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Site specific PEGylation of β-lactoglobulin at glutamine residues and its influence on conformation and antigenicity

Shunjing Luo, Xuli Lu, Chengmei Liu, Junzhen Zhong*, Lei Zhou, Tingting Chen

State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China

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ABSTRACT

β-lactoglobulin (β-LG) is one of the main allergens in milk. Polyethylene glycol (PEG) modification (PEGylation) was found to have the ability to reduce the antigenicity of proteins. To determine the effect of site specific PEGylation on β-LG antigenicity and conformation, we applied 5 kDa methoxy polyethylene glycol-amine (mPEG-NH_2) to modify β-LG at glutamine (Gln) residues under the catalysis of transglutaminase. The antigenicity of β-LG was measured using rabbit IgG antibodies by indirect competitive ELISA. The result indicated that the antigenicity of β-LG was decreased from 72.2 μg/mL to 22.7 μg/mL after PEGylation. SDS-PAGE and MALDI-TOF-MS showed that the molecular mass of native β-LG was about 18.3 kDa while the PEGylated β-LG had a molecular mass of 23.4 kDa, which meant that mono-PEGylated β-LG was obtained after PEGylation and purification by cation exchange chromatography. Additionally, the circular dichroism spectrum of the PEGylated β-LG was approximately superimposed on that of β-LG and the secondary structure content of β-LG also had no significant changes after PEGylation, which indicated that the secondary structure of β-LG was preserved. After PEGylation, the intrinsic fluorescence intensity of β-LG decreased from 6361 to 5159 while the surface hydrophobicity increased, which indicated that the tertiary structure of β-LG was slightly changed. PEGylation site analysis result showed that Gln 155 or Gln 159 might be the most possible binding site. In conclusion, the decrease of the antigenicity of β-LG induced by the PEGylation is mainly due to the steric shielding effect of PEG chain rather than conformational changes of β-LG.

1. Introduction

Bovine β-lactoglobulin (β-LG) is a high-quality protein with high alimentation value and a variety of functional characters like foamability, emulsibility and gelation, and is usually used as food ingredient in food production (Chanphai, Bekale, Sanyakamdhorn, Agudelo, & Tajmir-Riahi, 2014; Chen, Chen, Guo, & Zhou, 2015; Kontopidis, Holt, & Sawyer, 2004). However, the native β-LG is also one of the main allergens in milk which has limited its application in the food industry (Chen et al., 2016). Recently, many methods have been studied to reduce the antigenicity of β-LG, including physical treatment, such as heating (Kleber, Krause, Illgner, & Hinrichs, 2004) and dynamic high-pressure micro-fluidization (DHPM) (Zhong et al., 2011), and chemical modification, such as glycation (Zhong et al., 2013; Zhong, Yue, Wei, Luo, & Liu, 2015) and PEGylation (Zhong et al., 2016). PEGylation is a valid strategy for reducing antigenicity and improving the biochemical properties of proteins (Mero, Schiavon, Veronese, & Pasut, 2011). Polyethylene glycol (PEG) with molecular mass above 1000 Da is non-toxic and the US Food and Drug Administration (FDA) has approved its application in foods, drugs and cosmetics (Liu et al., 2014; Zhong et al., 2016). At present, random PEGylation has gradually been applied to some enzymes used in food industry, such as glucose oxidase (Ritter, Roberts, & McShane, 2013), phospholipase (Fang et al., 2018) and trypsin (Liu et al., 2014), to enhance their thermal stability and catalytic efficiency. Moreover, it has also been applied to some food proteins to improve their functional properties or reduce their antigenicity (Yang et al., 2018). It has been reported that random PEGylation can reduce the antigenicity of β-LG (Nijs et al., 1997; Wróblewska & Jedrychowski, 2002; Zhong et al., 2016). However, the random PEGylation may often obtain a mixture of multi-PEGylated species due to

Abbreviations: β-LG, β-lactoglobulin; PEG, polyethylene glycol; Gln, glutamine; TGase, transglutaminase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry; ELISA, enzyme-linked immuno sorbent assay; mPEG-NH_2, methoxy polyethylene glycol-amine; mPEG-SC, monomethoxy polyethylene glycol-succinimidyl carbonates; ANS, 1-anilinonaphthalene-8-sulfonate; Trp, tryptophan; Tyr, tyrosine; Phe, phenylalanine

* Corresponding author at: State Key Laboratory of Food Science and Technology, Nanchang University, Jiangxi, Nanchang 330047, China.
E-mail address: zhongjunzhen@163.com (J. Zhong).

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non-specific conjugation at multiple sites (positional isomers) that is difficult to purify and characterize (Khameneh et al., 2016). The uncertainty of random PEGylation sites also makes it difficult to study the relationship between binding sites and antigenicity changes of β-LG during PEGylation. Besides, under random PEGylation conditions the conformation of β-LG will also be changed (Zhong et al., 2016). Our previous researches indicated that the antigenicity of β-LG was obviously related to the conformational changes induced by different treatments (Zhong et al., 2011; Zhong et al., 2014; Zhong, Tu, Liu, Luo, & Liu, 2015). Due to the above drawbacks of random PEGylation, the mechanism of antigenicity changes of β-LG induced by PEGylation is still not clear.

Enzymatic PEGylation is a new promising site specific PEGylation technique making use of transglutaminase (TGase). TGase is an enzyme that can catalyze the transfer of acyl groups between or within proteins using γ-carboxamide group of glutamine (Gln) residues as acyl donor and primary amine which generally is the ε-amino group of lysine residues as acyl acceptor (Aeschlimann & Paulsson, 1994; Spolaore et al., 2016). The advantage of enzymatic PEGylation is that a more homogeneous PEGylated product can be obtained because there are only a few Gln(s) of all those present in a protein are substrate of TGase (Fontana, Spolaore, Mero, & Veronese, 2008). Besides, compared to random PEGylation, the modification condition of enzymatic PEGylation is milder thus may have no significant influence on the conformation of proteins (Scaramuzza et al., 2012). Therefore, enzymatic PEGylation can overcome the drawbacks of random PEGylation and can contribute to further study the mechanism of antigenicity changes of β-LG induced by PEGylation. At present, enzymatic PEGylation has been widely used for site specific modification of many pharmaceutical proteins with methoxy polyethylene glycol-amine (mPEG-NH_2) used as acyl acceptor (da Silva Freitas, Mero, & Pasut, 2013; Yokoyama, Nio, & Kikuchi, 2004). However, the research of its application on food proteins especially on β-LG has not been reported up to now.

In this study, we applied enzymatic PEGylation to modify β-LG at Gln residues and investigated its influence on the antigenicity and conformation of β-LG aiming to further study the mechanism of its antigenicity changes induced by PEGylation. In this work, β-LG was site specific PEGylated by 5 kDa mPEG-NH_2 under the catalysis of TGase. The mono-PEGylated derivate of β-LG was purified by cation exchange chromatography. After purification, the structural characterization and antigenicity analysis was performed on the obtained PEGylated β-LG. The PEGylation extent of PEGylated β-LG was confirmed by SDS-PAGE analysis and MALDI-TOF-MS. The antigenicity changes were determined by indirect competitive ELISA. The conformational changes were characterized by circular dichroism, fluorescence spectroscopy and surface hydrophobicity analysis. Combining with the conformational changes and the previous research reports, the PEGylation site was predicted according to the three-dimensional structure of β-LG handled by the program PyMol, which helped to analyze the mechanism of its antigenicity changes induced by PEGylation.

2. Materials and methods

2.1. Materials

β-LG from bovine milk (L3908) was purchased from Sigma-Aldrich (Shanghai, China). 5 kDa mPEG-NH_2 was obtained from Jenkem Technology (Beijing, China). TGase from guinea pig liver (200 U/g) was provided by Shanghai yuanye Bio-Technology (Shanghai, China). The relative enzymatic activity of TGase measured in 0.1 M phosphate buffer (pH 7.0) containing 25% ethanol was 119.2% considering the activity of TGase in 0.2 M Tris-HCl buffer (pH 6.0) as 100%. The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) kit was from Beijing Solarbio Science and Technology (Beijing, China).

2.2. Preparation of β-LG conjugated with 5 kDa mPEG-NH_2

According to the method of Mero et al. (2011) with some modification, β-LG (1.0 mg/mL) was dissolved in 0.1 M phosphate buffer (pH 7.0) containing 25% ethanol (v/v). 5 kDa mPEG-NH_2 was added at a 8-fold molar excess and TGase was then added at enzyme/substrate ratio of 1:1 (w/w). The reaction mixture was stirred at the temperature of 25 °C for 12 h and then the solution was adjusted to pH 4.5 with acetic acid. The native β-LG dissolved in 0.1 M phosphate buffer (pH 7.0) was set as a control group. In order to study whether 25% ethanol was responsible for the antigenicity and structural changes of the protein, β-LG treated by 25% ethanol only was set as another control group. Before a series of analyses, 25% ethanol was removed and replaced with 10 mM phosphate buffer (pH 7.0) by ultrafiltration with 10 kDa Amicon Ultra-15 centrifugal Filter Unit (Ultrace-l5 membrane, Millipore).

2.3. Purification of PEGylated β-LG

According to Zhong et al. (2016) and Yu et al. (2007), the reaction mixture was purified by cationic exchange chromatography using SP Sepharose Fast Flow column. The column was first equilibrated with 0.04 M sodium acetate buffer (buffer A, pH 4.0) until the column effluent showed stable conductivity and UV baseline, then the reaction mixture was loaded. The free PEG reagent and other impurities were eluted with buffer A, subsequently, the adsorbed reaction products were successively eluted with a linear gradient of 0–100% buffer B (0.04 M sodium acetate buffer containing 1.0 M sodium chloride, pH 4.0) for 180 min at a flow rate of 2.0 mL/min. Absorbance of the effluent was measured at 280 nm. The peak corresponding to the PEGylated β-LG was collected and the solution was concentrated by centrifugation at 6000 rpm for 20 min at 4 °C with 10 kDa Amicon Ultra-15 centrifugal Filter Unit (Ultrace-l5 membrane, Millipore). PEGylated β-LG at 100 μg/mL was used for the following experiments if not mentioned otherwise.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to Laemmli (1970), the gel consisting of 5% stacking gel and 12% separating gel was used for electrophoresis here. 30 μL protein sample was mixed with 10 μL 4 × loading buffer (with β-Mercaptoethanol). The samples were boiled at 100 °C water path for 7 min and centrifuged for 1 min before electrophoresis. Electrophoresis was run for 15 min at 80 V for stacking gel and 40 min at 120 V for separating gel. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid for at least 30 min, then destained with a water solution of 5% methanol and 7.5% acetic acid. The Thermo Scientific Page Ruler Prestained Protein Ladder (ranging from 10 kDa to 180 kDa) was used as molecular mass standards.

2.5. Matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS)

The molecular mass of β-LG and purified PEGylated β-LG was measured by MALDI-TOF-MS according to Wu et al. (2018). 1 μL of the sample dissolved in 10 mM phosphate buffer (pH 7.0) was spotted on the sample target of the MALDI-TOF MS instrument (5800 MALDI-TOF/TOF, AB Sciex, USA), then 1 μL of sinapinic acid matrix solution was added to cover the sample and crystallized by air-drying. Before testing, the protein standards was used for calibration. The calibration and testing were both performed by linear method in positive ion mode with a pulsed nitrogen laser.

2.6. Indirect competitive enzyme-linked immunosorbent assay (ELISA)

The antigenicity changes of purified PEGylated β-LG were determined by indirect competitive ELISA. According to Zhong et al. (2011) and Kleber et al. (2004), the 96-well microtiter plate was coated with aliquots of native β-LG (2.5 μg/mL, 100 μL/well) and incubated at 4 °C overnight. After washing with PBST (300 μL/well) for 3 times, the plate was blocked with 1% pig gelatin in PBS (250 μL/well) for 1 h at 37 °C and washed for 3 times again. Then 50 μL of the diluted sample solutions in PBS (10 μg/mL) and 50 μL of rabbit anti-beta-lactoglobulin antibody (diluted to 1:1100 with PBS) were successively added to the wells and incubated for 1 h at 37 °C. After washing for 6 times as above, 100 μL of goat anti-rabbit IgG-HRP antibody (diluted to 1:7000 with PBS) was added to each well and incubated for 2 h at 37 °C. The plate was washed for 3 times, and the colour reaction was started by adding 100 μL chromogenic substrate containing 0.4 mg/mL *o*-phenylenediamine to each well. After reaction for 15 min at 37 °C in dark, the reaction was stopped by adding 100 μL of 1 M sulfuric acid. Finally, the absorbance was measured at 490 nm using an HF2000 microplate reader (Huaan Magnech, Beijing, China).

The antigenicity of the sample was calculated from a standard curve of β-LG whereas a linear logarithmic correlation was observed from 0.25 to 50 μg/mL β-LG, and finally expressed as β-LG concentration equivalent in μg/mL by multiplying with the dilution factor. All analyses were carried out in triplicate.

2.7. Circular dichroism spectroscopy

The circular dichroism spectroscopy of β-LG and purified PEGylated β-LG was measured according to de la Hoz and Netto (2008) using a MOS-450 spectropolarimeter (French Bio-Logic SAS, Claix, French). The samples at 0.1 mg/mL in 10 mM sodium phosphate buffer (pH 7.0) for far-UV were analyzed in a cylindrical quartz cuvette with path length of 0.1 cm at 25 °C under a nitrogen atmosphere. The spectra were recorded in far-UV region from 190 to 250 nm with step resolution of 1 nm, scan speed of 100 nm/min, 1.0 nm of bandwidth and nine scans, and corrected by subtracting the buffer baseline spectrum. The secondary structure was calculated by analyzing the spectroscopic data at the online Circular Dichroism Website (http://dichroweb.cryst.bbk.ac.uk/html/process.shtml) (Wu, Lu, Xu, Lin, He, Wu et al., 2018).

2.8. Intrinsic fluorescence analysis

The intrinsic fluorescence of β-LG and purified PEGylated β-LG was measured by using a Fluorescence Spectrophotometer F-7000 (Hitachi, Tokyo, Japan) equipped with a 10 mm square quartz cell. The protein concentration was diluted to 0.1 mg/mL with 10 mM phosphate buffer (pH 7.0) before measurement. The excitation wavelength was 280 nm, the emission spectrum scanning range was from 300 to 450 nm, the excitation and emission slits were 2.5 nm and 5.0 nm respectively, and the scan speed was 1200 nm/min. All samples were analyzed in triplicate.

2.9. Determination of surface hydrophobicity

The surface hydrophobicity of the samples was determined by Fluorescence Spectrophotometer F-7000 (Hitachi, Tokyo, Japan) using ANS (1-anilinonaphthalene-8-sulfonate) fluorescence probe methods according to Liu et al. (2018) and Dai et al. (2017) with some modifications. 4 mL of the samples (0.1 mg/mL) dissolved in 10 mM phosphate buffer (pH 7.0) were mixed with 20 μL ANS solution (8.0 mM in the same buffer). The extrinsic fluorescence intensity of the mixture was measured at the excitation wavelength of 370 nm and emission wavelength from 400 to 600 nm. The excitation and emission slits were both 5.0 nm and the scan speed was 1200 nm/min. The hydrophobicity of the samples was expressed as the fluorescence intensity.

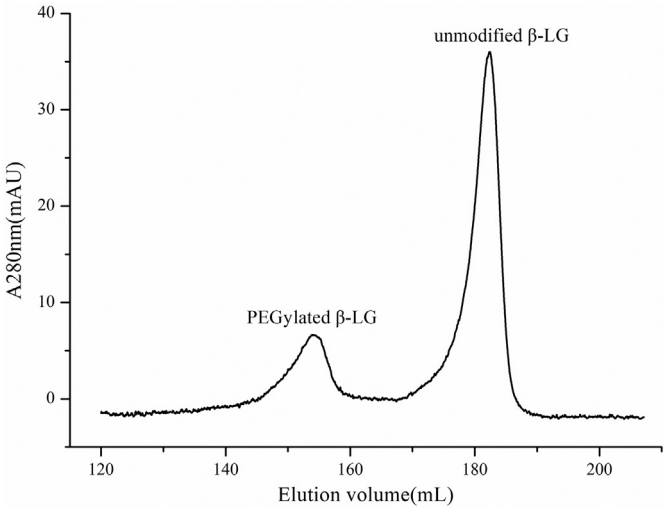

Fig. 1. Cation-exchange chromatography analysis of PEGylation mixtures.

2.10. Statistical analysis

All measurements were performed at least in triplicate and the data were expressed as the mean ± standard deviation (SD). The significant differences of data among samples were analyzed using SPSS Version 11.5 (SPSS Inc., Chicago, IL). $P \leq 0.05$ was considered statistically significant. All the figures were drawn by origin 8.0 (OriginLab Corporation, Northampton. USA).

3. Results and discussion

3.1. Preparation and purification of PEGylated β-LG

β-LG was modified by TGase at Gln residues using 5 kDa mPEG-NH_2. Then the PEGylated β-LG was purified by cationic exchange chromatography on a SP Sepharose Fast Flow column. The PEGylated protein was separated from the unmodified protein and other impurities. There were only two major peaks eluted from the column under the linear salt gradient of 0–1.0 M sodium chloride (Fig. 1). According to the reports of Seely and Richey (2001), the PEG chains conjugated to proteins will mask part of charges on the surface of the protein and weaken the interaction of the protein with the ion-exchange media, thus the PEGylated protein will be eluted before the unmodified protein. Therefore, the former elution peak in Fig. 1 would be the PEGylated β-LG and the latter would be the unmodified β-LG. The SDS-PAGE analysis also proved that the two fractions corresponded to the PEGylated β-LG and unmodified β-LG respectively (Figure not shown). The fraction corresponding to the PEGylated β-LG was collected and concentrated by ultrafiltration.

3.2. Molecular mass change of purified PEGylated β-LG

The molecular mass of purified PEGylated β-LG was determined by SDS-PAGE and MALDI-TOF-MS. The SDS-PAGE analysis under reducing conditions showed that the reaction mixture displayed two apparent bands at around 18 kDa and 28 kDa (Lane 1), while the native β-LG (Lane 3) and purified PEGylated β-LG (Lane 2) displayed a single band at the molecular mass of about 18 kDa and 28 kDa, respectively (Fig. 2). Each ethoxy unit of PEG molecule in solution can combine two or three water molecules, which significantly enhances the hydrodynamic radius of PEGylated products. Therefore, the apparent molecular mass of PEGylated protein usually equals to the molecular mass of the non-PEGylated protein plus about 2–3 times the molecular mass of per PEG molecule (Scaramuzza et al., 2012). As a consequence, the band observed at around 28 kDa was speculated to be the mono-PEGylated β-

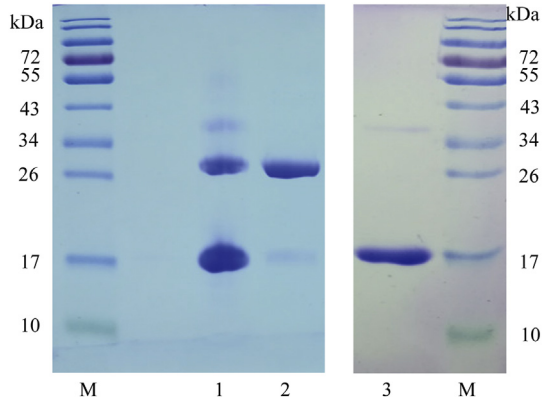

Fig. 2. SDS-PAGE analysis of mono-PEGylated β-LG purified by cation-exchange chromatography. M, protein markers; lane 1, reaction mixture; lane 2, PEGylated β-LG; lane 3, β-LG.

LG.

In order to verify above speculation, the MALDI-TOF-MS analysis was carried out to determine the true molecular mass of the native β-LG and PEGylated β-LG. The mass spectrum indicated that the molecular mass of β-LG was about 18.3 kDa (Fig. 3A). The PEGylated β-LG (Fig. 3B) had a molecular mass of about 23.4 kDa, which was approximately equal to molecular mass of one PEG (about 5 kDa) plus molecular mass of one β-LG molecule. MALDI-TOF-MS analysis confirmed that the obtained PEGylated β-LG was mono-PEGylated β-LG. In another study, tri- and di-PEGylated conjugates was obtained by random PEGylation (Zhong et al., 2016). Compared to random PEGylation, this site specific PEGylation method yielded a more homogeneous PEGylated product, the purification process of which was also easier.

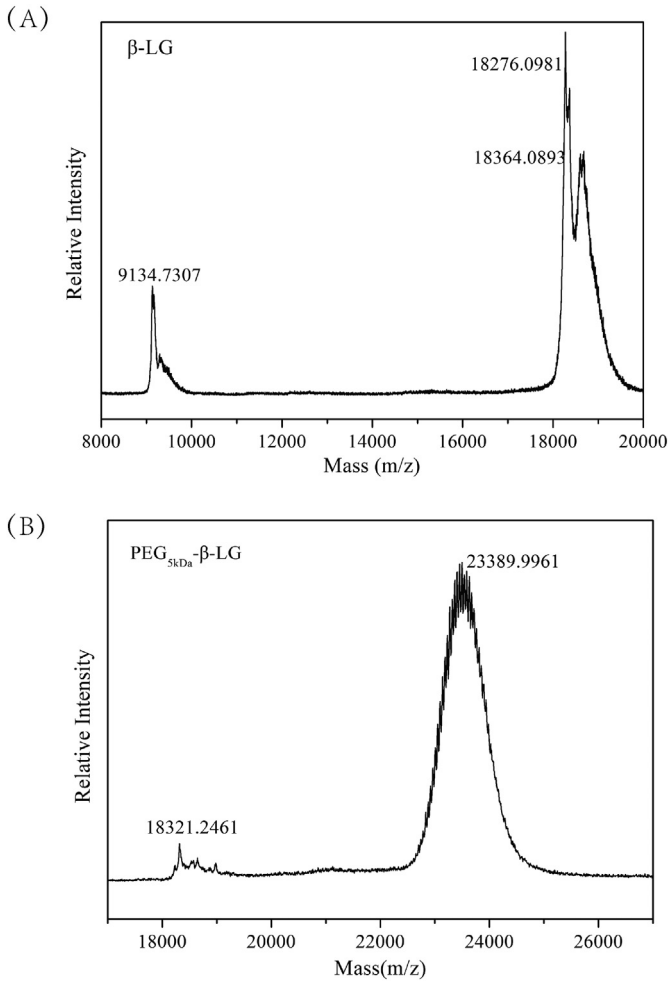

Fig. 3. MALDI-TOF-MS analysis of β-LG (A) and PEGylated β-LG (B).

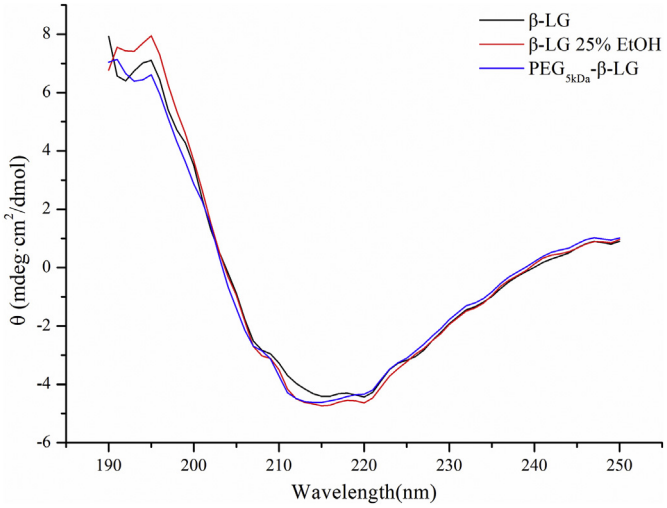

Fig. 4. Far-UV circular dichroism spectra of the native β-LG, PEGylated β-LG and β-LG treated by 25% ethanol.

3.3. Structural characterization of purified PEGylated β-LG

The secondary structure changes of PEGylated β-LG were investigated by circular dichroism analysis in Fig. 4 and Table 1. As shown in Fig. 4, the far UV circular dichroism spectra of β-LG exhibited a negative band at 214 nm which was the characteristic of the β-sheet structure of a protein (Zhong et al., 2016). This corresponded with the exposition of Zhong et al. (2014) that the native β-LG was mainly a β-sheet protein containing nine antiparallel β-strands and one main α-helix. The circular dichroism spectra of the PEGylated β-LG and β-LG treated by 25% ethanol were approximately superimposed on that of β-LG. As shown in Table 1, the secondary structure content of PEGylated β-LG and β-LG treated by 25% ethanol both had no significant changes compared with the native β-LG. This indicated that this PEGylation method didn't essentially affect the secondary structure of β-LG. The result was consistent with the research of da Silva Freitas et al. (2013) on PEGylation of human growth hormone. However, the present results differed from the research of Zhong et al. (2016) who found an increase in the percentage of β-strands after random PEGylation indicating a significantly change in the secondary structure of β-LG. The preservation of the secondary structure indicated that the PEGylation site might be on the surface of β-LG and the PEGylation condition didn't disturb its secondary structure.

Intrinsic fluorescence spectroscopy was applied to detect the changes in the tertiary structure of β-LG upon PEGylation. As shown in Fig. 5, both the PEGylation solvent and PEGylation slightly changed the tertiary structure of β-LG. When excited at 280 nm, the emission

Table 1
Effect of PEGylation on the percentage content of secondary structures of β-LG.

Sample	α-helix (%)	β-stands (%)	Turns (%)	Unordered (%)
β-LG	10.7 ± 0.78[a]	36.2 ± 0.85[a]	20.4 ± 0.46[a]	32.6 ± 1.01[a]
β-LG 25% EtOH	11.8 ± 0.35[a]	35.7 ± 0.91[a]	20.7 ± 0.40[a]	31.8 ± 0.35[a]
PEG_{5kDa}-β-LG	10.7 ± 1.11[a]	36.3 ± 0.32[a]	20.2 ± 0.70[a]	32.7 ± 1.47[a]

Means in the same column followed by the same letter denote no significantly different ($P > 0.05$).

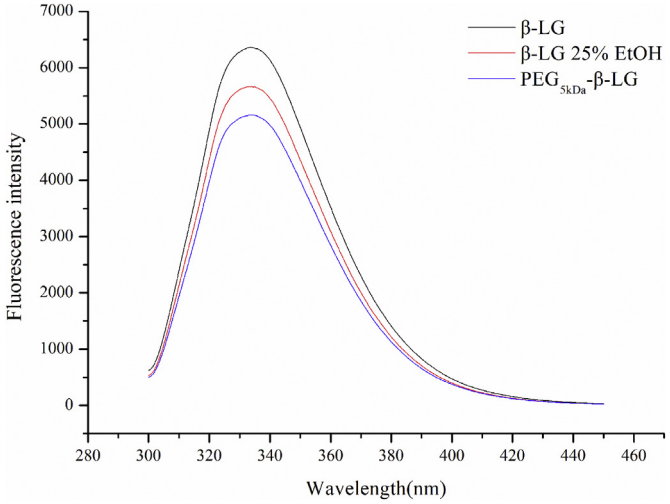

Fig. 5. Intrinsic fluorescent spectra of the native β-LG, PEGylated β-LG and β-LG treated by 25% ethanol.

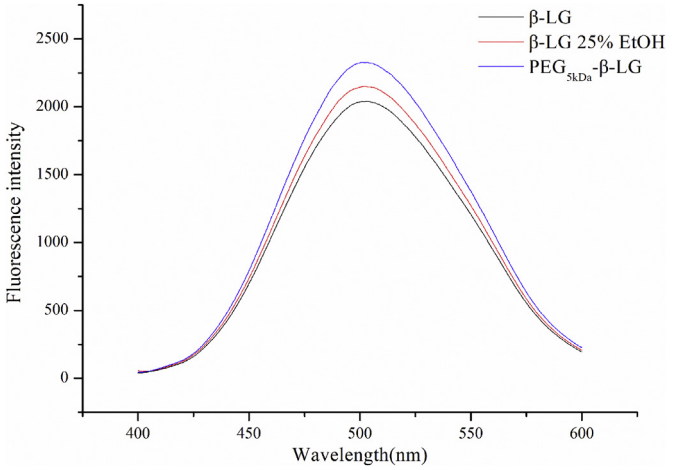

Fig. 6. Surface hydrophobicity of the native β-LG, PEGylated β-LG and β-LG treated by 25% ethanol.

fluorescence intensity of PEGylated β-LG and β-LG treated by 25% ethanol decreased from 6361 (control) to 5159 and 5665, respectively, without shift in the maximal emission wavelength (Fig. 5). The result was similar to the research of Liu et al. (2012) that the PEGylation catalyzed by TGase could slightly perturb the tertiary structure of staphylokinase. In addition, Zhong et al. (2016) also reported that the random PEGylation contributed to a decrease in intrinsic fluorescence intensity of β-LG. In proteins, some amino acids such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) have the ability to emit fluorescence (Ma, Wang, & Guo, 2018). The intrinsic fluorescence of β-LG is mainly excited from Trp residues (Zhong et al., 2013). According to Ma et al. (2018), the decrease of intrinsic fluorescence intensity of β-LG may be because of the exposure of Trp residues to solvents. Trp residues exposed to the less hydrophobic environment resulting in a decrease of fluorescence intensity (Zhong et al., 2016). Therefore, 25% ethanol added during PEGylation might make the structure of β-LG loose and because of which Trp residues used to be in a hydrophobic area exposed to solvents. In addition, Jimenezcastano, Lopezfandino, Olano, and Villamiel (2005) indicated that the shielding effect of ribose conjugated to Trp residues also led to the decrease of intrinsic fluorescence intensity of β-LG conjugation. As Zhong et al. (2016) mentioned, the proximity of the conjugated PEG chains to the Trp residues played an important role in the changes of fluorescence intensity. According to the research of Mu, Hu, and Yu (2013), PEG chain with high flexibility can form a characteristic hydrated layer around the proteins, thus leading to the steric shielding effect of PEG. Therefore, the decrease of intrinsic fluorescence intensity of PEGylated β-LG might be attributed to the combined action of the shielding effect of PEG chain as well as the exposure of Trp residues caused by 25% ethanol during PEGylation.

The surface hydrophobicity of proteins is usually determined by the fluorescent probe of ANS. As shown in Fig. 6, compared with β-LG (control), the extrinsic fluorescence intensity of PEGylated β-LG and β-LG treated by 25% ethanol both increased with the maximum at 501 nm. The result was in agreement with Liu et al. (2012), who reported that PEGylation induced increase in the extrinsic fluorescence intensity of staphylokinase. β-LG possesses a large number of hydrophobic amino acid residues located inside the molecule (Zhong, Tu, et al., 2015). The increase of the surface hydrophobicity of PEGylated β-LG may be mainly due to the breaking of hydrogen bonds and van der Waals forces during PEGylation, therefore the hydrophobic amino acid inside of the protein molecule exposed to the outside of the protein.

3.4. PEGylation site analysis

According to Fontana et al. (2008), the modification and PEGylation site(s) mediated by TGase can be predicted based on its structure and dynamics. However, surface accessibility of the Gln residues is not the mainly accordance to predict the TGase attack sites because there are also several Gln residues exposed on the surface of proteins can't react with TGase. Therefore, they concluded that only the Gln residues located in a flexible or unfolded region could be attacked by TGase. Additionally, they also proposed that the limited proteolysis regions were also the TGase attack regions because they held the characteristics of flexibility or local unfolding of the polypeptide chain (Fontana et al., 1986; Fontana et al., 2004; Fontana, Laureto, Filippis, Scaramella, & Zambonin, 1999). β-LG, a highly structured compact globular protein, consists of 162 amino acids including nine Gln residues (Gln 5, Gln 13, Gln 35, Gln 59, Gln 68, Gln 115, Gln 120, Gln 155 and Gln 159) and two disulphide bonds (Cys 66-Cys 160 and Cys 106-Cys 119) as well as a free thiol group (Cys 121) in each monomer (Hemung, Li-Chan, & Yongsawatdigul, 2009). The tridimensional structure of β-LG determined by X-ray crystallography (Fig. 7A) presents a flattened β-barrel formed by eight anti-parallel β-sheet (named A to H in Fig.7A) and another β-sheet (named I) as well as a three-turn α-helix (named S3) lies on the surface (Papiz et al., 1986). Previous studies have indicated that both C-terminal and N-terminal regions of β-LG are easier to be hydrolyzed compared to the parts located in the β-barrel since the terminal regions are relatively free (Cheison, Lai, Leeb, & Kulozik, 2011; Cheison, Leeb, Letzel, & Kulozik, 2011). Dalgalarrondo, Dufour, Chobert, Bertrand-Harb, and Haertlé (1995) also proposed that all the peptide bonds situated in the β-barrel core structure of β-LG were resistance to pepsin except for the two terminal peptides 2–10 and 157–162. Thus the PEGylation site of β-LG catalyzed by TGase might be one of the four Gln residues (Gln 5, Gln 13, Gln 155 and Gln 159) respectively located in the two terminal regions and outside the β-barrel core structure (see in Fig.7C).

According to Coussons, Price, Kelly, Smith, and Sawyer (1992b), the Gln 13, Gln 35, Gln 68 and Gln 120 were not prominently exposed to the solvent so it was difficult to be attacked by TGase (Fig. 7A.B). The Gln 5 and Gln 59 also seemed to be not the PEGylation site catalyzed by TGase, since they were close to the positively charged amino acids (Lys 8 and Lys 60 respectively) which were proved to hinder the TGase catalytic reaction by preventing the access of primary amine substrate (Coussons et al., 1991; Coussons, Price, Kelly, Smith, & Sawyer, 1992a). In conclusion, the Gln 155 and Gln 159 may be the most possible candidates for the TGase-catalyzed PEGylation sites. This speculation was also proved in the study of Coussons et al. (1992b) by reacting

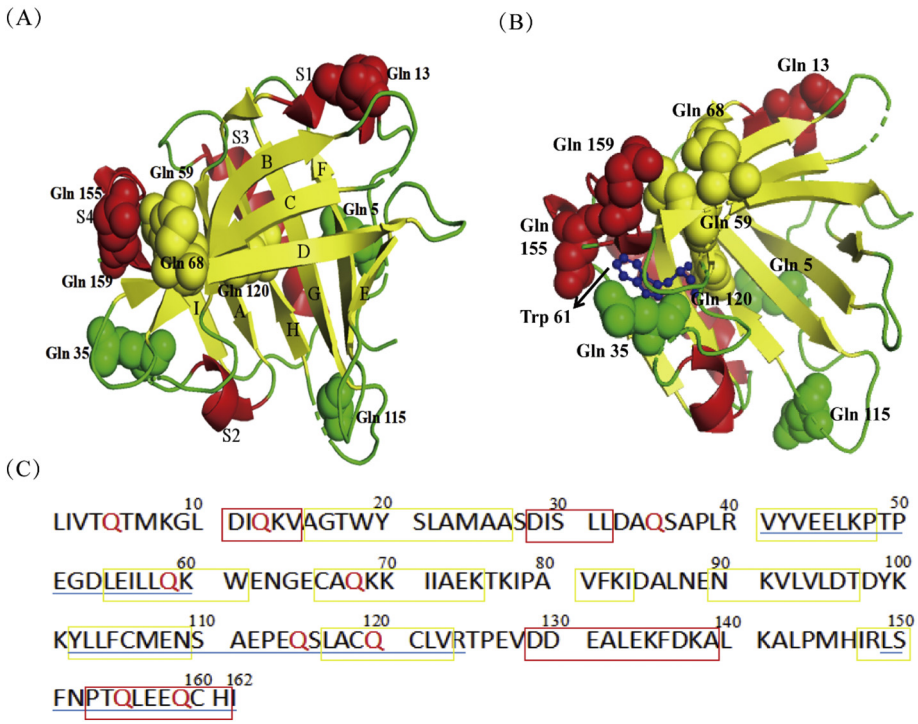

Fig. 7. TGase-mediated PEGylation of β-LG. (A) (B) Three-dimensional structure of bovine β-LG derived from the X-ray structure of the protein (file 3npo taken from the Protein Data Bank in Europe) using the program PyMol. The nine major β-sheet segments (A to I) are colored in yellow and the four α-helices (S1, S2, S3 and S4) in red. The nine Gln residues are showed as spheres. The Trp 61 is showed as balls and sticks colored in blue. (C) Amino acid sequence of bovine β-LG. β-sheet and α-helix regions are indicated by yellow and red boxes respectively. The nine Gln residues are colored in red and the three major epitopes AA41-60, AA102-124, and AA149-162 are underlined in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

putrescine and monodansylcadaverine with β-LG under the catalysis of TGase. As shown in Fig. 7B and Fig. 8A, the Gln 155 and Gln 159 were close to the fluorescence emitting group Trp 61. In addition, according to Palazolo, Rodríguez, Farruggia, Picó, and Delorenzi (2000) and the amino acid sequence of β-LG (Fig. 7C), the Gln 155, Gln 159 and Trp 61 were all near the disulphide bond Cys 66-Cys 160, which further illustrated that the two Gln residues were in the vicinity of the Trp 61. Therefore, the above speculation that the decrease of intrinsic fluorescence intensity of PEGylated β-LG was partly due to the steric shielding effect of PEG chain to the Trp residues seems to be possible.

3.5. Antigenicity analysis of purified PEGylated β-LG

As shown in Fig. 8B, after PEGylation the antigenicity of β-LG decreased from 72.2 μg/mL to 22.7 μg/mL, which was about 69% lower than that of control β-LG, while 25% ethanol had no significant influence on the antigenicity of β-LG. The result indicated that the reduction in antigenicity was mainly due to the PEG chains conjugated to β-LG rather than the reaction conditions. The result that PEGylation can significantly reduce the antigenicity of β-LG was similar to the research result of Zhong et al. (2016) who reported that after random PEGylation the antigenicity of β-LG reduced about 70%. Several studies suggested that the antigenicity of β-LG depended on both linear (continuous, sequential) and conformational (nonlinear) epitopes (Ball et al., 1994; Clement et al., 2002). Our previous studies also showed that the antigenicity of β-LG was associated with its conformational changes (Liu et al., 2011; Zhong et al., 2013; Zhong, Tu, et al., 2015). However, in this study, the reduction of the antigenicity of PEGylated β-LG had nothing to do with its conformation as its conformation had no obvious changes after PEGylation. In addition, as mentioned above, the flexible PEG chain can form a characteristic hydrated layer around the proteins, thus leading to the steric shielding effect of PEG to the proteins (Mu et al., 2013). Therefore, the reduction of the antigenicity of PEGylated β-LG might be mainly due to the steric shielding effect of PEG chain.

As Sélo et al. (1999) reported, the fragments AA41-60, AA102-124, and AA149-162 (shown in Fig. 8A) were the major epitopes of β-LG as recognized by 92%, 97% and 89% of sera respectively. Secondly, the fragments AA1-8, AA25-40 and AA92-100 could be recognized by 52%–65% of sera. And the less recognized epitopes AA9-14 and AA84-91 could be recognized by about 40% of sera (Wal, 2001). As shown in Fig. 8A, the epitope AA149-162 including Gln 155 and Gln 159 was on the surface of β-LG molecule which might be easier to be attacked by the specific antibodies. According to our binding site analysis above, the long PEG chain with a large molecular mass was most likely to be conjugated at Gln 155 or Gln 159 and covered on the surface of β-LG molecule. Therefore, the PEG chain masked the epitope AA149-162 and some other linear and conformational epitopes nearby thus prevented their binding to the specific antibodies, because of which the antigenicity of PEGylated β-LG reduced significantly.

4. Conclusions

We investigated a new method of reducing the antigenicity of β-LG by site specifically conjugating β-LG with PEG at Gln residues and clarified the mechanism of antigenicity changes of β-LG by studying its influence on antigenicity and conformation. The results showed that homogeneous mono-PEGylated β-LG with molecular mass of 23.4 kDa was obtained by site specific PEGylation. After PEGylation, the antigenicity of β-LG was decreased from 72.2 μg/mL to 22.7 μg/mL. Here, the reduction of antigenicity of β-LG could be due to the structural changes of β-LG or steric hindrance effect of PEG. However, we observed no obvious changes on the conformation of β-LG according to the results of circular dichroism, intrinsic and extrinsic fluorescence analysis. Therefore, we proposed that the steric hindrance effect of PEG was the main reason for the decrease of antigenicity of β-LG. In detail, the flexible and bulky PEG chain shielded some epitopes of β-LG and interfered with their binding to the specific antibodies thus reduced the antigenicity of β-LG. Compared to other chemical modification methods, such as glycation and random PEGylation, site specific PEGylation is a novel method for reducing the antigenicity of β-LG and at the same time with no tremendous influence on its conformation. In order to further explore the application of PEGylated β-LG in food industry, the subsequent studies can focus on investigating the effects of PEGylation on the digestibility, thermal stability and functional properties of β-LG such as foamability, emulsibility and gelation.

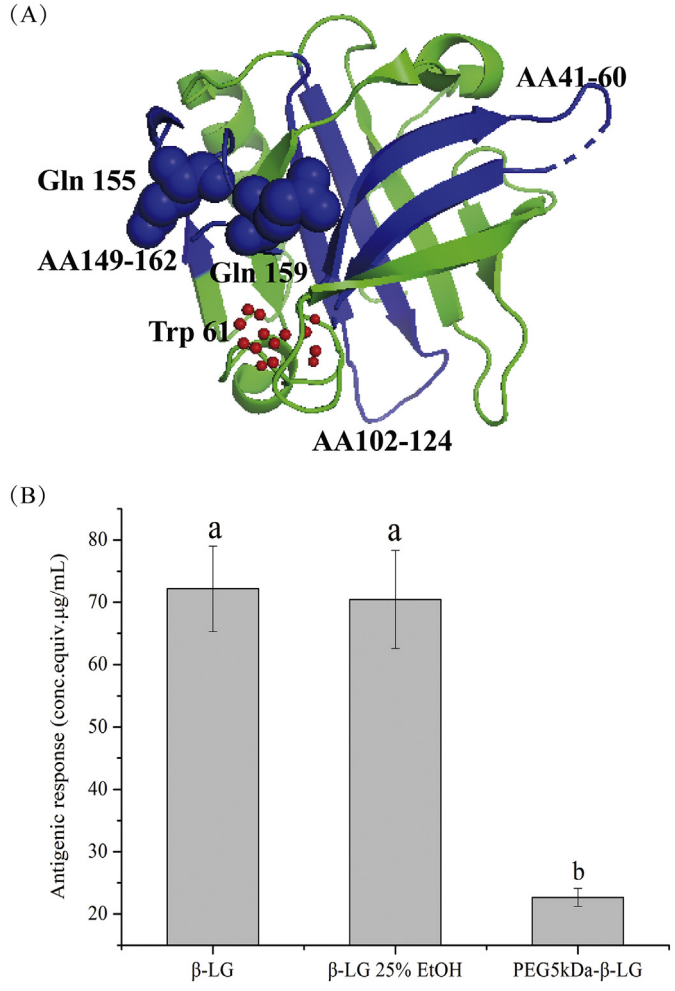

Fig. 8. The antigenicity analysis of β-LG. (A) Three-dimensional structure of bovine β-LG derived from the X-ray structure of the protein (file 3npo taken from the Protein Data Bank in Europe) using the program PyMol. The three major epitopes AA41-60, AA102-124, and AA149-162 are colored in blue. The Gln 155 and Gln 159 are showed as spheres. The Trp 61 is showed as balls and sticks colored in red. (B) Effect of PEGylation on the antigenicity of β-LG. Different letters (a-b) denote significant differences of different samples ($P \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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