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Thermochimica Acta 432 (2005) 106–111
thermochimica acta
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Structural changes in β-lactoglobulin by conjugation with three different kinds of carboxymethyl cyclodextrins

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Received 9 February 2005; received in revised form 31 March 2005; accepted 20 April 2005

Abstract

β-Lactoglobulin-carboxymethyl cyclodextrin (β-LG-CMCyD) conjugates were prepared by using water soluble carbodiimide. Three kinds of CMCyDs differing in molecular mass were used to investigate the effects of different CMCyD contents, net charge and hydrophobicity on the structural changes in β-lactoglobulin. The effect of CMCyDs on the structure of β-lactoglobulin was utilized to investigate the contribution of hydrophobic interactions to the stability of the protein. Spectroscopic studies suggested that the conformation around had not changed in either conjugate but the α-helix content of β-LG-CMCyD conjugates had markedly increased as compared with that of β-lactoglobulin. The differential scanning calorimetry technique confirmed that the addition of one glucose unit in β-LG-CMCyD conjugates, enthalpy change of calorimetry decreased and the denaturation temperature of each conjugate was higher than that of native β-lactoglobulin. The heat contents agreed well with the conformational transition measured by molar ellipticity at 222 nm ($[\theta]_{222}$) and Stoke's radius (R_S) values. Therefore, hydrophobic forces play an important role in stabilizing and shielding of the β-LG-CMCyD conjugates.

Keywords: Carboxymethyl cyclodextrins; β-Lactoglobulin; Stabilization; α-Helix induced; Hydrophobic force; Protein conjugation; Shielding; Compactness

1. Introduction

β-Lactoglobulin (β-LG), the predominant whey protein, is a globular protein of molecular mass 18,400 with two disulfide bridges as well as free cysteine containing plenty of essential amino acids [1]. Many physicochemical and physiological studies on β-LG have been carried out: higher ordered structure [2–6], denaturation mechanism [7–10], polymerization and gelation behaviour [11–13], foaming and emulsifying properties [14–17], immunological response [18–24], and so on. Results from X-ray crystallography [25] and protein sequence determinations [26,27] have shown remarkable similarly between β-LG and plasma retinol binding protein. The function of β-LG is tentatively thought to be binding and transportation of retinol, and it is categorized as a member of the lipocalin super family [28]. It is well-known that β-LG has good emulsifying properties above pH 5 [29]. Although β-LG is thought to be a valuable protein according to nutritional science, β-LG is known as a potent allergen of milk allergy; about 82% of milk allergy patient are sensitive to β-LG [30].

The fact that β-LG is stable at low pH and resistant to proteolysis is considered to be one of the reasons β-LG is allergenic [30–33]. Hence, it is very desirable to develop new means of decreasing the allergenicity and enhancing the functional properties of β-LG. To achieve this, Hattori et al.

Abbreviations: β-LG, β-lactoglobulin; β-LG-CM-α-CyD, β-lactoglobulin-carboxymethyl-α-cyclodextrin conjugates; β-LG-CM-β-CyD, β-lactoglobulin-carboxymethyl-β-cyclodextrin conjugates; β-LG-CM-γ-CyD, β-lactoglobulin-carboxymethyl-γ-cyclodextrin conjugates; CMCyDs, carboxymethyl cyclodextrins conjugates; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DSC, differential scanning calorimetry; CD, circular dichroism; R_s, Stoke's radius; [η], intrinsic viscosity

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doi:10.1016/j.tca.2005.04.014

studied neoglycoconjugates of β-LG [34]. Studies on neoglycoconjugates of proteins have been widely investigated in this decade, and various improvements to the functional properties of proteins have been reported. However, little is known about the structure of proteins with polymers. Conjugation of a protein with a polymer is thought to change the protein structure more effectively than conjugation with low molecular weight components. In particular, multiple changes to protein structure can be expected by conjugation with a charged polymer due to the difference in molecular weight or charge of the chemical species conjugated. To achieve low allergenicity, conjugation of a protein with polysaccharides is thought to be more effective than conjugation of a protein with low molecular weight molecules because it can be expected that polysaccharides will cover the epitopes of the allergens more effectively than low molecular weight molecules [35]. In the present study, we chose carboxymethyl α, β, γ-cyclodextrins as useful saccharides for conjugation with β-LG to study the structural properties of a protein. Cyclodextrins (CyDs) are cyclic carbohydrates which form inclusion complexes with various hydrophobic molecules and they are widely used in food and pharmaceutical applications [36–39]. Cyclodextrins are used in order to stabilize unstable materials and to solubilize insoluble or poorly soluble materials. Three CMCyDs are readily available: CM-α-CyD, CM-β-CyD and CM-γ-CyD having six, seven and eight glucose units, respectively. CyDs with fewer than six glucose residues are too strained to exist, whereas those with more than eight residues are very soluble, difficult to isolate and hardly studied to date. α-CyD, β-CyD and γ-CyD are commonly referred to as the native CyDs. Many very covalently modified CyDs have been prepared from native forms. We prepared β-LG-CMCyDs conjugates using water-soluble carbodiimide. Here, we describe the conformational changes in β-LG brought through its conjugation with three kinds of CMCyDs.

2. Materials and methods

2.1. Materials

Bovine β-LG and three kinds of CyDs (α-, β-, γ-cyclodextrins) were obtained from Sigma. The other substances of reagent grade were purchased from Merck. Other chemicals were of reagent grade. The molar mass for β-LG is 18,400 g mol^{-1} (Dalton).

2.2. Methods

2.2.1. Carboxymethylation of CyDs

CyDs were carboxymethylated by a method previously used for carboxymethylation of dextran [40], with some modifications. In brief, monochloroacetic acid (9 g) was dissolved in 48 ml of methanol, and 10.5 ml of NaCl solution (1 g ml^{-1}) was added with gentle stirring. After adding methanol (12 ml), CMCyD (3 g) was added to the solution and the mixture was incubated at 45 °C for 48 h. After washing with 70% methanol, filtration and lyophilization, CMCyD was obtained.

2.2.2. Preparation of the β-LG-CMCyD conjugates

The β-LG-CMCyD conjugates were prepared by the method of Hattori et al. [40] (method 1) and by referring to the method of Kitabatake et al. [41] (method 2).

2.2.2.1. Method 1. Each of CMCyDs (1 g, α, β, γ) was dissolved in 100 ml of distilled water, and 100 ml of an EDC solution (10 mg/ml) was added as separately. After the pH was adjusted to 5.5 with 1N HCl, 100 ml of a β-LG solution (10 mg/ml) was added. The reaction mixture was incubated at 4 °C for 6 h while gently stirring. The reaction was stopped by adding 18 ml of acidic acid and the solution was dialyzed against distilled water to form a separated oily phase of a coacervate. Crude β-LG-CMCyDs conjugates were obtained after lyophilization of the coacervate.

2.2.2.2. Method 2. β-LG and CMCyDs were dissolved in 30 ml of distilled water and adjusted to pH 4.75 with 1 N HCl, and EDC solution (413 mg/ml of distilled water) was added gradually during 30 min while maintaining the pH at 4.75 with 1 N HCl. The reaction mixture was incubated at 25 °C for 3 h. The reaction was stopped by adding 2 ml of a 2 M sodium acetate buffer at pH 5.5. After dialysis against distilled water and lyophilization, crude β-LG-CMCyDs conjugates were obtained.

Free CMCyDs were removed by salting out. The crude conjugates were dissolved in a 0.067 M phosphate buffer at pH 7.0 at a concentration of 5 mg/ml and the proteinaceous component was salted out with ammonium sulphate to a final concentration of 5 M. The participate, which was recovered by centrifuging at 18,000 rpm for 15 min at 25 °C and washing with the 0.067 M phosphate buffer at pH 7.0, containing 5 M ammonium sulphate, was next dissolved in the 0.067 M phosphate buffer at pH 7.0. After dialysis against distilled water and lyophilization, the β-LG-CMCyDs conjugates without free CMCyDs were obtained.

Free β-LG containing polymerized β-LG was removed by ion-exchange chromatography. A DEAE-Toyopearl 650 S column (Tosoh, 2.2 i.d. × 20 cm) was equilibrated with the 0.067 M phosphate buffer at pH 7.0 and at a flow rate of 4.0 ml/min. Each β-LG-CMCyD conjugate after salting out (30 mg/3 ml) was applied to the column and eluted with a linear gradient from 0 to 1 M NaCl concentration. To detect the protein and CMCyD, the absorbance was monitored at 280 and 490 nm after colouring according to the phenol–sulfuric acid method [42], respectively.

2.2.3. Differential scanning calorimetry (DSC) measurements

DSC experiments were performed on a Scal differential scanning microcalorimeter (Institute for Biological Instru-

mentation, Pushchino, Russia) with cell volumes of 0.48 ml at a scanning rate of 1 K/min (was kept constant in all experiments), interfaced with a personal computer (IBM compatible). Before the measurements, samples were degassed by stirring in an evacuated chamber at room temperature and then immediately loaded into the calorimeter cell; the final dialysis buffer (also degassed) was loaded into the reference cell. A pressure of 152 kPa (1.5 atm) of dry nitrogen was always kept over the liquids in the cells through out the scans to prevent any degassing during heating. The reversibility of the thermal transitions was checked by a second heating of the cool sample immediately following the first scan. The calorimetric traces were corrected for the instrumental background by subtracting a scan with buffer in both cells. The reactive errors of the values of molar enthalpy changes are in the range of 3% and the absolute errors of given transition temperatures T_m are 0.3 °C. The thermogram analysis and fitting were done based on Privalov and Potekhin theory [43] which was installed as DOS program in software package (named Scal-2) and supplied by Scal (Russia). Scal-2 program, which is installed in DSC instrument, enables to determine the native and denatured lines based on fitting error. The best fitting error is selected as a best thermogram.

2.2.4. Circular dichroism (CD) measurements

All measurements in this work were carried out at 20 °C with thermostatically controlled cell holders. CD spectra were measured with a JASCO J-715 spectropolarimeter (Japan) equipped with an interface and a personal computer. The instruments were calibrated with ammonium D-10-camphorsulfonic acid [44]. The results were expressed as the mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100\theta_{obsd}/(lc)$, where θ_{obsd} is the observed ellipticity in degrees, c the concentration in residue mol/l, and l is the length of the light path in cm. The helical content of the β-LG and three kinds of β-LG-CMCyDs conjugates were calculated by the method of Chen et al. [45]. The CD spectra were measured in a 1 mm path length cell from 250 to 190 nm. The rotatory contributions of a protein can be determined by $X = f_H X_H + f_\beta X_\beta + f_R X_R$ where X can be either the ellipticity or the rotation at any wavelength. f is the fractions of the helix (f_H), beta form (f_β) and unordered form (f_R); the sum of f is equal to unity and each f is greater than or equal to zero. With the f values of five proteins obtained by X-ray diffraction studies, the X of the protein at any wavelength is filled by a least-squares method, which defines the X_H, X_β and X_R. The CD for the helix, beta and random forms determined thus can be conversely used to estimate the secondary structure of any protein with X at several wavelengths for the same equation. The α-helical content (f_H) was estimated from the ellipticity value at 222 nm ($[\theta]_{222}$) as described as follows [45,46]:

$$f_H = -\left([\theta]_{222} + \frac{2340}{30300}\right) \quad (1)$$

2.2.5. Fluorescence measurements

Fluorescence measurements were made on JASCO SP-6200 spectrofluorometer at an excitation wavelength of 283 nm. Trp fluorescence emission was followed at 339 nm [34]. The temperature of the cell compartments was kept constant at 20 °C by water circulation.

2.2.6. Measurements of viscosity and Stoke's radius

The viscosity was measured using a Haake D8 (W. Germany) microviscometer. The intrinsic viscosities, $[\eta]$ and Stoke's radii, R_S, of the β-LG-CMCyDs conjugates were determined using the equation [47]:

$$\frac{\eta_{sp}}{c} \cong [\eta] = \lim_{c\to 0}\left[\left(\frac{\eta/\eta_o - 1}{c}\right)\right] = \frac{2.5N_A}{M}\left(\frac{4}{3}\pi R_S^3\right) \quad (2)$$

where η_{sp} is the specific viscosity, c the protein concentration in g ml^{-1}, N_A Avogadro's number, M the molar mass of the protein and π is equal to 3.14.

3. Results and discussion

The conjugation of β-LG and three different kinds of CMCyDs were confirmed by the coincidence of the protein and saccharides strained bands by SDS-PAGE and by the shift of the isoelectric point (p*I*) of β-LG to the acidic side (data not shown).

Fig. 1 shows the DSC profiles for β-LG and three different kinds of β-LG-CMCyDs conjugates. DSC is the method of choice for the determination of ΔH and ΔC_p associated with the heat-induced denaturation of proteins. The reason for saying this is that both thermodynamic parameters

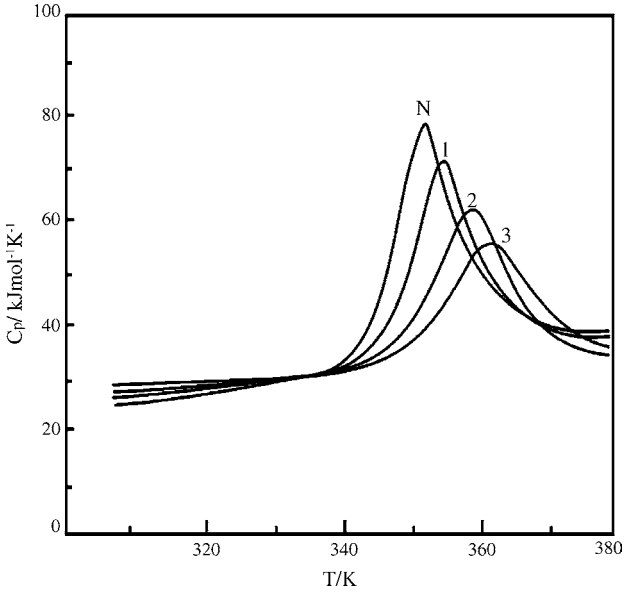

Fig. 1. DSC thermograms for the native β-LG and various kinds of β-LG-CMCyDs conjugates in 67 mM phosphate buffer. N, native state of β-LG; 1, β-LG-CM-α-CyD conjugate; 2, β-LG-CM-β-CyD conjugate; 3, β-LG-CM-γ-CyD conjugate.

can be determined directly and no assumption regarding the mechanism of denaturation is required for the analysis of the endotherm. The native state of β-LG showed a sharp heat absorption peak with a $383.1 \pm 1.4\,kJ\,mol^{-1}$ calorimetric enthalpy change value (ΔH) representation of cooperative melting of the native structure as reported by Burova et al. [48], thus implying a close structural similarity (see Fig. 1). The transition point (T_m) of native state of β-LG was $350.8 \pm 1.4\,K$. DSC profiles of the various kinds of β-LG-CMCyDs conjugates (CM-α, CM-β and CM-γ CyDs) exhibited small and broad curves with 354.3 ± 2.1, 316.6 ± 1.8 and $296.1 \pm 1.3\,kJ\,mol$ calorimetric enthalpy change values, respectively. The T_m of β-LG-CM-α, CM-β, CM-γ-CyDs conjugates were 354.4 ± 1.1, 358.6 ± 1.5 and $361.7 \pm 0.3\,K$, respectively (see Fig. 1). Since CM-α-CyD, CM-β-CyD and CM-γ-CyD were thermally inactive in the temperature range for the present DSC run, it can be said that these DSC curves detected the thermal denaturation of β-LG in the conjugate. One of the good diagnosis of the stability and globularity of the protein is T_m [49–52]. The values of the T_m show that the most stable form is the β-LG-CM-γ-CyD conjugate state. No tendency to aggregate was observed for any of the states. The DSC scan was repeated twice, keeping the same sample solution in the cell. The unfolding transition was reversible for native β-LG, β-LG-CM-α-CyD, β-LG-CM-β-CyD and β-LG-CM-γ-CyD conjugates. Due to reversibility of these states, it is possible to determining the ΔH for them which is shown in Fig. 1 and Table 1. T_m for three kinds of conjugates was much higher than that of native β-LG. Kitabatake et al. [41] have reported that covalent binding of gluconate to amino groups of β-LG enhanced the solubility of β-LG at high temperatures. Conjugation of saccharides to a protein is thought to bring about an improvement in the heat stability of a protein. However, ΔH values for three different kinds of CMCyDs conjugates (β-LG-CM-α-CyD, β-LG-CM-β-CyD and β-LG-CM-γ-CyD) decreased than to the value for native β-LG. These decreases in ΔH values are thought to have mainly been due to the change in secondary structure such as β-sheet in β-LG by conjugation with different kinds of CMCyDs. It is apparent from Table 1 that the ΔH values of the conformational transitions induced by CM-α-CyD, CM-β-CyD and CM-γ-CyD conjugates are not identical; as the one glucose unit of CMCyD increases, the values of ΔH decrease. The stability of the β-LG-CM-γ-CyD conjugate is more than other states and its ΔH value is less than others, therefore it is clearly that it's secondary structure changes more than other conjugates.

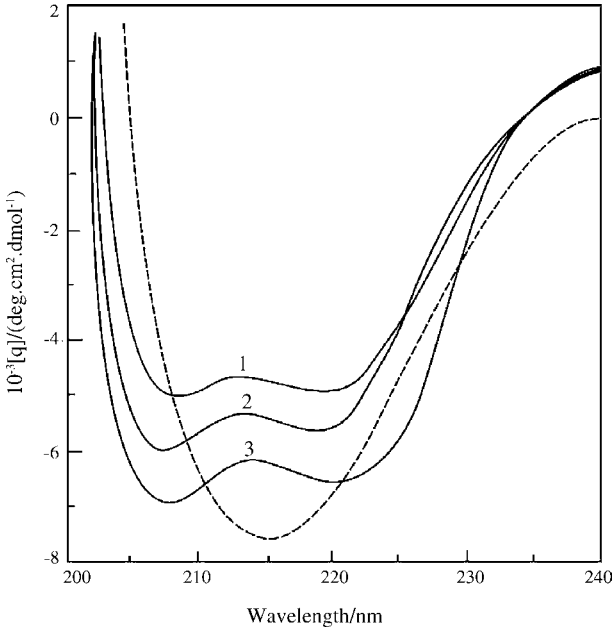

Fig. 2. Far-UV CD spectra of native β-LG and various kinds of β-LG-CMCyDs conjugates in 67 mM phosphate buffer. 1, β-LG-CM-α-CyD conjugate; 2, β-LG-CM-β-CyD conjugate; 3, β-LG-CM-γ-CyD conjugate. Dashed curve shows the native state of β-LG in 67 mM phosphate buffer.

The far-UV CD spectra of the β-LG and three kinds of conjugates are shown in Fig. 2. Native β-LG had a negative maximum at 216 nm, since β-LG was rich in β-sheet. In the case of the β-LG-CMCyDs conjugates, the broad negative maximum and the blue shift show that the β-sheet regions had been changed by conjugation with CMCyDs. The α-helix content of native β-LG estimated according to the method of Chen et al. [45] was 9.3%, corresponding to the results of X-ray crystallography [5]. However, the α-helix contents of β-LG-CM-α-CyD, β-LG-CM-β-CyD and β-LG-CM-γ-CyD conjugates were 14.4, 19.6 and 23.1, respectively, on the basis of the ellipticity values at 222 nm as calculated by the method of Chen et al. [45]. Table 1 shows the molar ellipticity at 222 nm ($[\theta]_{222}$) for native β-LG and three kinds of conjugates. These results indicate that the lowest value of $[\theta]_{222}$ beyond conjugates belong to β-LG-CM-γ-CyD conjugate. It shows that the α-helix content of β-LG-CM-γ-CyD conjugate is more than two other conjugates. It strongly sup-

Table 1
Intrinsic viscosity ($[\eta]$), Stoke's radius (R_S), molar ellipticity at 222 nm ($[\theta]_{222}$) and enthalpy change of calorimetric (ΔH) values of native β-LG and different structure states of β-LG-CMCyDs conjugates

Protein states	$[\eta]$ (M^{-1})	R_S (nm[a])	ΔH ($kJ\,mol^{-1}$)	$[\theta]_{222}$ ($^\circ\,cm^2\,dmol^{-1}$)
Native β-LG	39.03 ± 0.3	1.83 ± 0.02	383.1 ± 1.4	–
β-LG-CM-α-CyD conjugate	30.40 ± 0.2	1.65 ± 0.02	354.3 ± 2.1	−4750
β-LG-CM-β-CyD conjugate	22.56 ± 0.2	1.51 ± 0.02	316.6 ± 1.8	−5100
β-LG-CM-γ-CyD conjugate	16.65 ± 0.2	1.27 ± 0.02	286.1 ± 1.3	−6500

ΔH is dependent on the instrument scan rate; these data use a DSC scan rate of $1.0^\circ\,min^{-1}$.

[a] The values were calculated from equation: $\frac{\eta_{sp}}{c} \doteq [\eta] = \lim_{c \to 0} \left[\left(\frac{\eta/\eta_o - 1}{c} \right) \right] = \frac{2.5 N_A}{M} \left(\frac{4}{3} \pi R_S^3 \right)$.

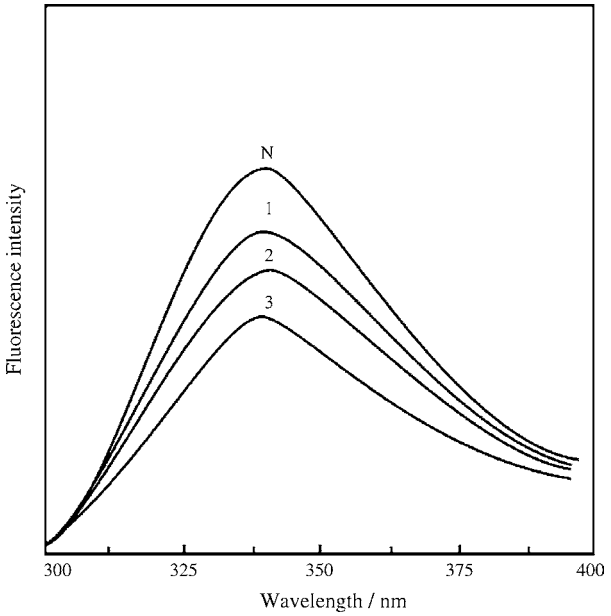

Fig. 3. Intrinsic fluorescence of native β-LG and three kinds of β-LG-CMCyDs conjugates in 67 mM phosphate buffer. N, native state of β-LG; 1, β-LG-CM-α-CyD conjugate; 2, β-LG-CM-β-CyD conjugate; 3, β-LG-CM-γ-CyD conjugate. The emission spectra of the β-LG-CMCyDs conjugates were measured with an extinction wavelength of 283 nm.

port the view that CM-γ-CyD with eight glucose unit induces more α-helix content with conjugation of β-LG than to CM-β-CyD and CM-α-CyD (see Fig. 2, Table 1). It means that CM-γ-CyD with hydrophobic residues play an important role in inducing α-helix content with conjugation of β-LG.

The fluorescence emission spectra of the native β-LG and β-LG-CMCyDs conjugates are shown in Fig. 3. When excited at 283 nm, native β-LG exhibited a fluorescence emission maximum at 336 nm. It has previously been clarified that the fluorescence intensity increases with red shift of the wavelength for maximum emission as the conformation of β-LG changes [53,54]. In this experiment, each conjugate also exhibited the same emission maximum wavelength. Hence, the conformation around Trp residues (19 and 61Trp) of the conjugates seems to have been the same as that of native β-LG. However, the fluorescence intensity of each conjugate was lower than that of native β-LG. This decrease is thought to have been due to the shielding effect by the polysaccharide chain bound to β-LG in each conjugate. This shielding effect on the Trp fluorescence was especially prominent in CM-γ-CyD conjugate. The fluorescence intensity of β-LG-CM-γ-CyD conjugate is lower than two other conjugates. It is noteworthy that the shielding effect of CM-γ-CyD bound to β-LG as conjugate is more than CM-α-CyD and CM-β-CyD shielding effects, therefore hydrophobic residues in CM-γ-CyD is more effective than two other conjugates in the shielding of β-LG.

Protein folding is a process in which an extended polypeptide chain acquired a maximally compact structure through formation of specific secondary and tertiary architecture. Large number of hydrophobic residues is buried inside the molecule upon protein folding, resulting in decreased heat capacity. However, a significant and opposite contribution by the burial of polar groups is also evident. A key parameter for characterizing the different states of protein is compactness or globularity, but only limited data have been reported on the direct measurement of this quantity [55–58]. The intrinsic viscosity and Stoke's radius, R_S, [59–61] are crucial measurements of the compactness of protein states. The intrinsic viscosity and R_S values in Table 1 show the most compact state is the β-LG-CM-γ-CyD conjugate.

The relationship beyond ΔH, $[\theta]_{222}$ and R_S values have been shown in Table 1 for native β-LG and three kinds of conjugates. These values determine that the most change of secondary structure is related to β-LG-CM-γ-CyD conjugate. On the other hand, the T_m value of β-LG-CM-γ-CyD conjugate is more than others; it means that β-LG-CM-γ-CyD conjugate is more stable than native β-LG and two other conjugates. Therefore, hydrophobic groups in CM-γ-CyD play an important role in shielding, stabilizing and inducing of α-helix content of β-LG.

4. Conclusions

In this study, we prepared β-LG-CMCyDs conjugates with different structures. The conjugates were more stable to heating than native β-LG. The α-helix content of β-LG by conjugation with CM-γ-CyD is more than two other conjugates, on the other hand, the intrinsic viscosity and R_S values show that the most compact state is belong to β-LG-CM-γ-CyD conjugate than to CM-β-CyD and CM-α-CyD conjugates. The T_m values determine that the β-LG conjugate with CM-γ-CyD is more stable than other states. Therefore, CM-γ-CyD with more hydrophobic groups than to CM-β-CyD and CM-α-CyD, play an effective role in shielding and stabilizing of β-LG.

Acknowledgments

The financial support from the Research Council of the Azad University of Mashhad and University of Tehran are gratefully acknowledged.

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