The reduction in the immunoglobulin G and immunoglobulin E binding capacity of β -lactoglobulin via spray-drying technology

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ABSTRACT

Bovine β -lactoglobulin (β -LG) is the major allergen in milk powder. The IgG/IgE binding capacity and structural characteristics of β -LG after spray drying in the presence or absence of α -lactose at 120 and 180°C were investigated by ELISA and mass spectrometry. At a drying temperature of 120°C, no change was found in the IgG/IgE binding capacity of β -LG and no change was observed in free amino group content, fluorescence intensity, or detectable glycation. At a drying temperature of 180°C, aggregation of β -LG occurred, leading to a decrease in the IgG/IgE binding capacity. When α -lactose was also present, 7 lysine side-chains in β -LG were modified by glycation and the IgG/IgE binding capacity was further decreased. Therefore, the glycation and structural changes in β -LG were responsible for the reduction in the IgG/IgE binding capacity during high temperature (180°C) spray drying.

Key words: β -lactoglobulin, immunoglobulin G and immunoglobulin E binding capacity, glycation, spray drying

INTRODUCTION

Milk contains abundant nutrients, including lipids, proteins, lactose, AA, vitamins, and minerals, and has long been regarded as an excellent food for infants and young children (Arpagaus et al., 2010). Half a liter of milk can provide a significant proportion of the required daily intake of nutrients (Haug et al., 2007). In addition, milk is often processed into yogurt, infant milk powder, milk powder for the elderly, and other products to satisfy the needs of different consumer groups, especially infants requiring milk powder formula instead of,

or in addition to, mother's milk. Nevertheless, cow milk is also a common cause of food allergy in early childhood. Bovine β -LG is recognized as a major allergen in cow milk, which can maintain high immunogenicity even after gastric digestion (Bonomi et al., 2013).

β -Lactoglobulin is the main component of whey protein and the major allergen causing IgE-mediated cow milk allergy (CMA; Yang et al., 2017, 2018). β -Lactoglobulin is a 162-AA globular protein with a molecular weight of 18.36 kDa. It contains 2 intramolecular disulfide bonds (Cys 66–Cys 160 and Cys 106–Cys 119), which stabilize the protein's secondary structure (Schuck et al., 2013; Rahaman et al., 2016). Its 3-dimensional structure seems to play an important role in maintaining linear and conformational epitopes, resulting in protein anaphylaxis (Bouaouina et al., 2006). Sélo et al. (1999) identified human IgE allergy epitopes, including Leu 1–Lys 8, Ala 25–Arg 40, Val 41–Lys 60, Tyr 102–Val 124, and Arg 149–Ile 162; of these, the most common epitopes are the last 3, which have been identified in more than 90% of CMA cases (O'Riordan et al., 2014).

Milk powder has a much longer shelf life and lower transportation costs than milk, so milk is usually processed into milk powder for sale and especially for export (Arpagaus et al., 2010; Singh and Dixit, 2014; Suzihaque et al., 2015). At present, milk powder is commonly produced through spray drying (Suzihaque et al., 2015; Carter et al., 2018). In the spray-drying process, the liquid material is atomized in a drying tower, then vaporized quickly with hot air to obtain a dried product. Physical shearing and chemical modification of milk components may occur during spray drying, but compared with other hot-air drying methods, the advantage of spray drying is that it is easy to control the drying conditions and adjust the product quality, especially for heat-sensitive products (Tanaka et al., 1988). Solutions or emulsions can be directly dried into powdered or granular products.

Reducing sugars, such as lactose, which is the only simple sugar in cow milk, may react with proteins

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Table 1. Information of cow milk allergy patients

No.	Sex	Age (yr)	Clinical symptoms	Other allergies	IgE (kU/L)
1	Female	38	Allergic rhinitis	Most foods	9.58
2	Male	28	Allergic rhinitis	Casein	91
3	Male	60	Received allergy shots	Fish, nut allergy	13.8
4	Female	64	Allergic rhinitis	Egg white	96.6
5	Female	47	Nose wheezing (breathing through the nose is not smooth)	Animal allergy	9.58
6	Female	62	Hives, wheezing	Most foods	29.8

during spray drying to form glycation products, consequently modifying their IgG/IgE binding capacity, thereby increasing or reducing the allergenicity of proteins. Research on the heat treatment of milk protein allergens in solution is of current interest (Tong et al., 2012), but to our knowledge, no reports are available on the effects of spray drying on the allergenicity of proteins.

The essence of glycation is the Maillard reaction, which forms covalent bonds between reducing sugars and α - or ϵ -amino groups in proteins. Glycation has been evaluated by traditional methods, including SDS-PAGE, UV absorption, and intrinsic fluorescence spectroscopy. However, these methods are not sensitive enough to accurately assess the degree of glycation and changes in glycated sites at the molecular level. Compared with traditional methods, liquid chromatography high-resolution mass spectrometry (**LCHR-MS**) is helpful to obtain accurate measurement data on mass, with high resolution and sensitivity. Our previous report has provided many resources to study glycation through MS. For example, a cationic map of the AA sequence can be obtained by LCHR-MS. According to the mass transfer and the relative abundance of glycated peptides, the properties and degree of glycation of the protein can be analyzed in detail (Tu et al., 2017). In general, high-energy c-trap dissociation (**HCD**) can identify glycated sites precisely for computational modeling of sugar interactions (Singh et al., 2012; Zhang et al., 2014). Therefore, the glycated sites and the degree of each glycation point of β -LG can be accurately identified by LCHR-MS.

Formation of glycated adducts and changes in the IgG/IgE binding capacity of whey proteins, are thus, expected after spray drying in the presence of lactose (Villa et al., 2018). The aim of this study was therefore to investigate the relationship between the IgG/IgE-binding capacity and glycation modification of β -LG under spray drying conditions at different inlet temperatures of 120°C (low-temperature spray-drying) and 180°C (normal drying temperature), respectively. It was also desirable to provide a better theoretical understanding of β -LG glycation and to create a basis for

studying the effect of spray-drying treatment on β -LG immunogenicity.

MATERIALS AND METHODS

Materials

β -Lactoglobulin from bovine milk (L3908), α -lactose, rabbit antisera (43413-1G), and goat anti-human IgE-HRP conjugate (A9667) were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG-HRP conjugate (bs-0295G-HRP) was purchased from Beijing Biosynthesis Biotechnology (Beijing, China). Other chemicals were of analytical grade from local suppliers. Distilled water was used throughout this study, from a water purification system (Lingde, Leader-A1, Shanghai, China).

Serum from CMA patients was purchased from PlasmaLab International (Everett, WA) and frozen (-80°C) until needed. Their specific IgE levels ranged from 9.58 to 96.6 kU/L (Table 1).

Preparation of β -LG Samples

Both of β -LG and α -lactose were dissolved in 10 mmol/L PBS (pH 7.4) to form a 5% solution, at a weight ratio of 1:1. The β -LG/ β -LG-lactose solution was treated using a spray drier (YM-015, Yuming Instrument Co. Ltd., Shanghai, China) at constant air inlet temperatures of 120 and 180°C, and air outlet temperatures of 60 and 90°C, respectively, and an injection flow rate of 10 mL/min. After treatment, the samples were placed into a freezer to stop any further reaction. Once cooled, the samples were dissolved in PBS and the unreacted α -lactose was removed by dialysis against PBS, using 3,000 Da molecular weight cutoff dialysis tubing, then stored at -20°C until needed. Native β -LG was spray dried at 120 and 180°C, and named **β -LG-120** and **β -LG-180**, respectively. The mixture of native β -LG and α -lactose was also spray dried at 120 and 180°C, and named **β -LG-L-120** and **β -LG-L-180**, respectively. Native β -LG was used as the control in all analyses and named **β -LG-N**.

IgG/IgE Binding Capacity

The IgG/IgE binding capacity of the β -LG samples was evaluated through indirect competitive ELISA with rabbit antibodies and antisera from patients with CMA, as described previously (Tong et al., 2012; Xu et al., 2015; Yang et al., 2017). First, a 96-well ELISA plate was coated with native β -LG (2 μ g/mL, 100 μ L per well) at 4°C overnight. Then, 1% fish gelatin sealant (250 μ L) was added to each well, followed by incubation for 1 h at 37°C in the incubator. After washing, the samples (8 μ g/mL, 50 μ L per well) were added into the wells. Rabbit antiserum (diluted to 1:38,400 in PBS) or antisera of patients with CMA (diluted 1:8, 50 μ L per well) was added to the wells, followed by incubation at 37°C for 1 h, then goat anti-rabbit IgG-HRP conjugate (100 μ L, diluted to 1:2,500 in PBS) or goat anti-human IgE-HRP conjugate (100 μ L, 1:2,500) was added and incubated at 37°C for 1 h. After each step, the ELISA plate was washed thrice with PBST (0.05% Tween-20 in 10 mmol/L PBS, pH 7.4). After 1 h, tetramethylbenzidine (100 μ L) solution was added and the plate was incubated at 37°C for 15 min. Finally, sulfuric acid (50 μ L, 2 mol/L) was added to stop the reaction. The absorbance at 450 nm was read immediately with a microplate reader (Synergy H1, BioTek Instruments, Winooski, VT). The inhibition rates of IgG/IgE binding capacity were calculated with the following equation (Meng et al., 2017):

$$\text{Inhibition} = \frac{\text{total reactivity} - \text{remaining reactivity after inhibition}}{\text{total reactivity}} \times 100\%.$$

Determination of Free Amino Groups

The ortho-phthalaldehyde (OPA) method was used to determine the degree of glycation of β -LG samples. The procedure was adapted from a previously reported method. The OPA reagent was prepared by mixing 150 mL of solution A (7.62 g of sodium tetraborate decahydrate was mixed with 200 mg of SDS in distilled water) with solution B (160 mg of OPA and 176 mg of dithiothreitol dissolved in 4 mL of 95% ethanol; Fayle et al., 2001). The final volume was adjusted to 200 mL with distilled water. Protein solution samples (200 μ L) were mixed with OPA reagent (4 mL) and incubated at 37°C for 2 min in the dark. Absorption was measured at 340 nm. Working standards were prepared by serial lysine dilutions to final concentrations between 0 and 0.5 mg/mL.

Intrinsic Fluorescence Spectroscopy

Intrinsic fluorescence spectra were obtained with a Hitachi spectro-fluorimeter (F-7000, Hitachi, Tokyo, Japan), with a protein concentration of 1 mg/mL in PBS (10 mM, pH 7.4). The emission spectra were scanned from 300 to 360 nm at a slit width of 5 nm and an excitation wavelength of 270 nm (Liu et al., 2017).

LCHR-HCD-Tandem Mass Spectrometry and Identification

Our method was based on previous reports (Tu et al., 2017; Liu et al., 2018a; Zhang et al., 2018). The MS analysis was conducted using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA) mass spectrometer in positive ion mode. After pepsin digestion, small peptides for subsequent MS analysis were generated. Twenty fragment maps [2 tandem mass spectrometry (MS/MS) scans] reflecting the mass-to-charge ratio of the peptides and their fragments were collected after each full scan. The ions detected during precursor ion scanning were further subjected to HCD fragmentation.

Statistical Analysis

All the analyses were performed in triplicate and the results are presented as the mean value $\pm$ standard deviation. The data analysis was performed using Origin-Pro 9.0 (OriginLab Corp., Northampton, MA).

RESULTS AND DISCUSSION

IgG/IgE Binding Capacity Analysis

The IgG/IgE binding capacity of the samples was evaluated through inhibition ELISA with rabbit antisera and antisera of CMA patients. The IgG binding capacity of β -LG-120 and β -LG-L-120, like that of native β -LG and the lowest IgG binding capacity, was detected in β -LG-L-180 (Figure 1A). The same trend was observed in the variation of IgE binding capacity (Figure 1B). The IgE binding capacity of β -LG-120 and β -LG-L-120 was similar to that of β -LG-N. Therefore, spray drying at 120°C did not change the IgG/IgE binding capacity of β -LG. A previous report stated that the IgG binding capacity begins to decrease after dry heat at 130°C for more than 5 min (Liu et al., 2016). When the inlet temperature of the spray drying tower was increased to 180°C, the IgE binding capacity of β -LG-180 was markedly reduced and the capacity of β -LG-L-180 was the lowest. The decreased IgG/IgE

binding capacity of β -LG-180 may be attributable to the aggregation of β -LG, possibly through the formation of intermolecular disulfide bridges, which may change the protein conformation and mask conformational epitopes on the surface of the protein molecule. A similar conclusion was reported previously (Kleber and Hinrichs, 2007). The reduction of IgG/IgE binding capacity of β -LG-L-180 may also result from the epitope-shielding effect of the Maillard reaction (Tong et al., 2012) and consequent changes to the protein conformation induced by glycation. Glycation can modify both linear and conformational epitopes, consequently preventing binding to their corresponding antibody and resulting in reduced binding capacity (Taheri-Kafrani et al., 2009; Villa et al., 2018).

In general, IgG and IgE antibodies can bind to epitopes on β -turn, random coil, α -helix, and β -sheet structures (Wróblewska, and Jedrychowski, 2002; Tong et al., 2012). To better understand the reason for the decrease in the IgG/IgE binding capacity of β -LG after spray drying treatment, structural analysis of β -LG was undertaken through fluorescence spectral analysis and MS (see below).

Free Amino Groups

No significant difference was observed in the free amino group content between β -LG-N, β -LG-120, and β -LG-L-120 ($P > 0.05$, Figure 2). After spray drying at 180°C, the free amino content decreased significantly ($P < 0.05$), especially that of β -LG-L-180. It is notable that the trend of decreasing free amino group content in Figure 2 is very similar to the trend in IgG and IgE binding in Figure 1.

Measurement of the free amino group content also provides an estimate of the extent of glycation. No significant change was observed in the free amino group content of β -LG during spray drying at 120°C, whereas at 180°C, the amino group content decreased markedly, suggesting that some amino groups were masked by aggregation or conformational changes in the protein structure. Moreover, the free amino group content of β -LG-L-180 was significantly lower than β -LG-180, suggesting that spray drying at 180°C promoted the glycation reaction between β -LG amino groups and lactose.

Fluorescence Spectral Analysis

Fluorescence emission spectroscopy was applied to characterize the conformational changes in the protein (Huang et al., 2019; Figure 3). When excited at 270 nm, β -LG-N exhibited a maximum fluorescence emission (λ maximum) at 335 nm. The intensity of

Trp-related fluorescence was decreased in β -LG-L-180, suggesting that the secondary structure of β -LG was changed by glycation. Thus, conformational changes, including the shielding of glycated β -LG, occurred near the Trp residues, at least partly as a result of glycation. Some reports indicate that the fluorescence intensity of β -LG conjugates is lower than that of natural β -LG and this may result from the binding of reducing sugars to Lys residues near the Trp residues, which results in

Figure 1. The IgG (A) and IgE (B) binding capacity of β -LG-N, β -LG-120, β -LG-180, β -LG-L-120, and β -LG-L-180. P1 to P6 refer to the antisera of 6 patients with cow milk allergy. Means with different letters (a–c) in the bars indicate significant differences ($P < 0.05$). β -LG-120 and β -LG-180 = native β -LG spray dried at 120 and 180°C, respectively; β -LG-L-120 and β -LG-L-180 = mixture of native β -LG and α -lactose spray dried at 120 and 180°C, respectively; β -LG-N = native β -LG (control). Error bars represent SD of data from 3 independent experiments.

the shielding of Trp-19 and Trp-61 (Jiménez-Castaño et al., 2005; Chen et al., 2016; Liu et al., 2018b). The fluorescence intensity of β -LG-180 was higher than that of β -LG-120. The difference may be the result of the aggregation of protein molecules caused by the increased temperature. The AA containing fluorescent chromophores were more shielded, resulting in enhanced fluorescence intensity. This agrees with a previous report that heat treatment at a high temperature can increase the fluorescence intensity of proteins (Stănciuc et al., 2012). The fluorescence intensity of β -LG-L-180 was the lowest of all the samples. The insignificant difference (Figure 3) between intrinsic fluorescence emission spectra of β -LG spray dried without α -lactose present suggests that aggregation was not extensive. The decreased fluorescence intensity of β -LG-L-180 suggests that glycation of the protein may result in changes to its secondary structure, resulting in a greater shielding effect.

Peptide Mapping and Identification of Glycated Peptides

Glycation of proteins can be monitored by MS because of the increase in mass resulting from the addition of sugars to the protein (Sakaguchi et al., 2016; Liu et al., 2018b). Mass spectrometric analysis of β -LG-120 and 180 samples was carried out to assess glycation levels resulting from the different treatments. In addition, β -LG-L-120 was compared with β -LG-N to elucidate the reason for their insignificant difference in IgG/IgE

Figure 2. The free amino group content of β -LG-N, β -LG-120, β -LG-180, β -LG-L-120, and β -LG-L-180. β -LG-120 and β -LG-180 = native β -LG spray dried at 120 and 180°C, respectively; β -LG-L-120 and β -LG-L-180 = mixture of native β -LG and α -lactose spray dried at 120 and 180°C, respectively; β -LG-N = native β -LG (control). Error bars represent SD of data from 3 independent experiments. Means with different letters (a–c) indicate significant differences ($P < 0.05$).

Figure 3. The intrinsic fluorescence spectra of β -LG-N, β -LG-120, β -LG-180, β -LG-L-120, and β -LG-L-180. β -LG-120 and β -LG-180 = native β -LG spray dried at 120 and 180°C, respectively; β -LG-L-120 and β -LG-L-180 = mixture of native β -LG and α -lactose spray dried at 120 and 180°C, respectively; β -LG-N = native β -LG (control).

binding capacity. It was possible that residual lactose in the spray drying tower could have caused glycation of β -LG-N in the absence of lactose in the feed solution. The β -LG-L-180 was analyzed because of its significant change in indirect competitive ELISA. Pepsin digestion was initially performed, and LTQ-Orbitrap Velos MS analysis was then carried out. Pepsin was chosen because of its relatively high selectivity for peptide-bond cleavage sites (Wang et al., 2016). Therefore, moderate numbers of small peptides, suitable for subsequent MS analysis, could be generated.

In theory, if a peptide is mono-glycated by α -lactose, the corresponding m/z peaks with charges of 1 or 2 display an increase in mass of 324.2965 or 162.1483 Da, respectively. To identify the glycated AA residues, the glycation sites were analyzed by MS/MS with HCD after pepsin digestion. No glycated site was detectable in the mass spectra of β -LG-120 or β -LG-180. Therefore, glycation of β -LG by residual lactose in the spray drying tower can be excluded.

Four glycated peptides derived from β -LG-L-180 are shown in Figure 4. For example, m/z of the unglycated peptide 10–19 (Figure 4A) was 565.8149²⁺, whereas the corresponding m/z of its glycated form was 727.3938²⁺. The corresponding m/z of the glycated peptide exhibited an increase of 162.1483 Da. Similarly, the peaks with m/z of 663.8437²⁺, 516.8085²⁺, and 525.3034²⁺ were identified as unglycated peptides 43–45, 74–82, and 134–142 (Figures 4B, C, and D), whereas the glycated derivatives had m/z at 825.8958²⁺, 678.8613²⁺, and 687.3612²⁺, respectively. This result indicated that these peptides were glycated with α -lactose.

The position of glycation in each peptide was determined by the HCD MS/MS spectrum, with *b* and *y* ions generated by the cleavage of the peptide bond backbone before and after the glycation sites. For example, Figures 5A and B show the HCD MS/MS spectra of the mono-glycated peptides 10–19 (sequence LDIQKVAGTW at m/z 404.3938²⁺) and 43–54 (sequence VEELKPTPEGDL at m/z 825.8958²⁺). Considering the complete matching of *b* and *y* ions, K14 and K47 were identified as the glycation sites. Similarly, Figures 5C and D show the HCD MS/MS spectra of the mono-glycated peptides 74–82 (sequence EKT-KIPAVF at m/z 678.8613²⁺) and 134–142 (sequence EKFDKALKA at m/z 687.3612²⁺). The corresponding glycation sites were identified as K75, K77, and K135. The 7 detected glycation sites of β -LG-L-180 are listed in Table 2.

The glycation sites obtained from peptide mapping of β -LG-L-180 were located at K8, K14, K47, K75, K77, K100, and K135, showing that glycation occurred exclusively on Lys side chains. This result is consistent with a previous report (Milkovska-Stamenova and Hoffmann, 2016) of the identification of glycation sites in different milk products; 13 Lys residues of β -LG in infant formula were glycated.

In a previous report (Sélo et al., 1999), human IgE allergy epitopes in β -LG were identified, including AA1–8, 9–14, 25–40, 41–60, 84–91, 92–100, 102–124, and 149–162. Immunoglobulin G major allergy epitopes have also been identified, including AA 51–64, 67–88, 129–144, and 139–156 (O’Riordan, et al., 2014). These main allergic epitopes contain the glycation sites of β -LG-L-120/ β -LG-L-180 (Figure 6). The K8 was in a β -turn region, K47 and K75 were in a β -sheet region, K77 and K100 were in random coils, and K14 and K135 were in an α -helical region.

DISCUSSION

Spray drying can promote glycation of β -LG and decrease the IgG/IgE binding capacity, which is related to changes in β -LG structure. The structural changes in spray-dried β -LG/lactose mixtures were investigated to understand the mechanism of the reduction in IgG/IgE binding capacity of β -LG.

Spray drying technology can remove 95 to 98% of the water in the feed liquid in a few seconds. After spray drying at 120°C, the structure of β -LG underwent negligible change, and no significant change occurred in the free amino group content and no glycation site was

Figure 4. Mass spectrometry spectra of the representative glycation peptides of the mixture of native β -LG and α -lactose spray dried at 180°C (β -LG-L-180). (A) Peptide 10–19 at m/z 565.8149²⁺, (B) peptide 43–54 at m/z 663.8437²⁺, (C) peptide 74–82 at m/z 516.8085²⁺, and (D) peptide 134–142 at m/z 575.3034²⁺. Glycation is indicated by double-headed arrows. The mass difference between the glycated and unglycated peptides is indicated above the arrows.

Figure 5. The high-energy c-trap dissociation MS/MS spectra of the peaks at m/z 727.3938²⁺ (A), 825.8958²⁺ (B), 678.8613²⁺ (C), and 687.3612²⁺ (D) of glycosylated peptides derived from the mixture of native β -LG and α -lactose spray dried at 180°C (β -LG-L-180). The sequence of each peptide is shown at the top of each spectrum. *b* and *y* ions are shown in the mass spectra, and 2 glycation sites in one peptide are marked by different colors.

observed in the mass spectrum. Consequently, the IgG/IgE binding capacity of β -LG-120 and β -LG-L-120 were similar to that of native β -LG.

Spray drying at the higher temperature (180°C) decreased the IgG/IgE binding capacity of the samples. The IgG/IgE binding capacity of β -LG-180 decreased, possibly because of the aggregation of protein molecules. This apparently contributed to the loss of accessibility of some epitopes initially located on the surface of β -LG. No glycation sites were found in this sample. β -LG-L-180 exhibited protein aggregation and decreased free amino group content and glycosylated Lys side-chains were detected by MS, mostly in the known epitope regions. A marked reduction also occurred in IgG/IgE binding capacity. No glycosylated Arg

was detected, which was to be expected because the nitrogen atoms in the Arg side chain sterically hinder each other and prevent any reaction, in agreement with previous reports (Gooding et al., 2004; Lifschitz and Szajewska, 2015). Seven glycation sites were discovered in β -LG-L-180, mostly in epitope regions of the protein, suggesting that glycation blocks access to the epitopes and reduces the binding capacity of IgG/IgE to β -LG.

CONCLUSIONS

Spray drying at a high temperature (180°C) reduced the IgG/IgE binding capacity of β -LG. However, a lower temperature (120°C) treatment resulted in no detectable change in protein structure, extent of glyca-

Table 2. Glycosylated peptides and sites of the mixture of native β -LG and α -lactose spray dried at 180°C (β -LG-L-180); underlined letters indicate glycosylated lysine (K)

Peptide location	m/z	Δ ppm	Sequence	Glycosylated peptide m/z	Glycosylated site
1–11	609.8423 ²⁺	1.22	(–)LIVTQTMKGLD(I)	771.3169 ²⁺	K8
10–19	565.8149 ²⁺	2.02	(G)LDIQK <u>V</u> AGTW(Y)	727.3938 ²⁺	K14
42–54	745.3759 ²⁺	1.65	(V)YVEELKPTPEGDL(E)	907.9293	K47
74–82	516.8085 ²⁺	1.08	(A)EK <u>T</u> KIPAVF(K)	678.8613 ²⁺	K75/K77
96–104	579.8063 ²⁺	1.04	(L)DTDYK <u>K</u> YLL(F)	741.8588 ²⁺	K100
134–142	525.3034 ²⁺	0.526	(L)EKFDKALKA(L)	687.3612 ²⁺	K135

Figure 6. Ribbon diagram of the mixture of native β -LG and α -lactose spray dried at 120 and 180°C (β -LG-L-120 and β -LG-L-180, respectively; Protein Data Bank identification number 1BSQ). The glycation sites are colored as follows: gray = framework of β -LG; red = glycation sites of native β -LG.

tion, or IgG/IgE binding capacity. The spray-drying feed solution takes only a few seconds to pass through, so the protein is heated very briefly. Only the higher temperature generated chemical reactions (i.e., protein aggregation and glycation of Lys side chains in epitope regions of the protein), which resulted in reduced IgG/IgE binding capacity. These results suggest that spray drying should reduce the allergenicity of β -LG to the potential benefit of people who suffer from CMA. Future research should include investigations of whether Maillard reactions and protein structure changes, which can modify the allergenicity of cow milk, occur in other processes, such as pasteurization, sterilization, or UHT treatment.

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REFERENCES

Arpagaus, C., N. Schafroth, and M. Meuri. 2010. Laboratory scale spray drying of lactose: A review. *Tuberculosis*. 3:4.

- Bonomi, F., H. Frokiaer, L. Henningsen, M. Gentilini, S. Iametti, M. Marengo, and M. Miriani. 2013. Denaturation at interfaces affects immunoreactivity of β -lactoglobulin and its cellular uptake by monocytes. *Miami Winter Symposium: The Molecular Basis of Metabolism and Nutrition*. February 2013, Miami, FL. <http://hdl.handle.net/2434/221961>.
- Bouaouina, H., A. Desrumaux, C. Loisel, and J. Legrand. 2006. Functional properties of whey proteins as affected by dynamic high-pressure treatment. *Int. Dairy J.* 16:275–284. <https://doi.org/10.1016/j.idairyj.2005.05.004>.
- Carter, B., H. Patel, D. M. Barbano, and M. Drake. 2018. The effect of spray drying on the difference in flavor and functional properties of liquid and dried whey proteins, milk proteins, and micellar casein concentrates. *J. Dairy Sci.* 101:3900–3909. <https://doi.org/10.3168/jds.2017-13780>.
- Chen, Y., Z. Tu, H. Wang, L. Zhang, X. Sha, J. Pang, P. Yang, G. Liu, and W. Yang. 2016. Glycation of β -lactoglobulin under dynamic high pressure microfluidization treatment: Effects on IgE-binding capacity and conformation. *Food Res. Int.* 89:882–888. <https://doi.org/10.1016/j.foodres.2016.10.020>.
- Fayle, S. E., J. P. Healy, P. A. Brown, E. A. Reid, J. A. Gerrard, and J. M. Ames. 2001. Novel approaches to the analysis of the Maillard reaction of proteins. *Electrophoresis* 22:1518–1525. [https://doi.org/10.1002/1522-2683\(200105\)22:8<1518::AID-ELPS1518>3.0.CO;2-U](https://doi.org/10.1002/1522-2683(200105)22:8<1518::AID-ELPS1518>3.0.CO;2-U).
- Gooding, J. J., C. Wasiowych, D. Barnett, D. B. Hibbert, J. N. Barisci, and G. G. Wallace. 2004. Electrochemical modulation of antigen–antibody binding. *Biosens. Bioelectron.* 20:260–268. <https://doi.org/10.1016/j.bios.2004.01.010>.
- Haug, A., A. T. Høstmark, and O. M. Harstad. 2007. Bovine milk in human nutrition – A review. *Lipids Health Dis.* 6:25. <https://doi.org/10.1186/1476-511X-6-25>.
- Huang, T., H. Zhao, Y. Fang, J. Lu, W. Yang, Z. Qiao, Q. Lou, D. Xu, and J. Zhang. 2019. Comparison of gelling properties and flow behaviors of microbial transglutaminase (MTGase) and pectin modified fish gelatin. *J. Texture Stud.* 50:400–409. <https://doi.org/10.1111/jtxs.12405>.
- Jiménez-Castaño, L., R. López-Fandiño, A. Olano, and M. Villamiel. 2005. Study on β -lactoglobulin glycosylation with dextran: Effect on solubility and heat stability. *Food Chem.* 93:689–695. <https://doi.org/10.1016/j.foodchem.2004.09.050>.

- Kleber, N., and J. Hinrichs. 2007. Antigenic response of β -lactoglobulin in thermally treated bovine skim milk and sweet whey. *Milchwissenschaft* 62:121–124.
- Lifschitz, C., and H. Szajewska. 2015. Cow's milk allergy: Evidence-based diagnosis and management for the practitioner. *Eur. J. Pediatr.* 174:141–150. <https://doi.org/10.1007/s00431-014-2422-3>.
- Liu, F., M. Teodorowicz, M. A. van Boekel, H. J. Wichers, and K. A. Hettinga. 2016. The decrease in the IgG-binding capacity of intensively dry heated whey proteins is associated with intense Maillard reaction, structural changes of the proteins and formation of RAGE-ligands. *Food Funct.* 7:239–249. <https://doi.org/10.1039/C5FO00718F>.
- Liu, J., Z. Tu, G. Liu, C. Niu, H. Yao, H. Wang, X. Sha, Y. Shao, and I. A. Kaltashov. 2018a. Ultrasonic pretreatment combined with dry-state glycation reduced the IgE/IgG-binding ability of α -lactalbumin revealed by high-resolution mass spectrometry. *J. Agric. Food Chem.* 66:5691–5698. <https://doi.org/10.1021/acs.jafc.8b00489>.
- Liu, J., Z. Tu, Y. Shao, H. Wang, G. Liu, X. Sha, L. Zhang, and P. Yang. 2017. Improved antioxidant activity and glycation of α -lactalbumin after ultrasonic pretreatment revealed by high-resolution mass spectrometry. *J. Agric. Food Chem.* 65:10317–10324. <https://doi.org/10.1021/acs.jafc.7b03920>.
- Liu, G., Z. Tu, W. Yang, H. Wang, L. Zhang, D. Ma, T. Huang, J. Liu, and X. Li. 2018b. Investigation into allergenicity reduction and glycation sites of glycated β -lactoglobulin with ultrasound pretreatment by high-resolution mass spectrometry. *Food Chem.* 252:99–107. <https://doi.org/10.1016/j.foodchem.2018.01.086>.
- Meng, X., Y. Bai, J. Gao, X. Li, and H. Chen. 2017. Effects of high hydrostatic pressure on the structure and potential allergenicity of the major allergen bovine β -lactoglobulin. *Food Chem.* 219:290–296. <https://doi.org/10.1016/j.foodchem.2016.09.153>.
- Milkovska-Stamenova, S., and R. Hoffmann. 2016. Identification and quantification of bovine protein lactosylation sites in different milk products. *J. Proteomics* 134:112–126. <https://doi.org/10.1016/j.jprot.2015.07.021>.
- O'Riordan, N., M. Kane, L. Joshi, and R. M. Hickey. 2014. Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology* 24:220–236. <https://doi.org/10.1093/glycob/cwt162>.
- Rahaman, T., T. Vasiljevic, and L. Ramchandran. 2016. Effect of processing on conformational changes of food proteins related to allergenicity. *Trends Food Sci. Technol.* 49:24–34. <https://doi.org/10.1016/j.tifs.2016.01.001>.
- Sakaguchi, Y., T. Kinumi, T. Yamazaki, and A. Takatsu. 2016. Quantification of glycated N-terminal peptide of hemoglobin using derivatization for multiple functional groups of amino acids followed by liquid chromatography/tandem mass spectrometry. *Biomed. Chromatogr.* 30:280–284. <https://doi.org/10.1002/bmc.3524>.
- Schuck, P., C. le Floch-Fouere, and R. Jeantet. 2013. Changes in functional properties of milk protein powders: Effects of vacuum concentration and drying. *Dry. Technol.* 31:1578–1591. <https://doi.org/10.1080/07373937.2013.816316>.
- Sélo, I., G. Clément, H. Bernard, J. Chatel, C. Créminon, G. Peltre, and J. Wal. 1999. Allergy to bovine beta-lactoglobulin: Specificity of human IgE to tryptic peptides. *Clin. Exp. Allergy* 29:1055–1063. <https://doi.org/10.1046/j.1365-2222.1999.00612.x>.
- Singh, C., C. G. Zampronio, A. J. Creese, and H. J. Cooper. 2012. Higher energy collision dissociation (HCD) product ion-triggered electron transfer dissociation (ETD) mass spectrometry for the analysis of N-linked glycoproteins. *J. Proteome Res.* 11:4517–4525. <https://doi.org/10.1021/pr300257c>.
- Singh, S., and D. Dixit. 2014. A review on spray drying: Emerging technology in food industry. *Int. J. Applied Eng. Technol.* 4:1–8.
- Stănciuc, N., I. Aprodu, G. Răpeanu, and G. Bahrim. 2012. Fluorescence spectroscopy and molecular modeling investigations on the thermally induced structural changes of bovine β -lactoglobulin. *Innov. Food Sci. Emerg. Technol.* 15:50–56. <https://doi.org/10.1016/j.ifset.2012.03.001>.
- Suzihaque, M. U. H., A. Norafiqah, and A. R. Norekanadirah. 2015. Fruit-milk powder: A review of spray drying technology. *Advanced Materials Res.* 1113:328–333. <https://doi.org/10.4028/www.scientific.net/AMR.1113.328>.
- Taheri-Kafrani, A., J. C. Gaudin, H. Rabesona, C. Nioi, D. Agarwal, M. Drouet, J. M. Chobert, A. K. Bordbar, and T. Haertle. 2009. Effects of heating and glycation of β -lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. *J. Agric. Food Chem.* 57:4974–4982. <https://doi.org/10.1021/jf804038t>.
- Tanaka, K., H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida, and T. Matsuo. 1988. Protein and polymer analyses up to m/z 100000 by laser ionization time-of flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 2:151–153. <https://doi.org/10.1002/rcm.1290020802>.
- Tong, P., J. Gao, H. Chen, X. Li, Y. Zhang, S. Jian, H. Wichers, Z. Wu, A. Yang, and F. Liu. 2012. Effect of heat treatment on the potential allergenicity and conformational structure of egg allergen ovotransferrin. *Food Chem.* 131:603–610. <https://doi.org/10.1016/j.foodchem.2011.08.084>.
- Tu, Z., B. Zhong, and H. Wang. 2017. Identification of glycated sites in ovalbumin under freeze-drying processing by liquid chromatography high-resolution mass spectrometry. *Food Chem.* 226:1–7. <https://doi.org/10.1016/j.foodchem.2017.01.038>.
- Villa, C., J. Costa, M. B. P. P. Oliveira, and I. Mafra. 2018. Bovine milk allergens: A comprehensive review. *Compr. Rev. Food Sci. Food Saf.* 17:137–164. <https://doi.org/10.1111/1541-4337.12318>.
- Wang, H., Z. C. Tu, G. X. Liu, L. Zhang, and Y. Chen. 2016. Identification and quantification of the phosphorylated ovalbumin by high resolution mass spectrometry under dry-heating treatment. *Food Chem.* 210:141–147. <https://doi.org/10.1016/j.foodchem.2016.04.082>.
- Wróblewska, B., and L. Jedrychowski. 2002. Effect of conjugation of cow milk whey protein with polyethylene glycol on changes in their immunoreactive and allergic properties. *Food Agric. Immunol.* 14:155–162. <https://doi.org/10.1080/09540100220145188>.
- Xu, Q., J. Shi, M. Yao, M. Jiang, and Y. Luo. 2015. Effects of heat treatment on the antigenicity of four milk proteins in milk protein concentrates. *Food Agric. Immunol.* 27:401–413. <https://doi.org/10.1080/09540105.2015.1117059>.
- Yang, W., Z. Tu, H. Wang, L. Zhang, I. A. Kaltashov, Y. Zhao, C. Niu, H. Yao, and W. Ye. 2018. 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.* 9:417–425. <https://doi.org/10.1039/C7FO01082F>.
- Yang, W., Z. Tu, H. Wang, L. Zhang, S. Xu, C. Niu, H. Yao, and I. A. Kaltashov. 2017. Mechanism of the reduction in the 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.* 65:8018–8027. <https://doi.org/10.1021/acs.jafc.7b02842>.
- Zhang, N., Z. Tu, H. Wang, G. Liu, Z. Wang, T. Huang, X. Qin, X. Xie, and A. Wang. 2018. Liquid chromatography high-resolution mass spectrometry identifies the glycation sites of bovine serum albumin induced by D-ribose with ultrasonic treatment. *J. Agric. Food Chem.* 66:563–570. <https://doi.org/10.1021/acs.jafc.7b04578>.
- Zhang, Q., Z. Tu, H. Wang, X. Huang, X. Sha, and H. Xiao. 2014. Structural changes of ultrasonicated bovine serum albumin revealed by hydrogen-deuterium exchange and mass spectrometry. *Anal. Bioanal. Chem.* 406:7243–7251. <https://doi.org/10.1007/s00216-014-8136-6> <http://dx.doi.org/110.1007/s00216-014-8136-6>.

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