

Comparative studies of interaction of β -lactoglobulin with three polyphenols

Jinhui Xu, Minghao Hao¹, Qifan Sun, Lin Tang^{*}

Key Laboratory of Food Nutrition and Safety of SDNU, Provincial Key Laboratory of Animal Resistant Biology, College of Life Science, Shandong Normal University, Jinan 250014, PR China

ARTICLE INFO

Article history:

Received 18 March 2019

Received in revised form 3 June 2019

Accepted 9 June 2019

Available online 20 June 2019

Keywords:

β -Lactoglobulin

Polyphenol

Allergenicity

Multi-spectrometry

Binding

ABSTRACT

To investigate the interaction mechanism between bovine protein β -lactoglobulin (β -LG) and theaflavin (TA), chlorogenic acid (CA) and delphinidin-3-O-glucoside (D3G), multi-spectrometry analytical methods and molecular modeling were applied. Fluorescence experiments proved that polyphenols strongly quenched the intrinsic fluorescence of β -LG mainly through static quenching and the main interaction force was hydrophobic interaction. Moreover, Fourier transform infrared (FTIR) and circular dichroism (CD) indicated that polyphenols changed β -LG secondary and tertiary structure. Enzyme-linked immunosorbent assay and molecular modeling study manifested that complex of β -LG with polyphenols could significantly reduce the IgE-binding capacity of β -LG due to the polyphenol binding site directly obscures the IgE linear epitopes. In conclusion, polyphenols had impact on the structure and potential functionality of β -LG, which would be valuable in dairy processing industry and food nutrition security.

© 2019 Elsevier B.V. All rights reserved.

1. Introduction

Plant polyphenols are a class of secondary metabolites with polyphenol structures that are widely distributed in plants. It is found in common plant foods such as coffee beans, tea, wine, vegetables and fruits. Many studies have shown that polyphenols have the function of promoting human health, such as antioxidant, anti-inflammatory, antiglycation [1,2], preventing cardiovascular and cerebrovascular diseases, preventing cancer and neurodegenerative diseases, preventing Crohn's disease and ulcerative colitis [3], even preventing psychological dysfunction and cognitive impairment [4]. Black tea is the most widely consumed tea product around the world and the theaflavin is the product of oxidation polymerization of catechins in the fermentation process of black tea. Theaflavin is a class of benzo tropolone compounds containing multiple hydroxyl or phenolic hydroxyl groups. It has been reported that theaflavin showed excellent activities on anti-inflammatory, anti-cancer, anti-microbial and antiviral [5]. Chlorogenic acid is a phenylpropanoid compound formed by caffeic acid and quinic acid. Chlorogenic acid is widely found in coffee beans, honeysuckle and other plants [6]. Chlorogenic acid showed excellent activity in areas such as inhibiting thrombus formation and human platelet activation, protecting nervous system and reducing diabetic retinopathy [6–8]. Delphinidin-3-O-glucoside is a type of anthocyanidin, which is a

flavonoid compound of anthocyanin and various sugars with glycoside bonds. Delphinidin-3-O-glucoside is widely found in grapes, blueberries, mulberries and other plants. Previous articles have reported that it did not only combat hyperglycemia, prevent cardiovascular, cerebrovascular and Alzheimer's disease, but also resisted proliferation, oxidation and scavenges free radicals [9–14].

Bovine β -lactoglobulin (β -LG) is the major protein in whey. It is a globular protein which is constituted of 162 amino acids with a molecular weight of 18.3 kDa. Many previous studies shown that β -lactoglobulin can be used as an effective nano-carrier or microcapsule for the delivery of drugs [15]. Kanakis et al. studied the interaction of β -LG with catechin, epicatechin, epicatechin gallate and epigallocatechin gallate by fluorescence spectroscopy, circular dichroism and molecular docking techniques. The results showed that the four polyphenols could interact with β -LG. The main forces were hydrophilic and hydrophobic forces. The conformation of β -LG had changed and both β -sheet and α -helix had increased [16]. Liang et al. studied the interaction of resveratrol with β -LG by UV–Vis absorption spectroscopy, circular dichroism and fluorescence spectroscopy. They found that resveratrol could interact with β -LG to form a 1:1 complex. After the interaction of β -LG with resveratrol, the secondary structure of the β -LG did not change significantly, but some of the tertiary structure was destroyed [17]. In addition, β -LG is generally considered to be the major cow's milk allergen, and 82% of milk allergy patients are sensitive to it.

Functional dairy products (milky tea, coffee milk, fruit milk, etc.) is a new type of dairy products, mainly produced by adding probiotics or active substances, so that dairy products have corresponding

^{*} Corresponding author at: School of Life Science, Shandong Normal University, 88 East Wenhua Road, Jinan, Shandong Province 250014, PR China.

E-mail address: tanglin@sdnu.edu.cn (L. Tang).

¹ These two authors are co-first authors.

physiological activities. Some articles studied the interactions of malvidin-3-O-glucoside with β -casein and β -lactoglobulin for functional milk, tea, coffee, fruit and other products contain polyphenols, and other articles reported the interaction between chlorogenic acid and milk proteins [18–20]. The interactions between polyphenols and bovine milk proteins have attracted great interest. Few papers, however, have focused on the comparison of the effects of different polyphenols interacting with milk proteins on protein conformation. As the main executor of biological function, the change of conformation of protein directly affects its function. Therefore, it is necessary to study the effect of functional ingredients in functional dairy products on milk proteins, in particular, the effects on allergic proteins in milk. Recently, there are researches finding that phenolic-IgE complexes with the major egg allergen ovalbumin and major peanut allergen Ara h1 by interactions, which reduced their allergenic capacity [21,22]. Hence, in this study, we investigated the interactions of theaflavin (TA), chlorogenic acid (CA) and delphinidin-3-O-glucoside (D3G) with bovine β -lactoglobulin (β -LG) and reduction of their allergenic capacity. The differences of their interactions were well compared, using ultraviolet-visible (UV-Vis) absorption spectroscopy, multi-fluorescence spectrophotometry, dynamic light scattering (DLS) measurements, Fourier transform infrared (FTIR), circular dichroism (CD) spectroscopy, enzyme-linked immunosorbent assay (ELISA) and molecular modeling study. The interaction between polyphenols and the bovine milk protein, and how they affect the structure and function of the protein should be validated. These results would be valuable in dairy processing industry and food nutrition security.

2. Materials and methods

2.1. Materials

Series standard reagents of theaflavin (TA), chlorogenic acid (CA) and delphinidin-3-O-glucoside (D3G) were obtained from BIYU BIOTECHNOLOGY (Shanghai, China), MedChem Express (Princeton, NJ, USA) and Tokiwa (Tokyo, Japan), respectively. Their purity is all >98%. β -Lactoglobulin (β -LG, purity $\geq$ 98%) from bovine milk was obtained from Sigma Chemical Company (St. Louis, MO, USA). Goat anti-human IgE-HRP conjugate was purchased from Immunology Consultants Laboratory Inc. (Oregon, Portland). Human sera: sera of patients allergic to bovine milk were purchased from PlasmaLab International (Everett, W.A., USA) and stored at -80°C until used. All other materials were of analytical grade.

2.2. Preparation of stock solutions

The β -LG solution was formulated into a stock solution of 1×10^{-4} mol/L in PBS buffer with pH 6.6. During the experiment, β -LG stock solution was diluted to 1×10^{-5} mol/L working solution in PBS buffer. TA, CA and D3G were all formulated with absolute ethanol at a concentration of 5×10^{-3} mol/L. Polyphenols were added dropwise to β -LG in a molar ratio required for different experiments to form a complex. The double distilled water was used for preparing other solutions. Reagents that need to be protected from light were placed in brown reagent bottles and all reagents were stored in 277 K refrigerator.

2.3. UV-Vis absorption spectroscopy

The β -LG-TA/CA/D3G system was monitored using the T9 Series Double beam UV-Vis Spectrophotometer (Beijing, China) scanning over the wavelength range of 190–800 nm with the help of a Quartz cuvette of 1 cm and the slit width was set at 2 nm.

2.4. Fluorescence spectroscopy

Fluorescence investigations were detected on an F-7000 fluorescence spectrophotometer (Hitachi, Japan) at three temperatures (290 K, 300 K and 310 K) equipped with a 10 mm path-length quartz cell. The excitation wavelength was 280 nm and the excitation and emission slit widths were fixed at 5 nm. The emission wavelength was recorded in the range of 290–500 nm with scan rate at 1200 nm min^{-1} . Synchronous fluorescence spectra were all recorded in the range of 200–350 nm ($\Delta\lambda = 15 \text{ nm}$; $\Delta\lambda = 60 \text{ nm}$) at 290 K. Post-collection data were processed by Origin8.6. Polyphenol blanks have been deducted during subsequent experimental processing.

2.5. Fluorescence data analysis

The fluorescence quenching mechanism was analyzed by the Stern-Volmer equation [23]. The double-reciprocal equation was used to calculate K_a (binding constant) and n (the number of binding sites) [24]:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{sv} \quad (1)$$

$$\log[(F_0 - F)/F] = \log K_a + n \log[Q] \quad (2)$$

where F_0 , F , K_q , $[Q]$, K_{sv} are the fluorescence intensities without TA/CA/D3G, fluorescence intensities with TA/CA/D3G, biomolecular quenching rate constant, quencher concentration and Stern-Volmer quenching constant, respectively. τ_0 is the average lifetime of the biomolecule without a quencher ($\tau_0 = 10^{-8} \text{ s}$).

The Van't Hoff equation was used to obtain the ΔS (entropy change), ΔH (enthalpy change) and ΔG (free energy change) [25]:

$$\ln K_a = -\Delta H/RT + \Delta S/R \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

where R and T are the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) and the experimental temperature, respectively.

The equation of Förster theory was used to calculate the E (efficiency of energy transfer) [26]:

$$E = 1 - F/F_0 = R_0^6 / (R_0^6 + r^6) \quad (5)$$

$$R_0^6 = 8.8 \times 10^{-25} \text{ k}^2 \text{ N}^{-4} \Phi J(\lambda) \quad (6)$$

$$J(\lambda) = \frac{\sum F(\lambda)\epsilon(\lambda)\lambda^4\Delta\lambda}{\sum F(\lambda)\Delta\lambda} \quad (7)$$

where r , R_0 , k^2 , N , Φ , J , $F(\lambda)$ and $\epsilon(\lambda)$ are the distances between TA/CA/D3G and β -LG, the Förster critical distance at 50% transfer efficiency from β -LG to TA/CA/D3G, the spatial orientation factor of the dipole of β -LG and TA/CA/D3G, the refractive index of medium, the fluorescence quantum yield of β -LG and the spectral overlap between the emission spectrum of β -LG and the absorption spectrum of TA/CA/D3G, the fluorescence intensity of β -LG at wavelength λ and the molar absorption coefficient of TA/CA/D3G at wavelength λ , respectively. For β -LG, $k^2 = 2/3$, $n = 1.36$ and $\Phi = 2.52$ [27].

2.6. Particle size of β -LG and β -LG-TA/CA/D3G

The average particle sizes of β -LG and β -LG-TA/CA/D3G were determined by dynamic light scattering (DLS) using a Winner 802 instrument (Winner Particle, China) according to the descriptions of Jia et al. [27,28]. 2.5 mL solution system of β -LG (1.0×10^{-5} mol/L) and β -LG-TA/CA/D3G was placed in the test vessel to test the particle size of β -LG respectively. The molar ratio of TA, CA and D3G to β -LG in the mixed system was 1:1. All measurements were repeated three times.

2.7. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of protein and of protein-polyphenols complexes were recorded using a TENSOR II FTIR spectrometer (BRUKER, Germany). The experimental conditions of infrared spectroscopy were 4 cm^{-1} and 32 scans with the attenuated total reflectance method. The β -LG sample concentration was $1.0 \times 10^{-5}\text{ mol/L}$, and the molar ratios of β -LG to TA/CA/D3G were 1:6. The infrared spectra of free β -LG was gain by subtracting PBS buffer solution from the spectra of the protein solution, and the different spectra of β -LG were acquired by subtracting the spectra of TA/CA/D3G from that of β -LG-TA/CA/D3G at the same concentration.

In order to analysis of the secondary structure of β -LG and β -LG-TA/CA/D3G complexes, the spectral region between 1600 and 1700 cm^{-1} (amide I absorption band of the peptide backbone) was selected. Fourier self-deconvolution and secondary derivative were applied to estimate the number, position and areas of component bands.

2.8. Circular dichroism (CD) spectroscopy

The CD spectra were recorded in a chirascan circular dichroism spectrometer (Applied PhotoPhysics, Surrey, UK) at 290 K. TA/CA/D3G was added dropwise to β -LG in quartz cuvettes of 0.1 cm path length to prepare a sample mixture having a molar ratio of 6:1. Far-UV CD spectra were recorded from 200 to 260 nm. The contents of secondary structures of β -LG with or without TA/CA/D3G were analyzed by the SELCON3 algorithm of CDPro.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The IgE-binding capacity of β -LG-TA/CA/D3G samples was estimated by an inhibition ELISA and indirect ELISA with modification [29–31]. Firstly, 96-well micro-plates were added $5\text{ }\mu\text{g/mL}$ $100\text{ }\mu\text{L/well}$ of β -LG and were incubated overnight (about 12 h) at $4\text{ }^{\circ}\text{C}$. After washing 4 times with PBS (0.01 M pH 7.4)/Tween (0.4% Tween) solution, the wells of micro-plates were blocked with 1% fish gelatin in carbonate buffer solution (0.05 M pH 9.6 CBS) for 2 h at $37\text{ }^{\circ}\text{C}$. Equal volumes of β -LG-TA/CA/D3G samples and 1:10 diluted human serum were mixed and hatched for 1 h at $37\text{ }^{\circ}\text{C}$. Then, after washing 4 times with PBST, the wells were added $100\text{ }\mu\text{L}$ of each β -LG-TA/CA/D3G samples and antisera samples mixture and incubated for 2 h at $37\text{ }^{\circ}\text{C}$ for inhibition ELISA. After washing 4 times with PBST, $100\text{ }\mu\text{L}$ of purified goat anti-human IgE-HRP conjugate diluted 1:5000 in PBS (0.01 M pH 7.4) was added and the plates were incubated for 1 h at $37\text{ }^{\circ}\text{C}$. However, for indirect ELISA, $100\text{ }\mu\text{L}$ of 1:30 diluted human serum was added to the wells and incubated for 2 h at $37\text{ }^{\circ}\text{C}$ similarly. After washing 4 times with PBST, $100\text{ }\mu\text{L}$ of purified goat anti-human IgE-HRP conjugate (diluted 1:200 in PBS) was added and the plates were hatched for 1 h at $37\text{ }^{\circ}\text{C}$. Finally, after washing 4 times with PBST, $100\text{ }\mu\text{L}$ of tetramethylbenzidine solution was immediately added to each wells and incubated for 30 min at $37\text{ }^{\circ}\text{C}$ in dark. A solution of $100\text{ }\mu\text{L}$ 2 M sulfuric acid was added to stopping the reaction. The absorbance was measured at 450 nm using the Microplate Reader. The inhibition rate was calculated using the following equation: $\text{Inhibition \%} = (1 - B/B_0) \times 100$, where B_0 and B are the absorbance values of the well without and with the inhibitor, respectively [30].

2.10. Molecular modeling study

The β -LG molecule model (PDB ID: 3NPO), TA model, CA model and D3G model were used for ligand docking. TA model, CA model and D3G model were drawn by Chemdraw. Docking simulations were executed using AUTODCK 4.2.6 and Lamarckian genetic algorithm was applied to count the possible conformation of the ligand molecule and macro-molecule. The conformer with the lowest binding free energy was used for further analysis [32].

3. Results and discussion

3.1. Interaction between β -LG and TA/CA/D3G

3.1.1. UV-Vis absorption spectroscopic investigations

The tryptophan, tyrosine, phenylalanine and sulfur-containing amino acid have absorption peaks in the wavelength range of 230–310 nm. Among them, tryptophan, tyrosine and phenylalanine have different absorption spectra due to their different color groups. The conjugated double bond of tryptophan and tyrosine residues has an absorption peak at 280 nm wavelength. The phenylalanine has a characteristic absorption peak near the wavelength of 257 nm. The polypeptide backbone structure has a strong absorption peak near the wavelength of 210 nm [32]. The UV-Vis absorption spectra of β -LG with or without TA/CA/D3G were shown in Fig. 1 (A/B/C). There was a strong absorption peak near the wavelength of 210 nm and a clear absorption peak at 280 nm, which represent the polypeptide backbone structure and amino acid residues, respectively. With gradual addition of TA/CA/D3G to protein solution, the absorption intensity of β -LG was all increased. There were new complex formations according to the new absorption peaks after 290 nm with the addition of TA/CA/D3G, which can reflect binding interactions existed between β -LG and TA/CA/D3G. It was obvious that the maximum peak position of the complexes was blue shifted slightly after added TA; it was red shifted obviously with gradual addition of CA. Whereas the position of the maximum peak was not changed with gradual addition of D3G to the protein solution. These results showed that the hydrophobicity of the microenvironment of aromatic amino acid residues increased when the TA existed. On the contrary it was decreased with gradual addition of CA. And the hydrophobicity of the microenvironment of aromatic amino acid residues was not changed when the D3G existed. It could be the polarity of the microenvironment near the aromatic amino acid residues decrease or increase [33]. Amino residues were located in a more or less hydrophobic environment and embedded in the proteins or exposed to the solution. Therefore, the binding interactions between proteins and TA/CA/D3G existed. For β -LG-TA system, two new peaks appeared at 370 nm and 470 nm. For β -LG-CA system, there was a new peak at 342 nm. For β -LG-D3G system, there were two new peaks appeared at 447 nm and 562 nm. These results manifested that the structure of the new complexes was related to the type of polyphenols. In addition, it could be speculated that the quenching mechanism of β -LG-TA/CA/D3G were static quenching, because dynamic quenching only effected the excited state of quenching molecule while it would not change the absorption spectra of quenching substances.

3.1.2. Analysis of fluorescence spectra of β -LG on addition of TA/CA/D3G

The binding information of polyphenols and proteins, such as quenching mechanism, binding site, binding constant and type of force, can be obtained by the analysis of fluorescence intensity and so on. β -LG contains two tryptophan (Trp) residues Trp-19 and Trp-61. Trp-19 makes 80% contributes to total fluorescence and it is in an apolar environment, while Trp-61 makes a minor contribution to Trp fluorescence and it is partially exposed to aqueous solvent [16]. The fluorescence emission spectra of β -LG with the gradual addition of TA/CA/D3G were shown in Fig. 2 (A/B/C). The maximum peak positions of β -LG near the wavelength of 340 nm with the excitation at 280 nm, the intrinsic fluorescence intensity value of β -LG decreased with the addition of TA/CA/D3G, the intrinsic fluorescence intensity value of β -LG decreased 84.8%, 76.5% and 64.6% respectively by counting at the TA/CA/D3G concentration of $60\text{ }\mu\text{M}$. These results suggested that TA/CA/D3G could bind with β -LG and the quenching ability on the intrinsic fluorescence intensity value of β -LG was $\text{TA} > \text{CA} > \text{D3G}$. Meanwhile, with the addition of CA, the maximum peak position of the complexes was clearly red shifted, which implied that the hydrophobicity of the microenvironment of aromatic amino acid residues decreased. Although the maximum peak position of the complexes with the addition of TA and

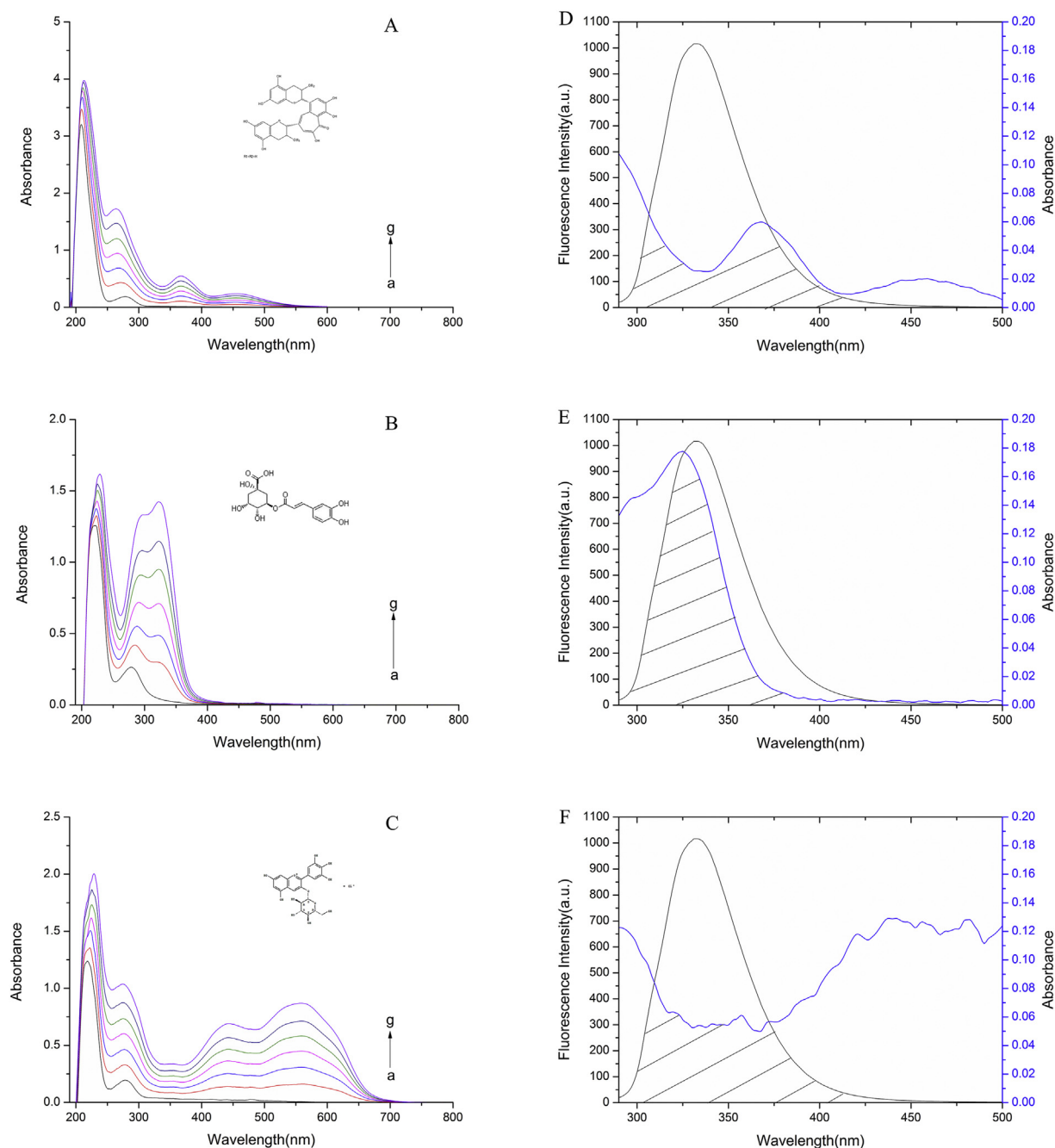


Fig. 1. A/B/C: UV-Vis absorption spectra of β -LG without and with TA/CA/D3G. D/E/F: Spectral overlaps of the fluorescence spectra of β -LG with the absorption spectra of TA/CA/D3G. a–g: $c(\beta\text{-LG})/c(\text{TA/CA/D3G}) = 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0$.

D3G was not altered, the clear decreased in intrinsic fluorescence intensity value may due to the conformational changes in the microenvironment.

3.1.3. The fluorescence quenching mechanism and binding constant

It is often used to study the interaction between biomacromolecules (such as proteins) and small molecules by fluorescence quenching (i.e. fluorescence material interacted with solvent or solute molecules to cause a decrease in fluorescence intensity). The Stern-Volmer equation was used to analyze the β -LG fluorescence quenching data at three different temperatures (290 K, 300 K and 310 K). From Table 1, the value of biomolecular quenching rate constant (K_q) was much higher than maximum diffusion collision quenching constant value (2.0×10^{10} L/mol/s), which indicated that the quenching type of β -LG caused by TA/CA/D3G belonged to static quenching. As shown in

Fig. 2 (D/E/F), the curves presented good liner relationships at 290 K, 300 K and 310 K, and the values of n (binding sites) and K_a (binding constant) were shown in Table 1. The values of binding sites were close to 1 when the TA/CA/D3G existed, so there was single binding site on β -LG. For the binding constant (K_a), their sequence was $\text{TA} > \text{CA} > \text{D3G}$ at 300 K and 310 K. But at 290 K, their sequence was $\text{TA} > \text{D3G} > \text{CA}$ which was indicative of the fact that the binding affinity of TA towards β -LG was stronger than that of CA and D3G. Therefore, we speculated that β -LG have much stronger affecting ability of digestibility of proteins and functional properties and bioavailability for TA than CA and D3G. Besides, the values of K_a increased with increasing temperature for the complexes of β -LG-TA and β -LG-CA, indicated an endothermic reaction. However, the values of K_a for the complexes of β -LG and D3G decreased with the increasing temperature, which could be indicated that their binding reaction was exothermic. Similar result was reported by

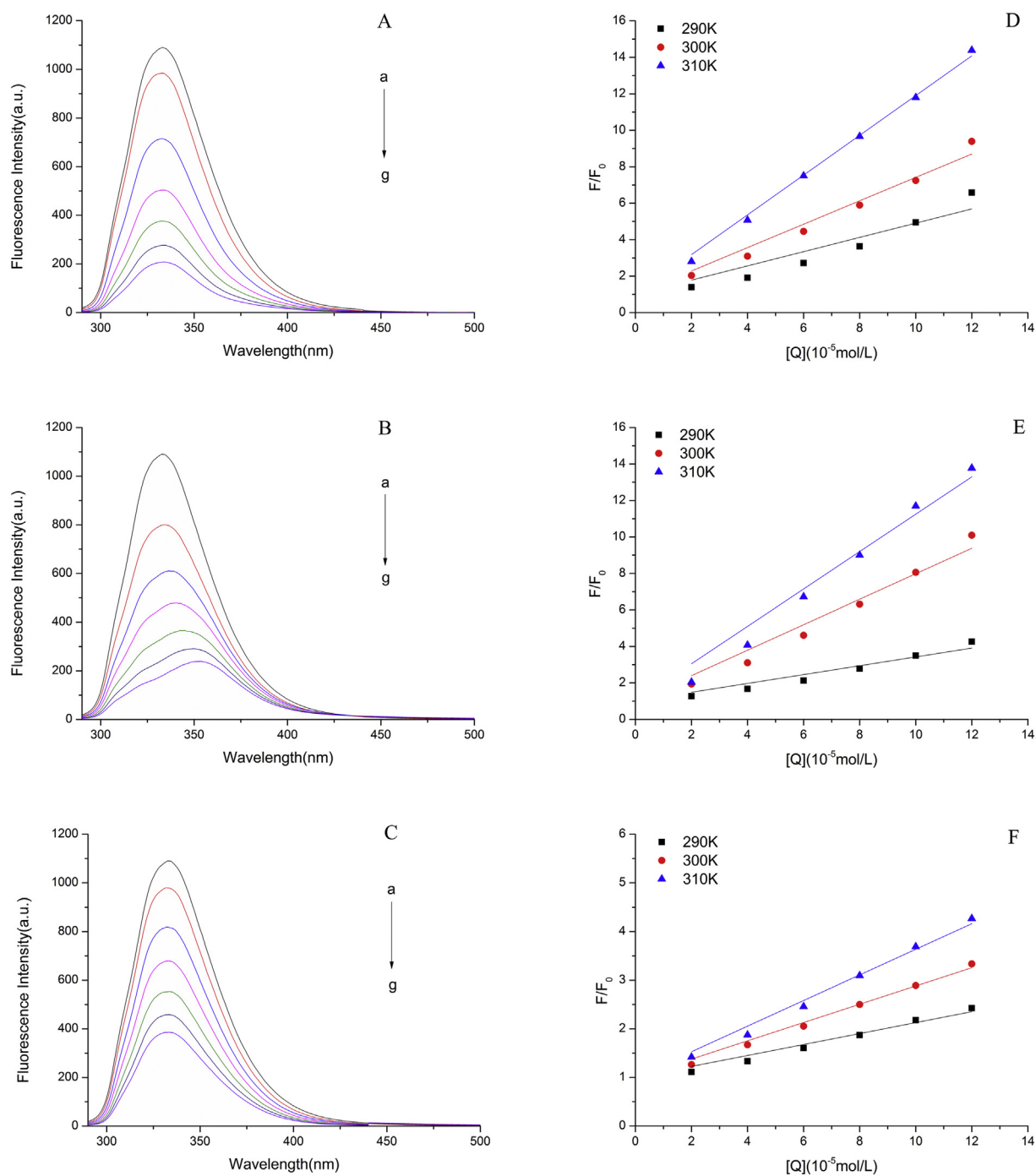


Fig. 2. A/B/C: Effect of TA/CA/D3G on fluorescence spectra of β -LG. D/E/F: The Stern-Volmer plots for the quenching of β -LG by TA/CA/D3G at different temperatures.

Table 1

The quenching and bind constants and thermodynamic parameters for the interaction of β -LG with TA/CA/D3G at different temperatures.

Compound	T (K)	K_{sv} (10^4 L/mol)	K_q (10^{12} L/mol)	R^2	K_a (10^5 L/mol)	n	R^2	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/mol)
β -LG + CA	290	2.425	2.425	0.9898	8.915	1.391	0.9976	137.62	-32.76	587.52
	300	7.409	7.409	0.992	42.771	1.438	0.999		-38.64	
	310	10.245	10.245	0.9931	3.545×10^2	1.605	0.9987		-44.51	
β -LG + D3G	290	1.335	1.335	0.9975	22.35	1.553	0.9998	6.68	-28.94	122.81
	300	2.105	2.105	0.9989	0.844	1.157	0.9965		-30.17	
	310	2.788	2.788	0.9983	1.95	1.211	0.9969		-31.39	
β -LG + TA	290	3.84	3.84	0.9994	32.727	1.482	0.992	100.89	-36.15	472.57
	300	7.031	7.031	0.9942	71.77	1.509	0.9939		-40.88	
	310	11.085	11.085	0.9992	4.842×10^2	1.672	0.9974		-45.6	

Cheng, J., et al., who reported that the interaction of β -LG with cyanidin-3-O-glucoside was tuned by hydrophobic forces and their binding reaction was exothermic [34]. The speculation would be explored in next thermodynamic parameters experiment.

3.1.4. Thermodynamic parameters

There are four types of non-covalent forces between the biomacromolecules and small molecules, including hydrogen bonds, electrostatic interactions, hydrophobic interactions and van der Waals forces. These forces can be determined by thermodynamic parameters (ΔS , ΔH and ΔG). The entropy change (ΔS), enthalpy changes (ΔH) and free energy change (ΔG) were calculated by Eqs. (3) and (4). These results were shown in Table 1. According to the previous literature, when $\Delta H < 0$, $\Delta S > 0$, the main forces are electrostatic forces; when $\Delta H > 0$, $\Delta S > 0$, the hydrophobic forces play a major role; when $\Delta H < 0$, $\Delta S < 0$, the main forces are hydrogen bonds and van der Waals forces [35]. It could be seen that the enthalpy change (ΔH) and the entropy change (ΔS) of β -LG were positive, which suggested the hydrophobic forces played a major role in the interactions of β -LG with TA/CA/D3G. However, the free energy change (ΔG) of β -LG was negative. It suggesting the binding of β -LG with TA/CA/D3G was spontaneous.

3.1.5. Energy transfer from β -LG to TA/CA/D3G

According to the theory of Förster fluorescence resonance energy transfer, the conditions are: there is a sufficient overlap area between the fluorescence emission spectrum of the donor (protein) and the UV absorption spectrum of the receptor (ligand), and the distance between the donor and the receptor is < 7 nm [26,27]. On the basis of the Förster theory, the efficiency of energy transfer (E), the distance between donor and acceptor (r) and the critical distance when the transfer efficiency is 50% (R_0) were calculated by Eqs. (5), (6), and (7), and these results were shown in Fig. 1 (D/E/F) and Table 3. The values of r were all smaller than 7 nm, which could be highly possible that a non-radiative energy transfer from β -LG to TA/CA/D3G occurred. Overall, these results were consistent with the previous results that there was a static quenching mechanism between β -LG and TA/CA/D3G, which were consistent with the previous results of fluorescence quenching mechanism.

3.2. Effect of TA/CA/D3G on the structural change of β -LG

3.2.1. Dynamic light scattering (DLS) measurements

Based on the literature, β -LG micelles have size below 100 nm [36]. Supplementary Table 1 showed that the results of particle size distribution of β -LG-TA/CA/D3G complexes were still in nano-size region. The particle size of β -LG and β -LG-TA/CA/D3G complex was 8.37 nm, 24.56 nm, 14.82 nm and 21.76 nm respectively. This indicated that the interaction of TA/CA/D3G and β -LG formed new complexes which made the particle size larger than before and the conformation of β -LG might have changed.

3.2.2. Fourier transforms infrared (FTIR) spectroscopy

The protein and polypeptide repeat units give rise to nine characteristic infrared absorption bands, namely, amide A, B, and I–VII. Of these, the two most prominent vibrational bands of the protein backbone are the amide I and II bands [37]. The amide I band (1700 – 1600 cm^{-1}) is the most sensitive spectral region to the protein secondary structural components, which is due almost entirely to the C=O stretch vibrations of the peptide linkages. The amide II derives mainly from in-plane NH bending and from the CN stretching vibration [38]. Supplementary Fig. 1 (D/E/F) described the original infrared spectra (amide I and II bands) of the free protein and the protein-polyphenol complexes. In the presence of TA/CA/D3G, the infrared spectra of pure protein had been transformed, suggesting that the secondary structure of β -LG was changed. In other words, TA/CA/D3G was not only interacted with β -LG, but also altered the secondary structure of the β -LG. In Supplementary Fig. 1 (D/E/F), it could be found that the TA changed the secondary structure of protein

was the most obvious in the three types of polyphenols. In order to further study the specific changes of TA/CA/D3G on the secondary structure of β -LG, we conducted circular dichroism spectroscopy.

3.2.3. Circular dichroism (CD) spectroscopy

Far-UV CD spectrum is an important spectroscopic method for the determination of the structure changes of protein. The different secondary structures of proteins have different absorption bands and different positions of CD spectra in the far ultraviolet region. From Supplementary Fig. 1 (A/B/C), it showed the CD spectrum of β -LG had two negative bands at 208 nm and 218 nm, which represented the characteristic α -helix structure of the protein [39]. Table 2 showed the fractions of α -helix, β -sheet, β -turn and random coil were estimated by CDNN. It could be seen that the fractions of α -helix was increased instead of the fractions of β -sheet was decreased, which indicated the β -LG-TA/CA/D3G complexes changed β -LG secondary structure inducing β -sheet to α -helix transition. The fractions of β -turn and random coil were all decreased. These results implied the secondary structure of β -LG was more firming after forming complexes. In addition, we compared the three groups of polyphenols data and found that the secondary structure of β -LG interacts with TA greatly changes, which could also be significantly found in Supplementary Fig. 1. In the presence of TA, there were two decreases between 208 nm and 218 nm. This indicates that TA has the greatest influence on the conformation of β -LG compared to the other two polyphenolic substances.

3.2.4. Synchronous fluorescence analysis

Synchronous fluorescence spectrometry is usually used to reveal the effect of protein and ligand on the tertiary structure of protein. When the wavelength interval is kept at 15 nm or 60 nm, the synchronous fluorescence spectra shows the conditions near the tyrosine (Tyr) and tryptophan (Trp) residues, respectively [40]. With the gradual addition of TA/CA/D3G, the protein fluorescence intensities decreased shown in Fig. 3. The protein fluorescence intensities decreased with the gradual addition of TA/CA/D3G, suggesting the combination of polyphenols with β -LG resulted in more exposure of chromophore to the solution environment and decreased fluorescence intensity. And the fluorescence intensity quenching of Trp residues ($\Delta\lambda = 60$ nm) was much stronger than that of Tyr residues ($\Delta\lambda = 15$ nm), which could be indicated that Trp residues contributed more to the quenching of the intrinsic fluorescence than Tyr residues. When the CA formed a complex with β -LG, the maximum emission wavelength with $\Delta\lambda = 60$ nm was red shifted, which was consistent with the analysis of UV–Vis absorption spectra and the fluorescence emission spectra above.

3.3. Effect of TA/CA/D3G on the IgE-binding capacity of β -LG

By calculating the inhibition rate, the inhibition rate of TA to β -LG reached 70.5%, CA reached 71.7% and D3G reached 68.0%, respectively. Their sequence was CA > TA > D3G. The allergenic capacity of β -LG was reduced after binding with TA, CA and D3G, with CA exhibiting the most reduction in IgE binding than TA and D3G.

β -LG belongs to the lipocalin family which are binding and carrying hydrophobic ligands. There have a much conserved sequences in their N-terminal parts, and the tryptophan invariably present at position 19. They form a very similar conical shaped structure called calyx, with the same preparations of 8 antiparallel-strands. There is a high allergenic

Table 2
CD spectra parameters of β -LG in the absence and presence of TA/CA/D3G.

Compound	α -Helix	β -Sheet	β -Turn	Random coil
β -LG	19.2	34.7	19.7	45.2
β -LG + CA	20.1	33.0	19.5	43.8
β -LG + D3G	20.2	33.0	19.5	43.7
β -LG + TA	25.4	26.1	18.3	40.3

Table 3Binding parameters of β -LG with TA/CA/D3G systems at 300 K.

Compound	K_a (10^5 L/mol)	E	r (nm)	R_0 (nm)
β -LG + CA	8.915	0.213	1.90	1.53
β -LG + D3G	22.350	0.101	2.01	1.40
β -LG + TA	32.727	0.280	1.61	1.37

potential in these kinds of molecules [41]. The IgE binding capacity is related to both linear and conformational epitopes of β -LG. Tyr102–Val124, Val41–Lys60, Ala25–Arg40 and Leu1–Lys8 are on the β -LG surface and are major IgE epitopes of β -LG. There are 14 lysine residues in β -LG and 12 locate in the major IgE epitope [42]. If the main epitope is combining with TA/CA/D3G, the allergenicity would decrease largely. Therefore, the predicted polyphenol binding site directly obscures the IgE linear epitopes

or near the epitopes, preventing IgE from approaching the β -LG. After treatment, the allergenicity of β -LG was significantly reduced, but the binding with IgE was not completely inhibited, indicating that the linear epitopes present or the epitopes retain allergenicity. Moreover, conformational epitopes are associated with changes in secondary or tertiary structure of β -LG. The secondary and tertiary structure could obviously affect the IgE binding capacities of β -LG. In this study, TA/CA/D3G could induce some changes on the secondary or tertiary structure of β -LG. Some structure of the conformational epitopes may have been affected, thus reducing the allergenicity of β -LG [43].

3.4. Molecular modeling study

Experiments on molecular modeling studies showed that TA, CA and D3G bound to β -LG in the hydrophobic regions. The best energy level

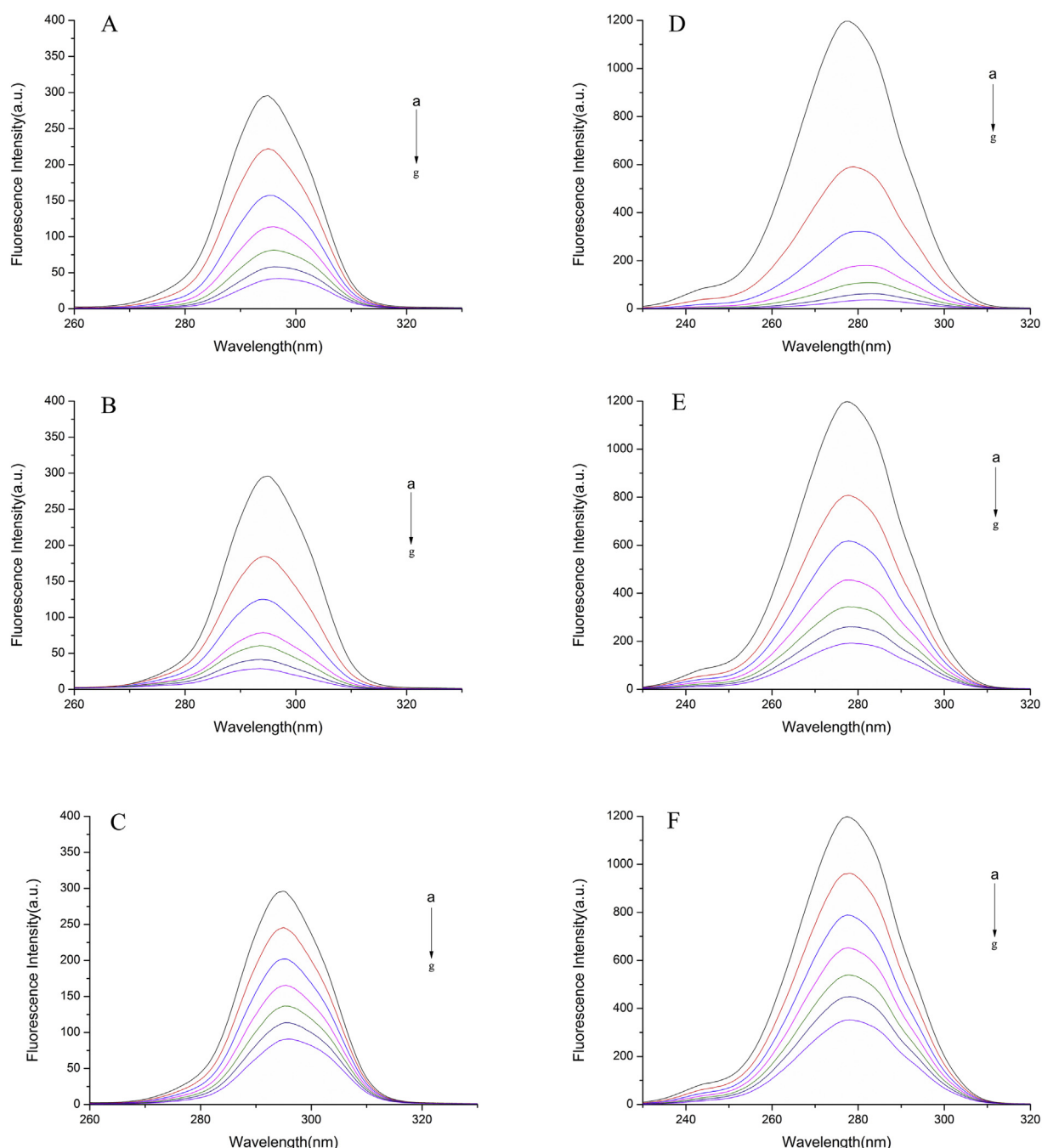
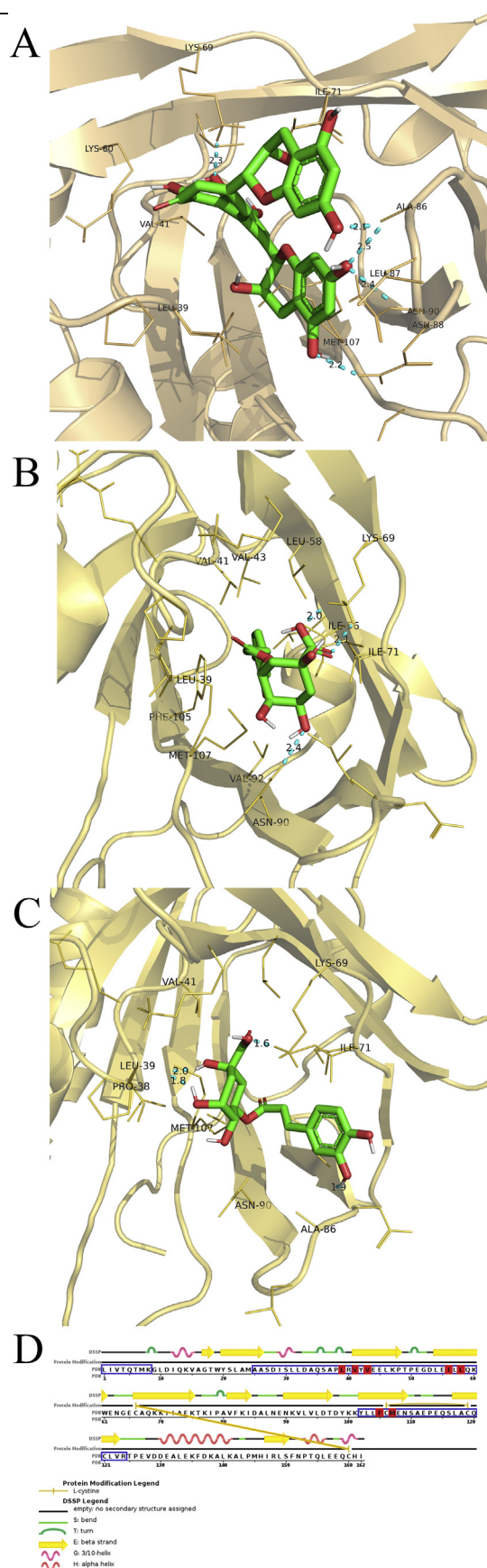


Fig. 3. Synchronous fluorescence spectra of β -LG with varying the concentration of TA/CA/D3G (A/B/C: $\Delta\lambda = 15$ nm, D/E/F: $\Delta\lambda = 60$ nm).



ranking results for the combination of β -LG and TA/CA/D3G were shown in Fig. 4. According to the experimental data, there were nine (Leu39, Val41, Lys 60, Lys69, Ala 86, Leu87, Asn88, Asn90 and Met107), ten (Leu39, Val41, Val43, Leu58, Ile56, Lys69, ILE71, Asn90, Phe105 and Met107) and eight (Leu39, Val41, Pro38, Lys69, Ile71, Ala86, Asn90 and Met107) amino acid residues involved in the binding between β -LG and TA/CA/D3G, respectively. Besides, by the type of amino acid residue and the binding properties of the lowest binding energy, the software also predicted the presence of hydrogen bonds in the β -LG-TA/CA/D3G system to enhance the stability of the complex. They were five hydrogen bonds present in the β -LG-TA system, including Lys69 (bond length - 2.3), ALA86 (bond length - 2.1; 2.5), ASN90 (bond length - 2.4), ASN88 (bond length - 2.2); three hydrogen bonds present in β -LG-CA system, including LYS69 (bond length - 2.1), ASN90 (bond length - 2.4), ILE56 (bond length - 2.0); four hydrogen bonds present in β -LG-D3G system, ALA86 (bond length - 1.9), MET107 (bond length - 2.0; 1.8), LYS69 (bond length - 1.6).

On the basis of previous studies, Tyr102-Val124, Val41-Lys60, Ala25-Arg40 and Leu1-Lys8 were on the β -LG surface and were major IgE epitopes of β -LG [42]. Therefore, for TA and D3G, there were three (Leu39, Val41 and Met107) overlapping amino acid residues involved in both binding interactions and IgE epitopes; for CA, there were seven (Leu39, Val41, Val43, Leu58, Ile56, Phe105 and Met107) overlapping amino acid residues involved in both binding interactions and IgE epitopes. Based on the above results, we could speculated that the polyphenol binding site directly obscures the IgE linear epitopes or near the epitopes, preventing IgE from approaching the β -LG. As a results, the allergenicity decreased might be due to the partial epitopes were combined with TA/CA/D3G. The allergenic capacity of β -LG was reduced after binding with TA, CA and D3G, with CA exhibiting the most reduction in IgE binding than TA and D3G, which because CA covers more IgE epitopes than TA and D3G.

4. Conclusion

The results of multi-spectroscopy demonstrate that polyphenols (theaflavin, chlorogenic acid and delphinidin-3-O-glucoside) caused the fluorescence quenching of bovine milk protein (β -LG). The mechanism of fluorescence quenching was mainly static quenching, which was induced by the formation of the protein-polyphenols complexes. According to the counted intrinsic fluorescence intensity value, the quenching ability was TA > CA > D3G in β -LG. The results of UV-Vis absorption spectra, DLS measurements, FTIR spectra, CD spectra, synchronous fluorescence spectra showed that the TA/CA/D3G affected the secondary and tertiary structure of β -LG, the fractions of α -helix was increased instead of the fractions of β -sheet was decreased. The binding interaction of β -LG with TA/CA/D3G did existed and the hydrophobicity of the microenvironment of aromatic amino acid residues was changed. The interaction between β -LG and TA/CA/D3G could observably reduce the IgE-binding capacity of β -LG. Molecular modeling study showed that the polyphenol binding site directly obscures the IgE linear epitopes or near the epitopes, preventing IgE from approaching the β -LG, which can provide a viable method for reducing the allergenicity of β -LG. The presented study is truly valuable in dairy processing industry and daily life.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2019.06.053>.

Fig. 4. Molecular modeling study predicted orientation of the binding conformation of β -LG with TA (A), CA (B) and D3G (C). (D): Sequence alignment of β -LG amino-acids involved in binding to TA/CA/D3G and IgE epitopes. Amino acids that compose both the TA/CA/D3G binding sites and IgE epitopes are highlighted blue.

References

- [1] W.-J. Yeh, S.-M. Hsia, W.-H. Lee, C.-H. Wu, Polyphenols with antiglycation activity and mechanisms of action: a review of recent findings, *J. Food Drug Anal.* 25 (1) (2017) 84–92.
- [2] S. Xie, J. Zhou, Harnessing plant biodiversity for the discovery of novel anticancer drugs targeting microtubules, *Front. Plant Sci.* 8 (720) (2017).
- [3] E. Riboli, A.J. Cross, H. Ward, Y. Lu, G. Hallmans, P. Karling, O. Grip, T. Key, H. Boeing, M.M. Bergmann, K. Overvad, D. Palli, G. Masala, M.-C. Boutron-Ruault, A. Racine, F. Carbonnel, V. Andersen, A. Tjønneland, A. Olsen, R. Kaaks, R. Tumino, A. Trichopoulou, A. Scalbert, R. Zamora-Ros, P. Jakszyn, S. Chan, A.R. Hart, R. Luben, K.-T. Khaw, J.L. Opstelten, B. Oldenburg, Dietary polyphenols in the aetiology of Crohn's disease and ulcerative colitis—a multicenter European prospective cohort study (EPIC), *Inflamm. Bowel Dis.* 23 (12) (2017) 2072–2082.
- [4] L. Ward, G.M. Pasinetti, Recommendations for development of botanical polyphenols as “natural drugs” for promotion of resilience against stress-induced depression and cognitive impairment, *NeuroMolecular Med.* 18 (3) (2016) 487–495.
- [5] M. Ohba, T. Oka, T. Ando, S. Arahata, A. Ikegaya, H. Takagi, N. Ogo, C. Zhu, K. Owada, F. Kawamori, Q. Wang, L.J. Saif, A. Asai, Antiviral effect of theaflavins against caliciviruses, *J. Antibiot.* 70 (2016) 443.
- [6] Y. Mikami, T. Yamazawa, Chlorogenic acid, a polyphenol in coffee, protects neurons against glutamate neurotoxicity, *Life Sci.* 139 (2015) 69–74.
- [7] E. Fuentes, J. Caballero, M. Alarcón, A. Rojas, I. Palomo, Chlorogenic acid inhibits human platelet activation and thrombus formation, *PLoS One* 9 (3) (2014), e90699.
- [8] X. Mei, L. Zhou, T. Zhang, B. Lu, Y. Sheng, L. Ji, Chlorogenic acid attenuates diabetic retinopathy by reducing VEGF expression and inhibiting VEGF-mediated retinal neovascularization, *Vasc. Pharmacol.* 101 (2018) 29–37.
- [9] M. Hidalgo, C. Sánchez-Moreno, S. de Pascual-Teresa, Flavonoid–flavonoid interaction and its effect on their antioxidant activity, *Food Chem.* 121 (3) (2010) 691–696.
- [10] S. Hogan, H. Chung, L. Zhang, J. Li, Y. Lee, Y. Dai, K. Zhou, Antiproliferative and antioxidant properties of anthocyanin-rich extract from açai, *Food Chem.* 118 (2) (2010) 208–214.
- [11] M.N. Ștefănuț, A. Căta, R. Pop, C. Tănăsie, D. Boc, I. Ienașcu, V. Ordodi, Anti-hyperglycemic effect of bilberry, blackberry and mulberry ultrasonic extracts on diabetic rats, *Plant Foods Hum. Nutr.* 68 (4) (2013) 378–384.
- [12] Y. Yang, Z. Shi, A. Reheman, J.W. Jin, C. Li, Y. Wang, M.C. Andrews, P. Chen, G. Zhu, W. Ling, H. Ni, Plant food delphinidin-3-glucoside significantly inhibits platelet activation and thrombosis: novel protective roles against cardiovascular diseases, *PLoS One* 7 (5) (2012), e37323.
- [13] N. Chorfa, S. Savard, K. Belkacemi, An efficient method for high-purity anthocyanin isomers isolation from wild blueberries and their radical scavenging activity, *Food Chem.* 197 (2016) 1226–1234.
- [14] F. Liu, Y. Yang, J. Gao, C. Ma, Y. Bi, A comparative transcriptome analysis of a wild purple potato and its red mutant provides insight into the mechanism of anthocyanin transformation, *PLoS One* 13 (1) (2018), e0191406.
- [15] P. Chanphai, P. Bourassa, C.D. Kanakis, P.A. Tarantilis, M.G. Polissiou, H.A. Tajmir-Riahi, Review on the loading efficacy of dietary tea polyphenols with milk proteins, *Food Hydrocoll.* 77 (2018) 322–328.
- [16] C.D. Kanakis, I. Hasni, P. Bourassa, P.A. Tarantilis, M.G. Polissiou, H.-A. Tajmir-Riahi, Milk β -lactoglobulin complexes with tea polyphenols, *Food Chem.* 127 (3) (2011) 1046–1055.
- [17] L. Liang, H.A. Tajmir-Riahi, M. Subirade, Interaction of β -lactoglobulin with resveratrol and its biological implications, *Biomacromolecules* 9 (1) (2008) 50–56.
- [18] J. Liu, Q. Wang, H. Zhang, D. Yu, S. Jin, F. Ren, Interaction of chlorogenic acid with milk proteins analyzed by spectroscopic and modeling methods, *Spectrosc. Lett.* 49 (1) (2016) 44–50.
- [19] Z. He, H. Zhu, M. Xu, M. Zeng, F. Qin, J. Chen, Complexation of bovine β -lactoglobulin with malvidin-3-O-glucoside and its effect on the stability of grape skin anthocyanin extracts, *Food Chem.* 209 (2016) 234–240.
- [20] Z. He, M. Xu, M. Zeng, F. Qin, J. Chen, Interactions of milk α - and β -casein with malvidin-3-O-glucoside and their effects on the stability of grape skin anthocyanin extracts, *Food Chem.* 199 (2016) 314–322.
- [21] J. Ognjenović, M. Stojadinović, M. Milčić, D. Apostolović, J. Vesić, I. Stambolić, M. Atanasković-Marković, M. Simonović, T.C. Velickovic, Interactions of epigallocatechin 3-gallate and ovalbumin, the major allergen of egg white, *Food Chem.* 164 (2014) 36–43.
- [22] S.-Y. Chung, E.T. Champagne, Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds, *Food Chem.* 115 (4) (2009) 1345–1349.
- [23] D. Li, J. Zhu, J. Jin, Spectrophotometric studies on the interaction between nevadensin and lysozyme, *J. Photochem. Photobiol. A Chem.* 189 (1) (2007) 114–120.
- [24] H. Zhang, D. Yu, J. Sun, H. Guo, Q. Ding, R. Liu, F. Ren, Interaction of milk whey protein with common phenolic acids, *J. Mol. Struct.* 1058 (2014) 228–233.
- [25] A. Al-Hanish, D. Stanic-Vucinic, J. Mihailovic, I. Prodic, S. Minic, M. Stojadinovic, M. Radibratovic, M. Milcic, T. Cirkovic Velickovic, Noncovalent interactions of bovine α -lactalbumin with green tea polyphenol, epigallocatechin-3-gallate, *Food Hydrocoll.* 61 (2016) 241–250.
- [26] P. Daneshgar, A.A. Moosavi-Movahedi, P. Norouzi, M.R. Ganjali, A. Madadkar-Sobhani, A.A. Saboury, Molecular interaction of human serum albumin with paracetamol: spectroscopic and molecular modeling studies, *Int. J. Biol. Macromol.* 45 (2) (2009) 129–134.
- [27] J. Jia, X. Gao, M. Hao, L. Tang, Comparison of binding interaction between β -lactoglobulin and three common polyphenols using multi-spectroscopy and modeling methods, *Food Chem.* 228 (2017) 143–151.
- [28] V. Carnovale, M. Britten, C. Couillard, L. Bazinet, Impact of calcium on the interactions between epigallocatechin-3-gallate and β -lactoglobulin, *Food Res. Int.* 77 (2015) 565–571.
- [29] X. Meng, Y. Bai, J. Gao, X. Li, H. Chen, Effects of high hydrostatic pressure on the structure and potential allergenicity of the major allergen bovine β -lactoglobulin, *Food Chem.* 219 (2017) 290–296.
- [30] Y. Chen, Z. Tu, H. Wang, L. Zhang, X. Sha, J. Pang, P. Yang, G. Liu, W. Yang, Glycation of β -lactoglobulin under dynamic high pressure microfluidization treatment: effects on IgE-binding capacity and conformation, *Food Res. Int.* 89 (2016) 882–888.
- [31] J.H.W.M. Rombout, G. Yang, V. Kiron, Adaptive immune responses at mucosal surfaces of teleost fish, *Fish Shellfish Immunol.* 40 (2) (2014) 634–643.
- [32] H. Bi, L. Tang, X. Gao, J. Jia, H. Lv, Spectroscopic analysis on the binding interaction between tetracycline hydrochloride and bovine proteins β -casein, α -lactalbumin, *J. Lumin.* 178 (2016) 72–83.
- [33] L. Tang, H. Zuo, L. Shu, Comparison of the interaction between three anthocyanins and human serum albumins by spectroscopy, *J. Lumin.* 153 (2014) 54–63.
- [34] J. Cheng, J.-H. Liu, G. Prasanna, P. Jing, Spectrofluorimetric and molecular docking studies on the interaction of cyanidin-3-O-glucoside with whey protein, β -lactoglobulin, *Int. J. Biol. Macromol.* 105 (2017) 965–972.
- [35] P.D. Ross, S. Subramanian, Thermodynamics of protein association reactions: forces contributing to stability, *Biochemistry* 20 (11) (1981) 3096–3102.
- [36] A.-A. Moenifshari, A. Zarabi, A.-K. Bordbar, Exploring the interaction of naringenin with bovine beta-casein nanoparticles using spectroscopy, *Food Hydrocoll.* 51 (2015) 1–6.
- [37] J. Kong, S. Yu, Fourier transform infrared spectroscopic analysis of protein secondary structures, *Acta Biochim. Biophys. Sin.* 39 (8) (2007) 549–559.
- [38] W. He, H. Mu, Z. Liu, M. Lu, F. Hang, J. Chen, M. Zeng, F. Qin, Z. He, Effect of preheat treatment of milk proteins on their interactions with cyanidin-3-O-glucoside, *Food Res. Int.* 107 (2018) 394–405.
- [39] X. Wu, R. Dey, H. Wu, Z. Liu, Q. He, X. Zeng, Studies on the interaction of -epigallocatechin-3-gallate from green tea with bovine β -lactoglobulin by spectroscopic methods and docking, *Int. J. Dairy Technol.* 66 (1) (2013) 7–13.
- [40] S. Bobone, M. van de Weert, L. Stella, A reassessment of synchronous fluorescence in the separation of Trp and Tyr contributions in protein emission and in the determination of conformational changes, *J. Mol. Struct.* 1077 (2014) 68–76.
- [41] T. Sophia, D. Kostas, N.P. Kostas, Cow's milk allergenicity, endocrine, metabolic & immune disorders - Drug Targets 14 (1) (2014) 16–26.
- [42] X. Li, S. Yuan, S. He, J. Gao, H. Chen, Identification and characterization of the antigenic site (epitope) on bovine β -lactoglobulin: common residues in linear and conformational epitopes, *J. Sci. Food Agric.* 95 (14) (2015) 2916–2923.
- [43] X. Wu, Y. Lu, H. Xu, D. Lin, Z. He, H. Wu, L. Liu, Z. Wang, Reducing the allergenic capacity of β -lactoglobulin by covalent conjugation with dietary polyphenols, *Food Chem.* 256 (2018) 427–434.

Supplementary Table 1

Particle sizes of β -LG with TA/CA/D3G systems.

Compound	Size (d.nm)
β -LG	8.37
β -LG+ CA	14.82
β -LG+ D3G	21.76
β -LG + TA	24.56