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Amyloid fibril formation by native and modified bovine β-lactoglobulins proceeds through unfolded form of proteins: A comparative study

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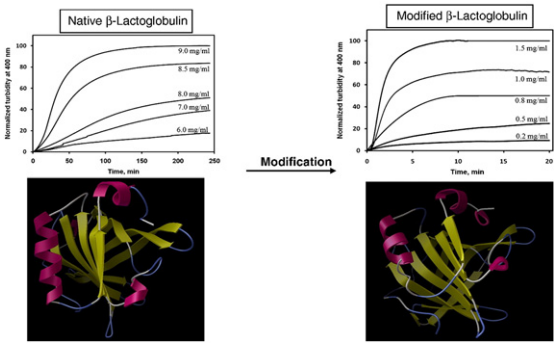
HIGHLIGHTS

► Modified β-lactoglobulin displayed an increased propensity to aggregation.
► Stability of β-lactoglobulin has a determinant role in protein aggregation.
► Importance of some of the lysine residues has been documented in the aggregation process.

GRAPHICAL ABSTRACT



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ABSTRACT

The misfolding and extracellular amyloid deposition of specific proteins are associated with a large family of human pathologies, often called protein conformational diseases. Despite the many efforts expended to characterize amyloid formation in vitro, there is no deep knowledge about the environment (in which aggregation occurs) as well as mechanism of this type of protein aggregation. Recently, β-lactoglobulin (β-lg) was driven toward amyloid aggregation under specific extreme conditions. In the present study, citraconylation was employed to neutralize the charges on accessible lysine residues of β-lg and different approaches such as turbidimetry, thermodynamic analysis, extrinsic fluorimetry and theoretical studies have been successfully used to compare the different behaviors of the native and modified proteins. Kinetic analyses of native β-lg aggregation showed a gradual development of turbidity, whereas the modified β-lg displayed an increased propensity toward aggregation. Our results clearly demonstrated that the stability of modified β-lg is markedly reduced, compared to the native one. Using of TANGO and WALTZ algorithms (as well as modelling softwares) which describe aggregation tendencies of different parts of a protein structure, we suggested critical importance of some of the lysine residues in the aggregation process. The results highlighted the critical role of protein stability and elucidated the underlying role of hydrophobic/electrostatic interactions in lactoglobulin-based experimental system.

Abbreviations: β-Lg, β-Lactoglobulin; BSA, Bovine serum albumin; SDS, Sodium dodecyl sulfate; TEM, Transmission electron microscopy; ThT, Thioflavin T; CR, Congo red; DSC, Differential scanning calorimetry; PSH, Protein surface hydrophobicity; ANS, 1-Anilinonaphthalene-8-sulfonate; PDB, Protein data bank; HSA, Hot spots area; TNBS, 2, 4, 6-trinitrobenzenesulfonic acid.

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1. Introduction

It is estimated that ~50% of the human diseases, including cancer and degenerative diseases, are caused by folding defects [1]. Protein misfolding followed by fibrillar amyloid aggregation is now well recognized to be a major contributing factor in a group of pathologic states known as amyloid diseases [2]. Images provided by transmission electron microscopy (TEM) show that amyloid fibrils are long, rigid and unbranched fibrils which usually consist of a number of typically 2–6 protofilaments [3] and are characterized by an extended cross-β structure, as revealed by X-ray fiber diffraction [4].

Different polypeptide chains have substantially different aggregation propensities, but it is well-known that the formation of amyloid fibrils is an intrinsic and generic polypeptide property [5,6]. It has been shown that a large number of proteins with no sequential and structural homology [7,8], could be converted into amyloid fibrils, under various conditions. These results support previous findings suggesting the independency of amyloid fibrils formation propensity on a consensus or specific protein sequence [9,10]. In this context, it has been shown that a series of unrelated peptides, corresponding to individual β-strands of β-lg, could form amyloid aggregates, independently [11]. One of the holy grails in understanding the molecular mechanism of protein fibrillogenesis lies in identifying, characterizing and controlling the formation of aggregation-prone species at the nucleation phase of fibrillar aggregation and now there is a strong motivation to elucidate the type of involved interactions and characterizing the conditions where the fibril formation can be controlled, kinetically [12]. On the other hand, there is a growing pile of data concerning the effect of altering the chemical properties of amino acids, either by chemical modification or mutation, on the propensity of proteins for amyloid formation [13–15]. It is common to use different methods to change the amino acid composition of proteins in order to explore their roles in aggregation and, especially, amyloid fibrillation [16]. Furthermore, the effects of various medium conditions on protein fibrillogenesis have been examined to gain more insight into this type of protein aggregation using various (disease and non-disease related) proteins [17]. However, very little is known about the conformational events involved in the early stages (such as the pre-nucleation phase) of protein fibrillogenesis.

Bovine β-lg, a known globular whey protein (18,400 Da, pI 5.2) with well-defined structure, has been found to be converted readily to amyloid fibrils upon heating at acidic pH [18–20]. In aqueous solution, a reversible dimerization occurs which its extent depends on the genetic variant, pH of the medium, protein concentration, temperature, and the degree of screening of the electrostatic repulsions [21]. Upon heating above 50 °C, the native structure of β-lg is partially altered: a decrease in the amount of ordered zones [22] and an increase in the exposure of the tryptophan and the free thiol groups [23,24] have been reported. The protein possesses 8 lysine residues, all of which appear to be surface accessible (PDB 3NPO). Some studies have attempted to highlight the important conformational events, specific interactions as well as protein stability in amyloid fibrillogenesis [25,26]. To the best of our knowledge, such comparative studies on native and modified β-lg have not been reported. We found that citraconylation of lysine residues in β-lg triggers amyloid formation and decreases the nucleation time, dramatically. Since native and modified β-lactoglobulins have different propensities to self-assemble into amyloid structure, it is suggested that the resulting data can be useful in providing mechanistic insights to develop potential intervention strategies (in vivo) against protein conformation diseases.

2. Experimental

2.1. Materials and equipments

Citraconic anhydride, 2, 4, 6-trinitrobenzenesulfonic acid (TNBS), Congo red (CR) and Thioflavin T (ThT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 1-Anilinonaphthalene-8-sulfonate (ANS) was purchased from Merck (Darmstadt, Germany) and urea was obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and used as obtained from the suppliers. All solutions were prepared with double distilled water. Unless otherwise stated, all solutions were made in 50 mM sodium phosphate buffer (pH 7.4). A stock solution of ThT (1 mM) was freshly prepared in water and stored in dark, at 4 °C. A Cary-100 Bio (Varian) spectrophotometer was used for protein determination, CR binding assays and turbidimetric measurements. All fluorescence measurements were performed in the ratio mode by using a 1 cm cell on a Cary Eclipse (Varian) spectrofluorimeter, equipped with a 150 W xenon lamp and a thermostated cell holder in which temperature was adjusted by an external thermostated water circulation. All the reported results (except for the relative data) were averages of 2–3 separate experiments whenever the coefficients of variation were less than 5%.

2.2. Protein purification/determination

β-Lg was purified according to the method described by Alomirah and Alli [27]. SDS-polyacrylamide gel electrophoresis was used to confirm the protein purity. The protein samples were loaded on a 12% slab gel under nonreducing conditions according to the method of Laemmli [28]. The protein concentrations were measured by UV spectrophotometry at 278 nm using an extinction coefficient of $\varepsilon_{278}^{1\%} = 0.96\ \mathrm{L \cdot cm^{-1} \cdot g^{-1}}$ [18] and also according to Lowry's method [29]. In the latter method, standard curve was generated using bovine serum albumin (BSA).

2.3. Modification of lysine residues

Citraconic anhydride was used to modify lysine residues in β-lactoglobulin, according to the procedure previously reported [30]. Protein samples were then dialyzed extensively against 50 mM sodium phosphate buffer, pH 7.4. To determine the extent of lysine modification, the free amino groups in the protein was measured using the TNBS method as previously described [31,32].

2.4. Turbidity measurements

Temporal development of relative turbidity at 400 nm and at specified concentration of native and modified β-lg was followed at 80 °C. Temperature was controlled within ±0.1 °C by a temperature controller. The pathlength of the sample cell used was 10 mm. Bovine β-lg samples were heated at 80 °C in 50 mM sodium phosphate buffer containing 6–9 mg/ml of the native protein at pH 2.0 and 0.2–1.5 mg/ml of the modified protein, at pH 4.6 (rapid aggregation of modified β-lg was observed only in this pH). Immediately, the increase in the turbidity was recorded in the kinetic mode by measuring the absorbance at 400 nm as a function of time relative to the appropriate blank solution. Whenever needed, turbidity values were normalized through dividing them by the maximal limiting turbidity observed. The kinetic data were also fitted to the model described by Kurganov [33].

2.5. Dye binding assays

ThT shows enhanced fluorescence at 482 nm when bound to amyloid fibrils [34]. To investigate kinetically whether native and (especially) modified β-lactoglobulins were converted to amyloid-like fibrils, a method based on that of Le Vine [35] was employed. Briefly, 200 μl of β-lg samples (1 mg/ml), was taken at varying time intervals from incubated protein on water bath and then placed on ice. Afterwards, the sample was added to 1.8 ml of 20 μM ThT solution (from 1 mM ThT stock solution in 20 mM sodium phosphate buffer, pH 7.4), mixed thoroughly, and incubated for 5 min. The assay solutions were excited at 450 nm and the emissions were measured over a range that included the wavelength of 482 nm. Excitation and emission slit widths were both set at 10 nm.

Congo red binding assays were performed according to Nilsson method [34]. Congo red stock solution (20 mM) was dissolved in 20 mM sodium phosphate buffer, pH 7.4, and then filtered. A pre-heated 300 μl aliquot of the well-mixed protein sample (2 mg/ml) was added to the same volume of CR solution to yield a final CR solution of 10 mM and final protein concentration of 1 mg/ml. Absorbance spectra were recorded over a range of 400–700 nm after incubation at room temperature for at least 10 min [34].

2.6. Transmission electron microscopy (TEM)

Samples of fibrillar dispersions were prepared for TEM using negative staining. A droplet of the native β-lg and modified sample (each 20 mg/ml), which had been diluted 1:10 in water, was deposited onto carbon support film on a 400 mesh copper grid, separately. Excess liquid was removed after 1 min, using filter paper. A drop of staining solution (0.1% (w/w) phosphotungstic acid) was added, and excess solution was removed after 30 s with filter paper. Electron micrographs were taken using a Philips EM208 field emission scanning electron microscope. Bright field TEM images were acquired at 80 kV [36].

2.7. Circular dichroism spectroscopy

Circular dichroism (CD) spectra in the far-UV (190–260 nm) region were obtained by an AVIV 215 spectropolarimeter at 25 °C using 1 mm-pathlength in 50 mM sodium phosphate buffer, pH 7.4. Protein concentration was 0.3 mg/ml. Protein secondary structure was determined by the CDNN program, version 2.1.0.223 (http://www.photophysics.com) [37].

2.8. Calorimetric studies and thermal stability determination

Irreversible thermal denaturation of native and modified β-lg (16 mg/ml) was studied by differential scanning calorimetry (DSC). The experiments were performed using Nano-DSC II differential scanning microcalorimeter (Setaram, USA). All measurements were performed in 50 mM sodium phosphate buffer, pH 7.4. The protein solutions were heated at a constant scan rate of 2 K. min^{-1} from 10 to 95 °C and under a constant pressure of 2 atm over 0.342 ml capillary glass cells. The reversibility of the thermal transition of native and modified proteins were tested by checking the reproducibility of the calorimetric trace in a second heating of the sample immediately after cooling [38]. The calorimetric data and the temperature dependence of the excess heat capacity, C_p, (in $kJ \cdot K^{-1} \cdot mol^{-1}$) was further analyzed and finally plotted as a function of temperature by Cpextract software. The T_m of the denaturation process was calculated as the temperature with the maximal excess C_p.

2.9. Urea denaturation studies

Urea-induced protein denaturation was studied in 50 mM sodium phosphate buffer, pH 7.4, by placing the protein solution in different urea concentrations (0–9 M). Absorbance of protein solutions both, in the presence and absence of urea, were recorded at 278–289 nm against their respective blank solutions. The solution for each sample was prepared by mixing a fixed volume of protein solution with the appropriate volume of urea solution. Difference extinction coefficients for each denaturation point were plotted against urea concentration. This yielded the denaturation curve for the native and modified β-lactoglobulins [39].

2.10. ANS fluorescence and PSH analyses

The polarity-sensitive fluorescent probe, ANS, was used to investigate the protein surface hydrophobicity (PSH), prompted by protein modification. Titration of protein solutions (0.2 mg/ml) in the presence of increasing concentrations of ANS provides information about differences in the ANS binding properties of native and modified β-lg. In this treatment, F_{max} is the maximum fluorescence intensity at the saturated ANS concentration [40] which indicates the number of surface hydrophobic sites of the protein. K_d^{app} is the apparent dissociation constant for ANS and $1/K_d^{app}$ is the binding affinity of ANS to the protein which can be obtained from the slope of the titration curve. The assay solutions (0.2 mg/ml protein in the presence of various concentrations of ANS) were excited at 390 nm and the emissions were measured over a range (400–600 nm) that included the wavelength of 470 nm. The protein surface hydrophobicity of the native and modified β-lactoglobulins can be calculated from the following equation:

$$PSH = F_{max}/[protein]K_d^{app}.$$

ANS was added from a stock solution (1 mM) to a final concentration range from 2 to 140 μM. The increase in fluorescence emission was recorded at 470 nm until no further increase in fluorescence was observed. The excitation and emission slit widths were both 10 nm.

2.11. Aggregation hot spots prediction

The β-lg amino acid sequence was given to the web server based programs TANGO (http://tango.crg.es), AGGRESCAN (http://bioinf.uab.es/aggrescan) and Waltz (http://waltz.vub.ac.be). The outputs of the programs include the aggregation propensity, number of hot spots area (HSA) and amyloidogenic region, respectively. The HSA is defined as the area of the aggregation profile above the hot spot threshold in a given hot spot, calculated with trapezoidal integration [41]. In order to assess the effect of changing lysine residues to acidic ones, the one letter code of that (E) was inserted in the amino acid sequence of β-lg instead of lysine residues (K) and the sequence was again submitted to the server. Since there is strong correlation between aggregation "hot spots", aggregation propensity and amyloidogenic regions and since it is proposed that a higher area over the threshold promotes a more specific aggregation reaction, only TANGO/Waltz outputs are reported, in this work.

2.12. Molecular modeling

The crystal structure of native β-lg (PDB 3NPO) was downloaded from the protein data bank (PDB) as a template for modeling the modified β-lg. An accessible residue will have a relatively fast modification rate, whereas a buried one will have a relatively slow modification rate. For these experiments, we chose lysine surface residues, which have >30% accessible surface area assessed in Swiss-PDB Viewer (version 4.0.1) [42]. The 'Hyperchem' program (Hyperchem 8.0) was used for a theoretical modification and the molecular dynamics simulations analysis of the native and modified β-lg. The atomic coordinates of the modified lysine residues were separately built using Hyperchem in PDB format [43], CHARMM 27 force field [44] was used for the simulations according to the previously described procedure [45]. The energy minimization of the proteins was carried out under implicit solvent conditions using the conformational analysis programs. Afterwards, the lowest energy conformations were solvated with TIP3P water explicitly, and finally the overall system was energy minimized using the Polak-Ribiere conjugate gradient method until convergence of the gradient ($0.01\ kJ \cdot mol^{-1}$) [46]. The structures visualization and figures generation were performed with Python Molecular Viewer (version 1.5.4) program package and PyMol (version 0.99 beta06) (http://www.pymol.org) [47].

3. Results and discussion

In vitro fibrillogenesis is generally primed by heating, buffer acidification and chemical or physical denaturation (or combination of them), however these are rare or impossible conditions to be achieved in vivo. In the present study, amyloid fibrillation by the native and modified

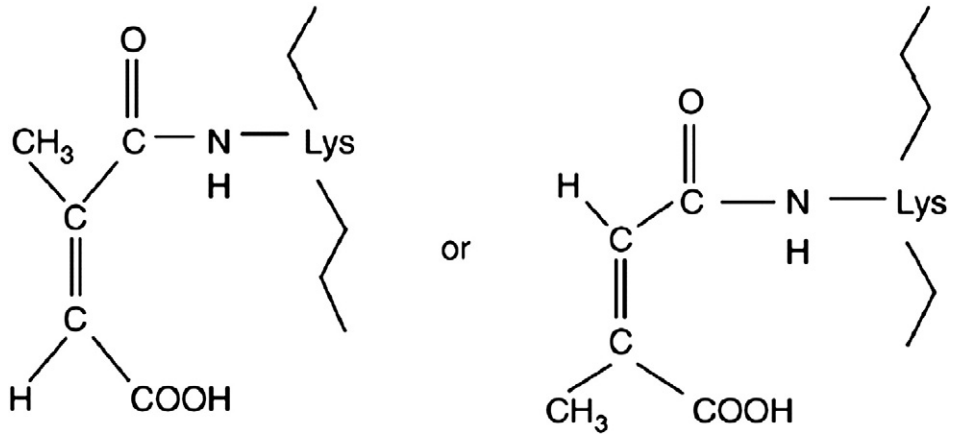


Fig. 1. Modified forms of lysine residue after modification by citraconic anhydride.

bovine lactoglobulins was investigated. A highly significant inverse correlation between the rate of aggregation and the overall net charge of proteins has been previously reported [48]. β-Lg has 15 lysine residues which account for a small part of the total 162 residues of the protein, 8 of which are surface exposed. Charge modification of lysine residues was achieved by citraconic anhydride (Fig. 1).

3.1. Effect of protein concentration on the kinetics of heat-induced β-lg aggregation

Since the sequence, 3D structure (for example see 3NPO in PDB) and biochemical/biophysical properties of β-lg have been well characterized and several reports exist on its capability to form fibrils under simple and different laboratory conditions [18,49,50] (and its amyloidogenic fragments have been identified [11]), it may be anticipated that this protein may provide a useful model for study of amyloid aggregation. To drive β-lg toward amyloid fibril formation, several experimental conditions have been developed [11,18,19,36].

The aggregation of β-lg at 80 °C was studied under the effect of different protein concentrations. Fig. 2 demonstrates a gradual development of turbidity at 400 nm along with increase in the native and modified β-lactoglobulins concentration, in the range of 6–9 mg/ml (for native protein, Fig. 2A) and 0.2–1.5 mg/ml (for modified protein, Fig. 2B). The sigmoidal pattern of the turbidity data for the native and modified proteins reaches to the maxima after about 100 and 10 min, respectively. Fig. 2 clearly indicates that formation of amyloid fibrils involves the typical three phases of the fibrillation process: an initial lag phase (nucleation), a subsequent growth phase (elongation) and a final saturation phase (equilibration). At the protein concentration of 8.5 mg/ml, as demonstrated by the nature of the curve presented in Fig. 2A, duration of the first stage of native β-lg fibrillation was relatively large and reciprocally dependent on initial protein concentration, $[P]_0$. Due to the large repulsion between β-lg monomers, nucleation is the rate limiting step; however, once a critical size is reached, the growth process would be relatively fast [19]. Adversely, the corresponding time for the citraconylated protein, at same concentration range (6–9 mg/ml), was difficult to assign by the method employed in the present investigation, due to a sharp increase in the rate of fibrillation of the modified protein. The initial nucleation process which is a thermodynamically unfavorable event may be facilitated following a number of approaches [19] and some reports suggest its possible dependency on charge neutralization [18]. A gross difference in dependency of aggregation onset/rate on protein concentration is evidenced by comparing aggregation kinetics in Fig. 2A and B. So that, while no fibril structure was detectable up to 20 min for the native protein [18], the modified structure provided extensive fibrillation, reaching its maximum value at 20 min of incubation.

Since the TANGO [51] and WALTZ algorithms were more informative compared to AGGRESCAN, only their related outputs were reported to predict the aggregation propensity and the data of the latter

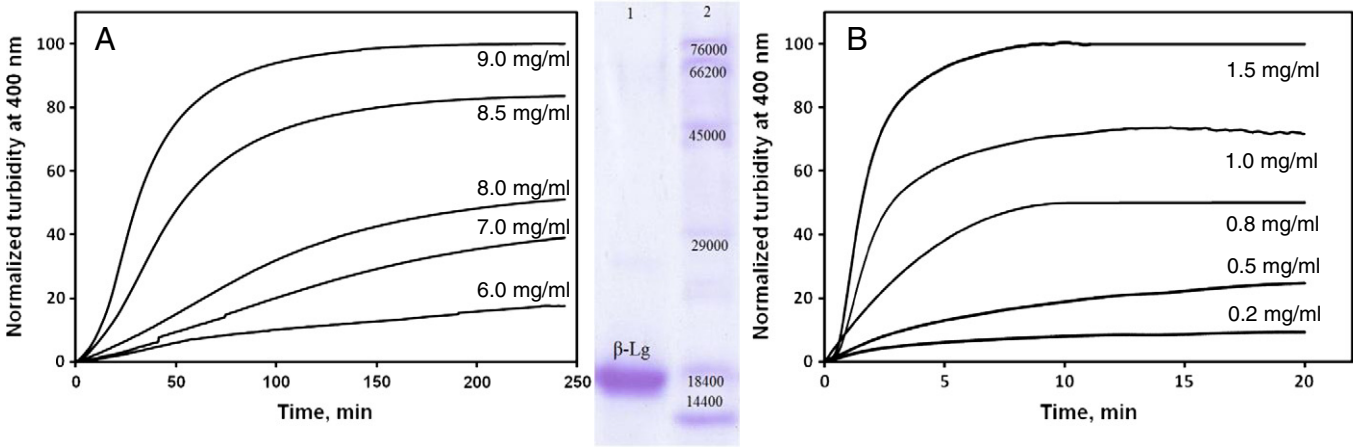


Fig. 2. Effect of protein concentration on the kinetics of heat-induced β-lg aggregation at 80 °C. (A, Left) The final concentration of the native protein (pH 2.0) was 6.0, 7.0, 8.0, 8.5 and 9.0 mg/ml. (B, Right) The final concentration of the modified protein (pH 4.6) was 0.2, 0.5, 0.8, 1.0 and 1.5 mg/ml. Turbidity changes were normalized according to maximal change observed. (Middle) SDS-PAGE pattern of purified β-lg (lane 1) and molecular size markers (lane 2). Further details are given in Experimental procedures.

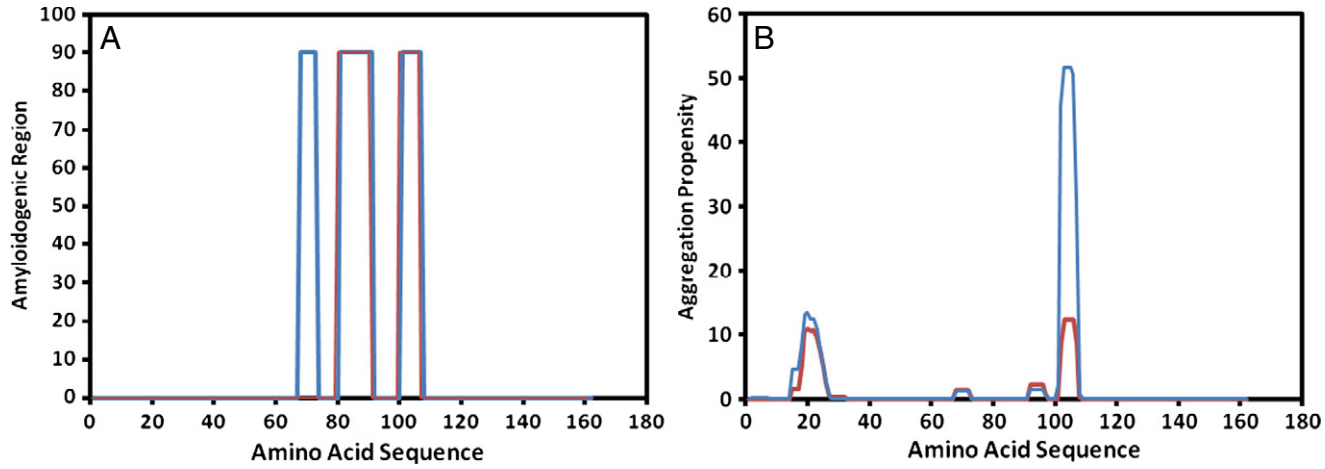


Fig. 3. The effects of replacing of some lysine residues on amyloidogenic (A) as well as aggregation propensity (B) behaviors, as obtained using the Waltz and TANGO algorithms, for native (red color) and modified (blue color) bovine β-lactoglobulins. X-axes depict number of amino acid residues of the protein.

algorithm were not shown. As indicated in Fig. 3A, modified protein presented more amyloidogenic behavior. Additionally, the propensities of the corresponding regions of the modified protein susceptible to aggregate are substantially higher than those of the native sequence (Fig. 3B).

3.2. Amyloid fibril formation by the native and modified β-lactoglobulins

As discussed earlier, native β-lactoglobulins have been found to be converted readily to amyloid fibrils under various experimental conditions [18,19,36,52]. In this investigation, to drive β-lg toward amyloid fibril formation, the native solutions at pH 2.0 [18] and modified protein solutions at pH 4.6 were incubated at 80 °C. Thereafter, formation of amyloid fibrils by the native and modified β-lactoglobulins was verified using ThT fluorescence spectroscopy (Fig. 4) [53] as well as Congo red binding assay (Fig. 5) [54,55]. Also, additional characterization of amyloid fibrils was performed using TEM (Fig. 6). As indicated in Fig. 4A, the fluorescence intensity of ThT upon binding to the fibrillar aggregates of native β-lg was increased significantly which is a characteristic of amyloid aggregation. Also, the same observations were made with citraconylated β-lg (Fig. 4B), confirming that modification of exposed lysines had no negative effect on amyloid formation by this amyloidogenic protein. Furthermore, the absorbance spectra of Congo red (Fig. 5) were increased significantly and showed a red shift of the maximum absorbance from 490 nm to 520 nm in the presence of the aggregated forms of the native and modified proteins. However, there was a relative increase in the Congo red absorption intensity at the maximum wavelength in the presence of the modified protein (Fig. 5B), which is in full agreement with the ThT data, probably coinciding with the increased formation of fibril structure by modified β-lg. Fig. 6A and B shows TEM images of the native and modified β-lg incubated for 400 min at pH 2, 80 °C and 30 min at pH 4.6, 80 °C, respectively.

3.3. Effect of citraconylation on CD spectra of β-lg

As discussed earlier, citraconylation of the lysine residues promoted amyloid formation, resulting in a dramatic decline in the nucleation time (Fig. 2). For several structurally unrelated proteins (such as β-lg and immunoglobulin light chains), fibril formation arises from a completely unfolded conformation [52,56]. Additionally, several amyloidogenic proteins are intrinsically unstructured and completely unfolded (with random coil structure) under physiological conditions [57,58]. So, it may be possible that ordered secondary structure of β-lg is reduced upon protein modification. To examine the effect of lysine modification

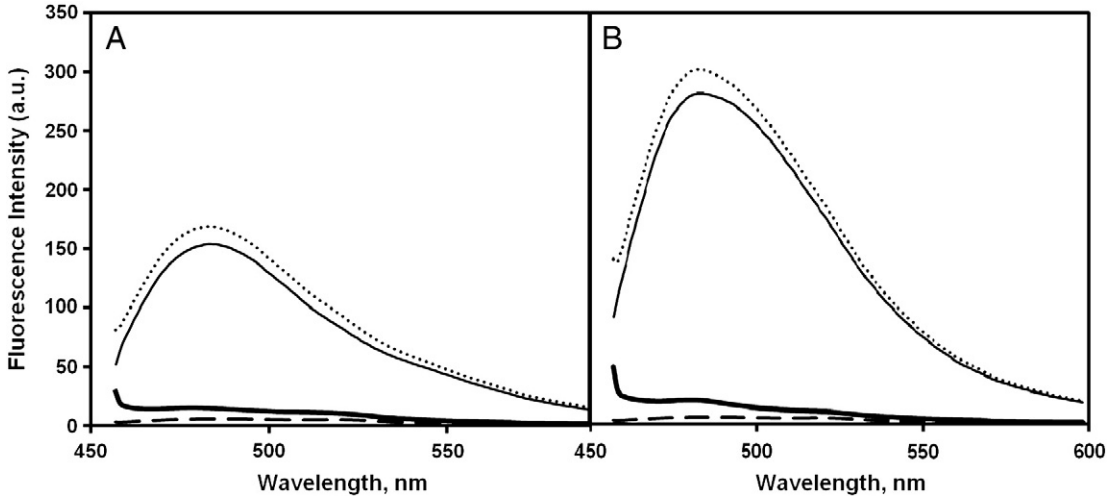


Fig. 4. Effect of chemical modification on β-lg amyloid fibrillation. ThT fluorescence intensity spectra of the native (A) and modified (B) β-lactoglobulins. Fluorescence intensity spectra of ThT alone (dashed line), a suspension of 1 mg/ml incubated β-lg alone (bold line) and in the presence of 20 μM ThT (dotted line). The difference spectrum (thin line) is obtained by subtracting the spectrum of β-lg alone from the spectrum of ThT in the presence of the protein. Data shown are representative of three independent experiments and standard deviations were approximately within 5% of the experimental values.

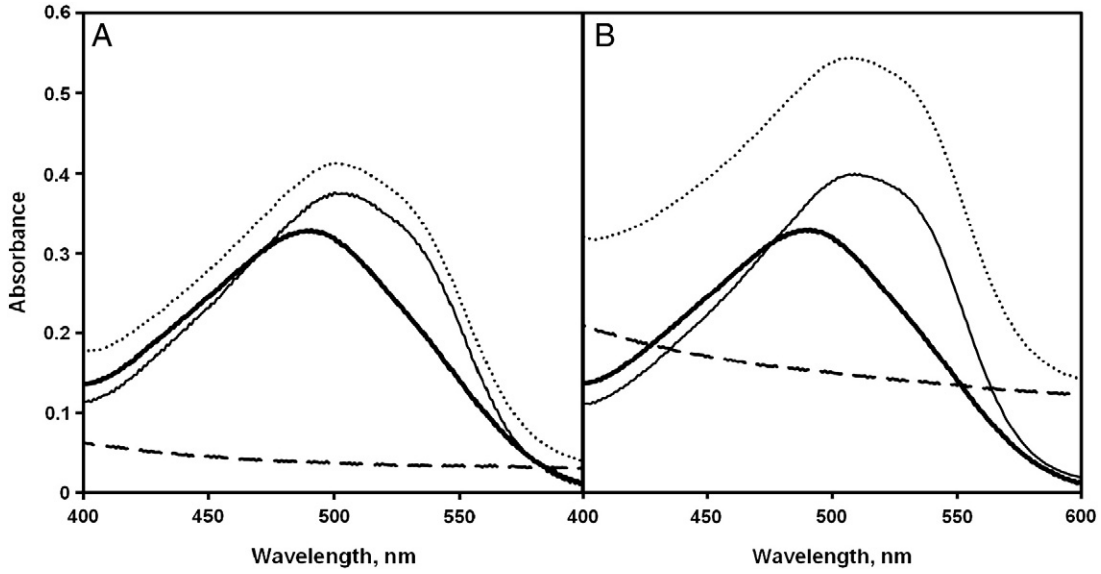


Fig. 5. Absorbance spectra of CR in the presence and absence of native (A) and modified β-lactoglobulins (B). Absorbance spectra of a suspension of 2 mg/ml incubated β-lg alone (dashed line) in the presence of 20 μM CR (dotted line) and of CR alone (bold line). The difference spectrum (thin line) is obtained by subtracting the spectrum of β-lg alone from the spectrum of CR in the presence of the protein. Data shown are representative of two independent experiments. For more details, please see the text.

on the secondary structure of β-lg and to investigate whether (or not) secondary structure content of the protein may play any determinant role in β-lg fibrillogenesis, the far-UV CD spectra of the native and modified proteins were compared.

CD spectra of the native and modified forms of β-lg obtained in 50 mM phosphate buffer, pH 7.4 are shown in Fig. 7. The far-UV CD spectra of the native and citraconylated β-lactoglobulins show significant differences in their secondary structure.

On other hand, as it is evidenced from the inset of Fig. 7, both native and modified β-lactoglobulins represent a β-rich conformation (with some α-helixes), so that the far-UV CD spectra of both proteins show a negative signal around 217 nm (typical of β-sheet structure). Furthermore, to analyze the features of the structural changes, secondary structure compositions of the proteins were calculated from the CD spectra by CDNN program [37]. As shown in Fig. 7 (inset table), the α-helical content was decreased and the content of β-structure (and random coil) was increased upon protein modification. These results suggest that the conformation of β-lg transforms from an α-helical structure in the native form to β-sheet structure in citraconylated protein. Additionally, these structural rearrangements may affect the degree of protein stability (As will be discussed later). Also, the results of molecular modeling are in full agreement with CD data (Fig. 8).

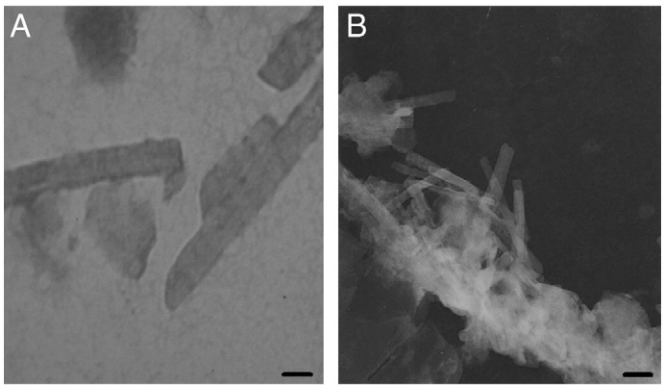


Fig. 6. Overview of β-lg fibrils by TEM. Negatively stained electron micrographs of non-modified (A) and modified (B) proteins. Samples (20 mg/ml) were incubated at pH 2.0 (for native β-lg) or pH 4.6 (for modified β-lg) and heated at 80 °C for specified time. Scale bars represent 50 nm for the left and 100 nm for right images.

3.4. Effects of modification on thermal denaturation of β-lg: its relation to amyloid aggregation

There are some reports suggesting that the first event in the aggregation process is often the unfolding of the native conformation [60]. Also, the ability of some proteins to form amyloid fibrils is primarily a result of their reduced thermodynamic stability, allowing unfolding to take place [52,56,61]. For example, thermally induced fibrillogenesis in lysozyme [62] sensitively depends on the enzyme instability. Thus, there is this possibility that the modified proteins

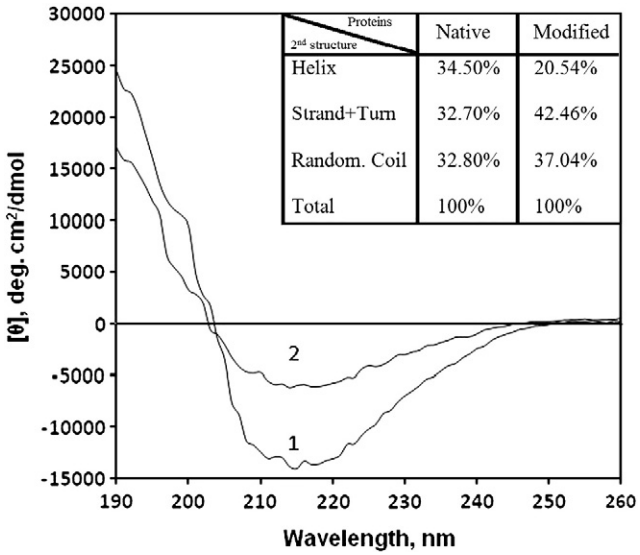


Fig. 7. Far-UV CD spectra of native (Curve 1) and modified β-lactoglobulins (Curve 2). Each experiment was repeated three times and an average CD spectrum was input automatically. The spectra were recorded at 25 °C after all necessary corrections were made for background absorbance. The concentration of protein was approximately 0.3 mg/ml. Further details are given in Experimental procedures (inset) [37].

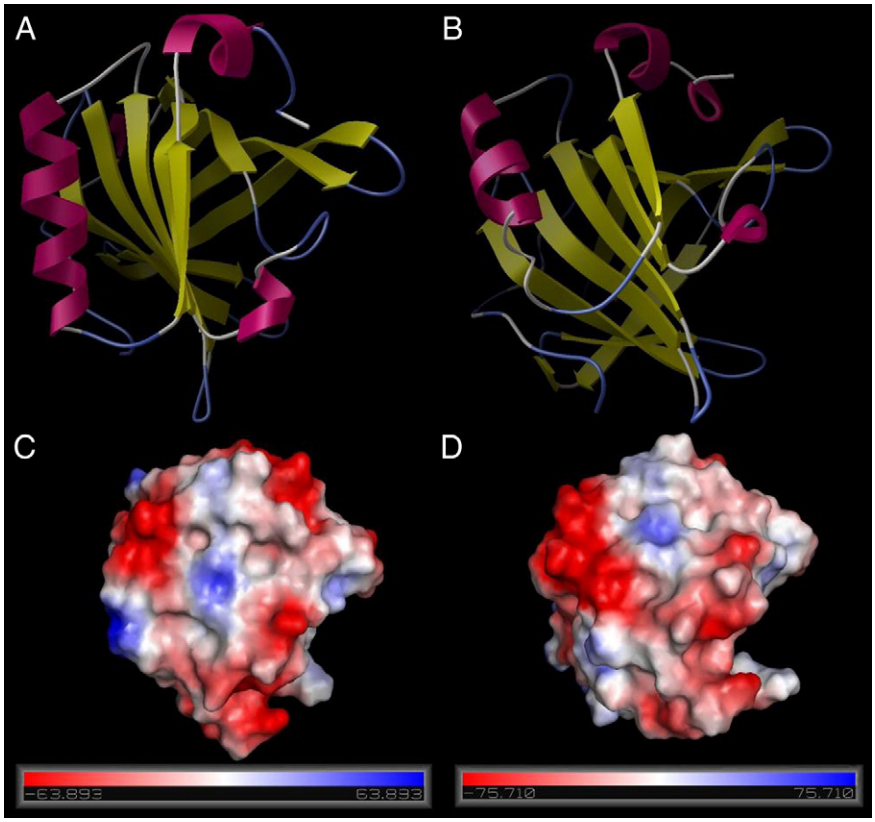
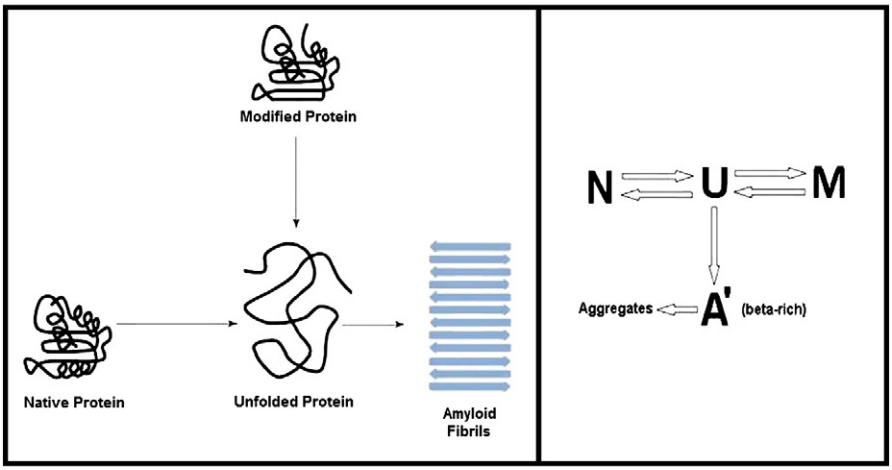


Fig. 8. Structural and physicochemical properties of β-lg: A structural model of (A) native (PDB: 3NPO), and (B) modified β-lactoglobulins, the protein backbone is shown in the "cartoon" representation. This structural model was prepared using the Python Molecular Viewer (version 1.5.4) program package. Native and modified β-lactoglobulins models are colored according to the distribution of electrostatic potential, of native β-lg (C) from red (−63 kT) to blue (+63 kT) and distribution of electrostatic potential, of modified β-lg (D) from red (−75 kT) to blue (+75 kT). This figure of electrostatic potential was provided using the program PyMol [47], the electrostatic potential was calculated using the APBS tools plug in to PyMol [59].

with lower conformational (thermodynamic) stability, can aggregate more easily. As mentioned earlier, several structurally unrelated proteins such as β-lg [52], immunoglobulin light chains [56] and insulin [61], fibril formation arises from a completely unfolded conformation (see Scheme 1). Additionally, several amyloidogenic proteins are also known to be intrinsically unstructured and completely unfolded under normal solution/physiological conditions [57,58]. It is reported that modification of lysine residues changes protein pI toward lower amounts, concomitant with alteration in solubility and stability [63] of β-lg. The net charge of the unmodified β-lg is higher at pH 2.0 (+17/monomer) than at neutral pH (approximately −6/monomer) [64] which causes an increased intermolecular repulsion between the native molecules, and hence a slower onset of aggregation. Accordingly, after modification with citraconic anhydride, the net charge of the protein molecule was diminished through replacing the positive charges of exposed lysine ε-amino groups by the potentially negative charges of carboxylic groups (At pH 4.6, the net charge of the modified β-lactoglobulin, with negatively charged carboxylate groups on modified lysines and positively charges on intact lysines, appears to be close to zero). Therefore, modification of lysine residues changes solubility,



Scheme 1. Proposed mechanisms for heat-induced amyloid aggregation for the native and modified β-lactoglobulins. Aggregated, unfolded, native and modified proteins are denoted by A, U, N and M, respectively.

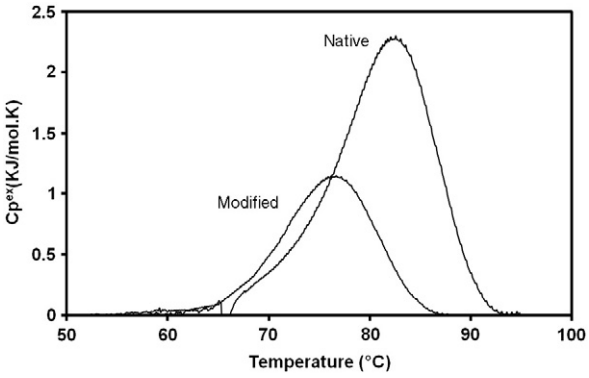


Fig. 9. Effects of lysine modification on irreversible thermal unfolding of β-lg, as followed by monitoring the changes in excess C_p (apparent heat capacity). DSC profiles of the native and modified β-lactoglobulins (16 mg/ml) were obtained, indicated by excess C_p as a function of temperature. The excess C_p unit was $kJ \cdot K^{-1} \cdot mol^{-1}$. DSC profiles were obtained with heating rate of $2\ K \cdot min^{-1}$. Data shown are representative of three independent experiments. For more details, please see the Experimental procedures.

stability [63] and probably affect aggregation propensity of β-lg. To examine the effects of lysine modification on β-lg thermodynamic stability and to investigate whether (or not) protein conformational stability plays any determining role in the heat-induced fibrillogenesis, T_m and enthalpy of the native (aggregation-resistant) and modified (aggregation-prone) β-lactoglobulins were determined, using DSC.

The irreversible thermal unfolding of native and modified β-lactoglobulins registered by DSC is characterized by sharp endothermic peaks with maxima at 82.9 °C and 76.7 °C, respectively, indicating the shift of maximum position (apparent T_m) toward lower temperatures, upon modification (Fig. 9). Also, the apparent calorimetric enthalpy changes of the native and modified proteins were 276 and 130 $kJ \cdot mol^{-1}$, respectively. As indicated in Fig. 9, modification of lysine residues resulted in a 6.2 °C decrease in T_m value and a prominent decrease in the apparent enthalpy change of β-lg unfolding. As it is evident, the stability of modified β-lg shows remarkable reduction, compared to the native one.

It is accepted that aggregation of proteins may be caused by a combination of different interactions [65]. Aggregation process can occur after a perturbation in the structure of protein which may result in protein destabilization. In this way, a critical role was suggested for intra-/inter-molecular electrostatic interactions. It may be this possibility that these types of interactions destabilize a transition state involved in heat-induced structural changes of the native β-lg. Additionally, we found that the aggregation extent/rate was triggered in lysine modified protein. There is the possibility that these intramolecular (destabilizing) interactions are rearranged in favor of faster aggregation of the modified β-lg. In other words, it is reasonable to assume that heat-induced structural changes are controlled thermodynamically. In this respect, as evidenced by Fig. 9, since the modified protein, with lower conformational stability, had potential to aggregate, it may be inspired that heat-induced β-lg amyloid formation is essentially initiated from completely unfolded state.

3.5. Determination of free energy for the denaturation process

In order to understand the details of the modification impact on β-lg stability, chemical denaturation was performed on both the native and modified proteins, using urea as an efficient chemical denaturant. To do so, the profiles of absorption changes at 280 nm versus urea concentration were obtained for the native and modified proteins. The determination of free energy (ΔG^0) as a criterion of conformational stability of a globular "module" (e.g. protein, domain, etc.), is based on the assumption that the native module (N) can be directly converted to the denatured state (D) via a reversible process [66,67]. Assuming a two state mechanism for each transition, one can analyze the process by monitoring the changes in the absorbance, and calculate the free energy (ΔG^0), as previously described [39]. The simplest method for estimation of the conformational stability in the absence of denaturant, $\Delta G^0_{(H_2O)}$, is extrapolating the linear plot of ΔG^0 versus [denaturant] to zero denaturant concentration [39]. Fig. 10A and B shows the plots of ΔG^0 as a function of urea concentration for the native and modified proteins, respectively. The apparent ΔG^0 of the native and modified proteins were determined as 8.7 and 6.0 $kJ \cdot mol^{-1}$, respectively. In full agreement with DSC data, these results also suggest the lowered stability of the modified β-lg, compared to the native protein.

3.6. Determination of changes in protein surface hydrophobicity, induced by lysine modification, using ANS fluorescence measurements

Since the apolar residues generally make up 30% to 50% of most proteins, a considerable amount of protein (stability and) folding driving force must be originated from hydrophobic interactions [68]. Moreover, regarding kinetic competition between correct folding and undesired aggregation [2]; it appears reasonable that hydrophobic effects should be also involved in unproductive protein misfolding and aggregation. In this respect, several authors have considered a key role for apolar interactions in the early stages of amyloid formation [25,26,65].

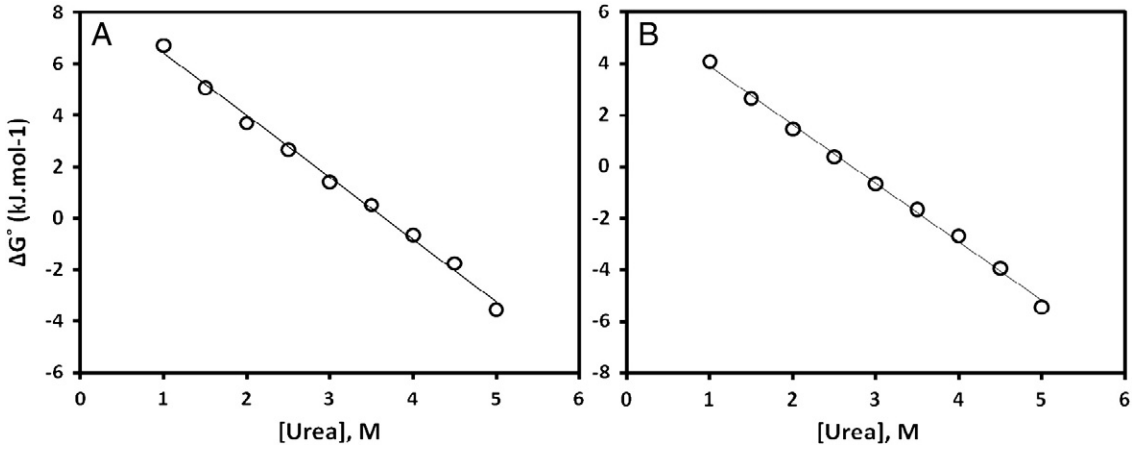


Fig. 10. Free energy changes for chemical denaturation of the native (A) and modified β-lactoglobulins (B) in 50 mM phosphate buffer, pH 7.4 and 25 °C.

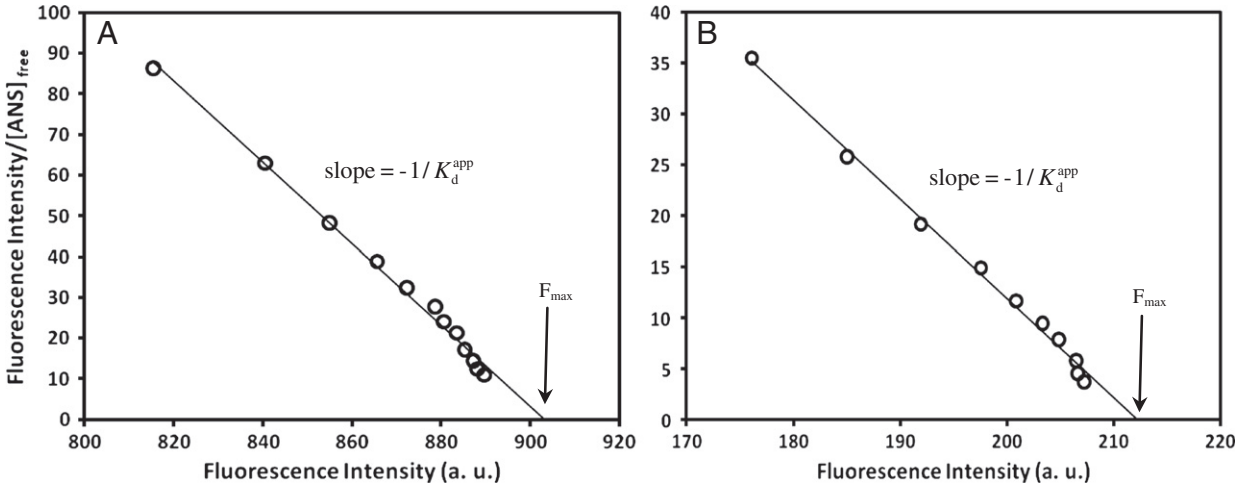


Fig. 11. Scatchard plots for the titration of native (A) and modified (B) β-lactoglobulins (0.2 mg/ml) with increasing concentrations of ANS. Fluorescence emission intensity was measured at 470 nm with excitation at 390 nm. Data shown are representative of three independent experiments and standard deviations were within 5% of the experimental values. Further details are given in Experimental procedures.

Using suitable probes, such as ANS [25], it is also possible to obtain a quantitative estimate of protein surface hydrophobicity (PSH) parameters. By performing ANS-titration experiments, we detected significant differences in the ANS binding properties of β-lg in its native and modified forms. It is noteworthy that ANS, at the used concentration range, had no significant effect on the native and modified β-lg tertiary structure (data not shown). From the Scatchard plot (Fig. 11), we determined the apparent dissociation constant for the probe (K_d^{app}) and the maximum fluorescence intensity (F_{max}) at saturating probe concentration. Thereafter, PSH indexes were calculated as described in Experimental procedures.

PSH values were 81 and 20 for the native and modified β-lactoglobulins, respectively. In particular, modified β-lg shows a ~75% decrease in exposed hydrophobic surfaces compared to the native protein. In addition, the exposed sites in the modified protein showed looser binding of the probe, as it can be inferred from the increase of K_d^{app} value.

As stated earlier, the modified protein had more potential to aggregate. As a possible mechanism, it can be suggested that accessible lysine residues (or electrostatic interactions) may be directly involved in the heat-induced structural changes and aggregation. As an alternate (possible) mechanism, there is also this possibility that some other types of inter- and intra-molecular interactions, with critical destabilizing role of transition states, are attenuated/corroborated due to modification of some accessible lysine side chains. Based on the ANS binding results, it can be suggested that the heat-induced structural changes of β-lg may involve a transition state in which mainly destabilizing electrostatic interactions occur, and lysine modification may destabilize this transition state(s). Therefore, we can conclude that the hydrophobicity has no major role in the formation of amyloid fibrils and probably other forces such as electrostatic interactions are involved in β-lg amyloid aggregation. Additionally, loose binding of ANS to the modified β-lg may be resulted from repulsion electrostatic interactions between sulfonate groups of ANS and exposed naturally/artificially negative moieties on the surface of the modified protein at pH>7.

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