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Reduced allergenicity of β -lactoglobulin in vitro by tea catechins binding

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Bovine β -lactoglobulin (β LG) is a major allergen in cow's milk. It is crucial to develop new methods that would reduce the allergenicity and not destroy the functional properties of β LG. To reduce allergenicity of β LG, tea catechins were bound to β LG to form β LG–catechin complex. The structure of β LG–catechin complex was analysed by circular dichroism (CD) spectra and fluorescence spectra assays. The results suggested that β LG was bound by catechin without apparent disruption of native conformation. Enzyme-linked immunosorbent assay (ELISA) indicated that the IgE-binding and IgG-binding activities of β LG were decreased upon catechin binding. Our study may be helpful for development of a new method to prepare milk proteins with reduced allergenicity.

Keywords: β -lactoglobulin; catechin; protein-polyphenols interactions; reduced allergenicity; IgG/IgE-binding

Introduction

Bovine β -lactoglobulin (β LG) is a major whey protein of MW 18,400 with two disulfide bridges and one free cysteine residue. This protein is one of the best characterised lipid-binding proteins and as such it is capable of binding a wide range of molecules including retinol, β -carotene and fatty acid (Flower, 1996). β LG is ubiquitously used as a food additive for its multiple functions in food processing (Foegeding, Kuhn, & Hardin, 1992; Shimizu, Saito, & Yamauchi, 1985). However, β LG is a major allergen in cow's milk, and about 82% of milk allergy patients are sensitive to this protein (Spies, 1973). Many attempts to reduce the allergenicity of proteins have been made by enzymatic digestion and denaturation (del Val et al., 1999; Ishizaka, Okudaira, & King, 1975; Kurisaki, Nakamura, Kaminogawa, & Yamauchi, 1982; Watanabe, Suzuki, Ikezawa, & Arai, 1994). However, these methods might destroy useful functions of the proteins and have adverse effects of their taste (Monaci, Tregodt, Hengel, & Anklam, 2006). Therefore, it is crucial to develop new methods that would reduce the allergenicity and not destroy the functional properties of β LG.

Polyphenols of green tea are widely investigated for their multifarious properties and potential health benefits attributed to their strong antioxidative activity (Jaziri,

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Slama, Mhadhbi, Urdaci, & Hamdi, 2009). A considerable amount of research has reported that phenolic compounds have an ability to interact with milk proteins (Kanakakis et al., 2011; Fox, 2001; Svetli, Guy, & Heidar-Ali, 2010; Zerrin, Elif, & Yasar, 2010). Tea polyphenols contribute to the functionality of the milk products (Batra & Saxena, 2005; O'Connell & Fox, 1999). Binding interactions of green tea polyphenols with milk proteins can be used for manufacturing of novel milk products with desired textural properties as well as antioxidant capacity (Zerrin et al., 2010). It is thought that the interacting ability of green tea polyphenols with milk protein was vital in modification of textural properties of milk products. Therefore, we expect that through binding interaction, tea polyphenols not only alter functions of β LG such as emulsifying, foaming and gelling properties but also can change its immunoreactivity.

Herein we prepared a complex of β LG by binding β LG with green tea catechins. After exploring the structures of this complex, we analysed IgE-binding and IgG-binding capacity of β LG before and after treatment of catechin by enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Preparation of β LG–catechin complex

β -lactoglobulin was from Sigma Chemical Co., (St. Louis, MO, USA). Green tea catechins were purchased from Chengdu Biopurify Photochemicals Ltd. (Chengdu, China). The β LG–catechin complex was prepared according to the method of Prigent et al. (2003). In brief, 0.1 g of β LG was dissolved in 100 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.0). Fifty mole catechin per mole protein was added to the β LG solution and incubated for 1 h at room temperature with stirring. Blanks without catechin were also incubated at the same time. After incubation, all samples were dialysed overnight in Slide-A-Lyzer minidialysis units (Pierce Chemical Co., Rockford, IL, USA.) with a molecular weight cut-off of 10 kDa against distilled water at 4°C to remove the free catechin.

Production of anti- β LG polyclonal antibodies

Anti- β LG polyclonal antibodies were prepared in mice by using native β LG as a soluble antigen. The antigen protein in Freund's complete adjuvant was intraperitoneally injected into BALB/c mice. Booster injections of the same amount of the antigen protein in Freund's incomplete adjuvant were administered twice at two-week intervals. The antiserum was prepared after centrifugation to remove blood clots and analysed by western blotting to test its IgG binding ability toward β LG. The antiserum was stored at -20°C until use.

Human sera

Pooled human sera were collected from 15 children. Individual serum samples from children with proven allergy to milk proteins were collected the Shenzhen Children's Hospital. All patients showed specific IgE antibodies towards milk proteins, which were determined by the Uni-CAP System (Pharmacia Diagnostics, Uppsala,

Sweden). All sera were analysed by western blotting and demonstrated IgE-binding ability toward β LG. Pooled normal sera from patients without a cow's milk hypersensitivity reaction were used as negative controls. All serum samples were given informed consent and authenticated by the ethical board of Shenzhen University and stored at -20°C until use.

Conformational analysis of the β LG–catechin complex by spectroscopic methods

The circular dichroism (CD) spectra for native β LG and β LG–catechin complex were measured. The samples were recorded at 25°C with a Jasco-810 spectrophotometer (JASCO Co., Tokyo, Japan) in cells of 1.0 mm path length. Each sample was dissolved in PBS at a protein concentration of 1.5×10^{-6} M. The spectra were recorded in the range of 190–250 nm with a scan rate of 30 nm/min and a response time of 1 s. Three scans were accumulated for each spectrum.

The intrinsic fluorescence of native β LG and β LG–catechin complex dissolved in PBS at a 1.0×10^{-6} M (as the protein concentration) were recorded on a Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo, Japan) in a 1-cm quartz cell using an excitation wavelength of 280 nm. The excitation and emission bandwidths were 5 nm. The emission spectra were recorded from 310 to 450 nm.

The retinol-binding activity of β LG and β LG–catechin complex was investigated by fluorescence titration with the Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo, Japan) according to Hattori, Ogino, Nakai, and Takahashi (1997).

ELISA experiments

The IgG binding of native β LG and β LG–catechin complex was evaluated by competitive ELISA. Aliquots of β LG solution (100 μL ; 0.001% β LG in PBS) were added to the wells of a polystyrene microtitration plate and plates were incubated at 4°C overnight. The β LG solution was then removed. The wells were washed with PBST (0.05% Tween 20 in PBS). This washing system was used after each incubation step. And 3.0% BSA in PBST (0.02% Tween 20 in PBS) for 2 h at 37°C was added. The plate was again incubated at 37°C for 2 h and washed. β LG or β LG–catechin at various concentrations (50 μL) was added to the wells, then anti- β LG polyclonal antibodies (50 μL) were immediately added, and the plate was further incubated at 37°C for 1 h. After removing the solution and washing the wells, plates were then incubated with 100 μL per well of horseradish peroxidase (HRP) labelled Goat Anti-Mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) diluted 1:10,000 in PBS solution. The plate was incubated for 45 min at 37°C . Fifty μL of substrate containing 0.4 mg/mL *o*-phenylenediamine and five μL H_2O_2 were added for colour development. Fifty μL of 2 M H_2SO_4 was added to stop the enzyme reaction. Optical densities (OD) were read at 450 nm on an automated ELISA plate reader (Multiskan MK3, Thermo Labsystems, Vantaa, Finland).

The IgE-binding assay was performed similarly to that of IgG but with slight modifications. Briefly, wells were coated with 100 μL of antigen as described earlier. After the blocking step, β LG or β LG–catechin at various concentrations (50 μL) was added to the wells, plates were then incubated with 50 μL per well of human

sera (serum at 1:50 diluted in PBST solution) for 1 h at 37°C. 100 μ L per well of HRP-conjugated antihuman IgE (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) were added at a 1:1000 dilution in PBST solution. Finally, colour development was performed as described earlier.

The ELISA determinations were carried out in triplicate and measurements averaged.

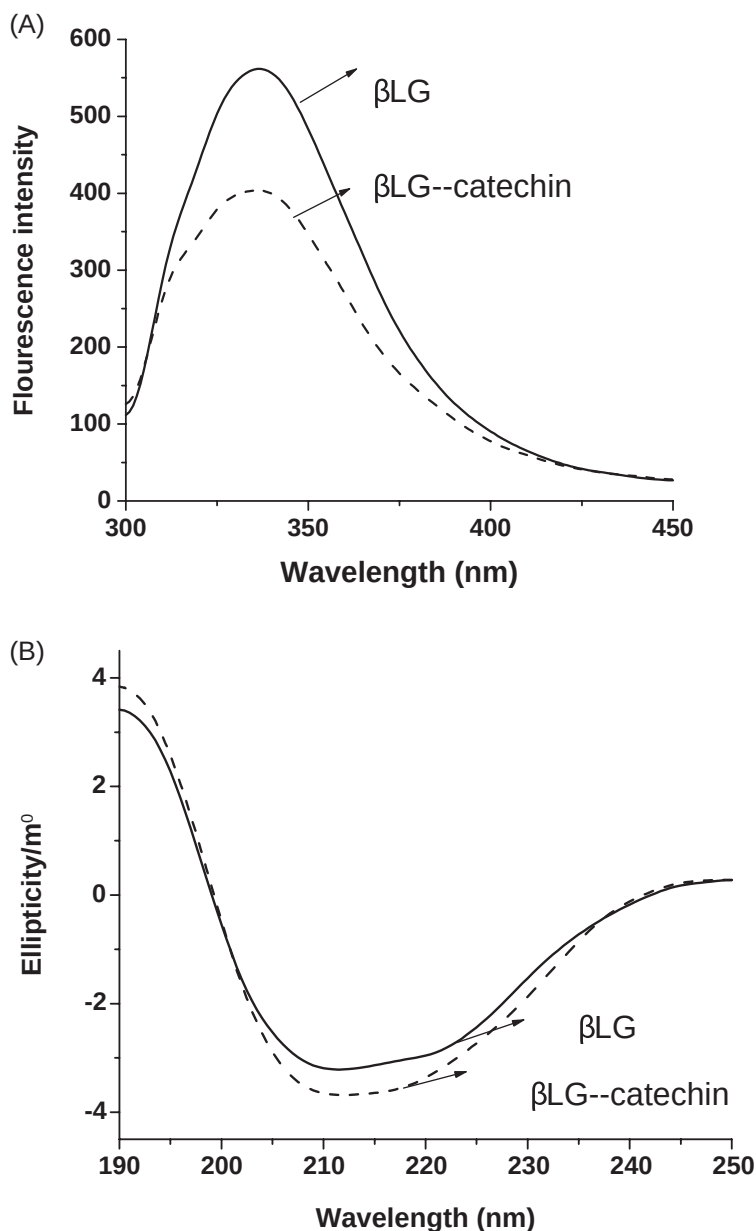


Figure 1. Spectroscopic analysis of the β LG–catechin complex. (A): intrinsic; (B) CD spectra.

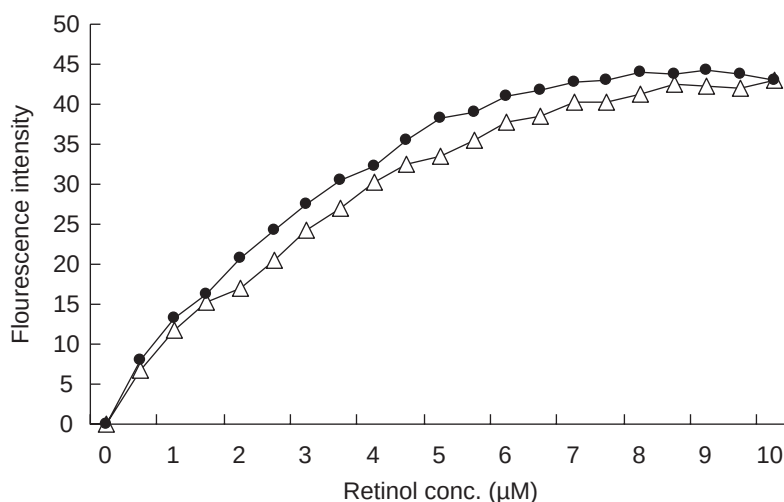


Figure 2. Retinol-binding activity of the β LG–catechin complex: (●): β LG; (Δ): β LG–catechin.

Results and discussion

Structural features of the β LG–catechin complex

The fluorescence emission spectra of the β LG–catechin complex are shown in Figure 1A. The fluorescence emission maximum wavelength was 337 nm for native β LG and 336 nm for the complex. It has been previously shown that the fluorescence intensity decreases with the blue-shift of the wavelength for maximum emission as the conformation of β LG changes. As only a slight blue-shift was observed in the complex, the conformation around the tryptophan (Trp) residues of the β LG–catechin complex was considered to be maintained in the native form. The fluorescence intensity of β LG–catechin complex was lower than that of native β LG, which suggested that the catechin shielded the area around the Trp residues (Hattori, Nagasawa, Ametani, Kaminogawa, & Takahashi, 1994; Hattori et al., 1997, 2000a; Lakowicz & Weber, 1973).

The CD spectra of the β LG–catechin complex are shown in Figure 1B. The CD spectra of samples exhibited two negative bands in the ultraviolet region at 208 nm and 218 nm, this is characteristic of α -helical structures of a protein (Venyaninov & Vesilenko, 1994). The spectrum for the complex was similar to the spectrum for native protein. The secondary structure was determined using CONTIN method in DICHROWEB (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>). By this method, it was found that the α -helical content of the protein changed from 21% to 24% upon binding with catechin. This suggested that the binding interaction of catechin with β LG only caused slight conformation change of protein.

β -lactoglobulin belongs to the lipocalin superfamily and can bind two molecules of retinol (Fugate & Song, 1980; Pérez & Calvo, 1995). Change in the retinol-binding activity of β LG after binding by catechin was investigated by fluorescence titration (Figure 2). The retinol-binding activity of β LG was reduced a little by conjugation

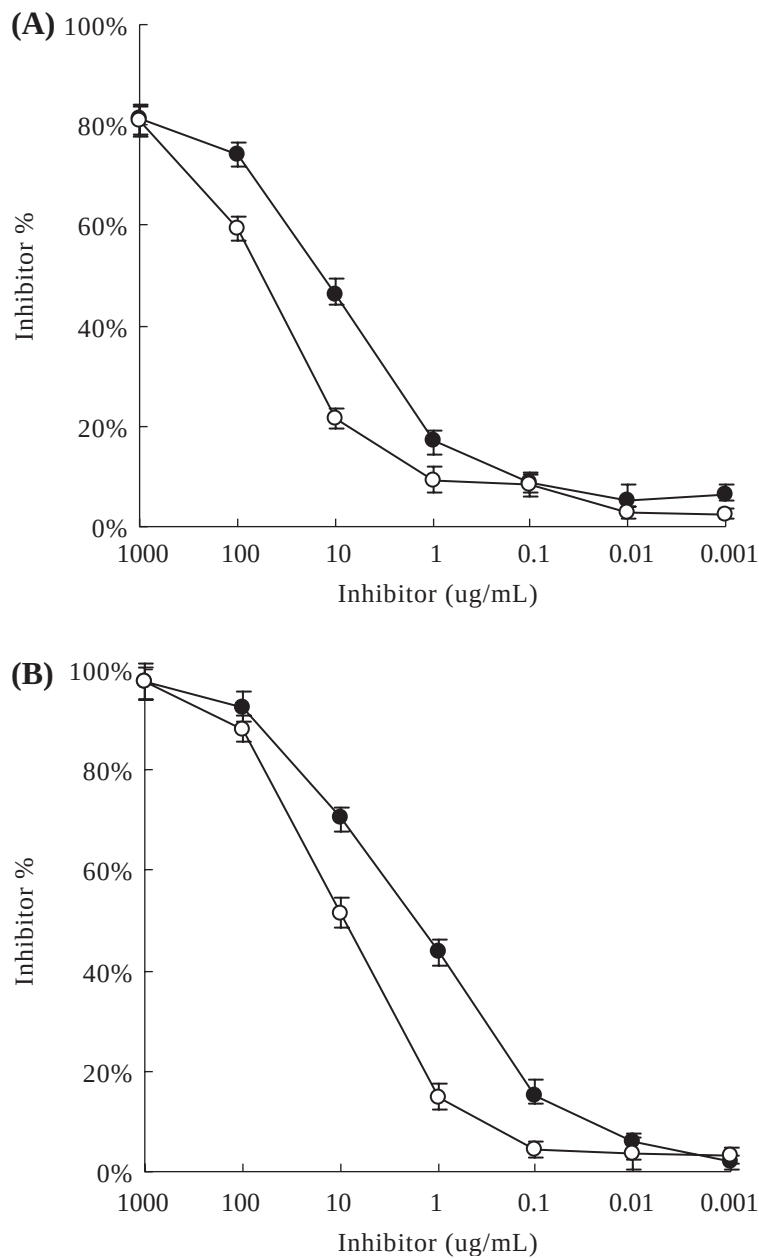


Figure 3. Indirect ELISA. Inhibition of the bind of polyclonal anti-βLG IgG (A) and polyclonal anti-βLG IgE (B) to immobilised βLG-catechin complex. (●): βLG; (○): βLG-catechin.

with catechin. The structure involved in retinol-binding in the βLG-catechin complex was considered to have been maintained in the native form.

Therefore, βLG binding by catechin maintained the native-like protein structure, although slight changes in the structure were observed.

ELISA experiments

The IgE-binding and IgG-binding capacities of β LG and β LG-catechin were measured by ELISA. Figure 3 shows that the reactivity of native β LG with IgG antibodies and IgE antibodies was apparently decreased after binding by catechin.

The molecular basis of changes in allergenic activity is the inactivation or destruction of epitope structures or increased inaccessibility to epitopes (Besler, Steinhart, & Paschke, 2001). According to structural analyses, binding catechin did not disrupt the native conformation of β LG greatly. β LG almost maintained the native-like protein structure. Therefore, the reduced allergenicity of β LG may not cause by significant structures changes. The reduction of the binding affinity of β LG–catechin complex may due to the shielding action of the β LG epitopes induced by the attachment of catechin to β LG.

As for the IgE response to β LG, ⁴¹Val-⁶⁰Lys, ¹⁰²Tyr-¹²⁴Val, ¹⁴⁹Arg-¹⁶²Ile are reported to be major epitopes (Selo et al., 1999). And ⁵¹Glu-⁶⁴Asp, ⁶⁷Arg-⁸⁸Asn, ¹²⁹Asp-¹⁴⁴Pro, ¹³⁹Arg-¹⁵⁶Thr are major epitopes for the IgG (Jarvinen et al., 2010). All these epitopes are on the protein surface. The results of structural analyses according to the spectroscopic experiment s indicated that the surface of β LG was covered by catechin. Catechin in β LG–catechin complex may be attached directly to the epitope(s) or region(s) in the proximity, which thereby prevent anti- β LG antibodies from accessing the antigens.

Kanakakis et al. (2011) observed that tea polyphenols could bind to β LG in solution. The catechins in green tea with antioxidant activity include epicatechin, epigallocatechin, epigallocatechingallate and epicatechingallate. Both hydrophobic and hydrophilic interactions were observed in the polyphenol– β LG complexation (Kanakakis et al., 2011).

It is essential to develop a new method that can reduce the allergenicity and maintain the functional properties of β LG. Proteins can combine with non-allergenic materials, simultaneously decreasing the allergenicity and improving the function of the proteins (Hattori et al., 2000b). Although a little conformational change in β LG was observed after binding by catechin, the complex maintained the retinol-binding activity of protein. Our work is the first study to find that the catechin effectively shielded epitopes of β LG, which reduced IgE-binding and IgG-binding capacity of β LG in vitro. However, the mechanism behind the reduced immunogenicity of β LG through binding by catechin needs more study. We hope that this method will contribute to preparation of novel proteins with desired functional properties, but reduced allergenicity.

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