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Probing the conjugation of epigallocatechin gallate with β -lactoglobulin and its *in vivo* desensitization efficiency

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Epigallocatechin gallate (EGCG) and β -lactoglobulin (β Lg) were conjugated by covalent bonds to form EGCG- β Lg conjugates. This conjugation causes structural and bioactivity changes in β Lg, which in turn can be used as a possible approach for desensitization to allergens. In this study, the desensitization mechanism was investigated by monitoring β Lg secondary structure and immunoglobulin E (IgE) combining capacity changes on the basis of the conjugation mechanism. Furthermore, the desensitization efficiency *in vivo* was evaluated through animal experiments. The results show that temperature influenced the conjugation by decreasing the binding affinities (K_a) and binding numbers (n) of EGCG. The conjugation of EGCG decreased β Lg's IgE combining capacity by decreasing the β -sheet component and imparted antioxidant properties by the introduction of hydroxyl groups. In addition, animal experiment results indicated that β Lg induced significant changes in the levels of IgE and inflammatory cytokines, and the relative abundance of small intestinal flora, linked to the inflammatory lesions and anaphylaxis symptoms. EGCG- β Lg conjugates can suppress the allergic response, attenuating serum IgE and relieving the anaphylaxis symptoms.

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Introduction

Food-allergic reactions have caused an increasing health concern worldwide, particularly cow's milk allergen (CMA), which results in severe symptoms, such as swelling, rash, breathing difficulty, itchiness, and even life threatening reactions.^{1–3} Among milk proteins, β -lactoglobulin (β Lg) is identified as one of the main allergenic proteins in terms of persistence and severity of reaction, contributing to immunoglobulin E (IgE) mediated milk allergy.^{4,5} β Lg contains 162 amino acid residues with a molecular mass of 18.4 kDa.^{4,6} Three main IgE epitopes were located at AA41–60, AA102–124, and AA149–162, assigned to the β -sheet composition, and were stabilized by a barrel structure formed by disulfide bridges, hydrogen bonds, and hydrophobic interactions.⁷ Destroying the structure of these epitopes and inhibiting their IgE combining capacity is the main approach to the desensitization of β Lg.⁸ Numerous traditional methods, such as heating, high

hydrostatic pressure and enzymatic treatments, were used to decrease the allergenicity through changing the conformation of β Lg.^{9–11} However, these methods have not been satisfactory, as they could destroy the nutritional value, and influence the flavor and storage properties of dairy products.^{12,13} Thus, developing a novel desensitization method to strengthen the function or impart new characteristics to the products is important for food safety.

Dietary polyphenols, such as epigallocatechin gallate (EGCG), are known for their significant bioactivities, being associated with antimutagenic, anti-inflammatory, anti-diabetic and anti-viral functions.^{14,15} And their incorporation offers opportunities in solving some bottleneck problems in the field of biomedicine and food.¹⁶ Recently, polyphenolic compounds have been reported for their strong interaction with globular proteins to form protein-polyphenol conjugates by covalent or non-covalent bonds when added into liquid and dairy food products.^{17–19} This conjugation could alter the conformation and functional properties of proteins, and result in conjugates with polyphenol characteristics,^{20,21} while providing a possible approach for the allergic desensitization of β Lg.

The conjugation and desensitization mechanisms are the preconditions when EGCG is exploited as a desensitizer. Many studies have been performed to investigate this conjugation and its influential factors from different aspects. El-Maksoud *et al.* studied the covalent conjugation of β Lg with caffeic acid

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(CA) and found that β Lg-CA conjugates showed superior water solubility and thermal stability than native β Lg.²² Wu *et al.* confirmed covalent bonding between chlorogenic acid (CHA) and β Lg, and revealed that this conjugation is effective in reducing the IgE combining capacity of β Lg.⁸ Xu *et al.* found that the covalent conjugation of CHA may cause an unfolding of the whey protein isolate (WPI), resulting in a lower IgE combining capacity.¹³ Generally, the protein-polyphenol conjugates have been proven to change the protein's functional properties and impart new characteristics from the polyphenols.^{23–25} However, this formation is a complex process and is influenced by the nature of the polyphenols and the proteins, as well as the conjugation environment (temperature, pH, ionic strength, *etc.*). Temperature is an important, but “ignored” factor of the protein-polyphenol conjugation, although it is one of the key parameters considered in food processing.^{16,26} Furthermore, the *in vivo* desensitization efficiency remains not fully understood, especially the changes in the small intestinal microbiota, which are expected to be affected by the polyphenols that are employed as desensitizers. The small intestinal microbiome is an integral part of the human physiology and is widely considered an important mediator between health and disease, since the small intestinal microbiota is inextricably linked to immunity, metabolism, and endocrine functions.^{27–29}

In this study, the conjugation of EGCG with β Lg and the influence of temperature were investigated to address the desensitization efficiency *in vivo*. The desensitization mecha-

nism for polyphenols was investigated from the aspects of binding affinity (K_a), binding numbers (n) and secondary structure changes. Furthermore, the IgE combining capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability, and surface hydrophobicity were measured in order to assess the changes of the functional properties of the conjugates. In addition, animal experiments were performed by considering the levels of IgE and cytokines, histological analysis, and intestinal flora to test the desensitization efficiency *in vivo*. The findings of this study will provide a fundamental understanding of the desensitization of β Lg by EGCG *in vitro* and *in vivo*, which in turn might be beneficial for the dairy industry.

Results and discussion

Thermodynamic parameters

Isothermal titration calorimetry (ITC) is a straightforward method for studying the association interaction, and allows the determination of the binding affinity (K_a) and the binding enthalpy (ΔH) simultaneously.^{30,31} The thermodynamic parameters of stoichiometry, K_a , and ΔH can be calculated by monitoring the released heat. The free energy change (ΔG) and entropy change (ΔS) can be calculated using eqn (1) and (2) below:³¹

$$\Delta G = -RT \ln K_a \quad (1)$$

$$\Delta G = \Delta H - T\Delta S \quad (2)$$

where R is the gas constant and T is the absolute temperature.

The raw data for EGCG titration in β Lg solutions at different temperatures are shown in Fig. 1. The results show that the conjugation of EGCG and β Lg is strongly exothermic ($\Delta H < 0$), and gradually decreased with increasing injection of EGCG. When the molar ratio reached ~ 8 , the conjugation reached an interaction equilibrium at 4 °C; however, the molar ratio reached at equilibrium decreased with the increase in temperature. The thermodynamic parameters, namely, K_a , stoichiometry, ΔH , ΔS and ΔG , are derived using non-linear least-squares fitting of the reactions and are summarized in Table 1. The formation of EGCG- β Lg conjugates is an entropy-driven process ($\Delta S < 0$).³² The results showed that the higher temperature induced lower ΔH and ΔS in the formation of the conjugates. K_a was $7.3 \pm 2.1 \times 10^6 \text{ M}^{-1}$, and the stoichiometry was 3.6, signalling that there are about 3.6 EGCG molecules which are conjugated on a single β Lg, when incubated at 4 °C.

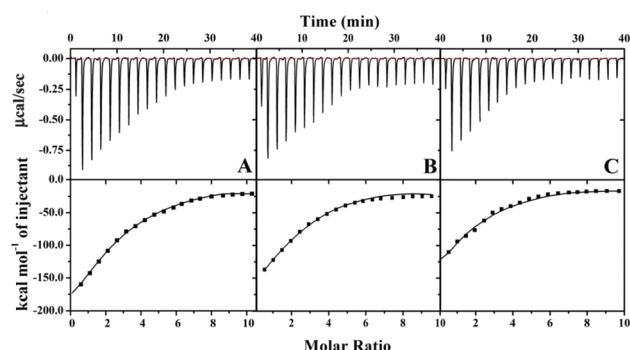


Fig. 1 Titration of epigallocatechin gallate (EGCG, 4 mM) into β -lactoglobulin solution (β Lg, 0.1 mM) at different temperatures. Upper figures are the raw data and the lower figures represent the integrated heats for each injection plotted against the EGCG : β Lg ratio. The temperatures are: (A) 4 °C, (B) 24 °C, and (C) 44 °C.

Table 1 Thermodynamic parameters of epigallocatechin gallate (EGCG) conjugated to β -lactoglobulin (β Lg). Stoichiometry of K_a , ΔH and ΔS as determined by isothermal titration calorimetry

T	Stoichiometry ^a	$K_a^a (\times 10^6 \text{ M}^{-1})$	$\Delta H (\times 10^5 \text{ J M}^{-1})$	$\Delta G^b (\times 10^4 \text{ J})$	$\Delta S^b (\times 10^3 \text{ J M}^{-1} \text{ deg}^{-1})$
4 °C	3.6	7.3 ± 2.1	-6.7 ± 2.3	-3.6	-2.3
24 °C	2.8	6.1 ± 2.2	-5.8 ± 2.8	-3.8	-1.8
44 °C	2.1	4.3 ± 1.7	-4.6 ± 1.9	-4.0	-1.3

^a Derived from one site binding model. ^b Calculated according to eqn (1) and (2).

However, K_a and stoichiometry decreased to $4.3 \pm 1.7 \times 10^6 \text{ M}^{-1}$ and 2.1, respectively, when the temperature increased to 44 °C, indicating a weakening of the conjugation at higher temperatures.

Binding number

Mass spectrometry is a commonly used method to investigate conjugation by measuring the mass-to-charge ratio (m/z) to determine molecule weight (MW) and profile of the protein and its conjugates.³³ The protein can be ionized into intact and isolated molecules in the gas phase, and then separated according to its MW after migration in an electric field. Soft ionization techniques, such as matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS),⁶ are considered as effective methods for protein conjugate identification without the need for prior purification.³⁴

In order to investigate the EGCG conjugated number more accurately, MALDI-TOF-MS was further used to study the MW changes of β Lg before and after conjugation at different temperatures (Fig. 2). The main MALDI-TOF MS peaks are representative of the β Lg molecule and its conjugates with the EGCG molecules. The results show that the MW of β Lg was about 18.413 kDa (Fig. 2A). While, a series of new peaks are observed at the m/z of 19.3, 19.7 and 20.2 kDa, corresponding to the masses of β Lg conjugated with 2, 3 and 4 EGCG, respectively (Fig. 2B–D). In general, the quadruple (~ 20.2 kDa) and triple (~ 19.7 kDa) conjugated β Lg with EGCG were the most abundant components when incubated at 4 °C (Fig. 2B). The triple (~ 19.7 kDa) and double (19.3 kDa) conjugated β Lg with EGCG show a higher intensity than quadruple (~ 20.2 kDa) conjugation when incubated at 24 °C and 44 °C (Fig. 2C and D). Thus, it could be speculated that the temperature plays an important role in the protein conjugation. The lower temperature induces more EGCG conjugation, which is consistent with the stoichiometry results determined by ITC.

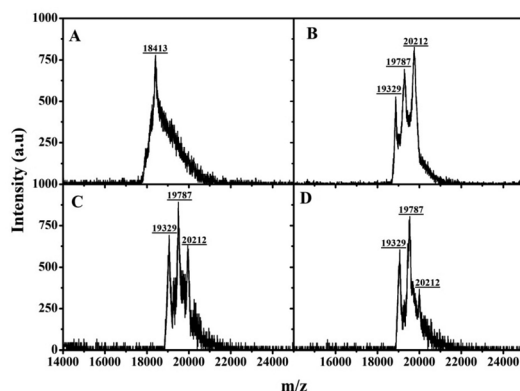


Fig. 2 Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) spectra of β -lactoglobulin (β Lg) and β Lg conjugated with epigallocatechin gallate (EGCG) at different incubation temperatures. (A) β Lg alone, and conjugates obtained at (B) 4 °C, (C) 24 °C and (D) 44 °C.

Secondary structure

Fourier transform infrared (FTIR) spectroscopy is an effective vibrational technique for the structural analysis of protein and its conjugates in aqueous environments.³⁵ The second-derivative spectrum of the amide I band (1700–1600 cm^{-1}) has been widely used to analyze the secondary structure of proteins and protein conjugates without being limited by the samples' size or their physical state.^{36,37}

The curve-fitted inverted second-derivative amide I spectra of β Lg and EGCG- β Lg conjugates prepared at different temperatures are shown in Fig. 3 and their resulting secondary structural components are summarized in Table 2. The β -sheet (1628 cm^{-1}) is the main secondary structural component of β Lg (Fig. 3A). The FTIR spectrum shows significant changes in the secondary structure of β Lg after it is conjugated with EGCG. The intensities of the β -sheet and 3_{10} -helix (1663 cm^{-1}) remarkably decreased, whereas those of the α -helix (1658 cm^{-1}) and β -turn (1667 and 1675 cm^{-1}) increased. In this covalent conjugation, EGCG oxidized to quinone, and then reacted with amine or sulfhydryl side chains, which are located in the β -sheet component. Moreover, the stereo-hindrance effect of EGCG induced spatial structural changes of β Lg, which increased the random coil bands (1649 cm^{-1}). The results show that the lower temperature lead to more structural changes because of the higher conjugated number of EGCG onto a single β Lg molecule. The structural changes induced by the conjugation would bring the changes in the β Lg functional properties, which might provide a novel approach to allergy desensitization.

Functional properties of the conjugates

β Lg is the main allergenic protein in milk, which can be recognized by human IgE, and can induce type I hypersensitivity reactions.^{4,38} The main rule to reduce anaphylaxis is changing the structure and spatial conformation of the allergen,

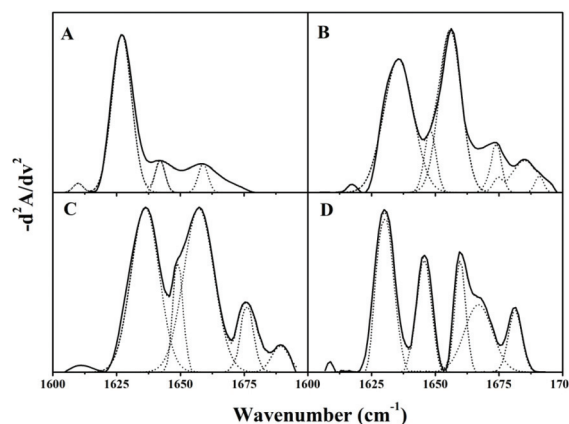


Fig. 3 Curve-fitted inverted second-derivative amide I band (1600–1700 cm^{-1}) from the FTIR spectra of β -lactoglobulin (β Lg) and epigallocatechin gallate (EGCG)- β Lg conjugates obtained at different incubation temperatures. (A) β Lg alone, and EGCG- β Lg conjugates obtained at (B) 4 °C, (C) 24 °C and (D) 44 °C.

Table 2 Secondary structural component of β -lactoglobulin (β Lg) and epigallocatechin gallate (EGCG)- β Lg conjugates at different incubation temperatures as detected by Fourier transform infrared (FTIR) spectroscopy

	3_{10} -Helix ^a	α -Helix ^b	β -Sheet ^c	β -Turn ^d	Side chain ^e	Random coil ^f
β Lg	17%	0	79%	0	4%	0
4 °C	0%	36%	38%	10%	5%	11%
24 °C	0	30%	29%	15%	6%	20%
44 °C	0	23%	31%	18%	5%	23%

^aThe 3_{10} -helix is at 1663 cm^{-1} . ^bThe α -helix is at 1658 cm^{-1} . ^cThe β -sheet are at 1628 and 1637 cm^{-1} . ^dThe β -turn are at 1667 and 1675 cm^{-1} . ^eThe side chain is at 1617 cm^{-1} . ^fThe random coil is at 1648 cm^{-1} in the Fourier transform infrared spectrum.³⁵

thereby, decreasing the IgE combining capacity.³⁹ The conjugation of EGCG induced significant changes in the secondary structure of β Lg, which created the possibility of desensitization. Moreover, these conjugates can also possess some characteristics of EGCG.

The functional properties of the EGCG- β Lg conjugates, namely, IgE combining capacity, antioxygenic property and surface hydrophobicity, were determined by ELISA, DPPH radical scavenging activity and the 1-anilinonaphthalene 8-sulfonate (ANS) probe, respectively (Fig. 4). The results show that the IgE combining capacity was inhibited after the formation of EGCG- β Lg conjugates. Three main IgE allergenic epitopes are located at AA41–60, AA102–124, and AA149–162 in the β -sheet component.⁷ The decrease of β -sheet components would destroy the conformation of these epitopes. Thereby, IgE is prevented from accessing β Lg and the stimulus of anaphylaxis decreased. Conjugation temperature influences the IgE inhibition rate through changing the EGCG binding number since the inhibition decreased with the increase in the conjugation temperature; more structural changes are achieved at a lower conjugation temperature (Table 2). Moreover, the EGCG- β Lg conjugates show excellent antioxidant activities (Fig. 4A). The introduction of a higher number of hydroxyl groups is responsible for the improved antioxidant activities of the conjugates.⁴⁰ The change trend of the antioxidant activity as a function of the conjugation temperature was similar to that of the IgE inhibition rate. In addition, the surface hydrophobicity decreased with the conjugation with EGCG (Fig. 4B), probably due to the blocking of

lysine and/or arginine residues of the protein.⁴¹ Moreover, surface hydrophobicity induced the cleavage of hydrogen bonds and van der Waals forces and the exposure of hydrophilic amino acid residues in the protein inner site, involved in the conformational stability of the IgE epitopes.⁴²

In vivo experiments

The allergy symptoms induced by β Lg were estimated and scored according to the anaphylactic response.⁴³ Some symptoms, such as diarrhea, hematochezia and convulsions, were observed in the β Lg group (anaphylactic response, 4.1 ± 0.6 points, Fig. 5A). In contrast, anaphylactic symptoms were considerably suppressed in the EGCG- β Lg conjugate group (anaphylactic response, 1.3 ± 0.5). The level of total IgE was 742 ng ml^{-1} in the sensitized group, which is significantly higher when compared to the control group (213 ng ml^{-1}). While, the IgE level decreased to 284 ng ml^{-1} in the conjugate group, a level comparable to the control group (Fig. 5B). The conjugation of EGCG destroyed the spatial conformation of IgE epitopes in β Lg, hindering their recognition by the specific Fc ϵ R1-bound IgE and avoiding Fc ϵ R1 aggregation, which prevented the degranulation of the mast cell.⁴⁴

The intestinal profiles of the anaphylactic response induced by the administration of sterile water, β Lg and EGCG- β Lg conjugates were evaluated according to the mRNA expression level of inflammatory cytokines (Fig. 5C). Compared with the control group, the levels of cytokines in the serum (IL-4, IL-5, IL-10 and IL-13) significantly changed with an increase in IL-4, IL-5 and IL-10, while IL-13 decreased after oral feeding with β Lg. It is suggested that a subset of Foxp3⁺ Treg cells are feed-

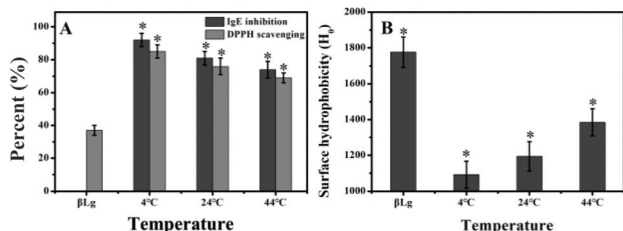


Fig. 4 Changes in the functional properties of conjugates prepared at different incubation temperatures. (A) Immunoglobulin E (IgE) combining capacity and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. (B) Surface hydrophobicity. Compared to β -lactoglobulin (β Lg), * means that p -values (<0.05) were considered significantly different.

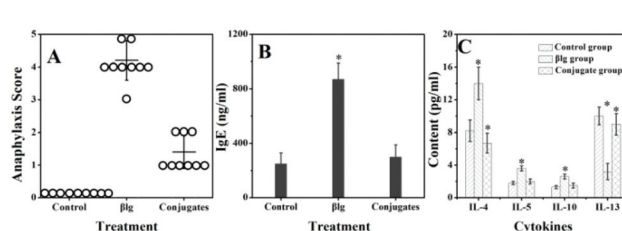


Fig. 5 Anaphylactic response of the mice after administration of the control group, β -lactoglobulin (β Lg) administered group, and conjugate administered group. (A) Anaphylaxis score; (B) immunoglobulin E (IgE) levels; and (C) the levels of inflammatory cytokines. Compared to the control group, * indicates that p -values (<0.05) were considered significantly different.

back activated by the overexpressed IL-4, impairing oral tolerance and promoting the development of food allergy.^{29,45} This is consistent with the anaphylactic symptoms observed in the β Lg group. The conjugates decrease the IgE combining capacity and reduce allergic symptoms, with a level of inflammatory cytokines comparable to those of the control group.

To assess the potential organ toxicity of the allergy induced by β Lg and the EGCG- β Lg conjugates, histopathology of the small intestine (jejunum) was analyzed after hematoxylin-eosin staining as shown in Fig. 6. The villi of the jejunum in the control group were intact and tightly arranged. As expected, pathology analysis showed that the jejunum morphology was affected and induced inflammatory lesions in 100% of the mice after the oral administration of β Lg. Compared with the control group, observed inflammatory lesions in the β Lg group included the degeneration and denudation of villous epithelia, the collapse of villi, surface erosion with exuberant inflammatory exudate, and the necrosis of lamina propria fibrosis (Fig. 6B), whereas the administration of EGCG- β Lg conjugates did not result in pathological effects, since no significant changes are observed in the histopathology (Fig. 6C).

Allergy has been reported to induce significant changes in intestinal flora compositions.⁴⁶ To further probe the desensitization effect of the EGCG- β Lg conjugate, the taxonomic compositions at different levels were compared in the three groups and are presented in Fig. 7. Nine phyla (relative abundance > 0.1%) were taken into account; of which, Firmicutes and Bacteroidetes accounted for more than 80% of the intestinal flora in the control group at the phylum level, similar to the results of Liu *et al.*⁴⁷ Although their proportion did not vary, Bacteroidetes was increased and Firmicutes was decreased significantly by β Lg, compared to the control group. Notably, the intestinal flora in the EGCG- β Lg conjugate remain comparable to the control group (Fig. 7A). At the class level, Bacteroidia and Bacilli decreased from 51.20% and 23.96%, respectively, in the control group, to 37.74% and 9.76% in the β Lg group. Conversely, Clostridia increased from 19.38% to 45.37% after β Lg administration, and is the predominant microbiota. As expected, EGCG- β Lg conjugates did not show similar effects (Fig. 7B). At the family level, 10 families (relative abundance > 1%) were taken into account and 4 of them are dominant bacterial communities in the control group, with Muribaculaceae

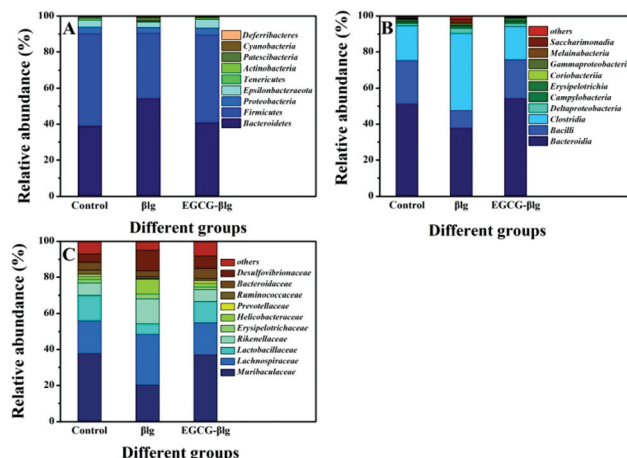


Fig. 7 Faecal microbiota of mice after 28 d. (A) Composition of flora at the phylum level; (B) composition of flora at the class level; and (C) composition of flora at the family level.

(37.59%), Lachnospiraceae (18.41%), Lachnospiraceae (14.04%) and Rikenellaceae (6.78%). Also, a similar microbiota ratio was observed in the EGCG- β Lg conjugate group. These 4 bacteria showed significant changes in the β Lg group. β Lg caused a significant increase of some pathogenic bacteria, such as Lachnospiraceae, Rikenellaceae and Desulfovibrionaceae, which were positively correlated with the organism immunity level.²⁷ In addition, the relative abundances of Muribaculaceae and Lactobacillaceae decreased (Fig. 7C). The conjugation of EGCG with β Lg might avoid FcεRI aggregation and decrease the levels of IgE and IL-4, compared with the mice group fed with β Lg, thus preventing stress on the mucosal immune system and maintaining tolerance against an invading antigen while inhibiting detrimental inflammatory reactions to the intestinal flora.^{29,44} Moreover, polyphenols can improve the imbalance of the Th1/Th2 immune response and can suppress antigen-specific IgE formation, attenuating the intestinal flora disruption and allergy symptoms.⁴⁸

Experimental

Materials

Bovine β Lg (purity $\geq$ 90%), EGCG (purity $\geq$ 99%), goat anti-human IgE-HRP, and ANS were purchased from Sigma-Aldrich (USA). All cow's milk allergen (CAM) patients' serum samples were generously provided by Shandong Children's Hospital (Jinan, China). Specific pathogen-free BALB/cJ female mice (3- to 4-week-old) were purchased from Jinan Peng Yue Experimental Animal Breeding Co. Ltd (Jinan, China). Other chemical reagents were of analytical grade and were purchased from Sinopharm Chemical Reagents Co., Ltd (Shanghai, China).

Formation of EGCG- β Lg conjugates

EGCG- β Lg conjugates were prepared by the free-radical induction reaction method with minor modifications.¹³ In brief,

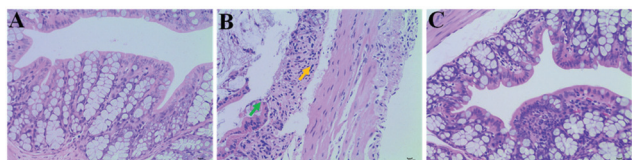


Fig. 6 Colonic histopathology of mice after administration for 28 d. (A) Control group, (B) the β -lactoglobulin (β Lg) administered group and (C) the epigallocatechin gallate (EGCG)- β Lg conjugate administered group. In B, the yellow arrow indicates colonic lamina propria necrosis and the green arrow indicates the loss of colonic epithelial cells.

50 μL H_2O_2 (5.0 M) and 100 μL ascorbic acid (Vc, 1.0 M) were added into 5 ml βLg solution (0.2 mM, pH 8.5), and kept at 20 $^\circ\text{C}$ for 2 h. Then, 0.5 ml EGCG solution (20 mM, pH 8.5) was added to the mixed solution and incubated for 6 h under continuous stirring at 4 $^\circ\text{C}$, 24 $^\circ\text{C}$ and 44 $^\circ\text{C}$, respectively. After incubation, the unreacted EGCG and Vc were removed by dialysis (8000 Da cut-off) against double-distilled water at 4 $^\circ\text{C}$ with 4 water changes.

ITC

All ITC experiments were conducted in 10 mM phosphate buffer solution (PBS, pH 7.2) at 4, 24 and 44 $^\circ\text{C}$ on a MicroCal ITC-200 system (GE Healthcare). βLg with H_2O_2 and EGCG solutions was thoroughly degassed and equilibrated at the desired temperature before titration. EGCG (40 μL , 4 mM) was titrated into βLg (200 μL , 0.1 mM) solutions. The reference cell contained 10 mM PBS (pH 7.2). Titration consisted of 20 injections, with 0.5 μL for the first injection and 2 μL for each subsequent injections.

MALDI-TOF

The samples were prepared by freeze-drying the EGCG- βLg conjugates prepared at different temperatures after resolubilisation in 10 μL deionised water. 1 μL of each solution was deposited on a MALDI target plate and dried at room temperature. A matrix of acetonitrile/water/trifluoroacetic acid (1 μL , 10 mg mL^{-1} , 50/49.5/0.5, v/v/v) was used to cover the dried sample and then dried again. The molecular weight of each sample was determined by MALDI-TOF MS analysis (MALDI Biotyper, Bruker, Germany). The average binding number (N) of EGCG on a single βLg molecule was calculated by the change in the molecular weight (MW) of EGCG- βLg conjugates divided by that of EGCG.

FTIR

FTIR spectra of βLg and EGCG- βLg conjugate samples were recorded at 20 $^\circ\text{C}$ using a Bomen MB series FTIR spectrometer (Quebec, Canada) equipped with a dTGS detector, purged constantly with dry air. A 128-scan interferogram was collected in a single-beam mode with 4 cm^{-1} resolution for each spectrum. The relative secondary structure contents were calculated according to the curve-fitting analysis of the amide I band from 1600 to 1700 cm^{-1} .

Functional properties of the conjugates

IgE combining capacity. The IgE combining capacity of βLg and EGCG- βLg conjugates was determined by an inhibition enzyme-linked immunosorbent assay (ELISA).⁴ 120 μL of βLg or EGCG- βLg conjugates (5 $\mu\text{g mL}^{-1}$) was added to coat the polystyrene MaxiSorp 96 U-well microplates (Roskilde, Denmark) at 4 $^\circ\text{C}$ overnight. After washing with PBS/Tween solution (PBST), 1% fish gelatin was added and incubated for 2 h at 37 $^\circ\text{C}$ to block the residual free-binding sites, and then, PBST was used to wash the plates. 100 μL of the CAM patients' serum (1 : 30 in PBST) was added and incubated for 2 h, after washing with PBST, and 100 μL goat anti-human IgE-HRP (1 : 200 in PBST)

was added and incubated for 60 min at 37 $^\circ\text{C}$. The absorbance was measured at 450 nm using a Bio-Rad microplate reader. The inhibition rate was calculated using the following equation: inhibition (%) = $(1 - B/B_0) \times 100$, where B and B_0 are the absorbance values of the well with and without the inhibitor, respectively.

DPPH radical scavenging activity. An aliquot containing 1 ml of conjugates (0.1 mM) and 1 ml of ethanolic DPPH solution (0.2 mM) was mixed and incubated at room temperature for 30 min. The control sample made of DPPH solution with distilled water was subjected to the same protocol. The absorbance was determined at 517 nm using a spectrophotometer (Infinite, M200 PRO, Tecan, Austria). DPPH radical scavenging activity (%) was calculated using the following equation: DPPH radical scavenging activity (%) = $(1 - A/A_0) \times 100$, where A and A_0 are the absorbance values of the control group and conjugate group, respectively.

Surface hydrophobicity. The surface hydrophobicity of βLg and EGCG- βLg conjugates was measured according to the methods of Haskard and Li-Chan with a slight modification.⁴⁹ 1-Anilinonaphthalene 8-sulfonate (ANS) was used as the hydrophobic probe. 20 μL of ANS (8 mM, pH 7.2) solution was mixed with 4 ml of samples (0.02 mg mL^{-1} to 1.0 mg mL^{-1}) and measured using a fluorescence spectrophotometer (F-7000, Hitachi, Japan). The emission spectra were recorded from 400 nm to 600 nm at the excitation wavelength of 390 nm with a slit of 5 nm. The surface hydrophobicity (H_0) was defined as the initial slope of the relative fluorescence intensity to the protein concentration (mg mL^{-1}).

In vivo determination of the desensitization effect

Animal experiments. All mice used in this study were cared for in accordance with the National Guidelines for Experimental Animal Welfare (Ministry of Science and Technology of the People's Republic of China, 2006). All experimental procedures were approved by the Animal Care Review Committee of Qilu University of Technology.

The specific pathogen-free BALB/cJ female mice (3- to 4-week-old) were acclimated to the new environment for 1 week before the start of the experiment. The mice were allowed free intake of food and purified water. The ratio of βLg was chosen based on the weight of the mice, as well as on preliminary experiments, which allowed determining the amount of βLg that can induce allergic symptoms in most of the mice in the βLg group.

The mice were divided into 3 randomly assigned experimental groups with 9 in each group and housed in an air-conditioned room (23 ± 2 $^\circ\text{C}$) for 28 days under a 12 h light/12 h dark cycle. The 28 days period was chosen based on a preliminary experiment, which showed that after 28 days the symptoms remained stable. Every day, the control group was administered 0.5 ml sterile water intragastrically, the βLg group was administered 0.5 ml βLg solution (2 mg mL^{-1}), and the conjugate group was administered 0.5 ml EGCG- βLg solution (prepared at 4 $^\circ\text{C}$, 2 mg mL^{-1}).

On day 28, all mice, except those in the control group, were orally challenged with β Lg (20 mg per mouse) and EGCG- β Lg (20 mg per mouse) in the case of β Lg and EGCG- β Lg groups, respectively. The retro-orbital plexus blood was taken after 60 min. Then, faecal and tissue samples from each mouse were immediately collected for further analysis.

Evaluation of the allergic reaction. The weights of the mice were recorded every day, and the allergic reaction was evaluated by observing the symptoms of the mice after each administration. Allergic symptoms were scored as 0 = no symptoms; 1 = hair up and scratching head or ear; 2 = reduced activity and swelling around the eyes and mouth; 3 = rest for more than a minute; 4 = loss of consciousness and no activity upon prodding; and 5 = convulsion or death.

Detection of the levels of IgE and cytokines. The retro-orbital plexus blood was taken at d 28, and then centrifuged at 4000g for 10 min at 4 °C. The supernatant was stored at -80 °C until use. The levels of serum IgE, IL-4, IL-5, IL-10 and IL-13 were determined by ELISA (NeoBioscience, Co. Ltd, Shanghai, China).

Histological analysis. The colon tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) for intestinal mucosal integrity and lymphocytic infiltration. All analyses were performed under a microscope at $\times 100$ magnification (1CX41, Sunny Optical Technology Company, China).

Detection of intestinal flora. The intestinal flora compositions were examined by detecting 16S rRNA gene sequencing according to the MiSeqPEX 300 bp platform (Itechgene, Shanghai, China). The faecal samples were collected and stored at -80 °C. The extraction and purification of DNA were performed according to the methods of Xue.⁵⁰ The V3-V4 hypervariable regions of the bacterial 16S rDNA were amplified by the primers (341F and 805R). Afterwards, the 16S rDNA PCR products were sequenced using a 300 bp paired-end model with the MiSeq system and the sequences clustered at 97% similarity were assigned to the same operational taxonomic unit (OTU).

Conclusions

Polyphenolic compounds interact with amino or sulfhydryl side chains in globular proteins to form protein-polyphenol conjugates. This conjugation mainly rely on covalent or non-covalent bonding, resulting in the structure and bioactivity changes of proteins, and impart new polyphenolic properties to the conjugates, which might provide a novel approach to desensitize allergens, such as β Lg.

In this study, the conjugation of EGCG to β Lg is a spontaneous exothermic process, in which the binding affinity parameter (K_a) decreased from $7.3 \pm 2.1 \times 10^6 \text{ M}^{-1}$ at 4 °C to $4.3 \pm 1.7 \times 10^6 \text{ M}^{-1}$ at 44 °C and the binding number decreased from 3.6 to 2.1 at the same temperatures. The conjugation of EGCG decreased β Lg's IgE combining capacity by decreasing the β -sheet component and destroying the conformation of IgE epitopes, and provided good antioxidant properties by the

introduction of a high number of hydroxyl groups. Animal experiment results indicated that β Lg caused changes to a series of basic immune parameters including change in the levels of IgE and inflammatory cytokines and the relative abundance of small intestinal microbiota, resulting in the destruction of villous epithelia and anaphylaxis symptoms, while all these changes were suppressed when β Lg was conjugated with EGCG. This study strongly indicates that conjugating β Lg with EGCG can suppress allergic response by attenuating serum IgE and relieving anaphylaxis symptoms. The *in vitro* and *in vivo* results obtained in this study can contribute to our fundamental understanding of the desensitization efficiency of β Lg through its conjugation with EGCG. Future research work should consider in more detail the modification of β Lg by EGCG and carry out further comprehensive *in vivo* experiments. Finally, it will be industrially important to investigate how the β Lg-EGCG conjugates perform in real food products such as infant formula.

Author contributions

Xiaoning Zhang: investigation, methodology, validation, formal analysis, and writing – original draft. Sining Li: data curation, formal analysis and funding acquisition. Xiaoqing Shao: data curation and formal analysis. Meifeng Li: supervision, project administration, funding acquisition and conceptualization. Yacine Hemar: methodology and writing – review and editing.

Conflicts of interest

The authors declare no competing financial interests.

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