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# Enzymatic Cross-Linking of β-Lactoglobulin: Conformational Properties Using FTIR Spectroscopy

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In this study, we use FTIR spectroscopy to probe the conformational changes of β-lactoglobulin (β-LG)—the main constituent of whey proteins—as subjected to enzymatic cross-linking by transglutaminase. We investigate both the amide I region (1600−1700 $cm^{-1}$) and the C−H stretching region (2800−3100 $cm^{-1}$). In the amide I region, spectra of denatured conformations of β-LG, known to be necessary for cross-linking, differ according to the denaturation procedure, i.e., chemical or thermal treatment. Denaturation by chemical denaturants, dithiothreitol (DTT) or β-mercaptoethanol, show no effect on the α-helix, while shifting the monomer dimer equilibrium toward higher monomer concentration. On the other hand, denaturing by thermal treatment dissociates the β-sheets in the native structure, leading to new intermolecular β-sheets being formed. Preheated then enzyme cross-linked β-LG molecules show very similar spectra in the amide I region to the molecules with no cross-linking, indicating minimal effects of the cross-links on the carbonyl stretching mode. However, chemically denatured (using β-mercaptoethanol) then enzyme cross-linked β-LG molecules show noticeable diminution in the α-helix band and formation of strong hydrogen-bonded intermolecular β-sheets. In the C−H stretching region, preheated then enzyme cross-linked β-LG molecules exhibit a different degree of exposure of aliphatic amino acids due to the enzyme action. The same behavior is observed for DTT-treated then enzyme cross-linked β-LG molecules. Generally, the changes in the C−H stretching region clearly indicate that hydrophobic interactions are altered upon enzymatic cross-linking.

## Introduction

Whey proteins are important food ingredients of high nutritional value and unique functional properties.[1−3] The functional characteristics of whey proteins depend primarily on the protein−protein interactions and protein−solvent interactions. These interactions are influenced by the chemical, structural, and conformational properties of the protein molecules. Gelation, emulsification, foaming, and thickening are typical functional properties of whey proteins that are a direct reflection of the molecular interactions. β-Lactoglobulin (β-LG) is the major constituent of whey protein, to which most of the whey protein properties can be attributed to.

Enzymatic cross-linking provides a powerful tool to tailor the functional properties of whey proteins. Transglutaminase has been widely used for this purpose.[4−17] Several studies have investigated the polymerization, rheology, texture, and microstructure of protein solutions and gels after cross-linking with transglutaminase. Transglutaminase has been shown to considerably increase protein molecular weight,[14,15] gel modulus, gel fracture strain and stress[15] and enhance film properties.[18] However, investigations into the conformational characteristics of protein molecules and their effect on the transglutaminase-catalyzed reactions are absent in the literature. Investigating the conformational characteristics of β-LG, the main constituent of whey proteins, can help in understanding its susceptibility to enzymatic cross-linking. The secondary and tertiary structures of β-LG in the native state may impede the enzymatic catalysis. Consequently, complete or partial denaturation of β-LG may be necessary to facilitate the enzymatic reactions.

Figure 1 shows a three-dimensional structure of β-LG[19] which exists as a dimer under physiological conditions.[20] The secondary structure consists of nine β-strands (∼50%), a single α-helix (∼15%), several turns (∼20%), and random arrangements (∼15%)[21] (see Figure 1). Transglutaminase cross-links β-LG molecules by inducing an acyl transfer reaction between the lysine and glutamine residues, producing ϵ-(γ-glutamyl)lysine bonds.[22] Glutamine and lysine residues per β-LG molecule are 9 and 15, respectively. The ϵ-(γ-glutamyl)lysine bonds cause aggregation of the protein and lead to high molecular weight polymers. However, previous work by us[15] indicates that the β-LG molecule must be partially or completely denatured to undergo enzymatic cross-linking. The denaturation can be induced by thermal treatment at elevated temperatures (>70 °C) or by addition of denaturants such as dithiothreitol (DTT) or β-mercaptaethamol (β-ME) that cleave the disulfide bonds. However, it is not yet fully understood what critical conformational changes occur to the β-LG molecule to allow for enzymatic cross-linking. Of equal importance are the conformational changes that result from enzymatic cross-linking. In this study, we address the conformational changes required to facilitate the cross-linking of β-LG by transglutaminase as well as the resulting conformational changes accompanied with cross-linking.

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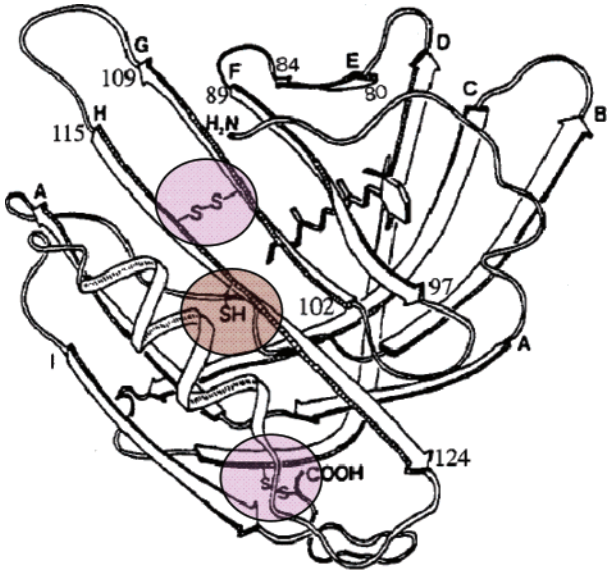


**Figure 1.** Three-dimensional schematic of a $\beta$-LG molecule, showing the $\beta$-strands, the $\alpha$-helix, the position of the disulfide bonds, and the free cysteine residue (reproduced with permission from ref 19).

## Materials and Methods

**Materials.** $\beta$-LG (>99%), N-ethylmaleimide (NEM), $\beta$-mercaptoethanol ($\beta$-ME), and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Deuterium oxide ($D_2O$) was obtained from Aldrich (Milwaukee, WI). A commercial transglutaminase was supplied (1% enzyme and 99% maltodextrin, by weight) by Ajinomoto Co. (Tokyo, Japan).[23] All materials were used as received.

**Preparation of Protein Solution.** $\beta$-LG was dissolved in deuterium oxide at concentrations of 3, 5, and 7 wt %. Samples were purged with nitrogen and kept under nitrogen atmosphere for 48 h to ensure complete hydrogen/deuterium exchange.[24] Some samples were heated at 80 °C for 1 h to denature the protein. Other samples were denatured by treatment with DTT (50 mM) or $\beta$-ME (4%). For samples treated with enzyme, the enzyme was added (50 U/g) and then thoroughly mixed after heat or thermal denaturation. The sample was then incubated at 50 °C for 12 h. Enzyme was found to lose activity completely after ~12 h. More details on these protocols can be found in previous publications and thesis.[15,25,26]

**FTIR Measurements.** Infrared spectra were recorded at room temperature using a Magna 760 Nicolet spectrometer (Madison, WI). Dry air was continuously run through the spectrometer. Samples (25 $\mu$L) were placed between two $CaF_2$ crystals with a 25 $\mu$m spacer. The infrared spectra were recorded at a resolution of 2 $cm^{-1}$. A total of 128 scan were recorded, averaged, and apodized with the Happ−Genzel function. Spectra in all the experiments were subtracted from the spectra of $D_2O$. No smoothing correction was invoked. Deconvolution of the spectra (1600−1700 $cm^{-1}$) was performed by Ominc 5.2 software with an enhancement factor of 3 and bandwidth of 37 $cm^{-1}$.

## Results and Discussion

**Cross-Linking of $\beta$-LG by Transglutaminase Enzyme.** Figure 2 shows an electrophoresis gel for different $\beta$-LG samples that were incubated with transglutaminase for 12 h at 50 °C. The temperature of incubation (50 °C) was chosen as it has been shown to be the optimum temperature for enzyme activity,[23] while the duration (12 h) was chosen based on the fact that the enzyme becomes inactive after 12 h at 50 °C. Details on the gel electrophoresis method can be found in our earlier publications.[15,25,26] The heat denatured sample (80 °C for 1 h, lane 4) and the samples treated with chemical

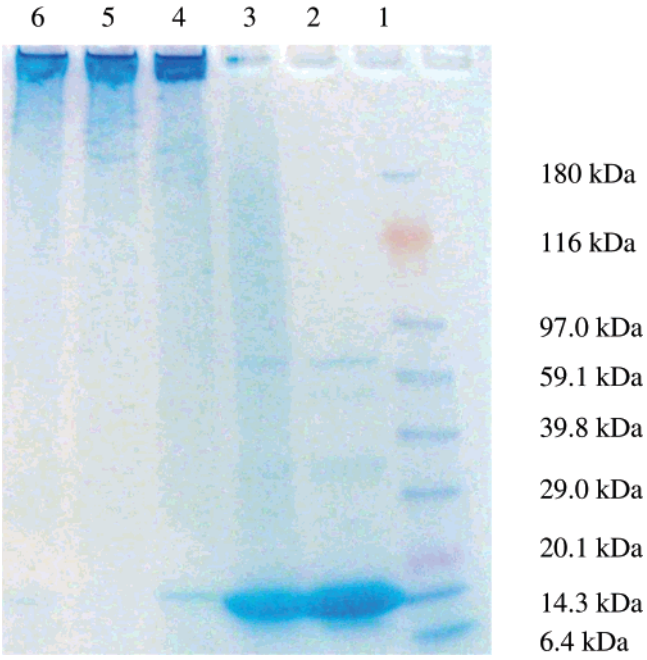


**Figure 2.** SDS−PAGE analysis of $\beta$-LG samples under reducing conditions. Lane 1, molecular weight marker; lane 2, native $\beta$-LG; lane 3, enzyme-treated native $\beta$-LG sample; lane 4, enzyme-treated $\beta$-LG sample that was preheated for 1 h at 80 °C; lane 5, enzyme-treated $\beta$-LG sample in the presence of DTT; lane 6, enzyme-treated $\beta$-LG sample in the presence of $\beta$-ME. Enzyme treatment was done by incubating the sample at 50 °C with transglutaminase (10 U/g protein) for 12 h.

denaturants (DTT and $\beta$-ME, lanes 5 and 6, respectively) were cross-linked by the enzyme to form large molecular weight aggregates and appear on the top of the gel. However, the native $\beta$-LG sample (lane 3) remains virtually unchanged. This behavior indicates that cross-linking by transglutaminase requires denaturation of $\beta$-LG either thermally or by chemical denaturants such as DTT or $\beta$-ME. We believe that this behavior is primarily due to the globular nature of $\beta$-LG, which has deep hydrophobic pockets. However, the mechanism of thermal denaturation is quite different from the mechanism of denaturation induced by chemical denaturants that cleave disulfide bonds, as will be evident later.

**Spectra of the Native $\beta$-LG Molecule.** The FTIR spectra of $\beta$-LG have been the subject of numerous studies,[27−37] specifically the spectral range of 1600−1700 $cm^{-1}$, which is known as the amide I region[38] and corresponds mainly to the peptide backbone C═O stretching mode in addition to the C−N stretching mode. Bands in this region correspond to $\beta$-sheets, $\alpha$-helix, turns, and random coils. The amide I region of the FTIR spectra of the native $\beta$-LG molecule at different concentrations is shown in Figure 3. [No FTIR spectra of transglutaminase-treated $\beta$-LG are shown as the enzyme has no effect on native $\beta$-LG, as observed from SDS−PAGE analysis in Figure 2.] The bands at 1621, 1634, and 1692 $cm^{-1}$ correspond to $\beta$-sheets and strands,[36,39] while that at 1649 $cm^{-1}$ represents $\alpha$-helix.[40] The band at 1677 $cm^{-1}$ corresponds to turns or $\beta$-sheets, and the one at 1663 $cm^{-1}$ corresponds to turns. The small shoulder appearing at 1605 $cm^{-1}$ reflects side chain residues. It is clear from Figure 3 that the protein concentration in the range shown (3−7 wt %) does not affect the band positions. However, it is reported elsewhere that at lower concentrations (less than 1%) some bands change dramatically.[32] Lefevre and Subirade[32] showed complete disappearance of the bands at 1621 and 1692 $cm^{-1}$, while the band at 1635 $cm^{-1}$ was shifted to 1629 $cm^{-1}$

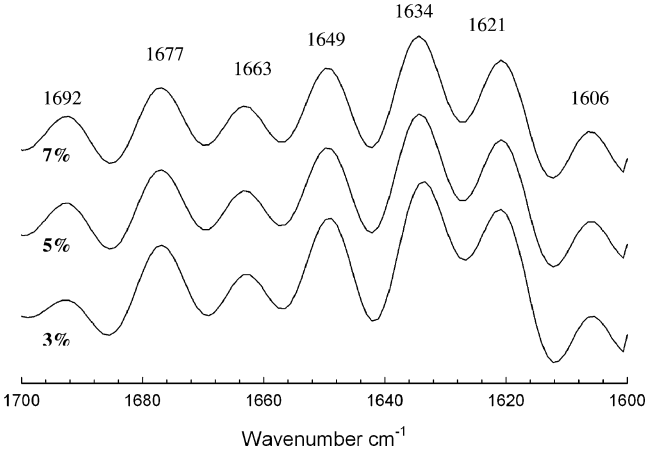


**Figure 3.** FTIR deconvoluted spectra of native β-LG at concentrations of 3, 5, and 7 wt %.

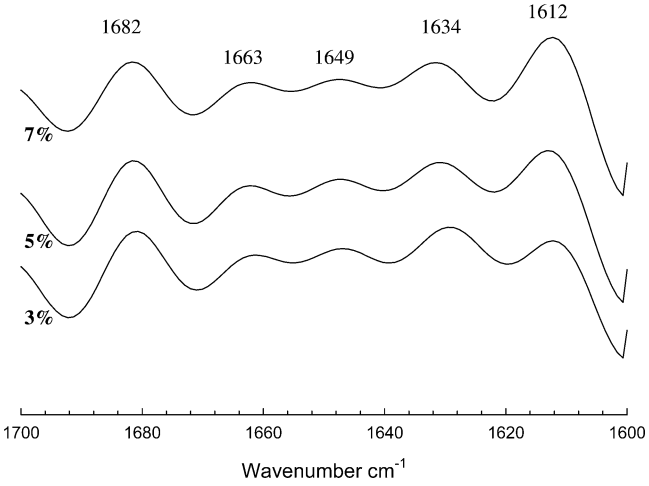


**Figure 4.** FTIR deconvoluted spectra of preheated (80 °C for 1 h) β-LG at concentrations of 3, 5, and 7 wt %.

at a concentration of 0.25%. This indicates that the bands correspond not merely to the secondary structures of the individual molecules but depend also to a certain extent on the interaction between the molecules. The change in spectrum with concentration was attributed to the dissociation of the native dimer to the native monomer.[32,41] This ratio of monomer to dimer changes with protein concentration, and hence the bands change from one concentration to another. However, the range of concentration of interest in this work does not seem to show this behavior as we effectively observe the same band positions and shapes for all the spectra in the range of 3−7% protein.

**Structural Changes upon β-LG Denaturation.** *Thermal Denaturation.* During thermal treatment the hydrogen bonds stabilizing the native structure of β-LG are disrupted, causing loss of the α-helix and β-sheets structures and creating new β-sheets arrangements.[21,42] These new β-sheets result from the intermolecular hydrogen bonding between the protein aggregates. Aggregation is induced primarily by disulfide interchange and hydrophobic interactions.[43,44] Figure 4 shows FTIR deconvoluted spectra of β-LG samples denatured by thermal treatment (i.e., heating to 80 °C for 1 h). Comparison of Figure 4 with Figure 3 shows that thermal treatment dramatically changes the amide I region of the FTIR spectra. Thermal denaturation diminishes the band at 1634 $cm^{-1}$, eliminates the band at 1621 $cm^{-1}$, and creates a strong band at 1612 $cm^{-1}$. Since the bands at 1634 and 1621 $cm^{-1}$ correspond to intramolecular β-sheets and that at 1612 $cm^{-1}$ to intermolecular

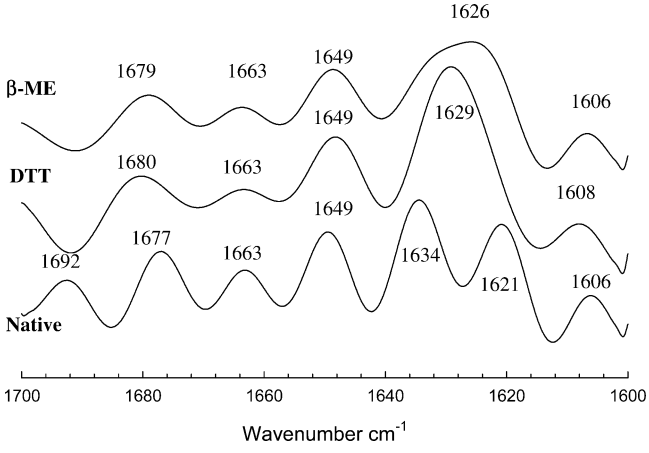


**Figure 5.** FTIR deconvoluted spectra of native, DTT-treated, and BME-treated β-LG samples, all at a concentration of 7 wt %.

β-sheets, these results indicate formation of intermolecular, β-sheet structures.[42] We also observe that heat treatment diminishes the bands at 1649 and 1692 $cm^{-1}$ corresponding to α-helix and β-sheets, respectively, and creates a band at 1682 $cm^{-1}$ indicative of antiparallel β-sheet formation.[38]

*Chemical Denaturation.* On the other hand, denaturation by DTT and β-ME proceeds quite differently as they disrupt the β-LG structure by cleaving the two disulfide bonds present in the native molecule: (Cys66-Cys160) that connects the C−D loop to the carboxyl-terminal region, and (Cys106-Cys119) that links strands G and H as shown in Figure 1. For DTT treatment at a specific β-LG concentration, the FTIR spectrum depends to a large extent on the concentration of DTT used (data not shown); therefore, an excess of DTT (200 mM) was used to ensure complete denaturation of β-LG. With DTT, the bands at 1621 and 1634 $cm^{-1}$ (Figure 5) move closer to each other and an intermediate band appears at 1629 $cm^{-1}$. In addition, the band at 1692 $cm^{-1}$ completely disappears. The addition of β-ME produces similar changes in the FTIR spectrum, with a broad intermediate band appearing at 1626 $cm^{-1}$ and the band at 1692 $cm^{-1}$ disappearing. This phenomenon can be attributed to the rearrangement of the β-sheets after cleavage of the disulfide bonds. The disappearance of the 1634 $cm^{-1}$ band may be interpreted as a decrease in the dimer content of β-LG.[45] This is confirmed by the fact that (Cys106-Cys119) is involved in dimer formation of β-LG.[40] For both DTT and β-ME, the band at 1649 $cm^{-1}$ exhibits minor changes, indicative of minimal modification to the α-helix structure. Likewise, the bands at 1663 and 1677 $cm^{-1}$ show a very minor change. Thus, the spectra of denatured β-LG by DTT and β-ME indicate minimal change of the α-helix with major changes occurring in the β-sheets structures, primarily in the bands at 1621, 1634, and 1692 $cm^{-1}$. This agrees with the fact that such denaturants cleave disulfide bonds, which do not interact with the α-helix.

Cleavage of the disulfide (Cys66-Cys160) bond will disconnect the C−D loop from the carboxyl-terminal region (see Figure 1), which is expected to open up the protein globule, as this bond attaches two distant amino acids. We also expect the C−D loop to participate in other β-sheet associations, possibly explaining the rearrangements of the bands at 1621 and 1634 $cm^{-1}$ observed in Figure 5. On the other hand, the cleavage of (Cys106-Cys119) disulfide bond will weaken the contact between strands G and H. The extent of this weakening and whether it is going to disrupt the antiparallel arrangement of strands G and H is not known and has not been addressed before. Intuitively, we can postulate that the (Cys66-Cys160) bond will

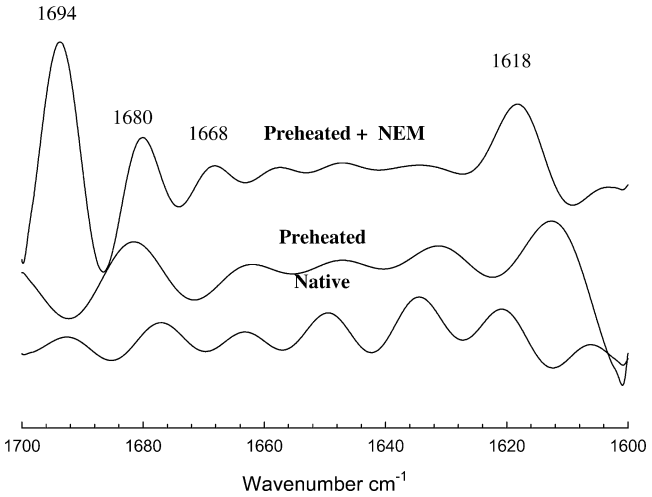


**Figure 6.** FTIR deconvoluted spectra of native, preheated, and preheated with NEM $\beta$-LG samples at a concentration of 7 wt %.

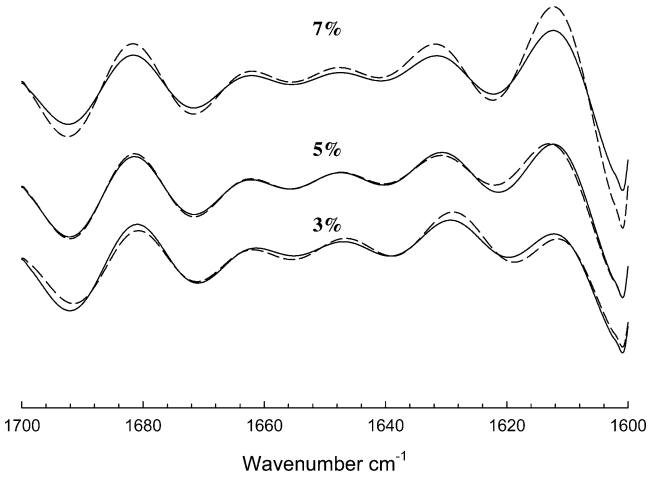


**Figure 7.** FTIR deconvoluted spectra of preheated (solid curves) and enzyme-treated (dashed curves) $\beta$-LG at concentrations of 3, 5, and 7 wt %.

have more of an effect in opening the protein structure than the (Cys106-Cys119) bond based on to the geometry of the molecule. However, an exact knowledge of the conformational changes corresponding to cleavage of each disulfide bond requires careful molecular modeling and is beyond the scope of this work.

*Thermal Denaturation in the Presence of a Cysteine Blocking Agent.* To study the thermal denaturation of $\beta$-LG in the absence of disulfide interchange, we added *N*-ethylmaleimide (NEM) to the protein solution. Chemical reagents such as NEM block the cysteine residues in the $\beta$-LG molecules and prevent disulfide interchange reactions.[46] Figure 6 shows the FTIR spectra of $\beta$-LG samples, preheated in the presence and absence of NEM. In the presence of NEM, the aggregate band at 1612 $cm^{-1}$ in the preheated sample shifts to 1618 $cm^{-1}$. The shift to a higher wavenumber may be explained in terms of the presence of weaker $\beta$-sheets as compared with those of the unblocked sample. In comparison with the native spectrum, we find the band at 1634 $cm^{-1}$ to disappear in the preheated sample, regardless of whether NEM is present or not, indicating that the dissociation of the $\beta$-LG dimer does not depend on the disulfide interchange reaction. In contrast, the band at 1682 $cm^{-1}$ that appears in the absence of NEM sharpens and exhibits a slight shift to 1680 $cm^{-1}$. The large band at 1694 $cm^{-1}$ that appears in the sample with NEM is due to the imide group of

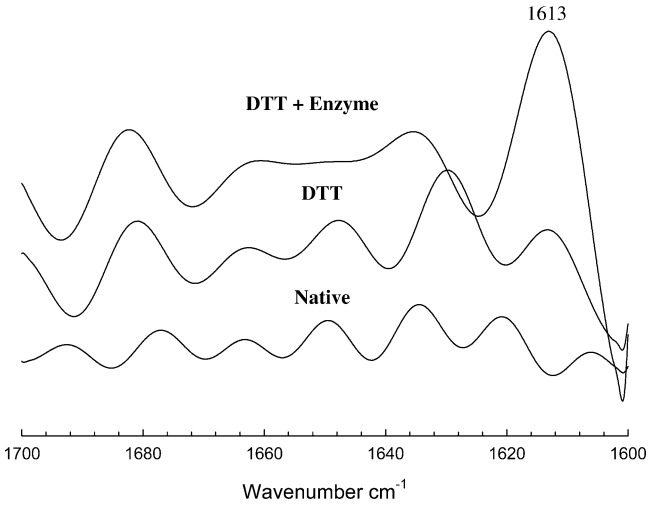


**Figure 8.** FTIR deconvoluted spectra of native, DTT-treated, and DTT followed by enzyme-treated $\beta$-LG samples at 7 wt % concentration. The sample with DTT was heated for 12 h at 50 °C to mimic the enzymatic incubation treatment.

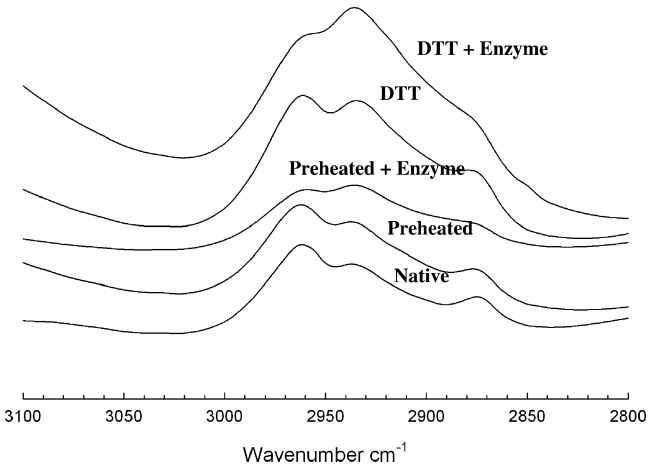


**Figure 9.** IR spectra of the C−H stretching region of different $\beta$-LG samples: native, preheated, preheated then enzyme treated, DTT treated, and DTT then enzyme treated. Sample concentration was maintained at 7 wt %.

the NEM. A separate run with NEM in solution shows a single strong peak at 1692 $cm^{-1}$ (data not shown). Therefore, it appears that a primary factor facilitating the cross-linking of $\beta$-LG by transglutaminase is the disruption of the $\beta$-sheet structures. However, the shift or disappearance of the band at 1634 $cm^{-1}$ under denaturing conditions indicates that the presence of $\beta$-LG as a dimer may be another factor impeding the cross-linking.

**FTIR Spectra of Cross-Linked $\beta$-LG Molecules.** The treatment of $\beta$-LG by transglutaminase is expected to cause some structural changes and affect the FTIR spectra. Figure 7 shows the FTIR spectra of $\beta$-LG after thermal denaturation or enzymatic treatment. We observe a very slight change in the spectra after enzymatic treatment in the amide I region. This indicates that the C═O stretching is hardly affected by enzymatic cross-linking, although the cross-linking reaction changes the microenvironment around the carbonyl side chain of the glutamine that reacts with lysine. This may be attributed to the low number of the bonds created with respect to the backbone bonds in the protein. However, in the case of chemical denaturation by DTT (Figure 8), we clearly observe a noticeable diminution in the α-helix band at 1650 $cm^{-1}$ upon cross-linking by transglutaminase. The α-helix of the $\beta$-LG molecule contains two lysine residues; so, the alteration of the spectrum is possibly

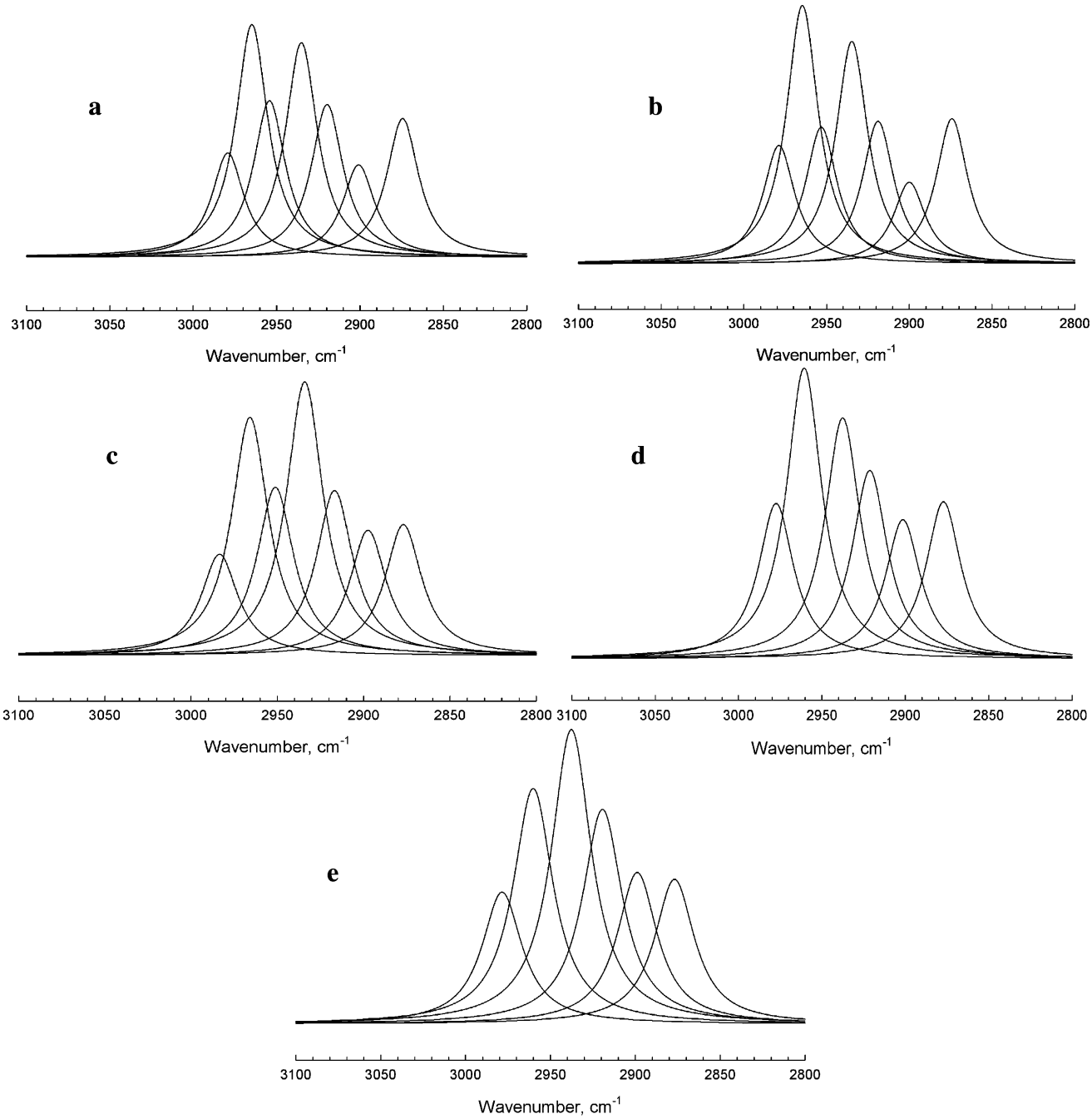


**Figure 10.** Deconvoluted IR spectra of the C−H stretching region of different β-LG samples: (a) native, (b) preheated, (c) preheated then enzyme treated, (d) DTT treated, and (e) DTT then enzyme treated. Sample concentration was maintained at 7 wt %.

**Table 1.** Bands of Deconvoluted IR Spectra of Various β-LG Preparations[a]

| native | | preheated | | preheated + enzyme | | DTT | | DTT + enzyme | |
|---|---|---|---|---|---|---|---|---|---|
| wavenumber $cm^{-1}$ | area % | wavenumber $cm^{-1}$ | area % | wavenumber $cm^{-1}$ | area % | wavenumber $cm^{-1}$ | area % | wavenumber $cm^{-1}$ | area % |
| 2874 | 12.5 4 | 2874 | 12.9 6 | 2877 | 10.7 4 | 2877 | 13.2 2 | 2877 | 12.16 |
| 2901 | 8.45 | 2900 | 7.37 | 2898 | 10.3 7 | 2901 | 11.8 4 | 2899 | 12.87 |
| 2919 | 14.0 0 | 2919 | 12.9 2 | 2917 | 13.7 3 | 2921 | 16.1 0 | 2919 | 18.34 |
| 2935 | 19.7 1 | 2935 | 20.1 8 | 2934 | 22.8 3 | 2938 | 20.6 4 | 2938 | 25.21 |
| 2954 | 14.3 8 | 2953 | 12.4 0 | 2952 | 14.0 4 | 2961 | 24.9 2 | 2960 | 20.16 |
| 2965 | 21.3 7 | 2965 | 23.4 5 | 2966 | 19.8 6 | | | | |
| 2979 | 9.56 | 2979 | 10.7 3 | 2983 | 8.42 | 2877 | 13.2 8 | 2979 | 11.26 |

[a] Deconvolution was done using the maximum likelihood technique based on a Lorentzian peak assumption and a full width at half-maximum (fwhm) of 25 $cm^{-1}$.

due to the cross-linking of one or both of the lysine residues. In addition, an intense band appears at 1613 $cm^{-1}$, indicative of strong hydrogen bonding in intermolecular β-sheet formation. It is worth mentioning here that the sample with DTT with no

enzyme was subjected to a similar thermal treatment (50 °C for 12 h) to isolate the effect of heating from that of enzyme cross-linking. We may also suggest that transglutaminase acts differently on aggregated $\beta$-LG molecules compared with nonaggregated (in the case of DTT treated) $\beta$-LG molecules, favoring the formation of additional intermolecular $\beta$-sheets in the latter case. Thus, cross-linking of $\beta$-LG does not change the amide I region of the FTIR spectra of the thermally denatured protein but does appear to change the $\alpha$-helix pattern and create intermolecular $\beta$-sheets in the case of chemical denaturation.

*Investigation of the C−H Stretching Mode after Cross-Linking with Enzyme.* Hydrophobic interactions play an important role in $\beta$-LG aggregation. We therefore examined the C−H stretching region, 2800−3100 $cm^{-1}$, of the various $\beta$-LG preparations. This region includes the $sp^3$, $sp^2$, and sp C−H stretching modes of the various C−H groups in the side chains of the constituent amino acids. Experiments conducted with different concentrations of DTT (60, 200, and 500 mM) and $\beta$-ME concentrations (2%, 4%, 6%, and 8%) showed no concentration effect on the C−H stretching bands of the spectra, thereby indicating that the modifications observed in Figures 9 and 10 for different conditions were not influenced by the presence of the chemicals. Figure 9 shows IR spectra of native, preheated, preheated then enzyme-treated, DTT-treated, and DTT then enzyme-treated $\beta$-LG solutions. Deconvolution of the spectra was done using maximum likelihood techniques based on a Lorentzian peak assumption and a full width at half-maximum (fwhm) of 25 $cm^{-1}$ as shown in Table 1 and Figure 10a−e. Although the Fourier self-deconvolution method has been successfully applied to resolve the bands of the amide I region,[47] the high sensitivity of the technique to noise[48] made it unsuitable for resolving C−H stretching bands,[49] and hence we used the maximum likelihood technique. The value of fwhm was chosen to be 25 $cm^{-1}$ to avoid over fitting as advised by Howell et al.[49] The deconvoluted bands in Table 1 and Figure 10, parts a and b, show that there is no noticeable change in the spectra upon preheating of $\beta$-LG. Howell et al.[49] found that upon heating $\beta$-LG the band area at 2940 $cm^{-1}$ increases considerably; however, they used a higher concentration (15% vs 7%) and temperature (90 °C vs 80 °C) compared to those of this work. The change in the spectra after heat treatment of proteins is still a contradictory issue; the increase in the band area close to ~2940 $cm^{-1}$ was suggested to be related to the unfolding of ribonuclease[50] due to the exposure of buried aliphatic amino acid residues. However, Bouraoui et al.[51] found that the band area at 2940 $cm^{-1}$ decreases after processing of Pacific whiting surimi into the heat-set kamaboko gel, unlike the observations made by Verma and Wallach.[50] It is important to mention that cystine—known to be formed upon preheating and polymerization of $\beta$-LG—shows a strong band at 2942 $cm^{-1}$.[49] So it is likely that the band at ~2940 $cm^{-1}$ is not solely a function of protein denaturation but is affected by the other protein interactions.

Upon cross-linking with enzyme (Figure 10c and Table 1), the band area at 2935 $cm^{-1}$ increases modestly and shifts slightly to 2934 $cm^{-1}$ reflecting a different degree of exposure of aliphatic amino acids and change in the $CH_2$ asymmetrical stretching mode. The change of the band at 2934 $cm^{-1}$ can also be due to the reaction of the lysine residue through the enzymatic catalysis. Although the lysine main band occurs at 2942 $cm^{-1}$ as an amino acid,[49] we expect the band to shift slightly to lower wavenumber when in protein, due to involvement in several interactions, possibly close to 2934 $cm^{-1}$. We also notice that the band at 2901 $cm^{-1}$ shifts to 2898 $cm^{-1}$ and its area increases indicating an alteration of the arrangements of the aliphatic amino acids.

On the other hand, the use of DTT to chemically denature $\beta$-LG affects the C−H stretching spectra as shown in Figure 10d and Table 1. We notice that the bands at 2954 and 2966 $cm^{-1}$ merge to form a new large band at an intermediate 2961 $cm^{-1}$. This behavior is possibly due to the alteration of the $\alpha$-$CH_3$ asymmetrical stretching and the change in the microenvironment of the cysteine and cystine that show medium and strong bands, respectively, in the region of 2956−2977 $cm^{-1}$.[49] Upon cross-linking with enzyme (Figure 10e and Table 1), we notice a large increase in the band area at 2938 $cm^{-1}$ and a large decrease in the band area at 2961 $cm^{-1}$. The increase of the band area at 2938 $cm^{-1}$ indicates that the enzymatic reaction affects the degree of exposure of amino acid residues and the hydrophobic interactions in turn. This observation was confirmed through our prior rheological work[26] where we clearly showed that enzymatic cross-linking modulated the hydrophobic interactions. The decrease in the band area at 2961 $cm^{-1}$ can again be due to the alteration of the $\alpha$-$CH_3$ asymmetrical stretching mode.

## Conclusions

The $\beta$-LG molecule needs to be denatured either by heat or by chemical denaturation (i.e., cleaving of disulfide bonds) to be susceptible to cross-linking by transglutaminase enzyme. Thermal denaturation proceeds quite differently from chemical denaturation, as manifested in the spectra in the amide I and C−H stretching regions. Cross-linking by transglutaminase of thermally denatured $\beta$-LG did not affect the spectra in the amide I region indicating essentially no change to the carbonyl stretching mode, while enzyme cross-linking of chemically denatured $\beta$-LG changes the structure of the $\alpha$-helix and induces intermolecular $\beta$-sheets. Investigation of the C−H stretching mode shows that cross-linking by transglutaminase of thermally denatured $\beta$-LG changes the band at 2934 $cm^{-1}$ indicating a different degree of exposure of aliphatic amino acids and change in the $CH_2$ asymmetrical stretching mode. On the other hand, denaturation using DTT results in an alteration of the $\alpha$-$CH_3$ asymmetrical stretching. Subsequent cross-linking with enzyme causes further change in the same region. In addition, enzymatic cross-linking affects the band at 2938 $cm^{-1}$ and hence the degree of exposure of amino acid residues and the hydrophobic interactions in turn.

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