Original Article

In Vitro Refolding Process of Bovine Allergen β-lactoglobulin by Multispectroscopic Method*

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Abstract

Objective To characterize the relationship between the refolding process of recombinant bovine β-lactoglobulin and its immunoreactivity for clinical purposes. To establish a spectral method which examine the extent of recombinant allergen renaturation.

Methods The refolding process of recombinant bovine β-lactoglobulin was investigated by using circular dichroism, fluorescence and synchronous fluorescence spectra. IgE-binding capacity of recombinant protein was analyzed by ELISA. In addition, bioinformatic methods were used to explain the spectral characteristics and analyze the relationship between the conformational changes and the immunoreactivity of the protein during renaturation *in vitro*.

Results Renaturation of recombinant bovine β-lactoglobulin resulted in a more compact structure resembling the natural counterpart with stronger IgE-binding capacity.

Conclusion The degree of protein renaturation correlated with the IgE-binding capacity of the protein. Results from this study may be of help for food allergy therapy and development of vaccination in the future.

Key words: Food allergen; β-Lactoglobulin; Protein folding; Spectroscopy; Immunoreactivity

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INTRODUCTION

Food allergy is an important public health problem, especially in industrialized countries where it has been estimated to affect about 1%-2% of the adult population and up to 8% of children below the age of 3 years[1]. Cloning and expression of allergens has enabled innovative approaches for allergy diagnosis and therapy[2-4]. Engineered recombinant allergens often exist as inclusion bodies with lower biological activities. Renaturation of these abnormally folded allergens results in variants with different structures that demonstrate different IgE-binding capacities. Therefore, it is essential to characterize the relationship between the refolding process of recombinant allergen and its immunoreactivity for clinical purposes.

Circular dichroism (CD) spectra can be used to probe protein folding and unfolding, which investigate the thermal and chemical stability of proteins[5]. With respect to allergen research, CD spectra have been proven to be very useful, because they allow for rapid determination of secondary

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structure of recombinant allergens and thus their structural integrity[6]. Fluorescence spectra can provide useful information for characterization of the chromophores in the protein molecule and its microenvironment. They have high sensitivity, specificity and simplicity. They can provide abundant information about photophysics[7]. Synchronous fluorescence spectra can distinguish the spectral characteristics between tryptophan (Trp) and tyrosine (Tyr)[8], which has be applied widely in studies of protein structure.

In the present study, we prepared recombinant bovine β-lactoglobulin (βLG), a major allergen of cow milk. The refolding process of recombinant protein was investigated by using circular dichroism (CD), fluorescence and synchronous fluorescence spectra. IgE-binding capacity of recombinant protein was analyzed by ELISA. In addition, bioinformatic methods were used to explain the spectral characteristics and analyze the relationship between the conformational changes and the immunoreactivity of the protein during renaturation *in vitro*.

MATERIALS AND METHODS

Materials

Recombinant bovine βLG was prepared according to the method of Jean et al.[9].

Native βLG, bovine serum albumin (BSA), goat anti-human IgG–horseradish peroxidase (HRP) conjugate, 3,3'-5,5'-tetramethylbenzidine (TMB), were obtained from Sigma (St. Louis, MO, USA). The nitrocellulose membrane, the sample pad, and the absorbent pad for colloidal gold immunochromatography assay GICA were obtained from Whatman (Maidstone, Kent, UK). All chemical reagents were of analytical grade.

Pooled human sera were collected from 15 children. Individual serum samples of children with proven allergy to milk proteins were collected from Shenzhen Children's Hospital. All patients showed specific IgE antibodies toward milk proteins, determined by the Uni-CAP System (Pharmacia Diagnostics, Sweden). Western blotting showed that all sera demonstrated IgE binding ability toward βLG. Normal serum from humans without cow's milk hypersensitivity reaction was used as an IgE-negative control. All serum samples were obtained by informed consent and authenticated by the Ethical Board of Shenzhen University, and stored at -20 °C until use.

Analysis of Protein Structure and Epitopes Information of βLG

Crystal structure of βLG has been resolved previously[10], and was downloaded from the Brookhaven Protein Data Bank (accession number: 2Q2M). Protein structure and distribution of chromophores were calculated by using UCSF-Chimera software[11] and GetArea online software[12].

Renaturation of βLG

Renaturation of recombinant βLG (2.0 mg/mL) was initiated by urea (3 mol/L) at 4 °C in Tris-HCl (pH 8.5). Dialysis included four steps and buffer was changed every 12 h. The urea concentration of dialyzing buffer was 3, 2, 1, or 0.5 mol/L. The samples with different concentration of urea were collected after each step and applied to CD, fluorescence and synchronous fluorescence spectra and ELISA.

CD Spectra

The CD spectrum for the sample was measured with a Jasco-810 spectrophotometer (JASCO, Tokyo, Japan) in cells of 1.0 mm path length at 25 °C. 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.

Fluorescence Spectra

Fluorescence spectra were recorded on a Hitachi-850 spectrofluorometer (Hitachi, Tokyo, Japan) in a 1 cm quartz cell using an excitation wavelength of 280 nm at 25 °C. The excitation and emission bandwidths were 5 nm. The emission spectra were recorded from 300 to 450 nm.

ELISA

The IgE binding of βLG was evaluated by indirect ELISA using the pooled sera of patients, using normal serum as a negative control. Recombinant βLG, renatured βLG and native βLG dissolved in PBS at a protein concentration of 1 μg/mL (100 μL) were added to the wells of polystyrene microtitration plates (Maxisorp; Nunc, Roskilde, Denmark). The plates were incubated at 4 °C overnight to coat the wells with each antigen. After removal of the solution, plates were washed with PBS containing

0.05% Tween 20 (PBST). This washing system was used after each incubation step. Residual free binding sites were blocked with 3.0% BSA containing 2.5% Tween 20 for 2 h at 37 °C. After the blocking step, plates were incubated for 1 h at 37 °C with 100 µL per well of human serum (diluted 1:20 in PBST). Plates were then incubated with 100 µL per well of HRP-conjugated antihuman IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) diluted 1:5 000 in PBS. The plates were incubated for 45 min at 37 °C. Finally, 50 µL substrate containing 0.4 mg/mL *o*-phenylenediamine and 5 µL H_2O_2 was added. Plates were incubated for 10 min at 37 °C, and the reaction was stopped by adding 50 µL per well of 2 mol/L H_2SO_4. The absorbance at 450 nm was measured by the microplate reader (Multiskan MK3, Thermo, USA). The ELISA determinations were carried out in triplicate and the average was calculated.

RESULTS

Structure and Epitopes of βLG

Structure and epitopes of βLG are shown in Figure 1. Fluorescence spectra are considered as a useful technique for the study of protein structure. Therefore, it is possible to use fluorescence spectra of the intrinsic Try (^{20}Tyr, ^{42}Tyr, ^{102}Tyr, and ^{99}Tyr) and Trp (^{19}Trp and ^{61}Trp) in βLG as a tool to study the alteration of the polarity around the chromophore microenvironment[19]. Trp was in a hydrophobic environment and in a random coil, ^{61}Trp was on the surface of protein in a β-sheet; 20Tyr, 42Tyr, and 102Tyr were in a β-sheet and 99Tyr was in a random coil; 20Tyr was on the surface of the protein and 102Tyr in a hydrophobic environment. 1Leu-8Lys, 25Ala-40Arg, 41Val-60Lys, 102Tyr-124Val, and 149Arg-162Ile are reported to be major IgE-reactive epitopes[13]. The secondary structure of 1Leu-8Lys and 25Ala-40Arg were largely α-helices, and the secondary structure of 41Val-60Lys, 102Tyr-124Val, and 149Arg-162Ile was largely a β-sheet.

ELISA

As shown in Figure 2, the recombinant βLG in the inclusion body had low IgE reactivity. The reactivities of human IgE against recombinant βLG improved steadily in the process of protein renaturation, and the completely refolded protein had similar IgE binding ability as its native counterpart. These results suggested that the structure

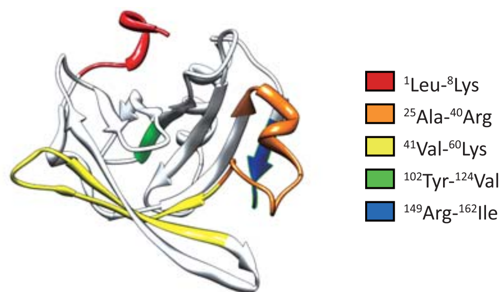

Figure 1. Structure and epitopes of βLG. ^{1}Leu-^{8}Lys, ^{25}Ala-^{40}Arg, ^{41}Val-^{60}Lys, ^{102}Tyr-^{124}Val, and ^{149}Arg-^{162}Ile were reported to be major IgE-reactive epitopes and shown in color.

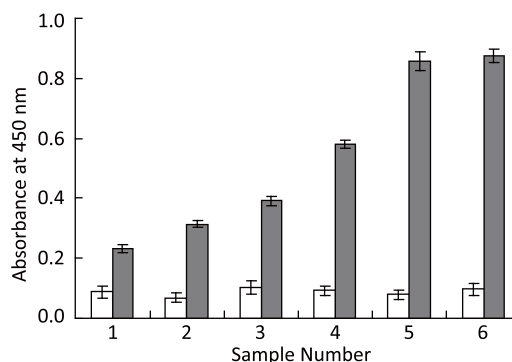

Figure 2. ELISA using patients' sera for βLG. 1: recombinant βLG; 2-5: recombinant βLG dialyzed against 50 mmol/L Tris-HCl (pH 8.5) containing 3, 2, 1, or 0.5 mol/L urea at 4 °C for 12 h; 6: native βLG.

of recombinant β-LG after renaturation was close to the native protein. Thus, its epitope sites recognized by human IgE antibodies were recovered after renaturation.

CD Studies

Proteins with different secondary structure had CD spectra with different spectral bands and intensity. The secondary structure information can be inferred from CD spectra of proteins[14]. CD spectra of βLG during the renaturation process are shown in Figure 3A. CD spectra of native βLG exhibited two negative bands in the UV region at 209 and 219 nm. The recombinant βLG in the inclusion body had a negative peak at 226 nm without any positive peak. The negative band widened and had a blue shift towards 219 nm as renaturation progressed. After complete renaturation, a positive peak was detected at 190 nm, with a negative peak at 209 nm and another at 219 nm, which resembled the spectrum of native βLG. This CD characteristic

spectrum indicated that the native protein existed mainly in secondary structure, whereas the recombinant protein showed significantly reduced folding. As renaturation progressed, the α-helix and β-sheet structures continued to increase. From the above CD analysis, it was evident that the conformation of recombinant βLG after renaturation was close to that of the native protein.

Fluorescence Studies

Trp, Tyr and phenylalanine (Phe) are able to emit fluorescence. Trp has the strongest fluorescence, followed by Tyr and Phe. The maximum emission wavelength of Phe is 257 nm. As the fluorescence of protein is excited at a wavelength of ≥280 nm, Phe is not excited under most laboratory conditions. The maximum wavelength and intensity of fluorescent amino acids are correlated with their hydrophobic environment, therefore, the change in the maximum wavelength and intensity can provide clues about the fluorescent amino acids and their structural alteration[15]. The fluorescent spectra of βLG with emission wavelength at 280 nm are displayed in Figure 3B.

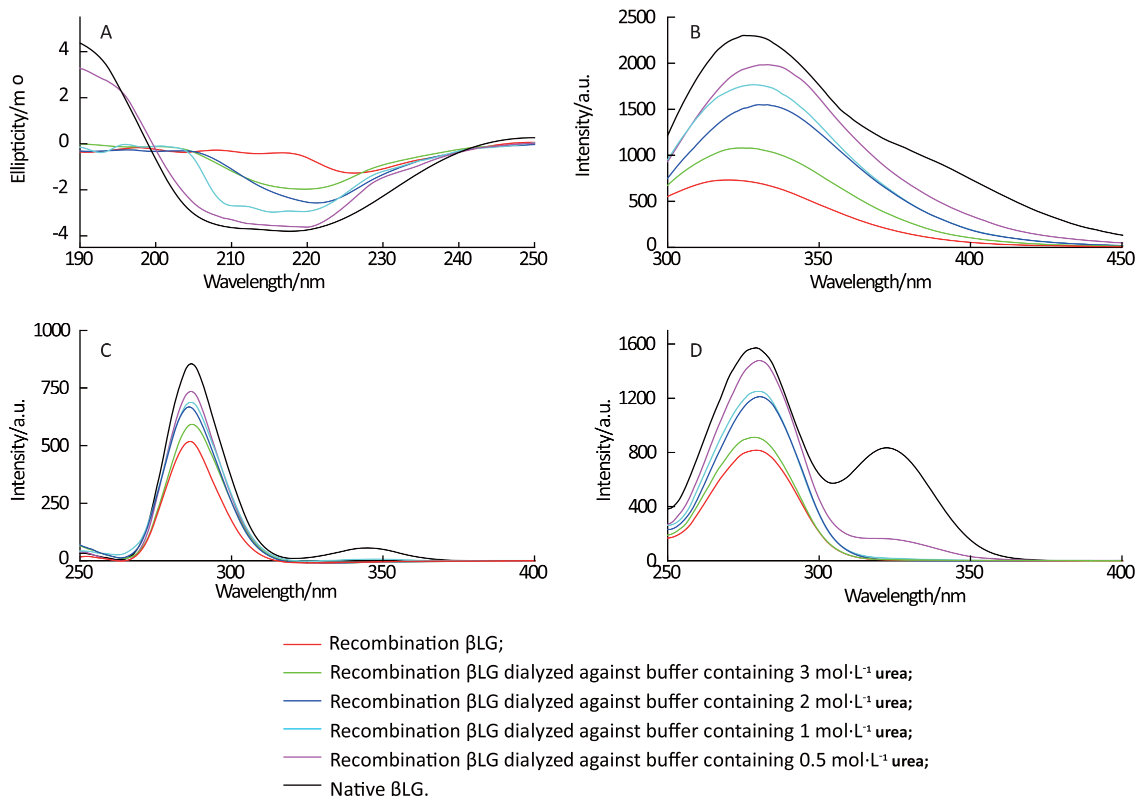

Figure 3. (A) Far-UV CD spectra of βLG; (B) fluorescence emission spectra of βLG; (C) synchronous fluorescence spectra of βLG at Δλ=15 nm; (D) synchronous fluorescence spectra of βLG at Δλ=60 nm.

The fluorescence intensity of βLG increased as renaturation progressed. It was indicated that the hydrophobic and hydrogen bonds that maintained the tertiary structure of the protein were not correctly formed and tended to be loose before renaturation. Thus, most chromophores were exposed and had weak fluorescence.

Synchronous Fluorescence Studies

To verify the alterations in chromophores during renaturation, synchronous fluorescence was utilized. Synchronous fluorescence is a simple and effective means to measure the possible shift in the maximum emission wavelength relative to alteration of the polarity around the chromophore microenvironment. When the fluorescence scanning interval between excitation and emission wavelength was stabilized at 15 and 60 nm, respectively, the synchronous fluorescence gives characteristic information about Tyr or Trp residues. If Δλ is 15 nm, synchronous

fluorescence yields information about the characteristics of Tyr residues, whereas when it is 60 nm, it provides characteristic information about Trp residues[16]. As shown in Figure 3C, the maximum emission wavelength changed from 286.6 to 286.4 nm. The fully renatured and native proteins had the same maximum emission wavelength. As shown in Figure 3D, the maximum emission wavelength of Trp shifted from 279.6 to 280.2 nm after renaturation. Tyr residues in the renatured and native protein had the same maximum emission wavelength. We observed that both Trp and Tyr had a blue shift after renaturation, whereas Trp had more significant shifts. At the same time, the intensity increase of Trp was more prominent than that of Tyr after renaturation. This indicated that the microenvironment around the Tyr or Trp residues had higher hydrophobicity after renaturation.

DISCUSSION

According to the tertiary structure of native βLG, ^{20}Tyr and ^{61}Trp were on the surface of the protein, and ^{102}Tyr and ^{19}Trp were in the hydrophobic environment. Through renaturation, the originally exposed chromophores on the surface of the recombinant protein in the inclusion body were buried inside the hydrophobic moieties inside the protein molecule. These chromophores could thus avoid collisions with the solution and resulted in stronger fluorescence.

On the other hand, β-sheets tend to contain many non-polar residues and form a hydrophobic core in the interior portion of the protein, but α-helices are always biphasic with hydrophilic residues on the surface and hydrophobic ones in the interior[17]. Four of the six major chromophores are in β-sheets according to the structure of native βLG. The change in secondary structure also revealed that the chromophores after renaturation were more prone to be hidden in the hydrophobic region, which resulted in increased fluorescent intensity.

The results indicated that the hydrophobicity building up around Trp was more significant. It could be inferred from the native protein structure that ^{19}Trp was in a hydrophobic environment and located in a random coil; ^{61}Trp was on the protein surface and located in a β-sheet; ^{20}Tyr, ^{42}Tyr, and ^{102}Tyr were located in a β-sheet and ^{99}Tyr in a random coil; and ^{20}Tyr was on the protein surface and ^{102}Tyr was located in a hydrophobic environment. There were more Tyr than Trp residues in βLG, and the β-sheets containing Tyr were more abundant than those containing Trp. Thus, the conformational changes around Trp were more prominent than around Try during renaturation.

The major IgE epitopes of bovine βLG were mainly located in α-helices and β-sheets. CD and fluorescent assays revealed that recombinant βLG in the inclusion body did not have apparent helix and sheet structures. These structures became more compact after renaturation and closer to those of the native protein. These changes resulted in higher IgE-binding ability, which was verified by ELISA.

This study examined the extent of protein renaturation by spectral methods and ELISA. Renaturation of recombinant βLG resulted in variants with different structures, which demonstrated different IgE binding capacities, and the degree of protein renaturation correlated with the IgE-binding capacity of the protein. The structural integrity of the recombinant allergen is an important indicator. βLG is a potent milk allergen and is categorized as a member of the lipocalin superfamily[18]. Many major allergens of animal origin belong to the lipocalin superfamily[19]. βLG has been used as a model to study how to change the immunoreactivity of food allergens[20-22]. Unfolding and refolding studies on recombinant βLG are thought to provide essential knowledge for a better understanding of the mechanism involved in its change of immunoreactivity. Therefore, results from this study characterize the relationship between the refolding process of recombinant bovine βLG and its immunoreactivity, and may be of help to food allergy therapy and development of vaccination in the future.

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