels, and one band was also observed between 3.3 and 6.5 kDa. This result could be due to β -Lg glycation, which hindered proteolysis, and steric hindrance caused by the covalent binding of Gal to the protein backbone limited the accessibility of the enzyme.

HPLC Profiles of the β -Lg Proteolytic Products. The chromatograms of each sample were analyzed by HPLC (Figure 2). The MW standard curve equation prepared by five standards was $\log(\text{MW}) = -0.1251t + 6.1704$ ($R^2 = 0.987$). The peptide ranges of different MWs (18.3–5 kDa, 5–3 kDa, 3–1 kDa, and <1 kDa) corresponded to the chromatograms of four different regions, namely, F1, F2, F3, and F4, respectively. The four regions were quantified relative to all peaks (Tot, Table S2 and Supporting Information file 1). The MWs of β -

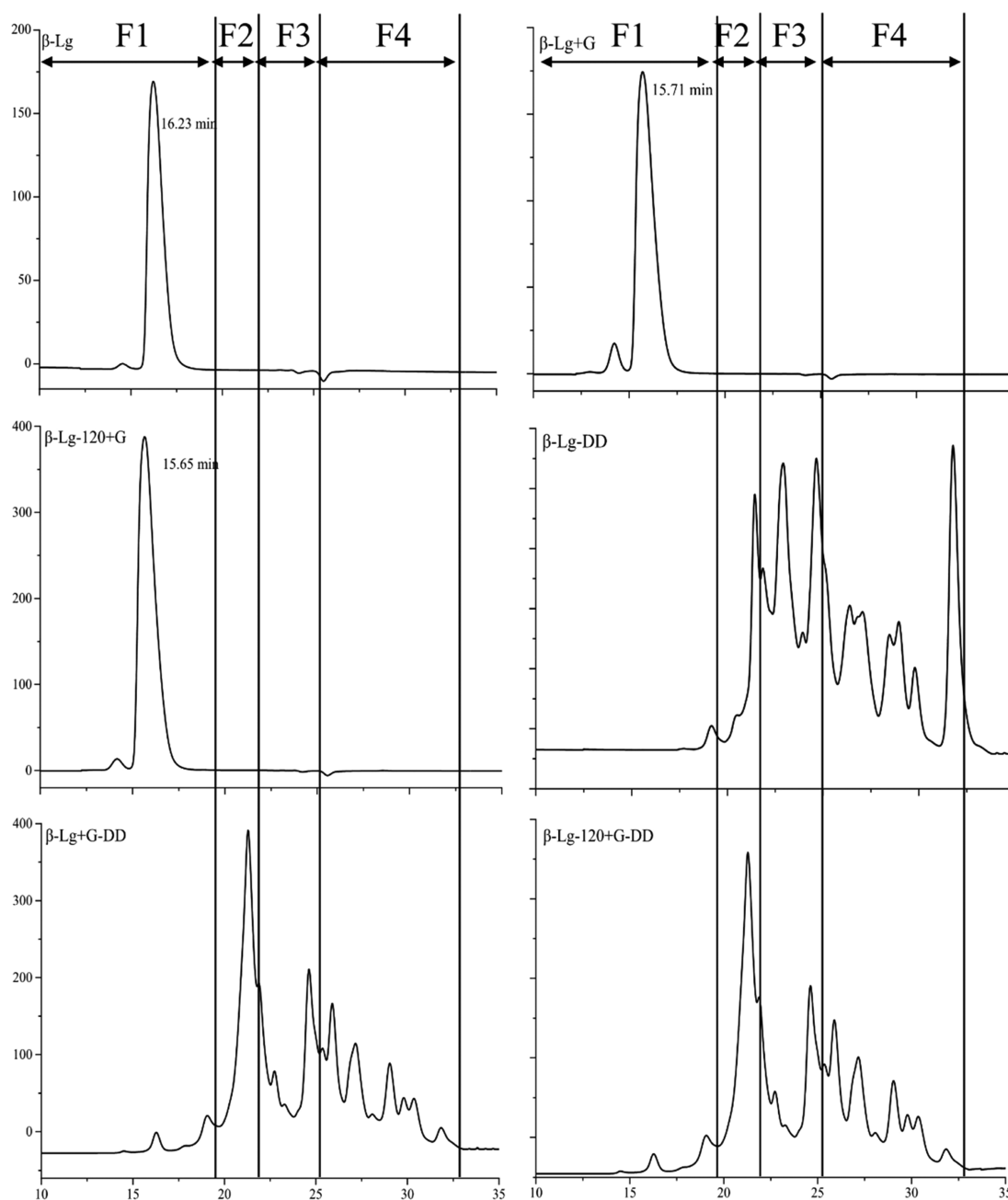


Figure 2. Effect of digested UCG-modified β -Lg on the HPLC chromatography.

Lg, β -Lg-G, and β -Lg-120-G were determined by HPLC. The peak time of β -Lg-G and β -Lg-120-G shifted to 15.71 and 15.65 min, respectively, which was lower than that of β -Lg at 16.23 min. Therefore, the MW of β -Lg-G and β -Lg-120-G was higher than that of β -Lg. This result was consistent with the SDS-PAGE results (Figure 1). After digestion through the GI tract, β -Lg-DD showed a high level of degradation, with a high ratio of fraction with a MW of 3–1 kDa (41.9%) and <1 kDa (46.1%). Many small molecular peptides were produced after the digestion of β -Lg in the GI tract. Compared with β -Lg-DD, the degradation levels of β -Lg-G-DD and β -Lg-120-G-DD were decreased, implying that glycation reaction can gradually reduce the fraction in the subsequent retention time, such as F3/F4, whereas F1/F2 increased. This result may be due to the glycation of β -Lg arginine, lysine, and Gal carbonyl, which were the site of trypsin hydrolysis, thereby reducing the enzymatic hydrolysis efficacy.²⁹ Compared with β -Lg-G-DD, the degradation levels of β -Lg-120-G-DD decreased. β -Lg-120-G-DD had a high peptide ratio of 18.3–5 kDa (3.3%) and 5–3 kDa (39.4%). However, the percentage of 3–1 kDa (26.6%) and <1 kDa (30.7%) peptide fractions was low. This result also indicated that the degree of glycation was high and the digestibility was poor. Moreover, this result was consistent with the results presented in Table S1; that is, the degree of glycation of β -Lg-120-G was higher than that of β -Lg-G.

Intrinsic Fluorescence Emission Spectroscopy. Figure 3 shows the intrinsic fluorescence spectra of each sample. As

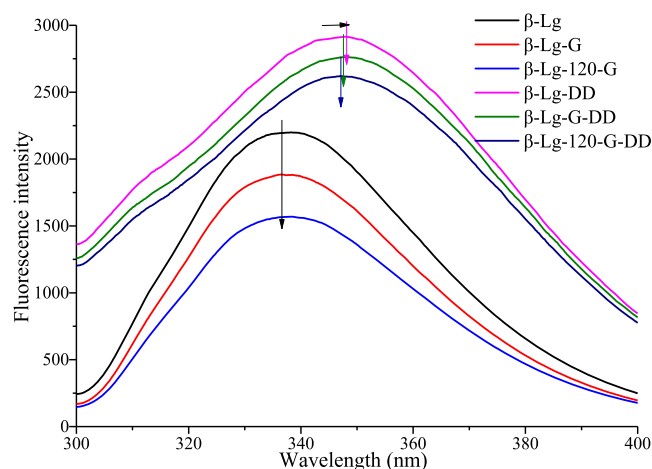


Figure 3. Effect of digested UCG-modified β -Lg on the intrinsic fluorescence emission spectra.

shown in Figure 3, when the excitation wavelength was 280 nm, β -Lg had a maximum relative fluorescence intensity of 2200 at 338.2 nm. The intrinsic fluorescence intensity of β -Lg-G and β -Lg-120-G decreased to 1881 and 1569, respectively, indicating that the glycation of β -Lg will affect its conformational structure. This result might be due to the shielding effect caused by the changes in the structure around the Trp residues caused by Gal. Moreover, the digested β -Lg showed an increase in relative intrinsic fluorescence intensity. The above-mentioned results were consistent with the expected results. In general, enzymatic hydrolysis would lead to the exposure of buried aromatic groups in the solvent, resulting in high fluorescence intensity.³⁰ λ_{max} was related to the microenvironment of Trp residues. If λ_{max} was >330 nm, then Trp residues were exposed to the polar environment. If λ_{max} was <330 nm,

then Trp residues were embedded in the nonpolar environment, which indicated solvent exposure.³¹ λ_{max} of β -Lg, β -Lg-G, β -Lg-120-G, β -Lg-DD, β -Lg-G-DD, and β -Lg-120-G-DD was greater than 330 nm, which indicated that Trp residues were exposed to the external polar environment of β -Lg. However, digestion could induce the red-shift of λ_{max} . In β -Lg-DD, β -Lg-G-DD, and β -Lg-120-G-DD, λ_{max} increased from 338.2 to 348.2, 347.4, and 347.0 nm, respectively. This result could indicate that digestion may have an effect on the structure of β -Lg, which was manifested by the expansion of Trp residues around β -Lg. The degree of increase of λ_{max} was decreased by glycation reaction, and λ_{max} of β -Lg-G-DD was larger than that of β -Lg-120-G-DD, which may be due to the low digestibility of glycosylated samples. These results were consistent with those shown in Table S2. Therefore, digestion increased the intrinsic fluorescence intensity of β -Lg, and glycation reduced the degree of digestion, thereby reducing the intensity and red-shift of intrinsic fluorescence.

IgG/IgE Binding Ability of β -Lg before and after In Vitro GI Digestion. As shown in Figure 4, ELISA was used to measure the IgE/IgG-binding ability of each sample. The IC_{50} value of β -Lg-G was higher than that of β -Lg, indicating that

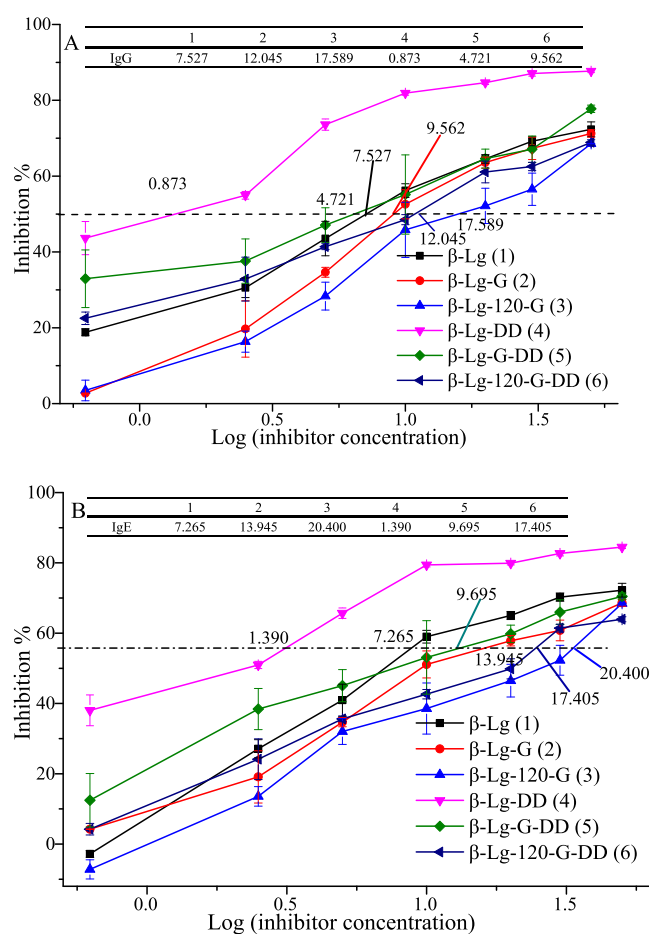


Figure 4. Changes of digested UCG-modified β -Lg on the IgG (A) and IgE (B) binding ability. IC_{50} is the concentration of inhibitors that causes a 50% inhibition of antibody binding ($\mu\text{g/mL}$). Pooled rabbit anti- β -Lg-sera or human serum pool (50 μL per well) was incubated separately with 0.625, 2.5, 5.0, 10.0, 20.0, 30.0, and 50.0 $\mu\text{g mL}^{-1}$ of β -Lg and β -Lg proteolytic products as inhibitors (50 μL per well).

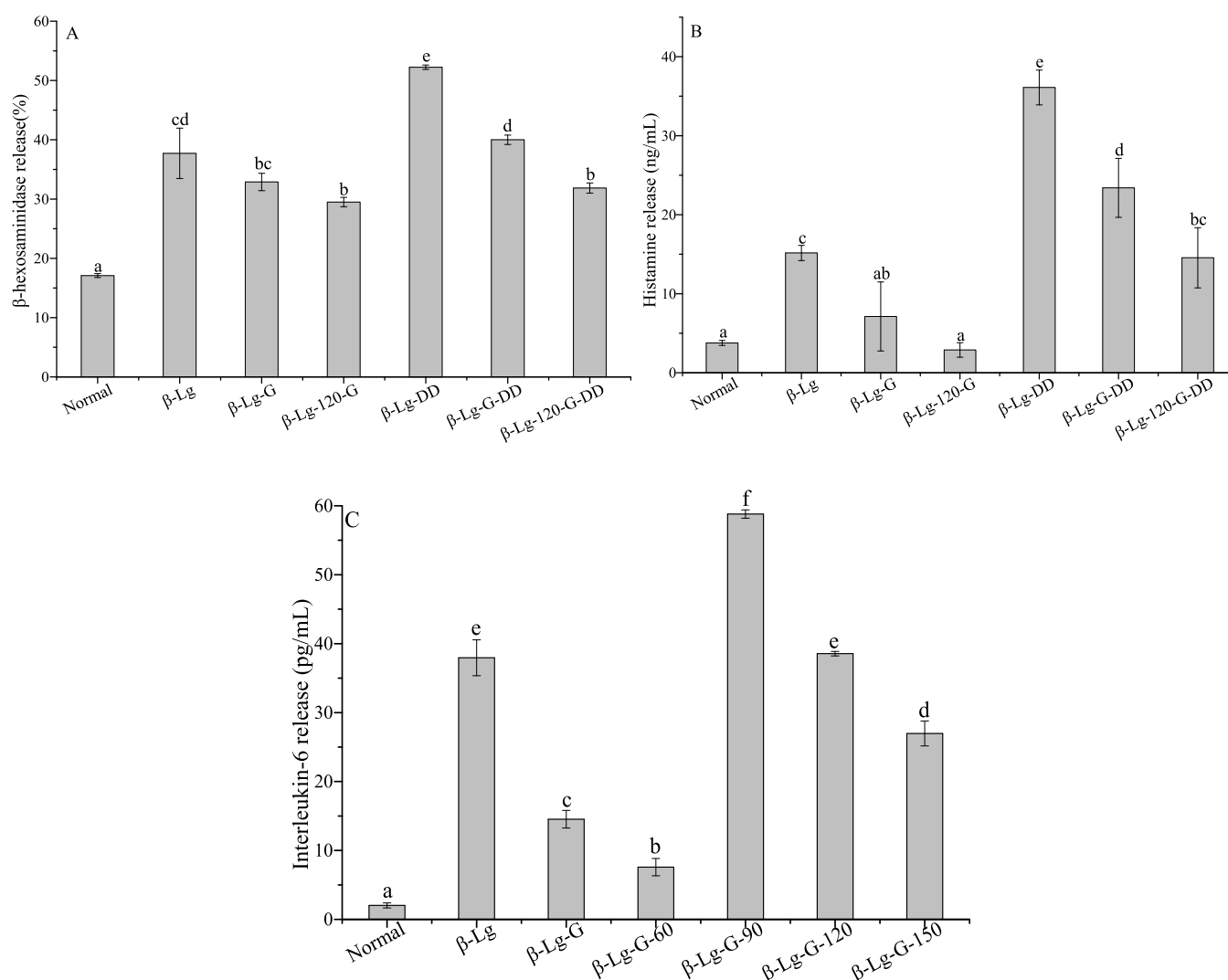


Figure 5. Effects of digested UCG-modified β -Lg in IgE-mediated allergic responses in KU812 cells sensitized with sera from patients allergic to milk. Different letters (a–e) in the bars mean significantly different ($p < 0.05$).

glycation affected the conformational structure of β -Lg and decreased the binding ability of IgG/IgE. This result was consistent with our previous studies.¹⁰ The IgE/IgG-binding ability of β -Lg-DD, β -Lg-G-DD, and β -Lg-120-G-DD was enhanced. The IC_{50} values of β -Lg-DD, β -Lg-G-DD, and β -Lg-120-G-DD shifted to 0.873, 4.721, and 9.562 μ g/mL, respectively, which were higher than the IgG-binding abilities of β -Lg (7.527 μ g/mL), β -Lg-G (12.045 μ g/mL), and β -Lg-120-G (17.589 μ g/mL), indicating that β -Lg had higher allergenicity after GI digestion (Figure 4A). A similar trend was observed in Figure 4B. This result may be due to the production of a large number of peptides, some of which had a complete structure and epitope, thereby exposing more allergic sites and antigen epitopes.^{32,33} Compared with β -Lg-DD, the IC_{50} values of β -Lg-G-DD and β -Lg-120-G-DD were higher, which may indicate that the glycation sites were consistent with the trypsin hydrolysis sites, which impaired the hydrolysis of β -Lg, thereby reducing the allergenic potential of hydrolysates. In addition, the complete protein was detected on the GI digestion products of glycosylated β -Lg (Figure 1), and some peptides with complete immunogenicity produced by hydrolysis may be reduced because of poor hydrolysis efficiency. In addition, galactosylation had a masking effect on β -Lg

allergenic epitopes, which could counteract the negative effect of digestion on the allergy. The IC_{50} value of β -Lg-G-DD was lower than that of β -Lg-120-G-DD, which may be due to the increase in the glycation degree caused by ultrasonic pretreatment; the higher the degree of glycation, the lower the degree of digestion. Meanwhile, the masking effect of β -Lg allergenic epitopes increased with the increase in the glycation degree.

Impact of β -Lg after In Vitro GI Digestion on IgE-Sensitized KU812 Cells. Samples were used to verify the effect of degranulation on KU812 cells and understand the allergenicity of the digested UCG-modified β -Lg at the cellular level (Figure 5). We found that glycation reduced the degranulation of KU812 cells, and this result was consistent with our previous study using rat basophil RBL-2H3 cells.¹⁰ Similarly, the degranulation of the cells before and after digestion was significantly different (Figure 5). After digestion, cell degranulation significantly increased compared with the predigested sample, which was reflected in the increase of β -hex release (Figure 5A), indicating that digestion affected the release of the mediators linked to allergic symptoms. Similar trends are found in Figure 5B,C. Digestion produced a large number of peptides, which exposed the allergenic epitopes

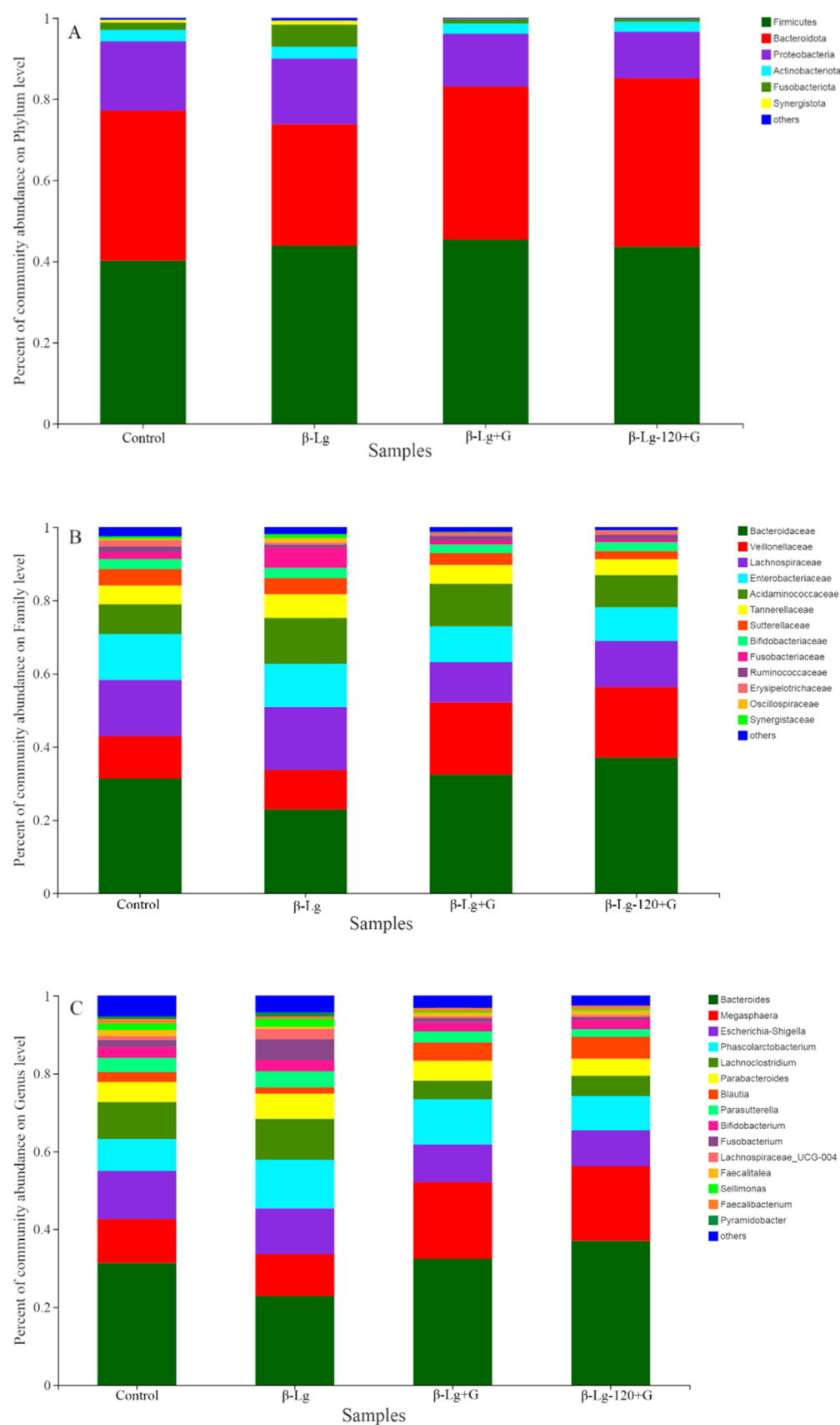


Figure 6. Effect of digested UCG-modified β -Lg on the relative abundance columnar of microbial on the phylum-level (A), family level (B), and genus level (C).

originally buried in β -Lg, which made binding with IgE on the surface of sensitized mast cells or basophils easier, resulting in the increase of mediator release.³⁴ This result was consistent with the result shown in Figure 4. Compared with β -Lg-DD, the release rates of β -Lg-G-DD and β -Lg-120-G-DD were significantly reduced, which indicated the poor digestibility of β -Lg-G-DD and β -Lg-120-G-DD, resulting in less exposure of allergenicity loci and antigen epitopes. In particular, the degree of degranulation of KU812 cells exposed to β -Lg-120-G-DD decreased compared with that exposed to β -Lg-G-DD. Moreover, the digested UCG-modified β -Lg may have a lower allergenic potential than glycation, and these changes can be confirmed by the reduced IgE/IgG-binding ability (Figure 4) and decreased cell degranulation at the cellular level (Figure 5).

Microbial Community Structure Analysis. After GI digestion, β -Lg is covalently linked with Gal to produce high-molecular-weight glycopeptide fragments, which may reach the distal colon region and be utilized by intestinal microbiota. Based on the species level of bacterial phylogeny, results at the threshold of nucleotide sequence identity over 97% operational classification units were obtained. The total microbial composition in fermented juice was compared with the phylum, family, and genus levels (Figure 6). The results showed that the microbial community was dominated by Firmicutes and Bacteroidota, followed by Proteobacteria, Actinobacteriota, Fusobacteriota, Synergistota, and others. Compared with the control group, the β -Lg group showed a decrease in the relative abundance of Bacteroidota and an increase in the Fusobacteriota. β -Lg-G and β -Lg-120-G showed an opposite trend, that is, the relative abundance of Bacteroidota improved significantly, whereas Fusobacteriota decreased, which had similar colony composition and relative abundance to the control group. Low Bacteroidota/Firmicutes ratios were observed in the β -Lg group compared with the control group, including β -Lg-G and β -Lg-120-G (Table S3). Therefore, β -Lg greatly affected the stability of the microbial community structure, which can be alleviated by glycation of β -Lg. Previous studies have shown that Bacteroidota in allergic infants is significantly reduced.¹⁹ As shown in Figure 6, a total of 13 families were identified in fermented juice at the family level. Bacteroidaceae, Veillonellaceae, Lachnospiraceae, Enterobacteriaceae, Acidaminococcaceae, Tannerellaceae, Sutterellaceae, Bifidobacteriaceae, Fusobacteriaceae, and Ruminococcaceae were the dominant families. Under the ratio of Enterobacteriaceae to Bacteroidaceae (E/B ratio), the β -Lg group was higher than the control group, whereas the relative abundance of Ruminococcaceae was lower. The E/B ratio of the β -Lg-G and β -Lg-120-G groups decreased, and Ruminococcaceae increased, reversing the impact of β -Lg on the community structure (Table S3). Azad et al.¹² found that the increase of E/B ratio and low abundance of Ruminococcaceae were associated with food allergy in infants with low intestinal microbiota abundance, and each quartile increase in the E/B ratio doubled the risk of allergenicity. As shown in Figure 6, the fermented juice contained 15 genera, among which *Bacteroides*, *Megasphaera*, *Escherichia*–*Shigella*, *Phascolarctobacterium*, *Lachnoclostridium*, *Parabacteroides*, *Blautia*, *Parasutterella*, *Bifidobacterium*, *Fusobacterium*, *Lachnospiraceae_UCG-004*, *Faecalitalea*, *Sellimonas*, *Faecalibacterium*, and *Pyramidobacter*. Notably, *Bacteroides* and *Faecalibacterium* in the β -Lg group decreased significantly. In the β -Lg-G and β -Lg-120-G groups, the proportion of *Bacteroides* and *Faecalibacterium* was

relatively high. As recently shown, the gut microbiota rich in *Bacteroides* played a positive role in preventing milk allergy.³⁵ *Faecalibacterium* may be beneficial in achieving tolerance to food allergens or alleviating symptoms of food allergy. Therefore, UCG modification and subsequent digestion could alter the structures of the microbial community and may affect allergic reactions, but the correlation between the two must be further determined.

Analysis of Reduction in Allergenicity and Altered Human Intestinal Microbiota. After food allergens were ingested, the allergenic proteins were hydrolyzed in the GI tract. The peptide with allergenic epitopes will interact with immune cells and cause allergic reaction after reaching the GI mucosa.³⁶ On the contrary, it will change the intestinal microenvironment and microbial structure, thereby affecting food allergy.³⁷ Our previous research results showed that UCG modification can reduce the allergenicity of β -Lg by changing linear or conformational epitopes of β -Lg, but the allergenicity changes and effect on the intestinal microbiota structure of UCG-modified β -Lg after digestion were not clear.

After digestion of allergenic proteins, many peptides with a complete structure and epitope were produced, resulting in great exposure of allergenic sites and antigen epitopes, which increased allergenicity.³¹ In this study, after UCG modification, the digestibility of β -Lg decreased, resulting in the production of intact proteins with a spatial structure or peptides with higher MWs but not completely exposed to the allergenic sites and antigen epitopes of β -Lg (Figures 1 and 2), which made the binding of digested UCG-modified β -Lg to IgG and IgE antibodies difficult. In addition, Gal on these glycopeptide fragments had a masking effect on the antigen epitopes of β -Lg, resulting in a high IC₅₀ value (Figure 4). As shown in Figure 5, the release of β -hex, histamine, and IL-6 in KU812 cells exposed to digested UCG-modified β -Lg decreased, which indicated that the allergenicity of the digested UCG-modified β -Lg peptide was related to its peptide MW. A recent study by Prodic et al.³⁸ reported that the allergenic potential of the peptide of the food allergen is related to its peptide MW. Meanwhile, the structure of UCG-modified β -Lg changed significantly after digestion, which was reflected in the increase of intrinsic fluorescence intensity and the red-shift of λ_{max} (Figure 3). Therefore, the changes in structure and MW were related to the allergenic potential of digested β -Lg by UCG modification.

Studies have shown that the occurrence of allergenicity is closely related to the structure of intestinal microorganisms.^{39,40} Given the antiprotease activity of β -Lg in the intestinal digestion and absorption, its peptide reached the intestinal cavity, which was fermented and utilized by intestinal microorganisms, resulting in the change in intestinal microorganisms. As shown in Figure 6, in particular, compared with the control group, the relative abundance of *Bacteroides* in the β -Lg group was significantly decreased, whereas that of Fusobacteriota was significantly increased. Similarly, the E/B ratio in the β -Lg group was significantly increased, whereas Ruminococcaceae was significantly decreased. Significant changes were observed in intestinal microbiota, and these specific genera/species were associated with food allergy.⁴¹ Compared with β -Lg, the glycation site of β -Lg-120-G was the same as that of trypsin, which resulted in low digestibility of β -Lg-120-G. These glycopeptides may reach the distal colon region and be used by the intestinal microbiota, resulting in changes in the structure of intestinal flora and varying degrees

of influence on the abundance of communities at the phylum, family, and genus levels, thereby increasing the relative abundance of Fusobacteriota and the E/B ratio, whereas Ruminococcaceae, *Bacteroides*, and *Faecalibacterium* decreased significantly (Figure 6). The results showed that UCG modification and subsequent digestion altered the structure of β -Lg, reduced the allergenicity of β -Lg, and affected the composition of the human intestinal microbial community. These results could provide a theoretical basis for studying the relationship between intestinal dysbiosis and cow's milk allergy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c03501>.

Free amino level and DG of β -Lg, β -Lg-G, and β -Lg-120-G; relative area of the peptide fraction obtained from the β -Lg digestion product; ratio of Bacteroidota/Firmicutes or Enterobacteriaceae to Bacteroidaceae; molecular weight of β -Lg, β -Lg-G, and β -Lg-120-G; and quantitative detection of common allergic sIgE in normal human serum (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Jun Liu – National R&D Center for Freshwater Fish Processing, and Engineering Research Center of Freshwater Fish High-value Utilization of Jiangxi Province, Jiangxi Normal University, Nanchang, Jiangxi 330022, China; orcid.org/0000-0002-8570-5741; Phone: +86-791-8812-7901; Email: lj_anyi@126.com; Fax: +86-791-8812-7901

Zong-cai Tu – National R&D Center for Freshwater Fish Processing, and Engineering Research Center of Freshwater Fish High-value Utilization of Jiangxi Province, Jiangxi Normal University, Nanchang, Jiangxi 330022, China; State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi 330047, China; orcid.org/0000-0001-8877-4681; Phone: +86-791-8812-1868; Email: tuzc_mail@aliyun.com; Fax: +86-791-8830-5938

Authors

Yan-hong Shao – National R&D Center for Freshwater Fish Processing, and Engineering Research Center of Freshwater Fish High-value Utilization of Jiangxi Province, Jiangxi Normal University, Nanchang, Jiangxi 330022, China

Yao Zhang – National R&D Center for Freshwater Fish Processing, and Engineering Research Center of Freshwater Fish High-value Utilization of Jiangxi Province, Jiangxi Normal University, Nanchang, Jiangxi 330022, China

Lu Zhang – National R&D Center for Freshwater Fish Processing, and Engineering Research Center of Freshwater Fish High-value Utilization of Jiangxi Province, Jiangxi Normal University, Nanchang, Jiangxi 330022, China

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.jafc.1c03501>

Funding

This work was funded by the China National Natural Science Foundation (21878135), Science Foundation for Young Scientists of Jiangxi Province (20202BABL215027), and

earmarked fund for China Agriculture Research System (CARS-45).

Notes

The authors declare no competing financial interest.

■ ABBREVIATION

β -Lg, β -lactoglobulin; UCG, ultrasonic combined with glycation; GI, gastrointestinal; GD, gastric digestion; DD, duodenal digestion; IgE/G, immunoglobulin E/G; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HPLC-ESI-QTOF-MS, high-performance liquid chromatography-electrospray ionization-quadrupole time of flight-mass spectrometry; Tricine-SDS-PAGE, tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MW, molecular weight

■ REFERENCES

- (1) Stanic-Vucinic, D.; Cirkovic-Velickovic, T. The modifications of bovine β -lactoglobulin: Effects on its structural and functional properties. *J. Serb. Chem. Soc.* **2013**, *78*, 445–461.
- (2) Yang, W.; Tu, Z.; Wang, H.; Zhang, L.; Xu, S.; Niu, C.; Yao, H.; Kaltashov, I. A. Mechanism of Reduction in IgG and IgE Binding of β -Lactoglobulin Induced by Ultrasound Pretreatment Combined with Dry-State Glycation: A Study Using Conventional Spectrometry and High-Resolution Mass Spectrometry. *J. Agric. Food Chem.* **2017**, *65*, 8018–8027.
- (3) Bu, G.; Luo, Y.; Zheng, Z.; Zheng, H. Effect of heat treatment on the antigenicity of bovine α -lactalbumin and β -lactoglobulin in whey protein isolate. *Food Agric. Immunol.* **2009**, *20*, 195–206.
- (4) Perusko, M.; van Roest, M.; Stanic-Vucinic, D.; Simons, P. J.; Velickovic, T. C.; Smit, V. T.; Smit, J. Glycation of the Major Milk Allergen β -Lactoglobulin Changes Its Allergenicity by Alterations in Cellular Uptake and Degradation. *Mol. Nutr. Food Res.* **2018**, *62*, 1800341.
- (5) El Mecherfi, K. E.; Saidi, D.; Kheroua, O.; Boudraa, G.; Touhami, M.; Rouaud, O.; Curet, S.; Choiset, Y.; Rabesona, H.; Chobert, J.-M.; Haertlé, T. Combined microwave and enzymatic treatments for β -lactoglobulin and bovine whey proteins and their effect on the IgE immunoreactivity. *Eur. Food Res. Technol.* **2011**, *233*, 859.
- (6) Bu, G.; Luo, Y.; Zhang, Y.; Chen, F. Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins. *J. Sci. Food Agric.* **2010**, *90*, 2015–2020.
- (7) Zhong, J.; Tu, Y.; Liu, W.; Xu, Y.; Liu, C.; Dun, R. Antigenicity and conformational changes of β -lactoglobulin by dynamic high pressure microfluidization combining with glycation treatment. *J. Dairy Sci.* **2014**, *97*, 4695–4702.
- (8) Yang, W.; Tu, Z.; Wang, H.; Zhang, L.; Kaltashov, I. A.; Zhao, Y.; Niu, C.; Yao, H.; Ye, W. The mechanism of reduced IgG/IgE-binding of β -lactoglobulin by pulsed electric field pretreatment combined with glycation revealed by ECD/FTICR-MS. *Food Funct.* **2018**, *9*, 417–425.
- (9) Chen, Y.; Tu, Z.; Wang, H.; Zhang, Q.; Zhang, L.; Sha, X.; Huang, T.; Ma, D.; Pang, J.; Yang, P. The Reduction in the IgE-Binding Ability of β -Lactoglobulin by Dynamic High-Pressure Microfluidization Coupled with Glycation Treatment Revealed by High-Resolution Mass Spectrometry. *J. Agric. Food Chem.* **2017**, *65*, 6179–6187.
- (10) Shao, Y.-H.; Zhang, Y.; Zhu, M.-F.; Liu, J.; Tu, Z.-C. Glycation of β -lactoglobulin combined by sonication pretreatment reduce its allergenic potential. *Int. J. Biol. Macromol.* **2020**, *164*, 1527.
- (11) Corzo-Martínez, M.; Avila, M.; Moreno, F. J.; Requena, T.; Villamiel, M. Effect of milk protein glycation and gastrointestinal digestion on the growth of bifidobacteria and lactic acid bacteria. *Int. J. Food Microbiol.* **2012**, *153*, 420–427.
- (12) Azad, M. B.; Konya, T.; Guttman, D. S.; Field, C. J.; Sears, M. R.; HayGlass, K. T.; Mandhane, P. J.; Turvey, S. E.; Subbarao, P.;

Becker, A. B.; Scott, J. A.; Kozyrskyj, A. L. Infant gut microbiota and food sensitization: associations in the first year of life. *Clin. Exp. Allergy* **2015**, *45*, 632–643.

(13) Corzo-Martínez, M.; Hernández-Hernández, O.; Villamiel, M.; Rastall, R. A.; Moreno, F. J.; Moreno, F. J. In vitro bifidogenic effect of Maillard-type milk protein-galactose conjugates on the human intestinal microbiota. *Int. Dairy J.* **2013**, *31*, 127–131.

(14) Hernández-Hernández, O.; Sanz, M. L.; Kolida, S.; Rastall, R. A.; Moreno, F. J. In vitro fermentation by human gut bacteria of proteolytically digested caseinomacropeptide nonenzymatically glycosylated with prebiotic carbohydrates. *J. Agric. Food Chem.* **2011**, *59*, 11949–11955.

(15) Ríos-Covián, D.; Ruas-Madiedo, P.; Margolles, A.; Gueimonde, M.; de los Reyes-Gavilán, C. G.; Salazar, N. Intestinal short chain fatty acids and their link with diet and human health. *Front. Microbiol.* **2016**, *7*, 185.

(16) Hooper, L. V.; Midtvedt, T.; Gordon, J. I. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr.* **2002**, *22*, 283–307.

(17) Townsend, G. E.; Han, W.; Schwalm, N. D.; Raghavan, V.; Barry, N. A.; Goodman, A. L.; Groisman, E. A. *Dietary Sugar Silences a Colonization Factor in a Mammalian Gut Symbiont*; Proceedings of the National Academy of Sciences, 2018.

(18) Abdel-Gadir, A.; Stephen-Victor, E.; Gerber, G. K.; Rivas, M. N.; Wang, S.; Harb, H.; Wang, L.; Li, N.; Crestani, E.; Spielman, S.; Secor, W.; Biehl, H.; DiBenedetto, N.; Dong, X.; Umetsu, D. T.; Bry, L.; Rachid, R.; Chatila, T. A. Microbiota therapy acts via a regulatory T cell MyD88/ROR γ t pathway to suppress food allergy. *Nat. Med.* **2019**, *25*, 1164.

(19) Ling, Z.; Li, Z.; Liu, X.; Cheng, Y.; Luo, Y.; Tong, X.; Yuan, L.; Wang, Y.; Sun, J.; Li, L.; Xiang, C. Altered Fecal Microbiota Composition Associated with Food Allergy in Infants. *Appl. Environ. Microbiol.* **2014**, *80*, 2546–2554.

(20) Wang, Z.; Han, F.; Sui, X.; Qi, B.; Yang, Y.; Zhang, H.; Wang, R.; Li, Y.; Jiang, L. Effect of ultrasound treatment on the wet heating Maillard reaction between mung bean [*Vigna radiata*(L.)] protein isolates and glucose and on structural and physico-chemical properties of conjugates. *J. Sci. Food Agric.* **2016**, *96*, 1532–1540.

(21) Vassilopoulou, E.; Rigby, N.; Moreno, F. J.; Zuidmeer, L.; Akkerdaas, J.; Tassios, I.; Papadopoulos, N. G.; Saxoni-Papageorgiou, P.; van Ree, R.; Mills, C. Effect of *in vitro* gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. *J. Allergy Clin. Immunol.* **2006**, *118*, 473–480.

(22) Haider, S. R.; Reid, H. J.; Sharp, B. L. Tricine-SDS-PAGE. *Electrophoretic Separation of Proteins*; Springer, 2019, pp 151–160.

(23) Irvine, G. B. High-performance size-exclusion chromatography of peptides. *J. Biochem. Biophys. Methods* **2003**, *56*, 233–242.

(24) Zhao, C.-B.; Zhou, L.-Y.; Liu, J.-Y.; Zhang, Y.; Chen, Y.; Wu, F. Effect of ultrasonic pretreatment on physicochemical characteristics and rheological properties of soy protein/sugar Maillard reaction products. *J. Food Sci. Technol.* **2016**, *53*, 2342–2351.

(25) Liu, J.; Tu, Z.-c.; Liu, G.-x.; Niu, C.-d.; Yao, H.-l.; Wang, H.; Sha, X.-m.; Shao, Y.-h.; Kaltashov, I. A. Ultrasonic Pretreatment Combined with Dry-State Glycation Reduced the Immunoglobulin E/Immunoglobulin G-Binding Ability of α -Lactalbumin Revealed by High-Resolution Mass Spectrometry. *J. Agric. Food Chem.* **2018**, *66*, 5691–5698.

(26) Appel, K.; Munoz, E.; Navarrete, C.; Cruz-Teno, C.; Biller, A.; Thiemann, E. Immunomodulatory and Inhibitory Effect of Immulina, and Immunologes in the Ig-E Mediated Activation of RBL-2H3 Cells. A New Role in Allergic Inflammatory Responses. *Plants* **2018**, *7*, 13.

(27) Kuehn, H. S.; Rådinger, M.; Brown, J. M.; Ali, K.; Vanhaesebroeck, B.; Beaven, M. A.; Metcalfe, D. D.; Gilfillan, A. M. Btk-dependent Rac activation and actin rearrangement following Fc ϵ RI aggregation promotes enhanced chemotactic responses of mast cells. *J. Cell Sci.* **2010**, *123*, 2576–2585.

(28) Lv, L.; Lin, H.; Li, Z.; Nayak, B.; Ahmed, I.; Tian, S.; Chen, G.; Lin, H.; Zhao, J. Structural changes of 2,2'-azobis(2-amidinopropane)

dihydrochloride (AAPH) treated shrimp tropomyosin decrease allergenicity. *Food Chem.* **2019**, *274*, 547–557.

(29) Zhong, B. Z.; Liang, Q. X.; Tu, Z. C.; Wang, H. Enzymolysis Reaction Kinetics and Liquid Chromatography High-Resolution Mass Spectrometry Analysis of Ovalbumin Glycated with Microwave Radiation. *J. Agric. Food Chem.* **2020**, *68*, 10596.

(30) Pain, R. H. Determining the fluorescence spectrum of a protein. *Ultrason. Sonochem.* **2005**, *43*, 227–236.

(31) Vivian, J. T.; Callis, P. R. Mechanisms of tryptophan fluorescence shifts in proteins. *Biophys. J.* **2001**, *80*, 2093–2109.

(32) Clément, G.; et al. Allergy to bovine β -lactoglobulin: specificity of human IgE to tryptic peptides. *Clin. Exp. Allergy* **2010**, *29*, 1055–1063.

(33) López-Expósito, I.; Chicón, R.; Belloque, J.; Recio, I.; Alonso, E.; López-Fandiño, R. Changes in the ovalbumin proteolysis profile by high pressure and its effect on IgG and IgE binding. *J. Agric. Food Chem.* **2008**, *56*, 11809–11816.

(34) Kaminogawa, S.; Hachimura, S.; Haruyo, N.-A.; Mamoru, T. Food allergens and mucosal immune systems with special reference to recognition of food allergens by gut-associated lymphoid tissue. *Allergol. Int.* **2010**, *48*, 15–23.

(35) Rodríguez, B.; Prioult, G.; Bibiloni, R.; Nicolis, I.; Mercenier, A.; Butel, M. J.; Waligora-Dupriet, A. J. Germ-free status and altered caecal subdominant microbiota are associated with a high susceptibility to cow's milk allergy in mice. *FEMS Microbiol. Ecol.* **2010**, *76*, 133–144.

(36) Huang, J.; Liu, C.; Wang, Y.; Wang, C.; Xie, M.; Qian, Y.; Fu, L. Application of *in vitro* and *in vivo* models in the study of food allergy. *Food Sci. Hum. Wellness* **2018**, *7*, 235–243.

(37) Gensollen, T.; Blumberg, R. S. Correlation between early-life regulation of the immune system by microbiota and allergy development. *J. Allergy Clin. Immunol.* **2017**, *139*, 1084–1091.

(38) Prodic, I.; Stanic-Vucinic, D.; Apostolovic, D.; Mihailovic, J.; Radibratovic, M.; Radosavljevic, J.; Burazer, L.; Milcic, M.; Smiljanic, K.; van Hage, M.; Cirkovic Velickovic, T. Influence of peanut matrix on stability of allergens in gastric-simulated digesta: 2S albumins are main contributors to the IgE reactivity of short digestion-resistant peptides. *Clin. Exp. Allergy* **2018**, *48*, 731–740.

(39) Savage, J. R.; Sordillo, J.; Sodergren, E.; Weinstock, G.; Gold, D. R.; Weiss, S. T.; Litonjua, A. A. A Prospective Microbiome-Wide Association Study of Childhood Food Sensitization and Allergy. *J. Allergy Clin. Immunol.* **2016**, *137*, AB96.

(40) Zhao, W.; Ho, H.-e.; Bunyavanich, S. The gut microbiome in food allergy. *Ann. Allergy, Asthma, Immunol.* **2019**, *122*, 276–282.

(41) Bunyavanich, S.; Shen, N.; Grishin, A.; Wood, R.; Burks, W.; Dawson, P.; Jones, S. M.; Leung, D. Y. M.; Sampson, H.; Sicherer, S.; Clemente, J. C. Early-life gut microbiome composition and milk allergy resolution. *J. Allergy Clin. Immunol.* **2016**, *138*, 1122–1130.