

Dielectric-barrier discharge (DBD) plasma treatment reduces IgG binding capacity of β -lactoglobulin by inducing structural changes

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ABSTRACT

The present study investigated the effects of dielectric-barrier-discharge (DBD) plasma treatment (12 kHz, 40 kV) at 1, 2, 3, and 4 min on the reduction of the immunoglobulin G (IgG) binding capacity of β -lactoglobulin (β -LG). The IgG binding capacity of β -LG was reduced by 58.21% following a plasma treatment time of 4 min, as confirmed by western-blot and ELISA analyses. The reduction in IgG binding capacity of β -LG was directly related to a stepwise change in its structure. The initial drop in the IgG binding capacity of β -LG was found to be caused by conformational alteration, free sulfhydryl exposure and cross-linkage of molecules induced by oxidation of NH₂-NH₂ functional groups of peptide bonds and of sensitive amino acid residues (Tyr, Trp) as confirmed by SDS-PAGE, surface hydrophobicity and multi-spectroscopic analyses. Plasma treatment of more than 3 min resulted in cleavage of disulfide bonds and fragmentation of β -LG that was confirmed by LC-MS/MS analysis, which resulted a further decline in the IgG binding capacity of β -LG. Plasma treatment therefore has great potential as a substitute treatment for enzymatic hydrolysis for the production of hypoallergenic milk protein-based products.

1. Introduction

Cow milk allergy can be a serious health risk in infants as well as in young children with a prevalence of 0.2–17% (Flom & Sicherer, 2019). Allergic reactions are a subset of immunological reactions, which manifest gastrointestinal, respiratory and dermatological problems (Villa, Costa, Oliveira, & Mafra, 2018). The main allergen associated with cow milk is β -lactoglobulin (β -LG) (Villa et al., 2018). β -LG naturally exists in milk of many lactating species as a non-covalent dimer (36 kDa) and each subunit has one free cysteine and two disulfide bridges. β -LG has a β -barrel core structure that creates a hydrophobic cavity in the center of the β -LG structure (Zhu, Li, Wu, Li, & Sun, 2020). Currently, diet restriction is the only approved and effective way to prevent cow milk allergy (Villa et al., 2018). However, due to the industrial and technological importance of cow milk proteins in a variety of processed foods/diets, the potential for exposure of infants and children in child-care and kindergartens to milk protein allergen is inevitable. Nowadays, different strategies are being investigated to eliminate

or reduce the allergenicity of cow milk, such as heat treatment, high pressure, glycation, enzymatic hydrolysis, chemical treatment and fermentation by lactic acid bacteria (Ekezie, Cheng, & Sun, 2018b; Lv et al., 2018, 2019; Bavaro et al., 2019; Oliveira et al., 2019; Xu, Sun, Lin, Ishfaq, & Li, 2020;). Among the studied technologies, enzymatic hydrolysis appears to be an efficient and reliable technique for producing hypoallergenic milk-based products. However, the high cost, longer processing time and the often bitter taste of the hydrolysates has restricted its application and commercial adoption of the technology (Xu et al., 2019). Therefore, novel methods to reduce the allergenicity of the milk proteins are urgently needed.

Recently, atmospheric cold plasma has attracted increasing attention from researchers as a promising and novel non-thermal treatment in the food processing field (Jiang, Cheng, & Sun, 2020; Sharma & Singh, 2020; Liu, Niu et al., 2021). Reactive oxygen species (ROS) ($\cdot\text{OH}$, $\text{OH}^\cdot$, $\text{O}_2^\cdot$ and H_2O_2) and reactive nitrogen species (RNS) ($\text{N}^\cdot$, $\text{N}_2^\cdot$, NO and $\text{NO}^\cdot$, etc.) are generated as a result of feed gas ionized in a high electric field, which results in the inactivation of microorganisms, and reduction of

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anti-nutritional substances and contaminants in foodstuffs (Cheng, Lv, Pan, & Sun, 2020; Tolouie, Mohammadifar, Ghomi, & Hashemi, 2018). Recent studies have demonstrated the ability of cold plasma to modify protein structure with subsequent improvement of physicochemical and functional properties. For example, allergenicity caused by soybean protein, shrimp protein and milk protein has been reduced by treatment with cold plasma (Ekezie, Cheng, & Sun, 2018a, 2019; Ng et al., 2020). A substantial reduction in the immunoreactivity of soluble soybean protein fraction was reported to be attained by direct exposure to plasma (Meinschmidt et al., 2016). Similarly, the hemagglutination activity of soybean agglutinin was reduced by 87.31% after exposure to plasma (Liu, Manzoor, Tan, Inam-ur-Raheem, & Aadil, 2020). Ng et al. (2020) reported that plasma treatment efficiently alleviated the antigenicity of casein and α -lactalbumin, but no significant effect on the antigenicity of β -LG was reported. Similar results were obtained by Tammineedi, Choudhary, Perez-Alvarado, and Watson (2013), who reported that plasma treatment had no effect on the IgE binding capacity of α -casein and whey proteins. However, the allergenicity of tropomyosin was found to be decreased by plasma treatment, on the basis that the binding capacities of IgE and IgG were decreased by 17.6 and 26.87%, respectively (Ekezie, Sun, & Cheng, 2019). Although numerous studies have been conducted in recent years, there is still a lack of important information/data about the relationship between the reduction of protein antigenicity and the protein structure modifications induced by plasma.

To gain more insight into the structural changes that can be caused by treatment with plasma and the effect on allergenicity, β -LG was used in this study to explore the underlying mechanism and relationship of the alleviation of antigenicity and the structural alteration induced by plasma treatment. Structural modification of β -LG was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Liquid chromatography–tandem mass spectrometry (LC–MS/MS) and multi-spectroscopic analyses. The antigenicity of β -LG was evaluated by western-blotting and enzyme linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Materials and chemical

β -LG (purity $\geq 95\%$) was procured from Macklin Biochemical Co., Ltd (Shanghai, China). Polyvinylidene fluoride (PVDF) (0.45 μ m) was obtained from Millipore (Merck Millipore Ltd, USA). Rabbit anti- β -LG antibody was produced in our laboratory. Goat anti-rabbit IgG conjugated to horse radish peroxidase (HRP) was obtained from Sangon Biotech Co. Ltd. (Shanghai, China). Primary and secondary antibody dilution buffer was bought from Biyotime Biotech. (Shanghai, China). The protein marker for SDS-PAGE was obtained from SMOBio-Technology Inc. (Taiwan) and 96-well microtiter plates were bought from Thermo Fisher Scientific (Waltham, MA). All other chemicals used were of the reagent grade.

2.2. DBD plasma treatment

Plasma treatment was conducted at 0–50 kV using an AC power source (CTP-2000 K) at 10 to 20 kHz frequency (Nanjing Suman Electronics Company Limited, Nanjing, China). The equipment included a monitoring system and treatment chamber. β -LG was dissolved in distilled water at 2 mg/mL. A 5-mL aliquot of the β -LG stock solution was transferred into a quartz vessel and placed between stainless steel electrodes spaced 50 mm apart, with the upper electrode enclosed with a quartz plate ($\phi 102$ mm $\times$ 1 mm) and air was used as the feed gas. After plasma treatment, the β -LG sample was diluted to 1 mg/mL with 0.5 M phosphate buffer solution, pH 7.3, and retained for subsequent analysis. All analyses were conducted within 24 h.

2.3. Electrophoresis

SDS-PAGE was utilized to analyze the pattern of proteins in plasma treated β -LG. Gradient SDS-PAGE gels (4–20%) were electrophoresed in a vertical gel electrophoresis unit (WIX-multiPRO2; WIX Technology Co., Ltd. Beijing, China) as described by Liu et al. (2020). Plasma treated and untreated β -LG samples at a concentration of 0.5 mg/mL were mixed with reducing and non-reducing loading buffer in a 1:4 (v/v) ratio and then heated at 90 °C for 10 min; then 10 μ L of the samples were loaded per well of the gel.

2.4. Western blotting analysis

The IgG binding capacity of the samples was assessed by western blotting (WB) analysis (Ekezie, Sun et al., 2019). The SDS-PAGE gel was transferred onto a 0.45- μ m PVDF membrane (Merck Millipore Ltd, USA) for 1 h at 200 mA using a transfer buffer in a WIX-fast BLOT System (WIX Technology Co., Ltd. Beijing, China). Tris-buffered saline (TBST; 0.1 M Tris, 0.15 M NaCl, 0.05% Tween-20, pH 7.5) was utilized as the washing buffer. After blocking the PVDF membrane with a blocking buffer (Biyotime Biotech., Shanghai, China) for 30 min, the membrane was washed thrice with the washing buffer. Then, the membranes were incubated overnight (4 °C) with primary antibodies (IgG rabbit sera raised against β -LG diluted 1:3000). Thereafter, the blots were washed thrice for 5 min each and then these were incubated with the secondary antibody (HRP-labelled goat anti-rabbit IgG (diluted 1:5000)) for 60 min at 25 °C. The membranes were photographed through enhanced chemiluminescence by using a chemiluminescent imaging device (ImageQuant LAS 4000 mini, Fujifilm corporation, Tokyo, Japan) after being washed with TBST.

2.5. ELISA assay

An indirect ELISA assay was used to further evaluate the IgG-binding capacities of the samples as described by Ekezie, Sun et al. (2019). The concentrations of both treated and untreated β -LG were briefly adjusted to 50 μ g/mL with carbonate buffer (50 mM, pH 9.6). 96-Well polystyrene ELISA plates (Costar, USA) were mounted with 100 μ L per well of the samples, covered with a sealing cover and incubated at 4 °C overnight. The plates were washed thrice using a TBST washing buffer and treated with the blocking buffer at 37 °C for 30 min. After washings, 100 μ L primary antibody (IgG rabbit sera raised against β -LG diluted 1:3000) were added to each well and incubated for 1 h at 37 °C, then the plate was washed 3 times with the TBST. The plates were then incubated for 1 h with HRP-labelled goat anti-rabbit IgG (diluted 1:5000). Fifty microliters of chromogenic agents A and B were added in order and allowed to react for 10 min at 37 °C in the dark. A 2 M H_2SO_4 solution (50 L/well) was then used to stop the reaction. After that, using a plate reader (Mutiscango Reader; Thermo Fisher Scientific oy, Vantaa, Finland), the absorbance was measured and recorded at 450 nm. Blanks were run in parallel as described above except that β -LG was not added. The IgG binding capacities were calculated as:

$$\text{IgG binding capacities (\%)} = \frac{A_0 - A}{A_0 - A_b} \times 100\% \quad (1)$$

where A_0 and A is the absorbance of untreated and treated sample, respectively; A_b is the absorbance of blank.

2.6. Carbonyl concentration, total free sulfhydryl (–SH) groups and surface hydrophobicity of β -LG

The concentration of carbonyl, total free sulfhydryl (–SH) groups and surface hydrophobicity of the treated and untreated samples were measured as described in a previous study (Liu, Niu et al., 2021; Liu et al., 2020; Liu, Zhou et al., 2021).

2.7. Intrinsic fluorescence spectroscopy, UV absorption analysis and Far-UV circular dichroism (CD) spectroscopy

These methods were investigated according to the method described in our earlier study (Liu, Niu et al., 2021; Liu, Zhou et al., 2021).

2.8. Measurement of particle diameter

At a temperature of 25 °C, a Zetasizer Nanosystem (Nano-ZS90, Malvern Instruments Ltd, Great Malvern, UK) was used to calculate the average particle size and zeta potential of the samples. The Malvern standard operating procedure (SOP) program (Malvern Instruments Ltd) was used to calculate particle size (nm). For each sample, three replicates were used in the study.

2.9. LC-MS/MS analysis

Lyophilized samples were dissolved in ddH₂O and transferred to a 3 kDa ultrafiltration spin filter unit (Merck Millipore, Bedford, MA) and centrifuged at 12,000g at 4 °C for 10 min. The filtrate was reduced by adding 10 mM DTT at 56 °C for 1 h and subsequently alkylated by 50 mM iodoacetamide at room temperature in the dark for 40 min. Then, the reduced and alkylated protein material was hydrolyzed by trypsin

overnight at 37 °C. After desalting the sample using an in-house C18 tip, the sample was evaporated on a rotary evaporator (Eppendorf plus, Hamburg, Germany). LC-MS/MS analysis was conducted on an Ultimate 3000 HPLC system coupled with a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, USA) with an ESI nano spray source. Peptide-containing solution (5 µL) was loaded and separated using a 150 µm × 15 cm column made in-house packed with reversed-phase ReproSil-Pur C18-AQ resin (1.9 µm, 100 Å; Dr Maisch GmbH, Ammerbuch, Germany) at a flow rate of 600 nL/min, with gradient elution using solvent A (0.1% (v/v) formic acid in water) and solvent B (0.1% (v/v) formic acid in acetonitrile). The LC-MS/MS data were analyzed and searched against the β-LG target protein database using Peaks Studio.

2.10. Statistical analysis

The study was carried out using three experimental replicates. One-way ANOVA was used to investigate the data and the results were presented as mean ± SD and a significance level at $p < 0.05$ was determined. PyMOL was used for 3D visualization of β-LG, as in Figs. 2, 3, 5, and 6.

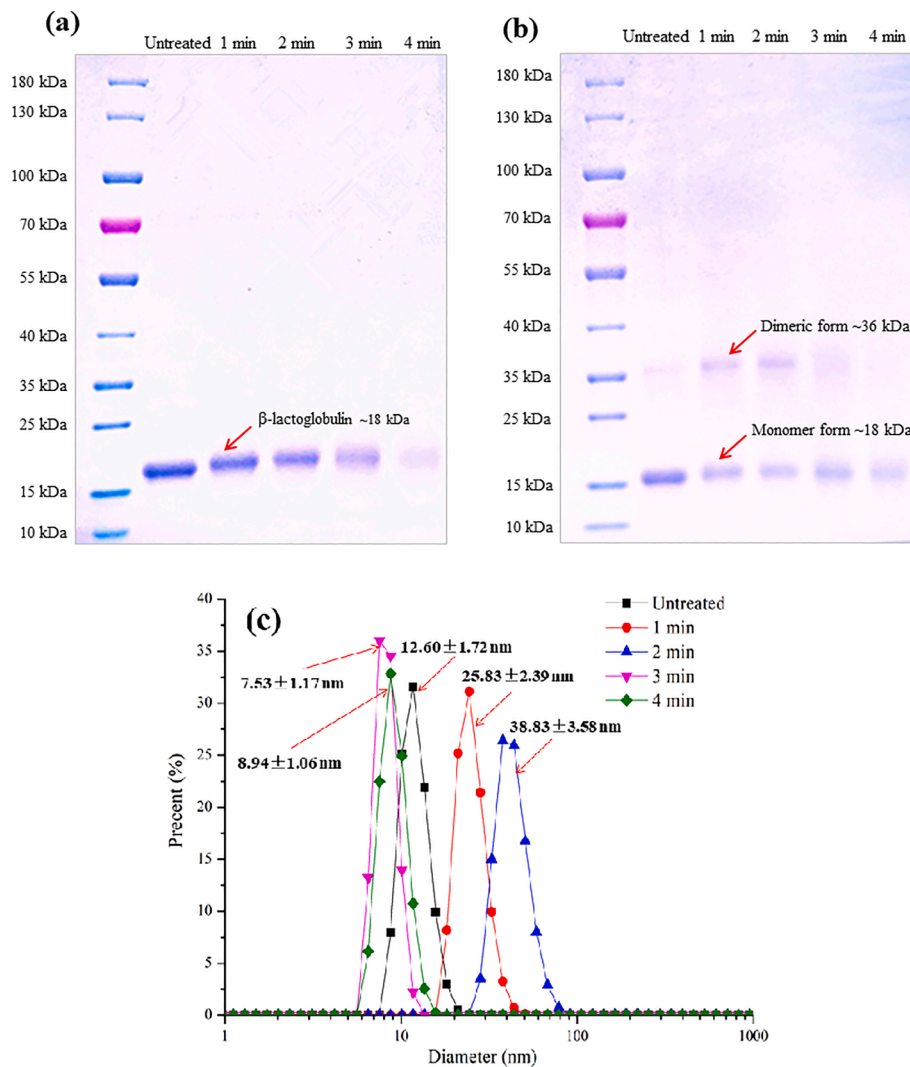


Fig. 1. The protein pattern of β-LG: (a) reducing condition; (b) non-reducing condition. Continuous gel (4–20%) was used to resolve the protein. β-LG with a concentration of 0.5 mg/mL was mixed with reducing/non-reducing loading buffer at a ratio of 1:4 (v/v); 10 µL sample was loaded each lane. (c) The changes of particle size distribution analysis of β-LG after plasma treatment.

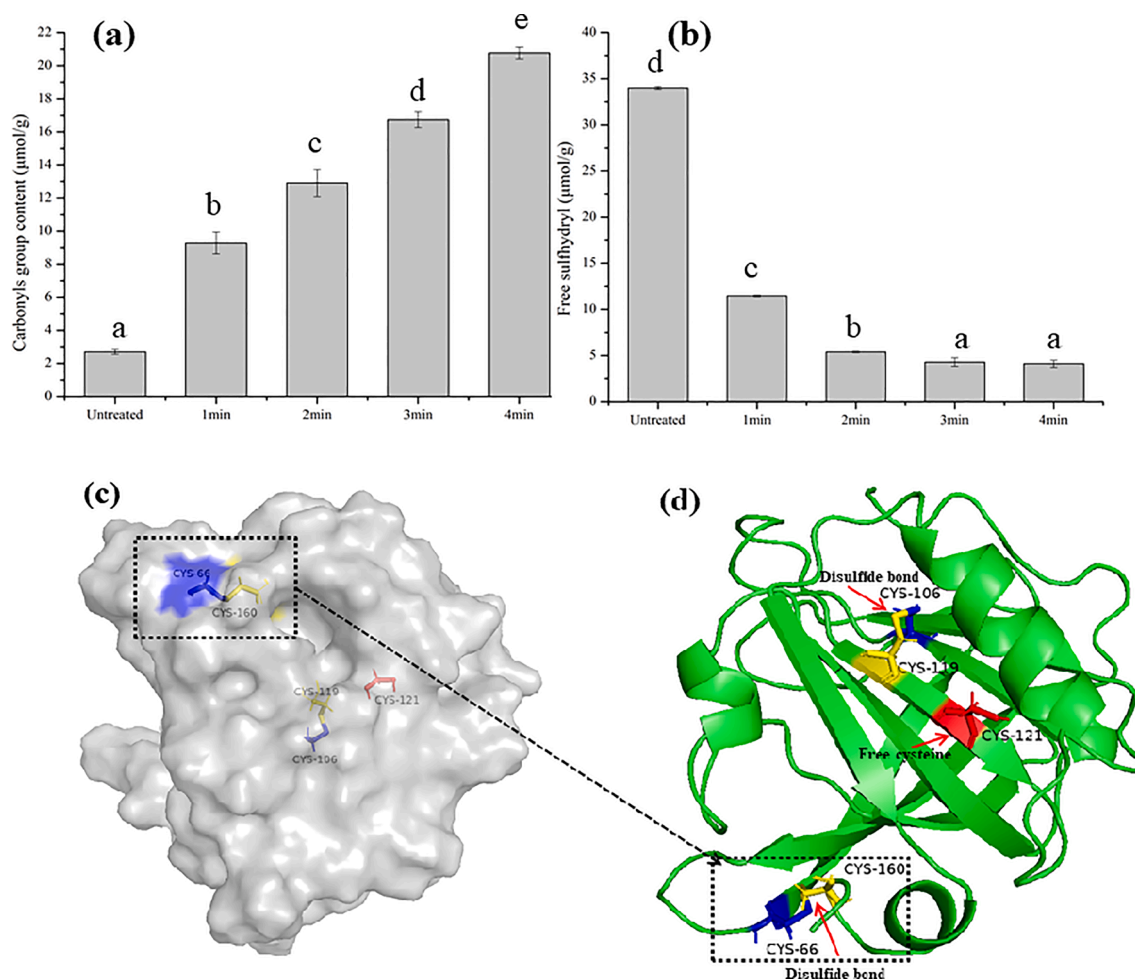


Fig. 2. The effect of plasma treatment time on the carbonyl (a) and free sulfhydryl (b) content of β-LG at 40 kV, 12 kHz. (c) Schematic representation of the structure of β-LG (PDB ID: 3NPO) showing the positions of the five cysteine residues and the generation of a disulfide bond. (b) The surface of β-LG, where the blue and yellow colors represent the exposure of cysteine residues on the surface of β-LG. (d) Showing as a cartoon. ANOVA is used, and statistically significant differences ($p < 0.05$) between values are indicated by different letters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Profile of β-LG and average size analysis

SDS-PAGE analysis was performed under non-reducing and reducing conditions to explore the effect of plasma therapy on the protein pattern of β-LG. Bovine β-LG is composed of 162 amino acids and has a molecular weight of 18.3 kDa. Under physiological conditions, the dominant form of β-LG is as non-covalent associated dimers, while at low pH β-LG exists in the form of monomers. As shown in Fig. 1, a specific band at ~18 kDa corresponding to the monomeric form of β-LG was observed. Compared to untreated native β-LG, the protein bands apparent on SDS-PAGE gradually increased in molecular weight as the duration of exposure to plasma increased, indicating that the molecular weight of β-LG increased. This phenomenon may be ascribed to the oxidation of amino acid residues in β-LG by radical species generated in the plasma, resulting in the addition of oxygen atoms to amino acids in β-LG (Xu et al., 2019). Additionally, a notable difference in the intensity of the untreated and treated β-LG sample bands can be observed in Fig. 1a. As compared to the untreated sample, the band intensity of β-LG gradually fades as the plasma treatment time increased up to 4 min. A marked decrease in the band intensity is evident after a treatment time of 2 min, and the band is not present after a treatment time of 4 min. These results suggest that plasma treatment could break the β-LG polypeptide chain as

a result of plasma treatment in the current study. This is in agreement with earlier studies (Li et al., 2017; Ng et al., 2020; Tammineedi et al., 2013) that reported a noticeable decrease in the intensity of protein bands after plasma treatment, compared to untreated samples.

More information about association/cross-linkage between β-LG can be observed in Fig. 1b. Two noticeable bands were observed under the non-reducing condition, which correspond to the dimer form (~36 kDa) and monomer form (~18 kDa) of β-LG, suggesting the association of β-LG molecules by forming disulfide bonds as a result of plasma treatment. Additionally, it was clear that a dynamic transition of intermolecular cross-linkage of β-LG existed, as the plasma exposure time increased. For the untreated sample, the monomer form of β-LG was the predominant form. After plasma treatment, cross-linkage of β-LG was progressively enhanced as in the samples treated with plasma for 2 min, which resulted in an increase in the intensity band of the dimer form, accompanied by a corresponding drop in the band intensity of the monomer form. Conversely, as exposure time extended to 3 min, the dimer form was disassociated into the monomer form (Fig. 1b). These results are similar to the findings reported by Krämer, Thulstrup, Lund, and Davies (2016), who found that a cross-linking of β-LG resulted in the formation of the dimer form which was formed by low concentrations of H_2O_2 (molar ratio β-LG: H_2O_2 1:2 and 1:10), but then decreased with larger excesses of H_2O_2 (1:2000).

The results obtained in the present study are consistent with the

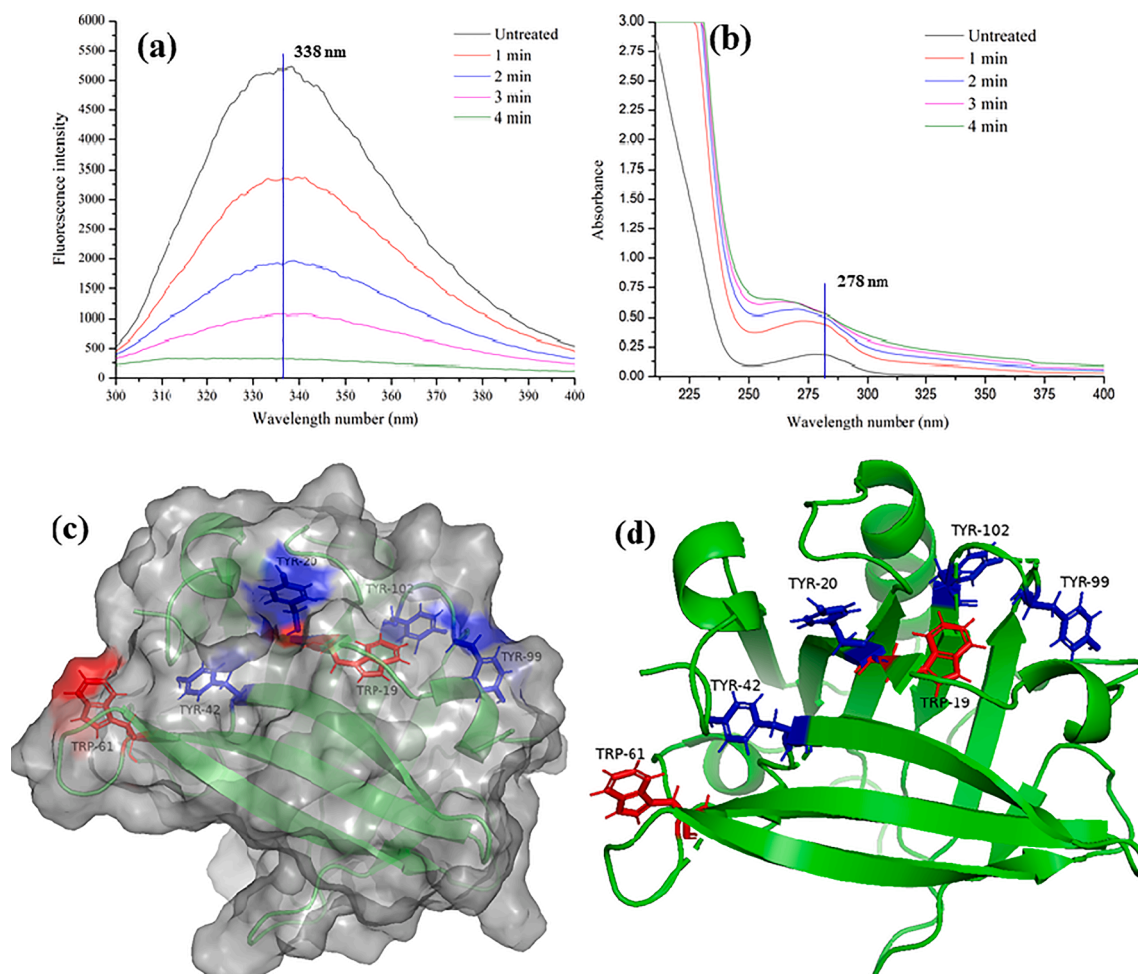


Fig. 3. Multi-spectroscopic analysis of β -LG after plasma treatment. (a) Intrinsic fluorescence emission spectra, (b) UV absorption spectra. Schematic representation of the structure of β -LG showing the location of two Trp and four Tyr residues, (c) showing the surface of β -LG, where red and blue color represents the exposure of Trp and Tyr residues on the surface of β -LG, respectively, (d) showing as a secondary structure of β -LG. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cross-linkage and cleavage of the polypeptide chain of β -LG as a result of plasma treatment. The cleavage of the polypeptide backbone of β -LG was observed in the samples at all treatment times accompanied by its association/disassociation. The cross-linkage of β -LG was ascribed to the generation of a disulfide bond between β -LG (Krämer et al., 2016). It is well established that β -LG contains 5 cysteine residues, of which four of the cysteines form two intramolecular disulfide bridges (Kurpiewska et al., 2019). We propose that in the initial stages, the conformational transformation of β -LG induced by plasma treatment results in exposing the free cysteine and oxidation of the -SH group. This subsequently leads to the formation of intermolecular disulfide bonds and aggregation of β -LG molecules. As the treatment time increases, the intermolecular disulfide bonds are likely broken down into sulfinic and sulfonic acid derivatives as the extent of oxidation increases. Previously, plasma-induced ROS, such as H_2O_2 , O_3 , $OH^\cdot$, $O_2^\cdot$, $^1O_2$ and $ONOO^\cdot$ in have been found to promote chemical reactions in amino acid polymer backbones as well as in amino acid side-chains, leading to protein polymerization, cleavage, and the oxidation process (Venkataratnam, Sarangapani, Cahill, & Ryan, 2019).

The association/disassociation of β -LG as a result of plasma treatment was also confirmed by particle size distribution analysis of β -LG. As depicted in Fig. 1c, the average particle size diameter increased significantly ($p < 0.05$) after 1 and 2 min of plasma treatment, from an initial value of 12.60 ± 1.72 nm to 25.83 ± 2.39 and 38.83 ± 3.58 nm, respectively. A subsequent decrease in the protein particle size from

38.83 ± 3.58 nm to 7.53 ± 1.17 nm and 8.94 ± 1.06 nm was attained at 3 and 4 min of treatment time, respectively. These data are consistent with the protein pattern of β -LG on PAGE under non-reducing conditions. The increase in the average particle size of β -LG in the initial 2 min was related to aggregation of β -LG molecules through disulfide bonding (Ekezie et al., 2018a). Following further exposure to plasma treatment, the disulfide bonds were oxidized to sulfinic and sulfonic acids (Tolouie et al., 2018), resulting in a decrease in the average particle size of β -LG in solution.

3.2. Change in carbonyl and free sulphydryl (-SH) content

Carbonyl compounds as intermediate substances during protein oxidation were extensively applied as an indicator to evaluate the formation and intensity of protein oxidation. In Fig. 2a, a gradual and significant ($p < 0.05$) increase from 2.71 ± 0.15 μ mol/g to 20.77 ± 0.36 μ mol/g in carbonyl content was detected with an increase in the plasma treatment time, suggesting that β -LG oxidation induced by reactive species had occurred. Carbonyls are formed by alteration of the amino acid chain, specifically by oxidation of NH_2/NH -cleavage of amide bonds (Segat, Misra, Cullen, & Innocente, 2015). In Fig. 2b, the free sulphydryl (-SH) content of β -LG is reported. Results indicate that a significant ($p < 0.05$) decrease in free sulphydryl content was attained as the treatment time increased. After 4 min plasma treatment, the -SH content declined from an initial content of 33.98 ± 0.15 to 4.09 ± 0.39

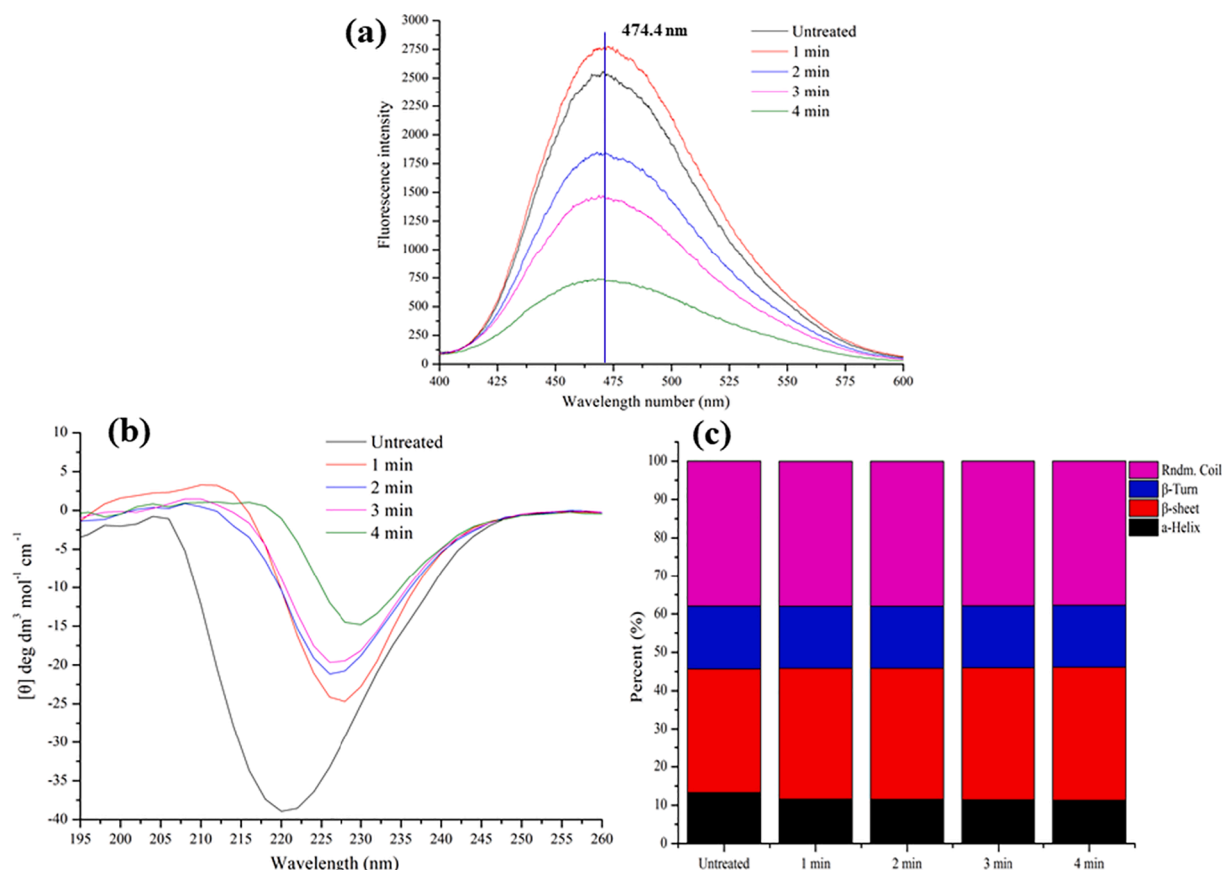


Fig. 4. The effect of DBD plasma treatment on the hydrophobicity and secondary structure of β -LG. (a) Hydrophobicity of β -LG, (b) Far-UV CD spectra, (c) secondary structure content.

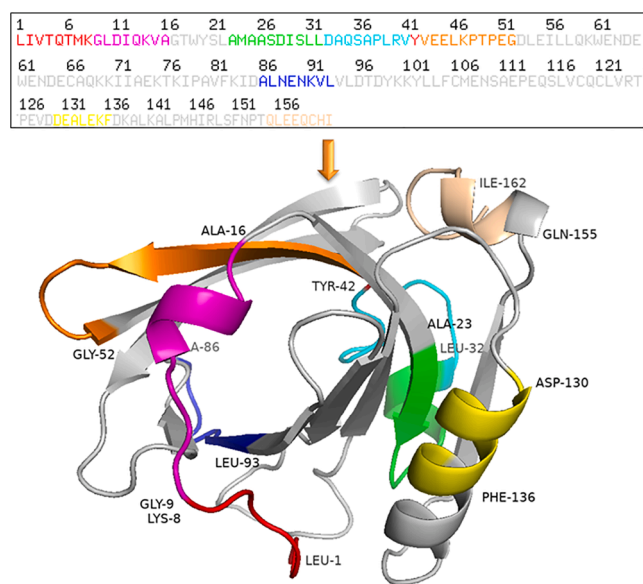


Fig. 5. Schematic representation for the location of peptides generated after 4 min plasma treatment in β -LG. The sequence of β -LG (above); presentation of the secondary structure of β -LG (below).

$\mu\text{mol/g}$. Especially in the initial first minute of plasma treatment, a substantial decline from 33.98 ± 0.15 to $11.45 \pm 0.09 \mu\text{mol/g}$ was observed. The reduction in the free sulfhydryl content implied the unfolding of β -LG and the formation of intra- and/or intermolecular disulfide bonds via disulfide interchange or -SH oxidation. These results

are in agreement with the protein pattern analysis on PAGE under non-reducing conditions (Fig. 1b) and are in line with earlier studies that demonstrated the content of SH groups declined with a prolonged plasma treatment time (Ekezie, Sun et al., 2019; Segat et al., 2015).

As shown in Fig. 2c and d, native β -LG (a globular protein) contains one free cysteine (C121) and two disulfide bridges (C106–C119 and C66–C160), while the secondary structure of β -LG is composed of nine β -sheets (A–I), and a three-turn α -helix on the surface (Fig. 2d). The free cysteine is on β -sheet H buried under the α -helix, and adjacent to the disulfide bridge (C106–C119), which connects β -sheet G and H (Fig. 2c). The other disulfide bridge links the strand D to the C terminus at the globular protein surface (Fig. 2c and d indicated by dashes). It is deduced that three stepwise changes were involved in the plasma-induced loss of -SH from the cysteine residual in β -LG. Firstly, the β -strand (H) region and a part of α -helix of the native conformation of β -LG were partially unfolded, which resulted in contact of the free sulfhydryl group with the surface of the globular protein structure. This exposed -SH was rapidly oxidized by the radical species in the plasma, which was indicated by the substantial decrease in the content of -SH. Secondly, a covalent dimer between two β -LG molecules was formed spontaneously as a result of the formation of the disulfide (-S-S-) intermolecular cross-linkage, as indicated by the increase in the band intensity of the dimer form of β -LG in the 2 min plasma treatment (Fig. 1). Finally, as the plasma treatment time increased, radical species gathering in the aqueous solution caused further oxidation of the disulfide, resulting in the formation of sulfinic and sulfonic acids, which caused the disassociation of the β -LG dimer, as indicated by the increase in the band intensity of the monomer form of β -LG after 3 min plasma treatment (Fig. 1).

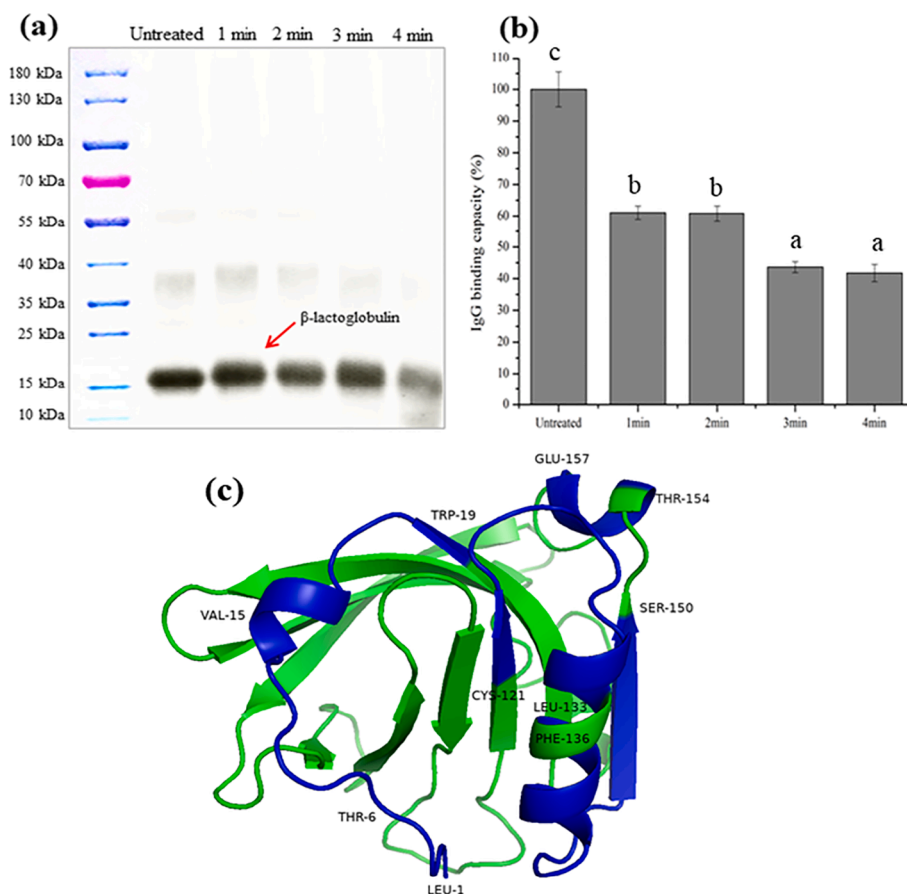


Fig. 6. The change of allergenicity of β -LG after plasma treatment. (a) Western blot analysis; (b) ELISA analysis. Pregel (4–20%) was used. β -LG with the concentration of 0.5 mg/mL was mixed with reducing loading buffer at a ratio of 1:4 (v/v); 10 μ L sample were loaded each lane. Primary antibody (IgG rabbit sera raised against β -LG diluted 1:3000) was used. Second antibody HRP-labelled goat anti-rabbit IgG with dilution 1:5000 was applied, (c) Schematic representation of the location of linear epitopes (Ig E and Ig G) reported in previous studies.

3.3. Intrinsic fluorescence analysis

Intrinsic fluorescence analysis of the protein was used as an effective tool to obtain information on the micro-environment of aromatic amino acids, which was principally obtained from tryptophan (Trp) residues, especially when the protein is excited at 295 nm, which can give information on the protein unfolding, aggregation and dynamic properties (Rodrigues, Avelar, Vicente, Petersen, & Pereira, 2020). The emission fluorescence spectra of plasma treated and untreated β -LG were obtained (Fig. 3a). It was observed that a maximal fluorescence intensity (FI_{max}) was obtained at 338 nm, and a gradual decline in FI_{max} occurred as the time of plasma treatment increased. The FI_{max} was decreased by 37.25 and 93.83% after 1 and 4 min of treatment time, respectively. There was no shift observed in FI_{max} wavelength (λ_{max}). Our results indicate that plasma treatment disturbed the microenvironment of Trp as a result of the structural variation and amino acid alteration of β -LG (Engelborghs & Fersht, 2006). There are two tryptophan residues present in β -LG at positions 19 and 61 (Fig. 3c). Trp 19 is near the end of β -sheet A, which is located at the bottom of the internal hydrophobic cavity of the β -LG β -barrel, adjacent to Tyr 20, and is relatively far from Trp 61. Trp 61 is located at the end of β -sheet C. Trp 19 is completely buried in the β -LG globular structure and is shielded from the surrounding solvent, unlike W61, which is exposed at the surface of the globule (Fig. 3c). Two reasons can be proposed for the quenching of the β -LG fluorescence: one is the unfolding of the protein resulting in the position of Trp residues, which were originally buried inside β -LG, shifting to the surface of the globular protein where the environment is more polar, after the plasma treatment. The other reason can be ascribed to the oxidation of the Trp residues, especially Trp 61, upon exposure to the plasma active species. Trp residues are sensitive and preferentially oxidized by plasma-induced active species as has been reported by Takai

et al. (2014), who reported that hydroxylation and nitration of the aromatic ring of tryptophan, phenylalanine and tyrosine upon exposure to plasma treatment, was detected by high-resolution mass spectrometry.

3.4. UV-visible spectroscopy

To further analyze the chemical and structural changes to β -LG caused by plasma treatment, the UV spectra were determined to monitor the micro-environmental of aromatic amino acids. Usually, proteins exhibit an absorption maximum from 275 to 280 nm, which is ascribed to the absorbance of the aromatic amino acids tryptophan (Trp, 280 nm) and tyrosine (Tyr, 275 nm). Variation in the UV absorption spectra has been reported to be extremely useful for monitoring changes in proteins after functional groups exhibiting electron resonance are subjected to different micro-environments (Wang et al., 2017). In Fig. 3b, a strong absorption peak was observed at 278 nm for native β -LG, due to the absorption of Trp and Tyr residues. The absorption intensity of β -LG was found to gradually increase with an increase in plasma treatment time, which was accompanied by a shift in the wavelength maxima of the absorption peak. A blue shift in the wavelengths of the maximum absorbance peaks occurred with plasma treated β -LG from 278 nm to 272 nm after 1 min of plasma treatment that suggests unfolding of β -LG and changes in the polarity of the micro-environment of amino acids (Ghisaidoobe & Chung, 2014). With further prolonged plasma treatment time (of more than 2 min), the gradual disappearance of the characteristic absorbance peak of Trp and Tyr residues was observed. These results imply that a partial oxidation of Trp and Tyr residues of β -LG occurred. The spectral analysis data are consistent with the results of SDS-PAGE of β -LG. The two Trp (W19 and 61) and four Tyr (Y20, 42, 99 and 102) residues in β -LG are depicted in Fig. 3d. The aromatic side chains of W61, Y20, Y99 and Y102 are located on the surface of the

globular β -LG protein whereas the others are buried (Fig. 3c). This infers that exposed amino acid residues are more accessible to the plasma active species, and hence are more susceptible to oxidation, resulting in the modifications mentioned above.

3.5. Hydrophobicity analysis

An analysis of the surface hydrophobicity of β -LG was carried out using the fluorescent probe ANS to monitor plasma-induced structural alterations. Conventionally, ANS is considered to bind to pre-existing hydrophobic sites on the protein surface through its non-polar anilino-naphthalene group and this increases the ANS fluorescence intensity (Matulis, Baumann, Bloomfield, & Lovrien, 1999). A substantial increase in fluorescence intensity was observed after 1 min of plasma treatment (Fig. 4a), indicating an increase in the hydrophobicity of β -LG due to additional hydrophobic sites being exposed on the protein surface. This indicates that plasma treatment causes a modification of the secondary structure of β -LG, and unfolding of the structure increased the ANS accessibility. Increasing the plasma treatment time from 2 to 4 min was found to cause a slow decrease in the fluorescence intensity, indicating that the number of hydrophobic sites on the surface of β -LG was reduced upon extended plasma treatment. This may be due to the oxidation of hydrophobic amino acids, Phe, Trp and Tyr, in β -LG. Takai et al. (2014) reported that Met, Cys, Trp, Tyr, and Phe, were oxidized by plasma species, and plasma treatment caused the hydroxylation and nitration of the aromatic rings of Tyr, Trp, and Phe. The results obtained from the hydrophobicity analysis were in agreement with the alteration in the intrinsic fluorescence emission and UV-visible spectroscopy of β -LG.

3.6. Secondary structure analysis

Circular dichroism (CD) in the far-UV is an adequate spectroscopic technique used to analyze the secondary structure of a protein (Rogers et al., 2019). The characteristic secondary structure of β -LG is that of a β -barrel protein consisting of nine β -strands (A–I) and one major α -helix on the surface of the globular protein (Kurpiewska et al., 2019). Fig. 4b depicts the CD spectra of plasma-treated and untreated β -LG. For untreated β -LG, there is a negative peak around 220 nm indicating the β -sheet structure of β -LG. During plasma treatment, the CD spectral negative ellipticity values were found to gradually decrease and there was an obvious shift of peak wavelength from 220 nm (untreated) to 230 nm (4 min), indicating disruption of the ordered structure of β -LG. In Fig. 4c, the amount of α -helix was found to decrease significantly ($p < 0.05$) from 13.26% to 11.58%, and was accompanied by an increase in β -sheet from 32.43% to 34.31%, respectively. No significant change in β -turn and random coil was observed as a result of plasma treatment. When the plasma treatment time was increased from 2 to 4 min, there was a reduction in the negative ellipticity value, but no significant changes were detected in the secondary structure. These results are consistent with the findings of hydrophobicity analysis. Changes in the secondary structure of β -LG may be ascribed to the oxidation of NH-/NH₂- of the peptide bond, which resulted in disruption of hydrogen bonds between amino acid residues, that constitute the key non-covalent force to retain protein's secondary structure (Tolouie et al., 2018). Earlier studies have reported that short-lived radicals such as OH, and ¹O₂ preferentially oxidize amino acid side-chains, -NH and -NH₂, resulting in a deamination reaction and generation of carbonyls (Cheng et al., 2020; Segat et al., 2015).

3.7. HPLC-MS analysis

More evidence for plasma oxidation-induced cleavage of the amino acid polymer of β -LG was obtained by LC-MS/MS. As shown in Supp. Table 1, fragmentation of the β -LG polypeptide chain occurred after exposure to plasma for 4 min, for which 8 fragmentation positions (¹LIVTQTM⁷; ⁹GLDIQKVA¹⁶; ²⁴MAASDISLL³²; ³³DAQSAPLRV⁴¹

⁴³VEELKPTPEG⁵⁹; ⁸⁶ALNENKVLV⁹⁴; ¹³⁰DEALEKF¹³⁶; ¹⁵⁵QLEEQCHI¹⁶²) and 25 peptides were detected. These results support the results obtained in the SDS-PAGE analysis. From analysis of the MS data and the peptide positions in β -LG, it was found that the breakage sites were not random, but appeared to follow a pattern (Fig. 5): 1) Peptide sequences containing Ala, Asp, Gly, Gln, Leu, Lys and Tyr, which were close to Met, Tyr and Phe, were found to be prone to oxidation by plasma-reactive species; 2) The cleavage of the polypeptide chain of β -LG was preferential near to the C-terminal and N-terminal, which is located on the surface of β -LG; and 3) The structure of the α -helix exhibited was sensitive to oxidation by the plasma treatment, more so than the β -sheet elements.

3.8. Antigenicity analysis

To evaluate changes in the antigenicity of β -LG after exposure to plasma, western-blotting (WB) and ELISA analyses were carried out. As depicted in Fig. 6a, a noticeable decrease in blot intensity was seen as a result of increasing the plasma exposure time, and was quite faint after 3 min of plasma treatment. Increasing the time of plasma treatment to 4 min resulted in no signal being evident on the blot, and was accompanied by a smearing in the lane. These results are in agreement with the change of protein pattern of β -LG analyzed by SDS-PAGE under reducing conditions. The change in the blot intensity indicated that the antigenicity of β -LG was considerably reduced by plasma treatment. To further verify and to quantify the antigenicity reduction of β -LG by plasma treatment, ELISA was conducted in parallel to the WB (Fig. 6b). The result indicated a gradual and significant decline in the IgG binding capacity of β -LG to $61.07 \pm 2.12\%$ after 1 min of treatment and to $41.79 \pm 2.73\%$ after 4 min of treatment compared to the untreated sample. These results were in agreement with those obtained by WB analysis.

Further analysis by ELISA revealed that two steps were involved in decreasing the IgG binding capacity of β -LG. The first step was observed after 1 min of plasma treatment where the IgG binding capacity substantially dropped to $61.07 \pm 2.12\%$ and was not changed after further plasma treatment up to 2 min ($60.83 \pm 2.37\%$). An increase in plasma treatment time to 3 min generated a further decline ($43.62 \pm 1.74\%$), but no significant change was observed as the plasma treatment time was increased to 4 min. This phenomenon can be explained by the results obtained in the structure analysis, suggesting that the IgG binding capacity of β -LG is directly correlated with its structural conformation. It is well known that the immunoreactivity of a protein allergen is mainly determined by linear (sequential) and conformational (discontinuous) epitopes. Linear epitopes consist of a continuous peptide fragment, while conformational epitopes are a three-dimensional motif formed by spatially adjacent amino acids (Ekezie et al., 2018b). Several authors have reported that the linear epitopes (for IgE and IgG), including the peptides ¹LIVTQTMKGLDIQKV¹⁵, ⁶TMKGLDIQKVAGTW¹⁹ and ¹²¹CLVRTPEVDDEAL¹³³, ¹³⁶FDKALKALPMHRLS¹⁵⁰, ¹⁵⁴TQLE¹⁵⁷ (Niemi et al., 2007; Villa et al., 2018; Williams, Badley, Davis, Puijk, & Meloen, 1998), are located mainly at the protein surface and α -helix of the β -LG (Fig. 6c). These regions of β -LG are susceptible to the actions of the reactive species as discussed in the LC-MS/MS analysis section.

Therefore, we deduce that a two-step decrease in the IgG binding capacity of β -LG was related to a stepwise change in its structure. In the first step, the oxidizing effect of plasma led to oxidation of NH-/NH₂- on the peptide bond, which was accompanied by oxidation of sensitive amino acid residues (Phe, Trp, and Tyr,) and disruption of hydrogen bonds and other non-covalent forces among the amino acid residues. Collectively, these changes result in structural modification, free sulfhydryl exposure and the cross-linkage of β -LG molecules (Segat et al., 2015; Tolouie et al., 2018), which have been confirmed in the present study by SDS-PAGE, surface hydrophobicity, and multi-spectroscopic analyses. In this step, the reduction of the IgG binding capacity of β -LG was mainly ascribed to disturbance of the conformational epitopes and/or the association of the molecule partly blocking the linear

epitopes. In the second step, the plasma reactive species accumulated in the aqueous solution upon increasing the treatment time (>3 min), causing advanced oxidation that led to the cleavage of the disulfide bond and the disintegration of the β -LG, and resulted in an unfolding of its structure as well as the exposure of aromatic amino acids (Trp and Tyr). In addition, cleavage of the polypeptide partially destroyed the linear epitopes, resulting in a further decline in its IgG binding capacity.

4. Conclusion

In the current study, an effective elimination of antigenicity of β -LG was achieved by plasma treatment, which was directly correlated with conformational alteration and cleavage of the β -LG polypeptide chain. Plasma treatment has great potential as an alternative technique to enzymatic hydrolysis for producing hypoallergenic food materials. Plasma treatment not only exhibits equivalent effects to enzymatic hydrolysis for reduction of linear epitopes, resulting from polypeptide chain breakdown of protein, but also has a unique advantage of eliminating the effect of key amino acids in epitopes by oxidation. Further studies should be conducted to examine the functionality and safety of plasma-treated proteins.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.129821>.

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Supplementary data

Supp. Table 1 LC-MS/MS data of peptides detected from β -LG after 4 min of DBD plasma treatment.

* The color of in the section of Sequence numbers is matched to the marked color in Fig. 5. The sequence between the dot represents the peptide. -10lgP: score of peptides, the higher is the value, the more is reliable; PTM: chemical reaction, (+15.99) represents the mass increase of methionine residue after oxidation

Sequence numbers	Peptide	-10lgP	Mass	Length	m/z	z	RT	PTM
1-7	A.LIVTQTM(+15.99).K	49.77	820.44	7	411.2	2	27.97	Oxidation
1-9	A.LIVTQTM(+15.99)K G.L	44.36	1005.6	9	503.8	2	21.89	Oxidation
1-8	A.LIVTQTM(+15.99)K. G	39.39	948.53	8	475.3	2	21.8	Oxidation
1-6	A.LIVTQT.M	28.06	673.4	6	337.7	2	29.3	
24-32	A.M(+15.99)AASDISL L.D	36.49	935.46	9	468.7	2	53.21	Oxidation
26-33	A.ASDISLLD.A	34.49	832.42	8	417.2	2	48.54	
26-32	A.ASDISLL.D	28.33	717.39	7	359.7	2	52.77	
27-32	A.SDISLL.D	21.84	646.35	6	324.2	2	52.74	
86-93	D.ALNENKVL.V	39.17	899.51	8	450.8	2	30.9	
86-94	D.ALNENKVLV.L	15.93	998.58	9	500.3	2	36.1	
130-136	D.DEALEKF.D	54.03	850.41	7	426.2	2	37.54	
10-16	G.LDIQKVA.G	31.15	785.46	7	393.7	2	32.87	
10-14	G.LDIQK.V	20.68	615.36	5	308.7	2	18.59	
9-14	K.GLDIQK.V	24.71	672.38	6	337.2	2	22.18	
9-16	K.GLDIQKVA.G	18.99	842.49	8	422.3	2	36.05	

9-15	K.GLDIQKV.A	17.25	771.4 5	7	386. 7	2	35.7 8
33-41	L.DAQSAPLRV.Y	64.03	955.5 1	9	478. 8	2	32.2 1
33-42	L.DAQSAPLRVY.V	47.32	1118. 6	10	560. 3	2	37.5 4
33-39	L.DAQSAPL.R	33.12	700.3 4	7	351. 2	2	24.8 7
32-40	L.LDAQSAPLR.V	37.52	969.5 2	9	485. 8	2	24.9 6
36-42	Q.SAPLRVY.V	42.27	804.4 5	7	403. 2	2	32.9
36-41	Q.SAPLRV.Y	33.59	641.3 9	6	321. 7	2	26.6 9
155-162	T.QLEEQCHI	47.77	1055. 5	8	528. 7	2	26.9 9
43-49	Y.VEELKPT.P	29.8	814.4 4	7	408. 2	2	19.2 1
43-52	Y.VEELKPTPEG.D	28.22	1097. 5	10	549. 8	2	24.2 4