

Reducing the allergenic capacity of β -lactoglobulin by covalent conjugation with dietary polyphenols



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ABSTRACT

To help produce hypoallergenic food, this study investigated reducing the allergenicity and improving the functional properties of bovine β -lactoglobulin (β LG) by covalent conjugation with (–)-epigallo-catechin 3-gallate (EGCG) and chlorogenic acid (CA). The covalent bond between the polyphenols and the amino acid side-chains in β LG was confirmed by MALDI-TOF-MS and SDS-PAGE. Structural analysis by fluorescence spectroscopy, circular dichroism (CD) and Fourier transform infrared (FTIR) indicated that the covalent conjugate of EGCG and CA led to the changed protein structure of β LG. Western blot analysis and enzyme-linked immunosorbent assay indicated that conjugation of β LG with these polyphenols was effective in reducing the IgE-binding capacity of β LG. The conjugates maintained the retinol-binding activity without denaturation of the protein and enhanced the thermal stability with high antioxidant activity. The study provides an innovative approach to producing hypoallergenic food.

1. Introduction

Bovine β -lactoglobulin (β LG), a major whey protein, is a globular protein with molecular weight 18,400 as a monomer. β LG consists of one α -helix and nine anti-parallel β -sheets to form a calyx-shaped β -barrel structure with two disulfide bridges and one free cysteine residue (Flower, 1996). β LG is a valuable protein with nutritional features and various functional properties such as foaming, gelling, and emulsifying and is frequently used as a food additive for its multiple functions in food processing (Kontopidis, Holt & Sawyer, 2004).

Although β LG is considered an important nutrient of dairy products, β LG is one of the major milk allergens responsible for cow milk allergy (CMA) (Shek, Bardina, Castro, Sampson & Beyer, 2005). Many processing methods have been used to modify β LG to decrease its allergenicity, such as heating, high hydrostatic pressure, and hydrolysis (Verhoeckx et al., 2015). However, heating and hydrolysis might destroy its useful functions and have adverse effects on taste (Wu, Liu et al., 2013; Wu, Dey et al., 2013). High hydrostatic pressure might increase the allergenicity of β LG (Meng, Bai, Gao, Li & Chen, 2017). Among the potential protein-modifying methods, conjugation methods might be safe as compared with other methods (Huang et al., 2012). They may modify β LG to change its antigenicity by causing irreversible structural modification, thereby covering up the antigenic epitopes. Such methods include glycation (Zhong et al., 2013; Zhong, Tu, Liu, Luo & Liu, 2015), dynamic high pressure combined with glycation

(Zhong et al., 2014; Chen et al., 2016), PEGylation (Zhong et al., 2016), and ultrasound pretreatment combined with glycation (Yang et al., 2017).

Dietary polyphenols may conjugate covalently or non-covalently with proteins (Ozdal, Capanoglu & Altay, 2013). β LG has been used as an ideal model to characterize the conjugation between polyphenols and globular proteins (Kontopidis et al., 2004). Some dietary polyphenols show strong non-covalent interaction with β LG (Gholami & Bordbar, 2014; Guo, Harris, Kaur, Pastrana & Jauregi, 2017; Jia, Gao, Hao, & Tang, 2017; Kanakis et al., 2011; Wu, Dey et al., 2013; Wu et al., 2011). This interaction with β LG may alter the structure and functional and nutritional properties of the complex, to increase the potential applications of this protein. Recently, polyphenols were found able to form complexes with the major egg allergen ovalbumin and major peanut allergen Ara h1 by non-covalent interactions, which reduced their IgE-binding capacity (Chung & Champagne, 2009; Ognjenović et al., 2014).

However, the non-covalent interactions between polyphenols and proteins are reversible; they may be easily affected by temperature, pH, and salt concentration etc. (Ozdal et al., 2013), so the complexes are unstable for food processing (Liu, Ma, Gao & McClements, 2017). Many polyphenols are able to be oxidized to their corresponding quinones and semiquinones, which can further undergo covalent reactions with an enormous number of nucleophiles such as cysteine or lysine on the protein (Rohn, 2014). Properties of the proteins might be severely

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affected after covalent conjugation of polyphenols (Rohn, 2014). Therefore, covalently linking polyphenol to protein is a way to generate a more stable conjugate for food processing (Liu et al., 2017). Recently, covalent interactions between β LG and caffeic acid generated a stable β LG–polyphenol conjugate with exceptional antioxidant properties (Abd El-Maksoud et al., 2018). However, we lack studies about whether the IgE-binding capacity of allergens might be affected after covalent conjugation of polyphenols. The covalent interaction with polyphenols may be a novel approach to producing hypoallergenic foods.

Tea and coffee are considered polyphenol-rich foods that provide a significant source of polyphenols in the diet (Kilmartin & Hsu, 2003). (–)-Epigallo-catechin 3-gallate (EGCG) and chlorogenic acid (CA) are the major active principles of green tea and coffee bean, respectively, and have beneficial effects on human health (Singh, Shankar & Srivastava, 2011; Tajik, Tajik, Mack, & Enck, 2017). In this study, we examined covalent interactions between β LG and EGCG and CA and characterized the covalent conjugates (β LG–EGCG and β LG–CA). We investigated the allergen-IgE antibody recognition of β LG and the antioxidant properties of β LG–EGCG and β LG–CA conjugates.

2. Materials and methods

2.1. Materials

β LG (protein content > 90%), EGCG (purity > 98%), CA (purity > 99%), Folin-Ciocalteu's phenol reagent, 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), 1,10-diphenyl-2-picrylhydrazyl (DPPH) and 3,3'-5,5'-tetramethylbenzidine (TMB) were from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Pooled human sera were collected from 10 children with CMA. As determined by the Uni-CAP System (Pharmacia Diagnostics, Uppsala, Sweden), all sera showed specific IgE antibodies toward cow milk proteins, and on western blot analysis, all sera demonstrated IgE-binding ability for β LG. Sera from individuals ($n = 5$) without CMA were pooled and used as negative controls. All serum samples were generously provided by Shenzhen Children's Hospital (Guangdong, China) and all were authenticated by the ethics board of the School of Medicine of Shenzhen University and stored at -80°C until further use.

2.2. Sample preparation

The free radical method is widely used in protein–polyphenol covalent conjugate preparation. Because of their chemical structure, phenolic compounds are highly reactive. They can be easily oxidized enzymatically or non-enzymatically. In the free radical method, hydroxide free radicals are generated by oxidation-deduction reactions between H_2O_2 and ascorbic acid; the free radicals can participate in non-enzymatic reactions to oxidize the phenolic compounds (Rohn, 2014). Covalent conjugates (β LG–EGCG and β LG–CA) were prepared by the free radical method as described (Liu et al., 2017) with some modification. In brief, the conjugates were prepared in a 100-mL glass flask; 0.2 g of β LG was dispersed in 100 mL distilled H_2O , then 1.0 mL of 5.0 M H_2O_2 and 0.25 g ascorbic acid were added, and the mixture was maintained at 25°C under atmospheric air. After 2 h, 0.6 mM polyphenols (EGCG or CA) was added for 24 h; unreacted polyphenols were removed by dialysis with 1000 molecular weight cutoffs for 48 h at 4°C with 10 changes of Milli-Q water. Control β LG was treated under the same conditions but without polyphenols. Finally, all samples were obtained by lyophilization.

Determining total phenolic content in the covalent conjugates involved the Foline-Ciocalteu method with EGCG and CA as individual standards. An amount of 2.5 mL Foline-Ciocalteu phenol reagent (0.2 M) was mixed with 0.5 mL diluted sample at 25°C for 5 min. Then, 2 mL sodium carbonate solution (7.5% (w/v)) was added. The mixture was vortexed and left in the dark at 25°C for 2 h. Finally, the

absorbance was measured at 760 nm. A standard curve of EGCG or CA was used for calculating the polyphenol content in the sample. The grafting efficiency was calculated as follows:

$$\text{Grafting efficiency \%} = \frac{\text{polyphenol content in sample}}{\text{polyphenol content in the reactants before dialysis}} \times 100$$

2.3. Characterization of β LG–polyphenol covalent conjugates

2.3.1. SDS-PAGE

SDS-PAGE was performed as described (UK, 1970). The samples (1 mg/mL) were denatured by heating at 95°C for 3 min before analysis, then mixed with the loading buffer. The aliquots were loaded on a 12% separating gel and 5% stacking gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250G overnight and destained with deionised water for protein visualization.

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

The molecular weight of β LG–polyphenol conjugates was determined by MALDI-TOF-MS as described (You, Luo & Wu, 2014). In brief, the sample was dissolved in 20 μL distilled H_2O . Then, 0.8 μL of this solution was placed on the target and covered with an 0.8- μL aliquot of matrix solution (with 2,5-dihydroxybenzoic acid as the matrix compound). After crystallization of the sample by air-drying, MALDI MS involved using a TM MALDI-TOF/TOF system (Bruker Daltonics, Billerica, MA, USA).

2.3.3. Determination of free amino groups

Determining free amino groups involved use of trinitro-benzene-sulfonic acid (TNBS) as described (Rawel, Rohn, Kruse & Kroll, 2002).

2.3.4. Determination of sulfhydryl thiol (SH) groups

Determining SH groups in samples involved use of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) as described (Liu, Sun, Yang, Yuan, & Gao, 2015). In brief, a 15-mg sample was dissolved in 5 mL of 50 mM Tris-HCl buffer (pH 8) containing 8 M urea. DTNB reagent (4 mg of DTNB dissolved in 1 mL of 50 mM Tris-HCl buffer containing 1 mM EDTA) was added to the sample solution and mixed rapidly. Then the mixture was incubated for 1 h. Finally, the absorbance of the samples was measured at 412 nm by using a Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo).

2.3.5. Determination of tyrosine groups

The content of tyrosine in samples was measured as described (Liu et al., 2015). In brief, 1 mL sample (1 mg/mL) was mixed with 1 mL nitric acid (16 M). After the mixture was heated at 50°C for 15 min, NaOH (5 M) and ethyl alcohol were added. The absorbance was measured at 360 and 430 nm by using a Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo).

2.4. Structural analysis of β LG–polyphenol conjugates

2.4.1. Fluorescence spectroscopy

Samples (0.1 mg/mL) in phosphate buffered saline (PBS) (pH 7.2) were analyzed by using a Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo) in a 1-cm quartz cell at 25°C . The fluorescence emission spectra were collected from 300 to 500 nm with excitation wavelength 295 nm. The emission and excitation bandwidths were 5 nm. All samples were analyzed in triplicate.

2.4.2. Circular dichroism (CD) spectra

Spectra in samples (0.015 mg/mL) in PBS (pH 7.2) were examined by using a Jasco-810 spectrophotometer (Jasco Co., Japan) in cells of

1.0-mm path length at 25 °C under a nitrogen atmosphere. The spectra were recorded from 190 to 250 nm with step resolution 0.2 nm and scan speed 100 nm/min. Three scans were accumulated for each spectrum. The spectroscopic data were analyzed at the online Circular Dichroism Website (<http://dichroweb.cryst.bbk.ac.uk>) to calculate the secondary structure.

2.4.3. Fourier transform infrared (FTIR) spectroscopy

FTIR study involved use of the FTIR-8300PCS spectrometer (SHIMADZU Co., Kyoto, Japan) with KBr pellets. The infrared spectra of the samples were collected from the spectral range of 400–4000 cm^{-1} with resolution 4 cm^{-1} and 32 scans at 25 °C.

2.5. IgE-binding assay of β LG–polyphenol conjugates and complexes

2.5.1. Western blot analysis

Samples underwent SDS-PAGE as described previously, then protein was transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked with 1% fish gelatin in PBST (0.05% Tween 20 in PBS) for 1.5 h, incubated with a 1:50 dilution (v/v) of pooled human sera in PBST, washed in PBST four times, and incubated with mouse IgG-anti-human IgE antibody (1:2000) (SouthernBiotech, Birmingham, AL, USA) for 1 h. The membrane was washed 4 times and incubated with NeutrAvidin-horseradish peroxidase (HRP)-conjugated antibody (1:2000) (Southern Biotech, Birmingham, AL, USA) for 1 h, washed, then protein bands were detected by incubation with ECL Reagent (GE Healthcare, Buckinghamshire, UK) and exposure to X-ray film.

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

IgE-binding was evaluated by competitive ELISA as we previously described (Wu, Liu et al., 2013). Briefly, aliquots of sample solution (100 μL ; 100 ng protein in PBS) were added to 96-well plates and incubated at 4 °C overnight. The wells were washed with PBST, then incubated with 100 μL 2% fish gelatin in PBST for 2 h and washed 4 times. Samples at various concentrations (50 μL) were added to wells, then 50 μL 1:50 dilution (v/v) of pooled human sera was immediately added. Samples were incubated at 37 °C for 1 h and washed as above, then incubated with 100 μL HRP-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) (1:10,000) for 45 min at 37 °C. The plate was washed 4 times, and 50 μL substrate containing 0.4 mg/mL *o*-phenylenediamine and 5 μL H_2O_2 was added. After 15 min, 50 μL of 2 M H_2SO_4 was added to stop the enzyme reaction. Finally, the absorbance was read at 450 nm in an ELISA plate reader. ELISA determinations were carried out in triplicate and measurements were averaged.

2.6. Functional properties of β LG–polyphenol conjugates and complexes

2.6.1. Differential scanning calorimetry (DSC)

Thermal stability was examined by using a DSC-60 thermal analysis system (Shimadzu, Tokyo). With a standard procedure, about 5.5 mg sample was placed inside an aluminum pan and sealed tightly with a perforated aluminum lid and heated from 30 °C to 180 °C at a constant rate of 5 °C/min with constant purging of dry nitrogen at a rate of 30 mL/min. An empty aluminum pan was used as a reference.

2.6.2. Measurement of DPPH radical scavenging activity

Antioxidant activity was measured by using DPPH radical scavenging as described (Gong et al., 2012). Briefly, 2 mL diluted sample (0.5 mg/mL) was mixed with 2 mL DPPH (1.75×10^{-4} M solution in methanol). After storage in the dark for 60 min, the residual DPPH concentration was determined at 517 nm by using a spectrophotometer. The DPPH radical scavenging activity was calculated according to a Trolox calibration curve, by the method mentioned above.

2.6.3. Measurement of ABTS^+ free radical scavenging activity

ABTS^+ free radical scavenging activity was measured as described (Liu et al., 2015). Briefly, 1 mL sample (0.5 mg/mL) was mixed with 3 mL ABTS reagent (absorbance 0.7 at 734 nm). After incubation at 25 °C for 1 h, absorbance was measured at 734 nm. ABTS^+ free radical scavenging activity was calculated according to a Trolox calibration curve as described previously.

2.6.4. Measurement of retinol-binding activity

Retinol-binding activity was measured as described (Hattori, Okada & Takahashi, 2000). Increments of retinol in ethanol (5 μL at a time) at 2.64×10^{-4} M were added to a cuvette containing 1.0 mg protein in PBS. After mixing for 1 min, the fluorescence intensity was measured by using a Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo) with excitation 330 nm and emission 470 nm.

2.7. Statistical analysis

All measurements were carried out at least in triplicate. Results are expressed as mean $\pm$ SD. Differences between groups were analyzed by the Duncan least significant difference test and ANOVA. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Molecular weight change of β LG

Molecular weight change of β LG–polyphenol conjugates was determined by SDS-PAGE and MALDI-TOF – MS. The main band in control β LG was about 18 kDa (Fig. 1) and the bands of β LG–EGCG and β LG–CA conjugates slightly migrated up, which suggests increased molecular weight of β LG–polyphenol conjugates. These high-molecular-weight conjugates were not separated by using SDS or mercaptoethanol, which suggests that the bonds formed between the molecules

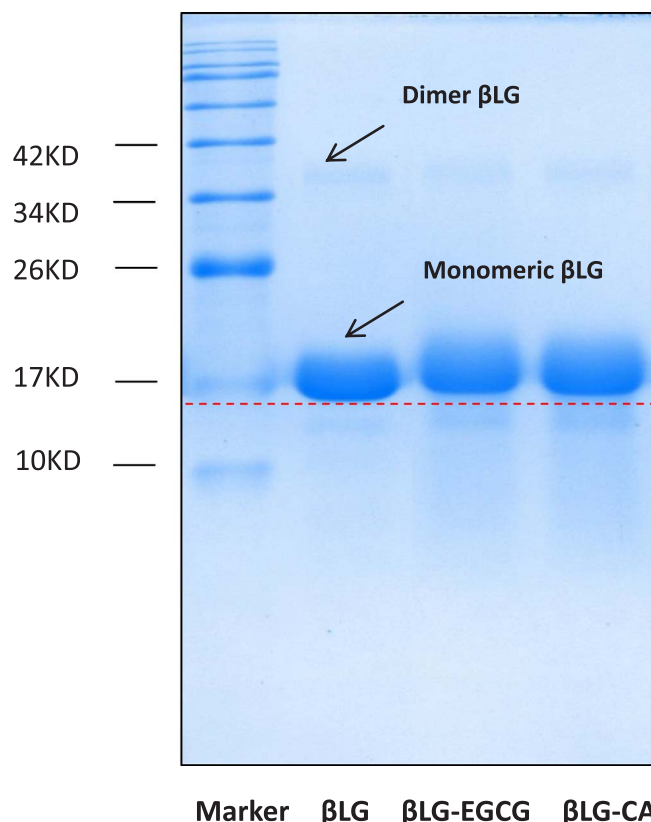


Fig. 1. SDS-PAGE of control β LG, β LG–EGCG and β LG–CA conjugates.

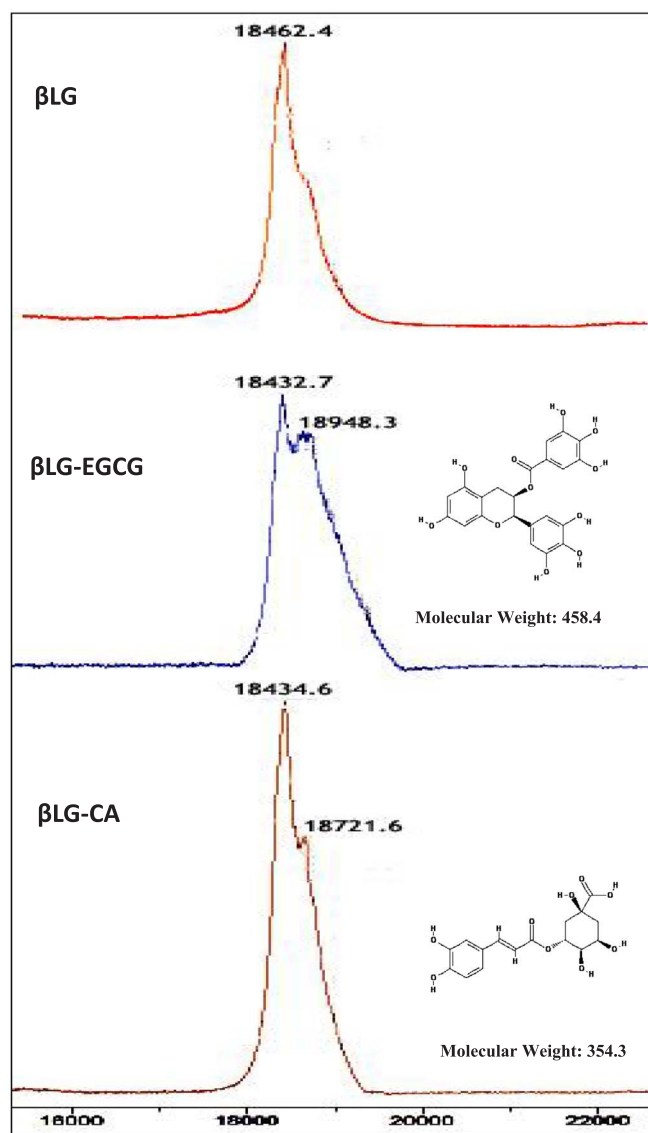


Fig. 2. MALDI-TOF-MS analysis of β LG, β LG-EGCG and β LG-CA conjugates.

were covalent (Liu et al., 2015). An increase in molecular weight of β LG conjugates after covalent conjugation with polyphenol compounds was previously reported (Ali, Homann, Khalil, Kruse & Rawel, 2013).

MALDI-TOF-MS was further used to study molecular-weight change of conjugates. The molecular weight of β LG was about 18.462 kDa (Fig. 2). The molecular weights of β LG-EGCG and β LG-CA conjugates both increased to 18.948 and 18.721 kDa as shown in the new peaks (Fig. 2). The difference in molecular weight between β LG and β LG-EGCG (~ 500 Da) and between β LG and β LG-CA (~ 300 Da) might correspond to the EGCG and CA molecules conjugated with β LG.

The SDS-PAGE and MALDI-TOF-MS results agreed with the determination of total phenolic contents of β LG-polyphenol conjugates (Table 1). The EGCG contents of the β LG-EGCG and β LG-CA conjugates were 34.29 ± 1.4 mg EGCG/g and 13.38 ± 0.9 mg CA/g, and the estimated conjugation efficiency was $8.5 \pm 0.35\%$ and $3.3 \pm 0.35\%$, respectively. These results confirmed the possible conjugation of β LG with EGCG and CA.

3.2. Content of free amino, thiol groups and tyrosine residue change of β LG

We further elucidated the interactions of phenolic compounds with β LG by examining contents of free amino and thiol groups and tyrosine residue changes (Table 1). The content of free amino groups in β LG decreased after conjugation with polyphenols, which suggests that oxidized phenolic compounds can undergo a nucleophilic addition to the amino acid side chains, thereby covalently modifying the proteins (Liu et al., 2015). Ali et al. found that the covalent reaction of ϵ -amino in lysine groups of β LG conjugated with 5-CQA reduced the free amino groups of the protein (Ali et al., 2013), which was similar to our results. In this study, 8 M urea was used to prevent the free thiol groups from converting to disulphide bond. Therefore, the reduction of free thiol groups suggested that the phenolic compounds covalently interacted with free thiol groups in β LG. Furthermore, the content of free tyrosine groups was lower in conjugates than in β LG. As reported, under aerobic conditions, the o-quinones generated from the phenolic compounds could react with nucleophilic groups, such as amino groups, methionine, cysteine, histidine, tryptophan, tyrosine and N-terminal proline in proteins (Kroll, Rawel, & Rohn, 2003). Prigent et al. found that the side chains were more reactive to tyrosine and lysine than histidine and tryptophan in covalent interactions between caffeoylquinic acid and proteins (Prigent, Voragen, Visser, van Koningsveld & Gruppen, 2007). These results indicate that β LG interacting with EGCG and CA occurred via covalent binding, with a decrease in the amount of free amino and thiol groups and tyrosine residues. EGCG was more effective in forming a β LG-polyphenol conjugate and showed greater reactivity and binding strength with free amino, thiol and tyrosine residue groups than did CA.

3.3. Structural analysis of β LG-polyphenol conjugates

Fluorescence, CD and FTIR spectroscopy were used to study β LG conformational changes before and after polyphenol conjugation. Fluorescence spectroscopy can be used to obtain information on changes in the tertiary structure of the proteins. β LG contains 4 tyrosine molecules. Therefore, the activity of tyrosine can be used to analyze the change in the intrinsic fluorescence of β LG to evaluate changes in the tertiary structure of β LG. The maximum emission wavelength for the control β LG was about 336.4 nm (Fig. 3A), which could be attributed mainly to tyrosine residues. The fluorescence intensity was lower for β LG-polyphenol conjugates than control β LG, which suggested that the EGCG and CA may have associated with the tyrosine groups on the protein. The maximum emission was 337.0 and 338.2 nm for the β LG-EGCG and β LG-CA conjugates, respectively. A red shift of maximum emission was observed in the conjugates, so the tyrosine residues transferred to a more hydrophilic environment. These findings could be related to a changed β LG structure after conjugation with EGCG and CA.

The secondary structure of β LG-EGCG conjugates was quantified by measuring the CD spectra. The spectra of samples exhibited a negative band at 208 nm (Fig. 3B), characteristic of α -helical structures of a protein. β LG-polyphenol conjugates had less absolute θ values at 208 nm than those of control β LG, which indicates a decrease of some α -helical structures in the control β LG (Wu et al., 2011). On quantitative analysis of the secondary structure content by using the SELCON3 method in DICHROWEB (Table 1), unmodified β LG contained 26.8% α -helices, 31.3% β -sheets, 31.9% unfolded structures and 10.0% turn elements. After conjugation with EGCG and CA, α -helical and β -sheet contents slightly decreased, with an increase in the unfolded structure content, which suggests that polyphenol conjugation caused the protein structure to become more unfolded, which agrees with the results of fluorescence spectroscopy.

FTIR was used to further explore the structural change of β LG conjugated with EGCG and CA. The protein in amide I bands (1600–1700 cm^{-1} , mainly C=O stretch) and amide II bands (≈ 1540 cm^{-1} , C–N stretch coupled with N–H bending mode) is

Table 1
Characterization of β LG and β LG-polyphenol conjugates.

Property/sample	Control β LG	β LG-EGCG	β LG-CA	P
Free amino groups (nmol/mg protein)	470.2 $\pm$ 9.4	391 $\pm$ 12.9 ^a	444.1 $\pm$ 12.1 ^{b,c}	0.002
Free thiol groups (nmol/mg protein)	20.9 $\pm$ 0.4	7.3 $\pm$ 0.4 ^a	18.0 $\pm$ 0.8 ^{b,c}	< 0.01
Tyrosine group (nmol /mg protein)	19.8 $\pm$ 0.8	15.3 $\pm$ 1.1 ^a	19.1 $\pm$ 0.6 ^c	0.001
Polyphenones bound (mg/g protein)	–	34.2 $\pm$ 1.4	13.3 $\pm$ 0.9 ^c	< 0.01
Grafting efficiency (%)	–	8.5 $\pm$ 0.35	3.3 $\pm$ 0.22 ^c	< 0.01
α -Helix (%) by CD	26.8 $\pm$ 0.2	25.1 $\pm$ 0.1 ^a	25.8 $\pm$ 0.3 ^{b,c}	< 0.01
β -Sheet (%) by CD	31.3 $\pm$ 0.2	30.7 $\pm$ 0.1 ^a	29.7 $\pm$ 0.1 ^{b,c}	< 0.01
Random coil (%) by CD	31.9 $\pm$ 0.1	30.8 $\pm$ 0.1 ^a	30.9 $\pm$ 0.3 ^b	< 0.01
Turn (%) by CD	10.0 $\pm$ 0.4	13.4 $\pm$ 0.2 ^b	13.6 $\pm$ 0.2 ^b	< 0.01
α -Helix (%) by FTIR	26.4 $\pm$ 0.1	24.6 $\pm$ 0.1	25.1 $\pm$ 0.1 ^{b,c}	< 0.01
β -Sheet (%) by FTIR	35.3 $\pm$ 0.1	34.4 $\pm$ 0.2 ^a	32.9 $\pm$ 0.2 ^{b,c}	< 0.01
β -Anti (%) by FTIR	34.7 $\pm$ 0.1	38.2 $\pm$ 0.1	39.0 $\pm$ 0.1 ^c	< 0.01
Turn (%) by FTIR	2.6 $\pm$ 0.2	2.9 $\pm$ 0.1	3.0 $\pm$ 0.1 ^b	0.03
DPPH scavenging activity (μ mol Trolox per g sample)	42.12 $\pm$ 3.35	82.45 $\pm$ 1.75 ^a	61.19 $\pm$ 5.40 ^{b,c}	< 0.01
ABTS scavenging activity (μ mol Trolox per g sample)	51.08 $\pm$ 5.40	139.89 $\pm$ 6.28 ^a	85.06 $\pm$ 5.18 ^{b,c}	< 0.01

^a Statistical difference ($p < 0.05$) between β LG-EGCG and control β LG.

^b Statistical difference ($p < 0.05$) between β LG-CA and control β LG.

^c Statistical difference ($p < 0.05$) between β LG-CA and β LG-EGCG.

related to the secondary structure of protein. The spectra of the unmodified β LG exhibited major bands at 1641.4 cm^{-1} (amide I) and 1534.8 cm^{-1} (amide II) (Fig. 3C). As compared with control protein, for β LG-EGCG, the amide I and amide II bands moved from 1641.4 to 1642.0 cm^{-1} and 1534.8 to 1536.6 cm^{-1} , respectively; the amide I and amide II bands of β LG-CA moved from 1641.4 to 1642.4 cm^{-1} and 1534.8 to 1537.0 cm^{-1} . These results implied a changed secondary structure of β LG in the conjugates.

The amide I band in protein is more sensitive to the change in

protein secondary structure than the amide II band. Therefore, we used quantitative analysis of the protein secondary structure by curve fitting to the range 1700–1600 cm^{-1} (Heino & Michael, 1896) (Table 1). For the control β LG, the content of α -helix was 26.4%, β -sheet 35.3%, β -anti 34.7% and turns 2.6%. For EGCG-CA, the secondary structure contents were slightly changed. These FTIR results were consistent with the fluorescence and CD spectroscopy findings.

The results indicated that the covalent conjugation of EGCG and CA led to changed protein structure of β LG.

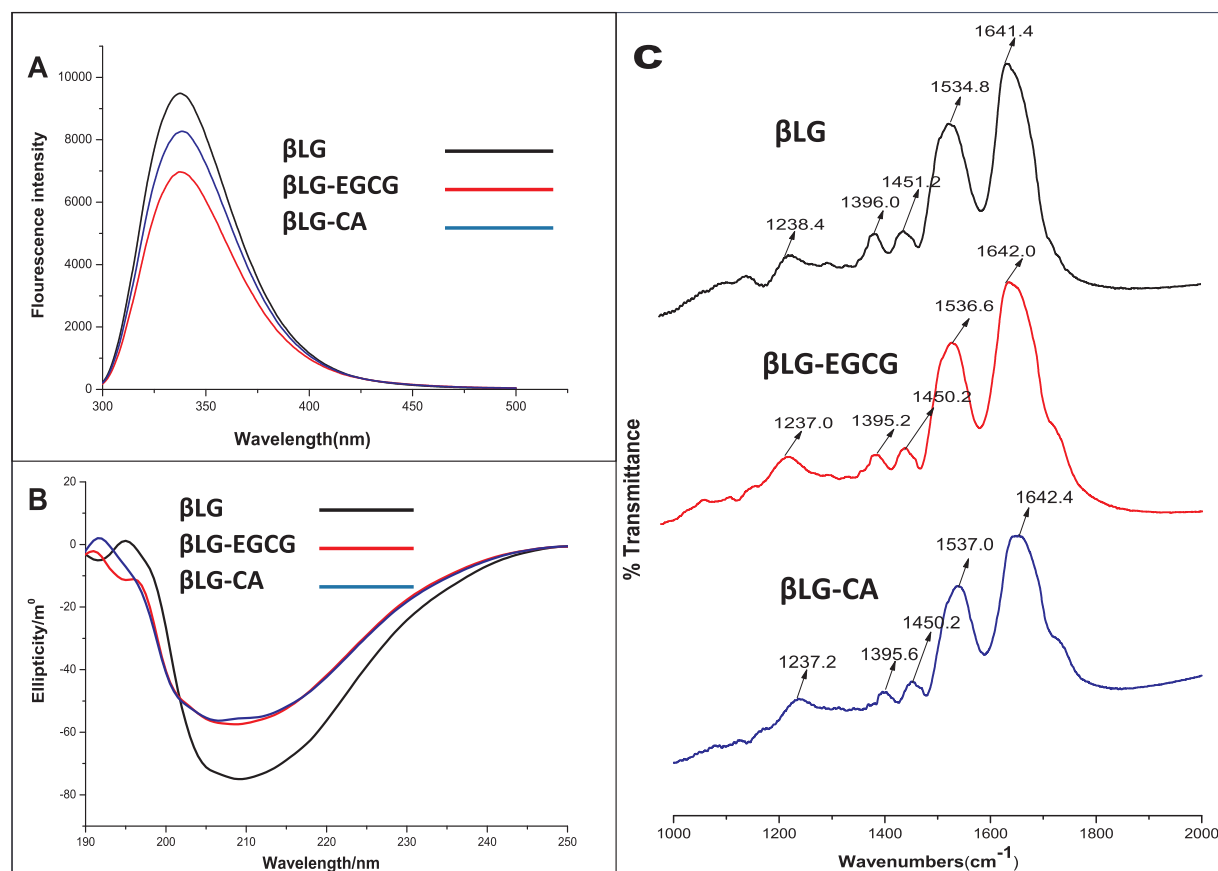


Fig. 3. Fluorescence (A), CD (B) and FTIR (C) spectra for β LG, β LG-EGCG and β LG-CA conjugates.

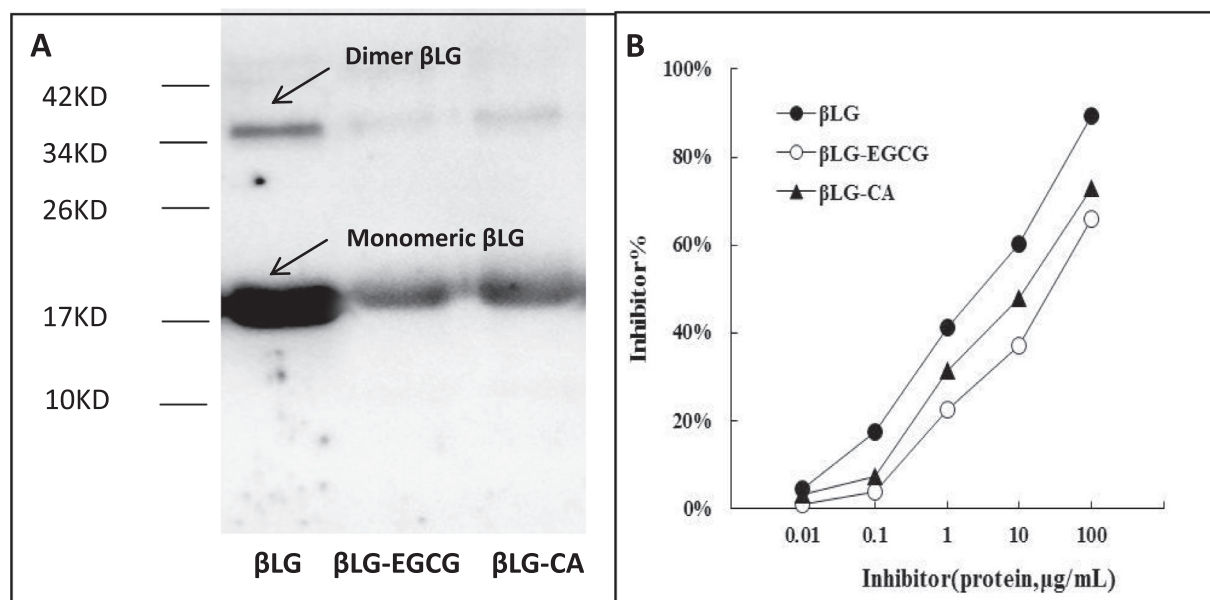


Fig. 4. Western blot analysis (A) and ELISA (B) of βLG, βLG-EGCG and βLG-CA conjugates.

3.4. IgE-binding capacity analysis of βLG–polyphenol conjugates

The IgE-binding capacities of βLG–polyphenol conjugates and complexes were measured by western blot analysis. Fig. 4A shows the band profiles of control βLG and βLG–polyphenol conjugates binding to IgE. Allergen bands were strongly recognized by IgE antibodies. However, a marked reduction in the βLG–EGCG and βLG–CA conjugate bands was observed, which indicates reduced IgE binding to the allergens. To further support the findings, on ELISA, both βLG–EGCG and βLG–CA conjugates exhibited a lower IgE inhibition curve than the βLG control (Fig. 4B). The allergenic capacity of βLG was reduced after covalent conjugation with EGCG and CA, with βLG–EGCG exhibiting more reduction in IgE binding than βLG–CA.

The molecular basis of changes in IgE-binding capacity is the increased inaccessibility to epitopes or destruction of epitope structures (Besler, Steinhart & Paschke, 2001). The IgE-binding capacity of βLG is related to both linear and conformational epitopes of protein; the linear epitopes are more important than the conformational epitopes (Besler et al., 2001; Sélo et al., 1999). Because proteins need to be denatured before loading in western blot analysis, IgE is used to analyze antibodies binding the linear epitope(s) of allergen.

Leu1-Lys8, Ala25-Arg40, Val41-Lys60 and Tyr102-Val124 are major IgE epitopes of βLG and are on the protein surface (Sélo et al., 1999). In the covalent interaction between protein and polyphenol, polyphenol oxidized to a quinone reacts with amino or sulfhydryl side chains of a protein (Kroll et al., 2003). The conjugation of EGCG and CA with βLG was monitored by the decreased contents of free amino, thiol and tyrosine residue groups in protein, which suggests that the polyphenol may form covalent bonds with these residues in βLG. These residues may locate in the IgE epitopes. βLG has 14 lysine residues; 12 locate in the major IgE epitope. The antigenicity would decrease most of them, if the main epitope is conjugated with compounds (Zhang et al., 2014). Thus, the predicted polyphenol-binding site in βLG directly masks the region of the linear IgE epitope(s) or is near the linear IgE epitope(s), which prevents IgE from accessing the protein. This situation might explain the weak IgE-binding capacity of βLG–polyphenol conjugates detected by western blot analysis.

Furthermore, the conformational IgE epitopes of βLG is linked to its secondary or tertiary structure (Chen et al., 2016; Kleber, Krause, Illgner & Hinrichs, 2004). The secondary structure could significantly affect the IgE binding capacities of βLG (Li et al., 2015). In this study,

EGCG and CA interacting covalently with βLG led to the subtle structural changes in the protein. Because the secondary structure changed after conjugation with EGCG and CA, some structure of the conformational IgE epitopes may have been affected, thereby reducing the allergenic capacity of the protein.

The decrease in IgE binding abilities of βLG may be attributed to changes in conformational structure induced by covalent conjugation. Moreover, the shielding of some epitopes by the covalent attachment of amino acid residues and polyphenols may be responsible for the decreased binding. Similarly, glycation could change the conformation of protein and shielding of some epitopes of βLG (Yang et al., 2017). To better understand the mechanism of reduced IgE binding of βLG on covalent conjugation with polyphenols, conjugation sites need to be further determined.

Although the βLG structural changes did not differ on EGCG and CA conjugation, EGCG was more effective in forming a βLG–polyphenol conjugate than CA, which may explain why βLG–EGCG exhibited more allergenic capacity than βLG–CA.

3.5. Functional properties of βLG–polyphenol conjugates

A biological function for βLG is binding two molecules of retinol (Perez & Calvo, 1995). We investigated the changes in retinol-binding activity of βLG after conjugation with EGCG and CA (Fig. 5A). The retinol-binding activity of βLG–polyphenol conjugates was ~70% of the control βLG activity. The retinol-binding activity of βLG is lost if the protein is denatured (Perez & Calvo, 1995). The βLG–polyphenol conjugates maintained most of the retinol-binding activity of protein, so βLG was covalently conjugated with EGCG and CA without apparent disruption of its native conformation (Kobayashi et al., 2001; Wu, Liu et al., 2013; Wu, Dey et al., 2013). Although the structure of βLG was altered slightly after covalent conjugation with EGCG and CA, βLG maintained the native-like protein structure.

DSC was used to study the thermal stability of the βLG–polyphenol conjugates (Fig. 5B). The control βLG showed thermal denaturation temperature at $120.7 \pm 0.2^\circ\text{C}$, whereas for βLG–EGCG and βLG–CA conjugates, thermal denaturation temperatures were severely increased, which suggests that the increased thermal stability of βLG was attributed to the covalent conjugation with polyphenol. This result agreed with a previous report that covalent conjugation with caffeineoylquinic acids increased the thermal stability of βLG (Ali et al.,

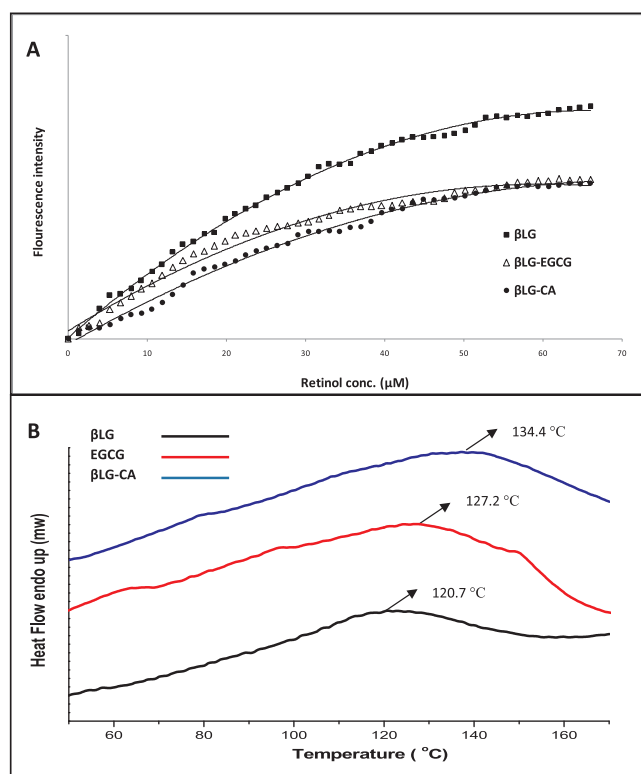


Fig. 5. Retinol-binding activity (A) and differential scanning calorimetry thermography (B) of β LG, β LG-EGCG and β LG-CA conjugates.

2013).

The antioxidant activities of β LG and β LG-polyphenol conjugates were evaluated in terms of ABTS and DPPH radical scavenging activities (Table 1). β LG-polyphenol conjugates inhibited DPPH and ABTS radicals more effectively than did control β LG, which suggests that the antioxidant activity of β LG was increased after conjugation with EGCG and CA. β LG-EGCG conjugates could reduce DPPH and ABTS radicals to a greater extent than could β LG-CA conjugates, which may be due to greater content of EGCG than CA.

Numerous methods have been investigated to reduce the allergenicity of β LG. The decrease in IgE-binding capacity of β LG caused by covalent methods, such as heat treatments and hydrolysis, can be explained by denaturation of the protein (Verhoeckx et al., 2015). However, denaturation of β LG reduces the protein's functional properties. Therefore, new methods are needed to reduce the allergenicity of β LG without destroying or by even improving the functional properties of this protein. The functional attributes of some food proteins can be extended by forming covalent bonds with polyphenols (Cao & Xiong, 2015; Liu et al., 2017; Yin, Yang, Zhao & Li, 2014; You et al., 2014). In this study, we prepared β LG-polyphenol conjugates by the free radical method. The conjugates showed reduced allergenic capacity without denaturing the protein, maintained the retinol-binding activity, and conferred enhanced thermal stability with high antioxidant activity. This study demonstrates a new possibility of reducing the allergenicity of β LG and improving protein functionality by conjugation with dietary polyphenols. However, this study was developed with *in vitro* experiments. *In vivo* studies in animals or the clinic should be performed to further confirm whether the β LG-polyphenol conjugates can reduce the allergenic properties of β LG.

4. Conclusion

We have investigated a method of potentially reducing the allergenicity of β LG by conjugating it with EGCG and CA. β LG interacting

with EGCG and CA occurred via covalent binding. β LG showed structural change after covalent conjugation with polyphenols. The conjugation between β LG and polyphenols was effective in reducing IgE-binding capacity. The conjugates maintained the retinol-binding activity and enhanced the thermal stability with high antioxidant activity. EGCG was more effective than CA in the formation of β LG-polyphenol conjugates. This study provided a novel method for reducing the allergenicity of β LG, which might have potential in producing hypoallergenic foods.

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